Best abstracts

1

DEFINITION OF THE BREAKPOINT FUSION PEPTIDES FOR HLA-A3 AND HLA-B8 IN CML CELLS . THEIR USE TO GENERATE TETRAMERS AND TO EXPAND AND DETECT PEPTIDE SPECIFIC T CELLS IN PATIENTS IN VIVO

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Chronic Myelogenous Leukaemia (CML), characterised by the presence of philadelphia chromosome (Ph) is the result of the t(9;22) translocation, which encodes for the bcr-abl fusion oncogene. The b3a2 junctional region peptides therefore represent potential immunogenic antigens. K562 is a Ph+, HLA class I negative cell line, which expresses b3a2 bcr-abl mRNA. These cells were transfected with single HLA-A*03011 or HLA-B*08011 alleles by electroporation. Acid elution of CML specific peptides from HLA-A3 or HLA-B8 K562 transfectants as well as from HLA-A3 (b3a2 +) CML patients, was performed. The resultant peptides were analysed by mass spectrometry with nanospray ionisation. Sequencing results obtained confirmed the presence of the HLA-A*03011 restricted peptide KQSSKALQR on both transfected K562 and primary CML cells. Tetramer of HLA-A3 and HLA-B8 with the corresponding eluted peptides were produced. These are currently being used to detect antigen-specific CTLs from HLA-A3 and HLA-B8 patients at various stages of their disease and treatment. These data demonstrate that (1) CML cells do express HLA-associated leukaemic-specific peptides and that (2) CML patients have circulating CTLs specific for b3a2 fusion peptides. (3)That these cells can be expanded with specific peptides in vitro. These findings provide the basis for Immunotherapeutic intervention in CML.

2

IN VIVO ANTI-LEUKEMIA SPECIFIC CD8 T CELL RESPONSE IN CHRONIC MYELOID LEUKEMIA.

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As a consequence of the t(9,22) translocation associated with chronic myeloid leukemia (CML), a novel bcr/abl fusion protein is produced. Several peptides from the junction or extra-junction region of the chimeric molecule bind with high affinity to HLA class I molecules and elicit CTL responses *in vitro*. To determine whether CTL responses against tumoral peptides exist in CML patients we used a panel of HLA-A2, -A3 and -B7 specific tetramers. In addition to the bcr-abl neoantigen, we studied 3 other tumoral antigens: - proteinase 3, a myeloid tissue-restricted serine protease overexpressed in leukemia cells, - the Wilm's tumor gene encoded transcription factor (WT1) normally expressed in immature CD34+ progenitor cells, and overexpressed in CML, and – telomerase reverse transcriptase, a universal tumor antigen. Longitudinal FACS analysis on frozen peripheral blood cells sampled at time of diagnosis, after treatment with IFNα or STI571, and during remission following bone marrow transplantation was performed. Our results indicate that a specific CD8+ T cell response directed to various tumoral peptides is observed in patients in remission (0.1 to 1.6% of circulating CD8 T cells), but is never observed in healthy individuals. Sorting of tetramer-positive cells will allow testing for cytotoxicity on autologous leukemia cells and may provide evidence that leukemia-specific CTL actively contribute to CML eradication, provided anergy mechanisms do not preclude the effector potential of these specific T cells. Strategies to boost immunity against leukemia antigens might thus be beneficial for the treatment of CML.

3

INTRAVENOUS INFUSION OF APOPTOTIC LEUKOCYTES WITH ALLOGENEIC BONE MARROW TRANSPLANTATION INDUCES TOLERANCE TO DONOR ANTIGENS.

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Apoptotic cells possess immunomodulatory properties. We recently report that intravenous infusion of apoptotic leukocytes enhances bone marrow (BM) engraftment across major histocompatibility barriers. We propose to define how apoptotic leukocytes induce tolerance and we evaluate the feasibility of a such an approach in a combined or sequential BM and solid graft transplantation after a non-myeloblative conditionning regimen. Sub-lethally irradiated recipient mice, received a restricted BM allograft from donor mice, with or without irradiated apoptotic leukocytes by flow cytometry. Then, skin allografts were performed on chimeric and non chimeric mice. Apoptotic cell injection favored allogeneic BM engraftment. Tolerant chimeric mice kept the capacity to reject third-party skin graft, while tolerating a skin graft provided by BM donors. The observed tolerance was restricted to allogeneic donor BM antigens, independently of apoptotic cell origin. Apoptotic cell infusion did not induce anti-H-2 and/or auto-immune antibody production. Immunohistochemical analysis of the tolerated skin allograft revealed a massive infiltrate of T lymphocytes without any alteration of the skin structure. These finings show that co-infusion of apoptotic cells from different origin with BM can favor tolerance to highly immunogenic allograft. We then evaluated the use of intravenous apototic leukocyte infusion to induce tolerance in a combined BM and solid graft transplantation after a non-myeloablative conditionning regimen. Prolonged survival of skin graft implanted with a limited amount of donor BM cells after a non-myeloablative conditionning regimen was observed. Such a new cell-based therapy could be of interest to induce prolonged survival in organ transplantation.

4

PCR AMPLIFICATION BY THE 5'-3' EXONUCLEASE ASSAY USING MINOR GROOVE BINDER PROBES: A STEP TO AUTOMATE SEQUENCING BASED TYPING OF HLA

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Exact typing of HLA polymorphisms can only be achieved through haplotype-specific Sequencing Based Typing (SBT) allowing the detection of known and previously unidentified alleles. In the first step, SBT requires the haplotype-specific amplification of a DNA fragment. This procedure is time-consuming, preventing a high throughput of DNA samples. In order to solve this limitation we have used the 5'-3' exonuclease assay, in which short fluorogenic probes hybridize with conserved parts of the respective HLA locus (HLA-A, -B, -C, -DRB1). Fluorogenic probes consisted of dihydrocyclopyrroloindole tripeptide (DPI_3) molecules (conjugated minor groove binder-oligonucleotides, MGB-ODNs) which fold into the minor groove formed by the terminal 5-6 bp. The high stability of this structure allows shorter and more specific fluorescence probes to be employed, resulting in lower fluorescent background. Since amplification was indicated by the change of fluorescence, the time-consuming agarose gel-based readout of the amplicons was avoided. In our study, 400 DNA samples were successfully analyzed. Fluorescence was measured by the TaqMan 7700 (Applied Biosystems) system. There was no interference of the MGB-ODNs with the cycle-sequencing procedure. In conclusion, amplification control by MGB-ODNs represents a reliable method which allows to integrate the PCR amplification step in the automated process of template preparation.

5

GLUTATHIONE S-TRANSFERASE T1 (GSTT1) POLYMORPHISM, A NEW HISTOCOMPATIBILITY SYSTEM IN LIVER TRANSPLANTATION.

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A *de novo* immune-mediated hepatitis is an infrequent but consistent finding after living transplantation Four patients of 283 liver-transplant recipients (1.4%) developed this *de novo* immune-mediated hepatitis approximately two years after transplantation. Antibodies showing an unusual liver/kidney cytoplasmic staining pattern were detected in the sera of all four patients and one of them was used to screen a human liver cDNA expression library with the aim to identify the antigenic target of these newly developed antibodies. After cloning and sequencing the gene, it was identified as the gene encoding the glutathion-S-transferase T1 (GSTT1), a 29 kD molecular weight protein, abundantly expressed in liver and kidney. Sera from the other three patients also contain anti-GSTT1 antibodies, two of them demonstrated by immunoblot analysis against the recombinant antigen and the other, that was negative by immunoblot, gave a positive reaction when used directly to screen the same library, suggesting to be directed to a conformational epitope. The GSTT1 enzyme is the product of a single polymorphic gene that is absent from 20% of the caucasian population. When we analyzed the GSTT1 genotype of the four patients described above, we found that this gene is absent from all of them. Three donor paraffin emboided DNA samples were available and showed to be positive for GSSTT1 genotype. In accordance with these results, we suggest that this form of post-transplant *de novo* immune hepatitis, that has been reported as autoimmune hepatitis by others, could be the result of an anti-graft reaction in individuals lacking the GSTT1 phenotype, in which the immune system recognizes the GSTT1 protein as a non-self antigen, being the graft dysfunction not the result of an autoimmune reaction, but the consequence of an alo-reactive immune response

6

DEVELOPMENT OF A PANEL OF SINGLE MHC CLASS I ANTIGEN TRANSFECTED CELL LINES FOR HLA ANTIBODY SCREENING.

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The prediction of a negative crossmatch for highly immunized patients is difficult, due to the complexity of their serum HLA antibodies. Study of these sera with conventional cell panels is complicated because most panel cells express 2 antigens encoded by each of the 3 MHC loci HLA-A, -B, and -C. To alleviate this problem, we are developing a collection of single antigen lines (SALs). To date, 16 SALs were prepared by transfecting cDNA of human HLA class I alleles into a cell line, K562, lacking class I cell surface expression. The validity of use of SALs for alloantibody screening was tested by flow cytometry (FC) with a panel of human monoclonal HLA antibodies (HuMAbs) developed in our lab. The panel consisted of 37 A-locus specific (12 IgG/25 IgM), 51 B-locus specific (20 IgG/31IgM) and 3 C-locus specific (all IgM) HuMAbs. The majority of HLA HuMAbs (24/32 IgGs and 54/59 IgMs) reacted with SALs in complete agreement with their CDC reactivity, which was determined against large panels (n>240) of PBLs. Irregular reactivity was observed with 3 HuMAbs, that reacted with all SALs as well as the untransfected K562 cells. The reactivity of the remaining antibodies contained extras not detectable by CDC. Ten allosera were tested with the current SAL panel, showing full concordance with the CDC specificity for 6 allosera, and additional alloreactivity for 4. The HLA-expression of the SALs was stable in short term culture; long term cultures are being studied. This panel of SALs offers good prospect for screening highly immunized patients by CDC and FC.

7

RELEVANCE OF KIR GENE POLYMORPHISMS IN BONE MARROW TRANSPLANTATION OUTCOME

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Natural Killer (NK) cells may be involved both in allogeneic Bone Marrow Transplantation (BMT) rejection and Graft-versus-Host-Disease (GvHD). The physiological functions of NK cells appear to be regulated by diverse non-inhibitory and inhibitory receptors including the Killer cell Immunoglobulin-like Receptors (KIR). Although HLA-epitope mismatches are well-known causes of NK alloreactivity, the role of KIR genes in transplantation remains to be further investigated. In this study, we have evaluated whether KIR genotype differences between donors and recipients of HLA identical - related and unrelated - compared to HLA non-identical unrelated BMT, had an impact on transplantation outcome. The results of KIR genotyping revealed that 70 different genotypes were found in all individuals (n=150) when 15 KIR genes were tested. When the donor KIR genotypes were compared to that of the corresponding recipients, 4 different combination patterns were observed. Interestingly, one of these combination patterns represented a high risk factor for the occurrence of aGvHD since 100% of unrelated BMT with the combination "recipient KIR genotype included in the donor KIR genotype" developed aGvHD as compared to 60% for all the other pooled combinations (p=0.012). In contrast, no aGvHD was observed in the related BMT with the same combination pattern (p=0.0001). Moreover, KIR2DS3 could represent a potentially high risk marker for aGvHD in unrelated BMT since it was mismatched in 8 out of 11 pairs with the combination "recipient KIR genotype included in the donor KIR genotype". Genotyping of this non-inhibitory KIR gene and characterisation of its putative HLA ligand should help in the selection of an appropriate donor in unrelated BMT.

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IMPACT OF KIR GENE POLYMORPHISM ON HLA GENO-IDENTICAL HAEMATOPOIETIC CELL TRANSPLANTATION OUTCOME.

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Killer cell immunoglobulin-like receptors (KIR) are a family of receptors expressed on natural killer and some T cells. KIR genes have been described on chromosome 19 and the number of KIR genes varies between individuals. We typed the donors and recipients of 84 consecutive HLA geno-identical related bone marrow transplantations for ten KIR genes (2DS1, 2DS2, 2DS3, 2DS4, 2DL1, 2DL2, 2DL3, 3DS1, 3DL1, 3DL2) using PCR-SSP techniques. This population was already typed for cytokines and HA-1 gene polymorphisms. The KIR gene frequencies as well as the observed KIR gene linkage disequilibriums were consistent with previous data. The impact of each KIR genes on acute GVHD (Grade II-IV) was evaluated using univariate analysis. We observed that mismatching for 2DL2 and 2DS2 (genes in linkage disequilibrium) were associated with GVHD (p=0.02 and 0.07 respectively). Moreover, an increased GVHD was observed when the number of matches KIR between donors and recipients is below 5 out of 12 (p=0.02). In multivariate analysis, 2DL2 mismatches and the number of matches KIR remained associated with GVHD independently to the already defined risk factors of GVHD : IL10 polymorphism, HA-1 mismatch and positive CMV serology of the donors. These results suggest that KIR polymorphism may have an impact on the occurrence of GVHD in geno-identical BMT.

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DONOR-SPECIFIC TOLERANCE: RESULT OF EXTRATHYMIC CLONAL DELETION IN A MURINE MODEL?

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A murine model has been developed, that involves engraftment of fully allogeneic T cell depleted donor BM cells in low dose irradiated and anti-CD3 treated recipient mice. These mice developed permanent stable multilineage mixed chimerism and donor-specific tolerance without GvHD. To study the mechanisms via which donor-specific tolerance is induced in our animal model we investigated: (1) the effect of high doses IL-2 *in vivo* and *in vitro*, (2) the production of Th1 and Th2 cytokines after re-exposure to the donor antigen *in vitro*, and (3) we performed adoptive transfer experiments. Finally, we investigated the role of clonal deletion. Results showed that neither clonal anergy, nor a Th1/Th2 shift or other active suppressor/regulatory processes play a prominent role in the maintenance of tolerance in our model. We found certain VβTCR deletions in our tolerant mice and, surprisingly, also in thymectomized recipient mice. We conclude that clonal deletion plays a role in the establishment of donor-specific tolerance, although the thymus is not required for this process.

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PROTEASOMAL CLEAVAGE ANALYSIS OF POLYMORPHIC PROTEINS TO PREDICT PRESENTATION OF MINOR HISTOCOMPATIBILITY PEPTIDES

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Minor histocompatibility antigens (mHags), derived from polymorphic proteins, are thought to be targets for graft-versus-host disease and graft-versus-leukemia reactions after allogeneic stem cell transplantation. To achieve a correct size for an optimal binding to MHC class I molecules, proteins have to be fragmented by proteasomal processing. We used the proteasomal cleavage prediction algorithm recently developed by Nussbaum et al. to analyze the known aa sequences of the autosomal mHags HA-1, HA-8, HB-1 and of the Y-chromosomal mHags SMCY, DFFRY and UTY for a properly proteasomal processing. Strikingly, the immunodominant HA-1H nonamer was correctly predicted at both the N- and C-terminus. By replacing histidine by arginine at position 3, an epitope-destroying cleavage site is inserted into HA-1. For the HA-8R nonamer and the HB-1H decamer slightly prolongated peptides at the N-termini were predicted which could be further N-terminally processed by cytosolic or ER-trimming proteases, but the C-termini of these mHags were correctly predicted. The replacement of arginine by proline in HA-8 as well as of histidine by tyrosine in HB-1 led to epitope destroying cleavage sites. For the Y-chromosomal mHags derived from SMCY und UTY, the algorithm predicted correct C-terminal cleavage sites but the predicted peptide fragments were shorter than the HLA-restricted CTL epitopes described for HLA-A2, B7, B8 and B60. The proteasomal cleavage analysis provides evidence that a part of mHag peptides predicted to bind to HLA class I molecules will never be generated by proteasomal cleavage and thus are without any clinical relevance.

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GENOMIC ORGANISATION AND HLA-RESTRICTED PEPTIDES OF CD13 (AMINOPEPTIDASE N)

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Minor Histocompatibility Antigens (mHags) are thought to be targets for graft versus host and graft versus leukemia (GVL) reaction as well as graft rejection after stem cell transplantation. This study was designed to identify new mHags which are involved in the immunotherapeutically desired GvL effect. Aminopeptidase N is a member of a family of membrane-bound metallopeptidases. This Protease is expressed on the surface of normal and malignant human myeloid cells, fibroblasts, hepatocytes, and the epithelial cells that form brush borders of the small intestine and kidney. Cell-surface metallopeptidases have specific functions, including potential roles in the control of growth and differentiation in hematopoietic and epithelial systems by participating in the final steps of digestion by cleaving peptides (peptide scavenging). An indirect consequence of genetic variation is the generation of polymorphic self peptides that may cause therefore histoincompatibility. By sequencing cDNA of Aminopeptidase N a 967-amino acid protein was predicted. We detected 17 single nucleotide polymorphisms, resulting in 13 amino acid exchanges. In order to develop a simple PCR-SSP strategy for these polymorphic sites the DNA was cloned into a pCR2.1 plasmid and sequenced. The gene coding for Aminopeptidase N has a length of 21,238 nucleotides and consists of 19 non-coding regions and 20 exons. To further characterise immunodominant mHags of CD13 we used the proteasomal cleavage prediction algorithm "PA-ProC" to analyse the polymorphic regions for a properly proteasomal processing and HLA peptide binding analysis "SYFPEITHI" for epitope prediction. By this we found two CD13 peptides (Q86R and I603M) predicted in the correct matter for peptide presentation in HLA-A*0201, HLA-B*0701, HLA-B*0801 or HLA-B*2705 molecules.

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Vδ1+, Vδ3+, Vδ5+ γδ T CELLS EXPAND IN SOLID ORGAN TRANSPLANTED RECIPIENTS AND IN HEALTHY SUBJECTS INFECTED WITH CYTOMEGALOVIRUS: A FUNCTIONNAL COMMUNITY FOR Vδ2NEGATIVE γδ T CELLS ?

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T lymphocytes can be separated in two subtypes as a function of the T cell receptor (TCR) they express. In human, αβ T lymphocytes constitute the major part of circulating T cells, and their antigen-recognition is MHC-restricted. γδ T cells represent generally less than 10% of T cells; their function and the ligands of the γδ TCRs are not definitively determined. The chains of the TCR are designed through rearrangements of V, D, and J segments, similarly to the immunoglobulins. For the δ chain, these genic segments are found inside the α locus between the Vα and the Jα segments. Most circulating γδ T cells express the Vγ9 and the Vδ2 segments in healthy individuals.

We show in solid organ-transplanted patients a dramatic expansion of circulating γδ T cells only when they develop a Cytomegalovirus infection (CMV+). This expansion concerned Vδ1, Vδ3 andVδ5-positive cells that expressed all the different Vγ segments, while these cells are normally barely detectable in healthy individuals. These cells display a restricted repertoire, indicating that their expansion is antigen-driven. Moreover, Vδ1+ and Vδ3+ cell expansion can occur in healthy subjects after CMV infection, but at a considerably lower level. Based on a previously published observation of a Vδ4+ population in a CMV+ bone marrow grafted patient, we postulate that a functionnal community might exist among human Vδ2neg T cells. They may be a part of the immune response directed to the CMV particularly relevant in an MHC-mismatch context.

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INCREASED PRODUCTION OF IL-10 AND IL-1 Ra BY PHOTOPHERESIS IN CHRONIC GRAFT VERSUS HOST DISEASE (GVHD)

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Mechanisms of immunosuppressive action of photopheresis in GVHD are incompletely understood. Patient's PBMCs recovered by leukapheresis are treated ex vivo by UVA + 8-MOP and reinjected to the patient. This induces a process of lymphocytes apoptosis that produces immunosuppression by activating monomacrophagic cytokines after phagocytosis of the debris. Manomacrophages are resistant to apoptosis. We studied 11 leucapheresis products from 6 patients suffering of severe chronic GVHD resistant to conventional treatment and undergoing photopheresis. By co-culturing these apoptotic lymphocytes with patient's monomacrophages, we observed an increased production of two immunosuppressive and anti-inflammatory cytokines IL-10 and IL-1 Ra as measured by RT PCR. In vitro addition of LPS markedly enhanced the process (IL-10 + 215%, p = 0.004; IL-1 Ra + 68%, p = 0.004). Increased endotoxinemia constitutes a feature of GVHD. We also found, by blocking experiments with an anti IL-10 Ab that production of IL-1 Ra, an agent that has been shown to be endowed with potent anti GVHD properties is under the control of IL-10.
We propose that photopheresis induced lymphocytic apoptosis activates monomacrophages possibly at the GVHD sites to release IL-10 and IL-1 Ra immunosuppressive cytokines that ultimately correct GVHD manifestation.

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TH1/TH2 POLARISATION IN CHRONIC VASCULAR REJECTION CORRELATED TO COMPATIBILITY IN MURINE ALLOGRAFTS.

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Chronic vascular rejection is a serious problem in organ transplantation. In order to study the pathology in different Th1/Th2 settings we have established a model of aortic allografts in mice using the sleeve technique. The donors are DBA/2 (H-2^d) or B10.A(2R) (H-2^{h2}) and the recipients C57BL/6 (H-2^b), either wild type or deficient for IL-6. The mice were sacrificed at 15, 30, 60 or 90 days after the graft. Typical histologic lesions with intimal thickening and proliferation of smooth muscle cells were found in allografts but not in isografts. Flow cytometry showed that very high alloantibody titres, mainly of IgG1 isotype (Th2), were produced when DBA/2 was donor (up to 10^{-5}), while only moderate and delayed antibody titres of IgG2a isotype (Th1) type were found after B10.A grafts. In IL-6 deficient recipients, the antibody response was as expected exclusively of Th1 type, but the response appeared surprisingly early and was very strong. ELISPOT assays revealed a direct alloresponse with IFNγ production in wild type mice with B10.A as donor while DBA/2 donors induced at the same time a direct IFNγ response and a partly indirect IL-4 response. At day 60, an indirect alloresponse was found in both combinations. IL-6 deficient mice displayed only a direct alloresponse. Morphometry (Cyberview) was performed on serial transversal cuts at 100 μm intervals on the totality of the length of the grafts (8-9 mm) and a computer program reconstituted a longitudinal image. The lesions differed in severity and kinetics according to the Th1/Th2 profile. Our model offers a unique opportunity to evaluate the effect of immunomodulation on chronic vascular rejection in situations where the alloresponse is clearly biased towards Th1 or Th2 profiles.

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PGE_2 SIGNALING IN MONOCYTE-DERIVED DENDRITIC CELLS

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PGE_2 has a prominent role in cytokine-driven differentiation of monocyte-derived dendritic cells (MDC). Recently, we demonstrated that PGE_2 is central to a DC-mediated inhibition of T-cell proliferation, exploiting indoleamine 2,3 dioxygenase (IDO)-dependent tryptophan depletion. Thus, we were interested to determine signaling pathways of PGE_2-dependent induction of IDO. Since PGE_2 receptors (EP) utilize cAMP/protein kinase A (PKA), as well as Ca^{2+}-dependent pathways, specific IDO mRNA levels were monitored upon treatment with specific PGE_2 agonists and inhibitors. Upon treatment with the non metabolisable cAMP analog, dibuturyl-cAMP, a concentration dependent induction of IDO mRNA was seen. Furthermore, PGE_2 (10^{-7} to 10^{-9} M) stimulated IDO mRNA expression was inhibited by the protein kinase A (PKA) inhibitor H89 (10 μM). Assuming a EP-mediated increase in intracellular Ca^{2+} concentration, MDC were treated with ionomycin (IONO). In the presence of various concentrations of IONO (10^{-6} and 10^{-7} M) an induction of IDO mRNA expression was seen. Similarly, phorbolester (PMA) also induced IDO mRNA expression upon addition to MDC on day 6 of differentiation. Since PMA activates PKC leading to activation of NF-kB, we pretreated MDC with pyrrolidine-dithio-carbamate (PDTC), a specific inhibitor NF-kB inhibitor to block this pathway. MDC pretreated with PDTC and subsequently challenged by various concentrations of PGE_2, demonstrated an inhibition of IDO mRNA induction.
In conclusion, we demonstrated that PGE_2 utilizes cAMP/PKA- as well as Ca^{2+}-dependent pathways to mediate IDO gene expression in MDC.

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MECHANISMS OF T-CELL PROLIFERATION INHIBITION BY INDOLEAMINE 2,3.DIOXYGENASE.

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Recent data indirectly suggest that the enzyme Indoleamine 2,3-Dioxygenase (IDO) in mice placental macrophages could play a role in maintaining maternal-foetal tolerance by enhancing tryptophan (Trp) catabolism. However, a direct demonstration of IDO action s lacking, and the mechanisms of this action are largely unknown. We have purified IDO from rabbit small intestine, and tested the purified enzyme in *in vitro* models of T cell proliferation. IDO was able to completely inhibit proliferation of PHA-activated T lymphocytes, and of alloreactive T cell lines. Inhibition of T cell proliferation did not occur in the presence of the IDO inhibitor mehyl-Trp. We found that IDO exerts its inhibitory effect on T cell proliferation by blocking the cells in the G1 phase of the cell cycle; instead, apoptosis was not significantly affected. We then tested the role of the levels of Trp and of its catabolites Kynurenine, Picolinic acid and Quinolinic acid in inhibiting T cell proliferation. In the presence of Trp only Kynurenine and Picolinic acid were able to inhibit T cell proliferation. In the absence of Trp T lymphocytes retained their proliferative capacity, however, under these conditions all the three catabolites were effective in inducing a dose-dependent inhibition of proliferation. In the case of L-Kynurenine and Picolinic acid inhibition was observed at concentrations well below the lowest concentration that was effective in the presence of thryptophan, in the case of Quinolinic acid the inhibitory capacity was acquired de novo. We therefore suggest that IDO exerts its effect on T cell proliferation via two different mechanisms. On one side it starts the cascade of biochemical reactions that produce the three catabolites. On the other side it depletes Trp, allowing all the three catabolites to put in action their inhibitory potential at concentrations that can be achieved in the extracellular microenvironment. We hypothesize usage of IDO or Trp catabolites in modulating alloreaction in transplantation.

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IMMUNOTOXINS CONTAINING HUMAN RECOMBINANT ANTI-CTLA-4 ANTIBODIES FOR THE INDUCTION OF TRANSPLANTATION TOLERANCE.

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Several recombinant human-derived scFv antibody fragments against CTLA-4 (CD152) have been generated by the phage display technology. Immunotoxins containing scFv reagents #83 and #40 linked to saporin, a type 1 RIP (ribosome-inactivating protein) , were prepared and tested on CD3/CD28 activated T lymphocytes, mixed lymphocyte reactions, CTLA-4 positive cell lines and hematopoietic precursors. Immunotoxins induced apoptosis in activated T lymphocytes and specifically inhibited mixed MLRs between T lymphocytes and dendritic cells or EBV cell lines. Toxicity tests on hematopoietic precursors showed little or no effects in inhibiting colonies growth. As the #83 scFv antibody was reactive also with activated mouse T-lymphocytes, #83-saporin was tested in a model of tumor rejection consisting of C57Bl/6 mice bearing a murine H.end endothelioma cell line, derived from DBA/2 mice. The lymphoid infiltration due to the presence of the tumor was reduced to a high extent, demonstrating that the immunotoxin was actually available and active in vivo. Thus, this study might represent a new breakthrough for immunotherapy, showing the possibility of targeting CTLA-4 to kill activated T cells, utilizing conjugates containing scFv antibodies and a type 1 RIP.

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ASSOCIATION BETWEEN CD14 PROMOTER POLYMORPHISM AND TOTAL SERUM IMMUNOGLOBULIN E LEVELS

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CD14 is an important myeloid pattern recognition receptor that is critical for initiation of the innate immune response induced by lipopolysaccharide (LPS). LPS-induced activation of antigen presenting cells has been implicated in the development of an adaptive Th1 response. Linkage and association studies suggested that genetic variation in CD14 may modulate the development of the Th2 driven allergic immunresponse.
We genotyped a single nucleotide polymorphism (SNP) in the promotor region of the CD14 gene on chromosome 5q31 (C-159T) in 200 healthy blood donors. All individuals were analyzed for serum IgE levels and soluble CD14 (sCD14). There was a significant association of the CD14 C–159T genotypes and total serum IgE levels.
Individuals homozygous for the -159T allele had significant higher total serum IgE levels as well as serum sCD14 levels than carriers of the other genotypes (p = 0.037, Mann-Withney-U test). Our results suggest that CD14 has a key immunoregulatory role in the pathogenesis of allergy.

	CD14-159 TT homozygotes	CD14-159 CT heterozygotes	CD14-159 CC homozygotes
IgE-level < 200 kU/l	37 22,0%	75 44,6%	56 33,3%
IgE-level ≥ 200 kU/l	12 41,4%	13 44,8%	4 13,8%

Chi-square-test (Pearson): p=0,033

Abstract session II Disease and cancer

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POSTTRANSPLANTATION LYMPHOPROLIFERATIVE DISORDERS: HOST-RECIPIENT ORIGIN INFLUENCE THE CLINICAL ASPECTS

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Post-transplantation lymphoproliferative disorder (PTLD) is a well-known complication of immunosuppression associated with solid organ transplantation. The donor/host origin of PTLD may influence the outcome of the disease since it has been reported that a donor origin may be associated with a better prognosis. The aim of the study was to determine the origin (recipient / donor) of 12 PTLD occurring in kidney transplant recipients and to correlate the results with clinical findings.

Methods. Origin of PTLD was determined using HLA DRB1 molecular typing, analysis of multiple short tandem repeat microsatellite loci and HLA class I antigens expression by immunohistochemistry.

Results. The results of the 3 techniques were concordant; 8 cases originated from the recipient and 4 cases originated from the donor.. All the donor-origin PTLD were strictly localized to the kidney graft, developed after a mean time of 5 months after transplantation, and regressed after reduction of immunosupression. On the opposite, 7 of the 8 recipient-origin PTLD presented as multisystemic disease, occurred after a mean time of 75.7 months after the transplantation and had a worse outcome (mortality, 5 deaths out of 8 patients, 62,5%).

Conclusion. These results suggest that PTLD originating from the donor arise in the first year following transplantation into the graft, and that recipient-origin PTLD develop later as an invasive disease. The determination of the origin of the tumors appears of value in the management of PTLD to predict the outcome and to adapt therapy.

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THE MINOR HISTOCOMPATIBILITY ANTIGEN CD31 CONTROLS DIFFERENTIATION OF BREAST CANCER CELLS.

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CD31, a minor histocompatibility antigen is involved in leukocyte extravasation through the implementation of a signaling pathway intertwined with the one controlled by integrins. Its role in tumor growth and migration was evaluated using as a model the transfection of the molecule in MDA-MB-231 breast carcinoma line. Results indicate that the CD31-transfected cells i) lose the expression of CD44 (the hyaluronate receptor), ii) acquire an organized three-dimensional architecture, and iii) grow less. These results were validated in nude mice injected either with CD31- or mock-transfected MDA-MB-231 cells. Histological sections from the $CD31^+$ tumors showed the presence of sparse nests of cells organized around a lumen and surrounded by collagen and laminin. CD44 was absent. Most CD31-transfected cells were blocked in the G1 phase, as inferred from the expression of Ki-67, Kip-1 and p21. Control mock-transfected cells grew in solid sheets, without stromal material and displaying high levels of CD44 and proliferation.
CD31 conferred to the MDA-MB-231 cells unique motility features, as inferred from i) the ability to form papillae *in vitro* and *in vivo*, and ii) to specifically invade normal murine breast ducts, without the compression and destruction evident in the mock-transfected specimen. Lastly, iii) the cells migrate more efficiently in *in vitro* assays.
Taken together, these results indicate that transfection of human CD31 induces differentiation of tumor cells, suggesting a role of this minor histocompatibility antigen in the determination of the tri-dimensional architecture of breast tissue.

21 **DISCREPANCIES IN THE PATTERN OF REACTIVITY OF MONOCLONAL ANTIBODIES AGAINST FREE HLA CLASS I HEAVY CHAIN AND ß2M-HEAVY CHAIN COMPLEX REVEALES ß2M GENE INACTIVATION IN COLORECTAL TUMORS**

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HLA class I total loss or downregulation (phenotype I) has been reported in different tumor types. This phenotypic alteration can be associated with ß2-microglobulin gene alterations.
We studied phenotype I in 124 colorectal cancers by immunochemistry using the following monoclonal antibodies: W6/32 directed against assembled ß2-microglobulin-HLA ABC complex; GRH1 directed against ß2-microglobulin, and HC10, which recognizes free HLA class I heavy chain. 14 of 124 tumors (11,2%) showed absence of reactivity with W6/32 and GRH1 monoclonal antibodies (phenotype I). However, we detected discrepancies between reactivity with W6/32 and HC10 in 5/14 (35,7%) tumors with phenotype I. These cases were W6/32-negative and HC10-positive. To explain this discrepancy we looked for ß2-microglobulin mutations in HC10-positive tumors by specific amplification of the ß-microglobulin gene and sequencing. We analysed 4 tumors and detected ß2-microglobulin mutations in all of these: 3 frameshift mutations [(A)5 ◊ (A)4 in exon 2 in one tumor and (CT)4 ◊ (CT)3 in two tumors] and one 4-bp deletion in one tumor. Probably, these mutations are the cause of ß2-microglobulin inactivation. Therefore, we conclude that ß2-microglobulin inactivation is the mechanism that underlies complete HLA class I loss in tumors with positive cytoplasmic expression of free HLA class I heavy chain.

22 HLA HAPLOTYPE LOSS IN LARYNGEAL CARCINOMAS

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The importance of alterations in the expression of HLA class I antigens in the escape of tumors from immune surveillance is well known. The first step in reveling these alterations is immunohistological analysis of tumors, using mAbs against HLA class I antigens. One of the molecular mecanisms of this losses is HLA haplotype deletion . To stablish the frequency of HLA haplotype loss in laringeal carcinomas , 7 STR marker from 6p21-3 region were used with DNA obtained from 55 laringeal carcinomas. DNAs were obtained by microdissection of cryostat sections previously analized with immunohistologic techniques. In adition, HLA genomic typing by sequence specific oligonucleotide analysis was done to define the HLA haplotype lost.

The immunohistological and molecular results showed that 45 (81,8 %) laryngeal carcinomas had HLA alterations and 10 (18,2%) were normal for HLA expression with the panel of mAbs. 34 (61,8%) laryngeal carcinomas had HLA haplotype loss (Fenotype II) and of this group the HLA haplotype could be defined in 21 (38,2%) laryngeal carcinomas. These cases showed complete loss of one allele from at least 3 informative STRs markers, and also lost of the pattern of one HLA- A-B haplotype. In the other 13 cases (23,6%) a decrease of 25-50% was detected on one allele of three informative STRs; but by SSO the band pattern for locus A and B was normal. These cases may represent tumors that are heteregoneous for HLA haplotype or that contain residual infiltrating normal cells. 2 cases (3,6%) were HLA class I negative (Phenotype I), 4 cases (7,3%) showed loss of HLA locus B expression (Phenotype III) and 5 cases (9,1%) had lost only one locus B allele (Phenotype IV).

These results show that HLA haplotype loss is the most frequent HLA alteration on laringeal carcinomas.

23 TGF-B1 PROMOTOR AND CODON 10 T->C POLYMORPHISM ANALYSIS IN CLASSIC KAPOSI'S SARCOMA

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TGF-β is a multifunctional cytokine and three isoforms are known: TGF-β1, TGF-β2 and TGF-β3, coded by three different loci: TGFB1, TGFB2 and TGFB3. Two polymorphisms in the TGFB1 promoter are described: G-800A and C-509T, the latter indicated as primarily involved in TGFB1 plasmatic concentration. The presence of the T allele is associated with a higher plasma levels of TGFβ1. Another polymorphism in the TGFB1 codon 10 (codon 10 T->C, Leu->Pro) has been correlated with TGFβ1 plasma concentration, the CC genotype correlating with a increased production of TGFβ1. Among the different effects of TGFβ1, the direct stimulation of angiogenesis in vivo interested us because the neoangiogenesis is constant in the specific lesions of Classic Kaposi's Sarcoma. We have evaluated the TGF-β1 C-509T and codon 10 T->C polymorphism in 20 Kaposi's Sarcoma patients and in 58 normal controls belonging to the same area. The -509T and codon 10 C (Pro) alleles, showed frequencies significatively increased in the patients when compared to those observed in the control group ($p<0.0001$, RR=5.296 and $p = 0.0413$, RR=2.214 respectively). The homozygous genotype TT of the -509 promoter polymorphism showed a positive association with Kaposi's Sarcoma ($p=0.00112$, RR= 5.492) and the same was observed for the combined genotypes promoter/codon 10 TT/CC ($p=0.00112$, RR= 5.492). Our results suggest the involvement of -509 promoter and codon 10 T->C polymorphisms of the TGFB1 gene in the development of Kaposi's Sarcoma.

24 **SOLUBLE HLA-DR AS A POTENT PREDICTOR OF DISEASE PROGRESSION IN EARLY-STAGE MELANOMA**

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Because of increasing incidence of malignant melanoma (MM) and poor prognosis of metastasized patients a predictive marker is needed to identify patients at high risk of disease progression. Therefore our purpose was to analyse the significance of soluble HLA-DR (sHLA-DR) level as a host factor compared to the tumor factor S100 β in MM patients (N=183) in view of patients survival. sHLA-DR serum levels of 86 healthy individuals served as controls. We found appr 50 % lower sHLA-DR levels in MM patients than in controls (0.70 ± 0.08 SEM µg/ml vs. 1.38 ± 0.16 SEM µg/ml; $p < 0.0005$). Univariate analysis showed an association of sHLA-DR < 0.3 µg/ml and S100-β > 0.12 µg/l with poor overall ($p=0.021$ and $p=0.0009$) and progression-free survival ($p < 0.0005$ and $p=0.0025$) resp.. Multivariate analysis revealed disease stage ($p = 0.0093$) and tumor burden ($p < 0.0005$) as independent predictive factors for overall survival, and sHLA-DR ($p=0.0007$) and tumor burden ($p=0.0015$) for progression-free survival. In contrast to S100-β, sHLA-DR serum concentrations < 0.3 µg/ml were strongly associated ($p=0.0001$) with poor progression-free survival in a subgroup of 60 non-metastasized patients. Thus, our results suggest sHLA-DR as a prognostic serum marker in melanoma patients superior to S100-β revealing sHLA-DR but not S100-β as an independent predictive factor for patients' progression-free survival and as a potent predictive marker to identify early-stage melanoma patients at high risk for disease progression.

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IMPACT OF HLA CLASS I ALTERATIONS IN PATIENTS UNDERGOING T CELL SPECIFIC IMMUNOTHERAPY

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The potential impact of HLA class I abnormalities on the outcome of T cell-based immunotherapy for cancer has rekindled interest in the analysis of HLA class I antigen expression in malignant lesions and the molecular mechanism responsible for the HLA alterations. In the present study we analyzed the expression of HLA class I antigens in 15 different melanoma cell lines from nine patients with metastasic melanoma who were included in a T cell-based immunotherapy protocol (peptides and autologous cell vaccines). Eight of these patients have not responded to treatment, and the molecular study of cells lines, established from these patients showed different alterations in the expression of HLA class I molecules which were probably responsible for the failure of treatment. The most frequent HLA phenotypes in the tumor cells of the patients were: Phenotype I (3 patients with total loss of HLA class I molecules), Phenotype II (3 patients with haplotype loss), and Phenotype IIIb (2 patients with downregulation for locus HLA-B). The molecular mechanisms involved in the generation of these HLA altered phenotypes were also diverse: mutations in the *β2m* gene, hypermethylation of HLA class I genes, and genomic loss of HLA class I genes (Phenotype II). Only one patient responded well to therapy, this patient showed no alterations in the expression of class HLA I molecules. This study shows that analysis of HLA class I expression in tumoral cells is an indispensable element to establish an effective antitumoral T cell-based response and its correlation with the patient's clinical progress.

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ROLE OF G PROTEIN β3 SUBUNIT C825T AND HLA CLASS II POLYMORPHISMS IN THE IMMUNE RESPONSE AFTER HBV VACCINATION

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The G protein β3 (*GNB3*) subunit and HLA are candidate genes predictive of immune response capacity. We therefore studied the influence of both gene systems on cellular and humoral immunity against hepatitis B virus (HBV) in 79 HBV-booster vaccinated healthy volunteers and an independent group of 77 probands after HBV basic immunization. Lymphocyte *in-vitro* proliferation towards an HBV surface antigen after booster vaccination was 2.5-fold increased in *GNB3* 825T (TC+TT) vs. CC allele carriers ($P = 0.01$) and was not influenced by HLA-DRB1 or -DQB1 alleles. In addition, anti-HBs antibody titres in both groups were about 2-fold increased in TC and decreased in TT compared to CC allele carriers. However, antibody titres after HBV booster immunization were 1.7-fold elevated in HLA-DQB1*0301 carriers (P corrected = 0.027). Interestingly, the only non-responder in the booster vaccinated group displayed the *GNB3* CC genotype and HLA-DRB1*0301, DQB1*0201, all non-responders in the basic immunization group (3/77) showed the CC genotype. In summary, the *GNB3* 825T allele appears as a marker particularly predictive of cellular and HLA-DQB1*0301 of humoral immune responses following HBV vaccination.

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Psoriasis vulgaris and psoriatic arthritis share a 31-kb susceptibility region telomeric to HLA-C

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Psoriatic and psoriatic arthritis patients appear to share the same psoriatic susceptibility locus. However, this putative gene is in linkage disequilibrium with different HLA haplotypes in each disease, which may help to delimit the region associated to psoriasis. To map the susceptibility locus, patients with type I psoriasis (n=95) and psoriatic arthritis patients with classic psoriasis patterns of skin lesions (n=81) were included in this study. Cw*0602 was associated both with psoriasis (49% vs 21% pc=0.0006; OR=3.65) and with psoriatic arthritis (60% vs 21% pc=0.000001; OR=5.7). Polymorphic genes and microsatellite markers telomeric (*HLA-B*, *TNF*, C1_2_5) and centromeric (C1_4_4, *OTF3*, *HCR* and corneodesmosin gene) to *HLA-C* were also studied for association analysis. In psoriatic patients a susceptibility region telomeric to *HLA-C* that include C1_4_4 (56% vs 22% pc=0.00001; OR=4.39), OTF3 (85% vs 60% pc=0.0028; OR=3.76) and *HCR* (63% vs 26% pc=0.000001, OR=4.88) was observed. In psoriatic arthritis patients the psoriasis susceptibility region was delimit by *HLA-C* and C1_4_4 (388 allele 54% vs 22% pc=0.0002; OR=3.96) being both markers independently associated. Comparing the susceptibility regions associated with both diseases, an overlapping interval of 31 kb between *HLA-C* and C1_4_4, that might contains the psoriasis gene, can be define.

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LINKAGE DISEQUILIBRIUM BETWEEN A BASE PAIR EXCHANGE IN EXON 8 OF THE 21-HYDROXYLASE B GENE (318Gln→318Stop) AND HLA B AND DR

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We performed mutation analysis by SSO and RFLP in 258 families with at least 1 index patient with 21OHD and we will focus here on one of the 12 most frequent 21OHB mutations tested: 318Gln→318Stop, exon 8, codon 318. In one family, where the index patient is homozygous for a 21OHB deletion, the mother is compound heterozygous for a 21OHB deletion on one haplotype and both a 318stop mutation (318A) and a normal B sequence in codon 318 (318B) on the other haplotype. This indicates the presence of two 21OHB genes, one with a mutation and one with a normal sequence in codon 318.This assumption is also supported by RFLP analysis and it is also compatible with the fact that the mother is healthy.
Among the 258 families tested, we have found 9 families in which one of the healthy parents carries on the haplotype not inherited to the index patient two 21OHB genes, one with the mutation in codon 318 (318A) and one with a normal codon 318 (318B). In five cases we found the haplotype HLA-B*50-DRB1*07-21OHB318A-21OHB318B, in one case HLA-B*50-DRB1*X-21OHB318A-21OHB318B.
Among the index patients we have identified 19 patients with the 318 Stop mutation, but there was not a single haplotype which also carried the normal 318B sequence. 5/21haplotypes are B*50 positive, 2 of them B*50-DRB1*07. 8/21 haplotypes are DRB1*07 positive.
In our healthy unrelated control panel 6 individuals carry a 318 stop mutation, at least three with an additional normal 318B on the same haplotype. All six individuals are HLA-B*50 positive, three of them HLA-B*50-DR7.
We conclude that there are two different haplotypes 1)one carries two 21OHB genes, one with a 318stop mutation, the other with a normal.318B sequence so that the enzyme activity is not reduced. Here a fairly strong linkage disequilibrium exists with HLA-B*50 and DRB1*07 and 2)one haplotype with one 21OHB gene carrying a 318stop mutation, leading to a lack of enzyme activity. This haplotype is also associated with B*50 and DRB1*07.

Abstract session III Organ transplantation

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THE CLINICAL RELEVANCE OF HISTORICAL CROSSMATCHES IN CADAVERIC RENAL TRANSPLANTATION.

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In some transplantation centers graft survival of sensitised (≥ 50% PRA; panel reactive antibodies) and not sensitised (<10% PRA) patients is similar whereas in other centers sensitised patients have a significantly poorer prognosis. In order to define the reasons for these differences, we performed an analysis on the Eurotransplant database. From this database we analysed the transplantation results of first unrelated cadaveric kidney transplants performed in 24 centers between 1982 and 2000. We compared two groups of patients: Group I, 1208 transplants in patients with ≥ 50% PRA, and Group II, 28037 transplants in patients with <10% PRA. The differences in the 2 years graft survival between these groups were plotted per individual laboratory serving the centers. The difference (% graft survival of Group II - % graft survival in Group I) ranged between –7.6% to 31.0%. Thereafter we included in the analysis the information on the policy of the individual centers. Five centers included the results of historical crossmatches (HXM) in their decision making process while 11 only performed crossmatches with current sera (CXM). The policy of 8 centers was not available. The graft survival in the two groups were as follows: For the HXM centers a only a 2% difference was observed between sensitised and not sensitised patients in contrast to a 12% difference in the CXM centers. Furthermore, in the HXM centers 6% of all transplants concerned sensitised patients in contrast to only 3% in the CXM centers. These results clearly indicate that historical crossmatches are relevant in cadaveric renal transplantation.

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RELEVANCE OF HISTORICAL DONOR SPECIFIC HLA ANTIBODIES FOR RENAL TRANSPLANTATION

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The presence of donor specific HLA antibodies in the serum of transplant recipients at the time of transplantation is associated with hyperacute rejection (HR). The rate of HR has decreased significantly by the introduction of crossmatches (XM). The need to consider historical sera in the XM is still matter of discussion. Several transplantation centers are performing XM's with current sera only and discard the historical sera. We addressed the question on the relevance of historical sensitisation by selecting from the Eurotransplant data base all first, unrelated transplants with at least one HLA-A, B mismatch performed between 1973 to 2000 in which the patient's center ever reported an HLA sensitisation towards the HLA antigens of the donor (N=283). The serological XM with the serum of the patient at time of transplantation was in every case negative. The results were compared to those of a control group of all other first, unrelated transplants (N=36,403) of the same period excluding the zero HLA mismatched transplants. In an univariate analysis we observed that graft survival at 5 years was 36% in the group with historical donor HLA specific antibodies compared to the 62% for the other patients ($p<0.0001$). The results of a multivariate analysis considering all transplantation relevant parameters showed that the highest relative risk (R.R.) turned to be the presence of historical anti donor HLA specific antibodies (R.R. = 1.815). These results make it imperative that historical sensitisation should be taken into account for donor selection even if the current XM is negative. We conclude that in view of the scarcity of organs it is unethical to transplant in the face of historical donor specific HLA sensitisation.

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THE ACCEPTABLE MISMATCH PROGRAM IS AN EXCELLENT TOOL TO TRANSPLANT HIGHLY SENSITISED PATIENTS ON THE RENAL WAITING LIST

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Highly sensitised patients (HSP) are difficult to transplant and TEND TO accumulate on the waiting list. HSP have current (CUR) and/or historical (HIS) HLA alloantibodies with panel reactive antibody values (PRA) of ≥ 85%. Within Eurotransplant, HSP are offered the possibility to enter the Acceptable Mismatch (AM) Program. The principle of this program is that a negative crossmatch (XM) is predicted on the basis of the compatibility of the donor HLA-A,B,DR antigens with the patients HLA antigens in combination the AM. AM are HLA antigens towards which the patient never has formed antibodies. HIS and CUR sensitisation is considered equally important. AM are defined by analysis of the HLA typing of panel donors with negative screening reactions, which is only possible when the PRA is less than 100%. Additional AM are defined by performing XM with blood donors with one HLA mismatch to the patient's phenotype. In case a patient is selected via the AM Program, the exchange is mandatory. To date > 550 HSP entered the program and >400 have been transplanted. 60% of the AM patients received the offer within 21 months on the AM waiting list, compared to 18% if patients are not included in a special program. Since 1995, 152 HSP were transplanted. The 2 years overall graft survival was 84%, identical to not sensitised patients. No difference in graft survival was observed between HSP with CUR or HIS sensitisation only. Matching seems not to play a role. In conclusion, introduction of the AM program has shortened waiting of HSP and results in a similar graft survival as observed in non sensitised patients.

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VALIDATION OF THE HLA MATCHMAKER CONCEPT.

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HLA Matchmaker is a molecularly based algorithm to identify acceptable mismatches for highly sensitized patients. The algorithm is based on the concept that immunogenic epitopes are represented by amino acid triplets on those parts of the HLA molecules, that are accessible to alloantibodies.The principle of the program is that a patient does not make antibodies to self triplets. By both intralocus and interlocus comparisons of triplets present on the patient own HLA antigens and a mismatched HLA antigen it can predict the immunogenicity of that mismatch. If no triplet mismatches are present, the patient is supposed not to make alloantibodies.
We have validated this theoretical concept by checking the programs predictions with our extensive screening and crossmatch tests in highly sensitized patients. In 18 out of 18 HLA-A and 54 out of 54 HLA-B mismatches with zero triplet mismatches, the crossmatches were negative confirming the concept. Even more useful was the fact that HLA allo-antigens with only 1 triplet mismatch are hardly immunogenic: 25 out 25 HLA-A and 131out of 133 HLA-B mismatches resulted in a negative crossmatch.
Finally, we found a direct correlation between the number of triplet mismatches of a kidney donor and the incidence of HLA alloantibodies after transplant rejection. We conclude that the HLA Matchmaker concept is an excellent tool for the determination of acceptable mismatches in highly sensitized patients.

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PATTERNS AND OUTCOMES FOR MAINTENANCE IMMUNOSUPPRESSION IN THE UNOS RENAL TRANSPLANT REGISTRY

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The impact of changes in immunosuppressive and HLA matching is compared in a multivariate model. Of the 101,913 cadaveric renal allograft recipients transplanted between '91-'99, 26% received only Neoral (N) as a primary agent, and 12% Prograf (P). In '91, 4% of recipients received N or P; in '95 21% received N, 10% P. From '96-99 40% received N while P increased from 13-33%. Patients receiving P more likely were diabetic (34 vs. 26%), had PRA >50% (14 vs. 8%), 5-6 HLA mismatches (27 vs. 22%) and a re- (19 vs. 9%), or multi-organ transplant (17 vs. 5%) than those receiving N (all $p<0.001$). The incidence of rejection decreased from 50% to 10% from '91-99. Between '91-'94, 90% received azathioprine (A). Patients receiving Cellcept (C) increased from 11 in '95 to 77% in '99. From '96-99, Patients receiving C more likely had PRA >50% (11 vs. 7%), 5-6 HLA MM (24 vs. 21%), had a re- (13 vs. 9%) or multi-organ transplant (10 vs. 7%) (all $p<0.001$) than those receiving A.
The incidence of rejection was 16 for N vs. 20% for P, and 21 for A vs. 17% for C (all $p<0.001$). The 3-year graft loss rate was 11% for both N and P, and 18 for A vs. 10% for C ($p<0.001$). Logistic regression, adjusted for 12 covariates, indicate N had 34% lower risk of rejection vs. P but risk for C and A were similar. Risk of graft loss were similar for N and P but was 61% greater for A vs. C. After adjusting for immunosuppression protocol, patients with 5-6 MM had 90% increased risk of rejection and 53% increased risk of graft loss than those receiving a 0 MM graft.
Although new immunosuppressive protocols have resulted in a marked decrease in rejection rates, the risk of rejection and loss in HLA MM grafts remains high.

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THE CONSEQUENCES OF NON-CONSENSUS

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An unusual pattern of antibody specificity has been observed in four individuals who have rejected renal transplants. The HLA types of the patients are A1,B8,60DR7; A26,32,B14,DR7; A2,B57,62,DR7 and A1,29 B44,DR7 respectively. In all cases screening for HLA class II antibodies was performed using an ELISA kit, either GTI QUICK-ID or PRA-STAT. The results of these indicated that the only negative wells were those containing antigens derived from DR7 homozygous cells. Specificity for HLA DR1, 103, 15, 16, 17, 18, 4, 11, 12, 13, 14, 8, 9 and 10 was assigned in each case. These results suggest that some DR7 homozygous patients can produce antibodies which will react with all other class II antigens.
Analysis of the amino acid sequences of class II alleles shows that the DRB1*07 alleles possess glutamine at position 25 whereas all other class II alleles, except DRB1*1426 and DRB4*, have arginine at this position. This indicates that there is one residue which would be recognised as foreign in a DR7 DR53 homozygous individual which is common to virtually all other alleles. Individuals homozygous for DRB1*1426, or heterozygous for DRB1*1426,07, may not react in a similar manner due to the presence of arginine at position 25 of the DRB3* allele. Arginine at position 25 has previously been identified as the postulated antibody-binding site for monoclonal antibodies.
These results identify the possible epitope for an antibody produced by a number of DR7 homozygous individuals following renal allograft failure which results in very high levels of HLA sensitisation. This emphasises the importance of adopting policies which prioritise the allocation of DR homozygous donors to DR homozygous patients in renal transplantation, particularly in the case of DR7.

1. Marsh & Bodmer. Immunology Today 1989; 10:305

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RESULTS OF THE EUROTRANSPLANT QUANTITATIVE FLOW CYTOMETRY CROSSMATCH WORKSHOP 2001

G. Bein, D. Barz, M. Bengsston, M. de Bruyere, H. Grosse-Wilde, T. Horsburgh, W.R. Mayr, M. Müller-Steinhardt, E. Naumova, H. Otten, C. Schönemann, C. Stavropoulos-Giokas, J. Steinmann, C. Suberbielle-Boissel, Y. van Berkel, P.M. van den Berg-Loonen, R. Wassmuth, and I.I.N. Doxiadis (Institute of Clinical Immunology and Transfusion Medicine, Giessen, Germany, and Dept. of Immunohematology, LUMC, Leiden, Netherlands)

Previous Flow Cytometry Crossmatch (FCXM) Workshops revealed a low reproducibility of the FCXM techniques in the different centers. This workshop asked whether the use of calibration standards and standardized protocols results in reproducible quantitative detection of bound IgG on the same test cell. All 18 participating European centers received a complete set of reagents, control sera, test serum (anti-HLA-A2) as well as the same blood sample (HLA-A2,A2). Incubation of PBMC with control and test sera followed a detailed protocol. A monoclonal anti-human IgG PE reagent with standardized molar ratio between antibody molecules and PE molecules was employed for staining of cell bound anti-HLA antibodies. An anti-CD3 PerCP-Cy5.5 conjugate was used for gating of T cells. The number of bound IgG molecules on donor CD3+ cells was determined by converting the PE fluorescence intensity into the corresponding number of binding sites per T cell based on a calibrated bead standard curve (molecules of equivalent PE, MEPE). Eight laboratories entered the final analysis. The ratio between MEPE values of positive and negative control samples ranged between 46 and 348. Irrespective of the variation of MEPE values between different laboratories, this high signal to noise ratio is a good starting point for further optimization of quantitative and standardized FCXM.

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THE IMPORTANCE OF HLA-DR ANTIGENS AND MACROPHAGE INFILTRATION IN RENAL ALLOGRAFT BIOPSIES

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In normal kidney HLA-DR antigens are demonstrated on vascular endothelium, dendritic cells and mesangial cells but studies with HLA-DR antigens did not reveal DR antigen on renal tubular cells. To clarify the HLA-DR expression on renal tubular cells in renal allografts, we examined 90 patients with renal transplantation among whom 40 were in acute rejection (AR), 33 in chronic rejection (CR) and 10 in suspicious rejection (SR). Seven of 90 biopsy specimens showed no evidence of rejection. Sections were immunostained by the avidin biotin complex method with monoclonal antibodies specific for HLA-DR, LCA, CD3 and CD68. In addition to HLA-DR expression of tubular cells we studied the relationship between the interstitial lymphocyte, macrophage infiltration and expression of DR on the tubule epithelium and interstitium.
HLA-DR expression was marked in 35/40 cases of AR (87.5%), 22/33 cases of CR (66.6%), and 6/10 cases of SR (60%). The intensity of the lymphocyte and macrophage infiltration correlated well with the tubular and interstitial DR expression ($p<0.01$). Interstitial DR expression showed marked intensity around the lymphoid aggregates and perivascular macrophages. These findings suggest positive linear correlation ($p<0.01$) between HLA-DR expression and the degree of lymphocyte and macrophage infiltration.
These findings are consisted with the hypothesis that expression of HLA-DR antigens on renal tubular cells may be induced by the infiltrating macrophages and lymphocytes or be a consequence of tubular regeneration and remodelling following both phases of rejection or ischaemic damage due to chronic allograft nephropathy.

Abstract session IV Evolution. NK cells and receptors

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THE INFLUENCE OF SIMVASTATIN IN THE TREATMENT OF HIGHLY SENSITIZED DIALYSIS PATIENTS: THE LONG-TERM PRE AND POST-RENAL TRANSPLANTATION OUTCOMES

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One-year graft survival rates of sensitized kidney recipients have been shown to be lower than non-sensitized patients. We have recently shown that simvastatin had a significant immunosuppressive effect on panel reactive antibody (PRA) and/or crossmatch positivity. In this study, we present the long-term pre and post-transplantation outcomes of simvastatin in the treatment of these highly sensitized dialysis patients.
Twenty-eight patients (13F, 15M; age 32±9.6 years) were followed for a mean period of 22.7±6.1 months (range 5-31). The PRA and flow cytometric measurements were tested double blind and were performed at 3 to 6-months intervals. Simvastatin was administered with a beginning dose of 10-mg/day P.O. and the dose was increased monthly to 20-mg/day and to 40-mg/day in the nonresponder patients. Four patients drown out because of the noncompliance to the study and the mean PRA levels of these patients increased significantly after the cessation of simvastatin ($p<0.03$). Ten patients underwent successful kidney transplantation (8 living-related, 2 cadaveric). Simvastatin therapy with 10-mg/day doses was continued in these patients. The mean follow-up period was 16.1±8.2 months (range 3-28) after transplantation without hyperacute or acute rejection episodes and graft loss. In the remaining 14 patients on dialysis during follow-up, the mean PRA levels and/or the mean B and T cell positivity decreased significantly with regular simvastatin therapy ($p<0.01$).
In conclusion, our results indicate that the long-term continuous simvastatin therapy is effective in the treatment of highly sensitized dialysis patients. Meanwhile it has beneficial effect on 1-year graft survival in sensitized renal transplantation group.

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THE EXTREME COMPLEXITY OF THE MHC CLASS I REGION IN RHESUS MACAQUES.

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The rhesus macaque (*Macaca mulatta*) is an important animal model in studies on chronic and infectious diseases. For the use of these animals in immune-related diseases characterization of their MHC (*MhcMamu*) is necessary. We have sequenced MHC class I genes in a group of pedigreed and serologically typed rhesus macaques. Some of these animals were offspring of consanguinous mating. In the rhesus macaques more classical class I genes were detected than expected based on observations in the human population. Up to four different *A*-like alleles and various *B*-like alleles were detected per rhesus monkey. Alleles of non-classical class I genes were detected also. Based on these results we postulate that each haplotype contains at least one polymorphic *A* gene and one oligomorphic *A*-like gene. In addition at least three polymorphic *B* loci and two *B*-like oligomorphic loci, of which one was designated *Mamu-I*, are present per haploptype. Furthermore, the rhesus macaque possess the equivalents of the non-classical class I genes: *HLA-E, F* and *G*. Equivalents of *HLA-C* have never been found in the rhesus macaque. Sequencing is performed on amplificated cDNA, which indicate that all genes mentioned above are expressed. These studies show that the MHC class I region of the rhesus macaque is more complex than in humans. The *A* and *B* loci seem to be duplicated during evolution, leading to new polymorphic and oligomorphic loci within the class I region. As already described for the *DRB* region, the rhesus macaque uses an alternative evolutionary strategy to obtain complexity /polymorphism also in the class I region as compared to humans.

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BINDING OF ESCHERICHIA COLI ADHESIN AFAE TO CD55 TRIGGERS CELL-SURFACE EXPRESSION OF THE MHC CLASS I RELATED MOLECULE MICA.

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MICA are distant homologs of MHC class I molecules expressed in the normal intestinal epithelium. They are ligands of the NKG2D activating receptor expressed on most γ/δT cells, CD8+ α/βT cells and natural killer cells and therefore play a critical role in innate immune responses. We investigated MICA cell surface expression upon infection of epithelial cell lines by enteric bacteria and show here that MICA expression can be markedly increased by bacteria of the diffusely adherent *Escherichia coli* diarrheagenic group. This effect is mediated by the specific interaction between bacterial AfaE adhesin and its cellular receptor, CD55 or decay accelerating factor. It is extremely rapid after AfaE binding, consistent with a stress-induced signal. MICA induction on epithelial cells triggered IFN-γ release by the NKG2D expressing natural killer cell line NKL. This novel host-bacteria interaction pathway could play a role in the pathogenesis of inflammatory bowel disease, a condition which implicates a bacterial trigger in genetically susceptible individuals. This was supported by the increased MICA expression at the surface of epithelial cells in colonic biopsies from Crohn's disease affected patients compared to controls.

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NKG2D AND MICA EXPRESSION IN THE THYMUS

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MICA is an activating ligand for the NKG2D receptor expressed at the surface of NK cells, γδ T cells and CD8+ αβ T cells. MICA expression is observed at the surface of gut epithelial cells, but is upregulated in different types of tumors and in the context of intra-cellular infection. In CD8 T cells and γδ cells, NKG2D/MICA engagement delivers a costimulatory signal that complements TCR-mediated antigen recognition. Although MICA expression has been reported on thymic epithelial cells, nothing is known concerning the stage of acquisition of the NKG2D receptor on thymocytes. Thymocytes were freshly isolated from 5 normal thymi removed during cardiac surgery in children, and were analyzed by FACS using anti-CD3, CD8, C4, and MICA tetramers: NKG2D was absent from double-negative and double-positive thymocytes, but was expressed on 15% of CD4-CD8+ thymocytes, compared to 98% CD8+ peripheral lymphocytes and 95% CD8+ cord blood cells. In addition, MICA and NKG2D expression were analyzed by two-color immunofluorescence on frozen sections of normal or pathologic thymi using MICA monoclonal antibodies and MICA tetramers. In normal infant thymus, MICA molecules were expressed on Hassal's corpuscules. By contrast, MICA expression was observed on all epithelial cells in thymic hyperplasia, and was dramatically increased in thymoma. NKG2D+ CD8+ thymocytes were preferentially observed in the thymic medulla, in accordance with the late acquisition of NKG2D on CD4-CD8+ cells. Sorting NKG2D-negative cells will allow us to define whether cytokines or epithelial factors may induce NKG2D expression at the surface of thymocytes.

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ROLE OF NKG2D/MICA INTERACTION IN THE PATHOGENESIS OF CELIAC DISEASE.

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The NKG2D receptor is expressed at the surface of all circulating NK cells, γδ T cells and αβ CD8 T cells, indicating that it acts on both the innate and adaptive arms of the immune response. NKG2D recognizes ligands such as the stress-inducible MICA and MICB molecules, that have been associated with malignant transformation or intracellular infection. NKG2D-MICA interaction delivers a costimulatory signal that can complement TCR-mediated recognition of epithelial target cells. To determine whether MICA over-expression on gut epithelial cells might be involved in their destruction by tissue effector T cells during the course of celiac disease, NKG2D-MICA interaction was analyzed using MICA tetramers and anti-MICA monoclonal antibodies. Intestinal epithelial cells and intra-epithelial lymphocytes (IEL) were freshly isolated from small intestinal biopsies obtained from 6 patients with active celiac disease and 4 subjects without inflammatory disease. Staining with MICA tetramers showed that 65-70% of IEL expressed NKG2D, compared to 100% of circulating CD8+ T cells. Expression of MICA at the membrane of epithelial cells was increased (15-50%) in CD sections compared to normal intestine (<10%). Immuno-histochemistry on frozen tissue sections confirmed our FACS analysis. Furthermore, CD3-negative IELs overexpressed NKG2D following in vitro exposure to IL-15, and were able to lyse the MICA+ Hela cell line in a NK-like way. Cytotoxicity was partly inhibited by anti-MICA antibody, and was fully abrogated by blocking the NKGD2 receptor. Therefore, it appears that NKG2D/MICA engagement may well play a crucial role in the pathogenesis of celiac disease.

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THE HUMAN CD38 AND CD16 RECEPTORS ARE FUNCTIONALLY DEPENDENT AND PHYSICALLY ASSOCIATED IN NATURAL KILLER CELLS

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CD38, a monomorphic lineage unrestricted surface glycoprotein, is an ecto-enzyme (ADP ribosyl cyclase / cADPR hydrolase) regulating cytoplasmic Ca^{2+}. The molecule performs also as a receptor, modulating cell-cell interactions and delivering trans-membrane signals, despite a structural ineptitude to the scope. CD38 ligation by agonistic mAbs induces signals leading to the activation of the lytic machinery of adult NK cells: similar signals could not be reproduced in YT and NKL, two $CD16^-$ human NK-like lines.
The working hypothesis is that CD38 establishes a functional cooperation with professional signaling molecules of the NK cell surface. The present work answers the question on the molecule exploited by CD38 for signaling in NK cells, using as model $CD16^-$ NK lines genetically corrected for CD16 expression. The results indicate that a functional CD16 molecule is a necessary and sufficient requisite for CD38 to control an activation pathway, which includes i) Ca^{2+} fluxes, ii) tyrosine phosphorylation of ZAP70 and the MAPK; iii) secretion of IFN-γ and v) cytotoxic responses. FRET and co-capping experiments highlight also a surface proximity between CD38 and CD16.
These results were confirmed using the NKL cell line, where $CD16^+$ and $CD16^-$ variants were obtained without genetic manipulations.
The results obtained concur to portray CD38 as a unique kind of receptor molecule, which is unable by itself to signal but whose receptorial function is rescued by functional and physical associations with a professional signaling structure, which varies according to lineage and environment. This molecule is CD16 in NK cells.

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DIFFERENTIAL HIGH AND LOW DOSE EFFECT OF m-HLA-G ON T AND NK CELLS

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Recent studies on HLA-G reveal in vitro functions extending beyond that of classical class I molecules. Both membrane bound and soluble HLA-G appear potent immuno-modulatory molecules, affecting not only classical NK cell responses, but also determining the faith of activated T cell populations through apoptosis, inhibition of allo-induced cellular immunity and the maturation of antigen presenting cells.
Already, we observed inconsistent outcomes in the effect of membrane bound HLA-G (mHLA-G) on a class II mismatch induced alloresponse. We hypothesised that the effect of HLA-G depends on the number of HLA-G ligand interactions.
By co-culturing HLA-G transfectant cells with peripheral blood mononuclear cells (PBMC) we show that high doses of mHLA-G ($5.0\text{-}10.0\times10^4$ cells) inhibit the proliferation, IFN-γ and TNFα production of PBMC, whereas in contrast low doses of mHLA-G ($1.25\text{-}2.5\times10^4$ cells) stimulate these functions in a mixed cell population.
Enrichment of either T or NK cells, as well as CFSE labelling studies reveal that mHLA-G has in fact distinct effects on T and NK cell function. Whereas T cells are inhibited, NK cells are stimulated.
Our findings imply a finely tuned modulatory effect of mHLA-G on T and NK cell functions revealing a mechanism for modulating a respons of allo/auto-reactive T cells.

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LRC HAPLOTYPE SEGREGATION AMONG HLA MATCHED SIBLING BMT FAMILIES: 2 HAPLOTYPES MATCHING PROMOTES aGvH REACTION.

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Leucocyte Receptor Cluster (LRC) is a genetic region (19q13.4) encoding KIR and ILT receptors . The products of these genes are involved in target-cell recognition by NK cells. Variability in the number and in the sequences of individual KIR genes has been found. The role of KIR polymorphism in Bone Marrow Transplantation (BMT) is controversial.The aim of the present study was to analyse segregation of parental LRC haplotypes and investigate wether the degree of LRC haplotype compatibility influences the NK cell recovery and aGvHD occurence after HLA matched BMT.
Four STR (Short Tandem Repeats) markers were selected to reach closest proximity to KIR genes and highest heterozygosity: D19S418, D19S887, D19S926, D19S210. The STR markers were amplified by PCR using fluoro-labelled primers and run in denaturating gel on PE ABI-Prism 377.The recovery of NK cells 30-100 days post-transplant was followed by flow cytometry.
On the basis of STR polymorphism we divided 49 recipient-donor pairs into 3 groups. 13 pairs were matched for both, 23 for one and 13 for none of the parental LRC haplotypes. Segregation of LRC (chr.19q13.4) was independent of MHC haplotypes. The fractions of aGvHD>1 cases in groups matched for 2, 1 and 0 haplotypes were: 58%, n=12; 21%, n=23 and 15%, n=13; respectively. Matching for both LRC haplotypes resulted in significantly higher risk of developing aGvHD>1 than for one- (p=0.04) or none (p=0.02). Grading of aGvHD also showed significant differences in 2 haplotypes matched *vs* mismatched group (p=0.03). Higher severity of aGvHD in 2 haplotypes matched group was accompanied by the appearance of CD56+ CD16- lymphocytes. CD56/CD16 ratios were significantly different from hypothetical value=1 (p=0.01) in this patients and significantly higher (1.35 ± 0.15 N=8) than in the mismatched group (0.94 ± 0.06 N=9) (p=0.005). CD56/CD3 ratios in respective groups were 1.34 ± 0.37 N=9 and 0.63 ± 0.15 N=11.
Recipients sharing with their donors both haplotypes of LRC had higher risk of developing grade 2-4 aGvHD, accompanied by higher CD56/CD16 and CD56/CD3 ratios. This suggests involvement of NK cells and KIR genes polymorphism in the pathogenesis of aGvHD.

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KIR and HLA-Cw genotyping in related and unrelated recipient/donor pairs for stem cell transplantation

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Killer cell immunoglobulin-like receptors (KIR) are glycoproteins expressed on natural killer (NK) cells and some T cells. KIRS specifically recognise certain HLA class I molecules and upon binding either inhibit or stimulate the cytotoxic activity and/or cytokine production of the effector cell. Although it has become evident that different KIR haplotypes exist, their distribution in the population is not clear yet. Also the functional relevance of differences in KIRS between recipient and donors in allogeneic stem cell transplantation (SCT) remains unknown.

In the current study KIR genotypes were determined in (multigeneration) families of patients waiting for an allogeneic SCT as well as leukemic patients and their potential voluntary unrelated donors (VUD). We implemented a PCR-SSP typing protocol for KIR typing according to Uhrberg et al. with minor modifications, focussing on KIRS that interact with HLA-Cw (i.e. KIR 2DL1, 2DL2, 2DL3, 2DS1 and 2DS2). In addition to routine HLA-typing (identification of haplotypes in related patient/donor pairs and high resolution typing in unrelated P/D pairs), all subjects were typed at intermediate resolution for HLA-Cw.

Within families differences existed in the number of KIRS present in a person, varying from two up to all five HLA-Cw specific KIRS. There was no correlation between HLA-Cw genotype and KIR genotype. In families with multiple HLA identical sibs differences existed in KIR genotypes whereas HLA-non-identical sibs on occasion were matched for KIRS with the patient. Obviously, in unrelated combinations the differences were even more outspoken with differences in KIRS and HLA between patient and donors.

Matching for HLA does not guarantee KIR matching. Since KIR genotype will affect the allorepertoire of NK cells, differences in KIRS may affect the alloresponse after SCT. Whether matching or mismatching for KIRS is either beneficial (graft-versus-leukemia) or harmfull (graft-versus-host disease) for transplant outcome is currently under investigation.

Uhrberg M et al. Human diversity in killer cell inhibitory receptor genes. Immunity 1997:7:753-763.

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Assessment of Killer Cell Immunoglobulin-like Receptor (KIR) expression and corresponding HLA-class I phenotypes demonstrates heterogenous KIR expression independent of anticipated HLA-class I ligands.

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Killer cell immunglobulin like receptors (KIR's) on NK cells are highly polymorphic and are clonally distributed on NK cell populations within individuals. The KIR´s are independently segregated from their ligands, the HLA-class I alleles. However, mechanisms regulating the expression of matching KIR´s for the individual HLA-class I phenotype are not well understood. We have analyzed 99 healthy blood donors for 12 different thus far known KIR´s, using sequence specific primers. NK cells were isolated using Rosettesep (Cellsystems, D) and analyzed for KIR expression using flow cytometry and mAb specific for KIR 150a/b, KIR 158a/b and KIR70, respectively. Our results demonstrate that KIR genes are transmitted very heterogeneous. We found two patterns of KIR-genotypes to be predominant in the caucasian population, type A (49,5% with four subtypes) and type B (50,5% with 18 subtypes). A linkage disequilibrium exists for several gene combinations. KIR expression studies of groups exhibiting different HLA-Class I phenotypes demonstrates that four distinct populations exhibiting different densities of CD158a and/or CD158b positive NK cells coexist in all individuals. A correlation between KIR expression and currently known ligands was not observed. In conclusion, KIR genes are segregated independently, with two major groups of individuals, representing KIR genotype A and genotype B individuals respectively. Moreover, at least one gene for an inhibiting and one gene for an activating KIR was present in all cases. The surface expression of KIR´s in particular individuals with different HLA-Class I phenotypes indicate that others than the discribed HLA-Class I phenotypes might serve as ligands for KIR's.

Abstract session V Bone marrow transplantation and registries

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NATURAL KILLER CELL RECEPTOR GENOTYPES FAVOURING IMMUNE-ESCAPE ASSOCIATED WITH LEUKEMIA.

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Natural killer (NK) activity is regulated by a delicate balance between inhibitory and activatory receptors. The ligands for these receptors are distinct HLA class I molecules on target cells. Disruption of the dominant inhibitory signal by lowered expression of HLA class I on the target will trigger cytotoxicity mediated by activating receptors with subsequent destruction of the target cell. Tumor cells frequently show altered HLA class I expression and should become targets of NK cells. Because this doesn't happen, it was the aim of this study to investigate the possibility that leukemic patients could have an NK cell receptor (NKR) genotype in favour of escape from NK lysis. Ninety-eight leukemic patients (40 CLL-B, 30 AML, 20 CML and 8 ALL-B) and 51 normal, healthy Belgian controls were molecular typed for 13 different NK receptors: 2DL1-3, 3DL1-2, 2DS1-5, 3DS1 and CD94/NKG2a/c. In our control population the individual NKR's frequencies and AA, AB and BB genotype frequencies were comparable to those from other caucasoid populations. The following observations could be made in our leukemic population. First, an increased frequency of the more inhibitory AB genotype was detected in all leukemic groups. Second, the number of unusual (aberrant) genotypes was high: 25/98 (>25%). Third, although the diversity in NKR genotypes in the general population, is high, some types are clearly increased in leukemia: together the AB5 and AB9 NKR genotypes accounted for 15% of all our leukemic patiens. Interestingly, both types display all inhibitory KIR's. These results suggest that a substantial number of leukemic patients possess an NKR genotype with major inhibitory capacities, that is not able to destroy leukemic cells with altered HLA class I. These findings could also have major implications in understanding the graft versus leukemia effect (or absence) in allogeneic stem cell transplantation.

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IMPACT OF HIGH RESOLUTION HLA-A,B,C DONOR/ RECIPIENT MATCHING ON CLINICAL OUTCOME OF HEMATOPOIETIC STEM CELL TRANSPLANTATION (HSCT) FROM UNRELATED DONORS.®

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At our institution unrelated donors for HSCT are routinely matched by DNA-based PCR-SSP low resolution ("two digit level") HLA-A,B,C and high resolution ("four digit level") HLA-DRB1,DQB1-typing with the respective patients. To answer the question, whether HSCT outcome might be improved by high resolution HLA-A,B,C, i.e. sequence based typing (SBT), a retrospective single center study was performed. Enrolled were 145 adult unrelated transplants for early (N=87) and advanced (N=58) hematological malignancies. In all study patients the conditioning regimen used and the protocol for prophylaxis of graft-versus-host disease (GvHD) were identical and all enrolled donor/recipient pairs were fully HLA-matched according to the routine HLA-typing procedure outlined above. For the purpose of this study, HLA-A,B,C SBT was performed in all donor/recipient pairs and revealed only 39 (26,9 %) of the 145 transplants to be HLA-identical at the allelic level. In contrast, 39 %, 22 %, 10 %, and 3 % of the transplants exhibited 1, 2, 3 or even 4 HLA-A,B,C allele mismatches respectively. Uni- and multivariate statistical analysis showed overall survival after HSCT to be comparable from HLA-A,B,C-identical (N = 39) and partially HLA-mismatched donors exhibiting 1 through 4 HLA-class I allele disparities (N = 106). The cumulative risk of clinically relevant GvHD was 42 ± 11 % in the HLA-identical and 49 ± 6 % in the HLA class I allele-mismatched subgroup (p>0,05). The cumulative risk of graft lost after transplants from fully HLA-matched and HLA class I allele mismatched donors was 0 % (0/41) and (5/ 104), respectively (p>0,05). Thus, if present at all, the beneficial effect of prospective HLA-A,B,C SBT typing for donor selection seems to be modest, since it could not even be demonstrated in our retrospective approach enrolling 145 well selected transplants.

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DRB-DQB1 DIVERSITY IN THE ANALYSIS OF 1930 DONORS TYPED BY SEQUENCE BASED TYPING (SBT).

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Allelic and haplotype diversity are the most remarkable characteristics of the HLA system. However, it can be argued that only a few alleles account for the majority of the gene counts in a population. We examined the distribution of DRB1/3/4/5 alleles of 1930 NMDP donors selected for International Searches. We performed DRB typing by SBT and DQB1 typing by SSOP hybridization. Only 59 DRB1 alleles were detected. The DRB1*04 group presented 12 alleles. Most of the DRB1 groups presented multiple alleles with exception of DRB1*07011, 09012 and 10011 that were the only subtypes of their groups. Only a few alleles were detected in less than 2 occasions (DRB1*0409, 0440,08042, 08044, 0807, 0811, 1115, 1118, 1311, 1407, 15022) We observed only 135 DRB1-DRB3/4/5-DQB1 haplotypes. Several DRB1 alleles presented had associations predominantly with a single DQB1 allele, however we observed exceptions for each common DRB1 allele. Some DRB1 alleles formed multiple haplotypes, for example DRB1*13011 was observed forming 8 distinct haplotypes. DRB1*04051 and 11012 formed haplotypes with 6 different DQB1 alleles each, and none was predominant.The evolutionary relationship between sequence-related alleles became even more apparent with the observation of identical associations with alleles at DRB3/4/5 and DQB1 loci (e.g. DRB1*15022 and DRB1*15021 have identical DRB5-DQB1 associations. Other relationships between low and high frequency alleles are manifested by identical associations. These data appears to indicate that some of the low frequency alleles may have a recent origin from mutations in the DRB1 gene of the most frequent haplotypes found in a population.

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ONLY ONE OF THREE PATIENTS QUALIFYING FOR AN UNRELATED STEM CELL TRANSPLANT RECEIVE ONE. WAYS AND MEANS TO IMPROVE THIS!

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Registries of unrelated stem cell donor volunteers have been in operation since the early 70's and have shown a spectacular growth since the late eighties. Worldwide almost 7.5 million stem cell donors are available, while in the year 2000 for over 17.000 patients a search was initiated on www.BMDW.org.

The annual report of the World Marrow Donor Association (WMDA) reports however that less than 5000 patients received a stem cell transplant from an unrelated donor. This appears to imply that less than 1/3 patients eligible for an unrelated stem cell transplant actually received one. This observation is in accord with figures which can be extracted from the annual reports of the EBMT transplant activities (A.Grathwohl et al 1991-1999).

A preliminary analysis indicates that the failure of 2/3 of the patients to reach transplantation is due to a number of causes such as: incomplete HLA typing of the donors, under usage of (mismatched) donors, cord bloods and members of the extended family and inability to cope with the complexity of HLA immunogenetics.

A proposal will be presented to improve the present situation. This is a matter of some urgency not only because more patients should and can be helped, but also because of the gigantic financial investments made in the registries and cord blood banks (over 3 billion EURO).

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ASSESSMENT OF CELLULAR PERMISSIVITY BETWEEN HLA- A*2402 AND HLA- A*2403 AT THE CLONAL LEVEL

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Detection of cellular permissivity should allow extension of donor registries and contribute to treat more patients. Therefore, in our department, limiting dilution assays are performed when a single HLA-antigen mismatch is found among donor/recipient pairs.

Methodology: Permissivity between HLA-A*2402 and HLA-A*2403 antigens was initially detected by the use of the cytolytic T lymphocyte precursor (CTLP) assay. Indeed, CTLPs were found negative in both directions among two different donor/recipient pairs expressing the HLA-A*2402/HLA-A*2403 Ag mismatch. To further assess this cellular permissivity, T cell clones specific for HLA-A*2403 Ag were generated. With this aim, peripheral blood mononuclear cells expressing HLA- A*2403 were used to stimulate HLA-A*2403 negative APCs sharing with them 5 out of 6 HLA class I antigens. Cytotoxicity was assessed by the ^{51}-chromium release assay.

Results: 4 T cell clones specific for HLA-A*2403 were obtained. Our results show that in addition to lysis of HLA-A*2403 expressing antigen presenting cells (APCs), these T cell clones were also able to lyse any APCs expressing HLA A*2402. Supporting the specificity of the permissivity, HLA A*2416 expressing APCs were not susceptible to lysis, neither nor APCs expressing full HLA class I and class II mismatched haplotypes.

Conclusion: Here, by generating T cell clones, we have demonstrated cellular permissivity between HLA-A*2402 and HLA-A*2403 antigens. The methodology we used should allow acceptance of this mismatch in bone marrow transplantation.

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UNRELATED HEMATOPOIETIC STEM CELL TRANSPLANTATION: HLA-C AND MINOR HISTOCOMPATIBILITY ANTIGEN DISPARITIES RESULT IN LOWER CTLp FREQUENCIES COMPARED TO HLA-A/B MISMATCHES

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The functional relevance of individual HLA class I mismatches in unrelated hemotopoietic stem cell transplantation (HSCT) is still controversial. The *in vitro* frequency analysis of the cytotoxic T-lymphocyte precursors (CTLpf) represents one approach to address the relative contribution of HLA-A, B, and -Cw mismatches in the alloresponse. We have evaluated 402 CTLp assays relative to the HLA class I compatibility status determined by high resolution DNA typing. Single HLA-Cw mismatches (n=61) correlated with lower CTLp frequencies (mean: 7 precursors/10^6), compared to single A/B mismatches (mean: 26 p/10^6) or to combined HLA-A/B or B/Cw incompatibilities (mean: 47 p/10^6). Of 19 patients that have been transplanted with an HLA-Cw-mismatched donor, 8 (42%) are still alive (follow-up: 1-10 years). Survival did not correlate with the degree of Cw-KIR epitope matching, nor with patient's age or time for donor identification. In 14/170 (8%) CTLpf-positive combinations, high resolution molecular typing, including sequencing of all class I loci, did not reveal an HLA-A/B/Cw/DRB1/B3/B5/DQB1-mismatch, indicating that the CTL activity found was directed against minor histocompatibility antigens (miHA). None of the two patients transplanted with a marrow from a weakly CTLpf-positive donor suffered from GVHD. Altogether these results suggest that HLA-C or miHA disparities may be of lower importance in unrelated HSCT.

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ALLORESPONSES TO HLA-DP DETECTED BY THE MEASURE OF HTLP FREQUENCY BEFORE BMT COULD PROVIDE INDICATIONS ON THE RISK OF DEVELOPMENT OF GVHD.

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HLA-DP is considered as a transplantation Ag by certain authors, due to the isolation of HLA-DP specific T cell clones post-bone marrow transplantation (BMT) into patients suffering from GVHD. But for others, statistical analyses did not favour HLA-DP mismatch as a cause of GVHD. In this study, by measuring helper T lymphocyte precursor frequency (HTLP) among blood donor pairs matched by high resolution typing for HLA class II molecules, albeit HLA-DP, we show that HLA-DP mismatch can give rise to high (superior to 1/50 000), intermediate (between 1/50 000 to 1/100 000) or negative (inferior to 1/100 000) frequencies of immunoreactive T cells, depending on the combination pair tested. Moreover, when we isolated T cells from a skin biopsy in a patient suffering from GVHD following BMT from an unrelated donor matched by high resolution typing for HLA-class I and class II molecules, except for HLA-DP at a single locus (0401 /1701), we found that these cells were specific for HLA-DP Ag. This was assessed by the use of mAb against HLA-DP and of several APCs expressing HLA-DP 1701 or not. Mixed lymphocyte reactions and HTLPs performed in this donor/recipient pair before transplantation showed that MLR was weak, i.e : R.R =10%, whereas HTLp frequency was high, i.e : 1/5492. Altogether, our results support the notion that a single HLA-DP mismatch can give rise to a strong reaction and allow us to suggest that the measure of HTLP frequencies could help in the selection of

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MULTICENTRIC ANALYSIS OF HEMATOPOIETIC CHIMERISM

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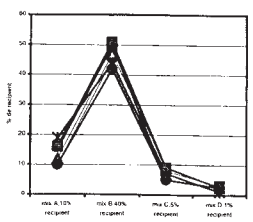
Analysis of hematopoietic chimerism has been emphasized in order to evaluate donor cell engraftment in allogeneic hematopoietic stem cell transplantation and to adapt immunosuppressive or cell therapy. Full donor or persistent mixed chimerism is essential for sustained engraftment and prevention of relapse. Precise analysis of chimerism requires comparison of methodological approaches and data reports. The French working group on hematopoietic chimerism was first constituted by 7 labs and performed comparison of the technical protocols and quality control exchange. Chimerism is analysed by competitive PCR with a panel of STRs in 5 laboratories followed by semi-quantification of individual cell populations is assessed using automatic DNA sequencer and Genescan® software (both from Applied Biosystems®). One laboratory uses nonradioactive hybridization of PCR-amplified VNTRs. Absolute chimerism quantification is performed by real-time PCR with Taqman probes (Applied Biosystems®) in 1 laboratory. Quality control exchange consisted in 4 mixes each containing a known ratio of recipient and donor's DNAs. As shown in figure, evaluation of chimerism was concordant between the labs with variation not exceeding 26%. These data stress the usefulness of quality control exchanges, especially including sorted cells, and standardization of the protocols.



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STRATEGY FOR MAPPING THE POTENTIAL OF CD33 AS MINOR HISTOCOMPATIBILITY GENE INVOLVED IN GRAFT VERSUS LEUKEMIA EFFECTS

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T cells recognising endogenous peptides presented by HLA class I molecules on the surface of hematopoietic progenitor cells are believed to play an important role in the graft-versus-leukemia effect after transplantation. Such alloantigens may be derived from leukemia-specific proteins or polymorphic self proteins which behave as minor histocompatibility antigens (mHags). To identify new mHags, which can be used to generate leukemia-specific T cell response we looked for genetic polymorphisms in the CD33 gene. CD33 is expressed by human monocytes, myeloid blasts, some acute undifferentiated leukemias, and occasionally by acute lymphoblastic leukemias. The alignment of cDNA sequences of CD33, obtained from original reports, indicated four polymorphic amino acids at positions I198M, L257V, S296R and N313K. In order to develop a simple PCR-SSP strategy we cloned and sequenced the non-coding regions (introns) of the whole gene. CD33 gene consists of 14,571 nucleotides. The predicted protein sequence of 364 amino acids has the typical features of a transmembrane protein. The gene could be divided into 7 exons and 6 introns. By sequence analysis we detected a new polymorphism at amino acid position G69R. To further examine whether the polymorphisms of CD33 could lead to the expression of peptides binding to HLA class I molecules we used the proteasomal cleavage prediction algorithm "PAProC" and HLA peptide binding analysis "SYFPEITHI". Nonapeptides from the polymorphism G69R were predicted to bind with high affinity to HLA-A*01 and HLA-B*2705. Peptides derived from I198M alleles were predicted to bind to HLA-A*0201 and HLA-B*2705. For peptides derived from polymorphic sites L257V, S296R and N313K high affinity to HLA-A*0301 molecules was determined. These binding predications are weaker than the binding of HA-1 peptide to HLA-A*0201, but may be sufficient for the in vitro induction of peptide-specific T cells.

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CONTINUED DEVELOPMENT OF THE IMGT/HLA DATABASE

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In 1998 the IMGT/HLA database was publicly released for the first time this provided a centralised repository for the sequences of the alleles named by the WHO Nomenclature Committee for Factors of the HLA System. Since this time the database has grown and is now the primary source of information for the study of sequences of the human major histocompatibilty complex.

The initial release of the database contained a limited number of tools. The latest releases demonstrate how the database has developed over the past two years. As a result of feedback from our users and developments in both bioinformatics and HLA we have been able to provide new tools and facilities. These have included the analysis of the ambiguous allele combinations as found in sequence based typing. The latest tools include online searches of the ambiguous typing combinations and polymorphism identification tools. The tools initially released with the database have also been updated to better suit the user's needs, for example allele searches can now be performed on both the allele name and component EMBL/GenBank/DDBJ entry's accession number.

The HLA sequences have also been extended to include intron sequences and the 3' and 5' untranslated regions in alignments and also the inclusion of new genes like MICA. On average an additional 100 new and confirmatory sequences are included in each quarterly releases of the database.

As well as the ongoing development of the IMGT/HLA database, we are collaborating with other research groups to develop parallel databases for other species.

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A NEW ALLELE FREQUENCY DATABASE

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There have been many publications on the frequency of HLA antigens in different populations. However these have not been centrally sourced and most investigators have had to rely on International Histocompatibility Workshop Reports when they have attempted to find the frequency of HLA antigens in a particular population. This problem has been further exaggerated by the need to know the HLA ALLELE frequency in a particular population.

In order to bring this data together a database has been created, www.AlleleFrequencies.net. This site enables the investigator to examine either the HLA frequencies in one population, or the frequency of one allele in several or all populations for which there is data. The latter can be performed if required by looking at the available populations in one country, one region or by ethinicity. In addition, examination can be made of all alleles at one locus or one or all allele family (i.e. 2 digit) groups. For those alleles for which no data has been submitted information will be provided on the population in which they were originally discovered.

Data can be added to the database directly via the website. Drop down boxes have been generated to ask the following questions of the population data being submitted; Country, Ethnic Origin, Region, Latitude and Longitude, Sample size, Urban or Rural, Whether grandparents or parents lived in same location, Contributor, Sample Date, Method used to determine allele, Reference (if published, but information does not have to be previously published). Data will then be reviewed before being available for subsequent retrieval searches.

Also included will be data from other polymorphic regions e.g. Cytokine, NK receptors, TAP, MICA etc. Direct links to IMG/HLA database, EFI, ASHI and BSHI websites will be available. The direct link to IMG/HLA database will enable new alleles to be automatically added to this database as "not tested" on each of the populations already entered. The success of this venture will depend on the whole HLA community submitting data. The site will be updated according to comments and ideas from the community. It is intended that summaries of new data added in the previous year would be published in a "HLA" journal.

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EVALUATION OF FREQUENT MITOCHONDRIAL DNA-POLYMORPHISMS IN 43 CELL LINES OF THE 12TH IHWS USING A NEW PCR-SSP CHIMERISM KIT

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Background: Molecular methods using mitochondrial DNA polymorphisms represent a future avenue for evaluating (micro-) chimerism after transplantation and evaluation of transfusions of homologous blood components. The advantage of such methods is that there is no need for additional labeling of cells. We developed a sequence-specific primer system enabling us to detect 20 frequent single nucleotide polymorphisms in the hypervariable regions (HVR1 and 2) of human mitochondrial DNA. We screened 43 cell lines for mtDNA-polymorphisms and the results were compared with a DNA-sequence analysis.
Study design and methods: MtDNA of 43 cell lines of the 12th IHWS was prepared (puregene extraction). For each MT-DNA polymorphism a primerpair was designed and reaction optimized. Each reaction volume contained a control Mt-DNA primerpair. PCR was performed in 10µl reaction volumes, using 30 pmol of extracted DNA. After PCR the detection was performed using EB-stained agarose gels and UV-illumination.
Results: With 20 primerpairs for frequent polymorphisms we were able to discriminate 40 % of the tested cell-lines. DNA-sequencing confirmed the specific single base polymorphisms targeted by SSP-PCR.
Conclusions: SSP-PCR analysis targeting HVR1 and HVR2 is a promising new method to potentially identify donor and recipient cells on the basis of mtDNA polymorphisms. The method can be optimized to quantify the amount of allogeneic cells.

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MICROCHIMERISM MONITORING OF ACUTE GRAFT VERSUS HOST DISEASE AFTER LIVER TRANSPLANTATION: REAL-TIME PCR BASED ON HLA-DRB1 MISMATCHES

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Here we report on a case of a liver allografted patient who developed an acute graft versus host disease (GVHD). After exclusion of other causes for isolated thrombocytopenia and maculo-papular skin rash of the face and trunk, the diagnosis of GVHD was clinically suggested. The diagnosis was confirmed firstly by immunophenotyping of lymphocytes in sections of the skin biopsy and secondly by PCR-SSP for HLA-A, B and DRB1. To evaluate the success of GVHD treatment a sensitive technique capable of analyzing quantitatively the donor microchimerism was required. Therefore, donor microchimerism was analyzed using real-time PCR (Sequence Detector 7700, Applied Biosystems). Allele-specific DRB1 primer pairs used in parallel amplified the donor (DRB1*11,16), the patient (DRB1*01,03) and the reference DNAs, permitting a relative quantification (%) of the extent of circulating donor lymphocytes. Thirty-one days after transplantation a maximum of 7.5% of donor lymphocytes was found in the peripheral blood and 16% in the bone marrow. After withdrawal of the immunosupressive therapy in order to force the immune system of the patient, a weekly decrease of the donor lymphocytes in the peripheral blood was observed (7,5%, 3,18%, 1.78%, 0.34%, 0%) allowing immunosupression to be continued. The quantitative results of donor microchimerisms correlated well with the severity of the disease. Thus, real-time PCR may be an effective tool to control the course of anti-GVHD therapy after solid organ transplantation.

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SEQUENCE-BASED TYPING OF THE HLA-B5/35 CROSS-REACTING GROUP INCLUDING EXONS 1, 4 AND 5

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The HLA-B5/35 cross-reacting group (CREG) is a complex set of antigens including HLA-B35, B51, B52, B53 and B78. The alleles encoding these molecules are closely related. In this study the HLA-B sequence-based typing (SBT) strategy designed by Voorter et al was tested for the HLA-B5/35 CREG[1]. In addition to the sequences of exons 2 and 3, the unknown nucleotide sequences of exons 1, 4 and 5 of several HLA-B*35, B*51 and B*52 alleles were elucidated by allele-specific SBT. Both forward and reversed sequencing of the exons was performed.
A total of 225 unrelated individuals were HLA-B typed by heterozygous sequencing of exons 2 and 3. In the B5/35 group 28 different alleles were identified, next to them a further 63 alleles were sequenced. The strategy was proven to be reliable and efficient for high-resolution HLA-B typing of the B5/35 CREG. The exons 1, 4 and 5 were determined for 55 individuals, 9 of them carried an allele for which these exon sequences were unknown. In addition, previously published sequences of exons 1, 4 and 5 were determined in at least 2 individuals for 19 alleles. This was done to validate the allele-specific SBT for these exons and to determine the level of polymorphism.
Three new alleles were identified. One allele showed a difference at position 200, which was previously considered a conserved position. The two other alleles showed exon 2 and 3 sequences identical with B*35011 and B*51011, but differences in exons 1 and 4, respectively. This shows again that more polymorphism might be present outside exons 2 and 3 then previously thought. [1] Voorter CEM, Vlies van der SA, Berg van den-Loonen EM. Sequence-based typing of HLA-B: the B7 cross-reacting group. *Tissue Antigens* 2000: 56: 356-62.

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COMPARISON OF INTRON 4 NUCLEOTIDE SEQUENCES BETWEEN HLA-B AND HLA-C ALLELES

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The sequence database of HLA class I genes focuses on the coding sequences, the exons. Little is known about the non-coding sequences of the different class I alleles. In this study we have determined the intron 4 nucleotide sequences of at least one representative of each major allelic group of HLA-B and -C. The intron 4 sequences were determined for 16 HLA-B and 14 HLA-C alleles by allele-specific sequence-based typing, using primers located in adjacent exons.
Overall, the intron 4 sequences of the different HLA-B and -C alleles display high similarity. However, the HLA-B alleles have a deletion of 20 nucleotides compared to HLA-C. This deletion enables the design of locus specific primer sites for sequencing exons 4 and 5. Several HLA-B alleles (*07, *08, *27, *39, *40 and *44) show a deletion of 11 nucleotides in the intron 4 sequence compared to other HLA-B alleles. HLA-Cw*07 alleles show several nucleotide differences with other Cw alleles, especially in the 5' end of intron 4. In comparison to HLA-Cw*07 and -Cw*17 all other HLA-C alleles show a deletion of three nucleotides. Surprisingly, the three nucleotides present in Cw*07 and Cw*17 are identical to the nucleotides of the HLA-B alleles at these positions. This suggests that the Cw*07 and Cw*17 alleles may have an ancestral relationship with HLA-B alleles.

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INSIGHTS INTO HLA-B AND -C INTRON POLYMORPHISM

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HLA intron sequences are not only a useful resource for the design and implementation of molecular based typing approaches but provide insight into the evolution of HLA polymorphism. Here we analyzed intron sequence data generated from a comprehensive DNA panel representing major allele groups of both HLA-B and -C loci. The HLA-B panel included 92 cell lines representing 31 different allele groups and the HLA-C panel consisted of 108 cell lines representing 13 different allele groups. Our findings are consistent with previously published data in that sequence variability is lower in Class I non-coding regions than in the adjacent coding regions and the amount of polymorphism decreases from the 5' to the 3' end of each gene. These results suggest that the evolutionary mechanisms that increase exon polymorphism also affect adjacent introns but with the opposite effect: i.e., there is a homogenization effect on these sequences. Also consistent with the concept that Class I alleles have evolved by gene conversion and recombination is the observation that both intron and exon sequence motifs are shared among allele groups and loci. This information further increases our understanding of HLA polymorphism and the ways in which the evolutionary mechanisms have affected both coding and non-coding sequences of HLA genes.

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THE NATURE OF INTRON 1 DEMONSTRATES STRONG LINEAGE SPECIFICITY IN HLA-DQB1 ALLELES

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For many HLA-DQB1 alleles coding sequences still need to be completed, and almost no sequence data about non-coding regions exist. In this study we have sequenced intron 1 of 30 haplotypes from samples of various ethnic groups, representing 16 alleles. Phylogenetic relationships, and synonymous (π_s) and nonsynonymous nucleotide substitution rates (π_a) were computed of intron 1 and the adjacent exons 1 and 2, respectively. The dendrograms showed a clear lineage specificity, as defined by the phylogenetic relationship in exon 2, in all three parts of the gene. This finding may be explained by the low CpG content in intron 1 (2.8%) and exon 1 (3.7%) which may reduce the probability of long interlineage recombination events in these parts of the gene. In the segments of exon 2 encoding the presumed antigen recognition site, π_a exceeds π_s, which thus demonstrates overdominant or balancing selection. The data obtained in our study for the first time provide additional evidence in support of this conjecture since π_s of exon 1 (0.07787) and exon 2 (0.08688) exceed π of intron 1 (0.06033), thus indicating intronic homogenization due to interallelic recombination and subsequent genetic drift.
Apart from further phylogenetic studies about HLA-DQB1 diversity, the sequence data obtained in our study may prove valuable for the development of a haplotype-specific sequencing strategy for exon 2, and for the explanation of recombination events in newly described HLA-DQB1 alleles.

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CLONING, SEQUENCING AND ANALYSIS OF FULL-LENGTH HLA-B AND –C GENES

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Currently most available HLA-A, -B and -C DNA sequences cover the polymorphic exons 2 and 3 with a limited number extending to other exons. Sequence data outside exons 2 and 3 is important to study for several reasons, including selection of annealling sites for PCR and sequencing primers, and to study evolution of both the polymorphic and non-polymorphic domains of HLA molecules. We have developed a rapid method for the accurate determination of full length genomic DNA sequences for HLA-A, -B and -C alleles. The method involves cloning of PCR amplified full-length HLA genes to separate alleles at heterozygous loci. To date we have sequenced full-length genomic sequences from representatives of all the major HLA-B and -C allele groups. We have selected DNA from which sequences (partial or cDNA only) were submitted to the WHO nomenclature committee and named officially. We have detected additional polymorphisms within introns for alleles which share identical exon sequences. These polymorphisms have been identified within intron 2, by reference strand conformation analysis (RSCA) of fragments containing exon 2, intron 2 and exon 3. All our sequences are officially recognised and are available to the scientific community.

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HLA-DRB FLUOROTYPING BASED ON SYBR GREEN MEDIATED MELTING CURVES

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The fluorotyping technique based on fluorogenic probes (5'-3' exonuclease assay) eliminates post-PCR steps and enables high throughput of DNA samples. Yet, this method can be only used for typing of certain HLA loci. The sequence motifs of the primers and of the probes are limited, which is an intrinsic problem of this technique since the fluorogenic probe needs to hybridize within a conserved region between the sequence-specific primers. This limitation extremely complicates the development of fluorotyping systems for highly polymorphic HLA loci. In order to overcome these problems, the fluorogenic probes were omitted; and DNA staining was performed using the fluorophore SYBR Green, which binds unselectively to double-stranded DNA during PCR. To discriminate between internal control and HLA-DRB specific amplification, the melting temperatures of the amplicons, as indicated by SYBR Green fluorescence, were recorded: Following the PCR, the temperature of the reaction mixture was increased from 55°C to 95°C over 20 min. During this ramp time, the decrease of fluorescence was reflected in the melting curves, with which internal control and HLA-DRB specific amplification were distinguished. Using this method, we have typed 100 DNA samples based on the amplicon melting curves. In all cases results were identical to those obtained by conventional gel electrophoresis. In conclusion, this method offers the advantages of the 5'-3' exonuclease assay but sorts out the problems related to the design of primers and fluorogenic probes. Hence, this method is a promising approach for complete HLA class I and class II automated typing.

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IDENTIFICATION OF HLA ANTIBODIES IN A SINGLE REACTION USING MICROSPHERES

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The LabMAP™ technology (Luminex Corp., Austin, TX, USA) provides an ideal format for developing multiplex HLA-antibody identification assays. Up to 100 different populations of antigen-coated microspheres, each with unique fluorescence signatures, can be mixed together and analyzed simultaneously. Thus, an assay equivalent to an ELISA, which employs 40 or more microtiter wells, can now be carried out in a single well. The assay protocol comprises: 1) incubating a serum sample with a mixed microsphere population, 2) washing to remove unbound sera, 3) incubating the bound sera with a PE-labeled secondary antibody, and 4) quantifying the captured PE using the LifeMatch™ Fluoroanalyzer. Up to 96 samples can be screened in one microtiter plate in less than 3 hours with minimal hands-on time. Using this technology, we have analyzed about 60 serum samples for the detection of HLA Class I antibodies using LifeMatch Class I ID and about 50 serum samples with a Class II ID product. The specificities of the assays were confirmed using previously characterized sera (e.g., NIH-CDC, AHG-CDC, ELISA, flow cytometry). The systems were further characterized using monoclonal antibodies and competition studies employing soluble antigens. Reproducibility was demonstrated among five laboratories. In addition to being rapid and user-friendly, these findings suggest that the LifeMatch assays are reproducible and as sensitive as ELISA methods for antibody detection.

Poster display I Immune response

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HISTORIC GENOMICS: AN HLA STUDY OF THE MEDITERRANEAN AREA

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HLA genes allele distribution has been studied in Mediterranean and sub-Saharan populations. Their relatedness have been tested by genetic distances, neighbour-joining dendrograms and correspondence analyses. The obtained population genetic relationships have been compared with the history of the classical populations (*historic genomics*) living in the area. A revision of the historic postulates would have to be undertaken, particularly in the cases when genetics and history are overtly discordant. HLA genomics shows that: 1) Greeks share an important part of their genetic pool with sub-Saharan Africans (Ethiopians and west Africans). The gene flow from Black Africa to Greece may have occurred in Pharaonic times carried by coloured people or when Saharan people emigrated after the present hyperarid conditions established (5000 years BC). Most classical Greek writers stated that Greek culture derived from the Egyptian civilisation. 2) Turks (Anatolians) do not significantly differ from other Mediterraneans, showing that the Asian Turks carried out an "elite" invasion with a cultural importance (language) but that is not genetically detected. 3) Kurds and Armenians are genetically very close to Turks and other Middle East populations. 4) There is no genetic trace of the so called Aryan invasion, which has only been defined on doubtful linguistic bases. 5) Iberians, including Basques, are related to north-African Berbers. 6) Present-day Algerian and Moroccan urban and country people show an indistinguishable Berber HLA profile.

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HLA CLASS II EXPRESSION IN HUMAN MONOCYTE-DERIVED DENDRITIC CELLS

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Introduction. Mature dendritic cells (DC) are characterized by high surface expression of HLA class II molecules, which is associated with increasing antigen presentation capacity. It, however, remains open to which extent HLA class II mRNA synthesis occurs during DC differentiation. Thus, we were interested to study the course of HLA-DR and -DQ mRNA and surface expression during generation of monocyte-derived dendritic cells. **Study design.** $CD14^+$ monocytes (MO) were cultivated in medium containing 10% FCS with GM-CSF and IL-4 for six days, followed by a maturation stimulus with TNF-α. During differentiation HLA-DRB1, -DQB1 and CIITA mRNA expression was carried out at different time points by real-time RT-PCR. In parallel, the surface expression of HLA-DR/-DQ was investigated by quantitative flow-cytometry. **Results.** In comparison to MO, a five-fold induction of mRNA of all genes was seen on day six of differentiation. After addition of TNF-α, mRNA levels declined. On the cell surface, increasing levels of HLA-DR and -DQ molecules were observed. However, a differential pattern of surface expression was seen for both isotypes. While absolute numbers were higher for DR compared to DQ, the induction, especially upon TNF-α treatment, was stronger for HLA-DQ. **Conclusion.** While during DC differentiation a steady increase of HLA class II mRNA and surface expression can be seen, a differential expression behaviour was observed upon maturation. The mRNA levels fall whereas surface expression is even further increased. This is compatible with a preexisting pool of peptide-loaded HLA class II molecules which can be rapidly exported to the cell surface.

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DIFFERENTIAL UPREGULATION OF HLA-DM AND CD83 ON MYELOID AND LYMPHOID DENDRITIC CELLS FROM PERIPHERAL BLOOD.

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Dendritic cells (DCs) form a heterogeneous population of cells and are the only antigen presenting cells capable of stimulating naive T cells. Two main subsets, known as lymphoid and myeloid DCs, have been described in peripheral blood. The myeloid subset is characterised by the presence of myeloid markers, e.g.CD11c or CD33, whilst the lymphoid subset is negative for these markers. The two subsets may perform different functions and have been defined as tolerogenic (the lymphoid subset) or immunogenic (the myeloid subset). The expression of HLA-DM molecules, which act as peptide editors in the antigen presentation process, was studied in freshly isolated lymphoid and myeloid DCs from peripheral blood. The results showed that the expression of intracellular HLA-DM was significantly different in both subpopulations ($p<0.001$, n=12). However, after culturing these cells in the presence of IL-3, TNFα and/or HSP-70, the expression of intracellular HLA-DM was upregulated to similar levels in both subsets. In addition, CD83 was not detected in any of these two subsets prior culture but its upregulation was also significantly different in both subpopulations of DCs ($p=0.008$, n=4). This differential pattern of DM and CD83 expression could be related to the function attributed to each subset.

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DOWNREGULATION OF FcαRI (CD89) DURING MATURATION OF HUMAN MONOCYTE-DERIVED DENDRITIC CELLS

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Introduction. Dendritic cells (DC) aquire exogeneous antigens to activate naive or primed T-lymphocytes. In part, antigen uptake is mediated by Fc receptors (FcR), especially FcγRII (CD32). Although, immunoglobulin (Ig) A is present at high serum concentrations, little is known about the myeloid IgA receptor (FcαRI, CD89) on DC. **Study design.** $CD14^+$ monocytes (MO) were cultivated in the presence of 10% FCS or 2% human plasma supplemented with GM-CSF and IL-4 for six days. Maturation was induced by TNF-α, or by a PGE_2-containing cytokine cocktail. Expression of CD89 was measured during differentiation by flow-cytometry and RT-PCR. In addition, FcαRI-dependent phagocytosis and antigen presentation assays were carried out. **Results:** Monocyte-derived dendritic cells (MDC) downregulated the CD89 molecule on their surface below the limit of detection (4000 molecules/cell), while it was present on MO (13.000 molecules/cell). This decrease in surface expression was paralleled by CD89 mRNA decrease. In contrast to macrophages, no FcαRI-dependent phagocytic antigen-uptake was seen with MDC. **Conclusion.** In this study, a decrease of CD89 surface and mRNA expression was observed upon cytokine-driven differentiation of MDC leading to a decline in CD89-dependent antigen-uptake. Thus, CD89 may only be involved in antigen-uptake during early stages of DC maturation.

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DIFFERENTIATION AND MATURATION OF HUMAN DENDRITIC CELLS ARE NOT ALTERED BY THE SECRETION OF SOLUBLE HLA-G, A NON CLASSICAL HLA CLASS I MOLECULE

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Dendritic cells (DC), professional antigen presenting cells, are essential for the initiation and control of the immune response. The specific expression of HLA-G, a non classical MHC class I molecule on placental trophoblast suggests an involvement of HLA-G in feto-maternal tolerance. Since HLA-G molecules are able to inhibit NK/T mediated lysis and to downmodulate both CD4 and CD8 responsiveness, HLA-G could act as an immunosuppressive molecule. The soluble isoform HLA-G5 was recently shown to be secreted during melanomas with a level correlated with advanced stages of disease. DC express NK inhibitory receptors such as ILT4, capable of interacting with HLA-G. In addition to suppressive effect on NK and T cells, HLA-G1 and G5 may have an inhibitory effect on human DC cells. Indeed, it was recently shown that maturation of dendritic cells was compromised in transgenic HLA-G mice. The aim of this work was to determine whether HLA-G5, a soluble isoform, may affect the differentiation or the maturation of human dendritic cells. Cells were differentiated from either peripheral monocytes, (n=2) or either CD34+ progenitors cells (n=2) in presence or in absence of purified soluble HLA-G (1 μg/ml) protein. Immunophenotypic analysis showed no difference of characteristics markers of immature monocytes or CD34-derived-dendritic cells differentiated in presence or not of HLA-G5. Moreover, soluble HLA-G has no effect on maturation of both monocytes or CD34-derived DC. Indeed, the markers of maturation and of costimulation and HLA-DR were positive with similar intensity level. These results suggest that soluble HLA-G5 which could be secreted during malignancies, has no effect on differentiation and maturation of human DCs generated from monocytes or CD34+ cells however further studies remain necessary to evaluate a potential role of HLA-G5 on functional capacity of DC cells.

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PCR-SSOP IDENTIFICATION OF CYTOKINE POLYMORPHISMS IN FIVE GEOGRAPHICALLY DIVERSE POPULATIONS.

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The release of cytokines plays an essential role in the regulation of the immune response. Polymorphisms in several cytokine genes affect gene transcription causing variation in cytokine production. PCR-SSOP detection methods for the identification of polymorphisms within IL-2 (-330), IL-6 (-174), IL-10 (-1082, -819 & -592), TNF-α (-308) and TNF-β (+252) have been established.

Application of the methods to various geographically diverse populations (Northern Ireland, Omani, South African Zulu, Singapore Chinese and Mexican) has identified ethnic differences within the cytokine gene frequencies for these groups. Most noticeable was the IL-6 -174 C mutation, which was either absent (Singapore Chinese) or present at low levels in the non-Caucasian groups, compared to 46% in the Caucasian Northern Ireland group. As a result the non-Caucasian populations predominate in presentation of the high producer IL-6 GG genotype. A similar trend was noted with respect to Caucasians within the IL-10 -1082 site in that the high producing GG genotype was reduced in the non-Caucasian groups (absent in Singapore Chinese). The low producing IL-6 -174 CC genotype and the IL-2 -330 GG genotype where both absent in the South African Zulu population. With respect to TNF-α -308 polymorphisms, similar allele and genotype frequencies were identified in the Northern Ireland and South African Zulu groups. While the TNF-α AA genotype is low in frequency within these two populations (7%) it is reduced in the Singapore Chinese (1%) and absent in the Omani and Mexican groups.

The introduction of PCR-SSOP for the identification of cytokine polymorphisms has proven to be a reliable and inexpensive typing method, easily adapted into established HLA typing laboratories.

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GENOMIC ORGANIZATION AND SINGLE NUCLEOTIDE POLYMORPHISM MAP OF THE RAT INTERLEUKIN-4 RECEPTOR GENE

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Interleukin (IL)-4 plays an important role in stimulating B-cell differentiation. It is crucial for the development of T-helper 2 cells. Its effects depend upon binding to and signalling through the IL-4 receptor (IL4R) complex. Polymorphisms in the IL4R gene have been reported to be associated with atopy and asthma. Genomwide screens for loci conferring susceptibility to collagen-induced arthritis (CIA) in rat models led to the identification of a candidate region on rat chromosome 1, where the IL4R gene is located. We investigated the genomic organisation of the rat IL4R gene as well as polymorphisms in seven important laboratory rat strains: BB/OK, SHR, DA, WOKW, BN, BB,KWR(1), LEW. The Gene ist devided into 12 exons and spans over 23 kb, the exons range in size from 50 to 2424 bp with introns ranging in size between 0,3 to 10 kb. We sequenced the upstream region up to position -1000. The following novel single nucleotide polymorphisms were identified: Promoter: T-349C, C-33A, 5´UTR: C2G, G3C, T4A, G7A, A153G, G170T, T171C, Exons: T376C, G1138A, C2108T, C2113T, 3´ UTR: I2950C, I3050C, G3104T. Phylogenetic examination revealed a striking homology of IL4R gene organization as well as the 5´flanking region in rat, mouse and human. The identified SNPs in the coding region of rat IL4R are silent, thus different susceptibility for autoimmune diseases of rat strains might be associated with genetic variation identified in the 5´flanking region.

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POLYMORPHISM IN THE FIRST INTRON OF IFN-γ GENE AND GVHD INCIDENCE IN HEMATOPOIETIC STEM CELL TRANSPLANT RECIPIENTS.

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IFN-γ gene characterises with polymorphic features due to: variable number of CA repeats and SNP in the first intron region (+812 - +932). Five microsatellite alleles (CA repeats from 11 to 15) and two alleles corresponding to T or A in position +874 are described.

103 HSCT recipients suffering from haematological malignancies (81pts), anaemia's (10pts), inborn immunodeficiences (7pts) and other disease (5pts) were examined with the use of both techniques: STR-PCR and SSP. Following alleles combinations were found:

PCR-SSP		STR-PCR		
genotype description	frequency (%)	number of repeats (CA)	genotype description	frequency (%)
T/T	17	12/12	2/2	17
T/A	49	12/13	2/3	42
		12/14	2/4	5
		12/15	2/5	2
A/A	34	13/13	3/3	25
		13/14	3/4	5
		13/15	3/5	4

In all patients the incidence and severity of transplant related toxicity, acute and chronic GVHD were recorded and investigated for association with IFN-γ intron I gene polymorphism. It was found that: (1) 12 CA repeats were associated with the presence of T in position +874. (2) There was no correlation with aGvHD and toxicity. (3) Individuals having allele 3 developed more frequently chronic GvHD than patients having other allele combinations (14/78 vs. 1/25; p=0.07, Fisher exact test). In addition only homozygous patients with alleles 3/3 developed fatal cGVHD (3/26 vs. 0/77; χ^2= 5.53, p=0.019).

It appeared IFN-γ intron I gene polymorphism was associated with the incidence and severity of cGvHD but not with toxicity.

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GENOTYPING FOR POLYMORPHISMS CYTOKINES ALLELE FREQUENCIES IN ITALIAN POPULATION

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Cytokines mediate inflammatory and immune response and may influence rejection and graft-versus-host-disease following solid organ or bone marrow transplantation. Recently it has been demonstrated that several cytokine genes bear polymorphisms which may affect gene transcription, causing individual variations in cytokine production. The majority of polymorphisms described are single nucleotide polymorphisms (SNPs). The allelic and genotype frequencies in one hundred healthy Italian subjects have been determined for the following cytokine genes: IL1α (T/C –889), IL1β (C/T –511, T/C +3962), IL12 (C/A –1188), IFNγ (A/T UTR 5644), TGFβ (C/T codon 10, G/C codon 25), TNFα (G/A –308, G/A –238), IL2 (T/G –330, G/T +166), IL4 (T/G –1098, T/C –590, T/C –33), IL6 (G/C –174, G/A nt565), IL10 (G/A –1082, C/T –819, C/A –592), IL1R (C/T pstI1970), IL1RA (T/C mspa1 11100), IL4RA (G/A –1903). All typings were performed with PCR-SSP assays using identical amplification and detection conditions, enabling rapid and cost-efficient analysis of multiple polymorphisms. The PCR-SSP tray kits were produced by Heidelberg University. Statistical analyses were performed calculating correlation coefficient r and chi square. All allele and genotype frequencies and linkage disequilibria were calculated and compared with those of others populations.

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IL10 AND TNF-α GENE POLYMORPHISMS IN PATIENTS WITH RECURRENT SPONTANEOUS ABORTIONS

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There is no recognised cause for pregnancy loss in approximately 50% of all recurrent abortion (RSA) cases, although data suggest immunological events resulting in embryo rejection may explain pregnancy loss in this group of patients. Cytokines are known to be important mediators of immune reponses, and may contribute to immune mediated pregnancy loss. Many data suggest the TH2 response is favoured over the TH1 response in normal pregnancy. In this study we investigated whether cytokine gene polymorphisms leading to variations in cytokine production may therefore influence the development of RSA.

The SNaPshot™ ddNTP primer extension technique and the ABI PRISM™ 310 (PE Biosystems, Warrington) was used to investigate the single nucleotide polymorphism at position -308 (G/A) of the TNF-A gene, in a cohort of 91 RSA patients, and IL10 polymorphisms at positions -1082 (G/A), -819 (C/T) and -592 (C/A) for 72 patients.

The TNF-alpha -308 genotype frequencies obtained in this study were G/G:65%; G/A:31%; and A/A:4%. The IL10 genotype frequencies were as follows: G/G:22.2%; G/A:55.6%; A/A:22.2%, at position -1082, C/C:63.9%; C/T:30.6%; T/T:5.5% at -819 and C/C:63.9%; C/A:30.6%; A/A:5.5% at -592.

This study showed no significant correlation between the development of RSA and genotype at position -308 of the TNF-A gene, or at positions -1082, -819, -592 of the IL10 gene, with no statistical differences observed between RSA patients and healthy controls.

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DEFINITION OF THE COMMON KIR HAPLOTYPES IN THE HUMAN POPULATION USING AN IMPROVED KIR PCR-SSOP TYPING SCHEME AND THE CHARACTERISATION OF NOVEL KIR VARIANTS.

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Recently we reported a PCR-SSOP typing scheme for the polymorphic killer immunoglobulin like receptor genes located in the leukocyte receptor cluster on chromosome 19 (Crum et al., 2000, Tissue Antigens 56: 313-326). We have since significantly modified and improved our original approach to facilitate identification of the newly identified KIR genes, 2DL5, 3DL3, X and Z. Additional probes, spanning the entire coding sequence, for each specificity have also been incorporated, such that new variants can easily be identified. The improved system has been re-applied to the original 90 individuals examined in the previous study and the updated results for the new genes and the relevant associations will be presented. While 3DL3 and X were found in all the individuals examined, 2DL5 was found in 51 individuals (56%) while Z was found in 87 individuals which were also positive for 2DL1. The improved system identified a 2DL2 allele variant in 18 individuals previously thought to be 2DL2 negative, increasing the documented frequency from 31% to 51%, and also discriminated between 2DL1v and 2DS1.

A large number of Irish families (n=26, 137 individuals) and 10 Black (Xhosa) South African families (33 individuals) have been KIR typed in an attempt to throw further light on the KIR haplotypes within the population. Our data on the eleven deduced KIR haplotypes from this analysis will also be presented. Novel KIR variants for 2DS4 (22bp deletion predicting a soluble KIR), 3DL1 and 3DS1 have now also been characterised revealing a range of different mechanisms involved in creating KIR diversity, and these findings will be presented.

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ALLELES OF THE KIR2DL4 RECEPTOR AND THEIR LACK OF ASSOCIATION WITH PRE-ECLAMPSIA.

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A survey of KIR2DL4 polymorphism revealed seven common sequences in the Australian population. The seven sequences encode three different amino acid sequences of the immunoglobulin domains. Two of the sequences encoding different amino acid sequences in the immunoglobulin domains also occur on some chromosomes with a single nucleotide deletion at the end of exon 6 which encodes the transmembrane domain (❒TM mutation) resulting in exon 6 skipping during mRNA production. Due to alternate splicing, a fraction of the mRNA produced by these alleles includes the transmembrane region but is missing the cytopolasmic region. The remaining two sequences differed only by synonymous substitutions. All of the exonic polymorphisms of KIR2DL4 could be detected by SSCP of individually amplified exons. The ❒TM mutation is in linkage disequilibrium with the KIR A haplotype and the wild type sequence is in linkage disequilibrium with the B haplotype. The frequencies of alleles with the ❒TM mutation or Ig-domain polymorphisms did not differ between women who experienced pre-eclampsia and normotensive controls. Similarly there was no difference in the KIR gene repertoire in pre-eclampsia and normotensive controls.

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HUMAN CD38 FUNCTIONS AS A SIGNALING MOLECULE IN DECIDUAL NATURAL KILLER CELLS.

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The establishment of human placenta in early pregnancy is characterized by the presence of a large number of perforin-rich NK cells within the maternal decidua in close proximity to the trophoblast. These cells have an unusual phenotype distinguishing them from peripheral blood NK cells. The hallmarks of decidual NK (dNK)cells are the absence of CD16 and an increased expression of CD38. CD38 - an ectoenzyme catalyzing the synthesis and degradation of cADPR - is a lineage-unrestricted signal transduction molecule which elicits signals relevant to circulating NK cell functions. This study analysed the events following CD38 ligation by agonistic mAb on human dNK cells. CD38 ligation i) induced a significant cell proliferation, as assessed in terms of ^{3}H-thymidine incorporation, ii) influenced the natural cytotoxicity of dNK cells against HLA-negative target cells, which appears reduced as compared to that of circulating NK cells and iii) was followed by a significant ADCC. Such effects were visible on dNK cells cultured in the presence of autologous adherent cells and IL-2, while the lysis obtained after ligating CD38 on resting dNK cells was negligible.
These data suggest that CD38 contributes to cytolytic machinery of dNK cells, which is primed for function during pregnancy but is simultaneously efficiently controlled to prevent accidental activation. A possible scenario is that multiple receptors may co-operate to induce optimal NK activation. This does not imply that all receptors function simultaneously, since their involvement may depend upon the presence and/or the epitope density of the proper ligands on the target cells.

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THE EFFECT OF IGM ENRICHED HUMAN IMMUNOGLOBULIN AND RABBIT ANTI- THYMOCYTE GLOBULIN ON THE STIMULATION OF MONONUCLEAR CELLS.

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Whether IgM-enriched intravenous immunoglobulin (Pentaglobin) is a useful adjunct treatment for GvHD prophylaxis in allogeneic stem cell transplantation is unclear. Clinical data with the use of a five-agent GvHD prevention regimen including Pentaglobin and ATG are encouraging. In vitro both have been reported to modulate alloreactive T cells. We compared their inhibitory effect on the phytohemagglutinin- induced lymphocyte proliferation. ATG blocked proliferation of lymphocytes at lower doses and much stronger than Pentaglobin. The combination of both was not different from ATG alone. In Pentaglobin, glucose used as stabiliser, caused the effect. Starting at a concentration of 40 mg/dl glucose, glucose alone showed a dose-dependent inhibition of PHA-induced proliferation. For the in vivo application of Pentaglobin, the results suggest that Pentaglobin does not inhibit the proliferation of T cells.

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FOURTEEN YEARS FOLLOW-UP OF AN AUTOIMMUNE PATIENT LACKING THE CD3γ SUBUNIT OF THE T-LYMPHOCYTE RECEPTOR.

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A familial defect of T cell receptor (TCR)/CD3 complex expression by T lymphocytes, due to a selective deficiency of the CD3γ subunit, was reported in 1986. Two brothers (V and D) had inherited mutations in both the paternal and maternal CD3γ genes that severely truncated the protein. V had a severe combined immunodeficiency and autoimmune enteropathy with gut epithelial cell autoantibodies. He died at 32 months of age after severe infections and autoimmunity. Still living sibling D shared the same genotype regarding CD3γ, but had milder laboratory immunological and clinical parameters. D was born in 1981 and started with clinical symptoms at 12 months of age, including infrequent bacterial infections, vitiligo, atopic eczema and a mild dilated cardiomyopathy. Several D alterations (vitiligo, DCM, specific thyroid autoantibodies) may certainly have an organ-specific autoimmune etiopathogeny. These results will be discussed within the context of the recently described CD3γ-deficient mice.

Immune function is apparently sufficient for the survival of the CD3γ lacking D patient, but continuing a close follow up will enable us to determine the real importance of adaptive *vs* non-adaptive immune mechanisms along individual's life and to study the mechanisms that relate autoimmunity and immunodeficiency.

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CYCLOSPORINE A SENSITIVITY OF T-LYMPHOCYTES: THE ASSESSMENT OF INDIVIDUAL PHARMACODYNAMC EFFECTS IN HUMAN WHOLE BLOOD

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The quantitative analysis of cyclosporine A (CsA) effects might be helpful to optimize immunosuppressive treatment after allogenic organ transplantation in the individual patient, since rejection occurs despite "therapeutic range" CsA blood levels. Previous investigations found that costimulation of the CD28 pathway generally mediates CsA resistant proliferation of TCR-activated T lymphocytes.
In this investigation we found considerable interindividual variation with respect to the immunosuppressive effects of CsA upon Anti-CD3/CD28 T cell costimulation in a human whole blood assay. The proliferation of T cells was inhibited by *in vitro* addition of CsA in all tested individuals (n=11). In contrast, the investigation of IL-2 mRNA expression profiles by a real-time RT-PCR protocol (TaqMan-PCR) revealed two groups of individuals: group I showed unaffected IL-2 mRNA expression (*low responder*) and group II was highly sensitive to CsA *in vitro* (*high responder*). The mRNA expression of IL-4, IFN-γ and TNF-α mRNA was unaffected by CsA in *low responders*, however, in *high responders* the expression of all cytokines was partially diminished. In patients undergoing CsA monotherapy before living donor kidney transplantation (n=4) we also observed individually distinct levels of CsA sensitivity *ex vivo*.
In conclusion, we cannot confirm a general CsA resistance of T cells upon Anti-CD3/CD28 costimulation, but our results rather suggest an individual degree of CsA sensitivity, which may correspond closely to the clinical experience. However, prospective studies are necessary to determine whether individual degrees of CsA sensitivity correlate with clinical events and are associated with low or high risk of transplant rejection.

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AN UNUSUAL A2 ANTIBODY.

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We have identified an antibody with HLA-A2 specificity in a renal patient typed as HLA A1,A2,B8,B49, DRB1*04, DRB1*0301/06/08. The original antibody detected was IgM demonstrable on both T and B cells. Two weeks later isotype switching had occurred and IgG activity was found against B cells. Autoantibody testing was performed on all positive sera and all tests were found to be negative.
The patient's HLA type was repeated and results were confirmed. High resolution A2 typing using polymerase chain reaction with sequence specific primers showed the patient to be A*0205.
The patient was first transplanted in December 1995 with a zero mismatched kidney HLA type A1,A2,B8,DRB1*0301/06/08; this graft was lost to chronic rejection within three years. The patient was listed for a second transplant in April 2000 when a weak antibody showing HLA-A2 specificity was identified. The patient had multiple transfusions in the six months before returning to the transplant list, previously to this there had been no transfusions for 2 years. The patient received a second graft in September 2000 with a 110 mismatched A1,A11,B8,B55, DRB1*04, DRB1*0301/06/08 kidney with a negative flow and cytotoxic crossmatch.
During post transplant monitoring one A2 panel cell out of a forty cell panel was consistently negative (nineteen were consistently positive) with patient's sera. High resolution A2 typing showed this cell to be A*0205.
The results indicate that HLA-A2 must be considered an unacceptable antigen for this patient. This finding has implications for future screening and matching protocols.

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DIFFERENTIAL IMMUNOGENICITY OF HLA MISMATCHES: ANALYSIS OF ANTIBODY PROFILES IN PREGNANT WOMEN

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We analysed the differential immunogenicity of HLA mismatches in a cohort of 1313 women who developed specific alloantibodies against paternal HLA antigens of their child.
The results show that women expressing HLA-A3 produce significantly more frequently HLA allo antibodies compared to women expressing other HLA-A antigens (P=0.024). Next to this we analysed how frequently women produce specific antibodies against individual HLA mismatches when these antigens are present as a mismatch in their children. A large variation was found, i.e. in 32% of the cases antibodies were found against HLA-A2 whereas only in 3% of the cases antibodies were found against HLA-A19. A similar situation was found for HLA-B mismatches although these differences were not significant, i.e. antibodies were produced against only 9% of the HLA-B18 positive children and against 26% of the HLA-B5 antigens of the children. Finally it was found that the immunogenicity of HLA mismatches is dependent on the HLA phenotype of the responder, i.e. against the mismatched HLA-A11 antigen of the child, antibodies were formed by 33% of the women expressing HLA-A9, 16% of the women expressing HLA-A2 and 5% of the women expressing HLA-A1. The current information may be useful for the determination of acceptable and taboo mismatches in case of donor selection for (higly sensitized) transplant recipients.

Poster display II Disease

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TRANSFUSION RELATED ACUTE LUNG INJURY (TRALI): LABORATORY INVESTIGATIONS

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TRALI is a serious, life threatening complication of blood transfusion commonly associated with the transfusion of donor plasma containing leucocyte antibodies that react with the recipient leucocytes, (6 fatalities in 19 cases reported to Serious Hazards of Transfusion Committee (SHOT) in 1999/2000). In addition to patient-specific leucocyte antibodies, TRALI is thought to arise when patients have a predisposing condition, such as active infection, recent surgery or cytokine therapy. The majority of cases result from the presence of donor HLA class I antibodies, however HLA class II and granulocyte specific antibodies have also been associated with the disorder

Laboratory investigations involve testing implicated donors for the presence of HLA and / or HNA antibodies. Not all transfusions from donors found to have leucocyte antibodies result in TRALI, therefore a flow cytometric crossmatch should be performed for all antibody positive donors, in order to confirm the diagnosis of TRALI.

We report a summary of the laboratory findings in a series of 24 TRALI cases referred to the National Blood Service, London and South East in the period 1996 - 2001. The blood components implicated included fresh frozen plasma, red cells, platelets and cryoprecipitate.

Donor leucocyte antibodies were detected in 23/24 cases, 18 involving HLA class I specific antibodies and 5 cases involving HNA antibodies only. Crossmatches were performed in 20 cases , 18 of which were positive, thus confirming specific patient-donor antigen-antibody interactions and supporting the diagnosis of TRALI. These laboratory investigations are critical for the future management of blood donors and understanding the true incidence of this serious hazard of blood transfusion.

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ROLE OF NIJMEGEN BREAKAGE SYNDROME PROTEIN IN SPECIFIC T LYMPHOCYTE ACTIVATION PATHWAYS

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Nijmegen breakage syndrome (NBS) is a genetic disorder characterized by immunodeficiency, microcephaly, and "bird-like" facies. NBS shares some clinical features with ataxia telangiectasia (AT), including increased sensitivity to ionizing radiation, increased spontaneous and induced chromosome fragility, and strong predisposition to lymphoid cancers. The mutated gene that results in NBS codes for a novel double-stranded DNA break repair protein, named nibrin.

In the present work, a Spanish NBS patient was extensively characterized at the immunological and the molecular DNA level. He showed low $CD3^+$ cell numbers and an abnormal low $CD4^+$ naïve cell/$CD4^+$ memory cell ratio, previously described in AT patients and also described in the present NBS patient. The proliferative response of peripheral blood lymphocytes *in vitro* to mitogens is deficient in NBS patients, but the possible link among NBS mutations and abnormal immune response is still unknown.

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HLA CLASS I DECREASE ARE THE MOST FREQUENT HLA ALTERATIONS IN NON-HODGKIN LYMPHOMA.

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HLA molecules play a major role in the recognition of human cells which is a key step in anti-tumoral response. HLA loss is observed in different malignancies, inducing escape from immune surveillance. HLA-G, a non-classical class Ib molecule, is actually considered as an immune tolerance inducing molecule. So its expression in cancer could be relevant for immune escape. Since few data are available in Non-Hodgkin Lymphoma (NHL), HLA molecules were prospectively studied in 614 cases using flow-cytometry with specific anti-HLA class I Monoclonal Antibody (MoAb W6/32) and anti HLA class II MoAb (B8.12.2; HLA-DR). Furthermore, a specific anti-HLA_2-G MoAb (87g) was tested in fifty cases, included 20 cases selected on their defective HLA class I expression. In sixty four cases (10,4%), lymphomatous cells exhibited a lower W6/32 mean fluorescence intensity compared to reactive cells. The characteristics of these cases were i) the diversity of histological entities (FL (20%); DLCL (56%); MCL; Burkitt, NK/T Lymphoma (6%)), ii) the frequency of relapse or transformation (34%), iii) the high incidence in high grade NHL (22,9%) compared to low grade (6,4%), iv) the severity of class I defect in 50% of the cases and mainly in high grade NHL. HLA-DR defect was always associated with severe class I defect (12 cases; 2%). HLA-G protein was only expressed in three class I defective cases with normal HLA-DR expression. Abnormalities of HLA molecules could be observed in NHL similarly to other malignancies. These HLA alterations frequently appeared as a second event at relapse or at transformation suggesting a role in lymphomagenesis. In addition, HLA abnormalities may favour tumour progression by (i) decreasing recognition of tumor cells (ii) altering apoptosis-induced HLA-DR in case of HLA-DR lack (iii) inhibiting NK cell lysis in case of HLA-G expression.

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ANALYSIS OF β2-MICROGLOBULIN ABNORMALITIES IN DIFFUSE LARGE B-CELL LYMPHOMA

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Human leukocyte antigen (HLA) class I molecules are expressed on the surface of nucleated cells and present antigens to cytotoxic T-cells, thereby initiating an anti-tumour immune response. We previously reported that loss of HLA class I in extranodal diffuse large B-cell lymphomas (DLCL) is a common event. Hemizygous deletions comprising the whole HLA region on chromosome 6p were observed. In this study we screened 15 DLCL cases with loss of HLA class I and β2-microglobulin (β2m) expression for mutations in the latter gene by direct sequencing. Mutations in the β2m gene were detected in two cases. In case T18 a 2bp deletion in a $(CT)_8$ repeat region in exon 2 caused a premature stop codon. In case T29 a 2bp deletion in a $(TG)_2$ repeat in exon 1 produced a frameshift mutation. LOH analysis showed loss of the remaining copy of the gene. We investigated whether there is a relationship between DNA-mismatch repair and mutations in the β2m gene. Low microsatellite instability as defined by the presence of frame shifts in >1 of 8 tested microsatellite markers was found in 8 of 15 cases. In three cases an intragenic MSH6 repeat was affected. Immunohistochemistry for MLH1, MSH2 and MSH6 proteins showed no loss of expression. We conclude that β2m mutations may account for loss of expression of β2m and HLA class I proteins in these DLCLs. So far the mechanism leading to these mutations is not clear, but our data suggest that an acquired defect in mismatch repair might play a role.

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HLA ANTIGENS IN PAPILLARY THYROID CARCINOMA

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It has been established that HLA antigens are susceptibility factors for different cancers, including thyroid tumors. We investigated HLA subtypes of Turkish thyroid carcinoma patients. 25 Turkish thyroid carcinoma patients and 175 healthy controls were typed for Class I (HLA-DRB* 1, DQB* 1) and Class II (HLA-A,-B,-C) antigens by PCR-SSP low resolution DNA technique. The phenotypic frequencies in the patients were as follows: DRB1* 13 (44%), DRB1* 15 (24%), DQB1* 3 (52%), DQB1* 5 (40%), HLA-A 24 (44%), HLA-A 2 (28%), HLA-B 35 (40%), HLA-B 51 (32%), HLA-Cw* 7 (32%), HLA-Cw* 1 (20%). Each phenotype frequency was compared to the control frequency of the general Turkish population. In previous reports with large number of patients it was found to be that both HLA Class I (B35) and Class II (DRB* 11) antigens were susceptibility factors only in the papillary tumor group of patients. In this study, no association was found between antigens of the HLA system and papillary thyroid tumors.

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INFLUENCE OF HLA CLASS II ALLELES IN THE DEVELOPMENT OF HEPATOCELLULAR CARCINOMA (HCC) IN HBV-INFECTED PATIENTS

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There is some evidence that immunogenetic factors may have a role in the mechanism of susceptibility to chronic hepatitis and development of HCC in HCV- or HBV-infected subjects.
In the present study, we have evaluated the distribution of HLA class I and class II alleles in 82 HCC patients with HCV infection and 41 HBV-infected patients compared to 210 healthy blood donors as control. All patients, consecutively analyzed in the period 1996 through 2001, underwent orthotopic liver transplantation. HLA class I typing was performed by serological method, whereas HLA class II typing was carried out by SSP-PCR. No significant difference in the frequencies of HLA class I antigens was observed. between HCC patients and controls. The comparison between HCC patients infected by HCV and the control group did not show any significant difference in the distribution of HLA class I and II antigens. On the contrary, the frequencies of DRB1*1104, DRB1*0701 and DQB1*03 alleles (p x n= 0.05, RR= 2.84; p x n= 0.045, RR= 2.41; p x n= 0.0041, RR =2.69) were higher in HCC patients with HBV infection than in control group; these alleles, therefore, could have a role in the susceptibility to the virus infection and/or in HCC development. The DRB1*13, DQB1*0201 and DQB1*06 frequencies were higher in the control group (p x n= 0.0083; RR= 0.12; p x n =0.017, RR =0.11; p x n=0.039, RR= 0.28), so suggesting that subjects expressing these alleles could be less susceptible to HBV infection and/or HCC development.

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INFLUENCE OF VASCULAR ENDOTHELIAL GROWTH FACTOR POLYMORPHISMS ON TUMOUR DEVELOPMENT IN MALIGNANT MELANOMA

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Vascular endothelial growth factor (VEGF) is a potent regulator of vasculogenesis and tumour angiogenesis. We have investigated whether expression-related VEGF -2578, -1154, +405 and +936 SNPs and associated haplotypes confer susceptibility to and/or influence prognosis in cutaneous malignant melanoma (CMM) skin cancer, by ARMS-PCR genotyping of 152 CMM patients and 266 controls. Strong linkage disequilibrium between the -2578, -1154 and +405 SNPs was detected (ρ = 0.488 - 0.965), but not between these SNPs and SNP +936 (ρ = 0.004 - 0.130). No SNPs or three SNP haplotypes (-2578, -1154, +405) were significantly associated with CMM susceptibility. However, the VEGF - 1154 AA 'low expression' genotype and -2578, -1154, +405 CAC haplotype were both significantly associated with less advanced (Stage I v Stage II-IV) disease (16.7% v 5.5%; P = 0.03, OR = 0.3 (0.1-1.1) for AA). In addition, the VEGF -1154 AA 'low expression' genotype was associated with thinner primary vertical growth phase tumours (31.8% v 5.0%; P = 0.002; OR = 0.1 (0.03-0.4) for tumours ± 0.75 mm thick), while the VEGF -1154 GG 'high expression' genotype was associated with thicker primary tumours (66.7% v 44.4%; P = 0.02; OR = 2.5 (1.1-6.0) for tumours ± 1.5 mm thick). Mean vertical growth phase tumour thickness was also significantly greater in patients of VEGF -1154 GG v AA genotype (3.1 ± 3.9 mm v 0.8 ± 0.5 mm; P = 0.002). These preliminary results indicate that VEGF genotype may influence tumour growth in CMM, possibly via the effects of differential VEGF expression on tumour angiogenesis.

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HLA ALLELIC AND HAPLOTYPE LOSS IN HUMAN MELANOMA AND DERIVED CELL LINES

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HLA class I allelic or haplotipe loss in tumor cell has been described in many tumours, however, the frequency of this defect is still unknown. This altered HLA class I phenotype can be one of the major tumor escape mechanism facing anti-tumor cell mediated immune response. Therefore, defects leading to these altered phenotype should be characterised to better address a possible anti-tumor immunotherapy. To further evaluate the molecular mechanisms underlying HLA down-regulation, we stabilised *in vitro* 22 human melanoma cell lines starting from surgical removed primary and/or metastatic tumors. Surface expression, studied by cytofluorimetric analysis, revealed melanoma associated antigens (MAA) in all cell lines whereas analysis of HLA class-I expression revealed total loss and low antigen expression in one and two cell lines respectively. Of interest is the finding that different HLA class I expression was observed in three cell lines derived from the same patient. DNA sequence analysis showed a β2-microglobulin mutation in the HLA class I negative cell line. The detected mutation was also present in the corresponding frozen tumor. Moreover, α-chain analysis by PCR-SSP of patient's PBL and derived cell lines revealed loss of the HLA A*24, B*49, C*w1 haplotype in one and loss of HLA A*03, C*w5 alleles in the second cell line. Our analysis showed HLA class I down-regulation only in 3 out of 22 (13.6%) melanoma derived cell lines, however, immunohistochemical analysis with allele specific MoAbs are in progress to detect HLA loss even in the frozen corresponding tissues.

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GENES IN THE MHC REGION AND THEIR ASSOCIATION WITH HEAD AND NECK SQUAMOUS CELL CARCINOMA.

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The HLA region on chromosome 6 contains many HLA-related genes which are highly polymorphic. Apart from HLA polymorphism short-tandem repeat (STR) markers are informative in disease association studies. The TNFb STR marker and the TAP1 gene, both located on chromosome 6, have been reported to be associated with head and neck squamous cell carcinoma (HNSCC). The goal of this pilot study is to investigate the short arm of chromosome 6 and define a region or find other genes which are associated with HNSCC or the development of cancer.

HNSCC patients (N=47) and a control group (N=50) were evaluated for the following STRs D6S291, -TAP1, -273, -TNFb, -TNFd, -MICA, -C125, -265 and -276 and STR allele lengths were evaluated by gene-scan analysis. In addition, HLA class I (HLA-A, -B and –C) and class II (HLA-DR and –DQ) typing at low resolution was performed for the same patient and control group by sequence-specific-priming (SSP).

Preliminary HLA typing results enabled us to speculate about a possible defined area on the short arm of chromosome 6 which might be associated with HNSCC. This area involves the DQ1 – DR6 – B40 – Cw3 haplotype. STR analysis has shown that the A5.1 and the A9 alleles of the STR marker D6SMICA, located near HLA-B, significantly are associated in patients compared to the control group. The STR markers D6S291, -265 and –276 did not show any association with HNSCC. Other STR markers distibuted over the p-arm of chromosome 6 are currently under investigation.

Preliminary results indicated that there is a defined area which is involved in HNSCC. Further refinement of this area, including SNP analysis and study on specific genes in this region may shed light on the gene(s) specifically involved in HNSCC.

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β2-MICROGLOBULIN EXPRESSION AND LOH IN HEAD AND NECK SQUAMOUS CELL CARCINOMA (HNSCC).

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Introduction: Malignant transformation is frequently associated with HLA class I and β2M downregulation or loss. Altered HLA class I expression affects the cytotoxic T-cell response. This may provide tumour cells with a mechanism to evade the cellular immune response. We studied in 53 HNSCC if LOH of β2M, using 6 STR's, is representative for loss of β2M expression.
Methods: Immunohistochemistry, using α-β2M, was performed on paraffin sections of HNSCC. LOH of β2M was determined using 6 STR markers on chromosome 15. STR PCR products from DNA isolated out of frozen sections were separated on a 6% polyacrylamide gel and analysed using GeneScan software.
Results: Out of 53 HNSCC, 33 showed positive β2M expression, 9 heterogeneous expression and 11 negative β2M$^-$ expression. Out of these 11 tumours, 7 showed LOH for the majority of STR markers and 4 tumours did not show LOH. These 4 are currently being analysed for mutations using SBMA on DNA isolated out of laser captured microdissected tumour fields. Remarkably, 5 out of the 33 β2M positive HNSCC showed substantial LOH for a variety of STR markers. Of these 5 tumours, 3 even showed β2M overexpression. As LOH determined by STR markers can not be distinguished from amplification, it may well be that these 5 HNSCC have a third β2M allele resulting in normal- or overexpression of β2M. We are currently analysing these 5 HNSCC for amplification of chromosome 15 using FISH on paraffin HNSCC sections. LOH of the 7 β2M negative HNSCC will also be validated by FISH.
Conclusion: This study makes clear that β2M is affected in many ways in HNSCC. This alters HLA Class I expression, which can affect cytotoxic T-cell response.

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EXPRESSION OF HLA-G MOLECULES IN PULMONARY DISEASES.

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HLA-G is selectively expressed in extravillous trophoblast of human placenta, which does not express classical HLA-A and -B molecules. Several studies show the role of HLA-G as a molecule involved in immune tolerance. By interacting with NK and T cells inhibitory receptors, HLA-G may down-regulate their cytotoxicity functions. To appreciate the biological and clinical relevance of HLA-G expression in lung diseases, we analyzed HLA class I and HLA-G expression in a panel of 36 *ex vivo* neoplastic tissues and 8 non-neoplastic lung tissues. We performed immunohistochemical analysis using a pan-HLA class I antibody (W6/32) and three different specific anti-HLA-G antibodies (87G, MEMG/9 and 4H84). Our findings showed that HLA-G products were not expressed in pulmonary structural cells. However, HLA-G molecules were detected in activated macrophages and dendritic cells infiltrating lung carcinomas (33%) and non-tumoral pulmonary diseases (25%). HLA-G expression was not correlated with classical HLA alterations. No statistical correlation was found between HLA-G expression and clinical or biological parameters except high tumor size. The expression of HLA-G in myelo-monocytic cells infiltrating lung pathological tissues could alter antigenic presentation and contribute to decrease immune response efficiency, subsequently favoring the progression of tumoral or inflammatory processes.

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INFLUENCE OF CYTOKINE POLYMORPHISMS ON SUSCEPTIBILITY TO AND/OR PROGNOSIS IN PROSTATE CANCER.

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Prostate cancer (PC) is one of the leading causes of male deaths in the western hemisphere. Polymorphisms in immune response genes may influence susceptibility to and/or prognosis in sporadic cases of PC, resulting from inter-individual differences in the anti-tumour immune response.
In this study, 250 prostate cancer patients and 256 controls were genotyped by ARMS-PCR for 5 polymorphisms: Interleukin (IL) -1β (-511, A/G), IL-8 (-251, A/T), IL-10 (-1082, A/G), Tumour Necrosis Factor-α (-308, A/G) and Vascular Endothelial Growth Factor (VEGF), (-1154, A/G). Patient-control genotype comparisons revealed that IL-8 AA (high producer) and VEGF AA (low producer) genotypes were significantly decreased in the patients when compared to the controls, (23.9% v 32.3%; p=0.04) and (6.3% v 12.9%; p=0.01) respectively. IL-10 AA (low producer) genotype was significantly increased in the patients when compared to the controls, (31.6% v 17.1%; p=0.001).
Within the PC group, IL-8 genotype showed a significant association with prostate specific antigen (PSA) level, (p=0.05).
Results suggest that genotypes associated with differential production of IL-8, IL-10 and VEGF are risk factors for PC. Genotypes associated with high IL-10 and low VEGF production may act via their influence on angiogenesis, necessary for metastasis. Therefore polymorphisms in cytokine genes may influence susceptibility to and prognosis in PC, indicating that a definitive investigation is required.

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HLA-E EXPRESSION IN TUMOR CELL LINES.

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The HLA class-I altered tumour phenotypes may have important consequences for tumour-cell resistance against specific CTLs. However, the loss of HLA class I molecules could make tumour cells susceptible to NK lysis. HLA-E molecule has been reported to be the major ligand for the natural killer inhibitory receptor CD94/NKG2A and protect cytomegalovirus infected cells from NK cell-mediated citotoxicity. The expression of this HLA class Ib on tumour cells may represent a similar strategy to evade the immune response, particulary in tumors with defects in HLA-ABC expression.
We analyzed the surface expression of the HLA-E in 40 tumour lines of different origin, using specific HLA-E mAbs. HLA-E cell surface expression was only evident in tumours of hematopoietic lineage. Analysis of HLA-E mRNA revealed high levels of HLA-E transcripts in all cell lines examined, indicating that a post-transcriptional mechanism should be regulating the expression of this molecule at the cell surface. In fact, HLA-E cell surface expression could be significantly increased in some melanoma cell lines after incubation with peptides derived from the signal sequence of HLA-A2403, and exogenous human β2-m.
The present study support that cell surface expression of HLA-E is not a frequent event in human tumour cell lines, and it is limited to hemapoietic cell lines. Weak HLA-E cell surface expression was also observed in other tumor cell lines that showed defects in the expression of HLA-ABC alleles indicating that peptides and competition for β2m may affect the cell surface expression.

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IL-6 CYTOKINE GENE POLYMORPHISM IN RHUMATOID ARTHRITIS.

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Introduction: It has been shown that polymorphism in Interleukin-6 (IL-6) gene (position - 174) is correlated with in vitro cytokine production level.
Objective: to determine the influence of this polymorphism on RA, independently from the HLA-DRB1 predisposing effect, IL-6 gene promoters were genotyped in individuals lacking the HLA-DRB1 RA associated shared epitope (SE) alleles.
Material and Methods: a Polymerase Chain Reaction using sequence specific primers was used in 47 RA patients and 32 controls selected to have HLA-DRB1 SE negative genotypes.
Results: 66% SE negative RA patients have an IL-6 low or intermediate producer genotype (-174 C/C or G/C), versus 34% in controls OR 3,7 (CI. 1,4 – 9,5).
The difference between IL-6 C/C or G/C genotypes and G/G genotypes distribution in patients and controls is significant ($p < 0,01$ $pc < 0,02$). Therefore, in the SE negative group studied, IL-6 G/G high producer genotype is acting as a protective factor. It is interesting to note that, in this particular group of patients in which no genetic marker has been described yet, a particular cytokine gene pattern allows to differentiate RA patients from controls.
Conclusion: IL-6 gene promoter genotyping may identify patients at risk for RA in DRB1 shared epitope negative individuals.

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DQCAR AND D6S273 POLYMORPHISM IN PATIENTS SUFFERING FROM RHEUMATOID ARTHRITIS

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Rheumatoid arthritis (RA) is a chronic articular inflammatory disease associated with DRB1 alleles that share a highly conserved amino acid sequence in the third hypervariable region of the DRB1 molecule. These associations are incomplete because not all patients carry the disease susceptibilty alleles and not all disease epitope positive individuals develop RA.
The aim of the present study was to define the genetic contribution of the DQCAR and D6S273 loci in RA development. One hundred and fifteen patients with RA and 141 controls were employed for this study, in which DQCAR, D6S273, and DRB1 allele typing were performed. All patients had definite classical seropositive RA. Statistical analyses were performed using a Fisher's exact test.
The frequencies of D6S273 alleles 132 (RR=1.3, $p<0.01$) and 138 alleles (RR=3.5, $p<0.001$) were significantly increased in RA patients compared with controls. On the other hand, 140 allele is the least frequent in RA patients as well as in controls (4.7% and 17%, respectively). No significant differences between RA and controls in terms of DQCAR allele frequencies could be found. Allele 138 was significantly higher in disease epitope negative patients in our population.
In conclusion, these data suggest that D6S273 alleles are probably a useful risk marker for RA susceptibilty.

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SOLUBLE HLA-DR ANTIGEN LEVELS IN SERUM CAN BE AFFECTED BY GENETIC FACTORS AND THERAPY IN RHEUMATOID ARTHRITIS.

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Correlations were investigated between soluble HLA-DR (SHLA-DR) molecules and clinical disease activity, ESR, CRP, rheumatoid factor (RF) and Waaler-Rose test (WaRo), in the presence or absence of disease-associated epitopes (Q)R/KRAA in the HLA-DRB1 chain, and during different types of therapy (254 samples from 88 RA patients). Weak but significant correlations of sHLA-DR levels with disease activity were detected. No significant differences in sHLA-DR levels or disease activity in groups treated with several disease modifying antirheumatic drugs, or with or without corticotherapy were found. However the combination of methotrexate (MTX) and prednisolone (Pr) was associated with lower sHLA-DR (205±21 ng/ml, n=34) than the mean value for all other available samples (306±16 ng/ml, n=217), $p<0.001$. This corresponded with significantly lower EULAR pain and swelling scores, ESR and RF titer ($p<0.02$ to 0.001) in the group with MTX and Pr, compared to the others. In contrast, we found significantly higher scores for pain, swelling, CRP, RF and WaRo titers ($p<0.05$ to $p<0.001$) in the patients treated with nonsteroidal anti-inflammatory drugs (NSAIDs), compared to those without. However, sHLA-DR was significantly lower during therapy with NSAIDs (267±15 ng/ml, n=182) than in those without NSAIDs (358±31 ng/ml, n=72), $p<0.01$. In the NSAID group, sHLA-DR was 320±21 ng/ml (n=115) in carriers of the disease-associated epitopes and 167±14 ng/ml (n=42) without the epitopes, and without NSAID therapy respectively 459±45 ng/ml (n=35) and 199±27 ng/ml (n=22) (both $p<0.001$). In conclusion, the combination of MTX and Pr reduces sHLA-DR and disease activity, while in contrast patients treated with NSAIDs have lower sHLA-DR levels in the presence of higher disease activity than without NSAIDs, indicating a different mechanism affecting serum sHLA-DR molecule levels.

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ASSOCIATION BETWEEN A POLYMORPHISM IN THE STROMELYSIN-1 (MMP-3) GENE PROMOTER AND SEVERITY OF RHEUMATOID ARTHRITIS (RA) IN A PROSPECTIVE STUDY

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Rheumatoid arthritis (RA) is an autoimmune disease characterised by chronic inflammation of the joints. Both genetic and environmental factors contribute to RA susceptibility and severity. Stromelysin-1 (MMP-3) is considered as the main metalloproteinase involved in articular cartilage degradation. We tested the possible association between a polymorphism in the MMP-3 gene promoter and susceptibility to, and severity of RA.
One hundred and three patients with early RA were included in this prospective study. A radiographic damage score was used to quantify disease severity at baseline and after 4 years of follow-up. The 5A/6A biallelic polymorphism in the MMP-3 gene promoter was analysed using a fluorescent-based polymerase chain reaction. One hundred and twenty-seven unrelated healthy individuals were used as controls.
Allele and genotype frequencies did not differ between patients and controls ($\bullet^2$ test). The distribution of the radiographic damage score was different across all three MMP-3 genotypes both at baseline ($p<0.01$) and after 4 years of follow-up ($p<0.05$) (Kruskal-Wallis test). The MMP-3 6A/6A genotype was associated with the highest radiographic damage score while the 5A/5A genotype was associated with the lowest. This association concerned both the erosive and the joint space narrowing components of this score.
These results provide the first evidence of an association between a polymorphism in the MMP-3 gene promoter and severity of rheumatoid arthritis.

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VITAMIN D RECEPTOR (VDR) POLYMORPHISM IN PATIENTS WITH RHEUMATOID ARTHRITIS: ASSOCIATIONS WITH CLINICAL AND LABORATORY PARAMETERS

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Recent studies have shown that VDR is involved in mineral and skeletal metabolism and plays an immunoregulatory role as well. On the other hand, Rheumatoid Arthritis (RA) strongly associated with HLA antigens, is also probably associated with other genetic factors.
In order to study the VDR polymorphism in Greek RA patients as well as any associations with clinical and/or laboratory parameters of the disease 156 RA patients (130 females, 26 males) and 112 controls (60 females, 62 males) were studied by molecular techniques for a) VDR polymorphism (using Apa I, Taq I and Bsm I restriction endonucleases) and b) HLA-DRB1* shared epitope (SE).
Results: A) The frequencies (%) of VDR genotypes (bb, BB, Bb, aa, AA, Aa, tt, TT, Tt) presented no statistical differences between 1) RA patients and controls, 2) patients with and without SE and 3) seropositive and seronegative patients. B) Patients with erosions compared to patients without erosions showed an increased frequency of BB (16.54% vs 7.41%, OR=2.48) and tt (14.96% vs 7.41%, OR=2.20) genotypes, the difference being more pronounced in patients with destructive arthritis (22.22% vs 7.41%, OR=3.57).
In conclusion, it seems that VDR although it is not associated with disease susceptibility, is rather involved in immunopathogenetic mechanisms for disease progression and severity.

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ASSOCIATION OF VITAMIN D RECEPTOR WITH TYPE 1 DIABETES MELLITUS USING *FOKI* IN THE DUTCH POPULATION.

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The vitamin D receptor (VDR) is a ligand–activated transcription factor. The VDR mediates the effects of 1,25-dihydroxyvitamin D_3 on gene expression.

Reports have described an association with genetic VDR variants and type 1 diabetes (T1D). Four major allelic variants of the VDR gene have been identified: *Fok*I in exon 2, *Bsm*I and *Apa*I in the intron separating exon 8 and 9 and a silent *Taq*I variant in exon 9. FokI is the only allelic variant leading to an expressed protein variant as a result of an alternative transcription initiation site.

We have collected 206 Dutch families with juvenile onset Type 1 Diabetes consisting of a patient with both parents. We have assessed the association of *Fok*I with T1D. Association was calculated by the Transmission-Disequilibrium-Test. A significant transmission of the "T" allele that leads to the expression of the protein with 3 additional amminoacids was found.

We conclude that our findings provide further evidence for the VDR gene as a locus marking susceptibility of T1D, and that the *Fok*I variant leading to expression of an alternative receptor is associated with T1D.

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HLA HAPLOTYPES DETERMINED AMONG CAUCASIANS AND ORIENTS AND CORRELATED TO TYPE I DIABETES

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We studied HLA-haplotypes associated to the type I diabetes in 2 Russians groups (Caucasians): group N1 – Moscovites, group N2 – Pomors from the North sea shore and 2 Orient groups: group N3 – Uzbecks from Tashkent (Middle Asia), group N4 – Tuvinians (Western Siberia). In group N2 the type I diabetes morbidity was twice higher as compared to both groups N1 and N3; and 4 times higher as compared to group N1. Our data on HLA haplotypes associated to the type I diabetes are submitted in a table below. It also reflects HLA profiles of the groups since we determined expressed differences in HLA alleles' frequencies (including type I diabetes-associated) between Moscovites and Pomors, and between Uzbecks and Tuvinians. It is noticeable that Pomors' HLA profile is alike to Finns. In Uzbecks the HLA-associations to the type I diabetes are thought the most expressed among Orients. In Uzbecks we detected high frequencies of some alleles and haplotypes typical for Caucasians.

HLA-haplotypes	Uzbeck (n=69)	Tuvinian (n=15)	Moscovite (n=79)	Pomor (n=48)
DRB1*03-DQA1*0501-DQB1*0201	**0,3986**	**0,2667**	0,2405	0,1711
DRB1*04-DQA1*0301-DQB1*0302	0,2536	0,1000	**0,4620**	**0,5921**
DRB1*15-DQA1*0102-DQB1*0602	0,0073	0,0667	0,0127	0,0

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THE MIC-A ALLELES' FREQUENCIES IN HEALTHY RUSSIANS AND IN TYPE I DIABETIC PATIENTS

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Besides the previously reported data on HLA-associations to type I diabetes there appeared the data on HLA MIC-A gene association to type I diabetes (G.Gambelunghe et al.) The aim of our study was to investigate possible association between the MIC-A gene polymorphism and type I diabetes in Russians. The MIC-A gene is located between the TNFA and the HLA-B gene.

Methods. Type I diabetic (n=65) and healthy (n=71) Russian subjects were genotyped for MIC-A exon 5. Exon 5 was PCR amplified. Followed amplification, a number of the GCT triplet repeat units were determined by fragment analysis using an automated DNA sequencer (Visable.Genetics, Canada). The results are submitted in a table below.

The MIC-A alleles' frequencies in diabetic patients and healthy controls

Alleles	Patients (%)	Controls (%)
A4	13%	18%
A5	16%	12%
A5.1	39%	38%
A6	10%	14%
A9	22%	18%

The differences in MIC-A alleles' frequencies among Russian diabetic patients as compared to healthy Russian controls were not determined.

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DIFFERENCES IN THE GENETIC BACKGROUND OF LATENT AUTOIMMUNE DIABETES IN ADULTS (LADA) AND TYPE 1 DIABETES MELLITUS

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According to the recent classification of diabetes mellitus the Latent Autoimmune Diabetes in Adults (LADA) belongs to the group of type 1 autoimmune diabetes, as a slowly progressive form. Our aim was to determine the prevalence of HLA-DRB1 and DQB1 genotypes, and the tumor necrosis factor (TNF) alpha promoter polymorphism at position –308 (the G→A substitution, designated the TNF2 allele) in patients with type 1 diabetes and with LADA compared to the healthy population.
42 type 1 diabetic and 39 LADA patients were tested. As control cadaver donors and samples of 138 volunteers were used.
The major histocompatibility complex (MHC) II genotypes and the TNF alpha promoter polymorphism were determined by PCR method. Both type 1 diabetes and LADA were positively associated with the HLA-DR4/DQ8 haplotype (p=0.00001, and p=0.001, respectively), and negatively associated with the DR11/DQ7 haplotype (p=0.0016, and p=0.009, respectively) compared to the control population. There were differences between the two disease entities in the frequency of the DR17(3)/DQ2 haplotype (p=0.009 vs p=0.237) compared to the control group.
The presence of TNF2 allele was significantly lower in LADA than either in type 1 diabetes (p=0.034) or in the control group (p=0.029).
Conclusion/hypothesis. Our findings indicate that there are marked differences in the genetic background of type 1 diabetes and LADA. The low presence of TNF2 allele (known to be associated with high amount of TNF alpha production) in LADA could be one of the factors responsible for the relatively slow progression.

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ASSOCIATIONS OF HLA DQB1 AND DRB1 ALLELES WITH THE LADA IN CZECH POPULATION.

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To examine the HLA - class II association with the type 1 diabetes mellitus in Czech adults (LADA) we performed a study of 188 patients diagnosed after 35 years of age and 99 control subjects. We compared our results with the HLA typing in Czech diabetic children. Patients were divided into three and two subgroups according the concentrations of C-peptide and antiGAD respectively. For genotyping of HLA DQB1 and DRB1 alleles we used polymerase chain reaction with sequence specific primers (SSP PCR). The significance of DQB1*0302, DQB1*0201, DRB1*03 and DRB1*04 alleles as risk factors to T1DM was evaluated by examination of their relative risks, that were computed by testing 2x2 tables considering uncorrected P<0,05 as significant. The DQB1*0302 (OR=4,80; group CP), DRB1*03 (OR=7,84; group antiGAD+) and DRB1*04 (OR=2,88; group antiGAD+) were significantly associated with risk to the T1DM. DQB1*0301 (OR=0,38; group antiGAD+), DQB1*0602 (OR=0,11; group antiGAD+) and DQB1*0603 (OR=0,00; group antiGAD+) alleles were significantly protective to the T1DM. The risk of the disease was higher associated with antiGAD+ group than with the group CP. Moreover, the importance of DQB1 and DRB1 alleles was age dependent. To summarize our analysis: the highest association of HLA class II alleles, particularly DRB1*03 allele, has been described in the Czech LADA antiGAD+ group of patients. The significance of all associations was lower in adult patients than in diabetic children.

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HLA-DQ POLYMORPHISMS MODIFY THE RISK OF THYROID AUTOIMMUNITY IN TYPE 1 DIABETIC CHILDREN

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Increased prevalence of thyroid autoimmunity (TAI) in patients with Type 1 diabetes mellitus (DM) is well known. Since sharing of genetic risk factors between the two autoimmune diseases is rather limited, we sought to determine the contribution of HLA-DQA1 and DQB1 polymorphisms to TAI susceptibility among diabetic children.
A group of 285 diabetic children (158 boys and 127 girls, with DM onset at the age of 7.7±4.3, mean±SD) was followed up for 5.9±3.7 yrs using yearly tests for autoantibodies against thyroid peroxidase and thyroglobulin. TAI was defined as repeated positivity of at least one of these autoantibody markers. The HLA-DQA1, -DQB1 genotype, and the DRB1*04 subtype were determined in 239 / 285 children using PCR-SSP. The phenotypic frequencies were compared between diabetic children with and without TAI, and the risk levels were expressed using odds ratio with 95% confidence intervals.
TAI was found in 45 / 285 diabetic children (16 %). The HLA-DQB1*0302 allele conferred susceptibility to TAI in diabetic children (OR 2.6, CI 95% 1.1-6.2); this effect was more apparent in children with later DM onset due to lower background prevalence of the allele. The DQB1*05 alleles act protectively (OR=0.24, CI 95 % 0.08 - 0.70), as well as the DQA1*01 allele (OR=0.30, CI 95% 0.13 - 0.70). No effect was seen for the DRB1*04 subtypes.
We demonstrate that the HLA-DQ polymorphisms modify TAI risk in diabetic children. However, clinical use of the association as a tool for differential TAI screening is rather unlikely due to its modest strength.

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HLA I AND II CLASS POLYMORPHISM IN MYASTHENIA PATIENTS WITH THYMIC HYPERPLASIA.

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Myasthenia Gravis (MG) is an autoimmune disease of the neuromuscular junction having multigene control. It seemed of interest to study the HLA-associations of this disease in patients of North-West region of Russia. Previously we have revealed, that in mixed group of unrelated patients with MG (thymoma and thymic hyperplasia, n=30) there was a significant increase of frequencies of the following HLA specificities: A1, B8, B18, DRB1*03 and DRB1*16, and decrease of A24, as compared to population control group (1st group, n=1011 for HLA-A и -B loci, and n=208 for DRB1). When the group of MG patients was marked out with histologically verified thymic hyperplasia, who undervent transsternal thymectomia (2nd group, n=14, average age 33,5 years, 64,3% -female) we have found, that HLA I и II class typing results on the whole confirmed the tendencies revealed for the mixed group. HLA frequencies are shown in the table. χ^2 values are calculated with Yates correction for small groups.

group	A1	A24	B8	B18	DRB1*03	DRB1* 16
1st	22,4%	20,9%	14,8%	14,2%	15,9%	5,8%
2nd	42,9%	0%	57,1%	35,7%	42,9%	21,4%
χ^2 1-2	1,9	2,1	14,1*	2,92	4,87*	2,92

* - $p<0,05$

Thus, in Noth-West Russian region in patients with MG with thymic hyperplasia , we have revealed statistically significant associations with HLA-B8 (RR=7,65, EF=0,45) and DRB1*03 (RR=3,98, EF=0,27). These data correspond to associations found by other researchers in Caucasoid population groups in Europe.

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PUTATIVE ROLE OF KIR GENES IN AUTOIMMUNITY

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Killer Cell Immunoglobulin-like Receptors (KIR) are membrane glycoproteins of the immunoglobulin (Ig) superfamily that modulates natural killer (NK) cell function upon recognition of HLA class I. In recent years, KIRs have emerged as a highly polymorphic multigene family. The number of KIR genes varies between individuals, resulting in different KIR haplotypes. Since the KIRs have opposing effects on signal transduction pathways and effector function, it is likely that polymorphisms in KIR genes can play a role in development of autoimmune diseases, including Type 1 Diabetes Mellitus. To determine the putative role of KIR in susceptibility to Type 1 Diabetes Mellitus, we performed PCR-SSP typing on a group of juvenile diabetic patients (n=130) and a random panel of the Dutch population (n=192). We typed for KIR genes responsible for inhibitory functions (2DL5), and activating signals (2DS1, 2DS2, 2DS3, 2DS5). These KIR genes appear as a cluster spanning approximately 50-kb of the KIR region. The results show significant increases and decreases of specific KIR profiles in the diabetic population as compared to the healthy controls. It is conceivable that these differences are dependent on MHC types.

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MCP-1 PROMOTER POLYMORPHISM IN SPANISH PATIENTS WITH SYSTEMIC LUPUS ERYTHEMATOSUS

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Systemic lupus erythematosus (SLE) is a multi-organ disorder characterized by an inflammatory response concluding in tissue damage, in which inflitrating mononuclear leukocytes may play a crucial role. The migration of leukocytes through vessels and tissues is dependent in part on chemokines. Monocyte chemoattractant protein-1 (MCP-1) is a β-chemokine that is thought to be responsible for monocyte and lymphocytes T recruitment in acute inflammatory conditions and may be an important mediator in chronic inflammation. Thus, genetic polymorphisms in the regulatory regions of the MCP-1 gene could be implicated in the susceptibility or progression of autoimmune diseases. A biallelic G/A polymorphism at position –2518 of the MCP-1 gene has been described. This polymorphism seems to influence the transcriptional activity because cells from individuals GG or AG produced more MCP-1 than those from individuals AA. The aim of this study was to investigate the possible role of this functional polymorphism in SLE. 276 SLE Spanish patients, among them, 99 with lupus nephritis (LN) and 55 with cutaneous vasculitis (CV), and 194 ethnically matched healthy controls were included. Genotyping for –2518 (A/G) MCP-1 gene was performed by PCR-RFLP. No association between –2518 MCP-1 polymorphism and susceptibility to SLE nor to LN was found. However, a significant increase in the frequency of genotype AG and a decrease in the frequency of genotype AA were found among patients with CV (51% of AG vs. 32% in individuals without CV; p=0.008, OR=2.2, 95% CI: 1.18-4.25; and 47% of AA vs. 64%; p=0.03, OR=0.5, 95% CI 0.27-0.96, respectively). These results indicate an association between the presence of G at position –2518 in the MCP-1 gene and the presence of CV among SLE patients. This polymorphism does not seem to influence the susceptibility to SLE nor the appearance of LN.

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HLA-DR AND DQ ASSOCIATIONS WITH MULTIPLE SCLEROSIS IN PORTUGUESE PATIENTS: INFLUENCE ON THE OUTCOME AND CLINICAL COURSE.

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Introduction: HLA-DR2 and more recently the haplotype DRB1*15–DQB1*06 were identified as susceptibility factors in Multiple Sclerosis in different populations, since 1972. The contribution of these alleles to MS in a Portuguese population has not been investigated so far.
Objectives: To identify HLA class II (DR e DQ) alleles in a MS population and to establish the relationship between the frequencies obtained and clinical and demographic data.
Methods: A total of 100 patients (65 women and 35 men), 79 with the relapsing-remitting form (RR) and 21 with the Secondary Progressive form (SP) were studied. A group of 100 unrelated healthy controls was used as a reference. HLA typing was performed by PCR-SSOP and PCR-SSP.
Results. HLA-DR2 and HLA-DRB1*15-DQB1*06 allele and haplotype frequencies were increased in the MS population (39% vs. 19,6%, p=0,0003, OR= 2,625), and (30% vs. 12% p= 0,0003,OR=3,143). A detailed analysis of data showed that this increase was significant only in the RR group (43% vs. 19,6% p=0,00008, OR=3,103). In the SP group, the frequencies of HLA-DR4 and HLA–DQ4 were increased (42,1% vs. 14% and 15,8% vs. 4,5% respectively). HLA-DRB1*13 (DR6) frequency was decreased in MS patients (15,8% vs. 31,2% in controls).
Conclusions: The results obtained confirm that HLA-DRB1*15-DQB1*06 represent a major susceptibility factor to MS in Portuguese patients. HLA-DR4 and DQ4 may be associated with increased severity of MS and HLA-DRB1*13 could have a protective role in MS.

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HLA DRB1*ALLELES AND AA 86 BETA CHAIN INFLUENCE ON CLINICAL COURSE AND IN MULTIPLE SCLEROSIS

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Multiple Sclerosis (MS) aetiology and physiopathology remain misunderstood but numerous authors agree that there are interactions between environemental and genetic factors. So HLA genes and particularly DRB1*1501 allele are associated with disease susceptibility. Myelin basic protein(MBP) seems to be an important target antigen presented to T cells by HLA DR molecules.We suppose that interaction between HLA DR and MBP exists through amino acid(AA) 86 of DRbeta chain.The aim of this study was to determine the possible influence of AA 86 , Valine or Glycine, on susceptibility and prognosis of MS.
HLA DRB1* alleles determined by PCR SSP in 89 patients were compared with 460 typing of unrelated controls from French Southern bone marrow registry. The presence of amino acid valine or glycine at residue 86 was infered from previous data obtained by HLA allele sequencing. Our study confirmed DRB1*1501 susceptibility to MS and its influence on disease course. Relapsing-remitting and secondary progressive(SP) forms were more frequent in DRB1*1501 sub-group (p<0,01) but there were no association with primary progressive(PP) forms.
Codon 86 type did not influence susceptibility to MS but could have a part on disease outcome. Patients with relapsing-remitting form were predominantly Val/Val (p<0,01),wereheas secondary progressive were Val/Gly(p<0,01)and primary progressive Gly/Gly (p<0,01).So, there is correlation between clinical severity and AA86.
Determination of DRB1 86 codon seems to be a predictive factor for disease course,complementarly to clinical and MRI data. These data could be important to adaptate treatment, as soon as possible, and so limit handicap development.

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CHILDHOOD CELIAC DISEASE AND HLA ASSOCIATION IN HUNGARY. FAMILY STUDY.

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The systematic care of celiac children has begun in 1978 in our region. The diagnosis of GSE was performed according to the criteria of ESPGAN. While the cumulated occurrence of GSE was observed among the family members of the celiac children patients, this care was extended to their family to find the potentially threatened, seemingly symptomfree individuals. Our care has focused on the non invasive investigations in these cases, namely: clinical investigations, anti-gliadin antibody and different kind of auto-antibody determinations, HLA-Class I and class II. determinations.
Now we demonstrate the HLA typing result of 149 cases (39 families, 43 GSE patients, and 106 first degree relatives). Methods: serological typing of HLA antigens, statistical evaluation was performed by the calculation of RR and Fischer's exact test. Controls: 542 healthy bone marrow donors representing the population of the S-W Transdanubian Region of Hungary.

HLA	BMD control	GSE children		Family members	
	%	%	P	%	P
B8	16,6	57,5	0,001	42,1	0,001
Cw7	37,29	55,9	0,0342	52,3	0,0147
DR3	31,53	80	<0,0001	56,5	0,001
DQ2	30,8	82,1	<0,0001	60,6	0,0001

The most interesting finding were those results, were the serologically defined HLA Class I. and Class II antigens were identical between the siblings, and one of them was celiac patient while the other, seemingly healthy (8 x2 brothers-sisters).Their more detailed analysis will occur (hopefully) at the forthcoming IHWG.

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MICA-A5.1 ALLELE IS ASSOCIATED WITH *ATYPICAL* FORMS OF CELIAC DISEASE IN HLA-DQ2-NEGATIVE PATIENTS

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We selected 38 consecutive celiac disease (CD) patients (from a group of 316 consecutive CD patients) and 91 healthy blood donors, all of whom were HLA-DQ2 (DQA1*0501/DQB1*0201) negative, and investigated the presence of classically associated alleles HLA-DQ8 and HLA-DRB4. We also studied the distribution of MICA TM alleles in the two clinical forms of the disease. For this reason, these 38 DQ2-negative patients were subdivided into two groups: 18 *typical* CD patients and 20 *atypical* ones.

No differences were found in the distribution of DRB4 allele between DQ2-negative patients and controls. HLA-DQ8 heterodimer (DQA1*03xx/DQB1*0302) was increased in CD patients (29%) compared with controls (10%), but no statistical differences were found. No differences were observed in the frequency of these alleles between either group of CD DQ2-negative patients. MICA-A5.1 was increased in atypical CD patients when compared with the *typical* forms of disease (p_c=0.03) and with healthy controls (p_c=0.002). No other MICA allele was found to be significantly increased in the groups under study. The presence of MICA-A5.1 in *atypical* CD DQ2-negative patients may indicate a possible role of this allele in the development of CD.

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AN ADDITIVE RISK FACTOR FOR CELIAC DISEASE (CD) IN THE DR3-B8 EXTENDED HAPLOTYPE

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Additive susceptibility factors in the HLA region, besides the well known DQB1*0201/A1*0501 heterodimer, have been suggested to play a role in CD susceptibility. To investigate this hypothesis we tested the association with CD of 7 microsatellites (TNFa, MICB, MICA, MIB, D6S265, MOGc and D6S2239) spanning the region from class III to HFE, 4.5 Mb telomeric of HLA-A. The association was tested by comparing allele frequencies in transmitted and non-transmitted haplotypes in CD families. The analysis was restricted to DR3 haplotypes (hp). There were a total of 49 transmitted (T) and 14 non-transmitted (NT) DR3 hps in the 50 tested DR3+ simplex families and 74 DR3 hps transmitted to both children (TT) and 15 never transmitted (NTNT) in the 102 tested DR3+ multiplex families. Significant results concern three loci closely linked to HLA-B (see table). Notably, MICA5.1 and MIB350 are both markers of the DR3-B8 extended haplotype. A significantly increased conservation of this hp from DR to MIB was observed among transmitted hps. In fact the haplotypic combination DR3-TNFa2-MICB24-MICA5.1-MIB350 was detected in 44/113 transmitted vs 4/27 non-transmitted hps (p=0.032, O.R.=3.67). Our data suggest that the DR3-B8 hp carries an additive factor affecting susceptibility to CD close to the HLA-B region.

	Simplex			Multiplex			Simplex+Multiplex	
	T	NT	p	TT	NTNT	p	p	O.R.
MICA5.1	22/47	4/14		44/70	5/15		0.02	2.88
MIB350	22/45	2/13		35/68	3/14		0.005	4.48
MICB15	1/47	4/13	0.006	1/71	1/15		0.003	0.08
MIB348	2/45	3/13		3/68	4/14	0.001	0.002	0.13

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CTLA-4 +49A/G DIMORPHISM IN ITALIAN PATIENTS WITH CELIAC DISEASE.

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The cytotoxic T-lymphocyte antigen 4 (CTLA-4) region on chromosome 2q33 has been reported in linkage and association with Celiac Disease (CD). In order to test the association between the biallelic polymorphism at position 49 of the CTLA-4 exon 1 and susceptibility to CD in the Italian population, we performed case-control and family-based studies. The +49 A/G dimorphism, leading to a Thr/Ala substitution in the leader peptide, was analysed in 177 CD patients, 302 relatives and 144 ethnically matched controls by PCR-RFLP method. The CTLA-4 A allele frequency was increased in patients compared with healthy controls (76% versus 65.6%; $p=2.6x10^{-3}$). This difference reflected the increase of AA genotype (57.6% vs. 45.8%; $p=2.3x10^{-2}$) and a decrease of GG genotype (5.6% vs. 14.6%; $p=6.1x10^{-3}$). The segregation analysis showed a preferential transmission of the A allele to the affected children (59.8%; $\chi^2_{TDT}=3.9$).
When the patients were stratified by the presence of the HLA-DQA1*05 and HLA-DQB1*02 alleles, coding for the high risk HLA-DQ2 heterodimer, the AA genotype raised to 78.3% in the DQ2 negative group showing a difference statistically significant versus both controls ($p=3.3x10^{-3}$) and DQ2 positive patients ($p=2.5x10^{-2}$). In the families where the proband was DQ2 negative, the A allele resulted transmitted in 6 of 7 cases. Altogether, these data support CTLA-4 as predisposing gene for CD in Italian population with a major role in patients not carrying the high risk HLA molecules.

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MICA GENOTYPES AND THE PROGRESSION OF HCV INDUCED LIVER INJURY.

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INTRO: The host immune response (IR)to HCV infection is thought to play a role in the pathogenesis of hepatic injury, and progressive liver injury has been associated with cytotoxic lymphocytes (NK and T cells). This cellular IR to HCV infection is regulated by a complex balance of cell surface receptors and engagement of activating receptors such as NKG2D results in Th1 cytokine secretion, and cytotoxicity. MHC class I chain-related (MIC) molecules have been described as stress-induced antigens, recognised by NKG2D and inducing IRs involving T cells and NK cells. MIC expression is regulated by polymorphic genes (MICA, MICB). The aim of this study was to analyze the relationship between MICA genotypes and HCV-induced liver injury.
METHODS: 136 HCV RNA positive individuals with histologically proven liver disease were analyzed for duration of infection, mode of transmission, fibrosis scores, and liver function tests. DNA was extracted by standard methods and MICA genotyping performed by PCR SSP for 21 MICA alleles. Controls were 122 caucasoid donors. The 136 individuals were scored for degree of fibrosis and divided into mild and cirrhotic/pre-cirrhotic groups. Histologic activity index (HAI) was also scored for degree of inflammation and divided into mild and severe. These groups were again divided according to duration of infection.
RESULTS: No apparent differences in the frequency of MICA alleles was observed between any of the groups and the control population.

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HLA and TNF POLYMORPHISMS IN SPANISH PATIENTS WITH CHRONIC HEPATITIS C.

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Infection with hepatitis C virus (HCV) causes acute and chronic liver disease with different severity and outcome. Genes located within MHC play a major role in influencing the immune response against infectious agents. The aim of this study was to investigate the possible role of MHC class I and class II, and TNF in predisposing to chronic HCV infection. Two hundred and ninety three patients suffering from biopsy-proven chronic hepatitis C with compensated liver disease were enrolled. All the patients were HbsAg negative, HIV negative, anti-HCV positive and serum HCV RNA positive, presenting raised ALT levels. 194 ethnically matched bone marrow transplantation donors were used as healthy controls. HLA-class I (A and B) was determined using complement dependent citotoxicity techniques. HLA-DRB1* low resolution typing was performed using Dynal RELI ™ SSO HLA-DRB typing kits. TNF –238 and –308 promoter genotyping was performed employing PCR-ARMS Frequency of HLA-B44 was decreased in patients (24.9% vs. 39.7%, OR=0.50; 95% CI 0.33-0.76, p<0.0006). Additionally, the frequency of the genotype AG at the position –238 of TNFα gene was increased in patients (13.7% vs. 4.1%; OR= 3.68; 95% CI 1.61-8.72, p<0.0006). No differences in the distribution of HLA-DRB1* specificities nor in the –308 TNFα promoter polymorphism were found among patients and controls. In conclusion, several loci located within the MHC region could to conditioned the immune response against HCV.

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NOVEL GENETIC POLYMORPHISM OF HUMAN INTERLEUKIN-21 RECEPTOR IS ASSOCIATED WITH ELEVATED IgE LEVELS

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The interleukin-21 receptor(IL-21R) was recently described as a novel member of class-I-cytokine-receptor family. Its ligand IL21 is produced by activated T- and B-cells and the biologic functions are currently under intense investigation. The IL-21R gene is directly adjacent to the encoding interleukin-4 receptor gene (IL-4R) – furthermore it shows strong sequence homologies to this IL-4R. IL-4R is assumed to be a candidate atopy-related gene and because of the close genetic relationship we analysed all exons of human IL-21R gene and its 5' promotor region in 20 healthy volunteers by direct DNA sequencing. We identified four novel single nucleotide polymorphisms(SNPs) and developed a PCR-SSP genotyping method to determine the allele frequencies in 300 healthy blood donors. Total serum IgE levels were measured in all blood donors and associated with the IL-21R SNPs. Results revealed an association of one polymorphism with elevated IgE levels (p=0.024). To specifically address the influence of this SNP on IgE levels, we analysed it in individuals that were wild-type at the other polymorphic loci. This subanalysis confirmed an association with elevated IgE levels (p=0.01) and a positive atopy medical history(p=0.018). In summary, we describe novel genetic variation of human IL-21R that suggests an involvement of the IL-21 pathway in IgE regulation.

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THE INFLUENCE OF QBP5.11 AND QBP5.12 PROMOTERS ON THE LEVEL OF EXPRESSION OF DQB1*0501 AND DQB1*0502 MOLECULES

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Our previous study on the contribution of the polymorphism of DR and DQ coding regions and DQB1 promoter region to the humoral immune response after vaccination with a recombinant hepatitis B antigen (HbsAg) revealed that the haplotype DRB1*1601, QBP5.11, DQB1*0502, DQA1*0102 was over-represented in a group of nonresponders when compared to responders. The same DQ haplotype QBP5.11, DQB1*0502, DQA1*0102 linked either to DRB1*1601 or to DR 14 was also over-represented in a group of Slovenian anti-Ro negative patients (Ro-neg) with systemic lupus erithematosus when compared to anti-Ro positive (Ro-pos) patients. All four possible combinations of DQB1*0501 and DQB1*0502 coding and QBP5.11 and QBP 5.12 promoter alleles were found in Slovenian population. While DQB1*0502 was linked only to QBP5.11 promoter in the group of nonresponders to HbsAg and Ro-neg patients, the same molecule was linked either to QBP5.11 or QBP5.12 in responders to HbsAg as well as in Ro+ patients. On the other hand, the haplotype DRB1*0101, QBP*5.12, DQB1*0501, DQA1*0102 was significantly more common in Ro-pos than in Ro-neg patients. It has been previously reported that QBP3.1 and QBP4.1 promoter polymorphism is associated with differential expression of HLA-DQB1 alleles. The aim of the present study was to investigate the influence of QBP5.11 and QBP5.12 promoters on the level of expression of DQB1*0501 and DQB1*0502 molecules. Total RNA was extracted and cDNA synthesised from a nonresponder to HbsAg vaccination homozygous for DRB1*1601, QBP5.11, DQB1*0502, DQA1*0102 and from a DRB1*0101, QBP5.12, DQB1*0501, DQA*0102 homozygous Ro+ patient. The level of DQB1 specific mRNA was determined using real time PCR.

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THE EFFECT OF HLA-DR AND CYTOKINE GENE POLYMORPHISM ON ANTIBODY RESPONSE TO HEPATITIS B VACCINATION

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Genetic polymorphisms were investigated in relation to humoral response following vaccination with two different hepatitis B vaccines, A and B (Herberviovac (n=162) and Engerix B (n=153)). Nine SNPs in six cytokine and receptor genes (IL-2, IL-4R, IL-6, IL-10, TNF-A and TGF-B1) were typed using multiplex SNaPshot™, a commercially available primer extension method. Individuals treated with vaccine B were also typed for HLA-DR using reverse SSO (Dynal, UK). Comparisons were made using antibody titre data obtained 30 days following the 3rd dose of vaccine. No difference was observed between vaccine A and B in antibody titre. SNPs in IL-2, IL-4R, IL-6, IL-10 and TGFB1 had no effect on antibody titre in either group. The presence of an A at the TNF-A–308 G/A polymorphism (TNF2 allele) was found to be associated with a lower humoral immune response in patients receiving vaccine B (p=0.001). HLA-DRB1*03 was also found to be associated with reduced humoral immune responses (p=0.008). Using an antibody level of <1000IU/l to define reduced response, the presence of TNF2 gave an OR=4.8 (1.6-13.8) for a reduced response and DRB1*03 gave an OR of 3.0 (1.0-8.8). DRB1*03 in combination with TNF2 gave OR=5.5 (1.6-18.6). These results confirm previous findings that the B8-DR3 haplotype is associated with reduced response to hepatitis B vaccine. The close linkage between DRB1*0301 and TNF2 means that a comprehensive genetic study of the HLA region is required to elucidate the causative gene(s) that lead to a reduced humoral response to certain hepatitis B vaccines.

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POSITIONAL MAPPING OF HLA PREDISPOSING GENES TO THE NONRESPONSIVENESS TO ANTI-HEPATITIS B RECOMBINANT VACCINE USING MICROSATELLITES.

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Microsatellites (repeats of a DNA base motif) are interspersed among structural genes and are polimorphic, thus we used this kind of analysis to define the centromeric and telomeric boundaries of the HLA region involved in the non responsiveness to hepatitis B vaccination. The nonresponsiveness to hepatitis B virus (HBV) vaccine is strongly associated with HLA-C4AQ0, DRB1*0301, DQB1*02 haplotype. We extended the vaccination schedule and microsatellite typing to the family members of 14 true non responder infants vaccinated at birth: antibody level steadily < 10mIU/ml after the booster dose. In order to get a better understanding of a possible parent-to-child trasmission of this trait, we selected the following microsatellites: DQCARII, G51152, D6S273, D6S291, TNFd, MICA-TM, MIB, D6S265, D6S2222 and D6S2239. We followed the XIII Workshop protocol. Family study allowed us to perform linkage analysis by means of TDT (Trasmission Disequilibrium Test) and Morton's LOD Score test. The TDT showed that the allele 126 of microsatellite TNFd, mapping in class III region, was transmitted from heterozygous parents to non responder children 100% of times (6/6 times; p=0.01). LOD score values were positive for all except two microsatellites (D6S2222 and D6S2239 located in the telomeric boundary of HLA region) but non higher than 3 (the value suggestive for linkage): the highest value was in HLA class III, 1.68 for the TNFd microsatellite. These results suggest to explore the polymorphism of TNF gene and to deepen the analysis to other microsatellites within the class III region.

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HLA-DRB1* AND DQB1* ALLELE FREQUENCIES IN CAT DANDER ALLERGIC PATIENTS IN COMPARISON TO NON-ALLERGIC BLOOD DONORS

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Background : IgE antibody responses to several common allergens have been linked to certain HLA class II alleles. We analysed the HLA-DRB1* and DQB1* profile of patients allergic to cat dander.
Methods : 143 patients allergic to cat dander as assessed by a positive skin prick test and specific IgE antibodies determined by CAP-assay, were further analysed by ELISA for IgE antibodies to Fel d1 and CSA (cat serum albumin). HLA class II DRB1* and DQB1* alleles were determined by allele specific PCR. 195 blood donors were included as controls.
Results : 38 of 143 patients (26.6%) had IgE antibodies to cat albumin, 142 patients (99.3%) had IgE antibodies to recombinant Fel d1.
Comparison of the blood donors with all 143 cat allergic patients showed an underrepresentation of DRB1*01 alleles (14.6 vs 7.8% ; p=0.0043, p_c=0.073) and DQB1*05 alleles (22.31 vs 12.6% ; p=0.0012, p_c=0.02). DRB1*04 alleles were significantly more frequent in the cat allergic population (20.6 vs 11.5% ; p=0.0010, p_c=0.017). Comparison of the subgroup of CSA positive patients with the blood donor group shows again an overrepresentation of DRB1*04 alleles (27 vs 11.5% ; p= 0.0009, p_c=0.0153) while in the complementary CSA negative group 18.3% of DRB1*04 alleles are found vs 11.5% in the blood donors, which is no longer significant.
Conclusion : IgE antibody response to Fel d1 was found to be negatively correlated with DRB1*01 and DQB1*05 alleles. The ability to raise IgE antibodies against CSA was correlated with the presence of a DRB1*04 allele.

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MOLECULAR TYPING OF HFE GENE MUTATIONS IN CENTENARIANS. A STUDY IN SICILY AND IN SARDINIA.

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Hereditary hemochromatosis (HH) is an HLA-linked inherited disease characterised by inappropriately high absorption of iron by the gastrointestinal mucosa. Recently it has been reported that cysteine-to-tyrosine substitution at codon 282 of the HFE encoding gene sequence, localised at 6p21.3-p22, is responsible for the disease. Other variants, as H63D and S65C, may modify the affinity of the protein for transferrin receptors. The C282Y substitution was mainly found in patients of Caucasoid north-European ancestry. On the contrary, the H63D or S65C substitutions seem not restricted to north-Europeans. In Italy frequency of C282Y has been estimated about 60%, with a decreasing North-South gradient (Caruso et al 2002). We have studied the distribution of HFE polymorphisms in two groups of centenarians from Sicily and from Sardinia to evaluate if HFE alleles might be differently represented in people selected for longevity. DNA samples were obtained from 206 controls (age range from 22 to 65) and 150 oldest old subjects (age range from 95 to 105). Samples were typed for C282Y , H63D and S65C alleles using sequence specific primers. Among the young individuals, none was heterozygous for the C282Y and only one for S65C mutation. About 10% were heterozygous for H63D mutation. Among the oldest old people, percentage of H63D heterozygous subjects was increased (20%) and, in Sicily, eleven were heterozygous for the C282Y mutation, whereas in the Sardinia centenarians we were unable to detect 282Y mutation. Present data seem to suggest that an increase of iron uptake capacity associated to heterozygous C282Y and H63D, significantly increases possibility to reach longevity adding another piece of evidence to the complex puzzle of genetic and environmental factors involved in control of life span expectancy in humans.

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LINKAGE DISEQUILIBRIUM OF HLA-CLASS I ALLELES AND HFE MUTATIONS IN TERCEIRA ISLAND-AZORES

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Background: Mutations of the hemochromatosis gene (HFE) were found to be in linkage disequilibrium (LD) with HLA antigens. Mutation C282Y was shown to be in strong LD with haplotype A3-B7 and H63D mutation with the haplotype A29-B44. Another important HFE mutation was recently described (S65C) suggesting other possible associations with HLA and allowing a better understanding of the disease. The objective of this study was to investigate LD between HLA-Class I alleles and three HFE mutations in Terceira island.
Methods: 213 individuals from Terceira were investigated for three HFE mutations (C282Y, H63D, S65C) by PCR-RFLP and typed for HLA-Class I (A, B) alleles by PCR-SSP. Genic frequencies and LD were estimated using Arlequin V 1.1 and Chi-square values with Microstat Software.
Results: Haplotype A3-B7 was associated with the C282Y mutation ($p<0.0001$) and haplotype A29-B44 with S65C mutation ($p<0.0001$). Mutations S65C and H63D were found in LD with B44 allele ($p<0.0001$).
Conclusions: As previously described, the HFE mutation C282Y was found in LD with haplotype A3-B7. The LD between haplotype A29-B44 and H63D mutation was not found in Terceira; however, this mutation was associated with B44 allele. This is the first study encompassing S65C mutation in Portugal and haplotype A29-B44 and allele B44 were shown to be in LD with this mutation.

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HLA DRB1 ALLELE POLYMORPHISM AND SISTER CHROMATID EXCHANGE FREQUENCY IN TURKISH PSORIASIS VULGARIS PATIENTS

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Psoriasis vulgaris is a skin disease with genetic and immunologic present in 1-3 % of the population. The disease has been shown to correlate well with certain HLA alleles. Several different studies have reported Class I and Class II alleles in common related to psoriasis vulgaris in different population. We performed PCR based typing with sequence-specific oligonucleotids for HLA-DRB1*. Fourty nine patients who were diagnosed and under control of outpatients clinic of Dermatology Department and healthy controls from bone marrow donor volunteers of Istanbul Med Fac. Bone marrow Bank were taken. In our study, positive association of DRB1*07 ($p=0.005$) has been shown with psoriasis vulgaris and other studies supports our findings.
Sister chromatid exchange (SCE), defined as the reciprocal exchange of homologous segments between two sister choromatids occurs as a result of direct or indirect DNA damage. Psoriasis vulgaris patients has been shown to be associated with increased SCE freqency. We performed SCE analysis before and after UV-therapy using by the method of Wolf and Perry Our study has been continued.

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HLA-Cw ALLELES IN PATIENTS WITH PSORIATIC ARTHRITIS

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Psoriatic arthritis (PsA) is an inflammatory arthritis associated with psoriasis. Case control studies of HLA-Cw polymorphism have found that Cw*0602 allele is associated with PsA. The aim of this investigation was to compare the frequency of alleles at the HLA-Cw locus in patients with PsA to that in the control population. Fifty consecutive patients attending the Clinic of Rheumatology between January and November 2001 and 144 unrelated healthy individuals were studied. HLA typing was performed by both serologic and PCR-SSP method, while Chi-squared test with Yate's correction and the Fischer's exact test were used to compare the frequency of HLA alleles in the patients and controls. HLA-Cw*07 allele was present in 20 patients and 16 controls: allele frequency of 21% in the patients versus 5.7% ($p<0.001$) in controls. HLA-Cw*0602 allele was present in 12% of the PsA patients, compared with 4.9% of controls ($p<0.01$). The relative risk (RR) for these two alleles was 5.3 and 3.3, respectively. No statistically significant difference for all other Cw alleles between two groups was observed.
In conclusion, study with larger number of PsA patients is necessary to confirm this finding and to elucidate the significance of HLA-Cw*07 allele in the susceptibility to PsA in our population.

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HLA-B/HLA-C REGION AS A MARKER OF PSORIASIS TYPE I IN CROATIAN PATIENTS

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Psoriasis (PV) is a cronic skin disease strongly associated with several susceptibility genes. Recent genome-wide linkage analyses have identified a locus susceptibility to PV (PSOR1) which is part of HLA region (HLA-B/HLA-C) maped on cromosome 6p21.3.
The aim of the study was to identify risk HLA alleles and haplotypes associated with type I PV according to the age of onset.
Total 138 unrelated patients with type I PV (early onset, <40 years, positive family history) were divided in two groups: 78 patients with 1a (<20 years) and 60 patients with 1b (>20 years). All patients and 181 controls (unrelated healty blood donors) were typed for HLA class I and II using high resolution PCR-SSP method.
Risk alleles in PV patients were: Cw*0602 with gene frequency of 0.298 vs. 0.074 in controls ($p<0.01$), B*57 0.126 vs. 0.016 ($p<0.05$), B*13 0.135 vs. 0.025 ($p<0.01$) and DQA1*0201 0.188 vs. 0.05 ($p<0.05$). Analysis of haplotypic association showed a significant increase of B*13-Cw*0602 haplotype in both PV group (30.77% in 1a and 25.0% in 1b) compared to 4.97% in control group ($p=0.006$ and 0.0218 respectively). 25.64% patients in group 1a and 18.33% in group 1b were positive for haplotype B*57-Cw*0602 compared to 3.31% in controls ($p=0.0139$ and 0.0177 respectively).
Haplotype analysis revealed that early psoriasis onset (<20 years) is more significantly associated with B*13-Cw*0602 haplotype ($p=0.006$ for type 1a) than with B*57-Cw*0602 ($p=0.0139$ for type 1a).

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NARCOLEPSY SUSCEPTIBILITY GENES: HLA AND TNFIIR

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Introduction: Narcolepsy is a sleep disorder associated with HLA-DR2 and HLA-DQ1. HLA-DRB1*1501-DQA1*0102-DQB1*0602 haplotype was identified in almost every Japanese patients, and in 70% of Caucasian and Afro-Americans. Mutations in hypocretin/orexin receptors or loss of hypocretin have been suggested to cause cataplexy and other narcolepsy symptoms. Recently a Japanese report suggested that a TNF-α and its receptor (TNFRII) are associated to narcolepsy susceptibility.
Objectives: To identify exon 6 polymorphism of TNFRII by PCR-RFLP, and HLA class II - HLA-DRB1, DQA1 and DQB1 - *loci* in patients with narcolepsy-cataplexy (NC), narcolepsy without cataplexy (N) and idiopathic hypersomnia (IH).
Methods: A total of 35 patients were diagnosed according to ASDA criteria. Genotyping was performed in 30 of 35 patients: 17/18 with NC, 4/6 with N and 9/11 with IH. Results were compared with a control population (CP).
Results: HLA-DRB1*15(2)-DQA1*0102-DQB1*0602 haplotype was observed in 65% of NC group vs. 12% in CP. HLA-DRB1*03, the most representative allele for IH, was identified in 36% of patients vs. 20.4% of CP. The frequency of 196M/196R genotype of TNFRII was increased in NC (47% vs. 36% in CP).
Conclusions: The results suggest that HLA-DRB1*1501-DQA1*0102-DQB1*0602 haplotype represents a genetic marker, important for distinction between NC and IH. The increase in frequency of M/R genotype of TNFRII in NC group may support the hypothesis of an autoimune response leading to hypocretin destruction in neuronal cells.

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TUMOR NECROSIS FACTOR α HIGH PRODUCER GENOTYPE IN ACTIVE WEGENER'S GRANULOMATOSIS

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Introduction: Wegener's Granulomatosis (WG) is a progressive systemic vasculitis characterised by necrotizing granulomatous lesions mainly affecting the respiratory tract and the kidney. The genetic factors predisposing and influencing to WG are not fully established.
Study design: 32 patients with WG suffering from chronic renal failure have been genotyped for HLA-DRB1/DQB1 and compared to 91 healthy blood donors (HC). In addition polymorphism of the genes encoding TNFα (promoter -238, -308, -376), TGFβ (codon 10 and 25), IFNγ (intron 1 +874), IL-6 (promoter -174), IL-10 (promoter -592, -819, -1082) and CTLA4 (promoter -319, intron 1 +49) were analysed by PCR and/or sequencing. A microsatellite analysis for CTLA4 (AT-repeat in the 3´ untranslated region of exon 3) was performed using fluorescent labeled primers.
Results: The significant difference we observed for HLA-DRB1/DQB1 was a decrease in the frequency of DQB1*0301 (WG = 9%, HC = 33%, p = 0.018). Additionally a trend towards a higher frequency of DRB1*03, DQ*0303, and a lower frequency of DQ*0604 in our patient cohort was found. By sequencing we detected a significant increase of the TNFα G-A genotype at position –238, associated with high production of TNFα. (WG = 19%, HC = 4%, p = 0.029).
Conclusion: This is the first study demonstrating an association of TNFα genotype high producer at position -238G/A in WG. This finding might help to identify patients who can benefit from an anti-TNF-antibody therapy.

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HLA-B27 CLASS I HAPLOTYPES IN PORTUGUESE FAMILIES

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Objectives: To investigate HLA Class I haplotypes in linkage desiquilibrium (LD) in HLA-B27 individuals from families in the Azores population.
Methods: 29 families with HLA-B27 individuals were investigated by direct interview. Ankylosing Spondylitis (AS) was diagnosed according to the New York criteria; pelvic x-rays were performed only if there was an history of inflammatory back pain. HLA Class I alleles were identified by PCR-SSP.
Results: 137 individuals were investigated. 23 families were B*2705, 2 were B*2703, 2 B*2707 and 2 B*2708. 30 patients had AS and 2 had uveitis. B*2703 was in LD with CW2, B*2705 was in LD with CW1/CW2 in 90% of cases, B*2707 was in LD with CW15 and B*2708 was in LD with CW0602. The table shows main A/B haplotypes found in these families.
Conclusions: 1. HLA-A9 (23,24), HLA-A2 and HLA-A19 (31,32) were present in pts and controls in B27 haplotypes and across different B27 subtypes.2. The presence of these alleles in B27 haplotypes probably do not add further susceptibility to AS.

Haplotypes	Nº Families	Nº pts	Nº controls
A9B27	12	11	13
A2B27	9	9	23
A19B27	5	9	7

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HLA B-27 SUBTYPES IN TURKISH PATIENTS WITH SPONDYLOARTHROPATHY AND HEALTHY CONTROLS .

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HLA-B 27 is a common HLA-B allele with a phenotype frequency of %9.4 among caucasians. The strong association between HLA-B27 and spondyloarthropathy (SpA) has been reported by several studies. B*2705 and B*2702 are the most frequent subtypes with a frequencies of about %90 and 5-10 % respectively in caucasians.
In this study, our aim was to investigate the frequency HLA-B27 in Turkish population and the distribution of subtypes of HLA-B27 in HLA-B27 positive controls and SpA patients. 2112 healthy Turkish bone marrow donors were studied by standart microlymphocytotoxicity technique. B27 subtyping was performed by PCR-SSP method using " Genovision - High resolution primers" in two groups: Group 1: 49 unrelated HLA-B27 positive Turkish patiens with the diagnosis of SpA according to the European Spondyloarthropathy Study Group Criteria and followed up by the division of rheumatology and Group 2: 55 HLA-B27 positive healthy controls.
The frequency of B27 was found to be 2.6 % in Turkish population. In patients with SpA three subtypes B*2705 (58.6%), B*2702 (38.5%), B2707 (27.3%) were more frequent. *2702 and B*2705 were the most common alleles with a frequency of 61.5% and 41.4 % in the control group. B*2705 in patients with SpA was significantly different compared to controls (p=0.008, OR= 2.9 with a 95% CI of 1.31-6.57). In this study B*2705 was found as the predominant allele among Turkish patients with SpA. Our study supports other reports from different populations which showed that B*2702 and B*2705 were more in SpA in caucasians.

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HLA PHENOTYPES IN HIV SERONEGATIVE AND SERO-POSITIVE EXPOSED FEMALE SEX-WORKERS IN BURKINA FASO

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HIV infection is known to be influenced by host genetic factors. Among them, particular HLA genes have been shown to independently interfer with HIV disease progression. Particularly, heterozygosity of HLA-A, B and C is associated with the delay onset of AIDS, although in Bw4 homozygotes the HIV viremia is strictly controlled. Such information is not available in sub-saharian Africa, where HIV infection is particularly frequent.

To analyze the influence of HLA component on HIV infection in African patients, a cohort of exposed 33 seronegative (ESN) with a mean follow-up of 24 ± 1.1 months and 55 seropositive (ESP) female sex-workers living in the Bobo-Dioulasso (Burkina Faso) area was studied. This group of individuals was homogeneous according to prostitution practice. Sixty-eight control individuals living in the same area were also included in the study. CCR5, CCR2, HLA class I and class II genes were genotyped by PCR-SSP.

As expected, the Δ32 CCR5 mutation was not found in this population. The CCR2 allele frequency was similar in sex-workers' group and in controls.

No particular HLA allele was found to be associated with HIV status. However some HLA phenotypes were specifically present in ESN or in ESP sex-workers. Among them A*6801-B*5301was significantly underepresented in ESN compared to ESP (p=0.02).

In conclusion specific HLA phenotypes could be associated with susceptibility or resistance to HIV infection in exposed individuals.

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IL-4, IL-10, TNF ALPHA GENES POLYMORPHISMS IN HIV INFECTION IN RUSSIAN POPULATION

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Allelic variants of genes of many cytokines is associated with predisposition to and course of some human diseases. 127 HIV-infected patients and 52 healthy individuals (control) have been examined for evaluation of distribution of polymorphic genes IL-4, IL-10 and TNF alpha and their possible association with HIV infection. Genotyping of C-590T IL-4, C-597A IL-10 and G-308A TNFalpha has been accomplished by PCR-RFLP analysis. Alleles are distributed as follows: 590C IL-4 with frequency 0.79 for HIV-patients and 0.75 for control (p=0.4619; OR=0.81; 95% CI 0.45-1.46); -597C IL-10 with frequency 0.87 and 0.80 (p=0.1635; OR=0.63; 95% CI 0.32-1.26); -308G TNF alpha with frequency 0.94 и 0.87 (p=0.06; OR=0.41; 95% CI 0.15-1.07) for HIV-patients and control, accordingly. Enlarged percentage of genotype G/A -308 TNF alpha contents for HIV- patients has been shown in comparison with control (26 % and 7.7 %, accordingly, p=0.007; OR=4.21; CI 95 % 1.37-17.21). Thus the genetic tendency to production of increased TNF alpha level because of presence -308A can change quality of immune response so that the individual has the increased risk of HIV-infection and the disease development that ends by fast progression to AIDS.
Also we reveal statistically significant differences in genotype distribution of investigated cytokines among the male and female in both groups (HIV and control). For example, the frequency of genotype C/C -597 IL - 10 for the men is higher. So taking into account the patients sex it is possible to personalize the prognosis of disease course.

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HLA AND HTLV-I ASSOCIATED DISEASE : ATL AND HAM/TSP

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HTLV-1 is a Human Retrovirus that is known to cause at least two diseases : adult T-cell leukemia/lymphoma (ATL) and HTLV-1 associated myelopathy/tropical spastic paraparesis (HAM/TSP) a chronic neurodegenerative disorder.
Endemic areas of HTLV-1 exist in southern Japan, The Caribbean, Central and South America and Africa.
Most infected people remain healthy but some patients develop (<1%) an HAM/TSP and rarely an ATL. It has been hypothesized that ATLL develops after a long latent period of 20-40 years following childhood infection primarily due to maternal transmission of HTLV1 via breast–feeding. Determinants of the pathogenesis of these two disease entities are not clearly understood.
Several studies have suggested the role of host related immunogenetic factors to influence the outcome of infection and particularly the role of HLA alleles.
Some authors have identified HLA class I and II associations with disease susceptibility (HAM/TSP & ATLL) and hypothesized that HLA alleles control HTLV-1 proviral load.
We compare HLA class I & II alleles among Martinican symptomatic HTLV-1 carriers, a group of TSP/HAM patients (n=96) and a group of ATL patients (n=29), with our population of healthy Martinican donors previously described (Tissue Antigens 2001:57:200-207).
The frequency of A*33 and DRB1*15 were increased among ATL patients but statistically not significant. Our first results within a black population from French West Indies differ from HLA antigens association studies previously reported essentially in southern Japan population.

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DISTRIBUTION OF HLA CLASS II ALLELES IN HELICOBACTER PYLORI INFECTED SUBJECTS WITH GASTRO PATHOLOGIES

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There is evidence that HLA class II molecules play a role in the susceptibility to the Helicobacter pylori (H. pylori) infection.
Aim of the study is to evaluate possible associations between HLA class II genes (DRB1 and DQB1), environmental exposures and H. pylori related disease (intestinal metaplasia, IM). DNA typing was carried out by PCR-SSP in 68 subjects with H. pylori infection (determinated by serology) affected by gastric intestinal metaplasia histologically confirmed and 70 healthy subjects without H. pylori infection used as controls. Furthermore, the presence of antibodies to cytotoxins CagA and VacA, expressed by more virulent H. pylori strains, were investigated. The control subject was individually matched to the case for sex and year of birth (±1 year). All subjects answered a detailed questionnaire collecting sociodemographic characteristics, smoking, alcohol drinking and dietary habits.
A significant association with HLA system was represented by the frequency increase of the allele HLA-DQB1*05 (OR 3.9; 95% CI 1.1-13.4) in metaplasia patients with CagA+ status over controls. In addition, metaplasia/control status was closely associated with environmental exposure: control group was associated with high consumption of olive oil, fresh fruit, vegetable and never smoking history and metaplasia was associated with cigarettes smoking and high consumption of eggs, meat and butter.

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THE HLA-B39 ALLELE CONFERS SUSCEPTIBILITY FOR OSTEOARTICULAR COMPLICATIONS IN HUMAN BRUCELLOSIS.

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Osteoarticular focal forms are the most common complication in human brucellosis. In order to establish a possible association between this complication and the HLA genes, 57 patients with brucellosis, 23 of whom had osteoarticular complications, and 73 healthy controls were genotyped for HLA class I and class II antigens. The patients with osteoarticular complications presented the HLA-B39 allele significantly more frequently than the patients without osteoarticular complications, in whom the frequency was the same as for the controls (35% versus 3%; P=0.0006, odds ratio 15·684, 95% CI: 3.453-71.231).

Thus, the HLA-B39 allele seems to confer susceptibility for osteoarticular complications in patients with brucellosis.

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CONNECTION OF THE POLYMORPHISM OF HSP70-2 AND HSP70-HOM GENES WITH THE DEVELOPMENT OF CHLAMIDIA and MYCOPLASMA INFECTIONS.

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We have considered Pst I polymorphism HSP70-2 and NcoI polymorphism HSP70-HOM genes of heat shock proteins which participating in processing of infection agents and may being the possible trigger of development sinovium pathology. 145 patients with Chlamidia trachomatis ,146 patients with Mycoplasma hominis and 284 health controls are inspected. The PCR-RFLP analysis determined "A" allele of HSP70-2 gene at presence of Pst I site and" B" allele at its absence. Similarly, for HSP70-HOM gene " A " allele was determined at presence of NcoI site and" B " allele at its absence. The statistical analysis was conducted by Fisher " s exact test.
We are shown the minor increased relative risk of development C. trachomatis infection only in patients with A/A variant of HSP70-2 gene ($P < 0,05$). There was not detected significant differences in allelic polymorphism of HSP70-2 and HSP70-HOM genes for patients with M. hominis. The analysis of frequencies HSP70-2/ HSP70-HOM has not shown differences between the healthy individuals and patients with this infections.

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FREQUENT MEFV MUTATIONS IN TURKISH FMF PATIENTS

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Familial Mediterranean Fever (FMF) is a recessive disorder characterized by episodes of fever and neutrophil-mediated serozal inflammation. The gene responsible for FMF was recently cloned and several disease-associated mutations have been described. A hundred sixty patients clinically diagnosed as having FMF underwent molecular genetic studies using PCR and restriction endonuclease digestion methods to detect the presence of the four mutations (M694V, V726A, M680I, E148Q). We correlated the presence of each mutation with clinical manifestations, disease severity and amyloidosis.
Allel frequencies were found to be as follows; M694V 53%, M680I 9%, V726A 7%, E148Q 5 %. The M694V allele accounted for 53% of the alleles studied and 20 % of the patients were homozygous. The distrubition of the four most common MEVF mutations found in healthy individuals were M694V 3%, M680I 5%, V726A 2% and E148Q 12 % and was different from that found in patients. There was no significant correlation between mutations and clinical manifestations such as arthritis, serozal inflammation, abdominal pain and amyloidosis. However patients with amyloidosis tend to have M694V mutation. Our results emphasis that, both clinical and genetic features are to be taken into account for patient diagnosis.

Poster display III Organ transplantation

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HUMAN LEUKOCYTE ANTIGENS AND LEPROSY ASSOCIATIONS IN TURKISH POPULATION

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Leprosy (Hansen's Disease) is a chronic infection of man caused by an intracellular microorganism. Genetic susceptibility to presentation and immunopathology of leprosy has been postulated for many years. The aim of this study was to determine whether there was HLA-linked susceptibility to leprosy and its different types. HLA class I (A,B,C) and II (DR,DQ) antigen frequencies in 80 patients with leprosy (35 BL, 25 LL, 15 BT, 5 TT) were compared with those in 120 healthy individuals. HLA antigens were determinated by microcytotoxicity technique. HLA-class I antigens A 9, A 10, A 32, B 5, B 21, Bw 4 ,Bw 6, Cw 1, Cw 2 and HLA-class II antigens DR 9, DR 10, DRw 52, DQ 1, DQ 3 were found to be significantly more frequent in patients with leprosy, while HLA-class I antigens A 3, B 44, B 49 and HLA- class II antigens DQ 5 were so in controls. However, there was no significant difference in HLA-class I and II antigen frequencies between subtypes of leprosy. HLA-A null antigen was found to have a weak expression in patients with leprosy.

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THE EUROTRANSPLANT EXPERIENCE WITH THE HIT (HIGHLY IMMUNISED TRAY) PROGRAM FOR HIGHLY SENSITISED PATIENTS.

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In Eurotransplant (ET) highly sensitised patients (HSP), having current HLA alloantibodies with panel reactive antibody values (PRA) of 85% or more can be included in the HIT program. In this "trial and error" program, current serum of the patients is crossmatched with all blood group compatible organ donors of the participating centers. Repeated HLA-A,B,DR mismatches are not accepted for retransplantation. In case of a By negative crossmatch in the donor center, and if a minimal matching of 1 HLA-B + 1 HLA-DR antigen, the kidney is mandatory shipped to the recipient center, where the decisive crossmatch is performed using current and historical sera, if available. During the last 4 years 400 patients were included in the HIT of which 187 have been transplanted. The 2 years graft survival was 79% slightly lower than not sensitised patients. About 30% of the HSP received the offer within 21 months on the HIT waiting list, in comparison to 18% for the highly sensitised patients, which have not been included in a special program. In conclusion, the HIT program is a good tool to enhance transplantation of HSP. The major drawbacks of the program are the high workload during the duty hours, and the high incidence of positive decisive crossmatches at the recipient center (>35%), leading to a reallocation of the organ and thus an increase of the cold ischeamia period. Due to these reasons ET decided to discontinue the program from 2002 on and convert the patients from the HIT to the Acceptable Mismatch Program.

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ENHANCED DETECTION AND CHARACTERIZATION OF HLA-SENSITIZATION IN RENAL TRANSPLANT RECIPIENTS.

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Dealing with sera from prospective transplant recipients, we need a sensitive and specific technique for proper determination of these patients' allosensitization status. A new flow-cytometry based assay (FlowPRA™) proved to be more sensitive in revealing the existence of HLA-specific IgG antibodies (Abs) than conventional citotoxicity (CDC) based assay. Using FlowPRA™ Screening and Specific tests we investigated humoral sensitization to HLA due to different immunizing events in 230 transplant candidates exposed to a single kind of immunizing event. Patient population was thus divided in three groups: Ts-group (110 transfused pts); Tx-group (41 previously transplanted pts); Pg-group (79 pts who had pregnancies and/or abortions). FlowPRA Screening highlighted a significantly higher incidence of sensitized subjects in both the Tx-group (36.6%, $P<0.00001$) and the Pg-group (27.8%, $P<0.00001$) than in the Ts-group (3.6%). Comparison of CDCPRA and FlowPRA™ screening results showed a higher incidence of "false negative" CDC results in the Tx-group and particularly in the Pg-group ($p=0.02$) compared to the Ts-group of patients. Analysis of HLA class I Abs specificity evidenced a high incidence of CREG Abs (mainly against CREG 1C) in the Tx-group (80%) and in the Pg-group (95.4%). Remarkably 11 of the 21 (52.4%) CREG-specific Abs evidenced in the sera of the Pg pts and 6 of the 15 (40%) found in the Tx pts were INTRA-CREG Abs. As regards HLA class II specificity, our study evidenced DR and/or DQ specific Abs in 61.5% of Pg pts and in 63.6% of Tx pts; Abs directed towards a public antigen (DR51, DR52, DR53) were present in 30.8% of Pg pts and 18.2% of Tx pts. In conclusion we demonstrated, by means of the sensitive and specific FlowPRA™ technique, that pregnancy had a strong immunogenicity, similar to that of transplants, but often underestimated by CDC assay and confirmed that pre-sensitization status must thus be attentively investigated before transplantation lowering risk of rejection and poor graft survival.

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PRODUCTION OF ANTI-HLA ANTIBODIES AFTER RENAL TRANSPLANTATION

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Many recent publications have been providing stronger and clearer evidence that anti-HLA antibody production after organ transplantation is associated with increased acute and chronic rejection and decreased graft survival. The purpose of this study was to investigate the production of such antibodies after renal transplantation and their clinical relevance on the graft outcome. A total of 268 recipients who received a primary renal graft between March, 1987 and September, 2001 were investigated. Collection and testing (ELISA screening and definition of antibody specificities) of sera samples began in December, 1998. Rejection was defined as any rejection treatment at any time after grafting and graft failure as loss of kidney function and return to dialysis. Anti-HLA antibodies were detected in 42 (15.7%) of the recipients: in 30.9% of these we detected only class I, in 47.6% only class II and in 21.4% both class I and II antibodies. Acute rejection, chronic rejection and graft failure was experienced by 30.9% of the antibody positive patients(app), while the rest 69% did not develop any such clinical problems.Of those app who developed rejection or graft failure, 46% had donor-specific antibodies. Both class I and II antibodies were found to be detrimental to the graft outcome. Patients with only class II antibodies had about 2-fold higher incidence of rejection and graft failure than those with only class I antibodies. Furthermore, the class II antibodies were highly correlated with chronic rejection. In conclusion, our findings support those of other investigators that the anti-HLA antibodies can be harmful to the renal graft and should not be ignored.

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ANALYSIS OF PATIENT ANTIBODIES AFTER KIDNEY TRANSPLANTATION

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Introduction: Recent literary data suggest that antibodies to HLA antigens as detected by sensitive methods significantly correlate not only with chronic but also with acute immunological complications after transplantation. **Aim and methods:** The aim of this study was to analyze patient antibodies in the early posttransplantation period according to their immunoglobulin class, HLA specificity and dynamics of development by the standard complement dependent cytotoxicity test, flow cytometry (FCXM) and the ELISA-LAT test.
Results: Sera from 131 patients after first and 16 patients after second (or third) cadaver kidney transplantation were analyzed. FCXM revealed antibodies in 19 patients (14.5%) after first transplantation and in 7 patients (43.7%) after second (or third) transplantation. Newly developed IgG antibodies to donor cells were shown in 11 patients (8.4%) after first transplantation and in 4 (25%) patients after second (or third) transplantation (-/+). Analysis of the specificity of those antibodies was performed by the ELISA-LAT test. All patients having polyspecific antibodies to HLA class I and class II antigens as determined by the ELISA-LAT test experienced severe rejection episodes after transplantation. **Conclusion**: Analysis of antibodies in patients after transplantation by both flow cytometry and ELISA may help predict the onset of immunological complications and consequently, may improve prognosis after organ transplantation.

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OBTAINING RELEVANT PRA VALUES USING SINGLE ANTIGEN BEAD PANELS

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In many if not most cases, PRA's are utilized in the organ transplant setting as a means of predicting the likelihood of a positive crossmatch with a random donor. In reality, most panels used for this are made up of individuals that do not reflect the antigen frequencies seen in cadaver donor populations. This has been further exacerbated by the introduction of technologies allowing the reactions against single antigens to be measured. In order to address this, we have developed a "Donor PRA". Using HLA types from some 1000 cadaver donors at our institution, it is determined by identifying reactions against a particular HLA antigen(s) using primarily single antigen beads, creating a "virtual" positive reaction against any of our cadaver donor population with that antigen identified. Examples are shown in the table with our T cell cytotoxic PRA using an in-house panel, as well as Flow PRA and Donor PRA's obtained using High Definition Flow PRA beads (One Lambda). When the reactions using the single antigen beads are used to calculate a Donor PRA, the anticipated likelihood a positive crossmatch in our donor population, i.e. PRA, was generally higher. The last patient had a donor PRA that was actually less than the Flow PRA obtained. This approach seems appropriate for any screen technology that results in an antibody specificity being clearly defined, and can produce a PRA that is generated using the transplant program's own donor population.

Serum	T CYTO	Flow PRA	Donor PRA
1 pr	0%	24%	50%
2 gn	0%	63%	96%
3 js	0%	45%	72%
4 gg	24%	82%	40%

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EARLY CONTROL OF DONOR-SPECIFIC ANTIBODIES IN A HIGHLY IMMUNIZED PEDIATRIC KIDNEY RETRANSPLANTATION PATIENT

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We report on a successful early control of donor specific antibodies in a highly immunized patient after cadaveric kidney retransplantation.

The primary renal disease of our patient was Prune Belly syndrome diagnosed at birth. Peritoneal dialysis was started at the age of two weeks. The first cadaveric kidney transplantation was performed at the age of 3 years. HLA-A+B/DR mismatches were 2/0 in the first transplantation. Chronic rejection and malignant hypertension indicated transplantectomy at the age of 8 years.

The patient was highly immunized (flow PRA I > 90%) which made it difficult to find a compatible donor for a second transplantation. At the age of ten years, a retransplantation was performed despite the 3/2 HLA-A+B/DR mismatches. The decision of retransplantation was based on the negative cytotoxic T cell cross match. Retrospectively, it was found that also T cell IgG and IgM flow cross matches were negative but B cell IgG flow cross match was positive in pretransplantation sera.

Immunosuppression after the retransplantation consisted of basiliximab, cyclosporine, steroid and azathioprine. Repeated plasmaphereses were started whenT cell flow cross matches converted to positive one week after the retransplantation.

The re-graft has been functioning well and no pathological findings of rejection in renal allograft have been detected during the early follow up time of two months. Clinical data together with laboratory results will be presented.

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PREDICTIVE VALUE OF ANTI-HLA ANTIBODIES IDENTIFICATION BY THE ELISA LAT IN SECOND KIDNEY TRANSPLANTATIONS

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ELISA is supposed to be more sensitive than complement dependent cytotoxicity (CDC) for identification of HLA antibodies in organ transplantation. However, the clinical relevance of the antibodies detected has to be demonstrated. We have compared the 2 tests in a retrospective study having included 147 recipients of a second kidney transplant, with < 70 % pre-graft PRA, transplanted between 1992 and 2000. The ELISA LAT-ID 1288 (One Lambda USA) has been used for identification of HLA antibody specificity. The current serum only (ie harvested < 3 months before transplantation) has been studied. The % immunization has been constantly found higher with LAT. LAT was unable to clearly identify antibody specificity in patients > 50 % PRA (n = 12). In those patients with < 50 % PRA, 56 and 33 % in LAT versus 42 and 20 % in CDC had respectively HLA class I and class II antibodies. Thirty eight had antibodies specific for the first kidney donor in LAT but not in CDC. Seven were retransplanted with at least one repeated mismatch (class I in all). No acute rejection occurred but graft survival was lower than in the patients without repeated mismatch (75 versus 90 % at 3 years). In addition, 3 patients over 7 had significant proteinuria at 1 year, associated with chronic rejection on biopsy. In conclusion : The antibodies detected by LAT seemed to have clincal relevance in renal transplantation. LAT should be used as a complement of CDC in immunized patients, female patients with previous pregancies and patients with poor immunological follow up.

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VALIDATION OF THE ELISA IDENTIFICATION TEST LAT FOR CLASS II ANTI HLA ANTIBODIES.

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We used routinely, in our laboratory, the Elisa screening test for class II anti-HLA antibodies (LAT-M) since January 2000. We have identified all the positive samples with lymphocytotoxicity (CDC), but the method is very long and delicate ; therefore we realised the validation of an Elisa identification test for class II (LAT). We studied 150 positive sera from kidney recipients waiting for a first or second transplantation. In 76.6% of cases (115/150) the sera were positive by both methods (CDC and Elisa), and in 9.4% of cases (14/150), sera were negative by both methods. In 14% of cases (21/150), there was a discordance between Elisa (positive) and CDC (negative). We have focused on positive Elisa samples (n = 136) : for each sera, we have found in the patient some immunisation risk factors : previous transplantation (n = 131), previous blood transfusion (n = 1) or previous pregnancy (n = 4). Identified antibodies are specific in 73.5% of cases (100/136) : 67% squared with the previous graft mismatch antigens, 6% squared with the husband antigens, and in 10% of cases an associated blood transfusion was found. In 11% of cases, the HLA class II typing of the first graft was unknown, and in 6%, the specificity didn't fit with any known antigens of the previous transplant. In 26.5% of cases (36/150), antibodies were non-specific.
In conclusion, in a high risk recipient population, the Elisa identification test LAT for class II allowed us to confirm specificity of antibodies identified by CDC in 44%, to give further information in 27% of cases. In 20% of cases, there is no additional information compared with the classical CDC test. The greater advantage of the Elisa test for class II is the simplicity of the method compared to the CDC : Elisa obviates the necessity to absorb sera on platelets and reduces the maintenance of a large B lymphocytes panel.

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ANTIBODY MEDIATED ACUTE REJECTION FOLLOWING RENAL TRANSPLANTATION: PREDICTION AND PREVENTION.

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Antibody mediated acute rejection (AMR) is associated with poor renal allograft survival. We reviewed cases of early (<1 month) AMR between June 1998 and December 2000. The aim of this study was to define the population at risk of this complication.

440 cadaveric renal transplants were performed during the study period, and AMR developed in 20 patients (4.5%). AMR was diagnosed based on histological features and positive direct Immunofluorescence or donor specific antibody in serum. One patient had no sensitising events, ten had previous allografts (range 1-5), 14 had been transfused and 8/11 women had at least one pregnancy. Seven patients had PRAs < 10%. Antibody screening by ELISA was positive in 17 of the patients who developed AMR.

Nineteen patients had negative current cytotoxic crossmatches (CXM). One patient had a B cell positive IgG CXM in current and historic sera, while four patients had historic positive CXM (2 IgM B cell, 1 IgG B cell & 1 IgM T + B cell). Sixteen patients had retrospective flow cytometry crossmatches (FXM) performed. 10 patients had positive FXM (3 T&B cell, 6 B cell only and 1 T cell only). The positive prediction value of a positive FXM was over 90%. All patients with positive FCM were positive by pre-transplant ELISA antibody screening. AMR can complicate renal transplantation even in unsensitised patients. Cytotoxic XM are insensitive predictors, however FXM could have prevented 65% of AMR in our series. FXM should be offered to all patients with anti-HLA detected by ELISA.

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WITH THE FLOW CYTOMETRY AVAILABLE SHOULD WE ALWAYS DO FCXM BEFORE THE KIDNEY TRANSPLANT ?

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It is broadly accepted FCXM (Flow Cytometry Crossmatch) should be used before transplant (Tx) in all presensitized patients and second Tx (high risk recipients), while in the primary unsensitized patients there is no follow-up difference between recipients FCXM positive or negative. It is also widely accepted that FCXM is more advantageous than standard crossmatch (XM) by CDC technique used for years in routine XM worldwide.
FCXM is more sensitive, objective and allows the simultaneous analysis of complement-activating and nonactivating allo-Ab.
We tested FCXM post-Tx in 116 patients of cadaver kidneys (16 retransplant and 1 third Tx). Organ allocation was based on the best HLA-matching. Spleen and lymph nodes cells from donor and a BD cytometer were used.
The receptor's median age was 43 years old (6-67). 66 male and 50 female with a follow up of 18 months. All receptors were T and B cells pre-TX XM negative by CDC.
From this group of 116 patients, only 3 were T cells positive pre-Tx FCXM. One lost the graft, one suffered an acute rejection crisis and the third, the only primary Tx remains rejection free. Of the remains 113, 20 were presensitized defined by PRA > 10% or ELISA positive (LATM One-Lambda) and 14 suffered one acute rejection, at least.
We use Chi-square test or the Fisher's test to compare the two populations, and the Odds Ratio to analyse the Relative Risk.
Patients positive FCXM reveal a greater risk (odds ratio=14,1 ; P=0.05) of developing an acute rejection/kidney loss, when compared to T negative FCXM. Patients presensitized are a higher risk (odds ratio=4,8 ; P=0.004).) of developing acute rejection episodes when compared with unsensitized patients.
Our data suggests T cells FCXM must be done in all retransplants patients. When positive, the recipient should be excluded for that donor.

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COMPARISON BETWEEN FCXM AND CDCXM PROCEDURES FOR DETECTING IgG ANTI-DONOR ANTIBODIES

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Flow cytometric cross match (FCXM) has become an increasingly utilized method to detect the low levels of anti-donor antibodies (e.g. anti HLA antibodies) in potential renal allograft recipient. These antibodies do not become apparent in the standart complement-dependent cross match (CDCXM), but are detectable by the FCXM. In addition to the FCXM, they can be detected both complement fixing antibodies and non complement fixing antibodies. Anti donor antibodies are often associated with increased episodes of graft rejection and the early graft failure.
In this study, we compared the result of FCXM and CDCXM. Totally 147 of pretransplant sera from renal allograft recipients (118 living related, 29 cadaveric) were tested with both methods. In the living related group, 3 cross match (%2.54) scored positive in CDCXM, 9 (%7.62) scored positive in FCXM. Antibodies were directed against T and B cells in 7 cases and against B cells in 2 cases. Concordance between the two methods was % 94.91. In the cadaveric group, only one cross match(%3.43) scored positive in CDCXM, 9 (%31.0) scored positive in FCXM. Antibodies were directed, against T and B cells in 4 cases, against T cells in 3 cases and against B cells in 2 cases. Concordance between the two methods was % 75.86. In the living related group, 62 patients
(all of tests performed by CDCXM were found to be negative , 60 tests performed by FCXM were found to be negative while 2 tests performed retrospectively by FCXM were found to be positive) were transplanted. Sixty of them have a functioning kidney while 2 patients lost their kidney, because of the hyperacute rejection. These patients had positive T and B in FCXM. In the cadaveric group, 5 patients (with a negative by CDCXM, 1 test positive by FCXM) were transplanted. Four of them have a functioning kidney, while 1 patient lost the kidney because of the infections. The patient had positive B FCXM.
In conclusion, FCXM, yielding clinical benefits especially in the idendification of patients at risk of early transplant rejection and early graft failure.

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FLOW- CYTOMETRY CROSS MATCH (FCM) TEST RESULTS ON LONG TERM RENAL ALLOGRAFT SURVIVAL

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The goal of this retrospective study was to evaluate the correlation between positive and negative FCM results on long term renal graft Survival. (from 5 to 12 years)
MATERIAL AND METHODS :
Immunised patients (LCT positive Test : PRA >25%) having received a cadaveric kidney between january 1989 and december 1996 have been included in this study.
All these patients have been grafted with a negative LCT cross match result on historical and current sera T cells.
A FCM test was retrospectively performed on T and B cells and a LCT cross match test was completed on the B cells.
RESULTS :

	Historical sera		Current sera	
	FCM-	FMC+	FCM-	FMC+
Fonctionnal kidney	26	8	31	3
No Fonctionnal kidney	7	11	14	4

When we analyse the graft survival, it appears that a negative FMC test result can be correlated with a graft survival of more than 80%.On the opposite , a positive FMC result has been found in a population with a survival rate of 50%, independently of other studied parameters
CONCLUSION :
This study confirms our previous results obtained on a shorter survival period (1997) on the fact that FCM positive results would not be obligatory associated to a bad long term graft outcome, even if it remains obvious that a negative result gives a better chance to grafted patients.

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CHARACTERISTIC OF THE CROSS - MATCH AT TRANSPLANTED KIDNEY RECIPIENT WITH SYSTEMIC LUPUS ERITHEMATOSUS

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Systemic lupus erithematosus (SLE) is a disease, which can cause renal failure.
This is a report about patient K.Lj. born 1980. She was affected by systemic lupus erithematosus (SLE) in 1991, as a result of which lupus nephritis occurred and led to chronic renal disease in 1998. She was treated with peritoneal dialysis and was transplanted in 2001.
Her mother was chosen to be the donor with whom she was haploidentical. The cross-match between the donor and the recipient was positive. Auto cross-matches with patient's sera and hers own lymphocytes at +4° and +18° were positive. Cross-match with sera treated with DTT(dithiothreitol) was negative.
The renal transplantation was successful and after it there was no kidney rejection.
These kinds of HLA antibodies are from Ig M type and are not influencing the transplantation but they are causing cross-match positive reactions.

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CD26 AS MARKER OF ACTIVATION IN GRAFT-INFILTRATING CELLS IN FINE-NEEDLE ASPIRATION BIOPSY OF RENAL TRANSPLANTS.

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CD26 is a widely distributed cell-surface glycoprotein, strongly expressed on epithelial and endothelial cells, as well as leucocyte subsets. This protein is involved in regulation of DNA synthesis and proliferation as well as in production of cytokines.
Our purpose was to study CD26 receptor expression in fine-needle aspiration biopsy (FNAB) during acute rejection of kidney transplants (Tx).
Thirty-three Tx from cadaver donors were studied, all treated with cyclosporin, AZA/MMF and prednisolone. They were divided into Group I (acute rejection, n=11) and Group II (rejection-free, n=22). FNAB was done on first day of acute rejection and on day seven post-Tx in Group II. Every rejection episode was confirmed by a classical core biopsy and all occurred during the first month post-Tx. Immunolabeling was performed by the avidin-biotin immunoenzymatic antigen detection system (LAB) using a 1/40 diluted mouse $IgG_1\kappa$ anti-CD26 antibody, from Pharmingen®. All CD26 positive cells were counted as well as all kidney parenchymal cells, in order to obtain the ratio of CD26 positive over renal cells.
Group I showed 67 ± 45 CD26+ and a ratio 0.307 ± 0.273, while in Group II we counted 12 ± 13 CD26+ and the ratio was 0.036 ± 0.056 A highly significant difference (p= 0.0006) was found in the expression of CD26+/renal cells between the two groups
Our data is in agreement with the already described in vitro co-stimulatory properties of CD26, where this molecule may enhance proliferation of allospecific cells and is associated with higher IFN-γ and IL-2 synthesis. We speculate that the higher CD26 expression can be one of factors responsible for up regulating IL-2 production which is usually observed in acute rejection

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CTLA-4 EXPRESSION IN FINE-NEEDLE ASPIRATION BIOPSIES ASSOCIATES WITH KIDNEY ALLOGRAFT REJECTION

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CD28-B7 interaction is one of the main costimulatory signal for T lymphocyte activation. However, CD28 homologue, CTLA-4, also known as CD152, appears to negatively regulate T cell activation. We studied CD28, CD152, CD80, and CD86 expression on fine-needle aspiration biopsy (FNAB) in kidney transplants (KTX).
Thirty-eight KTX from cadaver donors, treated with CsA, AZA/MMF and pred were divided into groups I (acute rejection [AR], n=11) and II (stable, n=27). FNAB was done on first day of AR and on day seven post-TX in group II. Every AR was confirmed by a classical biopsy and occurred during the first three weeks post-TX. Immunolabeling was done by the avidin-biotin immunoenzymatic antigen detection system (LAB) using diluted mouse $IgG1\kappa$ (CD28, CD86), $IgG2\kappa$ (CD152) and $IgM\kappa$ (CD80) from Pharmingen®. All positive cells were counted as well as all kidney parenchimal cells in order to obtain the ratio of labelled cells over renal cells.
No significant differences were found when comparing the demographic data for donor-recipient pair in group I versus II. There was an equal distribution of MMF-treated TX and CsA blood levels did not differ between two groups. We did not observe any positive cells for either CD80 or CD86. In group I, the ratio of CD28 positive/renal cells = 0.008 (± 0.01) whereas in II = 0.09 (± 0.04). For CD152, this ratio in I = 0.167 (± 0.152) and in II = 0.038 (± 0.069), Median test, p=0.012.
Contrary to expectations we observed a significant up-regulation of CD152 during acute rejection versus stable KTX followed by a non-significant increase in CD28, too. While our results may appear to contradict the more generalised concept of CD152 association with down-regulation of alloimmune response they may just point an unsuccessful counter-regulatory action of immune events during acute rejection.

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THE RELATIONSHIP BETWEEN HLA MISMATCHES AND BIOPSY RESULTS OF RENAL GRAFT IN KAYSERI REGION OF TURKEY

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It is well recognised that the degree of HLA mismatch between renal transplant recipient and donor can significantly affect the levels of sensitisation to HLA should the transplant fail. In this study, the effects of HLA mismatches in graft rejection or poor graft function was evaluated retrospectively. Serological cross-matches between donor and recipient pairs were negative. The needle biopsy was performed on 14 graft of 37 renal transplanted patients with poor graft function whereas they had treated with immunosupressive drugs for clinical rejection. Nine acute interstitial rejection (AIR), 3 acute tubular necrosis, 1 Cyclosporin toxicity, 1 hematoma, 1 renal medulla and 1 necrotic renal tissue were histologically observed respectively. Twice biopsy was performed on two of 14 patients. Three of 9 patients with AIR had cadaveric kidney and the others had living-related donor kidney. All of patients with AIR had 1 DR, five patients 1 B, four patients 1 A and one of them 2 A mismatches respectively. Two of 9 patients with AIR died and the other two also has been in need of hemodialysis in follow-up period. There is no problem in remaining 23 transplanted patients in follow-up and just only two of them were fullmatch and the others had at least one or more A/B//DR mismatches.
In conclusion, these findings were compared with data of poor graft function group and it was observed no parallesim between AIR and HLA mismatch.

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THE RELATIONSHIP BETWEEN HLA-A, B, DQ AND DR ANTIGENS AND INTERSTITIAL FIBROSIS IN RENAL ALLOGRAFTS

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The pathogenesis of interstitial fibrosis (IF) in renal allografts is believed to be a multifactorial process. Although patients have similar clinic and immunologic parameters, some cases have an early IF in renal allograft biopsies. The existence of early IF in these cases with similar clinic parameters suggests a genetic predisposition. Genetic influences could have an important role of modulating collagen metabolism in the kidney.
To evaluate the relationship between HLA antigens and diffuse IF, we reviewed HLA-A, B, DQ and DR phenotypes in 88 renal transplant patients. Only patients with similar clinic and immunologic parameters were included in the study. All biopsies of patients were re-evaluated for the presence of diffuse IF. The files of patients were examined and the mean time between transplantation and diffuse IF were given in months.
The existence of diffuse IF was found significantly earlier in patients with HLA B8, B27, DQ2, DQ5, DQ6, DQ7, DR4, DR13, and DR15 antigens when compared with cases without these HLA antigens ($p<0.01$). Patients who showed diffuse IF 6 and 12 months after transplantation have tend to show higher incidence of HLA B8, B27, DQ2 and DR 4 antigens ($p<0.05$).
These data suggest that the patients with these antigens are more susceptible to develop diffuse interstitial fibrosis in early period. We conclude that although there are multiple etiological agents in the pathogenesis of interstitial fibrosis, one can not exclude a HLA association in these cases.

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LONG TERM SURVIVAL ANALYSIS: TIME BIAS?

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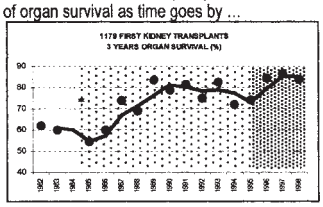
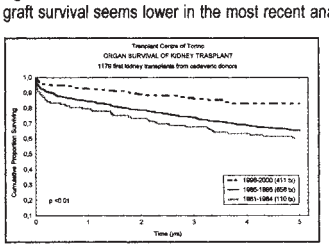
The Transplant Centre of Torino starts its activity at the end of 1981. At January 2001, 1344 kidney tranplants from cadaveric donors were performed. Kidney recipient's choice is mainly based on HLA compatibility between donors and patients and on additional parameters. Survival analyses to assess the quality of our kidney transplant centre were performed on the whole cohort of recipients, sometimes starting from 1985 (cyclosporin era). The results also influenced our allocation policy. The aim of this study is to show the difference of organ survival as time goes by ...



Looking at the trend figured, we identified three different periods in our transplant history. They show a good overlap to the introduction of new immuno-suppressive drugs, ciclosporina in eighties and tacrolymus in nineties. Here we analyse the organ survival according to the period when the transplants were performed, and a significant difference is evidenced. On the other hand, the HLA influence on graft survival seems lower in the most recent analysis (data not shown). These results, in our opinion, lead to the following considerations: a) analysis of the whole patients cohort on a single Transplant Centre could induce inaccurate results, dispite of a larger number of cases; b) the studies to decide new allocation policies must be performed on recent cohorts, to avoid time bias.



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TWO-YEAR ANALYSIS OF THE UNOS CREG ALLOCATION VARIANCE

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The specific aims of the UNOS crossreactive group (CREG) variance, initiated in 1999, were to determine whether the CREG variance increases access for non-white recipients, assess the impact of OPO size and ethnic make-up, and whether the variance improved outcome.
Twelve OPOs participated in this prospective study. Donors were randomized to two arms; one giving priority to recipients with 0-CREG, and 0 -DR mismatches (0 CREG MM), the other using the standard algorithm with priority given to patients with 0 B, DR mismatches. Through the end of 2000, 877 donors, 1241 transplants were enrolled in the CREG, and 1061 donors, 1400 transplants in the non-CREG arm. OPOs were categorized as small, medium or large (mean number waiting= 195, 640 and 2948 respectively), and by ethnic diversity (<70 vs. >=70% waiting White recipients).
The percentage of 0 CREG MM in the CREG arm was six fold higher than 0 BDR MM in the non-CREG arm of the study (8.8 vs. 1.2%). Of the 109 CREG matched transplants, 66 were allocated in large, and 79 in diverse OPOs. There was a significant increase in the percentage of CREG matched patients with OPO size (small: 2.4, medium: 9.1, large 14.0%, $P<0.0001$) and with ethnic diversity (5.1 vs. 12.0, $P<0.0001$). The incidence of positive crossmatch listed as a reason for not transplanting a patient was lower in the CREG study arm (14.0 vs. 17.3, $P<0.0001$). The mean number of A,B mismatches was 2.61 for 0 CREG MM transplants compared to 3.09 for CREG MM transplants ($P<0.0001$). There were too few cases to demonstrate differences in outcome.
Allocation based on HLA CREGs is more likely in large and ethnically diverse OPOs. Further analysis is necessary to evaluate the impact of this variance on graft outcome.

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ASSESSING THE USEFULNESS OF A MATCHABILITY SCORE FOR PATIENTS ON A TRANSPLANT WAITING LIST.

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A 'matchability' score could be used to predict how often an individual is likely to be offered a well-matched allograft.

Well matched grafts have better survival and cause less sensitisation when they fail. However, patient survival is strongly influenced by waiting time for transplantation. As patients with rare HLA types are highly unlikely to receive a well matched offer, prolonged waiting to obtain a good match is not beneficial.

A well matched graft is defined by the United Kingdom Transplant Service (UKTS) as a favourable match - i.e. no HLA-DR mismatch and no more than one mismatch at the HLA-A and HLA-B loci.
Our aim was to establish a system whereby such information could be provided to clinicians in an objective and easily interpreted form. The ultimate aim is to improve our rate of favourable transplants, while decreasing waiting time for patients with rare HLA types.

The current rate of favourable matches in our unit is 19% and using the matchability scores it is theoretically possible to increase that to > 40% by identifying patients who will get a favourable match if they wait for up to 1 year.
Using a database of 10,000 Irish HLA types a matchability score was calculated for each of our patients. When combined with the data on blood group frequency we can calculate the length of time it may take to get a well matched graft for each patient.

This information is now being supplied to the physicians and surgeons in the country and the impact of providing this information on the favourable match transplant rate is currently being audited.

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ACTIVATED HLA CLASS I REACTIVE CTLS ASSOCIATED WITH A POSITIVE HISTORICAL CROSSMATCH PREDICT EARLY GRAFT FAILURE .

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Alloantibodies in current sera against the mismatched donor HLA class I antigens, are believed to be detrimental for kidney graft survival. The relevance of a positive crossmatch with historical sera only is still a matter of debate. Previous studies showed a correlation between the presence of alloantibodies and the presence of alloactivated CTLs. We wondered whether the persistence of activated CTLs might explain the poor results in a proportion of patients with a historical positive crossmatch.
We tested in 10 sensitized patients whether activated CTLs persist when the antibodies disappear. Limiting dilution assays (LDA) were performed in the presence and absence of Cyclosporin A (CsA) to distinguish activated (CsA resistant) and naive (CsA sensitive) CTLs. Four patients had CsA sensitive CTLs, 3 patients had CsA resistant CTLs and 3 other patients had CsA sensitive CTLs for a particular HLA antigen and CsA resistant CTLs for another HLA antigen. In order to test the clinical relevance of persisting CTLs, 8 sensitized patients who underwent a kidney transplantation across a positive historical crossmatch, were retrospectively tested for the presence or absence of activated donor specific CTLs at the day of transplantation. Four patients with CsA sensitive CTLs at the day of transplantation were found to have a good graft function. In the other 4 patients the presence of CsA resistant donor specific CTLs was associated with rejection and early graft loss.
The present study suggests that determining the activation state of CTLs specific for the HLA mismatch against which antibodies were present in historical sera, can contribute to the success of transplantation.

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RELEVANCE OF CYTOKINE GENOTYPES ON RENAL ALLOGRAFT OUTCOME.

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Cytokines genes are one of the best targets to implement exhaustive research in order to locate new individual genetically based prognosis factors of renal allograft outcome. Allelic differences on gene promoter regions or codifying regions have been described as affecting gene expression regulation and consequently increase or decrease cytokine production or signal transduction over the same stimuli. This is the case for IL-10 (-1082 A/G, -819/-592 CT/CA), TGF-β (codon 10 C/T, codon 25G/C), TNF-α (-308 G/A), TNF-β (+252 A/G), IFN-γ (+874 T/A), IL-6 (-174 G/C) and IL-4Rα (+1902 G/A). To evaluate the influence of these cytokine genotypes on the development of acute or chronic rejection, we compare the genotypes frequencies on both recipients and cadaveric donors of kidneys grafts according to the clinical outcome. Were selected first kidney recipients and donors with 5 years follow up; at least 2 HLA-DRB compatibilities and a maximum of 25% anti-HLA sensitisation. These were grouped according to their clinical outcome as with stable functioning graft - NR (n=35), with acute rejection episodes - AR (n=31) and suffering from chronic rejection - CR (n=31). Subjects cytokine genotype polymorphisms were defined by PCR-SSP typing. Frequencies analysis on the receptors show a statistical significant prevalence for the IL-10 -819/-592 CT/CA genotype on CR individuals, while on donors TGF-β codon 10 CT genotype is significantly higher on AR grafts and IL-6 -174 CC genotype on RC affected grafts. Moreover, although non significant, we could see a strong predisposition of receptors TGF-β codon 10 CT genotype to CR and IL-4 Rα 1092 AA and TNF-β 252 AA to AR. Also on donors both IL-4 Rα -174 AA and TNF-α -308 AA genotypes are associated to worst clinical outcome of renal allografts. A study on a larger cohort is going on in order to confirm these preliminary results.

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CYTOKINE GENOTYPING IN RENAL TRASPLANTATION : CLINICAL APPLICATION

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Recent studies have demonstrated a correlation between variant genotypes of TNF-a, TGF-b1, IL10, IL6 and IFN-g with the expression levels of these cytokines. In kidney transplantation the individual variability of immune response is theorically determined by gene polymorphisms of cytokines production. These is growing evidence that polymorphism within the regulatory regions of cytokine genes may be associated wit graft survival. In this study the genotypes of cytokines were analysed for association with transplant outcome and acute rejection. **Material and methods** : one hundred one renal graft recipients were used ot study graft outcome. The follow up covered by the study was at last 6 months (52 ± 46) the genotypes of TNF-a TGF-b1, IL10 IL6 and IFN-g were determined by PCR-SSP (One lambda. Inc) analysis. Patients were classified as either high or low producers of TNF-a and IL6 and as High, Intermidiate or Low producers of TGF-b1, IFN-g and IL10. **RESULTS** . The frequencies are : TNF-a (Low : 64,4%, High : 35,6%) TGF-b1 (High : 64,4% ; Int : 30,7%, Low : 5%, IL6 (High 98%, Low : 2%) IFN-g (High : 16,8%, Int : 51,5%, Low : 32%) IL10 (High : 19,8%, Int : 44,6%, Low : 35,6%). 31 patients had rejection episodes (30.7%). There was nos significant difference in these genotypes frequencies of cytokines between the patients with acute rejection versus those without rejection. Considering conbined genotypes of recipients, TGF Higher, IFN-g low were associated with rejection episodes ($p<0,05$). TGF Higher was also associated with acute rejection in patients with single DR mismatched organs. Whereas intermidiate producer patients of TGF-b1 showed good graft survical (93% after 4 years VS. Higher producers 83% $p<0.05$). TNF-a and IFN-g did'nt correlate neither with survical nor acute rejection. Whereas Low producers patients of IL10 showed good graft survival 100% after 4 years versus Intermediate producers 80% $p<0.05$). Actually there were no clear correlation between cytokine genotypes and renal transplantation, testing larger numbers of patients may clarify the role of cytokine genotypes in predicting the outcome

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GENETIC SUSCEPTIBILITY FACTORS FOR CYCLOSPORIN INDUCED GINGIVAL OVERGROWTH IN RENAL TRANSPLANT PATIENTS.

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Introduction
Cyclosporin alters the production of cytokines involved in the fibrotic process, including TGFβ1, IL-10 and IL-6. Cytochrome CYP3A4 is involved in cyclosporin metabolism. Polymorphic variants of the genes encoding these molecules may contribute to gingival overgrowth in patients receiving cyclosporin.
Methods
Renal transplant recipients (n=152) on cyclosporin monotherapy were genotyped for the following polymorphisms: TGFβ1 - codon 10 (Leucine/Proline) and codon 25 (Arginine/ Proline), IL-10 (–1082, -819 and –592) ,IL-6 (–174) and CYP3A4 (–392). CYP3A4 gene screening was performed using WAVEMaker technology (Transgenomics, UK).
All subjects underwent a dental examination and severity of overgrowth was scored from plaster models.
Results
The TGFß1 C allele at codon 25 (p=0.05), and the GC genotype at IL-6 –174 were significantly increased (p= 0.00057) in patients with overgrowth (n=62), compared to patients with no overgrowth (n=67). Three previously unreported single nucleotide polymorphisms were detected in the CYP4A4 gene.
Conclusion
We have identified genetic markers that significantly influence susceptibility to cyclosporin-induced gingival overgrowth. . We have also identified three novel polymorphisms, which may exacerbate this condition.

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AN IN VITRO ASSAY TO ASSESS RESPONSE TO IMMUNOSUPPRESSIVE THERAPY.

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We have developed a novel *in vitro* system to assess individual responses to immunosuppressive therapy post transplant. Using a three colour flow cytometric assay, we are able to identify activated lymphocytes (CD69 +ve) in a given sample, and calculate the proportion of CD4 or CD8 positive cells. We stimulated PBLs with PMA + Ionomycin for 4 hours, after which 98.5% of CD8 cells and 99.3% of CD4 cells were activated.
Ciclosporin (csa) and tacrolimus (tac) can be included in the culture medium, and can prevent T cell activation in this system. Csa gives a reduction in stimulation of 71% for CD4 cells and 28% for CD8 cells, compared to the control. Tac results in 97% and 87% inhibition of CD4 and CD8 cells respectively.
In conducting the experiments we observed wide variation in individual responses to both drugs. CD4 lymphocyte response is reduced by between 30 and 70% using csa and between 18 and 85% using tac. We hypothesised that there may be a genetic basis for this range of response. Csa and tac inhibit T cell activation via the calcineurin pathway. In binding to their immunophilin binding proteins – cyclophilin and FKBP12 – they impede the intracellular cascade, reducing production of cytokines. We studied the genetic sequence of FKBP12 and have identified polymorphisms, which may contribute to the intra-individual differences in response observed in the cellular assay. A combination of this cellular assay and genetic screening could identify individuals at risk of adverse reactions to immunosuppressive therapy

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FACTOR V LEIDEN MUTATION AND EARLY LIVER GRAFT LOSS.

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Despite a growing body of data aimed to the clarification of inherited as well acquired risk factors for thrombosis, the cause of many vascular thrombotic episodes occurring after liver transplantation (LTX) remains largely unknown.The most commonly detected hypercoagulable state involves an abnormal Factor V protein, sythesized by the liver, in which arginine(R) at position 506 is replaced by glutamine(Q) as a result of a single-point mutation in the Factor V gene at nucleotide 1691(Factor V Leiden or FV506Q). LTX is complicated by hepatic vessel thrombosis in up to 15%-20% of cases, resulting in graft loss in most instances. The present study was performed to assess the contribution of Factor V Leiden (FVL) mutation to the risk of hepatic vessel thrombosis, including hepatic artery thrombosis, in patients (pts) with chronic failure undergoing primary LTX. A consecutive series of 109 recipients who received primary LTX at our center between January 1998 and April 2001,was analyzed. All transplanted pts had a minimum follow-up period of six months at least, after LTX. FV genotype was determined by PCR-SSP technique with two different sets of primers,one amplifying the wild gene and the other the FV mutation. 20 pts showed the FVL mutation whereas the remaining 89 appeared to have the wild type gene. The results demonstrated that the six-month graft survival (GS) of FVL mutation patient group was significantly lower than that of FV wild type cohort (50% vs 79.3% respectively; $p<0.005$). In particular, in the FVL cohort three pts out of eight underwent a graft loss for hepatic artery thrombosis whereas no such cases were observed in the FV wild type counterpart. In conclusion, FVL mutation seems to play a negative role on early liver GS, probably by acting as an additional co-factor risk in the pathogenesis of hepatic artery thrombosis.

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DETECTION OF HR_2 HAPLOTYPE POLYMORPHISMS IN THE FACTOR V GENE BY PCR-SSP

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It is well documented that a point mutation in the coagulation factor V gene, FV R506Q or FV Leiden is a common genetic risk factor for blood clotting disorders, due to activated protein C (APC) resistance. However, only a minority of individuals carrying this mutation develop venous thromboembolism and further components which may contribute to APC resistence in patients with VB have been investigated.

Recent studies have reported the detection of several polymorphisms in exon 13 of the coding region of the factor V gene which defines the HR_2 haplotype. Although HR_2 alone is not associated with VT, the relative risk of developing the disorder is increased three to four fold when co-inherited with FV Leiden.

We have successfully developed a PCR-SSP method based on the HLA phototyping protocol of Bunce et al to identify polymorphisms of the HR_2 haplotype of the FV gene. Using both sense and antisense primers specific for each substitution in 80 combinations the method is capable of assigning haplotypes by determining the cis or trans orientation of the mutations.

We will present results on DNA samples from individuals who are known homozygous/heterozygous carriers of the Factor V Leiden mutation and also haplotype frequencies obtained from 2 different racial groups.

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FACTORS INFLUENCING LYMPHOCYTE SUBSETS IN HEMODIALYSIS PATIENTS

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Several adverse reactions due to the bioincompatibility of the dialyser membranes can occur in HD (hemodialysis) patients. Recently effects of viral hepatitis on lymphocytes have been searched. We determined the influence of HD membranes and hepatitis infection on lymphocyte subsets in HD patients. Fifty-nine patients (age: 43,61±15,83 years, HD duration: 63,9±46,6 months) were included. Serology of the hepatit markers were: anti-HCV(+) in 23, Hbs Ag (+) in 13 and hepatitis markers were negative in 23 patients. Three patient groups were formed; 15 patients were dialysed with hemophane (GFSP 12, group 1), 26 with cuprophane (GFE 11, GFE 12, group 2) and 18 with synthetic membranes (MHP 120, group 3). Each group had similar distribution of anti-HCV (+), Hbs Ag (+) and hepatitis markers negative patients. Blood samples were obtained at the beginning and the end of the HD session. Total leukocyte count and lymphocyte subsets were analysed by flow cytometry: CD4, CD8, CD3 for T lymphocytes and CD19 for B lymphocytes. CD3 activation was significant with cuprophane membrane (CD3 positivity before and after HD were 54,74±14,16 % and 61,77±16,41 %, $p<0,03$). Interestingly CD3 activation with cuprophane membrane were observed only in hepatitis markers negative patients ($p=0,025$). Neither total leukocyte count nor the proportions of CD4, CD8, CD19 changed markedly after HD with either membrane. In conclusion; impaired cell-mediated immune response in ESRD can be associated with the activation of the dialyser membranes. Also viral hepatitis can maintain an additional supression effect on immune system in HD patients.

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CYTOKINE GENE POLYMORPHISMS IN TNF-α, IFN-γ, IL-6, IL-10 AND ACUTE REJECTION DEVELOPMENT IN LIVER TRANSPLANTATION

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Polymorphisms have been described for many human cytokine genes. Those polymorphism that occur within the regulatory regions of the cytokine genes are associated with high or low production *in vitro* of a given cytokine. In this analysis we retrospectively screened 96 liver allograft recipients to determine their genetic polymorphism for TNF-α, IL-10, IL-6 and INF-γ cytokine genes. The objetive for this study was to evaluate the significance of these cytokine genes polymorphisms, alone or in combination, as for the development of clinical acute rejection in liver allograft recipients.

A total of 96 liver graft recipients were included in this study. The acute rejection diagnosis was based on conventional clinical, biochemical and histological criteria. DNA was extracted from PBMC and biopsied material by routine methods. For TNF-α, IL-10, IL-6 and INF-γ promoter region polymorphisms DNA was amplified using PCR-SSP methods. Statistical analyses were performed using SPSS sotfware.

There was no significant association of IL-6, IL-10 and INF-γ genotypes or phenotypes with the incidence of rejection episodes. However, we found that patients who were high TNF-α producer phenotypes had a significantly higher incidence of acute rejection episodes compared to low TNF-α producer phenotypes. The TNF-α -308GA heterozygous genotype and the TNF-α –308A allele were also significantly associated with acute rejection ($p<0.05$).

These data show that TNF-α cytokine promoter polymorphism is significantly associated with acute rejection development in liver allograft recipients. IL-6, IL-10 and INF-γ cytokine genotype or phenotype were not asscociated with acute rejection.

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CYTOKINE GENOTYPE IN LIVER TRANSPLANTATION

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The immune system is regulated by the release of cytokines which influence cellular activation, differentiation and function. The production of cytokines is under genetic control. Individual differences in the inheritance of polymorphic cytokine genes lead to individual variation in the immune response to organ transplantation. Hence, a definition of cytokine genotypes can indicate how recipients will respond to their transplants and may provide an approach to design immunosupression in individuals.

Methods. We have studied allelic variation in polymorphic positions of three cytokine genes, namely, TNFα promoter –308 A/G ; TGFβ1 leader secuence codons-10 T/C and 25 C/G; IL-10 promoter –1082 A/G, - 819 T/C and – 592 A/C, in 97 liver recipients with their donors respectives. DNA was extracted from whole blood of liver donors and recipients and genotyped using the polymerasa chain reaction with sequence specific primers.

Results. Recipients with acute cellular rejection (30 of 97) show low levels of TNFα (63%) and IL-10 (90%). We haven´t found any individual with the homozygous TNFα – 308 genotype A/A.

Six recipients have developed tumor de novo, being their genotypes high levels for IL-10 (83%) and low for TNFα (83%).

No significant difference between donors and recipients was seen in polymorphisms for IL-10, TGFβ1, or TNFα, combined or separately.

Conclusions. It would been premature to predict transplant outcome using cytokine genotyping.

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DIFFERENTIAL EXPRESSION OF HLA-A AND B GENE PRODUCTS ON CULTURED HUMAN HEPATOCYTES IN THE PRESENCE AND ABSENCE OF IFN-γ

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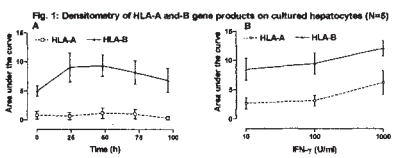
As HLA antigens are central structures for alloantigen recognition the expression of such molecules on hepatocytes might be important for induction alloresponsive CTLs after transplantation. As studies of HLA class I expression on hepatocytes are scarce we analysed systematically cultured hepatocytes from normal liver tissues of five patients with known HLA types in a time (0, 24, 48, 72, 96 h) and IFN-γ dose (0, 10, 100, 1000 U/ml) dependant manner by HLA class I specific immunoprecipitation, 1D-IEF followed by Western Blotting and densitometry. The densitometric evaluation of 1D-IEF gels revealed an expression maximum for HLA-A and –B gene products after 48 h of culture (Fig. 1a). Without IFN-γ HLA-A gene products were detectable only in 2 out of 5 cases and the HLA-B variants were present in all cases. In the presence of IFN-γ both the HLA-A and –B expression on hepatocytes increased dose dependently (Fig.1b). The HLA-B gene products, however, were found with an appr. 2.5fold higher expression than the HLA-A one ($p<0.001$). This differential expression pattern of HLA-A and B on hepatocytes might influence liver graft survival and T cell based immune therapy in patients suffering from liver cancer.

Fig. 1: Densitometry of HLA-A and-B gene products on cultured hepatocytes (N=5)



Poster display IV Bone marrow transplantation

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HLA-C POLYMORPHISM AND LIVER ALLOGRAFT TOLERANCE

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The biological role of HLA-C antigens in transplantation is still undefined because the detection of HLA-C antigen by CDC is not always clear. Therefore, the aim of this study was to investigate HLA-C genotype frequencies in liver recipients and to determine the possible influence of these antigens in the tolerance development of liver grafts. A total of 100 recipients and 100 controls was included in this study. The acute rejection diagnosis was based on conventional clinical, biochemical and histological criteria. HLA-C was typed by PCR-SSP methods. Statistics was performed using SPSS sotfware.

The frequencies of HLA-C alleles were analysed comparing their frequency in the total group of liver recipients against the observed frequencies in the controls. This analysis did not reveal any significant differences for any allele ($p>0.05$). When the liver recipients were clasified according to their rejection episode manifestations, and comparing HLA-C frequencies of the non acute rejection (NAR) group with respect to those observed in the acute rejection (AR) group or in controls, no significant differences, except for HLA-Cw*06 were observed (0% NAR vs 11.9% AR, $p=0.01$; 0% NAR vs 14%, controls $p=0.007$).

The influence of dimorphism at position 77 and 80 of the α1 helix of HLA-C molecules was also evaluated. In this case higher and significant frecuencies of the HLA-Cw*01, *03, *07, *08 group of alleles were observed in the patients with a better graft tolerance (NAR) when they were compared to the controls (55% NAR vs 34%, controls $p=0.014$).

Although these associations did not were significant after correction, these data suggest that the 77/80 dimorphism in HLA-C could be implicated in the development of liver allograft tolerance.

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PRIMARY AND SECONDARY CELLULAR PROLIFERATIVE RESPONSES AND CO-STIMULATORY MOLECULES EXPRESSION ON ALLOANTIGEN STIMULATED HUMAN UMBILICAL CORD BLOOD MONONUCLEAR CELLS AND T LYMPHOCYTES

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Allogeneic umbilical cord blood (CB) transplantation is associated with a lower degree of graft-versus-host-disease (GVHD) than bone marrow transplantation. Acute GVHD is thought to be a T cell mediated disease. In this study, total mononuclear cells (MNCs) from adult and cord blood were used to induce primary and secondary alloresponses on CB or adult blood MNCs and purified T lymphocytes. The magnitude of primary cell proliferation of total MNCs and purified T cells were similar in both CB and adult blood (AB), although slightly high responses were observed when total MNC were used as responder. However, reduced secondary proliferative responses, which could be overcome by the addition of exogenous rIL-2, were observed in both CB-T cells and CB-MNCs. Expression of the co-stimulatory molecules CD28, CD40L, CD152 were studied. Of all the markers studied, only CD28 is less expressed on CB T cells.

Conclusion: The slightly higher proliferation responses observed by total MNCs versus purified T cells could indicate the additional contribution of autologous APCs to the MLR responses. The lower expression of CD28 was seems to be a CB T intrinsic defect.

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DONOR CHIMERISM AND IMMUNE MODULATING EFFECT OF ALLOREACTIVE CELLS AFTER REDUCED TOXIC CONDITIONEN REGIMENT.

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The conditioning regiment is used to eradicate the malignancy and to allow engraftment by immunosuppression of the host. In patients undergoing allogeneic transplantation with reduced conditioning regimen the occurrence of transient mixed chimera is expected to be more frequent compared to patients after myeloablative conditioning. This conditioning regiment is also associated with a higher risk of acute Graft-Versus-Host-Disease (aGVHD). Monitoring the kinetics of donor cell engraftment by assessment of chimerism is of utmost importance. We studied a group of eight patient that received as conditioning Fludarabine+Busulfan+ Antithymocite Globulin. The median patient age was 49 years (range 41-60). The underlying disease was myeloid chronic leukemia in one, lymphocytic chronic leukemia in one, non-Hodgkin lymphoma in two, multiple myeloma in two and Acute Myeloid Leukemia in two. Chimerism was evaluated 30,90,180 days post-transplant or at individual time points of interest as part of the routine diagnostics, with a PCR-STR assay and detection in a ABI310 automated DNA sequencer. Compared to other published fludarabine based regimens , patients in our protocol rapidly achieved complete chimerism (75%). This fact may be beneficial for an effective Graft-Versus-Malignancy effect. On the other hand we have not found an incidence of a GVHD as high as other published studies as in our group the incidence of aGVHD grades II-IV was only 37%. The data may indicate that the antileukemic effect of allogeneic transplantation is achieved much more with an immune modulating effect of alloreactive cells of the graft than with the cytotoxicity of the conditioning regiment.

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MINIMAL RESIDUAL DISEASE AND CHIMERISM AFTER THREE DIFERENT ALLOGENEIC TRANSPLANTATION METHODS IN PATIENTS WITH CHRONIC MYELOGENOUS LEUKEMIA.

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Recently new allogeneic transplant methods have been developed for the treatment of haematologic malignancies as peripheral blood stem cell transplantation (PBSCT) after dose reduced conditioning and PBSCT CD34+ depleted of CD3-positive cells. Since 1999 three patients with LMC have undergone allogeneic PBSCT transplantation in our hospital, one the standard PBSCT, one the reduced conditioning procedure and the last received a CD34+ depleted PBSCT. Chimerism was evaluated +30,+90,+180 days post-transplant or at individual time points of interest as part of the routine diagnostics, with a PCR-STR assay and detection in a ABI310 automated DNA sequencer. Minimal residual disease was analyses by RT-PCR. The two patients who received the standard and the reduced conditioning transplant achieved a rapid situation of complete chimera and no molecular relapse occurred. The patient with the CD34+ PBSCT developed a molecular relapse (detection of at least two consecutive positive RT-PCR assays within a 4-week interval). Complete remission was obtained by infusion of donor lymphocytes(DLI). Although the little number of patients under study, the results agree with the idea that complete chimerism is a good marker of a better prognosis in regard to leukemia relapse than mixed chimerism. Also they agree with the fact that T cell depleted PBSCT is associated with an increased risk of relapse, as donor T cells are the most important effectors of graft-versus-leukaemia reactions, whereas patients who relapse have a high response rate to DLI.

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EXPANSION OF TRISOMIC 12 CELLS ASSOCIATED WITH PROGRESSIVE LYMPHOCYTOSIS AND DRAMATIC CHANGE IN CHIMERA STATUS IN A PATIENT WITH CHRONIC LYMPHOCYTIC LEUKAEMIA AFTER ALLOGENEIC TRANSPLANTATION

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Trisomy 12 is the most common chromosomal aberration in patients with B-cell chronic lymphocytis leukaemia (B-CLL). However its role in the course and pathogenesis of the disease remains unclear. We report a dramatic change in the progression of the disease in a patient with B-CLL who received an allogeneic haematopoietic cell transplantation. We monitored the number of CD23/CD5 leukaemic cells after transplant by cytofluorimetry and fluorescent multiplex PCR. After a period of stabilisation, the patient developed, over the 21 months that followed transplantation, progressive lymphocytosis that was resistant to pharmacological treatment. This lymphocitosis was associated with the detection and expansion of a trisomy 12 clone that was not observed in malignant cells obtained soon after allogeneic cell transplantation.. Our results suggest that the level of trysomic 12 cells is not constant, as previously published, and was associated with evidence of increasing numbers of lymphocytes and disease progression. These cells were probably expanded in the patient as a consequence of selection of a clone resistant to the immunologic graft-versus-leukaemia effect.

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VARIANTS OF SOLUBLE HLA MOLECULES IN BONE MARROW TRANSPLANTATIONS

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The significance of soluble HLA class I molecules (sHLA-I) was observing in prediction and differential diagnostics among posttransplantation complications (GVHD, graft rejection, infections). Even more sensitive indicator of immunologic changes could be the evaluation of sHLA-I variants. There are at least 3 variants of sHLA-I in serum with diverse molecular weights: 43, 39 and 35 kD. They are produced by different mechanisms and apparently have diverse immunomodulating effects. The sHLA-I is supposed to block anti-HLA class-I antibodies, to inhibit activity, to induct apoptosis of alloreactive cytotoxic T-lymphocytes, and to inhibit NK cells. The lymphocytes T and B actively secret the sHLA-I molecules after stimulation by mitogens, antigens and lymphokines.

We have introduced Western blot method for detection of sHLA-I variants in the blood serum. We have conducted a pilot study and examined the dynamics of sHLA variants in nearly 30 hematologic patients (about 300 blood samples) after bone marrow transplantation (BMT). Our results indicated that the dynamics of sHLA variant's levels were very specific for each patient and depended in addition to seriousness of posttransplant complications also on other factors, especially on used pharmaco- and immunotherapy. The concentrations of respective sHLA variants responded to post BMT complications in different manner. The 43 kD variants raised mainly in GVHD, the 35 and 39 kD variants increased rather in infections. sHLA-I levels mostly decreased in relapses, particularly the 35 and 39 kD variants. The correlations between levels of sHLA-I variants and the course of diseases are demonstrated as individual casuistics.

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AN INVESTIGATION INTO THE ASSOCIATION BETWEEN HLA-DPB1 AND COMMON HLA-A, -B, -C, DRB1, DQB1 HAPLOTYPES

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Current studies in unrelated haematopoietic stem cell (HSC) transplantation (Pay *et al*), using patient-donor pairs matched at five HLA loci (HLA-A, -B, -C, -DRB1, -DQB1) have looked into the role of HLA-DPB1 and the question of whether matching at this locus has implications for transplant outcome. Data available supports HLA-DP matching as beneficial for the outcome of HSC transplants (Pay *et al* and Petersdorf *et al*).
This aim of this study was to look at HLA-DPB1 diversity by examining the frequency of particular HLA-DPB1 alleles in two common caucasiod extended haplotypes, namely:

1. A1, - ; B8, - ; Cw7, - ; DR17(3), - ; DQ2, -
(125 samples)
2. A3, - ; B7, - ; Cw7, - ; DR15(2), - ; DQ6, -
(100 samples)

Using volunteer potential HSC donors, previously HLA typed at HLA-A, -B, and -DRB1, and as many as possible at HLA-C and -DQB1. High resolution HLA-DPB1 typing was carried out by Reference Strand Mediated Conformation Analysis (RSCA) and this information was used to work out the probability of HLA-DPB1 mismatching for these haplotypes.
Moreover, the observed frequencies of the DPB1 alleles are compared to the expected frequencies in a random Caucasoid population. Statistical analysis of allelic frequencies is used to establish whether any HLA-DPB1 alleles are in linkage disequilibrium with the other loci of the studied haplotypes.

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THE OUTCOME OF THE CZECH PATIENTS WITH ALL/AML/CML REFERRED TO THE CZECH NATIONAL MARROW DONOR REGISTRY (CNMDR) IN 1993 - 1999. A RETROSPECTIVE ANALYSIS.

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The unrelated stem cell transplantation (Tx) is the only curative option for CML and some ALL/AML patients (pts). This assumption should be proven in the random, unselected population of pts referred to unrelated donor (URD) registry for searching an URD. Is there any benefit if successfully searched (i.e. proceeding to URDTx) compared to the unsuccessfull ones? We thus retrospectively analyzed the outcome of Czech pts with ALL/AML/CML for whom CNMDR performed URD search during period of 1993-1999. Of 215 searched pts sufficient follow-up data were obtained from 142 pts (66%) – 50 ALL, 34 AML and 58 CML with proportion of 30, 26 and 41% respectively, being Tx. The medians of follow-up from search request were 26m for ALL, 38m for AML and 31m for CML and were not significantly different between groups of Tx and nonTx pts for each dg (p=0,32-0,91). Except for the younger age in the Tx pts with AML and CML (23vs37y, p=0,002 and 27vs35, p=0,014 respectively) the clinical characteristics among Tx and nonTx pts within each dg were not significantly different (i.e. time to search, phase of disease etc.) The median time from search request to URD Tx was 4 months for ALL (range 2-6) and AML (3-18) and 6 months (3-30) for CML respectively. As of 01/01/2001 98 pts (69 %) were dead. The 5 years OS probabilities from search request are as follows:

	ALL	p	AML	p	CML	p
Tx	51%		22%		46% (46% 8y)	
nonTx	12%	0,007	5%	0,09	32% (0% 8y)	0,77

As expected, the majority of nonTx pts died of leukaemia (96 % of deaths), on the other hand the TRM was leading mortality cause in group of Tx pts (80 %). Our results show the survival advantage if the searched pts proceed to URD Tx at least for ALL and AML, while for CML the longer follow-up obviously needed. The indirect evidence of effectiveness of CNMDR search operations is also demonstrated.

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TRANSPLANT OUTCOME USING CORD BLOOD UNITS PROVIDED BY THE LONDON CORD BLOOD BANK

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The London Cord Blood Bank (LCBB) currently has over 4000 units available for search by transplant centres world-wide. Transplant outcome data has been obtained on 30 of the 43 units issued between January 1998 and September 2001.

Cord blood units were issued to 11 countries and 20 transplant centres. Recipient disorders consisted of 77% malignancies and 23% bone marrow failure syndromes. Recipient age ranged from 0.7 to 47 years, with 70% being <15 yrs. The total number of nucleated cells (TNC) received averaged 3.8 x 10^7/kg, with a range of 1.2-11.3 x 10^7/kg. Median follow up time was 13 months (range 3-37 months).

Full neutrophil engraftment of 500/μl was observed in 22 recipients (79%), taking an average time of 28 days (range 11-60). Neither cell dose or recipient age had any significant effect on engraftment. Cell dose also appeared to have no significant effect on survival. However, recipient age did have an effect on survival, with 54% of patients ≤15 yrs still alive compared to only 11% of those >15 years ($p=0.005$). HLA matching also appeared to correlate with survival, with 43% of those receiving a 6/6 match still alive, compared to only 28% survival in patients transplanted a 4/6 matched cord unit. An overall survival rate of 40% has been achieved so far.

Although the numbers used in the analysis of units issued by the LCBB are small, the results compare favourably with those published by other transplant centres and cord blood registries. This clearly demonstrates the ability of the LCBB to provide viable cord blood units for transplantation for the treatment of a variety of recipient disorders.

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REFERENCE STRAND MEDIATED CONFORMATION ANALYSIS AS A MEANS TO MONITOR POST TRANSPLANT CHIMERISM IN A DOUBLE TRANSPLANT MODEL WITH KNOWN HLA CLASS I MISMATCHES.

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Reference Strand mediated Conformation Analysis (RSCA) involves the hybridisation of gene specific fluorescently labelled reference strands (FLR) to similarly sized, near complementary, unlabelled strands from alleles present in test samples.
The mobility of resulting heteroduplexes is measured after non denaturing PAGE providing a profile of mobilities obtained from a selection of FLRs, which on comparison with control values allows the calling of alleles present. The technique was initially developed to investigate polymorphism within exons 2 and 3 of HLA Class I alleles.
As part of a study to evaluate a double transplant model to investigate (among other things), the engraftment potential of ex vivo cultured cord blood (CB) cells, HLA-A and B RSCA was used to target known mismatches between patients simultaneously transplanted with a best matched CB donor and either a less well matched CB unit (expanded CD34(+)) or a haploidentical sibling donor (uncultured, selected CD34(+)). Electropherograms obtained from time course post transplant analysis of DNA isolated from patient peripheral blood and bone marrow detected chimerism and in turn aided the relative quantification of constituent cell origins.

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HLA CLASS I DISPARITIES IN UNRELATED BONE MARROW TRANSPLANTATION : PREVALENCE OF INDIVIDUAL SUBSTITUTIONS VARIES WITH MATCHING CRITERIA

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HLA matching at the allele level has been shown to correlate with improved clinical outcome in unrelated bone marrow transplantation. The extensive allelic polymorphism of HLA genes however decreases the chance of identifying a compatible donor. Search for permissive mismatches (MM) can rely on *in vitro* cellular assays, or on the clinical impact of individual loci disparites, or even on the analysis of specific amino acid substitutions in incompatible patient/donor pairs. MM may vary in different patient groups depending on the occurrence of less well conserved HLA haplotypes and on matching criteria. Based on 152 consecutive searches from 1998-2001 (697 donors tested), the distribution of individual residue incompatibilities on α1/α2 domains were analysed in the class I-mismatched patient/donor pairs. In 77 pairs incompatible for an A/B allele the 2 most frequent mismatched residues were found at positions 156 (61%) and 116 (36%). For 107 HLA-Cw disparities the most frequenty mismatched residues were found at positions 156 (72%), 77/80 (69%), 9 (56%), 97 (53%), and 116 (43%). In nearly half of these pairs a concomitant Cw-MM was identified, mostly due to B44-subtype incompatibilities. When B44+ donors were removed from the analysis because of double class I MM, both residues 116 and 156 were now equally involved in the A/B-MM, whereas for HLA-Cw MM residue 156 was still twice as frequent as residue 116. Thus depending on the matching criteria (i.e. A/B, B+Cw or Cw) and on the immunogenetic profile of the patient/donor groups, the occurrence of individual substitutions may vary widely, which could affect the relative importance of a given residue.

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"BACK UP DONOR":
HELP FOR UNRELATED STEM CELL DONORS AND PATIENTS IN ONE STEP

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Goal of the "Back Up Donor" strategy is to provide an alternative donor for the search community in case that a donor has completed a stem cell donation and is reserved for potential further donations for the same patient in a timeframe of actually 2 years.
First intention is to provide a donor as long as possible for "his" patient afterwards the 2 year suspension. This would enable us to provide second or further stem cell donations if required.
Second intention is to protect the donor from requests for multiple patients. It could be observed several times that post-donation donors are of increased interest for the search community and were requested for confirmatory testing (CT) multiple times. Providing alternative donors would take off the burden for a single donor.
Third intention is to fill up the gap in HLA-phenotypes when a donor is suspended from further donations within 2 years post-donation and to increase the number of high resolution typed donors in our donor center.
To find a "Back Up Donor" we perform up to 100 DR medium resolution or up to 5 DR high resolution typings on our own behalf for each donor post donation. Own searches and typings are repeated till success as long as a reasonable chance remains to find a "Back Up Donor".
End of 2001 over 9000 donors will be HLA-DR typed on medium or high resolution to provide a "Back Up Donor" for more then 560 donors.

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STRATEGIES FOR PROSPECTIVE HLA-DR TYPING: HOW TO EASE THE SEARCH PROCESS FOR AN UNRELATED STEM CELL DONOR

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The DKMS (Deutsche Knochenmarkspenderdatei) performed since 1994 more than 170.000 additional (prospective) HLA-DR typings on its over 825.000 stem cell donors without any patient initiated search. This accounts for 44% of all DR-typed donors (over 382.000). Efficiency is shown by the fact that more than the half of the over 3.200 stem cell donations resulted from prospective DR typed donors.
Therefore the DKMS can provide the search centers with a wide spectrum of nearly 150.000 different HLA-AB-DR typed donors.
Without providing further typed donors it is difficult to find a DR matched donor if the initial search for a patient returns several hundreds of only AB typed donors. Providing a higher number and diversity of DR typed donors does not only shorten the time for an donor search it also helps to reduce the costs for patients.
Donor center initiated prospective DR typing was always performed at DNA-level on an intermediate resolution level allowing us to narrow down the possible alleles from a donor by using the NMDP-Allele-Codes. This enables the search center to know in advance if there is a chance to find a specially required allele by high resolution DR typing if the donor and patient share the same serology.
Evidence will be presented why the DKMS favors the donor center initiated (prospective) DR typing of selected donors already in the database above the complete (HLA- A,B & DR) typing at time of recruitment and also the logic behind the donor selection using demographic data and observed genotype frequency is explained.

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REVIEW OF 400 RELATED BONE MARROW DONOR SEARCH

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Allogeneic bone marrow transplantation is a major therapeutic advance in some hematologic or oncologic disease.

HLA typing of the members of the patient's family is the first stage of the search. But we often hear that actually chance to find a HLA genotypic identical donors is reduce because they are less children per family.

In our center during the last 10 years ,400 family have been studied to search an HLA identical donor for a patient.
So we decide to analyse our data to estimate the real frequencie of related HLA identical donor in our patient's population.

For each patient, we retrospectively review age, sex,numbers of sisters and brothers who were HLA typed, numbers of HLA A-B-DR identical donors, their age and sex.
Results will be presented and analysed to identify if possible indicative factors.

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THE RELIABILITY OF SEROLOGICAL HLA-A,B HOMOZYGOSITY IN CZECH NATIONAL MARROW DONOR REGISTRY.

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Objective: Although DNA-based HLA class II typing generally replaced serology in bone marrow donor registries typings, it is assumed that the serological typing for class I is still valuable and effective in this setting. However, a concern exists regarding its reliability in "homozygous" samples and high error rate in these was convincingly demonstrated (e.g. Lorentzen et al. 1997). We thus tried to determine the accuracy of serological HLA-A,B homozygosity in the Czech National Marrow Donor Registry (CNMDR). **Methods and Results**: 120 consecutive Caucasian (West Slavonic) volunteer donors entered the CNMDR in 1999/2000 and being assigned serologically as both HLA-A and -B homozygous (regardless of DR , i.e. 2-loci homozygous) were DNA-retyped by PCR-SSP (Genovision, Olerup SSP™). As expected and reflecting the haplotype frequencies, majority of examined samples were originally A1B8 (29%) and A3B7 (10%) . After DNA retyping, we found that altogether 37 (31%) of these putative HLA-A -B homozygous had turned out to be heterozygous. 6 of them (16% of errors) for A, 24 (65%) for B and 7 (19%) for both loci. Moreover, 2 of them also with incorrectly assigned „homozygous" allele. There was no clear pattern of discrepancies, identified "blanks" within the same cross-reactive group as seeming homozygous antigens accounted for 17 (49%) of missed antigens. Although representing 29 % (i.e. 35) of evaluated samples, the A1B8 constituted only 5 % (2 errors) of all the false homozygous. The error rate for A1B8 serologic homozygotes was thus 6 % (2 errors from 35 samples) but for others 41 % (35 errors from 85 samples). **Conclusions:** HLA-A and -B serological homozygosity in CNMDR is encumbered with relatively high error rate (false homozygous rate of 31 %) confirming the overestimation of homozygosity in serology. This is even more significant in "non A1B8 " samples (41 % error rate). These facts could be forcibly utilized in the most effective Class I DNA retyping strategy i CNMDR and also could have implications in the searching for the "true" (genotypically) homozygous patients.

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BONE MARROW TRANSPLANTATION IN SOUTH AFRICA: CONTRIBUTION OF THE SOUTH AFRICAN NATIONAL BLOOD SERVICE

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South Africa is the only country in Southern Africa where bone marrow transplantation is performed routinely. HLA Class I serological typings of voluntary recruited donors for the South African Bone Marrow Registry (SABMR) are performed at 3 centres, namely, Johannesburg, Cape Town and Durban. These histocompatibility centres are also responsible for transmitting of donor results to the National SABMR Hub, based in Cape Town. National and International registry searches are directed from Cape Town.

Since January 1999, the Tissue Immunology Laboratory of the SANBS – Inland Region has HLA Class I typed approximately 12 000 of the 20 000 donors on the SABMR. Requests for HLA Class II typing, indicating a possible patient-donor match, increased from 64 requests in 2000 to 97 requests in 2001 (until October). A discrepancy rate of 14% was reported upon the retyping of HLA Class I of donors for requests for year 2000 to October 2001.

A total of 409 HLA Class I and II serological typings (147 patients and 262 family members) were performed during the same period. 39 Cases were matched at DRB and DQB medium resolution level. Locus A, B, C DR and DQ DNA testing was fully implemented in September 2001.

The Service has been harvesting autologous (80%) and allogeneic Peripheral Blood Stem Cells (PBSC) since 1996 and cryopreserving PBSC since April 1999. 208 harvests on 120 patients have been cryopreserved. Of these, 111 harvests (53.36%) from 55 patients have been reinfused, of which 110 engrafted.

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DRB RESOLUTION AND DIVERSITY OF DONORS LISTED IN BONE MARROW REGISTRIES (BMR)

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Volunteer donors listed in BMR are currently HLA typed by DNA-based methods. We investigated the degree of the HLA DRB genotype diversity complying with NMDP standards and code nomenclature to meet intermediate resolution level (IR). We utilized 31 DRB generic probes (DRG) and an additional amplification with DRB1*03/11/13/14 (GDR3/11/6) primers with a set of 21 SSO to resolve ambiguous genotypes. We typed 65,000 donors representative of all ethnicities in the U.S.A. The number of possible unique hybridization patterns within the DRG set is 60,000. We observed only 967 DRB generic patterns, in this study. Analysis of the cumulative frequencies showed that only 14 unique probe patterns (upp) are present in 30% of the unrelated donors; 50, 80, 95 and 99% of the donors are covered by 36, 130, 315 and 552 upp. A second amplification GDR3/11/6 was applied to 27.8% of the samples; 30% and 50% of the genotypes included in this group of donors were covered by 22 and 63 upp.
In spite of the great diversity observed in this study only 1.61% of the possible genotypes were found. This study allows us to pinpoint additional polymorphisms that may be required to further splitting the corresponding genotype to the most frequent patterns, which will allow to narrow down and optimize the search strategy.

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A GRAPHIC REPRESENTATION OF THE HAPLOTYPE DISTRIBUTION FROM THE TUSCAN REGION BONE MARROW DONOR REGISTRY

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Haplotyping of single individuals in the absence of pedigree information requires identification of sequence polymorphisms, which allow unambiguous chromosome phasing. Alternatively, computer-based inferential algorithms may be implemented.

As of 31 July 2001, the Tuscany Regional Registry counted 17,298 entries. Donors born in other Italian Regions or abroad were excluded from the analysis. We present results on i. conformity to Hardy-Weinberg expectations; ii. haplotype frequencies; iii. patterns of linkage disequilibrium; iv. haplotype distributions within the Tuscany of HLA class I polymorphism detected in 11,687 potential bone marrow donors. Only HLA-A and -B typing was considered, since most donors were HLA-DRB1 typed only when their I class phenotype was selected for compatibility with a recipient. A total of 20 HLA-A and 38 HLA-B specificities (most being split antigens) were evaluated. Therefore, if we include blanks, 819 A/B combinations were generated. Allele and haplotype frequencies were estimated by maximum likelihood methods (Excoffier and Slatkin, 1995). Delta values to measure linkage disequilibria between pairs of HLA-A and -B antigens and their statistical significance were calculated according to Mattiuz et al. (1970). Haplotypes were ranked according to their delta values and a score from 1 to 819 was assigned. These scores were used to pinpoint the most likely haplotypes present in each subject. Afterwards, those donors whose haplotypes did not reach the assigned level of significance ($p<0.05$, after correction for multiple comparisons), were excluded from further analysis. The remaining 9,472 donors (18,944 chromosomes) were grouped according to their birthplace and haplotype frequencies were calculated separately for 12 health districts and 34 geographic zones. Haplotype spreading was visualized by Epimap 2.0, a shareware available from the Internet, onto Tuscany charts.

Population studies can provide valuable information in defining likelihood estimates for finding donors and to address local resources for the unrelated hematopoietic stem cell transplant donor recruitment programs.

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GENETIC STRUCTURE ANALYSIS OF A SICILIAN BONE MARROW DONORS REGISTRY (IBMDR-PA02)

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The genetic structure of our bone marrow donor population was analysed by estimating the HLA–A, -B, -DR gene and haplotype frequencies . The aim of this study is to improve the quality of our registry to allow a more efficient recruitment of the donors. In 1999 S.Rendine et al. have shown that over recent years 95% of Italian patients found an HLA–A, -B compatible donor in the national registry IBMDR (Italian Bone Marrow Donors Registry). Therefore, to increase the probability of finding a final donor in the national registry, we need to have a wider number of different phenotypes increasing, for instance, the donors number from southern regions of Italy. Infact these regions present higher genetic heterogeneity and higher probability of providing donors with phenotypes not already present in the Italian registry. Moreover, we could improve the phenotype variability introducing more complete phenotypes. At the moment our registry counts about 3500 donors, mostly from western Sicily. They were typed by serological and molecular techniques. To calculate gene and haplotype frequencies we should select the broad HLA antigens, instead of the split, when the broad antigen frequency exceed 10%. HLA-DR typed donors sample was not random because HLA class II typing is usually performed when a potential donor is found to be HLA-A, -B compatible with a patient. Our observed HLA-A, -B phenotype frequencies distribution was compared with the expected one by the statistical method G^2. Moreover, we have calculated the absolute frequency of most common and most rare HLA phenotypes. At the end of this study we could evaluate the opportune strategies to improve our registry, and increase the probability that one of our donors could be selected for a patient.

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POPULATION GENETIC STUDY OF THE HLA POLYMORPHISM IN POTENTIAL BONE MARROW TRANSPLANT PATIENTS

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In this study we have analyzed HLA polimorphism in population of individuals with bone marrow disease referred to the National Tissue Typing Center in Belgrade.
The aim of this study was to estimate if there was some specific HLA gene or haplotype contributing to disease susceptibility.
The study was performed in 539 patients and 1827 family members. HLA antigens were defined by standard CDC and modified immunomagnetic techniques. Estimation of gene and antigene frequencies were performed on basis of Hardy-Weinberg equilibrium. Statistical significat results were comfirmed by chi- square test.
The analysis of gene, antigene and haplotype frequencies of HLA class I and class II have shown no significant diferencies compared with our healthy population. The most frequent genes in individuals with bone marow disease are: at HLA-A locus, A2 (0.2968); at B locus, B5 (0.1428); at HLA-DR locus, DR2 (0.2205). The most frequent haplotype is HLA-A1,B8 (0.0659). Analysis of HLA class II antigen frequencies in group of 105 patients including 28 individuals with ALL, 23 with ANLL, 19 with CML, 12 with MDS, 14 with AA and 9 with other hematological disorders show that HLA-DR2 is the most frequent (0.4667) and the similar DR2 frequencies are revealed in all mentioned group of patients. HLA-DR52 and HLA-DQ1 frequencies are detected as significant superior compared with their frequencies in healthy population ($p<0.01$) indicating their possible role in association with hematological disorders. In general, the data of HLA frequencies will be useful also in selection of unrelated bone marrow transplant donor and for establishment of National Bone Morrow Donor Registry.

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UNRELATED ALLOGENEIC BONE MARROW DONATION: SHORT FOLLOW-UP OF 79 VOLUNTEER DONORS.

RAFFOUX C and all French bone marrow donor Centres. (France Greffe de Moelle, Hôpital Saint Louis, Paris France.)

In order to improve French procedures developed in the marrow harvesting field, we analysed data given by donors, who completed a questionnaire focused on their physical status the day of and 10 days after the harvest.
Between January 1 and October 31 2001, 78 unrelated allogeneic bone marrow collections from 78 volunteer donors (38 females and 40 males; median age 39 years for females, 41.6 for males) were performed in 24 French harvesting Centres. The average volume of bone marrow collected was equivalent in both genders (1053 ml for females and 1054 for males), although the average post-collection blood volume depletion was higher in females, (respectively 18.03ml/kg and 14.45/kg in males). The average total number of nucleated cells was higher in females (19.74 10^8/ml and 16.16 10^8/ml in males).
The most common complaint was pain (29%) at the collection site, followed by the onset of fatigue (29%), aches (9%), nausea and vomiting (7%). In 35% of cases, no complaint was reported and in all cases no complaint at long-term was signalled.
Hospitalisation was short, less than 48 h. and donors started their normal daily activities after an average of 5.6 days. 7.6% of donors did not resume working for 10 days.
74% of donors left the hospital with prescribed drugs, iron in 43% of cases, analgesic in 42%, both in 18% of cases.
Our survey confirms our previous data (BMT 1997), in particular the safety of allogeneic bone marrow collection in volunteer donors.

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ISOLATION OF CD4/CD8 DOUBLE POSITIVE T CELLS THAT ARE SPECIFIC FOR A PUTATIVE MINOR HISTOCOMPATIBILITY ANTIGEN, WHICH IS NOT PRESENTED BY HLA MOLECULES.

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Minor histocompatibility antigens (mHag) are classically described as self-peptides derived from cellular proteins that are presented by MHC molecules and recognized by MHC restricted T cells. We have isolated donor T cells from a skin biopsy in a patient suffering from GVHD after HLA-identical BMT. Two distinct populations were found. The former was CD8+ and specifically recognized recipient APCs but two weakly to assess MHC restriction. The second was a double positive CD4/CD8 population which very specifically recognized recipient, but not donor neither nor foreign APCs, in proliferative assays. No need of MHC molecules was required in this recognition, as assessed by the use of mAb against HLA class I and class II molecules. Following stimulation with recipient APCs, the cytokine profile of these latter cells showed high levels of TGF beta and IL-10, intermediate levels of IL-4, but no secretion of IL-2. Moreover, these cells expressed CD25, CD62L, and CTLA-4, 7 days post-stimulation in the absence of IL-2. In conclusion, we show herein the first case of CD4/CD8 double positive T cells that are specific for a putative mHag. Interestingly enough, the Ag recognized by these cells is unlikely to be presented by MHC molecules, as classically described. Whether these cells are regulatory T cells is under investigation, at present.

This work was performed with the support of the Association for Research against Cancer, and the Etablissement Français des Greffes.

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ANALYSIS OF HA-1 AMINOACID SEQUENCE AND DETECTION OF A NEW INTRON VARIANT IN CAUCASIAN VOLUNTEERS LOCATED CLOSE TO EXON 22

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Introduction: Despite HLA-identity between donor and recipient, several patients develop acute graft-versus-host disease (aGVHD) after allogeneic hematopoietic stem cell transplantation (HSCT) due to incompatibilities for minor histocompatibility antigens (mHag). The high immunogeneic HA-1 nonamers VLH/RDDLLEA have been implicated as a cause of aGVHD, but the impact of putative variants other than the classical nonamers within the HA-1 gene at C19p13.3 remains to be determined. Our previous work could univariately associate HA-1 mismatches with increased aGVHD incidence in a cohort of HLA-B44 like superfamily positive CML patients and their HLA-identical donors (1). We screened the known HA-1 gene aminoacid sequence for putative peptides presented by HLA-B44 and other HLA-specificities (*HLA Peptide Prediction*, available at www.bimas.cit.nih.gov). Direct sequencing of exon 22 of the HA-1 gene in healthy volunteers (n=20) was executed to identify additional variants.
Results: We could identify two putative peptides with high binding affinities to HLA-B44, encoded by exon 22 of HA-1. Sequencing 109 bp of exon 22 in Caucasian volunteers revealed no variation within the coding sequence, but we could detect an thymine (0.24) to adenine (0.76) base exchange within the following intron sequence located 107 bp from the exon 22 boundary.
Conclusions: Our findings indicate that further efforts are needed to identify new variants within the HA-1 coding sequence in addition to the classical HA-1 nonamers followed by an evaluation of the putative impact to HSCT.
1. Heinemann et al., in preparation

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NO EVIDENCE FOR AN EFFECT OF DISPARITIES OF POLYMORPHIC CYTOKINE GENES AND SELECTED MINOR ANTIGENS ON GVHD AND SURVIVAL IN A VIENNESE PATIENT GROUP UNDERGOING BONE MARROW TRANSPLANTATION

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We were interested whether disparities of polymorphic cytokine genes and disparities of other putative minor antigens show an influence on the outcome of bone marrow transplantation with HLA identical donors. We typed the polymorphic cytokine genes of TNF-a, IL-10, IFN-γ, IL-6 and TGF-ß in 50 patients and their donors by the SSP technique. In addition we characterised polymorphisms of CD31, Hag-1 and IL1-RA genes with the same technique. Patients were grouped in respect to the severity of graft versus host disease (GvHD) and overall survival. We studied correlations of the presence of genomic polymorphic markers and disease outcome. In addition, the distribution of polymorphic alleles was compared in the various patients' groups. For the analysis of disparities of the Hag-1 antigen, patients were further grouped into HLA-A2-positive and –negative individuals. We could not find any statistically significant correlation of polymorphisms and the incidence of GvHD and survival. In addition, the distribution of genomic polymorphisms did not differ between patients' groups characterised by various grades of GVHD, or the survival. We conclude that we could not see a clear-cut effect of disparities of polymorphic cytokine genes and other putative minor antigens on the outcome of bone marrow transplantation in our patient sample. The number of patients was probably too small and larger studies are be necessary to prove any influence of those markers on transplantation outcome.

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MINOR HISTOCOMPATIBILITY ANTIGEN HA-1 : CLINICAL RELEVANCE IN HLA-IDENTICAL HAEMATOPOIETIC STEM CELLS TRANSPLANTATION.

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In HLA-identical haematopoietic stem cells (HSC) transplantation, graft versus host disease (GvHD) or rejection may be induced by disparities in minor histocompatibility antigens (MiAg) between the donor and the recipient. The MiAg HA-1 has been shown to be associated with GVHD in HLA identical sibling transplants.
In order to evaluated the clinical impact of the HA-1 disparity we included in a retrospective study 76 HLA-A*0201 recipients of HSC and their sibling confirmed to be HLA-A, B, DRB1, DPB1 identical. Most patients treated for hematologic malignancies, received cyclosporin and methotrexate for GvHD prophylaxis.
The alleles HA-1^H and HA-1^R were defined by PCR-SSP molecular typing. The allelic frequency of HA-1^H was found to be 0.414. Fourteen (18,4%) patients positive for HA-1^H were mismatched with their recipient negative for HA-1^H. In univariate analysis, HA-1 disparity was not significantly associated with an increased risk of acute GvHD, relapse or chronic GvHD. These results are in discordance with those previously published by others, although the discrepancy could arise from differences in the preventive treatment for GvHD of the patients.
In more general terms, they demonstrate the need to extend investigations to other polymorphic systems and to evaluate by multivariance analysis the clinical implication of MiAg.

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PRECURSOR HELPER T CELL ASSAY EVALUATION AT THE QUANTITATIVE LEVEL OF IL-2 PRODUCTION

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Bioassays based on limiting dilution analysis technique represent a powerful tool in assessing the functionality of cellular immune responses in vitro. Although the cell culture and immune reaction detection procedures are time and labour consuming, the results obtained are of great interest in histocompatibility testing especially in exploring the immunogenic potential of single or combined HLA mismatches. For frequency evaluation of helper T cell precursors, a sensitive human IL-2 dependent mouse cytotoxic cell line CTLL-2 is being used. We have developed a simple approach for calculating the amounts of IL-2 produced by T cells as detected by CTLL-2 in a single micro culture. By using non-linear regression model, robust equations can be generated, mathematically describing the dose response curve of CTLL-2 cells to defined quantities of human recombinant IL-2 in each experiment. When applied to experimental proliferation measurement data, not only frequencies of IL-2 producing T cells can be calculated in this way, but also the potency of each individual's alloimmune response can be evaluated.

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CYTOTOXIC T LYMPHOCYTE PRECURSOR FREQUENCY ANALYSIS IN HLA- IDENTICAL SIBLING BONE MARROW TRANSPLANTATION: A 68 CASE REPORT

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Acute graft-versus-host disease (aGvHD) still remains the most important complication in bone marrow transplantation (BMT) even in HLA-identical siblings. The donor/recipient alloreactivity is probably due to minor histocompatibility antigen differences, and can be assessed through *in vitro* evaluation of cytotoxic T lymphocyte precursor (CTL-p) frequency. In our previous experience (Affaticati *et al.*, *BMT* 2000), CTL-p frequencies were determined before BMT in 51 HLA-identical sibling pairs. This showed a significant correlation (p=.048) between high CTL-p frequencies (>1/100.000) and the development of severe (degrees II-IV) aGvHD. The aim of the present study is to confirm this result in a wider cohort of patients (n=68). Data showed that 17 out of 27 (63%) patients with high CTL-p frequency, and 15 out of 41 (37%) patients with low frequency, experienced severe aGvHD. A significant correlation between high CTL-p frequencies and the incidence of severe aGvHD (p=.047) was once again found. Our results confirmed the efficacy of the assay to evaluate the risk of severe aGvHD.

CTL-p frequencies were also correlated with sex mismatch (female donor/male recipient), which is considered a possible cause of GvHD. A greater (but not significant) number of high frequency CTL-p was observed in the mismatched pairs.

In conclusion, CTL-p assay may provide useful information both for selecting the best donor/recipient combination (if more than one HLA-identical sibling donor is available) and for deciding the level of GvHD prophylaxis when the only available donor shows a high CTL-p frequency.

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DETECTION OF MISSELECTED ALLOREACTIVE CD8+ T LYMPHOCYTES SPECIFIC FOR HLA-DPB1*0901 BEFORE BUT NOT AFTER ALLOANTIGENIC PRIMING IN VIVO

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The general immunological mechanisms involved in rejection of allogeneic tissue grafts are poorly understood. Elucidation of these mechanisms is important in order to improve the outcome of hematopoietic stem cell transplantation or of vector-transduced lymphocyte infusions for therapy of a variety of different disorders such as genetic diseases or cancer. We have previously reported on the isolation of cytotoxic CD4+ T lymphocytes recognizing allogeneic HLA-DPB1*0901 from the blood of a patient at the time of rejection of a hematopoietic stem cell allograft (ref.1) . Here, we have characterized HLA-DPB1*0901-specific CD8+ T cells from the same patient. The misselected T lymphocytes were obtained by purification of the patient's CD8+ T cells prior to transplantation and subsequent in vitro stimulation with donor-derived antigen presenting cells. Several donor-specific alloreactive CD8+ T cell clones were obtained, all of them specific for HLA-DPB1*0901. These CD8+ T cell clones were dependent on co-expression of a specific HLA-DPA chain for target cell recognition. Interestingly, HLA-DPB1*0901-specific CD8+ T cells could not be isolated from the CD8+ T cell population circulating in the patient at the time of hematologic stem cell graft rejection, suggesting that in vivo priming with the relevant alloantigen may have induced peripheral negative selection of the corresponding misselected T-cell population.

Poster display V Techniques

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AUTOMATIC INSTRUMENT FOR HLA CYTOTOXIC ANTIBODY SCREENING.

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Around 1000 patients are waiting for a kidney transplant in our centre. The periodical testing for HLA cytotoxic antibodies requires a hard work, that could be helped by automatic procedures. We experienced the Lambda Scan Plus II Fluoro, an advanced tool for automatic reading and information processing, and compared its performance with that of the traditional non-automatic procedure. In our experience:
1) some critical aspects of the Lambda Scan for satisfying results are the following: 1.1) the amount of lymphocytes needed is higher, up to the double than in traditional CDC. 1.2) A brilliant fluorescence both in living and dead cells should be obtained. 1.3) The operator should re-control the positive scores;
2) advantages of Lamba Scan are mainly: 2.1) the results of automatic reading are more reliable than those read by the operator. 2.2) The automatic movement of the tray is more precise and quick than that obtained by a manual movement. 2.3) The device is provided with a good software allowing a very fast analysis of the results.
In conclusion, if Lambda Scan is compared with a traditional microscope for CDC reading, is much faster and precise. If it is used for non-automatic reading, the time required is not longer than the automatic procedure (including re-controls), but the overall results are more reliable.

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Is ELISA LAT-M™ the appropriate pre-screening assay for detecting relevant HLA-class I and II antibodies?

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Aim: Patients free of IgG anti-HLA class I and II antibodies are not at risk for developing (hyper) acute antibody mediated rejection. A quick and reliable pre-screening for IgG-anti-HLA Class I and II for kidney transplantation candidates, followed by specificity tests should be an optimal approach for the detection of relevant antibodies. In our lab we used for pre-screening only anti class I ELISA- Quikscreen™ (QS). A panel of isolated B-cells from 30 selected donors in the presence of DTT is used in the CDC-assay for detection of IgG anti HLA class II. We investigated whether the ELISA LAT-M test could be an alternative for pre-screening of IgG-anti class I and II.
Results: LAT-M class I was compared with QS and LAT-M class II with CDC B-cells with DTT. The study included 263 sera.

	Quikscreen™			CDC (B cell + DTT)	
LAT-M™	Pos.	Neg.	LAT-M™	Pos.	Neg.
Positive	43	7		33	2
Negative	0	213		0	228

Concordance LAT-M:QS = 97,3 % Concordance LAT-M:CDC= 99,2 %
Discrepant LAT-M:QS = 2,7% Discrepant LATM:CDC = 0,8%
Discrepancies:
LAT-M class I and QS : 2 sera contain known specific HLA antibodies detected in CDC, 1 sera contain IgG anti-HLA reactivity in PRA STAT. 2 sera are currently investigated.
LAT-M class II and CDC: 2 discrepant sera are currently investigated.
Conclusion: Pre screening by LAT-M ELISA test for simultaneous IgG anti-class I and II seems to be the appropriate alternative. The test is more sensitive than the Quikscreen and time saving. So more effort can be invested efficiently in HLA-specificity determination.

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COMPARATIVE STUDY BETWEEN QUIK ID CLASS II AND FLOWPRA™ SPECIFIC CLASS II BEADS

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The ELISA based Quik ID Class II (GTI) and Flow PRA™ Specific Class II flow cytometry beads (One Lambda) are both kits which allow the detection and definition of IgG class II (-DR and –DQ) HLA specific antibodies. Our aim was to determine whether one method was more sensitive than the other for defining class II HLA specific antibodies in patient sera. We tested 37 sera from 37 renal transplant recipients (11 pre- and 26 post-transplant), which had previously tested positive for IgG HLA class II specific antibodies by ELISA (B-Screen, GTI). The sera were tested with both specificity definition kits, according to the manufacturers instructions.
The Quik ID Class II defined 68 specificities (59 HLA-DR and 9 HLA-DQ) and the Flow PRA™ Specific Class II beads defined 84 specificities (68 HLA-DR and 16 HLA-DQ). Of the specificities defined, 52.9% of the Quik ID Class II and 60.7% of the Flow PRA™ Specific Class II beads could be linked to previous sensitising events (previous transplant, pregnancy or transfusion). (Table 1).
These results show the increased sensitivity of FlowPRA™ Specific Class II beads over Quik ID Class II for the detection of HLA specific antibodies, particularly for pregnancy related antibodies as has been previously reported.[1]

	Tpx		Preg		Trf		Unknown		T
	DR	DQ	DR	DQ	DR	DQ	DR	DQ	
Quik ID	22	5	5	2	2	0	30	2	68
FlowPRA	23	9	14	3	2	0	29	4	84

References 1. Robson et al (2001) European Journal of Immunogenetics **28**, *471*

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HLA ANTIBODY IDENTIFICATION USING FLUORESCENTLY LABELED MICROSPHERES AND FLOW ANALYSER : A COMPARISON WITH CDC, ELISA AND/OR FLOW

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The characterization of anti HLA antibodies class I and II is very important in renal transplantation since it must identify patients with an increased probability of a positive crossmatch and of lower graft survival.
The goal of this study was to test a new method using fluorescently labeled microspheres (57 class I beads + 32 class II beads)and flow analyser (One Lambda/LABScreen and Luminex LabMAP).
We have tested 60 selected sera including 51 already tested for HLA class I and II antibodies with standard lymphocytotoxicity (CDC) with ≥ 35 cells and ELISA (LAT M 1288 : 56 class I wells + 32 class II wells) as well as 9 other sera which showed discrepancies between CDC, ELISA – LATM and/or Flow PRA screening. The 51 sera were issued from patients collected the day of their second kidney transplantation. Sera with PRA-LCT > 70 % were not included in our series and 3 groups were established on the basis of LCT results (0 – 10 % = SG1, 11 – 50 % = SG2 , 51 – 70 % = SG3).
Only reactions with scores ≥ 6 have been taken into account. The sensibility of detection is higher with LABScreen than CDC and LAT-1288, PRA with a good correlation between the specificity of the antibodies found and the mismatched antigen(s) from the first graft and/or the known identified specificities. Out of the 51 sera studied for class I , 29 sera were concordant with the 3 technics (56,8 %), 14 were negative with CDC and positive with LAT and LABScreen, 6 were negative with CDC and LAT and positive only with LABScreen and 1 was positive with LAT (4%) and negative with CDC and LABScreen whereas out of the 48 sera studied for class II, 31 sera were concordant with the 3 technics (64,6 %), 3 were negative with CDC and positive with LAT and LABScreen, 3 were negative with CDC and LAT and positive only with Labscreen and 1 was positive with LAT and negative with CDC and LABScreen, 2 were negative with LAT and positive with CDC and LABScreen.
The other 9 sera showed a better correlation between LABScreen and FLOW PRA than with LAT – M in class I and class II detection.

These first results allow us to confirm that LABScreen methodology which is very innovant, is very sensitive and specific for HLA antibodies identification. Moreover it takes less time than all other technics. It remains us to evaluate this technic in high immunized patients (>70 % PRA-LCT). It should be a very useful technic to follow the patients class I and class II immunisation especially when their follow up is not good after immunological events.

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INCREASING THE CHANCES OF RENAL TRANSPLANTATION

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In the UK cadaveric kidneys for transplantation are allocated initially on ABO blood group, unacceptable antigens and the degree of HLA matching. Allocation is then based on a points scoring scheme taking into account additional information such as time on waiting list, recipient age, donor/recipient age difference and balance of exchange. A maximum of 50 points are available for each patient, the average achieved is approximately 25. Three and a half points are available for clearly defined sensitisation data. A thorough antibody screening programme is therefore fundamental in helping to maximise the number of points.
Evaluation of the Luminex technology and Life Match kits has indicated that the use of this method for antibody screening may improve the specificity analysis of antibodies in patients awaiting renal transplantation. A total of 104 samples previously tested using an ELISA (QUICK-ID) and/or flow cytometric (Flow PRA) method were analysed using the Life Match kits. There was a good correlation between the methods for the detection of HLA specific antibodies (Chi square 50.2 p<0.0001). Antibody specificities were clearly defined by both Luminex and Flow/ELISA techniques for 41 samples with the specificities being concordant in 83%. However using Luminex techniques antibody specificies were assigned to a number of samples for which the other methods had failed to demonstrate any clear specificity. Overall 84% of Luminex positive samples had clear specificities compared to 75% of Flow/ELISA positive samples.
The adoption of a technique that improves specificity definition will clearly benefit patients awaiting renal transplantation. In addition to preventing unnecessary crossmatching it will also increase the proportion of patients on the waiting list who will receive maximum sensitisation points. This would, in effect, increase the access of individual patients to potential donors on an allocation system based on points scoring.

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EVALUATION OF THE LUMINEX SYSTEM FOR HLA ALLOANTIBODY DETECTION AND DEFINITION

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Recently a new technology (Luminex- LABMAPTM-Multi-Analyte Profile System) has been employed for the detection and definition of HLA specific antibodies. Fluorecently labelled microspheres are coated with purified HLA antigen, these are then incubated with patient serum/plasma, washed, and any HLA specific antibody bound is detected with an anti-human IgG conjugate. This technology permits simultaneous detection and definition of both HLA Class I and Class II allo-antibodies. In this study 2 commercially available luminex systems were compared with the CDC and ELISA techniques currently used in the laboratory. Selected sera (59 for Class I and 16 for Class II) were tested. Results for specificity assigned by both commercially available Luminex systems were then compared to the existing techniques.

	LABscreen	Lifematch
Concordant	65%	49%
Discordant	35%	37%
No previous specifity	not tested	13%

An increased sensitivity was observed using the Luminex methods in 24 % of the concordant results for LABscreen and 65% for Lifematch. 25% of results were discordant due to deficiencies in the commercial software. The Luminex software is a simple and robust technique that is potentially more sensitive.

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EVALUATION OF A LUMINEX ASSAY "LIFEMATCH ID" TO IDENTIFY HUMAN REACTIVE HLA CLASS I ANTIBODIES

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The study described here was performed to evaluate the quality of this new bead - based immunoassay on different patients sera already tested by techniques (LCT and Elisa).

MATERIAL :

96 sera have been included in this study: 14 identified "test" anti class I sera, 9 sera from the 2001 External Quality Control,60 sera isolated from renal transplantation tested by Elisa and LCT

PRELIMINARY RESULTS:

Test feasibility: The procedure contains very few and simple technical steps allowing numerous simultany tests in a very short time (96 tests performed in 3 hours). The computarised analysis is more time consuming and will be improved in the next future .

Reagents Quality: :The selected antigens panel allows the detection of most of the specificities but should be modified to respond to EFI Standards.

Test sensitivity : the analysis shows that the Luminex Assay appears to be more sensitive than the LCT test and as sensitive as the Elisa

Test specificity: Most of the Identified antibodies seem to be correlated to historical immunological events. LCT detected positive sera with a PRA>50% proved to show polyspécific reactivity with this test.

CONCLUSION : these preliminary study already allows us to point out the advantages of such a strategy , including the simplicity, robustness and ra idity of this assay .A more elaborate analysis is actually performed to evaluate the critical points which have to be improved for a routine based utilisation of this test in a lab

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FLUOROANALYSIS : HLA ANTIBODIES SCREENING AND IDENTIFICATION

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Microspheres color differing by ratio of two fluorescent dyes are coated with HLA antigens . For HLA screening ,they are mixed with sera to explore, washed and then incubated with a secondary antibody PE labelled. They are analysed one by one with LifeMatch Fluoroanalyser (Bionobis).
50 sera, previously studied by CDC with a panel of frozen cells and by ELISA (One Lamnda) were analysed with the Kit Life match Id Class I (Bionobis) for detection of panel reactive Class I HLA IgG antibodies. Our preliminary results showed no significant %PRA with CDC but more sensitive for identification sometimes explained by anterior events or cross reacting reaction . Like with Elisa computer analysis , a very sensitive method, we had, after Quicktype software used for results analysis, to select specificities after acute analysis of panel antigens distribution .LifeMatch is a simple method which gives fast results since one serum is studied against 60 different antigens in a single tube . It can be used either for urgency or for important runs as it is possible to test one to 96 samples at one time.

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HIGH RESOLUTION HLA DRB TYPING USING A MICROSPHERE-BASED MULTIPLEX SYSTEM.

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We have adapted the HLA SSO typing methodology to the LabMAP™ microsphere-based technology (Luminex Corp., Austin, TX, USA) to produce the LifeMatch™ DRB SSO Typing Kit. The LabMAP format is comparable to a reverse SSO of up to 100 probes in a single well of a 96-well microtiter plate. The process involves: 1) isolating genomic DNA , 2) PCR amplifying the DNA, 3) hybridizing the PCR product to a mixture of probes attached to microspheres, 4) quantifying hybridized PCR product using the LifeMatch Fluoroanalyzer and 5) assigning positive or negative attributes for each probe and typing the sample based on probe assignments. Using this process, HLA typing of about 20 samples can be completed in approximately 5 hrs.

The current typing kit contains reagents for the simultaneous amplification of DRB generic, DR52 & DR2 group specific products. These three reactions provide medium level resolution and resolve the vast majority of the most common serological ambiguities. More than 500 samples, previously typed by conventional SSO methods, were typed with the LifeMatch Typing Kit. The results did not show any significant typing discrepancies. The use of additional primer combinations will further enhance the resolution of the DRB typing kit. One approach consists of splitting the generic DRB amplification into two reactions based on the polymorphism at codon 86. The second approach involves amplification of only DRB1 alleles using a blend of group specific primers. The latter approach can also be broken down to provide additional group specific amplifications further enhancing DRB resolution.

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EVALUATION OF A NEW HLA CLASS II DNA TYPING STRATEGY BASED ON PCR/REVERSE SSO ON FLUORESCENT BEADS : THE LIFEMATCH HLA-DRB TYPING KIT

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MATERIAL AND METHOD

The Principle of this technique is based on selective hybridization of PCR amplified HLA DRB1*exon2 DNA with sequence oligonucleotide probes covently attached on fluorescent beads

3 different sets of beads have been selected to amplify DRB1*, DRB1*15,16 and DR52 associated alleles ; because of an asymetric PCR amplification, only one DNA strand will hybridize to the 86 probes; Detection of fluorescence and allele assignment are automatically performed on a LUMINEX100™ fluoroanalyser

155 DNA samples including selected cells (rare alleles), classical cells (common allelicombinations) and External Quality Control cells have been tested

RESULTS:

At least one positive hybridization signal has been obtained with 78 probes ; out of 9523 positive and negative signals, only 50 had to be manually transformed

154 typings have been correctly assigned at a generic or medium resolution level with the persistance of 12 allelic ambiguities all being due to the coexistence of DR52 specificities

24/29 DR2, 24/128 DR52 alleles have been already identified at an allelic level

CONCLUSION:

Beside the fact that the test is very simple and can include numerous typings , we con conclude from this preliminary study, that the typings obtained appear to be performant and reliable.

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A NEW REVERSE SSO HLA DNA TYPING METHOD USING FLUORESECENT MICROSPHERES AND FLOW ANALYZER.

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To improve throughput and accuracy, we have developed a new reverse SSO typing method for A, B, DRB1 and DQB1 loci using fluorescent microspheres and flow analyzer. The system consists of oligonucleotide probes immobilized on unique sets of microspheres (up to 100 per test) and biotinylated locus-specific PCR primers. Hybridization is detected by labeling with a streptavidin-PE conjugate. A flow analyzer capable of classifying the microspheres and detecting the PE fluorescence signal generates median fluorescence intensity (MFI) from 100~200 beads per probe per test. Interpretation software adjusts MFI and scores reactions positive or negative by comparing the MFI to pre-determined cut-off values. The software then assigns HLA allele(s) that best match the reaction patterns. The 46 probes for DRB1 exon 2 types DRB1 alleles at low to intermediate resolution. By using DRB1-specific amplification primers, we have been able to resolve typical ambiguities involving DRB1*15 and 16, and DRB5, and DRB3 (codon 86) in single amplification. Also, we have developed probes to establish a linkage of two polymorphisms to resolve ambiguities without an additional BW4 specific amplifications for A and B locus typing.

The microsphere-based SSO method provides high throughput, flexibility to increase the number of probes, excellent signal to noise ratio and statistically reliable data over the conventional methods. Assay using 96 well microplate and software-based data analysis allows 200-400 samples per day per a technician. This platform can also be used for many other bioassays such as HLA antibody screening.

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HLA-DRβ SSO TYPING USING LIFEMATCH® -LUMINEX-100® MICROSPHERE TECHNOLOGY.

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The Lifematch HLA DRβ typing system is a novel sequence-specific oligonucleotide probe (SSO) technique that uses fluorescently labeled beads as the solid phase.

The study incorporated 77 samples: 39 were derived from recent Eurotransplant quality control exercises (ETQC) and 38 were taken from our routine HLA laboratory. DNA from the latter samples was extracted using DNA E-Z Prep® kits (Lifecodes Europe, Nijlen, Belgium). DRβ typing results (sample 1-39) were known from a variety of different techniques (ETQC) or from the LiPA HLA DRβ1 and DRβ decoder (sample 1-77) reverse hybridization line probe assay from Innogenetics (Ghent, Belgium). All DNA samples were retyped using the Lifematch HLA DRβ SSO typing kit (Lifecodes Europe). This assay contains primers for a generic DRβ (45 probes), DR2 group-specific (18 probes) and DR52 (22 probes) separated amplification. Based on probe assignments typing results are obtained using the Quick-type (Lifecodes) and LiPA expert (Innogenetics) software.

All results between our routine LiPA assays and Lifematch were 100% concordant at the allele-group level of antigenic splits. The samples immediately resolved at the allele level were 31,8%. Most interestingly was the resolution of the DRβ1 15 and 16 groups with Lifematch. All, except one homozygous sample, could be typed at the allele level (27/28).

In conclusion, the multiplex microsphere DRB assay is an accurate approach for intermediate to high resolution DRB typing. To confirm our study, more samples are currently examined and will be included.

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HLA DRB* TYPING : LIFEMATCH KIT AND STANDARD SSO REVERSE DOT BLOT

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LifeMatch methodology is quite similar to that used in reverse dot blot (Innolipa) where several different probes are fixed to strips, PCR product hybridized and then catched product is detected by a colorimetric reaction .
LifeMatch used a mix of 100 microspheres, different by two internal fluorescent dyes ratio and coated with various SSO probes. After genomic DNA isolation, PCR amplification products, labeled with Cy3 are incubated 45 min in well plates with microspheres, for sequence specific hybridation and analyse with LifeMatch Fluoroanalysor .LifeMatch HLA DRB DNA Typing Kit (Bionobis) contains, not only primers for genomic DRB* typing but also DR2 specificities (DRB1*15, 16) and DR52 (DRB1*03,11,12,13,14).
We analysed 50 samples with Lipa and Life match among them, 35 results were identical by the two methods but LifeCodes could determined allele specific in 10 samples not determined by Lipa , 1 ambigous by Lipa ,4 were different or ambigous results.
Result are almost similar to those obtained by Lipa method but with the combinaison of probes present in LifeCods Kit, Quicktype software provided identification of DRB1* alleles at the same time.

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HLA CLASS I TYPING USING A MICROSPHERE-BASED MULTIPLEX SYSTEM

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We have developed a series of HLA Class I SSO typing methodology using the LabMAP™ microsphere-based technology (Luminex Corp., Austin, TX, USA). The initial focus was to develop SSO typing kits for HLA A & HLA B. The LabMAP format allows for the simultaneous detection of up to 100 probes in a single well of a 96-well plate. The amplification and assay conditions developed for HLA A & B are identical to those employed in the LIfeMatch™ DRB SSO Typing Kit, so that all three systems can be processed simultaneously. The entire process, from DNA isolation to final sample typing, can be completed in about 5 hours.

The HLA A SSO assay employs 39 probes and the HLA B assay employs 67 probes. Each locus requires two amplifications that are combined, added to the bead master mix and analyzed as a single tube in the instrument. A panel of 368 samples was assayed for each system. The results showed good concordance with previous typings performed with standard SSO methodology. For HLA A, 13% of samples could not be resolved at the serological level. For HLA B, 10% of 368 samples could not be resolved at the serological level. Off these samples, 2/3 will require the use of group-specific amplifications, which are currently being explored. Also, probes are being developed to resolve the remaining type of samples and to reduce the level of ambiguities for HLA-A.

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EVALUATION OF A SEQUENCE BASED TYPING KIT FOR HLA-DRB TYPING

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Sequence Based Typing (SBT) is currently accepted as a high-resolution (H.R.) method for Class I and Class II HLA typing. However in some cases the sequence obtained does not allow to discriminate among some allele combinations.
The aim of the present study was to evaluate the HLA-DRB BigDye™ Terminator SBT kit (Applied Biosystems, Foster City, CA, U.S.A.), based on a method consisting in a low resolution SSP-PCR followed by H.R. allele typing by automated DNA sequencer (ABI Prism 377 Sequencer in this case). The positive amplification reactions from the SSP-PCR are used as sequence templates to generate the H.R. allele typing information.
We analysed 175 sequences from unrelated bone marrow donors and patients, and from quality control DNA samples: 17 out of them failed.
The remaining 158 results showed some ambiguous allele combinations both for HLA-DRB1 (41.5%) and HLA-DRB3/B4 (92.9%). Most ambiguities were solved by H.R. SSP Dynal kit, except for DRB1*03/11/06, DRB1*07, DRB3 and DRB4 that were solved only in some cases. The failure reasons were due either to nucleotide differences located outside the amplified region, or to the fact that several allele pairs resulted in identical heterozygous sequences.
In general this typing protocol provides a good tool for H.R. HLA-DRB alleles recognition, though other methods, such as SSP, are required to solve most of ambiguous results.

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MONO ALLELIC SEQUENCE BASED TYPING (SBT) OF CLASS I ALLELES AS A VERY ATTRACTIVE COMPLEMENTARY TYPING STRATEGY IN A ROUTINE LAB

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Our experience in the lab has been to develop these last two years a monoallelic SBT strategy for A and B allelic identification conbined with a commercial SSP typing strategy ; a comparative study of results obtained by the two techniques is presented here
MATERIAL AND METHOD: Sequencing of class I exons 2,3 loci has been developped (exon4 under development) on an ABI Prism A310 and systematically performed on Proficiency Quality cells, Bone Marrow graft patients and donors cells, unusual typing results, rare alleles...all these typings being performed in parallel with a classical SSP technique.
RESULTS: Out of 40 different A and B identified alleles, 4 A and 6B could be considered as rare alleles, the SBT typing result being very useful to confirm a typing SSP result (based on a single distinctive signal).In 7 cases, the SBT typing was used to control ininterpretable or unusual typing results because of the presence of false negative or positive signals
Finally, SBT allowed us to identify 6 alleles not definitively assigned by PCR/SSP (several possible alleles, 1 ambigous allelic combination)
CONCLUSION:This comparative analysis shows that in most of the cases, SSP typing results are very reliable. However, it appears that a SBT typing strategy could be very helpful in a few cases, not only to control doubtful results but also to validate typing kits reagents (specially with rare alleles) and to resolve allelic ambiguities inherent to the constant HLA allele database updating.

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SEQUENCING BASED TYPING APPROACHES FOR RESOLVING AMBIGUOUS TYPING IN HLA CLASS I LOCI.

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Sequencing Based Typing (SBT) has become an established method for generating allele level typing results. On occasion, typing ambiguities still occur due to heterozygous sequencing or polymorphism outside of the region sequenced. We have developed a series of strategies that address both of these types of ambiguities in HLA Class I genes.

Resolving ambiguities due to heterozygous sequencing requires alleles to be separated and analyzed individually. At the B locus, we have designed a set of PCR reactions, each amplifying a defined group of alleles. Dependent on the allele combination, a group-specific PCR is chosen that will amplify only one of the two alleles in a given sample. Sequencing the hemizygous PCR product provides an unambiguous type based on homozygous sequence. At the HLA-A locus, we have applied a similar approach using an HLA-A*02 PCR and sequencing strategy.

The other type of ambiguity is due to defining polymorphism found outside of the region sequenced. Many HLA Class I typing approaches focus on exons 2 and 3. For HLA-A ambiguities, simply incorporating exon 4 data helps resolve many of the ambiguities at this locus. For HLA-C, certain alleles are defined through polymorphism in exons 5 and 6 (e.g. Cw*07011, Cw*07012, Cw*0706). We have developed an HLA-C PCR and sequencing strategy that allows analysis of exon 4 to 7 to help resolve such ambiguities.

We have successfully used these approaches to achieve allele level typing in response to higher levels of ambiguity due to increasing allele numbers.

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A UNIFORM STRATEGY FOR CLASS II SEQUENCING-BASED TYPING: ACHIEVING HIGH THROUGHPUT WITH MINIMAL AMBIGUITY.

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We have streamlined our Class II sequencing-based typing by designing a uniform approach for DRB1, DQB1, and DPB1. The assay for each gene is based on one amplification reaction followed by 3 sequencing reactions. The design minimizes ambiguous heterozygote results by anticipating the problem and routinely incorporating a partial solution. Most strategies rely on analysis of typings from a first step followed by additional steps to resolve ambiguities. Here, exon 2 of each gene is amplified with PCR primers that incorporate sequencing primer recognition sites into the amplicon. Forward and Reverse sequencing is performed with the same primers for all three genes. At each gene, a group-specific sequencing primer (GSSP) is also included. These primers recognize motifs present in approximately 50% of alleles and can generate homozygous sequences from templates heterozygous for the motif. Thus, in certain ambiguous heterozygote samples, an unambiguous allele assignment will be obtained. The GSSPs employed here are: DRB1, codon 86; DQB1, codon 93; and DPB1, codon 9 or 85. In a DRB1 study of an ethnically diverse population, ambiguity was less than 10%. At DQB1 and DPB1, we estimate similar or better results but actual levels of ambiguity will depend on allele frequencies within populations. While GSSP results may be uninformative in certain situations, for reasons of simplicity and throughput we have decided that it is preferable to do the extra sequencing up-front to minimize ambiguities rather than perform additional work upon obtaining ambiguous results.

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HLA-DPB1 ALLELIC TYPING USING A NOVEL TECHNOLOGY: PYROSEQUENCING™.

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Sequencing based typing using the dideoxy-chain termination reaction and automated fluorescence detection has been described for HLA allelic typing (1).

We describe here preliminary work using a novel technology, Pyrosequencing™ (2) which involves real-time DNA sequencing using detection of pyrophosphate release. This may offer a faster technology for SBT of HLA alleles. The objectives of this study were to investigate the quality of sequence data in particular, heterozygous positions, generated by Pyrosequencing™ and whether HLA allelic assignment was possible.

We typed 10 samples for HLA-DPB1 using Pyroseqencing™. DPB1 allele assignment was performed on the text sequence generated by Pyrosequencing™ using dedicated HLA assignment software, MatchTools™ (Applied Biosystems). The results of the Pyrosequencing™ were compared with those of established HLA SBT methods (1,3). The DPB1 allelic types were fully concordant for 8 out of the 10 samples. The two samples that were disconcordant differed by only 1 and 2 nucleotides respectively, these positions were ambiguous on the resulting pyrograms.

Preliminary results show that Pyrosequencing offers potential for HLA allelic typing subject to further development and optimisation.

1. Day et al (2001) European Journal of Immunogenetics 28: 340.
2. Ronaghi et al (1996) Real-time DNA sequencing using detection of pyrophosphate release. Anal Biochem 242, 84-89.
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MICROSTORAGE OF DNA ON FILTER PAPER: ITS EMPLOY ON QUALITY CONTROL FOR HLA GENOMIC TYPING

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The creation of a registry of samples of genomic material, usually requires a painstaking process to obtain the DNA and to store it at -85°C. All this implies a high cost in terms of staff, material, and storage, which limits the size and the number of archives.

The conservation of blood samples (or previously extracted DNA) on filter paper allows a great number of samples to be held within a small space without any maintenance, making it very easy to access the DNA of the sample and carry out different genomic studies, especially those that use PCR technique.

Several samples of 25 mcl of blood, or DNA previously extracted, on Whatman n° 3 paper, and dried at room temperature or in a heater at 45°C. Insert the filter paper of the sample into an envelope and label it. Enclose several of these envelopes within, in a plastic bag with hermetic closing and silica gel desiccant.

The spot on the filter paper for study is cut and placed in an Eppendorf tube, 200 mcl of a 4M solution of guanidine isotiocianate is added, and it is then heated at 55°C for 30 minutes. The DNA released from the paper can be extracted either with saline precipitation, phenol chloroform or cationic resins. The technique we have chosen was the Kit Direct II of Dynal, due to its ease and rapidity; it is based on a resin with a metallic core which permits the separation of the DNA-resin complex with an magnetic concentrator, and the complex is undone at 65°C for 5 minutes. After removing the imam with the resin, DNA in a liquid phase remains at a quantity of 1-4 mcg, ready to be employed. We have applied the DOP-PCR technique, when the amount of DNA available in a blood spot is not enough for our porpoises, such as a complete HLA genomic study.

We have stored our samples over filter paper for periods of over a year, using the DNA restored for the genomic typing of HLA class I and II, and also for coagulation factors, satisfactory results in both cases.

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APLIYING REAL TIME PCR FOR GENOTYPING OF THE LOCUS DQB1*

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Detection of amplified chains for genomic typing in the HLA system by PCR-SSP, is usually carried out by electrophoresis in agarose gels, which is a slow and labour-intensive method. The objective of our study is to detect the presence of possible amplified chains during the course of PCR-SSP by using SYBR green (Molecular Probes), a fluorochrome that is able to join double DNA chains while the PCR is being carried out, using a thermocycler equipped with a fluorometer. We have employed a commercial kit to assay DQB1* by PCR-SSP, changing the original conditions of the PCR-SSP to inhibit the control primers.
The loading buffer is similar to the one recommended by the manufacturer except for contains SYBR green at 0.5%.
The thermocycler employed was a System 5700 (PE Biosystem), and the amplifying conditions were: 95°C 2 mins, 1 cycle; 94°C 5 secs, 65°C 20 secs, 10 cycles; 94°C 5 secs, 61°C 20 secs, 72°C 30 secs, 20 cycles; 72°C 3 mins, 1 cycle. The products of the amplification were analysed using electrophoresis in agarose. A pararell study was done with the DNA problems using the conditions recommended by the manufacturer. 12 DNA samples were tested during the study.
The evaluation of the results graphics obtained by the thermocycler System 5700 in real time indicated two easily identified subsets of amplified chains (both appearing after three steps cycles). The first refers to tubes containing PCR with primers DQB, which are begun in all cases in cycle 12 and which correspond to the amplified DQB chains detected in the agarose gels. The second subset begins amplification at cycle 15 with agarose gel activity corresponding to the control primers.
The results obtained in both variants of PCR tested correlated perfectly, which suggests that it would be worthwhile to continue to improve application methods of SYBR green for HLA typing.

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OPTIMIZATION OF PCR-SSP CONDITIONS APPLIED TO LOW AND HIGH RESOLUTION HLA TYPING BY SSP

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Low and high resolution techniques of typing SSP for the HLA system, have the strength and sensibility required to make them essential for the studies of molecular biology by PCR.
A decisive factor is the length of the amplification process of the PCR, which limits the perfomance of the thermocycler, since the standard time parameters usually employed are the ones provided by the makers of the kit.
The kinetic of the PCR cycle is based on three steps: denaturation of the genomic DNA, primers annealing and extension. These steps are determined by the specific temperature for each one, and how the optimum temperature is reached is conditioned by the volume of the sample and the heat ramp that makes the thermocycler change from one temperature of the cycle to another. 10 mcl of sample, usually employed for the SSP, makes it possible to increase by 1°C/sec, top speed for most thermocyclers, and fast enough to reach the optimum temperature for each step of the cycle, and therefore able to reduce the length of time of the steps. All this has allowed us to reduce the duration of the technique by 30%, maintaining the reliability and resolution of the procedure. There have been analyzed in paralel 40 samples using both the recommended times by the manufacturer and the optimized by us.
We have employed for our study SSP Dynal kits for low and high resolution for the locus DRB1*-5*, DQA1*, DQB1* and DPB1*.
Standard PCR: 94°C 2min, 1 cycle; 94°C 10 sec., 61°C 60 sec., 10 cycles; 94°C 10 sec., 65°C 50 sec, 72°C 30sec, 20 cycles.
Optimised PCR: 94°C 2min, 1 cycle; 94°C 5 sec., 61°C 25 sec., 10 cycles; 94°C 5 sec., 65°C 25 sec, 72°C 10sec, 20 cycles.
The duration of the standard PCR is 1 hour 34 minutes while the optimised PCR takes 1 hour 3 minutes.
Results in both series offered identical typings.

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OPTIMIZATION OF PCR-SSP CONDITIONS FOR TYPING OF HLA CLASS I AND II WITH SMALL SAMPLES OF DNA

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The SSP method usually applied to HLA molecular typing requires quantities of DNA about 50 ng per tube, it makes necessary amounts of DNA about 15 mcg for a complete HLA class I and II molecular typing. This requirement, added to the necessity of storaging enough DNA samples for complementary studies, implies in the standard routine the extraction of large quantities of blood. Our procedure permits using the SSP method with about 1 mcg of DNA.
We took three aliquots of whole blood, 50-200mcg, from 20 different control people. Blood spots on 3MM filter paper from the first aliquot were dried and stored at room temperature. The second one was mixed 1:1 with ethanol, shaked and stored on a freezer. From the thirth aliquot, DNA was directly obtained and apropiately conserved.
DNA was stracted by two different procedures: salt precipitation and resin balls with methalic core (kit DNA Direct II from Dynal). We chosed the last one for its easyness and rapidity, obtaining 1-4mcg of DNA which was carried to a final volume of 1ml to prepare our working solution.
In all the 20 people, it has previously studied the following HLA class II locus: DRB1*-5, DQA1*, DQB1* and DPA1*, with the usual methodology in our laboratory.
We have changed some general conditions of the typing kits class I and II from the Dynal SSP method. We have modified the amount of DNA per tube to 2-4 ng and the concentration of the Taq Polymerase to 0,1 U/tube, instead of the 100 ng of DNA and 0,4 U /tube of Taq Polymerase recommended by the kit instructions. The conditions of the PCR had been: 1) 94°C, 2 min., 1 cycle. 2) 94°C 10 sec., 65°C 60 sec. 10 cycles. 3) 94°C 10 sec., 61°C 50 sec., 72°C 30 sec. 30 cycles. 4) 72°C 3 min.
The tests have successfully allowed us to study the HLA class II genotypes, getting similar results to the one's obtained with the quantities of DNA and the amplification conditions recommended by kits of Dynal.

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LOW AND HIGH RESOLUTION GENOMIC TYPING FOR THE HLA CLASS II WITH AMPLIFIED DNA BY DOP-PCR

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The SSP method usually employed for the molecular typing of the HLA system requires about 50 ng/mcl per tube. As a result we need quantities of about 40 mcg of DNA for a complete molecular typing of HLA class I and II. This requirement makes it more difficult to study from HLA of hipocelular samples such as medular aphasia and cells from the umbilical cord.
In order to overcome this difficulty, we have made a previous amplification of the sample with the DOP-PCR technique, based on the amplification with degenerated primers, creating a source of oligonucleotides that have as their hybridation target, fixed segments on the chains of genomic DNA. The DNA hold of the sample can be multiplied, obtaining enough genomic material to be employed for the complete study of HLA class II.
The samples, from 50 ng to 1mcg of DNA, were amplified using the DOP-PCR kit from Roche, obtaining 100 mcl in a concentration higher from 500 mcg/ml, and carried to a concentration of 50 ng/ml are used as preamplifier samples. At the same time, DNA from the same patients has been used as a parallel control.
For the genomic study of the system HLA class II, we have employed the kits for generic and specific typing, Dynal DRB1*, DQA1*, DQB1* and DPB1*.
The tests have had identical results in both parallel assays with "normal" DNA and DNA amplified by DOP-PCR.
The application of the previous amplification, for DOP-PCR, permits the use of very small quantities of DNA from hipocelular samples, blood smears, and blood spots on paper.

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HLA-DRB1 TYPING BY REFERENCE STRAND-MEDIATED CONFORMATIONAL ANALYSIS (RSCA) USING ABI PRISM 310 GENETIC ANALYSER (APPLIED BIOSYSTEM) .

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Allogeneic Bone Marrow Transplantation (BMT) is a widely used therapy for the most of Leukaemias and other haematological diseases. For this reason, more and more sophisticated HLA-typing systems are necessary to improve the outcome of BMT. Recently, our group has focused its attention to a new method of DNA typing, developed at the Anthony Nolan Research Institute in London. This method called Reference Strand mediated Conformational Analysis (RSCA) has been performed by our group using a capillary- based genetic analyser (ABI PRISM 310 Applied Biosystem). The application of RSCA to class I and II using ALFexpress (Amersham-Pharmacia Biotech) has been described. The application of RSCA to HLA-A and B using the capillary instrument (Applied Biosystem) has been described. In this study, we set up a database of mobility values using known DNA studied in high resolution by SSP. Every allele was run at least five times. Once having our mobility values, we used donors' DNA amplified with generic HLA-DRB1 primers and ibridizied with Fluorescent Labelled References (RLFs).The separation of the different alleles after electrophoretic run is due to the different association with the RLF used, therefore, the different combinations that every allele can form with the same reference cause different mobilities. We've just studied several known alleles tested in high resolution by SSP and by sequencing analysis in order to compare the results so having the RSCA as another useful test for HLA- typing. The benefit of RSCA is to have individualized a different kind of HLA typing that can be easily used giving a medium to high resolution and reduced cost.

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RESEARCH OF NEW TARGETS FOR MELANOMA IMMUNOTHERAPY BASED ON MICROARRAY TECHNOLOGY.

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The great development of gene expression technology allows the analysis of large number of genes at high resolution and has opened new possibilities for tumor studies. Using Affymetrix GeneChip we studied the gene expression profile of different melanoma cell lines to detect potential melanoma specific antigens. We extracted RNA from melanocytes and melanoma cell lines derived from the same patients. After an amplification process due to the small amount of starting material (50.000 – 200.000 cells), we obtained enough biotinilated cRNA for chip hybridization. We then compared the differences of expression level of about 12.600 genes and we selected few candidate genes using different software tools (RACE-A, SAM, dChip) for expression analysis. The genes of interest have been divided into some families according to their functions and the public databases. The following studies on selected genes are important to chose potential targets useful for tumor immunotherapy based on T cell.

Poster display VI Polymorphism

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SEROLOGY AND DNA BASED GENERIC TYPING FOR HLA-A;B;DR. ANTIGENS OF CADAVER ORGAN DONORS ARE CONCORDANT IN 98 % OF CASES IN A RETROSPECTIVE STUDY.

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The progress in molecular biology applied to HLA typing, the several advantages of this method over serology : lack of dependence of quality of cells and on cell surface expression, greater confidence of antigen assignment including antigens missed by serology had let us to consider the use of DNA technology, for routine in the typing of cadaver organ donors. We have addressed the question of how often cadaver organ donors are mistyped by serology in our laboratory. Retrospectively, we have performed blindly by DNA typing one hundred cadaveric donors typed by serology from 01/1999 to 06/2001. For microlympho-cytotoxicity we used our local alloantisera typing trays (class I + class II) in parallel with commercial available monoclonal trays. Lymphocytes were always extracted from lymphnodes. The number of HLA antigens tested were respectively A (17); B (28); DR (13); DQ (5). Retrospective DNA typing were performed by reverse SSOP. Ambiguities of this low level typing were resolved secondarily by PCR-SSP. We have noticed two discrepancies (2%). Sample "7414" found A*43 instead of A26 and sample "11052" found only DRB1*15 instead of DR15, 13. The concordance of serology and DNA typing is 98 % in this study. Undoubtedly for the determination of alleles, DNA typing represents the most accurate method. However in organ transplantation, rigorous pairing of recipient (R) and donor (D) is unrealistic due both to the polymorphism of HLA alleles and to the scarcity of donors which limits the choice. In our experience, analysis of A;B;DR. compatibilities of D/R pairs shows a compatibility of 2,7. In addition, the necessity of crossmatch tests with present and historic sera in immunised recipients imposes the isolation of donor T and B lymphocytes. In this study, routine DNA typing brings little improvement. The small % of discrepancies, the possibility of rapid recourse to targeted DNA typing in case of deficient serology contribute to maintain our present strategy. However, should a more flexible DNA technology be available, we would consider modifying our attitude.

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HLA CLASS II HAPLOTYPES DETERMINED BY A PROPOSITIONAL CALCULUS ALGORITHM

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Assigning a genotype (a pair of haplotypes) to a given HLA phenotype not only gives a genetic explanation to the analytical determination of HLA alleles, but it also provides a verification process. Genotype analysis requires haplotype frequency tables, which are deduced from family phenotypes or inferred from the distribution of alleles in large populations. A set of class II HLA haplotypes is presented here. The method used is based on the conceptualisation of HLA typing as a propositional logic. Each subject is represented as a vector in a logical space where the axes or dimensions of the space are of the form 'this subject shows allele X'. Such a space was created with 5 class II HLA loci. The determination of haplotypes was carried out by a heuristic algorithm to search this logical space and identify the best propositional function that account for a given DRB1 allele, where the arguments of the function are the presence or absence of the corresponding alleles for the HLA-DRB3, HLA-DRB4, HLA-DRB5 and HLA-DQB1 loci. The data source consists of high-resolution class II typing of 5,362 subjects from a bone-marrow transplantation programme, both patients and donors, both related and unrelated. (No claims are made about the racial distribution of alleles. This sample is just representative of the population it comes from.) The results include 169 haplotypes. The use of genotypes in HLA typing allow the identification of errors, provide a credibility measure to guide the confirmation process, and help in the donor search strategy.

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NUCLEOTIDE SEQUENCING ANALYSIS OF EXON 1 TO 8 OF A NOVEL HLA-C ALLELE

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We performed high resolution HLA-typing in a patient suffering from leukaemia. DNA was prepared from the peripheral blood by the salting out method. Sequencing analysis was performed on DNA amplification products including exons 1 and 2 and exons 2 and 3. Amplification of the whole HLA-C locus was performed with a blend of Taq-polymerases (Expand long PCR system, Roche, Mannheim). For sequencing of the long template, several primers situated in all exons and some introns were used. We found a novel HLA-C allele. Its sequence is identical to HLA-C*12021, except a C to G exchange at position 318 in exon 2. This nucleotide exchange leads to an amino acid exchange from arginine to proline. Confirmation of the novel allele was found by family analysis, when it was detected also in a sibling of the patient. Analysis of the "long" sequencing template revealed, that we had lost one allele. Solely the novel allele had become amplified.We conclude that it is possible to amplify the whole HLA-C locus in a single amplfication reaction. The possibility of allelic loss, however, has to be kept in mind. Sequencing of all exons of HLA alleles is probably the best approach to gain insight in the polymorphism of HLA molecules.

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IDENTIFICATION OF THE NOVEL ALLELE B*4427 AND A CONFIRMATORY SEQUENCE (B*44022)

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This report presents a novel allele, HLA-B*4427, which was identified in a bone marrow donor of Caucasian origin, and a confirmatory sequence (B*44022). Sequence analysis revealed the new allele differs from B*44021 by a single nucleotide exchange at position 668 (C→T), which is located in exon 4. At the protein level, it is the only B*44 variant to produce an Ala in place of a Val at codon 199. Its structure suggests that it may have originated from a point mutation in B*44021 or by gene conversion with a variety of HLA-B alleles (B*14,B*15,B*18, B*3701,B*3704,B*45,B*4601,B*49,B*50,B*5603,B*8202) in a region including the complete exon 4. To assign the new B*4427 allele to a certain B*44 allele group, we compared the new allele to the unknown exon 4 sequence of B*44022 allele that, after sequencing, appeared identical to B*44021 and to all other B*44 exon 4 sequences. The substitution found in exon 4 of B*4427 might modify the structure of the α3 domain, which associates with β2-m and interacts with the T-cell co-receptor CD8, and produce a different induction of cytotoxic immune response compared with all the other B44 variants.

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FIRST DETECTION OF THE DRB4*0103102N NULL ALLELE IN CZECH POPULATION

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HLA Class I antigens for Bone marrow registries and for solid organ transplantation are still typed by serology in the most of histocompatibility laboratories. Detection of HLA class II antigens is provided mainly by DNA-based typing only. Therefore identification of alleles with low or lack of protein expression on cell surface happens in routine practice more or less by chance. Their detection requires examination of large number of individuals by serology combined with molecular genetics methods. Particularly it involves DRB3, DRB4 and DRB5 genes encoding the DR52, DR53 and DR51. It is well known that DRB1*03, *11, *12, *13 and *14 alleles are associated with the DRB3 gene. The DRB4 is present in DRB1*04-, DRB1*07- and DRB1*09 - positive individuals. But DRB1*15 and *16 are associated with DRB5. Under the current HLA nomenclature, particular number of "null" alleles for DRB4 and DRB5 (not for DRB3) exists and their number increases year by year. So far it is known that 3 genes of DRB4 locus don't express DR53 protein on the cell surface (DRB4*0103102N, DRB4*0201N and DRB4*0301). The DRB4*0103102N allele seems to be exclusively present in DRB1*07-DQB1*0303, unusual association with DRB1*04-DQB1*0302 was reported in recent papers though.

Here we report the first detection of "null" DRB4 allele in Czech population. For the present 1 740 donors from Czech Bone Marrow Donor Registry and 200 healthy individuals from local panel were typed for HLA Class II genes. High resolution typing of two individuals (one donor from CBMD and one patient) using PCR-SSP method revealed the presence of DRB4*0103102N. Family studies showed it to be associated with DRB1*0701 - DQB1*0303 haplotype in both cases.

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IDENTIFICATION AND NUCLEOTIDE SEQUENCE OF A NEW NULL ALLELE, HLA B *3501N

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We report the definition of an HLA class I null allele, which has been identified within the B35 group by a combination of serology, PCR-SSP, PCR-SSO and SBT. This allele was detected in a foreign, potential unrelated donor of unknown ethnic origin selected as a probable match for an Irish patient. The presence of the null allele was initially suggested by the absence of B35 reactivity by serological typing. In contrast the donor was typed B*35, B*44 by PCR-SSP and PCR-SSO (Dynal). To define the apparent mutation causing the lack of B35 serological reactivity direct sequencing of an HLA-B specific PCR product containing exon 2, intron 2 and exon 3 was performed. The results demonstrated the presence of B*35011 and B*44031, with no indication of any null mutations. Further analysis involved the cloning of a 3.2kb PCR product encompassing the full length HLA-B genomic sequence. Clones containing the B*35 allele were selected by sequencing of exon 2 to distinguish between B*35 and B*44 alleles. Full length sequencing of three out of many possible B*35 clones identified a single nucleotide deletion at position 807 within exon 4 (numbering begins at the beginning of exon 1 and excludes introns). This deletion introduces a TGA stop codon at nucleotide 886 at the end of exon 4. The introduction of this stop codon most likely explains the lack of cell surface expression of the encoded protein. The nucleotide sequence for the HLA-B*3501 null allele was submitted to the WHO Nomenclature Committee in November 2001.

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A NUCLEOTIDE INSERTION IN EXON 4 IS RESPONSIBLE FOR THE ABSENCE OF EXPRESSION OF AN HLA-A*0301 ALLELE IN A PROSTATE CARCINOMA CELL LINE.

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In this report, based on a combination of cellular and molecular methods, we describe a nucleotide insertion responsible for the loss of expression of an HLA-A*0301 allele in the prostate carcinoma cell line DU145. HLA-class I expression on the cell surface, measured by indirect immunofluorescence, showed loss of expression of the HLA-A*0301 allele. RT-PCR analysis using specific primers for the HLA-A locus revealed the absence of transcript of HLA-A*0301. We therefore explored the HLA-A*0301 gene itself by PCR amplification of genomic DNA. Sequencing of the cloned fragments, showed the insertion of an additional C within a cytosine island located at the beginning of exon 4. This alteration leads to a premature stop codon at position 196, within exon 4 and it is likely to account for the absence of surface expression of the HLA-A3 antigen. The same insertion has been described in three different null alleles: A*0104N, A*2411N and B*5111N. Because nucleotide repeats are more susceptible to mutation in tumor cells that exhibit the mutator phenotype and these tumors display microsatellite instability, we analyzed the DU145 cell line for the RER phenotype using several mononucleotide repeat sequences. The DU145 cell line was unstable for three microsatellite markers. In conclusion, the mutational event that gives rise to loss of expression of HLA A*0301 in cell line DU145 may be interpreted in two ways: as1) the insertion of an extra cytosine generates a novel HLA-A3 null allele, or 2) the inactivating mutation arises from tumor genomic instability and is selected during tumor progression because it represents an immunological advantage

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PLATELET ALLOANTIGENS GENOTYPING: DISTRIBUTION OF HPA-1,-2-,-3,-5 IN A BLOOD DONOR PANEL

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Alloimmunization and associated refractoriness are adverse outcomes to transfusion therapy related to immunization to antigens HLA and to human platelet antigens (HPAs), through pregnancy or blood transfusion.
STUDY DESIGN: the aim of our work was to establish a panel of blood donors serologically typed for HLA class I and genotyped for the major platelet antigens, in order to find a compatible donor for both antigens HLA and HPA.
METHODS: 87 blood donors were typed for HLA class I by microlymphotoxicity assay (Onelambda-Biotest) and were genotyped for HPA -1,- 2, -3, -5 using PCR-SSP technique. Genomic DNA was isolated from EDTA whole blood samples by Salting Out method and amplified by PCR with specific primers described by Skogen et al (Transfusion 1994;34;955). Eight primer mixtures corresponding to the amplification of HPA-1a,-1b, -2a, -2b, -3a, -3b, -5a and -5b alleles respectively, were prepared using the primers for HGH as internal controls.
RESULTS: the frequencies of HPA-1,-2,-3,-5 among our group did not deviate significantly from those observed in other Caucasian populations; furthermore there was no significant deviation from the Hardy-Weinberg equilibrium for the four genetic systems.
CONCLUSIONS: the genotyping of platelet antigens by PCR-SSP represents a suitable method and can contribute to the establishement of panel HLA- and HPA- typed donors to provide a compatible transfusion for a good management of alloimmunized patients.

HPA–1a 0.816	HPA–2a 0.891	HPA–3a 0.632	HPA–5a 0.897
HPA–1b 0.184	HPA–2b 0.109	HPA–3b 0.368	HPA–5b 0.103

Poster display VII Population

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THE EVOLUTION OF *MHC-G* GENE DOES NOT SUPPORT A FUNCTIONAL ROLE FOR THE COMPLETE PROTEIN

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The study of the MHC-G gene evolution during nearly 50 million years does not support a role for the full molecule. New World monkeys MHC-G-like proteins are most probably classical presenting molecules, Old World Cercopithecinae monkeys do not have a full MHC-G molecule and human individuals homozygous for the HLA-G null allele are healthy and do not show birth pathologies.

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CYTOKINE GENOTYPES FREQUENCIES IN THE PORTUGUESE POPULATION

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Cytokines acting in a network regulates the immune response. Cumulative evidence indicates that single nucleotide polymorphisms (SNP) in regulatory regions of the gene or at codifying regions involved in postranslational control can influence cytokine production levels. Furthermore, several studies correlate the influence of individual differences in cytokine production levels with susceptibility to various autoimmune or other diseases and allograft outcome.
It is the aim of this study to evaluate the frequency of the polimorphic variants of 7 cytokine genes described as High, Intermediate or Low producers in a randomly selected population representative of the Caucasoid Portuguese population. This analysis will be important on subsequent studies of association between cytokines genotypes and some diseases susceptibility and severity. It was genotyped by PCR-SSP 124 Portuguese Caucasoids, 50 unrelated healthy donors and 74 cadaveric solid organ donors whose death cause was traumatic accidents without history of autoimmune diseases.

Percentage frequency of cytokines genotypes in a population of 124 individuals of Portuguese origin

IL-10 (-1082)	IL-10 (-819/-592)	TGF - β Codon10	TGF - β Codon 25	IL -4 Rα (+1092)	TNF - α (-308)	TNF - β (+252)	IL -6 (-174)	IFN - γ (+874)
GG 17.7%	TT/AA 5.7%	CC 15.3%	GG 84.7%	GG 61.3%	GG 73.4%	GG 8.9%	GG 46.8%	TT 14.6%
GA 46.0%	CT/CA 43.5%	CT 58.1%	GC 15.3%	GA 30.6%	GA 24.2%	GA 30.6%	GC 41.1%	TA 55.6%
AA 36.3%	CC/CC 50.8%	TT 26.6%	CC 0.0%	AA 8.1%	AA 2.4%	AA 60.5%	CC 12.1%	AA 29.8%

The frequency of the polymorphic variants of the studied cytokine genes in the probed population is consistent with those reported for other European Caucasoid groups while different when compared to Oriental or African populations. In summary, we present the distribution of 7 cytokines genotypes in a group of Portuguese origin, which is related to the one of other Caucasoid populations, in order to control possible associations with disease susceptibility.

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GENETIC RELATIONSHIPS BETWEEN THE MURCIA POPULATION AND IBERIAN, MEDITERRANEAN AND WORLD POPULATIONS

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The interest of this study is its contribution to the better knowledge of the origin of the peoples of the Iberian Peninsula. They constitute a natural prehistoric bridge between Africa and Europe, which can be extended to a cultural link between Europe and the American continent. HLA alleles were determined in 173 individuals from Murcia Region in the Southeast of Spain. Allele and haplotype frequencies, dendrograms and correspondence analysis were estimated and constructed by using the ARLEQUIN, DISPAN and VISTA sotfwares, respectively. The heterozygousity in Murcians was similar to those observed in other European population and showing higher values than in Basques, Sardinians and Spanish-gypsies. Ten haplotypes occurs with the greatest frequency in Murcians, five of them are shared with Spaniards, being four of these also present in Portuguese or Basque populations. Taking together both DA and the resulting tree, it can be observed that: first, the Murcians, Spaniards and Portuguese represent the closest branches; second, French, Italians, Algerians and Canadians (North Americans of European ancestry) constitute the following group; the third group includes Germans, Catalans and Spanish-Basques, followed by Cretans, Sardinians and Greeks, and in fourth place, at a greater genetic distance, there are the Senegalese, Spanish-gypsies, Bushmen and Japanese populations. Thus, Murcian population seems to belong to the Southwerstern European genetic pool, and in spite of their wide cultural differences, could have the same common origin as other Iberian and North African populations, especially due to the substantial gene flow from Paleo-North African populations to Iberia.

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HLA GENES IN MACEDONIANS AND THE SUB-SAHARAN ORIGIN OF THE GREEKS

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HLA alleles have been determined in individuals from the Republic of Macedonia by DNA typing and sequencing. HLA--A, -B, -DR, -DQ allele frequencies and extended haplotypes have been for the first time determined and the results compared to those of other Mediterraneans, particularly with their neighbouring Greeks. Genetic distances, neighbor-joining dendrograms and correspondence analysis have been performed. The following conclusions have been reached: 1) Macedonians belong to the "older" Mediterranean substratum, like Iberians (including Basques), North Africans, Italians, French, Cretans, Jews, Lebanese, Turks (Anatolians), Armenians and Iranians, 2) Macedonians are not related with geographically close Greeks, who do not belong to the "older" Mediterranean substratum, 3) Greeks are found to have a substantial relatedness to sub-Saharan (Ethiopian) people, which separate them from other Mediterranean groups. Both Greeks and Ethiopians share quasi-specific DRB1 alleles, such as * 0305, *0307, *0411, *0413, *0416, *0417, *0420, *1110, *1112, *1304 and *1310. Genetic distances are closer between Greeks and Ethiopian/sub-Saharan groups than to any other Mediterranean group and finally Greeks cluster with Ethiopians/sub-Saharans in both neighbour joining dendrograms and correspondence analyses. The time period when these relationships might have occurred was ancient but uncertain and might be related to the displacement of Egyptian-Ethiopian people living in pharaonic Egypt.

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THE ORIGIN OF PALESTINIANS AND THEIR GENETIC RELATEDNESS WITH OTHER MEDITERRANEAN POPULATIONS

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The genetic profile of Palestinians has, for the first time, been studied by using human leukocyte antigen (HLA) gene variability and haplotypes. The comparison with other Mediterranean populations by using neighbor-joining dendrograms and correspondence analyses reveal that Palestinians are genetically very close to Jews and other Middle East populations, including Turks (Anatolians), Lebanese, Egyptians, Armenians and Iranians. Archaeologic and genetic data support that both Jews and Palestinians came from the ancient Canaanites, who extensively mixed with Egyptians, Mesopotamian, and Anatolian peoples in ancient times. Thus, Palestinian-Jewish rivalry is based in cultural and religious, but not in genetic, differences. The relatively close relatedness of both Jews and Palestinians to western Mediterranean populations reflects the continuos circum-Mediterranean cultural and gene flow that have ocurred in prehistoric and historic times. This flow overtly contradicts the demic diffusion model of western Mediterranean populations substitution by agriculturalists coming from the Middle East in the Mesolithic-Neolithic transition.

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UNEXPECTED HLA-DRB1/DQB1 ASSOCIATIONS IN GREEK POPULATION

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A variety of HLA-DRB1/DQB1 haplotypes have been observed with different distribution in several populations, although a strong linkage disequilibrium and a rare recombination exists between HLA-DRB1 and DQB1 genes.
Since 1992, rare and unexpected DR/DQ associations observed , given the data concerning European Caucasoids, were recorded. Ninety-three (93) such unexpected DR/DQ associations among 15000 class II typings performed in our every day work were noticed. Twenty-eight (28) of them are not presented in this report, because in the meantime they have already been described as rare but existing haplotypes in other populations. The remaining 65 cases (31 males, 34 females) are presented.
All typings were performed by classical serological techniques and since 1994 by molecular techniques (PCR-SSP, PCR-SSOP).
Results. The following unexpected associations (the number of cases are reported in parentheses) were observed: DR4/DQ2 (20), DR11/DQ5 (10), DR11/DQ6 (8), DR17/DQ5 (8), DR7/DQ7 (3), DR17/DQ6 (2), DR12/DQ2 (2), DR17/DQ3 (2) DR10/DQ8 (1), DR8/DQ8 (1), DR11/DQ2 (1), DR13/DQ4 (1), DR14/DQ7 (1), DR12/DQ5 (1), DR4/DQ4 (1), DR3/DQ4 (1), DR3/DQ7(1).
In conclusion and according to the data from the 11th and 12th IHWCs, a few of the above mentioned associations have been reported in certain European Caucasoids, whereas most of them, mainly DR3/DQ1, DR11/DQ6, are observed in Asian as well as African Negroid populations.
This information may be of use for population genetics, disease associations and Bone Marrow Donor search.

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HLA POLYMORPHISM IN BONE MARROW DONORS IN CENTRAL ANATOLIA OF TURKEY

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Matching for allels of HLA loci is important in bone marrow transplantation as it has been shown to influence graft rejection and patient survival. In the present study, we have typed for HLA- A, B, DR B1* genes and haplotypes of 550 healty individuals in Central Anatolia of Turkey by PCR-SSP (sequence spesific primers) low resolution DNA technique.

The most frequent HLA-A genes are: A2 (45.45 %), A3 (20. 90 %), A24 (20), A1 (19. 09 %), A30 (12.72 %).

The most frequent HLA –B genes are: B35 (29.09 %), B51 (22.72 %), B38 (11.81 %), B8 (11.81 %), B7 (9.09 %), B18 (8.18) % .

The most frequent HLA-DRB1* genes are: DRB1* 11 (32. 72 %), DRB1* 04 (27. 27 %, DRB1* 15 (13.63 %) DRB1* 13 (10 %), DRB1* 01 (9. 09).

The most frequent HLA- A, B, DRB1* haplotypes are: A2 B35 DR B1* 11 (8.18 %), A2 B51 DRB1* 11 (4. 54 %), A2 B 35 DRB1* 04 (3.63 %).

We found the similar HLA genes and haplotypes in bone marrow donors in comparison to the previous data in Turkey.

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DNA TYPING OF HLA-AB ALLELES AT INTERMEDIATE RESOLUTION LEVEL OF 1902 TURKISH POPULATION

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Molecular typings of 1,902 subjects from Turkey Marrow donor registry were performed using PCR and sequence-specific oligonucleotide probes (SSOP) methods. Locus specific primers and 43 A SSOP and 61 B SSOP were used to type HLA-A, B alleles. These methods were designed to give alleles typing at intermediate resolution level. Those results were at serological level or higher resolution. Among the 1,902 subjects typed, we identified 26 HLA-A and 51 HLA-B alleles at allele level; and 12 HLA-A and 32 HLA-B alleles at serological equivalent level. At HLA-A, we observed the alleles A*3201, 0101, 2601, 2301, 6801, 3001, 0302, 3101 were at high frequency (Gf=0.0464, 0.041, 0.033, 0.030, 0.033, 0.021, 0.019, 0.018, respectively). At serological equivalent level, A*02XX, 24XX, 03XX, 01XX, 11XX, 26XX, 3201, 68XX composed 80% of allele coverage in this population studied. At HLA-B, B*4402, 4901, 3801, 0702, 5001, 5201, 0801, 3502, 3503, 1517 had the highest frequency (Gf= 0.043, 0.042, 0.039, 0.035, 0.032, 0.026, 0.024, 0.019, 0.017 and 0.016, respectively). At serological equivalent level, B*35XX, 51XX, 44XX, 18XX, 07XX, 4901, 3801, 15XX, 08XX, 13XX, 5001 made up 70.6% of the allele coverage in this population. Most of these alleles are common in other European and Middle Eastern populations. Interestingly, in the Turkish population we observed the occurrence of alleles previously thought to be exclusively found in other populations; we observed in a few instances A*0211, 2407, B*4601, 5401, 6701 common in Asians; and A*74XX, B*8101 common in Africans. These observations suggest that the population studied may contain alleles of different origins. Some A-B haplotype showed high frequency such as A*2301-B*4901, A*6802-B*1510, A*6601-B*4102, A*2601-B*3801 (D'=0.55, 0.52, 0.41 and 0.38, respectively) and are quite frequent in populations from the Middle East and Africa.

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HLA ALLELES AND HAPLOTYPES IN THE TURKISH POPULATION: RELATEDNESS TO KURDS, ARMENIANS AND OTHER MEDITERRANEANS

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Turkish and Kurdish HLA profiles are studied for the first time. The comparative study of their allele frequencies, characteristic haplotypes, genetic distances with other Mediterraneans is complemented by neighbor-joining dendrograms and correspondence analyses. Turks, Kurds, Armenians,Iranians, Jews, Lebanese and other (Eastern and Western) Mediterranean groups seem to share a common ancestry: the older "Mediterranean" substratum. No sign of the postulated Indo-European (Aryan) invasion (1200 B.C.) is detected by our genetic analysis. It is concluded that this invasion, if ocurred, had a relatively few invaders in comparison to the already settled populations, i.e. Anatolian Hittite and Hurrian groups (older than 2000 B.C.). These may have given rise to present-day Kurdish, Armenian and Turkish populations.

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HLA MOLECULAR MARKERS IN TUVINIANS: A POPULATION WITH BOTH ORIENTAL AND CAUCASOID CHARACTERISTICS.

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HLA class I and class II alleles have been studied for the first time in the Turkish-speaking Tuvinian population, which lives in Russia, North of Mongolia and close to the Altai mountains. Comparisons have been done with about 11000 chromosomes from other worldwide populations, ´and extended haplotypes, genetic distances, neighbor joining dendrograms and correspondence analyses have been calculated. Tuvinians show an admixture of Mongoloid and Caucasoid characters, the latter probably coming from the ancient Kyrgyz background or, less feasibly, more recent Russian Caucasoid admixture. However, Siberian population traits are not found and thus Tuvinians are closer to Central Asian populations. Siberians are more related to Na-Dene and Eskimo American Indians; Amerindians (from nowadays Iberian-America) are not related to any other group, including Pacific Islanders, Siberians or other American Indians. The 'more than one wave' model for the peopling of the Americas is supported.

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DETERMINATION OF HLA FREQUENCIES AND HLA-B/ HLA-C ASSOCIATIONS IN A BRITISH ASIAN POPULATION

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Matching potential haematopoietic stem cell (HSC) donors and patients for HLA-A, -B, -C, -DR, -DQ and -DP loci is desirable for optimum HSC transplant outcome. Due to linkage disequilibrium between HLA-B and HLA-C genes, matching for some HLA-B alleles will result in matching for HLA-C alleles. We have previously studied data from over 2000 HLA typed North European Caucasoid individuals to determine probable HLA-B/HLA-C haplotypes, and we found approximately 50% of HLA-B alleles studied to be in association with a particular HLA-C allele, the remaining HLA-B alleles demonstrating more promiscuous HLA-C associations.
In order to compare this data with other ethnic groups, we have analysed probable HLA-B/HLA-C haplotypes in 123 unrelated Asian potential HSC donors. These donors were previously HLA-A, -B, and -DRB typed. HLA-C typing was performed by the InnoLiPA (Abbott) reverse line SSO assay. HLA-B and HLA-C allele frequencies were calculated by direct counting. HLA-B/HLA-C haplotypes were determined by maximum-likelihood analysis. Gene and haplotype frequencies were determined using the linkage disequilibrium analysis. The results obtained have been compared with the North European Caucasoid population, and although frequencies of alleles differ between the two populations, no major differences in the HLA-B/HLA-C haplotypes were discovered. However, the rare HLA-Cw*1403 allele, which was originally defined in an Oriental individual, was found in an individual of Russian/Iranian ancestry.

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COMPARISON OF KIR GENES BETWEEN BULGARIANS AND OTHER POPULATIONS

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Here we attempt to compare the distribution of genes encoding 11 KIRs in 111 individuals from the Bulgarian population with those of the other populations published – UK Caucasians (n=136), Australian Caucasians (n=147), Irish (n=90), Thais (n=119), Palestinians (n=105), Vietnamese (n=59), Australian Aborigines (n=67). The frequency of KIR genes and KIR phenotypes were found different in the distinct populations. A correspondence analysis was performed to present graphically the relationships between the populations, according to differences of the KIR gene frequencies. The Bulgarians population is in the cluster of the other Caucasians except the Irish population, which is due to the more common KIR2DL2 in our population (51% vs 31%) and the less frequent KIR2DS1 (37% vs 56%). The Bulgarians were located closer to Palestinians and Thais, than to Vietnamese and Australian Aborigines. Some KIR phenotypes were observed more frequently in a given population. The AB9 profile is more common in the Bulgarians - 9.0%, than in the other Caucasians – 5.1% (UK Caucasians), 0.64% (Australian Caucasians), 0% (Irish). The AA6 phenotype was also more frequent in our population 3.6% vs 0.7% (Australians Caucasians) and 0% (Irish and UK Caucasians). We found only one BB7 profile, which seems to be rare in the Caucasians and absent in Palestinians, Vietnamese and Thais, but it was the most common KIR phenotype in the Australians Aborigines. More population studies are needed. These data demonstrate that the NK polymorphism could be useful to understand the relationships between populations.

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HLA POLYMORPHISM AT ALLELE LEVEL IN THE BULGARIAN POPULATION. COMPARISON WITH OTHER POPULATIONS.

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In this study we have analyzed HLA-A,-B,-DRB1/3/4/5 polymorphism at allele level in the Bulgarian population. Comparisons with European and Mediterranean populations were performed in order to clarify more precisely the origin of Bulgarians. Fifty-five randomly chosen healthy individuals were tested by PCR-SBT. Twenty HLA-A, 34 HLA-B and 24 HLA-DRB1 alleles were detected. Similarly to other European populations, particularly to those located in the Southern part of Europe, the most common alleles in Bulgarians were A*02011 (0.300), A*24021 (0.118); B*51011 (0.209); B*1801 (0.091); DRB1*11041 (0.155) and DRB1*16011 (0.155). Rare alleles, found in Southern part of Europe or Asia, were identified in our population with frequency ranging from 0.009 to 0.036: A*0211, A*0217, A*3004, A*8001, B*2707, B*3508, B*4405, B*4406, B*7301, DRB1*0410 and DRB1*1315. Correspondence and phylogenetic analyses based on DRB1 allele frequencies in 24 populations including Bulgarians were performed. Genetic distances and a neighbor-joining dendrogram showed that Bulgarians are more closely related to Macedonians and Greeks. Relatively short distances were observed with Romanians, Croatians, Slovenians, Armenians, Italians and Cretans. These results were in concordance with correspondence analysis. The most frequent haplotype in Bulgarians was A*02011-B*1801-DRB1*11041(0.036), followed by A*02011-B*51011-DRB1*16011, A*02011-B*51011-DRB1*13011 and A*02011-B*2702-DRB1*16011(0.027). Most of haplotypes observed in Bulgarians have been described in other European populations. Interesting haplotypes with significant linkage disequilibrium were identified for some of the rare alleles. In conclusion study of HLA polymorphism at allele level confirmed the place of Bulgarians in Southern European anthropological type and reveal significant genetic diversity of the Bulgarian population. Rare alleles and haplotypes are important to clarify more precisely the origin of the Bulgarian population.

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HLA II CLASS MOLECULAR POLYMORPHISM AND LINKAGE DISEQUILIBRIUM PARAMETERS IN SIBERIAN CAUCASOIDS.

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Siberian Caucasian population has been analyzed for evaluation of HLA class II loci polymorphism and linkage disequilibrium (LD) parameters. 80 individuals have been investigated for DRB1, DQA1 and DQB1 using multiple sequence-specific primers PCR (mSSP) and 119 individuals have been tested for DPB1 by restriction fragment length polymorphism technique (PCR-RFLP). Population contains 16 DPB1, 14 DRB1, 8 DQA1 and 11 DQB1 alleles. The most frequent alleles were DPB1*0401 (0.391), DRB1*01 (0.151), DQA1*0102 and *0501, observed in equal frequencies (0.206), and DQB1*0602-8 (0.256). Classic coefficient of LD (D) and its significance were computed between alleles from given loci, the significance of global LD between each pair of loci was also estimated. 6 DRB-DQA, 8 DRB-DQB and 8 DQA-DQB haplotypes were in significant disequilibrium and predominantly represented the most common haplotypes in population under study. Only DRB1*0301-DPB1*0101 was found in significant allelic association ($p<0.01$). This agreed with significant global disequilibrium detected between DRB1-DQA1, DRB1-DQB1 and DQA1-DQB1 loci ($p<0.001$). No LD was observed between DPB1 and any class II loci. Estimation of maximum likelihood DR-DQ haplotypes frequencies revealed their extraordinary diversity among Siberian Caucasoids. The most frequent three-locus haplotypes were DRB1*15-DQA1*0102-DQB1*0602-8 (0.131), DRB1*11-DQA1*0501-DQB1*0301 (0.100) and DRB1*01-DQA1*0101-DQB1*0501 (0.093). Present study provides the database for population genetic and HLA-diseases association research.

Poster display VIII Quality control

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HOW TO REACH THE RIGHT (OR WRONG) TYPE: A LESSON IN THE VALUE OF PROFICIENCY TESTING.

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Participation in proficiency testing programs provides a laboratory with an indicator of their performance in reaching a typing result. Such programs gauge performance of reagents, equipment, data quality, analysis and interpretation.

In a recent UCLA International HLA DNA Exchange, results from typing one sample at the HLA-A locus split the participating laboratories into two groups based upon typing methodology. PCR-SSP and SSOP labs typed the sample as A*1101, A*2403 whereas most sequencing based typing (SBT) labs came up with A*1104, A*2410.

Our laboratory submitted the SBT result. Seeing the discrepancy, we ventured to investigate the matter and found neither set of laboratories were correct. In fact the correct type was A*11011 A*2433.

Resolving the discrepancy between typing methods lead us to the following conclusions. Firstly, it is essential to faithfully update allele libraries and to include analysis of exon 4 at the HLA-A locus. Secondly, in terms of analysis, we became aware that in certain situations, the software could generate an incorrect type due to alleles with incomplete sequence in the database. This supports the need for a more complete sequence database including as many relevant polymorphic positions as possible.

Proficiency testing therefore provides a mechanism for finding problems with typing methods, which when addressed improve the capability of the laboratory. Even with established methods, we need to be alert to new problems and how to resolve them. The lessons we learned from this particular sample will hopefully help us reach the right type in the future.

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PROFICIENCY TESTING OF HLA CLASS I TYPING FOR CENTRAL EAST EUROPE (CEE) - REPORT

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20 laboratories from 8 countries (Bulgaria, Croatia, Czech Republic, Hungary, Lithuania, Poland, Russia and Slovakia) take part in the proficiency testing of HLA class I.

Five trials have been already completed and the results were discussed during a workshop organized in Wrocław in June 2001.

Each laboratory, which received four blood samples obtained from volunteer donors, was tested. Blood was taken on heparin and EDTA to make serology and DNA testing possible.

Results adequate to serological HLA class I typing / DNA low resolution were expected. It was up to the participating laboratory whether to use serology or DNA typing. Each laboratory was given a lab code.

Analysis of the QC of the Proficiency Testing results showed that:

(i) altogether 52 (6 %) from 868 specificities were false reported, (ii) the most erroneous specificities were A32 (7/18), A68 (5/18) and B 62 (8/21), B55 (4/17), (iii) false homozygocity constituted 27% of all false results, (iv) 19,2% of wrong results within CREG or cross reactive group of expected.

The Proficiency Testing was found to be productive and will be continued separately for serology and DNA typing.