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# Spontaneous cytotoxic activity of eosinophilic granule cells separated from the normal peritoneal cavity of *Dicentrarchus labrax*

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In this study the spontaneous *in vitro* cytotoxic activity to tumour cell lines, (K562), by unstimulated sea bass (*Dicentrarchus labrax*) leukocytes was examined by trypan blue exclusion test and lactate dehydrogenase release assay. A high anti-tumour cell line activity of resident peritoneal leukocytes was found at an effector to target ratio (E:T) of 25:1 after incubation for 2 h at 18° C. Rabbit and sheep erythrocytes were not lysed. A low activity was displayed by head kidney and spleen cell populations whereas blood leukocytes revealed no significant activity. The effect of E:T ratio on cytotoxicity as well as microscopy observations suggested that the cytotoxic reaction required effector-target cell contact. Eosinophilic granule cells, isolated on a Percoll density gradient from a peritoneal wash, appeared to be responsible for the *in vitro* cytotoxic activity. 

Key words: *Dicentrarchus labrax*, Teleostei, cytotoxicity, peritoneal cavity, eosinophilic granule cell.

## I. Introduction

Natural cellular immunity represents a first barrier against pathogens. It is a rapid and effective response that predominantly involves phagocytes and cytotoxic cells. Spontaneous cytotoxic activity of leukocytes against certain mammalian and fish cell lines (Hinuma *et al.*, 1980; Graves *et al.*, 1984; Suzumura *et al.*, 1994), virus-infected cells (Yoshinaga *et al.*, 1994) and a protozoan (Graves *et al.*, 1985) has been demonstrated in some species of fish. In elasmobranchs, spontaneous cytotoxicity requiring no *in vitro* activation is a property of macrophage-like cells (McKinney *et al.*, 1986). In teleosts the cytolytic activity of leukocytes seems to be mediated by various effector cells globally referred to as non-specific cytotoxic cells (NCC) (Evans *et al.*, 1988). Stuge *et al.* (1995) reported that the cytotoxic effector cells in catfish

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peripheral blood are different from those found in the pronephros. Furthermore, the cold target inhibition test showed that carp cytotoxic cells (NK-like) do not consist of a single population for target recognition (Suzumura *et al.*, 1994). In this species, NCCs consist of small agranular lymphocytes or monocytes in the blood (Meseguer *et al.*, 1994) or neutrophilic granulocytes in the head kidney (Kurata *et al.*, 1995). In *Oncorynchus mykiss* the effector cells seem to be small agranular lymphocytes in the head kidney (Greenlee *et al.*, 1991) and in the intestinal intraepithelial tissue (McMillan & Secombes, 1997).

In this study spontaneous cytotoxic activity to tumour cell lines and erythrocytes by Sea bass, *Dicentrarchus labrax*, leukocytes is reported. The killing activity and organ distribution of the effector cells were examined, with particular reference to resident peritoneal leukocytes. The peritoneal cavity, both in mammals (Appelberg *et al.*, 1991) and fishes (Afonso *et al.*, 1997), provides a model of distribution and mobilisation of cells that are quickly and extensively attracted by intraperitoneal injection of stimulants (Afonso *et al.*, 1998). In the case of fish, it has been reported that the sterile unstimulated peritoneal cavity of rainbow trout contains a significant population of resident macrophages but very few neutrophils (Afonso *et al.*, 1997). Bodammer (1986) showed that the principal cells recovered from the peritoneal exudate of striped bass (*Morone saxatilis*) challenged with bacteria are macrophages, eosinophilic granule cells and neutrophils. We found that the cell population in the unstimulated peritoneal cavity of *D. labrax*, obtained by simple washing with physiological medium, contains macrophages, neutrophils, lymphocytes and eosinophilic granule cells. These cell types were isolated on Percoll density gradients and tested for *in vitro* cytotoxic activity.

## II. Materials and Methods

### ANIMALS

Sea bass (*Dicentrarchus labrax*) (200–250 g) were obtained from a commercial fish farm (Petrosino, TP).

### COLLECTION OF EFFECTOR CELLS

The fish were anaesthetised with 0·05% MS222 (3-aminobenzoic acid ethyl ester) (Sigma) in seawater and sampled at the farm. The tissues and peritoneal cells were kept on ice and taken quickly to the laboratory. Blood was collected from the heart into a sterile plastic syringe containing 0·2 ml of heparin, and centrifuged at $800 \times g$ for 10 min at 4° C. The head kidney and spleen were surgically removed and placed in cold medium (Leibovitz L15 medium containing 2% fetal calf serum, 100 units penicillin $ml^{-1}$, 100 units streptomycin $ml^{-1}$, and 10 units heparin $ml^{-1}$). All culture medium components were from Gibco.

Peritoneal cavity cells (PCCs) were obtained as follows: after disinfection of the ventral surface of the fish with 70% ethyl alcohol, the peritoneal cavity was injected with 15 ml of isotonic (370 mOsm $kg^{-1}$) Leibovitz medium. After massaging the ventral surface of the fish for 10 min, the medium containing

the PCCs was harvested with a syringe. The PCCs were obtained by centrifugation at 400 × *g* for 10 min at 4° C. The fish serum and medium osmolarity was measured by an osmometer (Röebling).

### TARGET CELLS

The human erythromyeloid leukaemia-derived cell line (K562), human hystocytic lymphoma-derived cell line (U937), and Burkitt lymphoma derived cell line (Raji) were maintained in RPMI 1640 medium (Gibco) supplemented with 10% heat-inactivated fetal calf serum (Flow), gentamycin, streptomycin and HEPES buffer (Boerhinger), at 37° C with 5% $CO_2$. The culture was fed biweekly in order to maintain constant exponential cell growth.

The Zooprophylaxis Institute (Palermo, Italy) provided sheep erythrocytes. Rabbit erythrocytes (RE) were from Sclavo (Siena, Italy).

### DENSITY GRADIENT SEPARATION OF CELLS

Pronephros and spleen cell suspensions or blood were centrifuged on a 34/46% Percoll/Hanks balanced salt solution (HBSS: NaCl 190 mM, KCl 5·36 mM, glucose 5·54 mM, $KH_2PO_4$ 0·44 mM, $Na_2HPO_4$ 0·56 mM; pH 7·6, 370 mOsm $kg^{-1}$) density gradient. A band of enriched leukocytes was visible at the interface. Cellular debris was separated at the top of the gradient and the erythrocyte pellet was packed at the bottom.

To separate the leukocyte populations present in the peritoneal wash, a continuous density gradient was formed with 55% Percoll in HBSS by centrifugation at 25 000 × *g* for 20 min at 4° C. The PCC suspension was immediately layered on the preformed Percoll gradient and centrifuged at 2500 × *g* for 25 min at 7° C. After centrifugation two bands of cells were formed. The cells from each band were counted in a haemocytometer and the leukocytes adjusted to $2{\cdot}5 \times 10^5$ $ml^{-1}$ in HBSS.

### TRYPAN BLUE EXCLUSION TEST

Dead cells were determined by addition of 0·01% trypan blue to the medium. This test was also used to evaluate the cytotoxic activity against tumour cell lines, the dye was added into the reaction mixture after 2 h incubation.

To show target cell death following an *in vitro* cytotoxic reaction, the trypan blue was added to the medium 20 min after the leukocytes were mixed with target cells. Samples of the reaction mixture were smeared on slides and examined under the microscope.

### METHODS FOR STAINING LEUKOCYTE SMEARS

Samples (50 $\mu$l) of the leukocyte suspensions ($5 \times 10^6$ cells $ml^{-1}$) were layered on a clean slide, allowed to settle for 30 min and then fixed with undiluted methanol for 7 min at 4–8° C. Fixed smears were treated with May-Grünwald for 3 min and after washing, with Giemsa for 10 min. The slides

were rinsed with deionised water and, after ethanol dehydration and xylene treatment, were mounted in Permount (Sigma).

### CYTOTOXIC ASSAY

The cytotoxic assay against erythrocytes has been previously described (Cammarata *et al.*, 1997). Briefly, 200 $\mu$l of leukocyte suspension (E, $1{\cdot}5 \times 10^5$ cells) in phosphate buffered saline (PBS: NaCl 103·6 mM, $KH_2PO_4$ 1·46 mM, $Na_2HPO_4$ 0·8 mM, KCl 2·6 mM, $CaCl_2 2H_2O$ 0·9 mM, $MgCl_2$ $6H_2O$ 0·49 mM, pH 7·4) were mixed with an equal volume of freshly prepared rabbit erythrocytes (T, $8 \times 10^6$ cells) in the same medium. Spontaneous haemoglobin release never exceeded 5% of the total release obtained after keeping the erythrocytes in distilled water. The mixture was incubated at 18° C for 2 h, centrifuged at $200 \times g$ for 5 min at 7° C and the amount of released haemoglobin in the supernatant was estimated by reading the absorbance at 541 nm. The degree of haemolysis was determined according to the equation: Percent haemolysis = measured release – spontaneous release/complete release – spontaneous release $\times$ 100.

Cytotoxicity assay against tumour cell lines was performed using a cytotoxic detection Kit (Boehringer) based on determination of lactate dehydrogenase (LDH) activity released from lysed target cells (Korzeniewski & Callewaert, 1983). The target cells were washed and suspended in PBS supplemented with 1% bovine serum albumin (PBS-BSA, 370 mOsm $kg^{-1}$) at a concentration of $1 \times 10^5$ cells $ml^{-1}$. All tests were performed in triplicate with $1 \times 10^4$ target cells $well^{-1}$ and with appropriate effector-to-target ratios in V-shaped microplates (Nunc) in a total volume of 200 $\mu$l. Plates were centrifuged for 1 min at $100 \times g$ and incubated for 2 h at 18° C. The plates were then centrifuged for 5 min at $400 \times g$, and the release of LDH from lysed cells in 100 $\mu$l of supernatant from each well was determined by reading the absorbance at 490 nm in a microplate reader (Uniskan I, Labsystems). Spontaneous and maximum release were measured in 100 $\mu$l of supernatant from wells containing target cells only or target cells with 1% Triton X-100 (Fluka). Spontaneous baseline LDH release from target ($1 \times 10^4$ cells $well^{-1}$) and effector cells were used as controls. The values of the controls were subtracted from the degree of target cell lysis determined according to the equation: Percent lysis = (measured release – spontaneous target release) – spontaneous effector release/complete release – spontaneous target release $\times$ 100.

Appropriate controls showed that leukocyte mortality in PBS, under the experimental conditions employed, was lower than 5%. The living cells were observed through Nomarski differential interference contrast optics (DIC). Unless otherwise specified the cytotoxic reactions to both erythrocytes and tumour cells were carried out at 18° C for 2 h, at 25:1 E/T ratio, in 370 mOsm/$kg^{-1}$ medium.

### STATISTICAL METHODS

The data were examined by Student's *t*-test to determine the statistical probability, $P<0{\cdot}01$ being the lowest significance level.

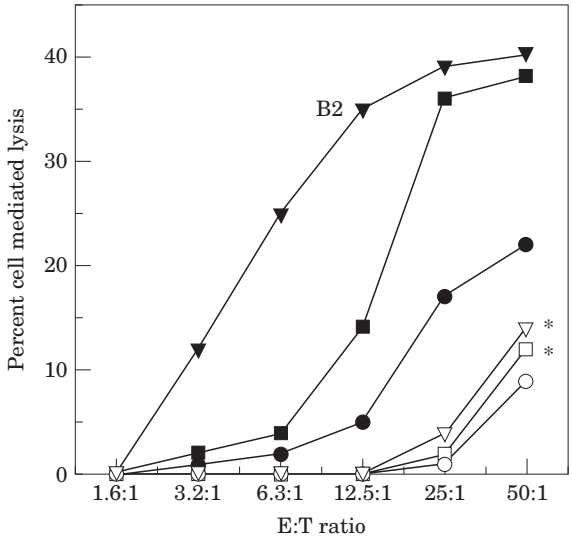


*Fig. 1.* Effect of E:T ratio on spontaneous cytotoxic activity of *Dicentrarchus labrax* by unseparated leukocytes or separated eosinophilic granule cells (EGCs) from peritoneal cavity co-incubated with K562 target cells. Lactate dehydrogenase release into the supernatant was used to calculate the percentage of target cell lysis. The percent of target cell lysis was also determined by trypan blue exclusion test (TBET). Unseparated leukocytes assayed by TBET (●); Unseparated leukocytes assayed by LDH release method (■); Percoll density enriched eosinophilic granule cells population (B2) (▼). *: The same number of cells contained in each reaction mixture were assayed for the spontaneous LDH release in the absence of target cells: (□) unseparated leukocytes; (▽) EGCs. ○ TBET of unseparated leukocytes in the absence of target cells. Percent cell lysis of target cells, assayed by LDH release method or TBET, were less than 5%.

## III. Results

### EFFECT OF OSMOLARITY AND E:T RATIO ON PCC CYTOTOXIC ACTIVITY

Leukocyte mortality of less than 5% and highest values of cytotoxicity ($36 \pm 8{\cdot}2\%$) were obtained when the osmolarity was $370 \pm 30$ mOsm kg$^{-1}$, the same as that measured in the serum of *D. labrax*. A significant decrease ($P<0{\cdot}001$) of the PCC cytotoxic activity against the K562 cell line ($10 \pm 6\%$) was found when the medium of the reaction mixture was at the physiological osmolarity of the target (280 mOsm kg$^{-1}$). In that medium leukocyte mortality was greater than 20%.

The time course and effector:target ratio experiments were carried out at 370 mOsm kg$^{-1}$ using both LDH release and trypan blue assays. The time course (0·25–12 h) experiments showed that the highest activity was reached after 2 h incubation at 18° C. Higher temperature values (20–37° C) significantly increased the leukocyte mortality.

To examine the cytotoxic mechanism, cytotoxicity was assayed at different E:T ratios (Fig. 1). The curve, obtained by plotting the numbers of unfractionated leukocytes against the lysis of K562 tumour cells, was sigmoidal in shape. A significant rise in lysis was found when $2{\cdot}5 \times 10^6$ leukocytes ml$^{-1}$ were

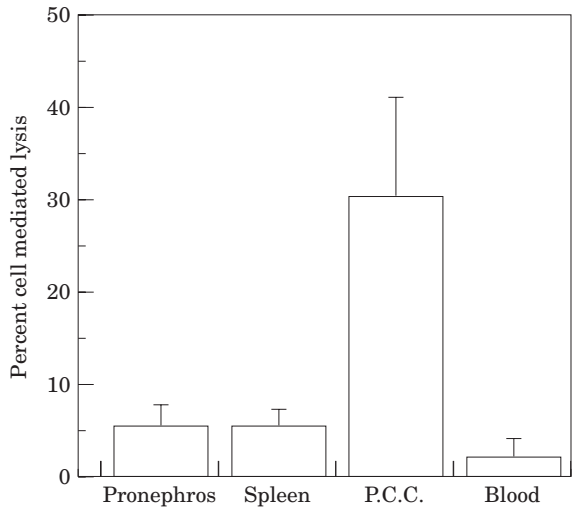


*Fig. 2.* Cytotoxic activity (LDH assay) of sea bass leukocytes from pronephros, spleen, peritoneal cavity (PCC) and blood against K562 tumour cell line (E:T=25:1).

incubated with $1{\cdot}0 \times 10^5$ target cells $ml^{-1}$ (E:T ratio 25:1). At the highest E:T values an increased background of spontaneous target lysis was evident.

### CYTOTOXIC ACTIVITY OF LEUKOCYTES FROM VARIOUS TISSUES EXAMINED BY LDH METHOD

Fig. 2 shows the anti-K562 cytotoxic activity of the leukocytes separated from the various tissues. PCCs exhibited the highest activity. A lower level ($P<0{\cdot}001$) of activity was shown by head kidney and spleen leukocytes, blood revealed no significant activity.

### CYTOTOXIC ACTIVITY AGAINST VARIOUS TARGETS

To examine the specificity range of the effector peritoneal cavity cells, LDH assays against Raji and U937 tumour cell lines as well as against sheep and rabbit erythrocytes were performed. As shown in Fig. 3, Raji and U937 cells appeared to be more sensitive than K562 cell lines. Both the erythrocyte types were unsensitive even when assayed at an E:T ratio of 100:1.

### PERCOLL ENRICHMENT OF EOSINOPHILIC GRANULE CELLS FROM PERITONEAL CAVITY

Microscopical observation revealed that cells morphologically identified as macrophages ($26{\cdot}4 \pm 7{\cdot}8\%$), neutrophils ($22{\cdot}8 \pm 6{\cdot}7\%$), lymphocytes ($32{\cdot}3 \pm 3{\cdot}4\%$) and eosinophilic granule cells (EGCs) ($18{\cdot}5 \pm 6{\cdot}4\%$) were contained in the unseparated leukocyte suspension from the peritoneal wash.

To identify and characterise the effector cells from the peritoneal cavity, the cytotoxic assays were carried out with leukocytes separated from each specimen on a continuous 55% Percoll/HBSS density gradient. After centrifugation, two distinct bands were visible within the Percoll gradient. The upper band 1 contained macrophages ($34 \pm 5\%$), neutrophils ($30{\cdot}8 \pm 5{\cdot}7\%$),

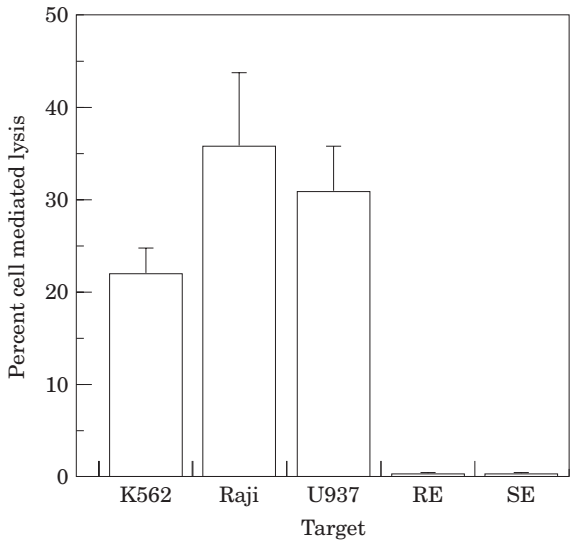


*Fig. 3.* Cytotoxic activity of sea bass peritoneal cavity cells against different tumour cell lines (Raji, K562, U937 assayed by a LDH assay) and erythrocytes (SE=sheep erythrocytes; RE=rabbit erythrocytes) (E:T=25:1)

lymphocytes (30·2 ± 3·4%) and other cells (5 ± 2%), while EGCs were enriched (85–98%) in band 2 situated below. The enrichment levels were obtained from 15 individuals. The EGCs were identified on the basis of the eosinophilic property of their granules. The Romanowsky type combination stained the granules a strong red colour (Fig. 4a).

Fig. 5 shows the cytotoxic activity of the leukocyte populations from the two separated bands measured with the LDH assay. The cytotoxicity of cells from band 2, enriched in EGCs, was significantly higher ($P$<0·001) than the unfractionated leukocytes, while a very low level of cytotoxicity was detected by the leukocytes from band 1.

A hyperbola was formed by the curve obtained by plotting enriched leukocyte populations from band 2 *v.* K562 tumour cell lysis (Fig. 1): a significant rise in lysis was found when $6{\cdot}3 \times 10^5$ cells $ml^{-1}$ were mixed with $1{\cdot}0 \times 10^5$ target cells $ml^{-1}$ (E:T ratio 6·3:1).

The DIC microscopical observations showed that the effector cells morphologically referred to as EGCs (Fig. 4b), came into close contact with the K562 target cells after 10–20 min (Fig. 4c, d). The effector cell then enfolded the cell target by cytoplasmic extensions containing granules. In addition, after about 1 h, some modification of the target cell membrane surface (from smooth to rough) became evident (Fig. 4d, e). Numerous tumour cells attacked by EGCs, after 60 min, stained with trypan blue (Fig. 4f).

## IV. Discussion

The current literature reports that spontaneous *in vitro* cytotoxic activity against tumour cell lines is a property of head kidney and spleen leukocytes

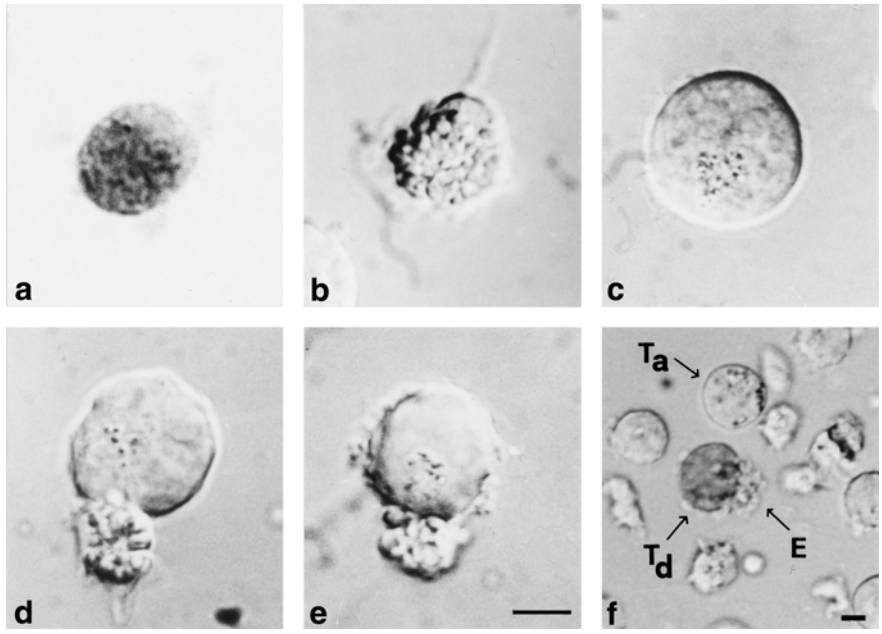


*Fig. 4.* Microscopic observations of cytotoxic activity of sea bass eosinophilic granule cell (EGC) against K562 tumour cell. (b)–(e): Living cells observed through Nomarski differential interference contrast optics. (a) EGC May Grunwald Giemsa stained; granules appeared red in colour; (b) EGC (effector); (c) K562 tumour cell (target); (d) Effector/target close contact (10–20 min); (e) Effector/target close contact (30–60 min), (a)–(e) bar=7 $\mu$m; (f) Effector/target close contact in the presence of trypan blue (60 min) bar=6 $\mu$m. Td=blue stained dead cell target, Ta=living cell target, E=living EGC.

from unchallenged fish. The present work has shown, by trypan blue and LDH assays, that a high level of cytotoxic activity was demonstrated by leukocytes from the normal peritoneal cavity of *Dicentrarchus labrax*. A low level of activity was displayed by head kidney and spleen cell populations whereas blood leukocytes had no significant activity. We cannot exclude that head kidney, spleen and blood cell activity may have been underestimated because the LDH method, although easy to use, may be less sensitive than the $^{51}$Cr release assay. With the LDH method a high cytotoxic level was displayed by the PCCs at 25:1 E:T ratio at 18° C.

The cytotoxic reaction may imply effector-target cell contact as shown by microscopical observations and indicated by the shape of the E:T curves. There is a direct relationship between cytotoxic activity and effector cell number (unfractionated or fractionated) up to an E:T ratio of 25:1. However, a similar level (about 35%) of target cell lysis was found even when the amount of leukocytes was doubled (50:1), whereas unfractionated leukocytes did not induce lysis below a ratio of 6:1. Both these results indicate that cytotoxic activity could be related to adequate effector-target contacts. Finally, the different levels of cytotoxicity observed with various tumour cell lines, as well as the insensitivity of sheep and rabbit erythrocytes, suggest a certain

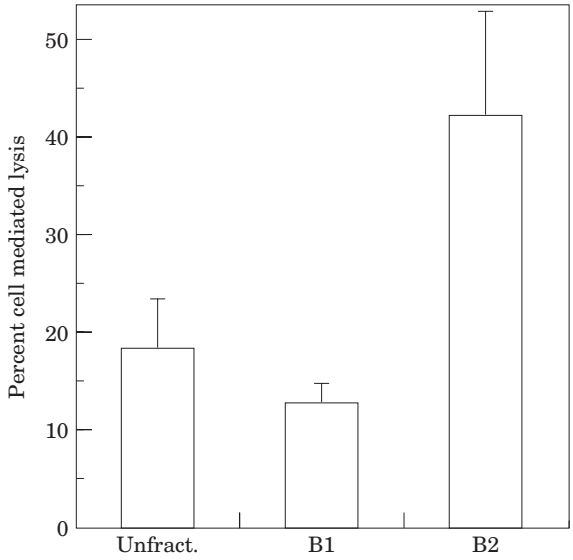


*Fig. 5.* Cytotoxic activity (LDH assay) of density gradient (Percoll 55%) separated peritoneal cavity cells from sea bass against K562 tumour cell line. Unfract.= unfractionated cells; B1=upper gradient band; B2=lower gradient band (85–98% EGCs).

degree of specificity which might imply the involvement of membrane receptor(s).

When various effector/target cell ratios were plotted against the percent cell lysis caused by leukocytes (unfractionated or fractionated in B2), it is evident that enriched populations of EGCs were more efficient in producing lysis. In fact, the degree of haemolysis was greater than 25% at 6·3:1 effector/target cell ratio whereas unfractionated cells caused only about 15% lysis at a higher (12·5:1) E/T ratio. The enrichment in effector cells probably allowed more frequent contacts with targets, and increased cytolysin release.

In vertebrates, cytotoxic activity may be a property of various cell types. Mammals possess a number of different kinds of cytotoxic cells which include cytolytic immune T cells (Shearer, 1974; Forquest *et al.*, 1990), monocytes or macrophages (Adams & Hamilton, 1984; Kessel *et al.*, 1986), granulocytes (Gerrard *et al.*, 1981; Korec *et al.*, 1980; Okuno *et al.*, 1986) and natural killer cells (Rosenberg *et al.*, 1974; Sendo *et al.*, 1975; Kiessling *et al.*, 1975). In *Rana pipiens* cytotoxic cells are similar in morphology to mammalian large granular lymphocytes (Ghoneum & Cooper, 1987). Peripheral blood mononuclear cells and granulocytes from chicken are able to lyse virus-transformed chicken cells (Sieminski-Brodzina & Mashaly, 1991; Mandi *et al.*, 1985). In fish, agranular cells (NCC) from the channel catfish, *Ictalurus punctatus* (Evans & Jaso-Friedmann, 1992; Evans *et al.*, 1988; Graves *et al.*, 1985) and neutrophilic granulocytes from the carp (*Cyprinus carpio*) head kidney were well defined for their spontaneous non-specific cytotoxic activity. On the other hand, this is a property of elasmobranch macrophage-like cells (McKinney *et al.*, 1986), carp blood small agranular lymphocytes or monocytes (Meseguer *et al.*, 1994)

and trout (*Oncorhynchus mykiss*) intestinal intraepithelial lymphocytes (McMillan & Secombes, 1997).

In the present work the effector cytotoxic cells of the sea bass peritoneal cavity were characterised as eosinophilic granular cells (EGCs). These cells, recovered from the unstimulated peritoneal cavity, could be identified on the basis of their morphology and cytochemical properties (*i.e.* myeloperoxidase, data not shown) as macrophages, eosinophilic granule cells and neutrophils. Bodammer (1986) reported that similar cells were present in the peritoneal cavity of striped bass injected with bacteria and that all three of the cell types were phagocytic. For the first time, we isolated the 'eosinophilic granule cells' from the peritoneal cavity and showed, by using the LDH release method and Nomarski contrast microscopical observations, that they are responsible for the spontaneous cytotoxic activity in the sea bass.

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