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THE ACTIVATION OF HUMAN PLASMA PREKALLIKREIN AS A HAEMOCOMPATIBILITY TEST FOR BIOMATERIALS.I.RESULTS OBTAINED WITH THE "END-POINT" METHOD.

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SUMMARY

The contact of blood with foreign surfaces causes the activation of factor XII to XIIa, which, through the conversion of plasma prekallikrein into kallikrein, starts the coagulation cascade.

The spectrophotometrical detection of the p-nitroaniline released in the reaction of kallikrein with the chromogenic substrate H-D-Pro-Phe-Arg-NH-Ph-NO_2 is a test able to measure the contact activation induced by materials to be employed in contact with blood.

The prekallikrein activation was evaluated by using the so called "end-point method", and the dependence of the activation on the geometry of the reaction vessels and on the volume of the plasma samples was found for silica-borate glass. Then, the activation as a function of plasma contact time was measured for glass, silicone, two commercials biomaterials, and a composite biomaterial of our production. The results obtained confirm the haemocompatibility of the biomaterials tested.

INTRODUCTION

In the last years the growing development of biomedical devices and artificial organs has stimulated the synthesis and the characterization of a variety of materials potentially applicable in contact with blood.

Antithrombogenicity is the necessary requirement for these materials and to measure the degree of the surface induced coagulation is perhaps one of the most critical problems in blood-material interactions (ref.1).

The activation of coagulation mechanism takes place after the adsorption of factor XII onto the foreign surface (contact phase)

(ref.2), and requires also the presence of a trimolecular complex of three proteins that is, factor XI, high molecular weight kininogen (HMWK) and prekallikrein (PKK) (ref.3).

The activated factor XII (XIIa) is efficient in converting PKK into kallikrein (KK), which in turn is the most potent activator of factor XII (ref.4); this cause not only the liberation of the powerful vasoactive peptide bradykinin from HMWK (refs. 5-6),but also the enzymatic cascade reaction implicated in the fibrin thrombus formation (Fig. 1).

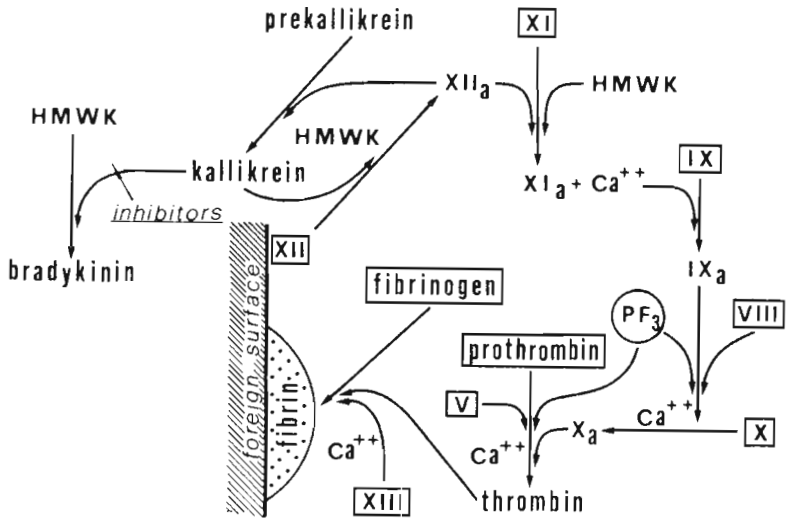

Fig. 1. Scheme of the contact activation mechanism.

The introduction of chromogenic peptide substrates with high specificity for plasma KK (ref.7) made easy the determination of the KK formed in the above described reaction. In the most common test, KK is allowed to react with the substrate H-D-Pro-Phe-Arg--pNA (S-2302 Kabi), which under the proteolytic action of KK, releases p-nitroaniline, spectrophotometrically detectable measuring its absorption at 405 nm (ref.8). Such a test, employed mostly for clinical and diagnostic purposes (refs.8-10), can also be used as

an in vitro assay to measure the activation of coagulation induced by foreign surfaces exposed to plasma samples under standard conditions (ref.11). The plasma PKK activation test can be therefore a useful haemocompatibility test for biomaterials.

This paper deals with the use of this test in the study of the haemocompatibility of some biomaterials. In particular, we report preliminary results pertinent to three different biomaterials in comparison with two reference materials: borosilicate glass as a high-activation reference and silicone as a low-activation reference. The three biomaterials tested are: Biomer®, a segmented poly (ether-urethane); Cardiothane 51®, a poly(ether-urethane) (90%) -poly(dimethylsiloxane) (10%) block copolymer; a composite of Cardiothane 51® and fibrin (1:1 ratio), recently obtained in our laboratories (ref. 12).

MATERIALS AND METHODS

Biomer® (Ethicon Inc., Somerville, NJ, USA) 20% dimethyl acetamide solution was diluted to 2% with dimethylacetamide and deposited onto the inner surfaces of plane-bottom glass tubes by evaporation of the solvent.

Cardiothane 51® (Kontron Cardiovascular Inc., Everett, MA,USA) was dissolved in 2:1 THF-dioxane mixture (2% concentration) and deposited in the same manner as Biomer®.

The composite of Cardiothane 51® and fibrin was deposited onto the inner surfaces of the glass tubes as follows: a solution of the copolymer in 2:1 THF-dioxane mixture and a solution of fibrinogen in a suitable aqueous buffer were simultaneously sprayed onto the surface; then the film so obtained was dried under vacuum to remove the solvents and reacted with a solution of thrombin, factor XIII and Ca^{++} ions to convert the fibrinogen into crosslinked fibrin.

At complete reaction the film was cleaned with H_2O to remove the crosslinking agents from the surface.

A 2 mM solution of the chromogenic substrate H-D-Pro-Phe-Arg--p NA (S-2302, Kabi Diagnostica, Stockholm, Sweden) was prepared

by dissolving 25 mg in 20 ml of H_2O.

The plasma samples used to carry out the PKK surface activation test were obtained as follows: 9 volumes of blood from a pool of 8 healthy donors were mixed with 1 volume of sodium citrate 0.1 M and centrifugated at 2000 g for 20 min at 25°C. In order to avoid low temperature induced PKK activation, the plasma so obtained was immediately frozen at -20°C and stored. Just before carrying out the test, the plasma was thawed at +37°C and kept at room temperature for all the time of the experiment; controls made at different time intervals show that it can be used for no more than three hours after thawing.

The PKK activation test was carried out as follows, by using the so-called "end point method": the plasma was diluted 1:10 with TRIS-HCl buffer (pH 7.8); a measured volume of the diluted plasma was placed in a plane-bottom glass tube, as such or internally coated with the material to be tested, and kept at 37°C under stirring at 1100 r.p.m. After different contact times samples of 0.1 ml were drawn and each was added to 0.1 ml of the 2 mM substrate solution and to 0.3 ml of TRIS-HCl buffer in a polystyrene test tube at 37°C. After a reaction time of 10 min (end point), the reaction was stopped by adding 0.2 ml of a 50% volume CH_3COOH solution and the absorbance value was read at 405 nm.

RESULTS AND DISCUSSION

Since PKK is activated by a substance formed following the contact between the plasma and the inner surface of the tube, the amount of PKK activated is strongly dependent on the ratio between the contact surface and the volume of the sample. The dependences of the surface to volume ratio (S/V) on the volume (V) in a suitable volume range for three tubes with inner diameters of 1.160 cm, 0.815 cm and 0.670 cm are shown in Fig.2. In each case, the S/V value decreases with increasing V and tends asymptotically to a constant value, equal to:

$$\lim_{V \to \infty} \frac{S}{V} = \frac{2}{r} \qquad (1)$$

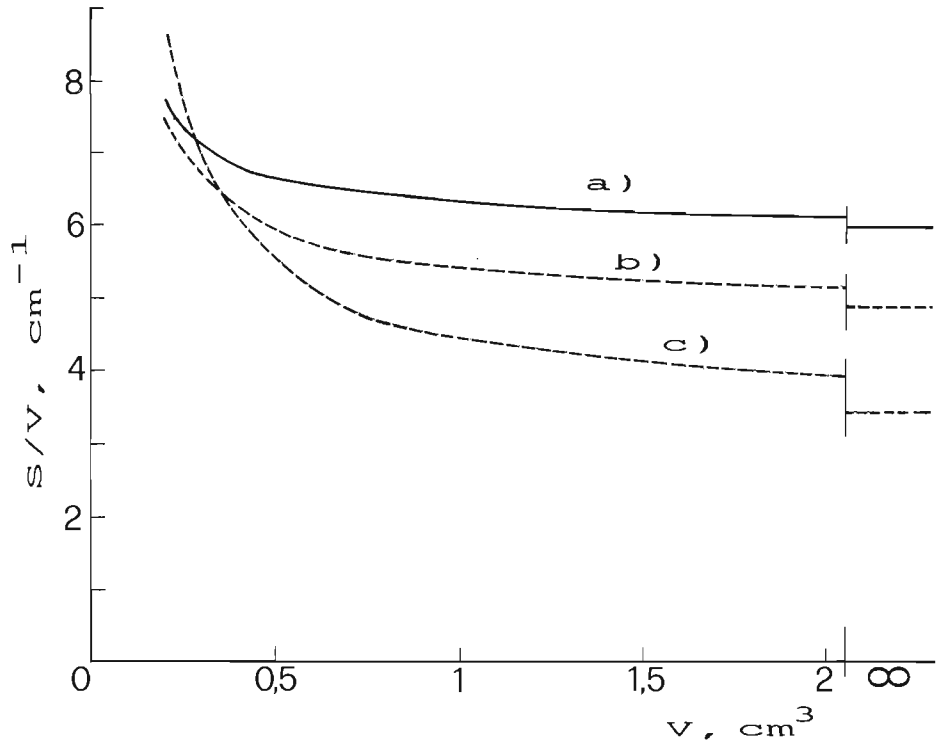

Fig. 2. Surface to volume ratio (S/V) vs. volume (V) curves for three plane-bottom test tubes with different inner diameters. a): inner diameter 0.670 cm; b): inner diameter 0.815 cm; c): inner diameter 1.160 cm.

being r the inner radius of the tube. The limiting values of $\frac{S}{V}$ for the three tubes are therefore 3.45 cm^{-1}, 4.91 cm^{-1} and 5.97 cm^{-1} respectively. Fig. 1 shows that, for the tube with 0.670 cm inner diameter, S/V values vary less than for the two other tubes in the volume range chosen; this tube is therefore the most suitable to introduce less variations in the system during the experiments.

The dependence of PKK activation on the S/V ratio has been evaluated by measuring the absorbances at the "end point" of diffe - rent volumes of diluted plasma put in contact with the test tubes of 0.670 and 0.815 cm inner diameters for 4 min. The results are

reported in Table 1.

TABLE 1

Dependence of prekallikrein activation on the volumes of the samples (V) and on the inner diameters of the tubes (d). Incubation time: 4 min. Stirring speed: 1100 r.p.m.

V,cm^3	$\frac{S}{V}$, cm^{-1}	$\frac{S}{V} - \frac{2}{r}$, cm^{-1}	A^a_{405}	d,cm
1.50	6.20	0.23	0.560	0.670
1.00	6.32	0.35	0.745	0.670
0.50	6.66	0.69	0.840	0.670
0.25	7.36	1.39	0.870	0.670
2.00	5.16	0.25	0.455	0.815
1.50	5.25	0.34	0.520	0.815
1.00	5.44	0.53	0.588	0.815
0.50	5.94	1.03	0.760	0.815
0.25	7.00	2.09	0.880	0.815

[a]Absorbance at 405 nm; reaction time with S-2302: 10 min.

The absorbance values obtained fit the equation:

$$A = A_o (1 - e^{-B(S/V - 2/r)}) \qquad (2)$$

where A_o is the limiting absorbance value to which A tends asymptotically when $S/V \rightarrow \infty$ (or $V \rightarrow 0$), $2/r$ is the limiting value of S/V from eq. (1), and B is a parameter of the dimension of a lenght measuring the "steepness" of the A vs. $(S/V - 2/r)$ curve.

An algebraic treatment of the data in Table 1 has permitted to evaluate A_o and B values for the two tubes employed in the test. The values, reported in Table 2, have been inserted in a computer simulation program based on eq. (2), by which theoretical curves for the two series of experiments have been calculated. The fitting between the calculated curves and the experimental points is shown in Fig. 3. As $(S/V - 2/r)$ tends to 0, i.e., by eq. (1), as V tends to ∞, the absorbance value tends to 0, that is. PKK acti-

vation decreases as the sample volume increases, and no activation would occur in a theoretical sample of infinite volume, as it is required by the surface activation mechanism.

TABLE 2

Limiting absorbance values (A_o) and "curve steepness" parameters (B) of the curve in eq. (2) for the activation of prekallikrein in two glass tubes of different inner diameters (d). Incubation time: 4 min. Stirring speed: 1100 r.p.m.

A_o	B, cm	d, cm
0.88	4.68	0.670
0.90	1.84	0.815

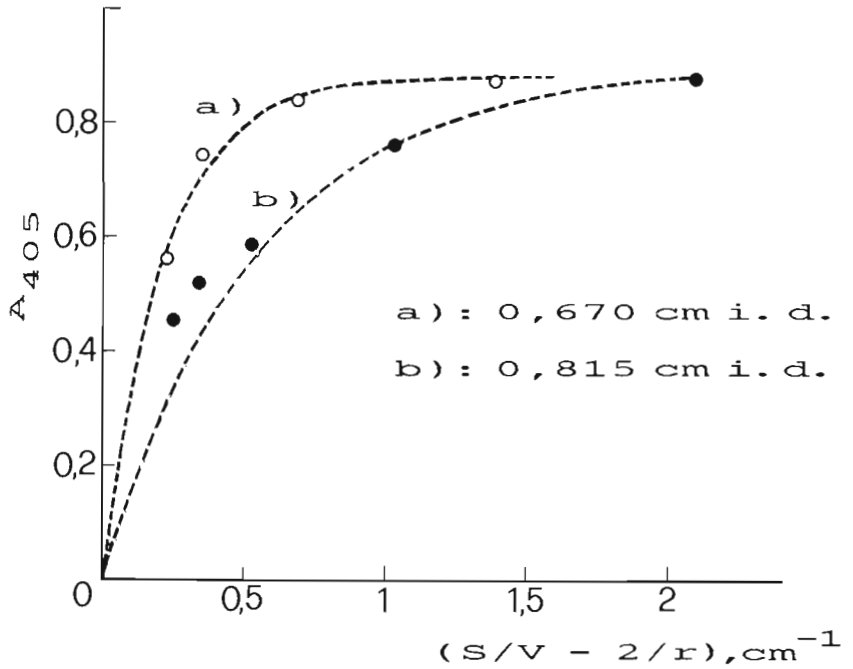

Fig. 3. Fitting between the absorbance values of Table 1 and the curves calculated from eq. (2) by using the A_o and B values of Table 2 (---) for two glass tubes with different inner diameters.

The asymptotical value A_o is approached at lower (S/V - 2/r) values by curve a) than by curve b), so confirming that the tube with 0.670 cm inner diameter is the most suitable to perform the contact activation test. A comparison between Figs. 2 and 3 suggests the choice of a sample volume of 0.5 ml to minimize the variations of S/V with V and of A with S/V.

The tests of plasma PKK activation have been carried out in 0.670 inner diameter test tubes by using 0.5 ml samples of diluted plasma. Fig. 4 shows the dependence of the 405 nm absorbance at the end point on plasma-material contact time for the two reference materials abd for the three biomaterials tested.

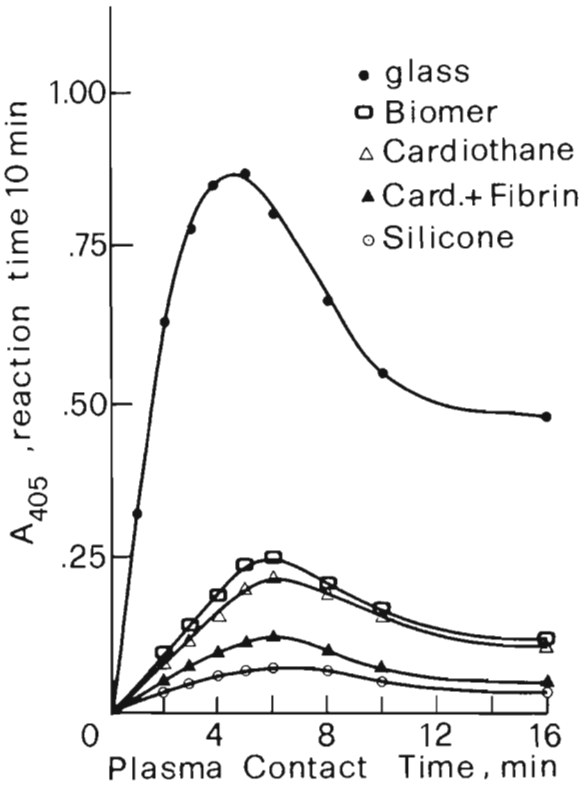

Fig. 4. Absorbance vs. plasma contact time curves for two reference materials and three biomaterials.

The KK-like activities increase in the first minutes of plasma contact time and then decrease, likely owing to the formation of KK-inhibiting substances in the activated plasma (ref. 10), setting at nearly constant levels at about 16 min plasma contact time, when an equilibrium between PKK activation and KK inhibition is reached, so giving an activation vs. time curve quite similar to that found by other authors for plasma activated by dextran sulfate (ref. 13). The maximum absorbance value, which is reached between 4 and 5 min plasma contact time for glass, between 5 and 6 min for the three biomaterials tested, and between 6 and 7 min for silicone, has been assumed as a measure of the PKK-activating power of the material tested, i.e. the greater such value, the less the haemocompatibility of the material. The results shown in Fig. 4, which for PKK activations measured by the "end-point" method are valid only in a first approximation (ref. 14), are in agreement with the different blood clotting powers of the materials tested, so confirming the validity of plasma PKK activation as a haemocompatibility test. In particular, the maximum absorbance of the fibrin-Cardiothane 51 composite, lower than those of the two commercial biomaterials and near to that of the low-activation reference silicone, is another result in favour of the haemocompatibility of this class of composites, already seen by other tests (ref. 15).

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