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Platelets in inflammatory exudates

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In exudates of implanted sponges in the rat there are apparent accumulations of blood platelets, assessed by the light microscope, and of radioactivity from labelled platelets administered systemically. The use of more specific techniques for platelet detection showed no evidence of intact blood platelets in the exudate and the radioactivity was not associated preferentially with cellular components in the exudate.

The possible roles of blood platelets in the initial development of inflammatory responses poses intriguing questions. Known mechanisms involved in the response of platelets to injury of the walls of small blood vessels by inflammatory insults include adherence of the platelets to the damaged endothelial tissue, aggregation and the release reaction (Mustard & Packham, 1975). A further example may be the migration of platelets into developing inflammatory exudates. It has been shown that platelets exhibit both random and active migration *in vitro* (Lowenhaupt, Miller & Glueck, 1973; Valone, Austen & Goetzl, 1974). They have also been reported to occur in a pleurisy exudate in the rat (Zawilska, Giroud & others, 1973) and to accumulate at the sites of renal allografts in sensitized animals (Lowenhaupt, Chanana & others, 1968). Their appearance in inflammatory exudates in severely thrombocytopenic rats has been interpreted as evidence of an active directional migration *in vivo* (Smith, Walker & others, 1976). The criteria used to detect and measure the platelets in exudate samples have been limited to observations with the light microscope. We have therefore re-examined the problem using more sensitive and specific procedures including the use of radioactively labelled platelets, platelet antiserum, immunofluorescent techniques and electron microscopy.

MATERIALS AND METHODS

Animals, exudates and platelet preparations

Female albino rats, 150–200 g, were obtained from Oxfordshire Laboratory Animal Colonies, Southern Ltd, and the sponge implantation method was that described previously (Walker, Smith & others, 1975) except that up to eight sponges, soaked in 0·9 g/100 ml NaCl solution, were implanted in each animal. The sponges were removed after 0·5, 3 or 5 h and the exudates were either used as such for the determination of red cell, total leucocyte and platelet-like body counts by light microscope methods (Walker, Smith & Ford-Hutchinson, 1976) or were fractionated as follows: initial centrifugation at 100 *g* for 15 min yielded supernatant A and pellet A (red and white cells), centrifugation of supernatant A at 2750 g for 30 min produced supernatant B (cell-free exudate) and pellet B (platelets and platelet-like bodies). Platelet-rich plasma was prepared from blood (4·4 ml) obtained from the aorta of anaesthetized rats and drawn into a syringe containing 0·6 ml of a solution containing in 100 ml trisodium citrate 2·4 g, citric acid 1·5 g and glucose 2·5 g. The mixture was centrifuged at 150 *g* for 15 min and the supernatant formed the platelet-rich plasma. Platelets were prepared from this fraction by centrifuging at 1200 *g* for 20 min.

Radioactively labelled platelets

Platelets from plasma were labelled with ^{51}Cr sodium chromate (Radiochemical Centre, Amersham, Bucks.) according to the directions of Radegran (1976). The preparation (0·5 ml) was administered by injection into the tail vein of each rat 18 h before implantation of the sponges. Groups of rats were then killed at 0·5, 3 and 5 h, blood samples obtained and the sponges removed. The exudate was squeezed out, the sponge washed with 0·9 g/100 ml NaCl solution and the washings and exudate combined and used for the preparation of exudate pellets A and B and exudate supernatant B. Total radioactivity was measured using an LKB Wallac 1280 automatic gamma counter in the whole exudate, in the washed sponges, in exudate pellets A and B, in exudate supernatant B and in the blood samples.

Platelet antiserum was prepared in rabbits against rat platelets according to the direction of Siemensma,

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Bathal & Penington (1975). Samples of platelet-rich plasma, platelets and exudate pellet B, resuspended in exudate supernatant B, were incubated with varying dilutions of platelet antiserum and the platelets counted in the mixtures, before and after incubation, using phase contrast microscopy.

Immunofluorescent techniques. Platelet pellets prepared from platelet-rich plasma and pellets B from the sponge exudates were suspended in phosphate buffer (pH 7·6; 0·01 M phosphate) containing 0·145 M NaCl centrifuged at 1200 *g* for 20 min and the sedimented cells washed twice with the buffer and resuspended in 0·5 ml of the buffer containing 3 mg of platelet antiserum. After incubating at 20° for 30 min the cells were separated by centrifugation as above, washed twice with buffer, suspended in 0·5 ml of a 1/10 dilution of anti-rabbit immunoglobulin conjugated with fluorescein (Wellcome Reagents, Ltd) in the buffer and incubated at 20° for a further 30 min. The mixtures were centrifuged at 1200 *g* for 20 min, the sediment washed twice with buffer, applied to microscope slides using an aqueous mounting medium and examined for fluorescent particles (Coons & Kaplan, 1950).

Electron microscope studies. The platelet pellet and pellets A and B, prepared from the exudates, were fixed in a mixture containing 2·5 g/100 ml glutaraldehyde and 2 g/100 ml formalin in cacodylate buffer (0·1 M pH 7·4) and postfixed using 1 g/100 ml aqueous osmium tetroxide. After dehydration the pellets were embedded in an epoxy resin and sectioned with an LKB ultratone III at 90 nm. The sections were stained with uranyl acetate and lead citrate and examined using an AEI Corinth 275 transmission electron microscope at 60 Kv.

RESULTS

The number of red cells, total leucocytes and platelet-like bodies, counted by the light microscope, in the sponge exudates at various time intervals are given in Table 1. If the migration of leucocytes and platelets was a passive process akin to that of the erythrocytes then the counts of leucocytes and platelets should have been in the same ratio to the red cells in the exudates as in the circulation, the values for these ratios in the circulation have been taken as red cells: total leucocytes, 2000:1 and red cells:platelets, 10:1. The calculated values for the cell types in the exudate have been included in the table as predicted values. The measurements of radioactivity in the exudate provide additional data in that they allow the number of platelets per unit volume of exudate to be calculated from the corresponding measurements in the whole blood sample. These values are also included in Table 1 as radiochromium results.

Table 1. *Counts of erythrocytes, leucocytes and platelets in sponge exudates.* Results given as number of cells per μl of exudate, each figure is the mean of at least five rats. Predicted values and radiochromium results are defined in the text.

Cell type	Method of measurement	Time of removal of sponge (h) 0·5	3	5
Erythrocyte	Light microscope	11650	9245	13925
Total leucocyte	Light microscope	0	1034	5586
	Predicted	6	5	7
Platelet	Light microscope	24120	26740	23718
	Predicted	1165	924	1392
	Radiochromium results	2013	3577	6353

The results in Table 1 show that both total leucocytes and platelet-like bodies, counted with light microscope methods, preferentially accumulate in developing exudates, i.e. that active migration of both cell types occurs. When the numbers of platelets are calculated from the accumulation of radioactivity in the exudate this degree of accumulation is very much less although it exceeds unity, indicating that an active, although greatly reduced, migration appears to have occurred.

The sponge exudates were fractionated to produce sediments containing either red cells and leucocytes (pellet A) or platelets and smaller particles (pellet B) and the cell-free exudate (supernatant B). The distribution of radiochromium, derived from the labelled platelets introduced into the circulation, in these fractions is given in Table 2.

Table 2. *Distribution of radiochromium in fractions of sponge exudates.* Results expressed as percentages of total radioactivity in sponge plus exudate, each figure is the mean of at least 25 sponges. Fractions are defined in the text.

Fraction	Time of removal of sponge (h) 0·5	3	5
Washed sponge	8·5	7·0	18·0
Pellet A	10·7	10·0	12·7
Pellet B	7·4	4·5	4·8
Supernatant B	73·4	79·0	63·5

The results show that up to 18% of the ^{51}Cr remained in the sponges after repeated washing, that smaller and approximately equal amounts of radioactivity appeared in the two exudate pellets but that 60 to 80% of the radiochromium was found in supernatant B.

The two exudate pellets were examined by a combination of methods designed to detect the presence of intact platelets. The use of platelet antiserum and phase contrast microscopy gave positive results with platelet-rich plasma and platelets suspended in supernatant B but no evidence of intact platelets in the exudate pellet B. The immunofluorescent procedure yielded characteristic results with platelet-rich plasma but although the very occasional fluorescent particle could be observed in pellet B these particles did not resemble those produced with intact platelets in the plasma. Electron microscopical examination revealed the presence of red cells and leucocytes only in pellet A and no evidence of intact platelets in pellet B. The only structures identified in pellet B were an occasional mitochondrion and non-specific cell debris.

DISCUSSION

The results of the present work show clearly that examination with the light microscope is an unreliable method for detecting and counting platelets in inflammatory exudates. Furthermore the use of radioactively labelled platelets may yield a misleading answer if radioactivity measurements are restricted to the whole exudate. The radioactivity which accumulated in the exudate was largely confined to the liquid phase and was not associated with a cellular fraction which should have contained platelets. What are best described as platelet-like bodies under the light microscope were not intact platelets when assessed by specific procedures involving the use of platelet antiserum and immunofluorescent coupling. Fractionation of the exudate by centrifugation showed that the sediment which should have contained platelets consisted of non-specific cell debris when examined under the electron microscope. These observations negate the findings of Smith & others (1976) that there is an active migration of blood platelets into the exudates of implanted sponges in the rat and throw doubt on the results of other workers who have identified intact platelets in inflammatory exudates by light microscopy methods. It is possible that intact platelets actively migrated into the exudate and then disintegrated into fragments. However, only 5% of the radio chromium from the labelled platelets was found in pellet B (Table 2). This is much less than would have been expected from the results of Steiner & Baldini (1970) who found that over 40% of ^{51}Cr from labelled platelets occurred in the stromal sediment obtained by centrifugation of the disintegrated platelets at 1000 *g*. Again more than the occasional fluorescent particle would have been observed in pellet B if such disintegration had occurred.

It must be concluded that blood platelets do not migrate actively into the exudates found in inert sponges implanted subdermally in the rat. If platelets contribute to inflammatory responses of this type this must be concerned with other aspects of platelet behaviour such as adherence, aggregation or the release reaction.

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