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Interactions of Macrophages and Erythrocytes

R. B. VAUGHAN AND S. V. BOYDEN

Department of Experimental Pathology, John Curtin School of Medical Research, Australian National University, Canberra, A.C.T., Australia

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Summary. An *in vitro* technique is described which demonstrates a difference in the interaction of fresh and stored, effete, red cells with autologous macrophages. The stored erythrocytes adhere to macrophages whereas fresh erythrocytes do not.

The effects on this difference of the medium in which the red cells are stored, and of the storage temperature, are described. Results are presented which indicate that serum opsonic factors are not necessary for the adherence of the stored red cells to macrophages.

INTRODUCTION

An essential feature of metazoan organization is the presence within the body of specialized phagocytic cells the main function of which is the disposal of unwanted particulate matter both 'foreign' such as bacteria, and damaged and dead cells of the organism itself. Clearly, the proper functioning of these phagocytes depends on their capacity to distinguish between healthy host structures on the one hand, and 'foreign' material and cell debris on the other. The mechanism of this discriminatory process has received little attention and is not understood.

A series of experiments is at present under way in our laboratory on this subject in relation to the uptake of both 'foreign' matter and autogenous cell debris. This paper concerns an investigation into the apparent capacity of macrophages to distinguish between healthy, fresh, autologous erythrocytes and autologous erythrocytes modified by storage *in vitro*. It seems likely that the changes occurring in red cells during storage *in vitro* are analogous to those taking place as the cells age naturally *in vivo*. A technique is described by which it is possible to demonstrate *in vitro*, in a semi-quantitative manner, a difference in affinity for macrophages between fresh and stored red cells. Some experiments are described directed towards an analysis of this difference in reactivity.

MATERIALS AND METHODS

Glassware

All glassware and the perspex slides used were cleaned in a hot solution of 'Haemo-sol' (Meineck & Co., Inc., 225 Varick Street, New York 14). They were rinsed in tap water and distilled water and dried by heat (not over 40° for perspex slides).

Guinea-Pigs

Animals of mixed stock and both sexes, weighing between 400 and 650 g., were used.

They were supplied by the Animal Breeding Establishment, Australian National University.

Macrophages

These were obtained from exudates induced by the intraperitoneal injection of a solution of glycogen in saline (10 ml. of a solution containing 0·2 mg. of Oyster glycogen B.D.H., England, per ml. in 0·9 per cent saline). The exudates were collected 48 hours after the injection of glycogen, by washing out the peritoneal cavities of the exsanguinated guinea-pigs with 20 ml. of Hanks's solution containing 5 I.U./ml. of Heparin. Differential cell counts were done on smears made between coverslips, and stained with MacNeal's tetrachrome stain. The total white cell count varied between 0·9 and $4{\cdot}5 \times 10^6$/ml., of which some 45 to 85 per cent were macrophages. For use in the experiments the exudate fluid was centrifuged at 80 ***g*** for 5 minutes, and the cells were resuspended to a concentration of $0{\cdot}8 \times 10^6$/ml. in 3 per cent autologous serum in Hanks's solution, except in the experiments on the effect of serum on the macrophage–red cell interaction where they were suspended in Hanks's solution.

Serum

In all cases this was from clotted blood obtained by cardiac puncture. It was stored at $-50°$ until used.

Erythrocytes

Blood was obtained from guinea-pigs either by sterile cardiac puncture or by aseptically incising a marginal ear vein. A 3 per cent sodium citrate solution containing antibiotics in the concentrations specified below for Hanks's solution was used as an anticoagulant (4 parts whole blood to 1 part citrate solution). Red cells to be stored in other media were washed aseptically three times in 10 volumes of sterile Hanks's solution and resuspended in the appropriate medium. Fresh red cells used in the experiments were collected in citrate solution when the guinea-pigs were exsanguinated. These cells were washed three times in Hanks's solution before being used. In all experiments the red cells were used at a concentration of 10×10^6/ml.

Balanced Salt Solution

Hanks's solution containing 50 I.U./ml. of penicillin G and 100 I.U./ml. of streptomycin sulphate was used. In those experiments designed to test the effect on the stored red cells of the composition of the storage medium, the following media were used: (1) Hanks's solution as above containing 1 per cent (w/v) 'Methocel' (Methyl cellulose, Dow Chemical International Ltd., Midland, Michigan), (2) Hanks's solution containing 0·5 per cent (w/v) Bovine serum albumin (BSA) (Bovine plasma albumin fraction V, Armour Laboratories, England), (3) Hanks's solution containing 0·5 per cent (v/v) Dextran ('Dextraven', Benger Laboratories Ltd., England), (4) Hanks's solution containing 20 per cent (v/v) autologous guinea-pig serum.

In vitro *Storage of Erythrocytes*

The cells were incubated in 10–15 volumes of the appropriate medium (all media contained antibiotics as above) in sterile, screw-capped glass tubes for up to 48 hours at various temperatures. The tubes were shaken from time to time. Before use, the stored cells were washed twice in 10 volumes of Hanks's solution.

Technique for Testing the Interaction of Macrophages and Red Cells

The tests were carried out in chambers made by fixing a glass coverslip (Assistent, Germany, thickness II) over a circular hole 1 cm. in diameter in the centre of a 1·25 mm. thick perspex slide. The coverslip was attached with a molten mixture of paraffin wax (m.p. 57°) 50 per cent and Vaseline 50 per cent. The chamber so formed was filled with the macrophage suspension and a second coverslip applied to close the chamber. This upper coverslip was held in place by surface tension forces due to the thin film of liquid which spread out between the coverslip and the slide. After 30 minutes incubation at 37° in a moist atmosphere containing 5 per cent CO_2 in air the macrophage preparation was washed by removing the top coverslip and immersing the slide, held vertically, three times in a beaker of Hanks's solution at 37°. This procedure leaves a clean, well-separated, layer of macrophages stuck to the lower coverslip. The chamber was then filled with the appropriate red cell suspension and incubated for a further 30 minutes. The slide was then inverted and placed at an angle of 20° to the horizontal, the tilting caused the red cells to collect at one side of the chamber and minimized the loss of quality of the phase contrast image during subsequent microscopy. After a further 15 minutes the preparation was examined with phase contrast optics and the number of red cells adherent to 200 randomly selected macrophages was recorded.

RESULTS

COMPARISON OF FRESH AND STORED, EFFETE, AUTOLOGOUS ERYTHROCYTES

This experiment illustrates the method used to study the interaction of macrophages and autologous red cells *in vitro*. Fresh red cells are compared with red cells that have been stored *in vitro* under conditions known to render them liable to prompt removal from the circulation if transfused back into the donor animal (Hughes Jones, 1961; Jandl and Tomlinson, 1958; Ross, Finch, Peacock and Sammons, 1947; Mollison, 1961, pp. 25–26; Noyes, Bothwell and Finch, 1960).

TABLE 1

EFFECT OF STORAGE OF RED CELLS AS CITRATED WHOLE BLOOD FOR 48 HOURS AT 37°

Red cells in suspension	*No. of cells sticking to* 100 *macrophages*
Fresh	3
Fresh	11
Fresh	7
Stored	390
Stored	400
Stored	420

Six slide preparations were used. To three of these a suspension of fresh red cells in 20 per cent autologous serum in Hanks's solution was added; a suspension of red cells which had been stored as sterile citrated whole blood for 48 hours at 37° was added to the other three chambers. The results of this experiment, which are given in Table 1, show that while there is little tendency for fresh red cells to stick to macrophages, large numbers of the stored red cells remain adherent to the phagocytes. The different appearance of two such preparations is shown in Figs. 1 and 2.

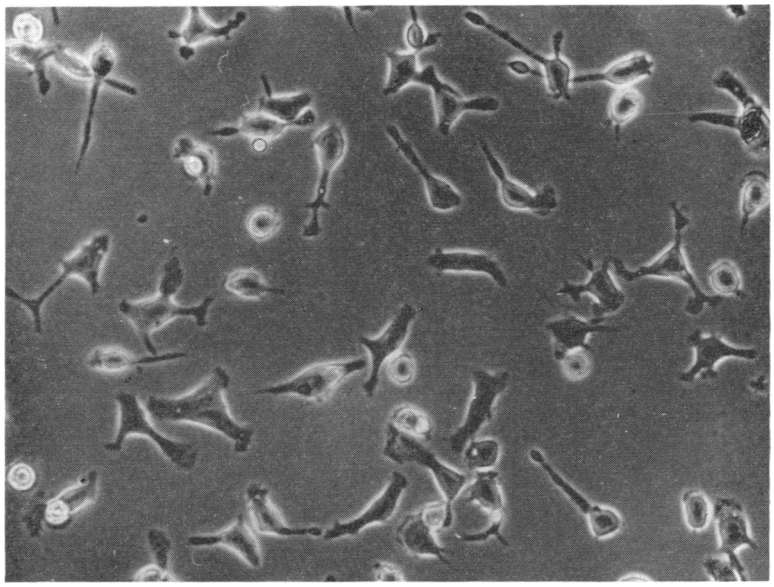

FIG. 1. Layer of macrophages to which fresh red cells were added before inversion. Phase contrast picture, ×112.

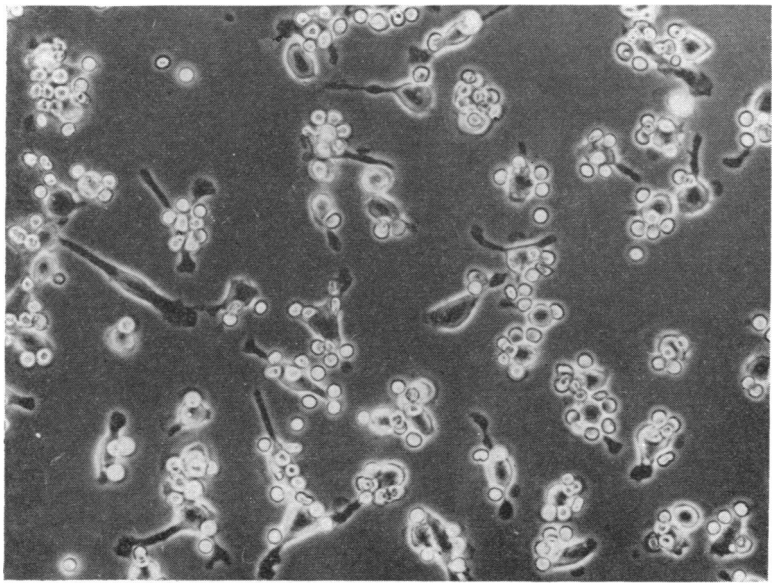

FIG. 2. Layer of macrophages to which stored red cells were added before inversion. Phase contrast picture, ×112.

EFFECT OF THE COMPOSITION OF THE STORAGE MEDIUM ON THE INTERACTION OF STORED ERYTHROCYTES WITH MACROPHAGES

The following experiment was designed to test whether the change in affinity of red cells for macrophages produced by storage *in vitro* at 37° is dependent on the presence of serum during the storage period.

The treatment of the erythrocytes was as follows:

(1) and (2): Stored for 48 hours at 37° as citrated whole blood.

(3) and (4): Stored for 48 hours at 37° suspended in 0·1 per cent 'Methocel' Hanks's solution.

(5) and (6): Stored for 48 hours at 37° suspended in 0·5 per cent Bovine serum albumin Hanks's solution.

(7) and (8): Stored for 48 hours at 37° suspended in 0·5 per cent Dextran Hanks's solution.

(9) and (10): Stored for 48 hours at 37° suspended in Hanks's solution.

(11) and (12): Stored for 48 hours at 37° suspended in 20 per cent autologous serum in Hanks's solution.

After storage, the erythrocytes were washed three times in Hanks's solution and then resuspended in 20 per cent autologous serum in Hanks's solution. Fresh erythrocytes, also in 20 per cent autologous serum, were added to preparations (13) and (14).

TABLE 2

EFFECT OF COMPOSITION OF STORAGE MEDIUM ON INTERACTIONS OF MACROPHAGES AND STORED RED CELLS

Red cells in suspension	*No. of red cells sticking to 100 macrophages*
(1) Stored as citrated whole blood	310
(2) Stored as citrated whole blood	300
(3) Stored in 0·1 per cent Methocel/ Hanks's solution	250
(4) Stored in 0·1 per cent Methocel/ Hanks's solution	300
(5) Stored in 0·5 per cent BSA/Hanks's solution	225
(6) Stored in 0·5 per cent BSA/Hanks's solution	250
(7) Stored in 0·5 per cent Dextran/ Hanks's solution	332
(8) Stored in 0·5 per cent Dextran/ Hanks's solution	352
(9) Stored in Hanks's solution	245
(10) Stored in Hanks's solution	195
(11) Stored in 20 per cent autologous serum/Hanks's solution	330
(12) Stored in 20 per cent autologous serum/Hanks's solution	313
(13) Fresh red cells	11
(14) Fresh red cells	17

The results of this experiment are given in Table 2. They show that the presence of serum during the period of storage is not necessary for the change affecting the red cells' affinity for macrophages to occur.

EFFECT OF STORAGE OF RED CELLS FOR DIFFERENT LENGTHS OF TIME ON THEIR INTERACTION WITH MACROPHAGES

The length of time for which stored red cells will remain in the circulation of an animal into which they are transfused after storage *in vitro* is known to vary with the length of the storage period (Ross *et al.*, 1947; Mollison, 1961, pp. 25–26). The following experiment was carried out to test the effect of the length of the storage period on the *in vitro* interaction of red cells and macrophages.

Erythrocytes were stored in three different media for varying periods of time and were then tested with preparations of autologous macrophages in the presence of autologous serum. The three storage media were: Medium A—Citrated whole blood; Medium B—Hanks's solution; Medium C—20 per cent autologous serum in Hanks's solution.

The cells were stored in these media at 37° for the following periods of time: (1) 1 hour; (2) 6 hours; (3) 24 hours; (4) 48 hours.

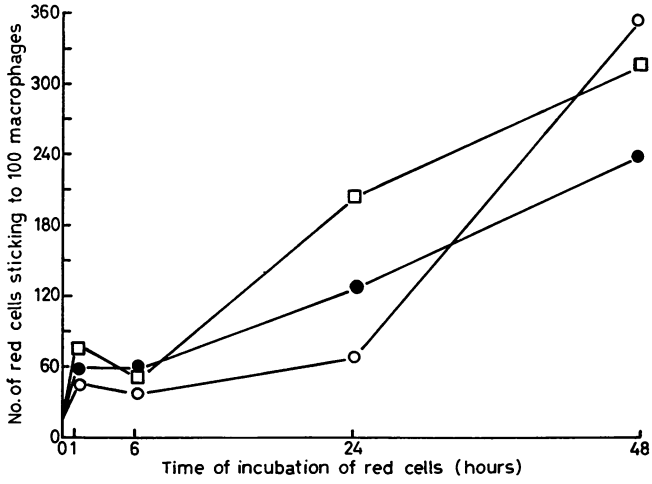

FIG. 3. Effect of duration of storage of red cells at 37° on their interaction with autologous macrophages. ○, Red cells incubated in 20 per cent autologous serum in Hanks's solution; □, red cells incubated as citrated whole blood; ●, red cells incubated in Hanks's solution.

The results are expressed graphically in Fig. 3. They show that the change in the red cells is a progressive one occurring with increasing rapidity over the period of time studied.

EFFECT OF TEMPERATURE DURING STORAGE OF RED CELLS ON THEIR SUBSEQUENT INTERACTION WITH MACROPHAGES

In man, red cells which have been stored *in vitro* at low temperature (0–5°) remain for a much longer period in the circulation when reinjected than do cells stored at 37° (Jandl and Tomlinson, 1958; Ross *et al.*, 1947; Mollison, 1961, pp. 24–26; Noyes *et al.*, 1960). In the following experiment the effect of storing red cells at 0° and 37° for different lengths of time, on their reaction with autologous macrophages, is tested.

The erythrocytes were suspended in three different media during storage: (a) As citrated whole blood; (b) in Hanks's solution; (c) in 20 per cent autologous serum in Hanks's solution. Specimens in all three media were stored for 24 and 48 hours at 0° and 37°.

It is evident from the results, which are shown graphically in Fig. 4, that the changes which occur in red cells on storage take place very much more slowly at 0° than at 37°.

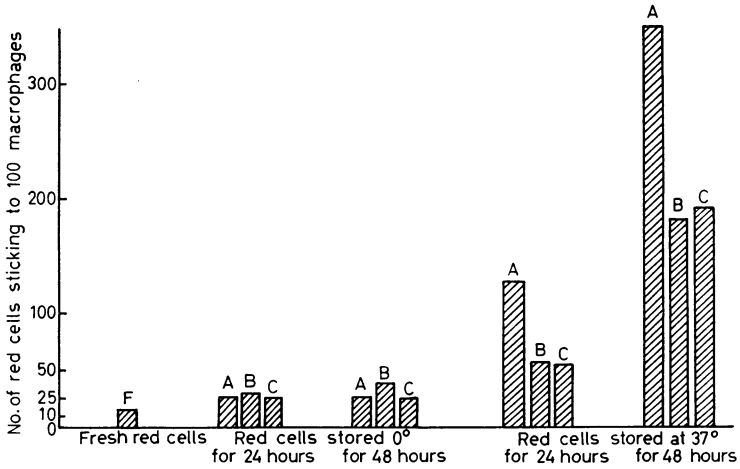

FIG. 4. Effect of temperature during storage of red cells on their interaction with autologous macrophages. A, red cells stored as citrated whole blood; B, red cells stored in Hanks's solution; C, red cells stored in 20 per cent autologous serum in Hanks's solution; F, fresh red cells.

EFFECT OF SERUM ON THE INTERACTION OF STORED RED CELLS WITH AUTOLOGOUS MACROPHAGES

An experiment was set up to determine whether the adherence of effete red cells to autologous macrophages was dependent on the adsorption onto the red cells of serum opsonic factors.

The red cells were stored for 48 hours at 37° in Hanks's solution (i.e. in the absence of serum). After storage, these cells were divided into two lots. One lot was incubated with serum at 37°. A mixture containing equal volumes of undiluted autologous serum and a 2 per cent suspension of red cells in Hanks's solution was incubated for 10 minutes and then washed twice with 10 volumes of Hanks's solution before adding to the macrophages. The other lot was incubated for the same length of time at 37° in Hanks's solution. As controls, two suspensions of fresh red cells from the same guinea-pig were similarly treated.

TABLE 3

EFFECT OF SERUM ON RED CELL–MACROPHAGE INTERACTION

Red cell suspension	*No. of red cells sticking to 100 macrophages*
(1) Fresh cells in Hanks's solution	26
(2) Fresh cells 'opsonized' with autologous serum	28
(3) Stored cells in Hanks's solution	180
(4) Stored cells 'opsonized' with autologous serum	186

The results of this experiment are given in Table 3. They indicate that the stored cells adhere to the macrophages in the absence of serum. Pretreatment of the stored cells with normal serum did not result in an increase in the number of red cells sticking to the macrophages. This experiment thus suggests that opsonic factors are not necessary for the adherence of the stored red cells to the macrophages.

DISCUSSION

The red cell–macrophage system was chosen as a model for the investigation of the mechanism of discrimination by phagocytes between healthy cells and effete cells mainly for convenience. Red cells are easy to obtain, to recognize and to handle. Moreover, the literature clearly indicates that macrophages play an important role in the removal *in vivo* of effete red cells (Smith, 1958; Essner, 1960; Hughes Jones, 1961; Mollison, 1961, p. 443; Hughes Jones and Cheny, 1961; Von Ehrenstein and Lockner, 1959), and although there has been some controversy over whether the cells usually lyse in the circulation or are taken up intact by the reticulo-endothelial system (Rous, 1923) there is little doubt that the reticulo-endothelial cells are in any case responsible for the removal of the cell membrane. There is considerable evidence indicating that the phagocytosis of 'foreign' matter, such as bacteria, heterologous red cells, and even carbon particles, by macrophages is dependent on the adsorption onto the surface of the particles of serum opsonins (Mudd and Mudd, 1933; Lucké, Strumia, Mudd, McCutcheon and Mudd, 1933; Whitby and Rowley, 1959; Howard and Wardlaw, 1958; Mackaness, 1960; Jenkin and Rowley, 1961; Murray, 1963). The question naturally arises whether the uptake of effete red cells is also dependent on the action of opsonic factors, reacting perhaps with newly exposed groups on the red cell surface. The experiments described above indicate that the adherence of stored red cells to autologous macrophages is not dependent on the presence of serum or plasma, either during the storage period or during the *in vitro* incubation with the macrophages. Experiments have recently been described (Jenkin and Karthigasu, 1962) in which, using an *in vitro* perfused liver preparation and red cells stored in the cold for several weeks, it appeared that in order for the stored cells to be 'cleared' from the perfusion fluid it was necessary for them first to be incubated with serum. Thus it is clear that further work must be done before any final conclusions can be drawn regarding the necessity for the presence of serum opsonic factors in order that the phagocytic cells of the reticulo-endothelial system may discriminate between healthy and effete erythrocytes.

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