## Demonstration of Specific Induction of Erythrocytes Phagocytosis by Macrophages from Normal, Non-Sensitized Rabbits by a Factor Released from Lymph Node Cells of Immunized Rabbits

During studies on the mechanism of delayed (cellular) type hypersensitivity, several authors have demonstrated that in the course of cultivation of lymph node cells from hypersensitive animals with antigen, a release of biologically active substances takes place capable of influencing the migration activity of mesenchymal cells from normal animals[1-3]. In our laboratory, we demonstrated that one of these factors was a substance possessing some properties of an antibody or antibody-like substance capable of passive transfer of a specific reaction to antigen in vitro to originally immunologically incompetent cells[3,4]. This substance is produced and released from hypersensitive lymphocytes.

We attempted to demonstrate the transfer of the specific reactivity in another system in vitro. As a model we used the specific adherence and phagocytosis of erythrocytes by macrophages.

Chinchilla rabbits were injected into the foot-pad with a mixture of sheep erythrocytes in complete Freund's adjuvant. In a control experiment, complete Freund's adjuvant alone was administered. The draining nodes were taken out after exsanguination of the animal on the sixth day following sensitization; suspension of lymphoid cells was prepared by the usual method[3]. Peritoneal exsudate from normal rabbits was collected 4 days after administration of 50 ml Indian ink suspension. As a medium we used Earle's solution with lactalbumin hydrolysate (Sevac) without serum.

Incubation was performed in Wassermann tubes and in cultivation chambers, which consisted of a ring of plastic material and a slide affixed with parafin as 'bottom'[5]. We followed the adherence either using Bürker's chamber in suspensions from Wassermann tubes or in cultivation chambers both 'in the wet state' and after stripping off the ring and drying the slide ('bottom') with air flow. Phagocytosis was observed also in these fixed slides after incubation at 37 °C. The cells were stained either supravitally with eosin (final concentration 2 mg/ml) or according to May Grünwald-Giemsa.

The principle of the experiment was the cultivation of a mixture consisting of sensitized lymphoid cells, normal macrophages marked by ingested carbon particles of Indian ink and erythrocytes. After 2, 6 and 20 h incubation, the following results were found: adherence of erythrocytes to lymphoid cells from sensitized animals as well as to macrophages from normal animals was observed. On the bottom of the cultivation chamber a great part of the macrophages contained phagocytosed erythrocytes besides the carbon particles (Figure 1).

Control experiments: normal non-sensitized macrophages after incubation with sheep or rooster erythrocytes contained hardly any phagocytosed erythrocytes. After incubation of rooster nuclear erythrocytes in a mixture with lymphocytes hypersensitive against sheep erythrocytes, sheep erythrocytes and normal macrophages, no phagocytosed rooster erythrocytes were found (Figure 2).


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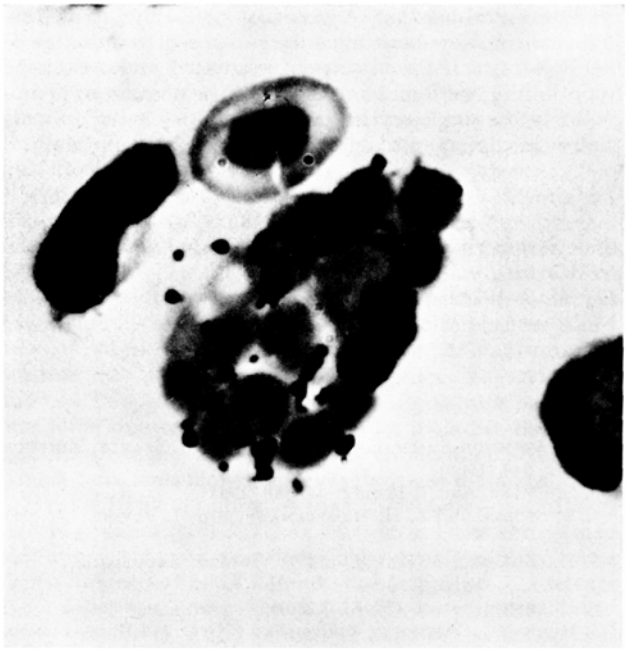

Fig. 1. Phagocytosis of sheep red cells by normal macrophage (in the centre) marked with in vivo phagocytosed carbon particles after 6 h cultivation with lymph node cells from a sensitized rabbit. In the vicinity of the macrophage a non-phagocytosed nucleated rooster erythrocyte. May Grünwald-Giemsa. × 1000.

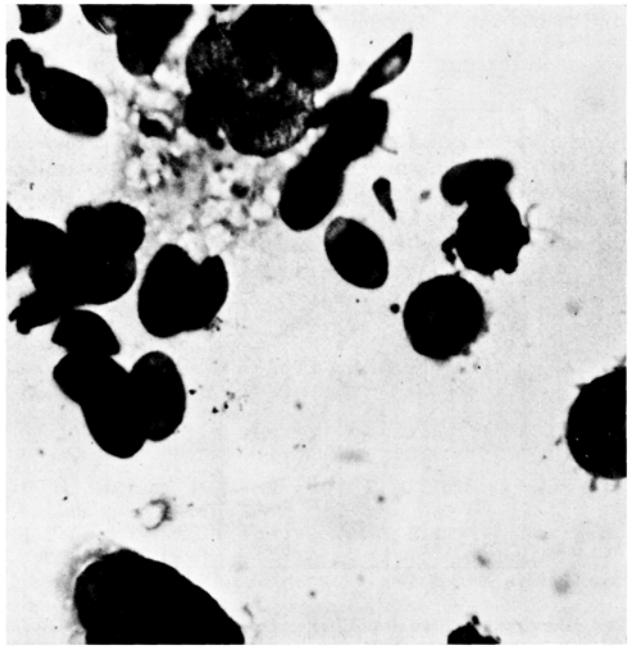

Fig. 2. Culture of rooster erythrocytes with normal macrophages (marked by phagocytosed carbon particles) and with lymph node cells from rabbits sensitized to sheep erythrocytes. The nucleated rooster erythrocytes are not phagocytosed. May Grünwald-Giemsa. × 400.

When lymphoid cell suspension was obtained from rabbits treated with Freund's adjuvant only, no specific phagocytosis of erythrocytes could be detected. On common incubation of killed staphylococci with hypersensitive lymphoid cells and erythrocytes no distinct influence of the degree of bacterial phagocytosis by macrophages was observed.

The incubation of erythrocytes with fresh sera of sensitized rabbits led to their partial destruction or damage. These erythrocytes, after washing, were not phagocytized. We suppose, therefore, that the factor in question is probably not a conventional opsonin.

Further experiments are in progress to analyse the distinct nature of the above-mentioned factor and its role in the mechanism of the reaction.

It was shown that on simultaneous incubation, a factor is released from sensitized lymphoid cells which is capable of inducing specific phagocytosis or adherence of erythrocytes to macrophages from normal animals. We think that these results are related to similar findings described in the studies of cell migration quoted in the introduction of our paper as well as to the problem of the so-called cytophilic antibodies[6-8] and possibly also of the so-called cellular immunity[9].

*Zusammenfassung.* Mit In-vitro-Experimenten wird gezeigt, dass in Anwesenheit von sensibilisierten Kaninchen-Lymphozyten Schaferythrozyten spezifisch an normale Kaninchen-Makrophagen adsorbieren und von diesen dann phagozytiert werden.

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## Alterations of the Erythrocyte Membrane Caused by Fluorinated Dinitrobenzenes

Acetylcholinesterase (ACHE) appears to be located at the outer surface of the human erythrocyte membrane[1,2]. We have recently shown that this enzyme was inactivated when red cells were treated with 1-fluoro-2,4-dinitrobenzene (FDNB) and 1,5-difluoro-2,4-dinitrobenzene (DFDNB)[3]. The effect was irreversible and characteristically distinct for each reagent. While ACHE inactivation was greater with DFDNB than with FDNB, exposure to the former rendered the residual activity more resistant to heat and urea than treatment with the latter. FDNB reacts with free amino, histidyl, tyrosyl or sulfhydryl groups to yield dinitrophenyl derivatives, and DFDNB reacts with 2 such groups, provided they are 5 Å apart, to give dinitrophenylene cross-links[4]. The increased stability of the residual ACHE activity following DFDNB treatment was ascribed to the formation of cross-linked derivatives[3]. Since ACHE activity is reduced in new-borns affected with ABO hemolytic disease[5] and inasmuch as blood-group specific antigenic receptors also are located at the red cell surface[6], we have investigated the influence of ABO antibodies on ACHE inactivation by FDNB and DFDNB and the possible changes in the agglutinability of cells thus treated.

Blood obtained from normal adult individuals of blood group A or B was centrifuged and the plasma and buffy coat were removed by suction. The erythrocytes were washed twice with 20 vol. of cold 0.15 $M$ NaCl and 5 times with 20 vol. of chilled 0.1 $M$ sodium-potassium phosphate buffer, pH 8.0. 5% stock solutions of FDNB and DFDNB in methanol were prepared daily and stored at 4 °C. A 0.5% suspension of A erythrocytes was incubated for 60 min at 25 °C and pH 8.0 with $5 \times 10^{-4} M$ FDNB and DFDNB. Methanol was added to the controls. Following incubation the cells were washed 5 times with 20 vol. of buffer and adjusted to a 50% suspension after the last centrifugation. ACHE activity was measured at 25 °C on replicate 0.1% cell suspensions in 0.1 $M$ phosphate buffer, pH 8.0 using acetylthiocholine iodide as substrate and 5:5′-dithiobis-(2-nitrobenzoic acid) as color reagent[5]. Residual enzyme activity was related to controls. Agglutinability was ascertained with human anti-A serum (Ortho Diagnostics). In some experiments, A or B erythrocytes were agglutinated with an excess of homologous human antiserum. The agglutinated cells were dispersed and washed 5 times with buffer prior to the exposure to FDNB and DFDNB. Finally, A or B erythrocytes were incubated with homologous human antiserum in amounts insufficient to cause direct agglutination, but sufficient to produce agglutination with rabbit anti-human globulin serum. Cells thus treated were washed with 0.1 $M$ phosphate buffer, pH 8.0 and then incubated with the reagents. Following this treatment and multiple washings with buffer, ACHE activity and agglutinability by rabbit anti-human globulin serum were determined. Agglutination was judged on a 1 to 4 scale.

In Table I it can be seen that while treatment of erythrocytes with $5 \times 10^{-4} M$ FDNB caused only a moderate reduction of ACHE activity, exposure to the same concentration of DFDNB and under identical conditions resulted in a much greater loss of activity. It can also be observed that whereas cells treated with FDNB were agglutinated by homologous antiserum, those incubated with DFDNB did not agglutinate. However, agglutination of FDNB-treated erythrocytes was invariably followed by hemolysis. When red cells were agglutinated with an excess of homologous antibody and then exposed to FDNB or DFDNB, reduction of ACHE

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