The author FREERK W. VAN DER MEULEN was born in 1947 in The Hague, The Netherlands. Initially he followed lectures in technical chemistry in Delft but moved in 1966 to Groningen to read medicine. In 1971 he obtained his masters degree and then he moved to the University of Amsterdam, where he qualified.

As a reserve officer in the Royal Netherlands Army, attached to the Military Blood Transfusion Service, he became due to his interest in the interaction between white and red blood cells.

After that period he was employed by the Central Laboratory of The Netherlands Red Cross Blood Transfusion Service and finished his research by writing this thesis in 1978.

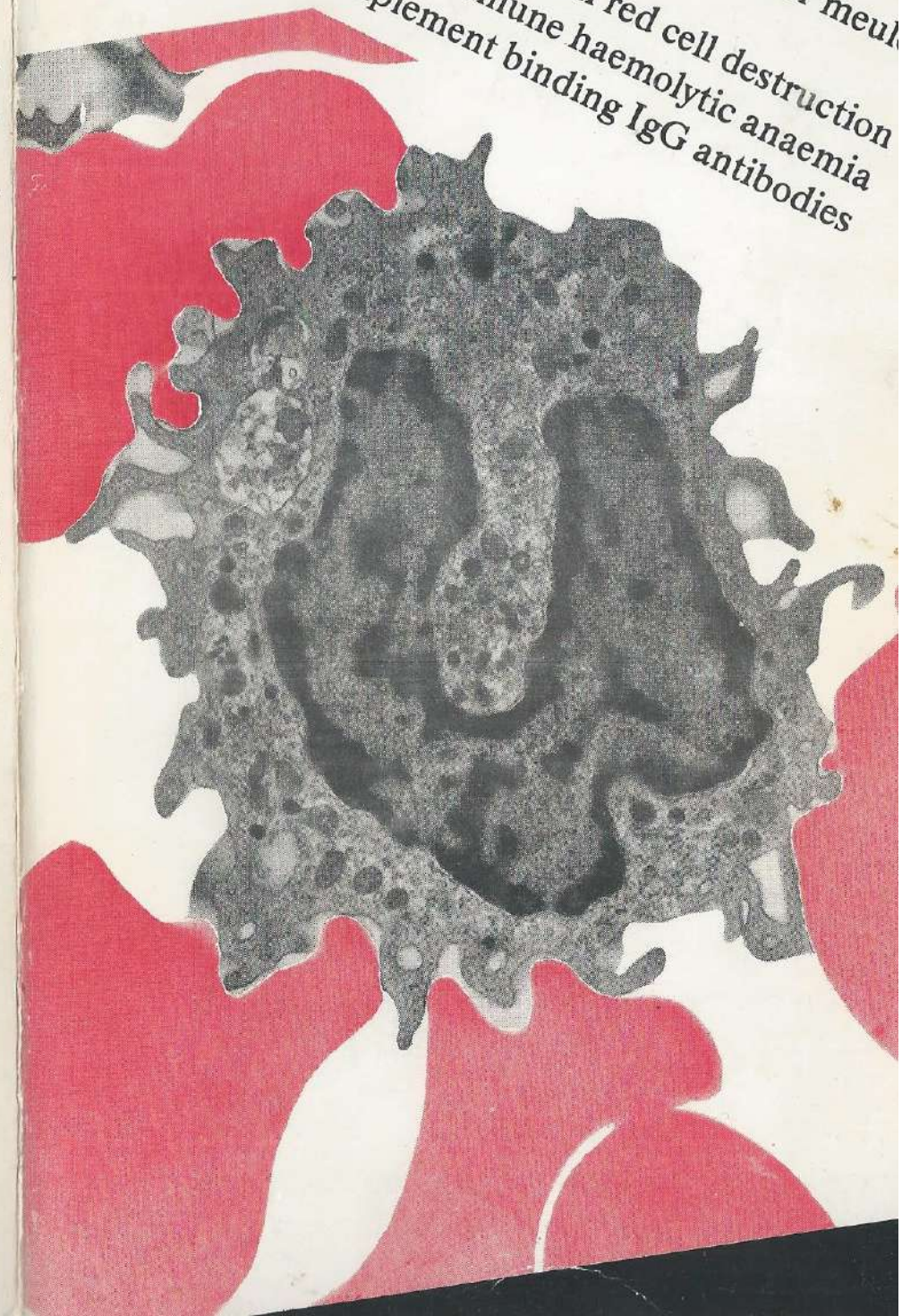
The author is publishing for some years articles relating to medical subjects in the Dutch magazine 'Viva'.

In his spare time he likes to play his favorite musical instrument, the trumpet.

freerk van der meulen ■ studies on red cell destruction in auto immune haemolytic anaemia with non-complement binding IgG antibodies

freerk van der meul

studies on red cell destruction
in auto immune haemolytic anaemia
with non-complement binding IgG antibodies



studies on red cell destruction
in auto immune haemolytic anaemia
with non-complement binding IgG antibodies

studies on red cell destruction in auto immune haemolytic anaemia with non-complement binding IgG antibodies

Academisch Proefschrift

ter verkrijging van de graad van doctor in
de Geneeskunde aan de Universiteit van Amsterdam,
op gezag van de Rector Magnificus Dr. G. den Boef,
hoogleraar in de faculteit der Wiskunde en Natuurwetenschappen,
in het openbaar te verdedigen in de aula der universiteit
(tijdelijk in de Lutherse Kerk, ingang Singel 411, hoek Spui)
op 26 oktober 1978 om 17.00

door

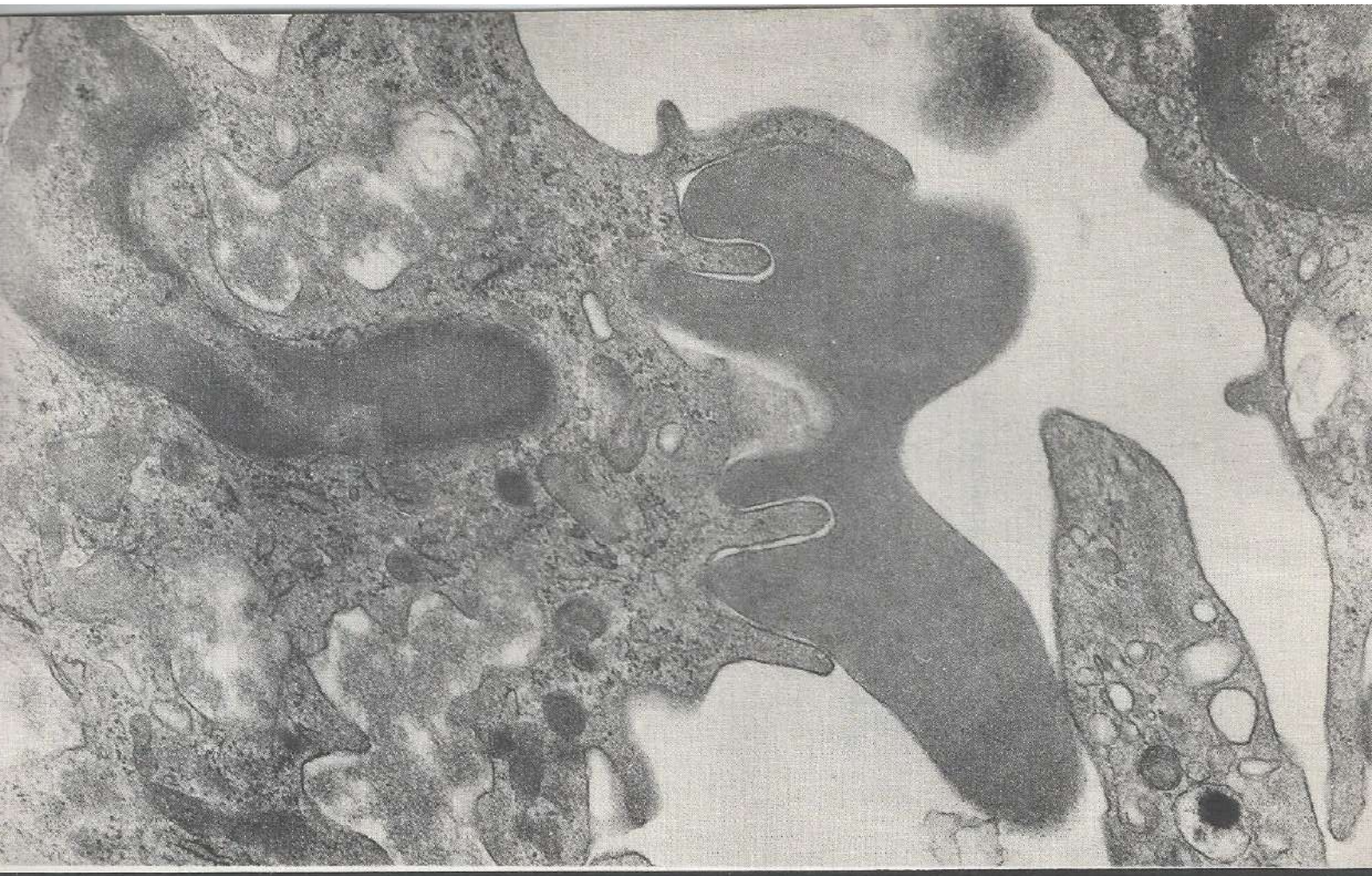
Freerk Walter van der Meulen

geboren te s'Gravenhage

Promotores : Prof. dr. C.P. Engelfriet
Prof. dr. J.J. van Loghem

Co-referent : Prof. dr. R.E. Ballieux

contents



introduction

Soon after the development of the antiglobulin test (AGT) by Coombs et al. (1945), Loutit and Mollison (1946) discovered that the red cells of some patients with acquired haemolytic anaemia are agglutinated by antiglobulin serum. Further investigations demonstrated that the so-called non-complement binding incomplete IgG warm autoantibodies, that may be detected in this way, are responsible for the red cell destruction in the vast majority of patients with autoimmune haemolytic anaemia (AIHA) (Dacie, 1962; Borne, 1971a). However, in some individuals who are otherwise haematologically normal, the presence of the above kind of antibodies is not associated with a shortened red cell lifespan in vivo (Weiner and Vos, 1963; Engelfriet et al., 1974; Issitt et al., 1976; Borne et al., 1977a).

The incubation of red cells with IgG antibodies in vitro does not lead to damage of these cells (Borne et al., 1971b) and since virtually all IgG incomplete warm autoantibodies are not capable of complement activation (Dacie, 1962; Engelfriet et al., 1974), another mechanism of red cell destruction must be responsible in the case of these antibodies.

In 1952, Zinkham and Diamond observed the binding of red cells to leukocytes in the peripheral blood of patients with signs of severe haemolysis. Then, LoBuglio et al. (1967) made the crucial discovery that in vitro red cells sensitized with IgG antibodies (EAIgG) were bound to, and subsequently damaged by, mononuclear phagocytes. From then on, the results of many studies pointed to an essential role of mononuclear phagocytes in the in vitro destruction of red cells under the influence of non-complement binding IgG allo- and autoantibodies. It appeared that adherence of EAIgG to mononuclear phagocytes leads to phagocytosis (Archer, 1965; Huber and Fudenberg, 1968; Abramson et al., 1970a) and cytotoxic damage and lysis outside the phagocytic cell (Holm et al., 1974; Fleer, 1978). Lymphocytes and granulocytes proved to only interact weakly with red cells sensitized with non-complement binding IgG antibodies (LoBuglio et al., 1967; Huber et al., 1969; Abramson et al., 1970a; Messner and Jelinek, 1970; Holm, 1972). It was also shown that the binding of EAIgG to mononuclear phagocytes in vitro is mediated by

the Fc-part, but not by the $F(ab')_2$ fragment of the sensitizing antibodies (Huber and Fudenberg, 1968) and Borne et al. (1977b) demonstrated the necessity of the Fc fragment of IgG anti-D antibodies for the destruction of red cells in vivo.

Since the consequences of the adherence of EAIgG to the membrane of mononuclear phagocytes was the only mechanism known by which red cells could be damaged under the influence of non-complement binding IgG antibodies, it was assumed that this mechanism is also active in vivo.

However, the adherence of red cells sensitized with IgG anti-D antibodies can be readily inhibited in vitro by free IgG in a concentration far below that in plasma and, therefore, the assumption that this adherence phenomenon is active in vivo needed support. In the studies described in this thesis we have endeavoured to produce such support by elucidating the factors that determine whether or not red cells are destroyed under the influence of IgG non-complement binding antibodies in vivo.

We have also studied the mechanism which is responsible for the cytotoxic damage of adherent EAIgG outside the macrophage membrane and, finally, we have evaluated the influence of corticosteroid therapy.

Huber and Fudenberg (1968), Abramson et al. (1970b) and Holm (1974) observed that only IgG of the subclasses 1 and 3, but not IgG of the subclasses 2 and 4, are able to establish adherence of EAIgG to, and thus lysis of these cells by, mononuclear phagocytes. In agreement with this was our finding (Engelfriet et al., 1974) that only patients with IgG3 and some patients with IgG1 autoantibodies show signs of increased haemolysis, whereas patients with IgG2 and IgG4 autoantibodies do not show such signs.

Further (Chapter I), we studied in patients with IgG non-complement binding autoantibodies the subclass composition of the antibodies on the patients' red cells in relation not only to the presence or absence of increased haemolysis in vivo, but also to the adherence of the patients' red cells to peripheral blood monocytes (PBM) of healthy volunteers in vitro. Monocytes were chosen as representatives of the mononuclear phagocytic cell population because they can be rather easily isolated from peripheral

blood and because they are the precursors of tissue macrophages (Furth and Cohn, 1968).

In Chapter II, these studies are further extended in a patient with predominantly IgG4 autoantibodies, who did not show evidence of increased red cell destruction in vivo.

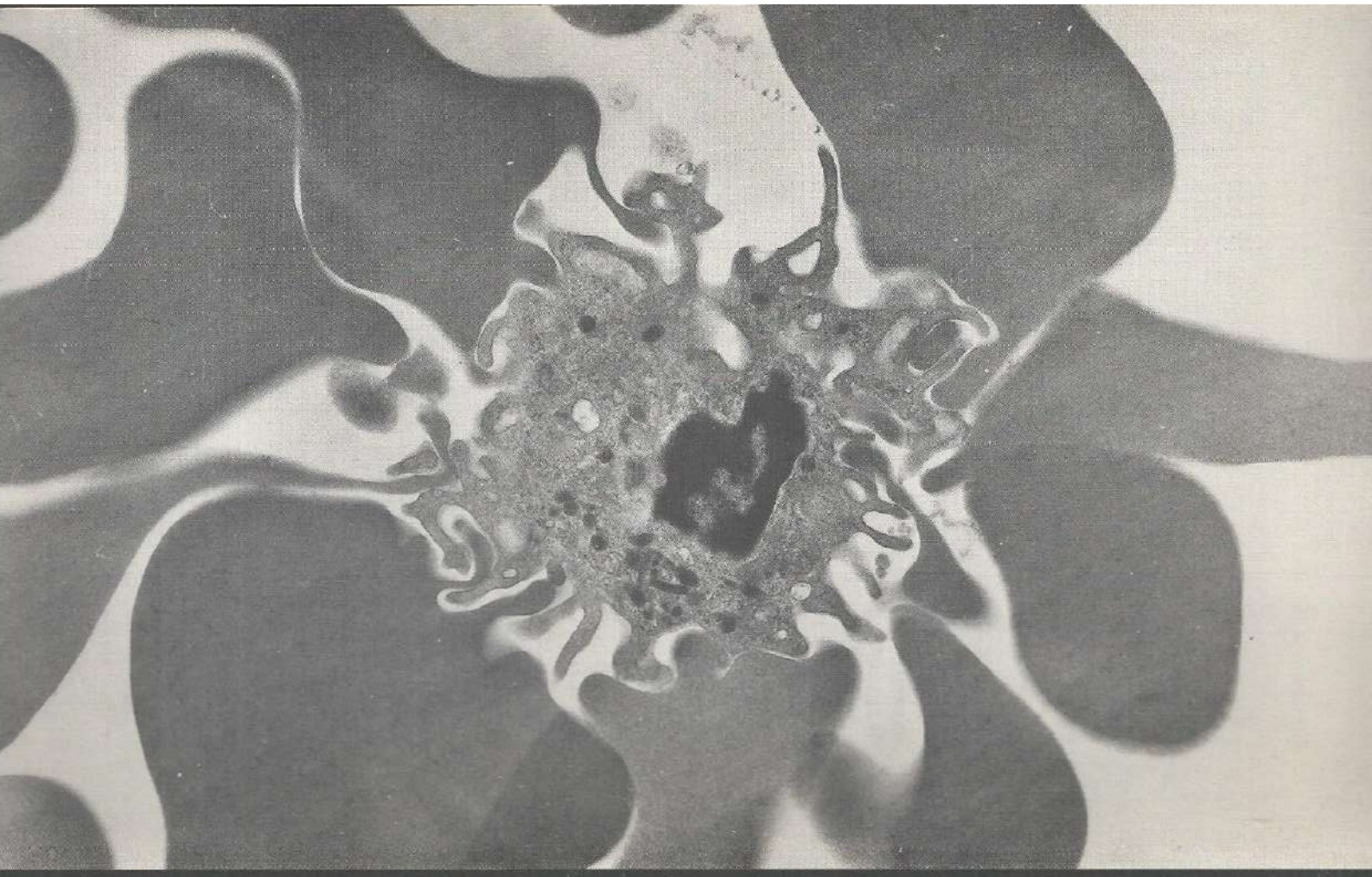
In Chapter III, we have studied the factors that influence the above-mentioned inhibition of adherence of EAIgG to monocytes by free IgG and we have tried to find an explanation why in the majority of patients the destruction of red cells sensitized with IgG non-complement binding antibodies is confined to the spleen (Jandl and Kaplan, 1960; Dacie, 1962; Mollison et al., 1965; Borne, 1971a). LoBuglio et al. already stressed in 1967 the importance of the special circumstances in this organ with respect to red cell destruction. For these studies, red cells sensitized with IgG anti-D alloantibodies (EAIgG anti-D) were used, since anti-D antibodies are known to resemble IgG non-complement binding autoantibodies in serological and immunochemical respect (Engelfriet et al., 1974). Since it is known that in the spleen a considerable haemoconcentration occurs (Jandl et al., 1957) and that in this organ the red cells are more strongly sensitized with antibodies than in the peripheral blood (Wagley et al., 1948; Rosse, 1971), the influence of the number of red cells per monocyte as well as of the antibody load present on the red cell surface on the inhibition by IgG in vitro was studied.

In some patients, IgG1 autoantibodies lead to increased red cell destruction in vivo, whereas in others the presence of antibodies of this subclass is not associated with signs of increased destruction of erythrocytes (Engelfriet et al., 1974). To determine whether this is due to a difference in the quality of the IgG1 autoantibodies of the various patients or merely to a difference in the number of sensitizing IgG1 antibodies, we compared the amount of IgG1 on the red cells of patients with and without haemolytic anaemia. To that end, we measured by means of a cytofluorograph -as is described in Chapter IV- the fluorescence obtained with the patients' red cells, sensitized with IgG1 autoantibodies only, after these cells were incubated with a fluorescein isothiocyanate (FITC)-labelled anti-IgG1 serum.

Results, recently obtained in our laboratory, strongly suggest (Fleer et al., in press,a,b) that extracellular cytotoxic lysis and damage by mononuclear phagocytes is of major importance in the destruction of red cells sensitized with IgG non-complement binding antibodies. We, therefore, investigated (Chapter V) the cytotoxic action of PBM of healthy volunteers towards these cells, obtained from patients with and without haemolytic anaemia. In a number of patients, the effect of corticosteroid therapy was determined by examining consecutively taken blood samples. In this longitudinal study, special attention was paid to the changes in the amount of antibody, in subclass composition and to the course of adherence and cytotoxic lysis of the patients' erythrocytes in vitro.

Viral and bacterial infections in patients with AIHA may be accompanied by an exacerbation of the haemolysis (Dacie, 1962; Borne, 1971a; Leddy and Swisher, 1971). To study whether the increase of the red cell destruction -at least partly- could be explained by an 'activation' of the mononuclear phagocytes in vivo, we measured, as described in Chapter VI, the cytotoxic activity of PBM from patients with acute infectious diseases (without AIHA) towards EAIgG in vitro. Simultaneously, the activity of granulocytes and lymphocytes from these patients was assessed to determine whether these cells could contribute to the increased rate of red cell destruction.

Finally (Chapter VII), the influence of cryopreservation on monocyte recovery and function was studied in order to determine whether these cells may be stored for long periods. Storage at -196°C of mononuclear phagocytes without much change in functional properties would be advantageous. In this way, one would be able to avoid the great variation in activity in several test systems, displayed by monocytes isolated from various normal individuals. Furthermore, it would provide the possibility to test the monocytes of one individual, collected during a longitudinal study, on one day. Finally, long-term storage of large quantities of monocytes would mean a gain of time, otherwise consumed by the daily isolation of these cells from small amounts of blood.



chapter I
the role of adherence to human mononuclear phagocytes in the destruction of red cells sensitized with non-complement binding IgG antibodies

F.W. van der Meulen, Mia van der Hart, A. Fleer,
A.E.G. Kr. von dem Borne, C.P. Engelfriet and
J.J. van Loghem

Central Laboratory of the Netherlands Red Cross
Blood Transfusion Service, and University Laboratory
of Experimental and Clinical Immunology, Amsterdam,
The Netherlands.

British Journal of Haematology, 1978, 38, 541-549.

summary

In patients with IgG incomplete non-complement binding warm autoantibodies, the subclass composition of the antibodies was studied in relation to the occurrence of increased haemolysis in vivo and the adherence of the patients' red cells to peripheral blood monocytes (PBM) in vitro.

The presence of IgG3 autoantibodies was almost always accompanied by haemolytic anaemia, but the presence of IgG1 autoantibodies only in some patients but not in others. IgG2 and IgG4 autoantibodies were not associated with increased red cell destruction.

A relation identical to that between subclass composition and increased haemolysis was found between subclass composition and adherence of the patients' erythrocytes to PBM and thus a strong correlation between positive adherence in vitro and increased red cell destruction in vivo.

These results strongly support an important role of adherence to mononuclear phagocytic cells in the destruction of red cells sensitized with non-complement binding IgG antibodies. Strong indications were found that IgG1 autoantibodies are of two kinds, only one of which causes adherence to phagocytes and thus increased red cell destruction.

introduction

The interaction of red cells with IgG antibodies per se has no effect on these cells (Borne et al., 1971) and, therefore, erythrocytes can only be destroyed under the influence of antibodies by mechanisms which are activated as a consequence of the interaction of the red cells with the antibodies. The best known of such mechanisms is of course complement activation, but not all red cell antibodies are capable of this activity. In fact, most IgG allo- and autoantibodies do not activate complement (Dacie 1962; Engelfriet et al., 1974).

In vitro red cells sensitized with non-complement binding IgG antibodies adhere to mononuclear phagocytes by the Fc-part of the antibody molecule. This process leads either to cytotoxic lysis of the red cells outside the phagocyte or to phagocytosis (LoBuglio et al., 1967; Huber & Fudenberg, 1968; Abramson et al., 1970a; Holm et al., 1974). However, whether adherence to phagocytes can take place in vivo is questionable as in vitro it is inhibited by IgG in low concentrations, presumably because IgG that is not bound to an antigen can also react with the 'Fc-receptor'. In vivo plasma IgG is present wherever sensitized cells can come into contact with phagocytic cells. Because the above process of adherence to phagocytes is the only one known to lead to red cell destruction under the influence of non-complement binding antibodies, it is important to find support for the assumption that this process can also take place in vivo and thus be responsible for red cell destruction.

The following observations are in favour of the above assumption: spherocytosis with an increased osmotic fragility of the red cells is a typical symptom of patients with autoimmune haemolytic anaemia with incomplete warm autoantibodies (Dacie, 1962; Borne et al., 1971) and LoBuglio et al. (1967) have shown that red cells sensitized with IgG antibodies turn into spherocytes when they adhere to monocytes in vitro.

Red cells, sensitized with non-complement binding IgG anti-D antibodies in vitro, are rapidly destroyed after reinfusion in vivo (Jandl et al., 1957). Borne et al. (1977a) showed that red cells sensitized with $F(ab')_2$ fragments of IgG anti-D molecules, survive

normally in vivo, which indicates that the Fc-part of the anti-D molecule is essential for red cell destruction in vivo. The above assumption would be further strongly supported if a relation could be established between the subclass of IgG non-complement binding antibodies and their activity in vivo.

It has been shown that only the IgG subclasses 1 and 3 can interact with the 'Fc-receptor' on phagocytic cells in vitro and if this interaction initiates red cell destruction in vivo, IgG1 and IgG3 antibodies should be active and lead to increased haemolysis while IgG2 and IgG4 antibodies should be inactive (Engelfriet et al., 1974). In this paper, results of studies concerning this relation between the subclass of IgG autoantibodies and their in vivo and in vitro activity are reported.

materials and methods

Erythrocytes

Erythrocytes of patients with IgG incomplete non-complement binding warm autoantibodies were investigated. The subclass or subclasses of IgG present on these cells were determined with subclass-specific antisera (Engelfriet et al., unpublished results). The red cells of 45% of the patients were also agglutinated by anticomplement serum. With specific reagents it could be shown that C3d was present on these cells, but not C4c or C3c. Red cells were stored in liquid nitrogen. This storage caused no change in either the subclass composition of the antibodies on the red cells or adherence of these cells to monocytes. After washing, the erythrocytes were suspended in Minimal Essential Medium (Gibco, Paisley, Scotland), buffered with Tris 0.025 M (MEM-Tris).

Isolation of monocytes

Defibrinated peripheral blood from healthy human volunteers was subjected to Ficoll-Isopaque density gradient centrifugation (density = 1.077 g/ml) (Bøyum, 1968). The interface layer, which consisted almost exclusively of mononuclear cells, was washed three times and the cells were suspended in MEM-Tris, containing 20% (v/v) heat-inactivated fetal calf serum (FCS)

(Gibco, Biocult, Paisley, Scotland) to a cell concentration of $10x10^6$ cells/ml. 2.5 ml of this suspension was pipetted carefully into the middle of plastic petri dishes (Falcon plastic tissue culture dishes, 3003, Oxnard, Cal., USA) and incubated for 1.5 h at 37°C. Non-adhering cells were removed by two washings and the monolayer was harvested with a piece of silicone rubber. Subsequently, the cells were washed and resuspended in MEM-Tris, containing 5% FCS. Suspensions were obtained containing 70-90% monocytes, as judged by morphological standards. Over 97% of the cells excluded trypan blue.

Adherence assay

The red cell suspension (0.05 ml, 10^8 cells/ml) and 0.1 ml of a monocyte suspension ($2.5x10^6$/ml) were mixed well with 0.15 ml MEM-Tris. The cells were centrifuged at 160 g during 8 min and incubated for 15 min at room temperature. After resuspension, the number of rosettes was determined in a Bürker counting chamber. Monocytes which had bound 3 or more erythrocytes were counted as rosettes. The number of rosettes formed by monocytes with red cells (OR_2R_2), sensitized with IgG non-complement binding alloantibodies anti-D, was taken as a positive control (250-300 rosettes/counting chamber). With unsensitized red cells (OR_2R_2) no rosettes were formed. All tests were performed in duplicate.

Criteria for haemolysis in vivo

At least four of the following parameters were used: blood haemoglobin level or haematocrit or erythrocyte count, erythrocyte osmotic fragility, serum haptoglobin level, serum LDH activity, erythrocyte ^{51}Cr $T_{\frac{1}{2}}$, reticulocyte count, bilirubin level and erythrocyte G6PD activity. Normal values used were those of Williams et al. (1972).

Statistics

Discriminant analysis was used as a statistical method (Williams, 1959) to evaluate the influence of the distinct IgG subclasses.

results

Among the first group of cases studied, in which the subclass of the IgG autoantibodies could be determined, there were 271 patients with IgG autoantibodies of only one subclass (Table I).

TABLE I

Presence or absence of increased haemolysis in patients with IgG incomplete warm autoantibodies of only one subclass

Number of patients	IgG1	IgG2	IgG3	IgG4	haemolysis
259	+	-	-	-	+ / -
2	-	+	-	-	-
8	-	-	+	-	+
2	-	-	-	+	-

It may be seen that in the 2 patients with only IgG2 and the 2 patients with only IgG4 autoantibodies, there were no signs of increased haemolysis. All the 8 patients with only IgG3 autoantibodies had overt haemolytic anaemia. So far, these results seem to support the presumed relation between subclasses and in vivo activity. However, the patients with only IgG1 autoantibodies -up to the present we have been able to examine a total of 259 patients- could be divided in 2 groups: patients in whom there were no signs of increased red cell destruction and patients with haemolytic anaemia. If adherence to phagocytes is indeed the mechanism which initiates red cell destruction in vivo, this means that IgG1 may be or may not be capable of interaction with the 'Fc-receptor' on phagocytic cells.

To investigate this point further, a new series of 61 patients was studied. This time we looked not only for the presence or absence of signs of increased red cell destruction, but we also studied the adherence of the patients' red cells to monocytes in vitro. Nineteen of the 61 patients were treated with prednisone. These will be considered separately for reasons outlined below.

a) Relation between the subclass of non-complement binding IgG autoantibodies and haemolysis in vivo

In this group also we studied the relation between the sublass of the IgG antibodies detectable on the patients' red cells and the occurrence of increased haemolysis in vivo (Table II). Among the 18 cases in which only IgG1 was detectable on the erythrocytes, again two categories were found: 5 patients showed signs of increased erythrocyte destruction and 13 did not.

Among the patients of this series, there were none with only IgG3 antibodies. However, from the results with 13 patients with IgG3 antibodies combined with antibodies of other subclasses, it was calculated with discriminant analysis (Williams, 1959) that the difference between the number of patients with haemolytic anaemia (i.e. 11) and the number of those without (i.e. 2) was significant ($p<0.025$). Patients with only IgG2 and IgG4 antibodies were not present either.

From the analysis of 15 cases in which such IgG antibodies were combined with antibodies of other subclasses, it appeared that in this group the percentage of patients with increased haemolysis did not differ significantly from that in the group of patients without IgG2 and IgG4 antibodies.

TABLE II

Relation between the subclass of non-complement binding IgG autoantibodies and haemolysis in vivo

Non-treated group

IgG subclass detected on the red cells	increased haemolysis	no increased haemolysis	number of patients
IgG1	5	13	18
IgG1+2	3	5	8
IgG1+2+3	2	0	2
IgG1+2+3+4	1	1	2
IgG1+2+4	0	2	2
IgG1+3	8	1	9
IgG1+4	1	0	1
Total	20	22	42

b) Relation between antibody subclass and adherence to monocytes in vitro

The relation found between adherence of the patients' red cells to monocytes in vitro and the antibody subclass composition was identical to the relation between subclass composition and increased haemolysis (Table III). Thus, the presence of IgG1 autoantibodies alone was accompanied by adherence in the same 5 cases which showed increased haemolysis, but not in the remaining 13. In a significant number of cases ($p<0.025$), sensitization with IgG3 autoantibodies -in combination with other subclasses- was found to be accompanied by a positive rosette-test. The presence of IgG2 and IgG4 autoantibodies did not exert a clear influence on rosette formation.

TABLE III

Relation between the subclass of non-complement binding IgG autoantibodies and adherence to monocytes in vitro

Non-treated group

IgG subclass detected on the red cells	adherence	no adherence	number of patients
IgG1	5	13	18
IgG1+2	3	5	8
IgG1+2+3	2	0	2
IgG1+2+3+4	1	1	2
IgG1+2+4	0	2	2
IgG1+3	8	1	9
IgG1+4	1	0	1
Total	20	22	42

c) Relation between haemolysis in vivo and adherence in vitro

Table IV shows that in all cases in which signs of increased haemolysis were present, the red cells adhered to monocytes in vitro and if there were no signs of haemolytic anaemia, no rosette formation occurred in vitro.

TABLE IV

Relation between haemolysis in vivo and adherence to monocytes in vitro

Non-treated group

adherence	increased haemolysis	no increased haemolysis	number of patients
present	22	0	22
absent	0	20	20
total	22	20	42

Prednisone-treated group

In a group of 19 patients, suffering from haemolytic anaemia with non-complement binding IgG autoantibodies and treated with prednisone for at least 10 days (with IgG autoantibodies still detectable on their red cells), 12 showed the same pattern as untreated patients, i.e. a strict correlation between monocyte adherence and signs of increased haemolysis, 6 being positive, 6 being negative (Table V). In 4 cases adherence took place while no signs of increased erythrocyte destruction were present, a finding possibly due to the prednisone treatment. However, in 3 patients adherence was negative while increased haemolysis was present, a completely unexpected finding.

TABLE V

Relation between haemolysis in vivo and adherence to monocytes in vitro

Prednisone-treated group

adherence	increased haemolysis	no increased haemolysis	number of patients
present	6	4	10
absent	3	6	9
total	9	10	19

discussion

As stated in Materials and Methods, the inactive fragment of the third component of complement -C3d- was detectable on the red cells of 45% of the patients. The presence of this component was not taken into account since it does not lead to a shortening of red cell lifespan in vivo (Evans et al., 1968; Borne et al., 1973; Atkinson & Frank, 1974; Jaffé et al., 1976), nor does it cause adherence to mononuclear phagocytes in vitro in the human system (Brown, 1973; Engelfriet et al., 1974; Reynolds et al., 1975).

The results of our studies show that IgG2 or IgG4 autoantibodies, when present alone, do not cause increased destruction of red cells in vivo. The presence of IgG3 autoantibodies on the red cells was accompanied by increased haemolysis in vivo in 19 out of 21 cases (Tables I and II). In the two patients with IgG3 antibodies without haemolytic anaemia, the titre with anti-IgG3 was low, but that with anti-IgG1, anti-IgG2 and anti-IgG4 was high in one, that with anti-IgG1 very high in the other. Since it was found that IgG2 and IgG4 and part of the IgG1 autoantibodies do not cause adherence to the 'Fc-receptor', it is possible that the adherence of the red cells of these patients was inhibited by the large number of inactive antibodies present on the cells and that, therefore, there was no increased red cell destruction.

Sensitization with IgG1 autoantibodies caused increased destruction of erythrocytes in some, but not in other patients, although there was no clear difference in agglutination titre with anti-IgG1 between these two categories. This finding made us study the relation between the subclass of IgG autoantibodies and the adherence of the patients' red cells to monocytes in vitro as well as the occurrence of increased red cell destruction in another group of patients. This group was deliberately chosen to perform a qualitative study and the figures are, therefore, not representative for the patient population as a whole. In 42 patients, not treated with prednisone, there was a close relationship between adherence of the patients' red cells to monocytes and haemolysis in vivo; thus the red cells of all patients with haemolytic anaemia adhered to monocytes in vitro while those of none of the patients without signs of increased haemolysis did. This was also the

case in the 18 patients with only IgG1 antibodies. Hence it seems justifiable to subdivide IgG1 autoantibodies into two kinds, only one of which induces adherence to monocytes in vitro and haemolysis in vivo.

It is of interest that 8 of the 13 patients, not treated with prednisone, with only IgG1 autoantibodies and without signs of increased red cell destruction, used α-methyldopa. 15% of patients treated with this drug develop IgG incomplete warm autoantibodies against red cells, but less than 1% of these patients show signs of increased haemolysis (Worlledge, 1973). It seems that α-methyldopa mostly induces the formation of inactive IgG1 autoantibodies.

The above results, particularly the absolute correlation between adherence of the patients' red cells to monocytes in vitro and increased haemolysis in vivo, strongly support the assumption that adherence to mononuclear phagocytes is an important mechanism in the in vivo destruction of red cells under the influence of non-complement binding IgG antibodies. How can we explain this in face of the fact that in vitro the adherence of sensitized red cells to mononuclear phagocytes is inhibited by free IgG in concentrations below that of plasma (Huber & Fudenberg, 1968)? Are there special circumstances under which this inhibition does not take place? In this connection, the following observations may be of importance: the spleen plays an important role in the destruction of red cells sensitized with non-complement binding antibodies (Jandl et al., 1957; Dacie, 1962) and in most patients with autoantibodies of this kind, red cell destruction is confined to the spleen.

If adherence to mononuclear phagocytes and the consequences thereof are essential for red cell destruction there must be circumstances in the spleen that prevent inhibition of adherence. On entering the splenic red pulp, the red cells are separated from the plasma (Prankerd, 1963). The haematocrit thus increases to 70-80%. LoBuglio et al. (1967) showed that inhibition of adherence by free IgG may be overcome by a rise in the concentration of sensitized cells. Further, it seems possible that macrophages in the spleen are more active in binding and damaging sensitized cells than peripheral blood monocytes. And, finally, sensitization of the erythrocytes increases in the spleen as autoantibodies are locally synthesized (Wagley et al., 1948), which may also hamper inhibition of adherence. This would be consistent with the finding that strongly sensitized red cells are removed from the circulation more quickly than weakly sensitized ones (Jandl & Kaplan, 1960; Mollison et al., 1965).
It seems plausible that the above circumstances together would allow adherence to take place in the spleen, while it is inhibited by plasma IgG in other parts of the circulation.

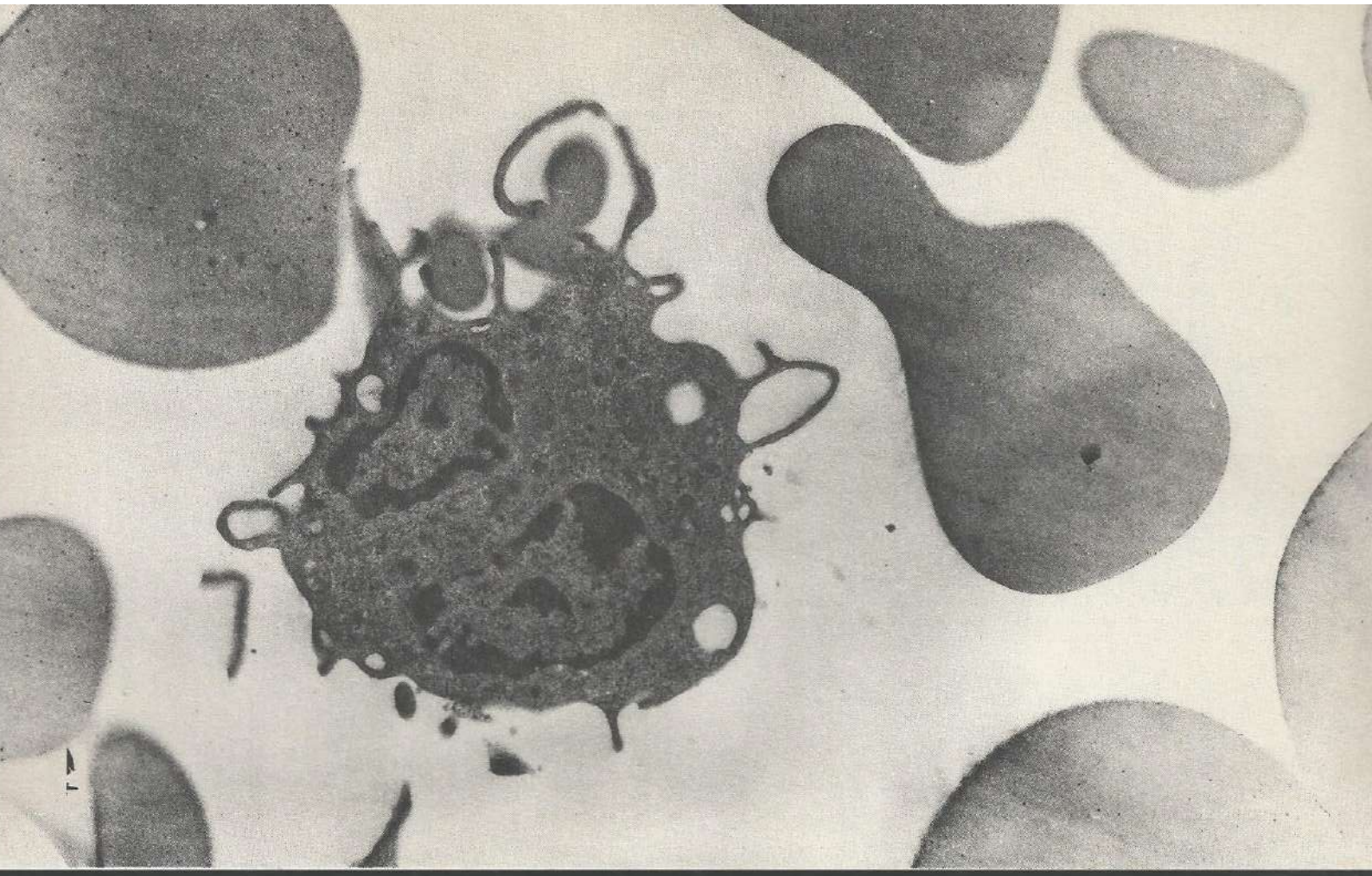
Six patients treated with prednisone, whose red cells adhered to monocytes in vitro, still had active haemolytic anaemia. Apparently, the therapy was not successful in these patients. In 4 other patients, also treated with prednisone, there were no signs of increased cell destruction, although their red cells adhered to monocytes in vitro. In these cases corticosteroids may have prevent the cytotoxic consequences of adherence, but not adherence itself.
It is important to realize that we used monocytes from healthy donors for this test. Whether corticosteroids do or do not influence the adherence phenomenon itself, either in vitro or in vivo, is still a matter of controversy (Abramson et al., 1970a; Rinehart et al., 1974; Schreiber et al., 1975). This is at present being further investigated.

In another 6 patients who had suffered from haemolytic anaemia and who received prednisone, the treatment was successful and the patients' red cells did not adhere to monocytes, although the direct antiglobulin test was still strongly positive (IgG1 in 5 patients, IgG1 + IgG2 in 1 patient). This is an important finding as it may imply that one of the possible effects of prednisone is that it induces a switch in the formation of adhering to non-adhering IgG antibodies (Borne et al., 1977b).

Finally, in 3 patients in whom IgG autoantibodies were still present, there were signs of increased haemolysis, whereas the patients' red cells did not adhere to monocytes. There are two possible explanations for this finding: (1) the increased haemolysis is due to spherocytic cells formed during the haemolytic episode previous to the treatment with prednisone and which had remained in the circulation; and (2) the autoantibodies in these patients (IgG1) are inactive and red cell des-

truction is due to other, non-immunological mechanisms. We hope that further studies of these patients may give an answer to the above questions.

notes



chapter II
IgG4 auto antibodies against erythrocytes without increased haemolysis

A.E.G. Kr. von dem Borne, Do Beckers, F.W. van der Meulen and C.P. Engelfriet

Department of Immunohaematology, Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, and University Clinic of Internal Medicine, Department of Haematology, Amsterdam, The Netherlands

British Journal of Haematology, 1977, 37, 137

summary

A patient is described who, notwithstanding a strongly positive direct antiglobulin test with anti-IgG serum, apparently did not suffer from haemolytic anaemia. The survival of the patient's red cells, measured with ^{51}Cr, was only slightly decreased.

In vitro, the sensitized cells of the patient showed only a minimal tendency to adhere to monocytes. The patient's spleen functioned normally, since ^{51}Cr-labelled donor erythrocytes, either sensitized with IgG-anti-D or damaged by heating, were eliminated from the circulation and sequestered in the spleen.

These apparently contradictory findings could be explained by the fact that the patient's red cells were sensitized with autoantibodies, mainly belonging to the IgG4 subclass. Only weak IgG1 and IgG3 autoantibodies were detectable. Since previously the patient had suffered from a severe haemolytic anaemia, it is postulated that a switch has occurred from 'active' to 'inactive' IgG autoantibodies, perhaps induced by prednisone therapy.

introduction

Although incomplete warm autoantibodies against erythrocytes may belong to different immunoglobulin classes (Engelfriet et al., 1968; Dacie & Worlledge, 1969) such antibodies are mostly of the IgG class (Engelfriet et al., 1974). With antisera specific for the subclasses of immunoglobulin G, the subclasses of IgG erythrocyte autoantibodies can be determined (Engelfriet et al., 1974). With such reagents, it was found that these autoantibodies mainly occur in the IgG1 subclass. IgG2, IgG3 and IgG4 red cell autoantibodies are less frequent and are mostly found in combination with IgG1. IgG2 and IgG4 autoantibodies alone are seldom observed. Several investigators have shown that in vitro destruction by mononuclear phagocytes of red cells sensitized with IgG antibodies is initiated by adherence to the Fc receptor of these cells. Such adherence can only be mediated by IgG1 and IgG3 since the IgG2 and IgG4 subclasses are inactive in this respect (Huber & Fudenberg, 1968; Abramson et al., 1970; Abramson & Schur, 1972; Hay et al., 1972; Holm et al., 1974).

This is most probably also the case in vivo (Borne et al., 1977), and since tests for the presence of erythrocyte autoantibodies are predominantly performed in patients suffering from acquired haemolytic anaemia, erythrocytes carrying only IgG2 and/or IgG4 autoantibodies are seldom found. The patient to be described initially came to our attention because of a severe haemolytic anaemia. The haemolysis responded well to therapy and eventually disappeared. However, strong IgG warm autoantibodies remained present.

CASE HISTORY

Mr. H. van E., born in 1893, had never been ill until 1956, when he began to complain of dyspnoea, a dry cough, tiredness and periods of fever. In 1958 it was found that he suffered from sarcoidosis of the lungs. He was treated with prednisone. This treatment was successful. Prednisone medication was gradually diminished and finally stopped in 1960. In 1961, however, he again became ill, complaining of dyspnoea, tiredness and palpitations. It was found that he was

anaemic and jaundiced and that the spleen was enlarged. An X-ray of the chest only showed signs of healed sarcoidosis. Laboratory investigations revealed a blood haemoglobin level of 7.9 g/dl and a reticulocyte count of 20%. The anaemia was normochromic and normocytic. Leukocyte and platelet counts were normal. There was also a hyperbilirubinaemia of the indirect type, ahaptoglobulinaemia and urobilinuria. Liver- and kidney-function tests gave normal results. The results of the serological analysis of his blood at that time are shown in Table I. No ANF were detectable. He apparently suffered from an autoimmune haemolytic anaemia (AIHA) of the IgG warm autoantibody type. Again treatment with prednisone was started and proved to be successful. The blood haemoglobin level became normal and his complaints disappeared.

TABLE I

Serological findings in patient van E. in 1961

Direct antiglobulin test on patient's erythrocytes:	
Anti-IgG	1024*
Anti-non-IgG	16*
Indirect antiglobulin test on patient's serum:	
Anti-IgG	64*
Anti-non-IgG	0*
Specificity not demonstrable	

*Reaction strength expressed as the reciprocal titre.

During the first years after the diagnosis of AIHA some signs of increased haemolysis were still present, i.e. ahaptoglobulinaemia and a slight splenomegaly. In spite of this the prednisone dosage was gradually diminished. In 1966 all signs of the haemolytic anaemia had disappeared although serological findings were unchanged. Prednisone therapy was stopped. The discordance between the clinical and laboratory findings initiated an extensive clinical and serological revaluation

in 1967 (v.i.). The patient remained well without any medication until 1971 when he was admitted to the hospital because of a gastrointestinal bleeding after using chefarine 4 (Chefaro International B.V., Rotterdam, Holland) because of a painful cystitis. Blood transfusion was necessary. The recovery was uneventful. In 1974 he was again admitted, this time with a large inferior wall infarction. This caused his death at an age of 81. He died without signs of haemolytic anaemia.

materials and methods

Patient's blood

Clotted samples as well as samples anticoagulated with heparin were obtained. Red cells prepared from the latter were stored in straws in liquid nitrogen, using glycerol as a cryopreservative. Before use, the frozen cells were thawed and washed with sorbitol (17.5%) and with phosphate-buffered saline. Thus, samples taken over a period of several years could be tested on the same day with the same reagents.

Antiglobulin reagents

Specific antisera, suitable for the antiglobulin test, against IgG, IgM, IgA, immunoglobulin light chain κ and λ, as well as anti-complement and anti-non-IgG sera were prepared in our laboratory and evaluated as described previously (Engelfriet et al., 1968); the preparation of antisera against the subclasses of IgG, specific in the immunoprecipitation test has also been described (van der Giessen et al., 1974). Specificity in the haemagglutination test was examined with red cells from patients with AIHA sensitized with antibodies of which the immunoglobulin class(es) was known or with complement, and with tanned red cells coated with different proteins. Further absorptions with purified proteins were always required to obtain antisera specific in the antiglobulin test. For this purpose, more or less pure myeloma proteins of the various immunoglobulin classes and IgG subclasses were used.

Serological tests

These tests were performed as described elsewhere (Engelfriet et al., 1968).

Monocyte adherence

A modification of the method of Huber & Fudenberg (1968) was used (van der Meulen et al., 1978). Red cells of the patient were washed three times and suspended in Tris-buffered minimal essential medium (MEM) in a concentration of 10^8/ml. Peripheral blood monocytes prepared from normal donor blood were suspended in the same medium, containing 5% heat-inactivated fetal calf serum, in a concentration of 2.5×10^6/ml. To 0.15 ml of Tris-buffered MEM, 0.05 ml of erythrocyte suspension and 0.1 ml of monocyte suspension were added respectively. After mixing the cells were spun at 160 g for 8 min in a clinical centrifuge and incubated for 15 min at room temperature. The sediment was resuspended with a pasteur pipette. The percentage rosette-forming cells was counted in a Bürker chamber. Non-sensitized normal OR_2R_2 red cells and OR_2R_2 cells sensitized with IgG anti-D were tested as controls. The sensitized cells were prepared by incubation of two parts of a 5% cell suspension with one part of MEM and one part of a specially selected anti-D serum. All tests were performed in duplicate.

Radioisotope methods

^{51}Cr survival studies with the patient's own erythrocytes were performed according to Mollison & Veall (1955). Organ sequestration was measured according to Jandl et al. (1956) and Hughes Jones & Szur (1957). Apart from the patient's own cells, normal donor erythrocytes of blood group OR_1R_1 were also used. However, the donor red cells were either sensitized with anti-D-antibodies or damaged by heat treatment before injection. The survival of these cells was measured separately in order to study the functional capacity of the patient's spleen. 4 ml of donor blood, anticoagulated with 1 ml of ACD was labelled with 50-100 µCi of ^{51}Cr Na_2CrO_4 under sterile conditions. After washing, the blood was either incubated for 30 min at 37°C with 1 ml of anti-D IgG, containing 100-125 µg anti-D antibodies, washed once with ice cold saline and suspended in 20 ml of saline, or directly suspended in 20 ml of saline and incubated for 20 min at 50°C. In both experiments, 10 ml of the labelled suspension was injected. Radioactivity in the blood,

above the heart, liver and spleen was measured at short intervals after injection. Radioactivity in the blood at time zero was calculated from the measured amount of injected radioactivity and from the blood volume of the patient. The blood volume had been measured previously.

results

The results of the extensive serological investigations on the blood of patient van E. performed in 1967 are summarized in Table II.

TABLE II

Serological findings in patient van E. in 1967

Direct antiglobulin test on patient's erythrocytes:	
Anti-IgG	1024*
Anti-IgM	0
Anti-IgA	0
Anti-non-IgG	0
Anti-complement	0
Indirect antiglobulin test on patient's serum:	
Anti-IgG	4*
Anti-complement	0
Indirect antiglobulin test on erythrocyte eluate:	
Anti-IgG	512*
Anti-complement	0
Specificity not demonstrable	

*Reaction expressed as the reciprocal titre.

During a period of about 7 years these were often repeated on fresh samples with essentially similar results. It is clear that his red cells were strongly sensitized with incomplete warm autoantibodies of the IgG class. IgA or IgM antibodies were never detectable. The reaction with anti-complement serum was negative. IgG autoantibodies were, although weakly, also present

in the serum and strongly present in eluates, prepared from the patient's red cells with the Rubin method (1963 The serum and eluate were tested with a panel of donor erythrocytes with various phenotypes of the rhesus system and other blood group systems as well as with red cells with deletions of various rhesus antigens. No specificity of the autoantibodies could be demonstrated.

The results of the ^{51}Cr-survival studies in 1967 are summarized in Fig. 1. At that time all signs of increased haemolysis had already receded. The ^{51}Cr $T_{\frac{1}{2}}$ of the patient's own cells was marginally decreased. A ^{51}Cr $T_{\frac{1}{2}}$ of 21 d was found (normal: 28.2±2.3 s.d.). Organ sequestration indices were normal. Donor red cells labelled with ^{51}Cr, and sensitized with anti-D IgG or damaged by heating, were rapidly eliminated from the blood with a $T_{\frac{1}{2}}$ of respectively 17.3 and 7.5 min, i.e. an initial clearance velocity of respectively 4.0% and 9.3% per minute. Surface counting showed that in both experiments the donor cells were mainly sequestrated in the spleen. These findings proved that in this respect the patient's spleen functioned normally.

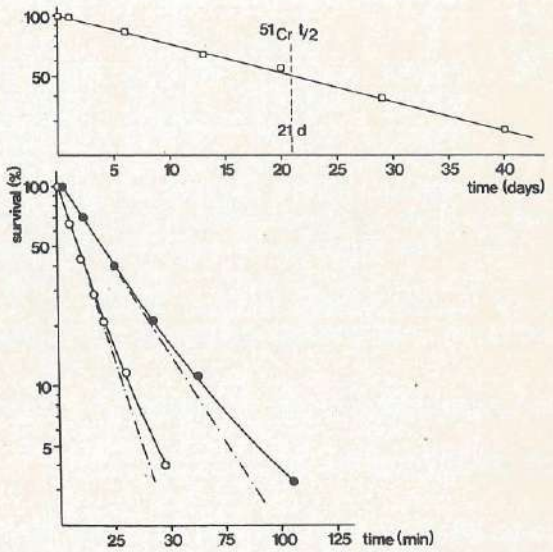


Fig. 1. ^{51}Cr-survival studies in patient van E. □, Patient's own erythrocytes; o, heated donor erythrocytes; ●, anti-D-sensitized donor erythrocytes.

The results obtained with antisera specific for the subclasses of IgG and with light chain-specific antisera in the direct antiglobulin test on patient's red cells during a time span of 7 years are shown in Table III. The tests were performed on the same day on freshly-thawed samples. From these results, it is clear that the IgG autoantibodies, bound to the patient's red cells, consist mainly of IgG4 although IgG1 is also present at lower concentrations and often traces of IgG3. Both light chain types were detectable. The reaction pattern seems rather constant throughout the years.

TABLE III

Direct antiglobulin test on patient van E.'s red cells with IgG subclass and light chain-specific antisera*

	1968†	1970	1971	1972	1974
Anti-IgG	1000/42	1000/43	2000/48	2000/48	2000/40
Anti-IgG1	128/10	512/36	256/19	256/19	256/5
Anti-IgG2	0	0	0	0	0
Anti-IgG3	0	32/16	16/9	16/7	4/1
Anti-IgG4	1000/41	4000/71	2000/48	2000/50	2000/46
Anti-κ	256/37	1000/53	512/44	1000/47	512/37
Anti-λ	256/17	512/47	256/21	512/35	256/18

The scoring system used in Tables III and IV is that described by Race & Sanger, Blood Groups in Man, 2nd edn, p. 275. The degrees of agglutination are recorded as +++, ++, +, (+), ±, and -. These reactions have been given the following values: 10, 8, 5, 3, 2 and 0. The results of a titration expressed as a number are added.

*Reaction expressed as the reciprocal titre and as the score.

†Year of collection of the sample.

Table IV shows the results of cross absorption experiments, performed to prove that the obtained reactions were in fact specific. The results shown were obtained with the 1972 sample. These experiments seem to confirm the specificity of the obtained reaction

TABLE IV

Results of absorptions of the IgG subclass-specific antisera with IgG myeloma proteins of different subclasses*,†

		xIgG1	xIgG2	xIgG3	xIgG4
Anti-IgG1	256/19	0	128/13	128/17	128/17
Anti-IgG2	0	0	0	0	0
Anti-IgG3	16/7	16/3	0	0	0
Anti-IgG4	2000/50	512/23	1000/43	1000/38	16/10

*Reaction expressed as the reciprocal titre and as the score.

†Experiments performed on red cells samples obtained in 1972.

patterns. When eluates of the patient's red cells were tested in the double immunodiffusion test in agar gel with the various IgG subclass-specific antisera, a precipitation line was only obtained with anti-IgG4 which confirms that much more IgG4 was present on the patient's red cells than either IgG1 or IgG3.

The results of the monocyte adherence test are shown in Table V. It is clear from this table that some adherence to monocytes took place. There was some variation in the results obtained with the two different monocyte suspensions. However, rosette formation was weak and the rosettes could easily be disrupted by more vigorous resuspension in contrast to the rosettes obtained with the positive control cells, red cells sensitized with IgG-anti-D.

discussion

It seems very likely that in autoimmune haemolytic anaemia (AIHA), with IgG warm antibodies, the erythrocyte autoantibodies are responsible for the increased red cell destruction. The underlying mechanism of this destruction is, however, not entirely clear. These autoantibodies are not capable of binding complement to the red cells. Binding of the antibodies per se does not lead to damage of the cell, nor does it have any

TABLE V

Adherence (%) to monocytes in vitro of patient van E.'s red cells

		1968	1970	1971	1972	1974	Positive control
Donor 1*	(a)	10	20	3	11	2	70
	(b)	6	2	1	0	0	65
Donor 2*	(a)	0	10	12	10	0	50
	(b)	0	0	0	0	0	54

*Monocytes from two different donors, after careful resuspension (a) or vigorous resuspension (b) (see text).

influence on its metabolism (Borne et al., 1971).

When incubated in vitro with mononuclear phagocytes, however, cells sensitized with IgG antibodies become rapidly spherocytic and subsequently lysis and phagocytosis occurs (LoBuglio et al., 1967; Huber & Fudenberg, 1968, 1969; Holm, 1972; Holm & Hammerström, 1973). It is generally accepted that initiation of these processes takes place by adherence of the sensitized cells to the phagocytes through the so-called Fc-receptor. Adherence in vitro can be strongly inhibited by small amounts of normal IgG. This does not necessarily mean that this process cannot occur in vivo. Pepsin-digested, $F(ab')_2G$, anti-D still binds to rhesus-D-positive cells, but has lost the capacity to bind to monocytes in vitro as well as to eliminate red cells in vivo (Borne et al., 1977). This implies that in vivo the elimination of red cells, sensitized with non-complement binding IgG antibodies, may be effected by similar mechanisms. Apparently, IgG inhibition can be easily overcome in the spleen.

In vitro binding to the monocyte Fc-receptors is only possible with IgG of the subclasses IgG1 and IgG3, not with IgG2 and IgG4 (Huber & Fudenberg, 1968, 1969; Abramson et al., 1970; Abramson & Schur, 1972; Hay et al., 1972; Holm et al., 1974).

In this context our patient is of great interest. This case is an argument for the hypothesis that the

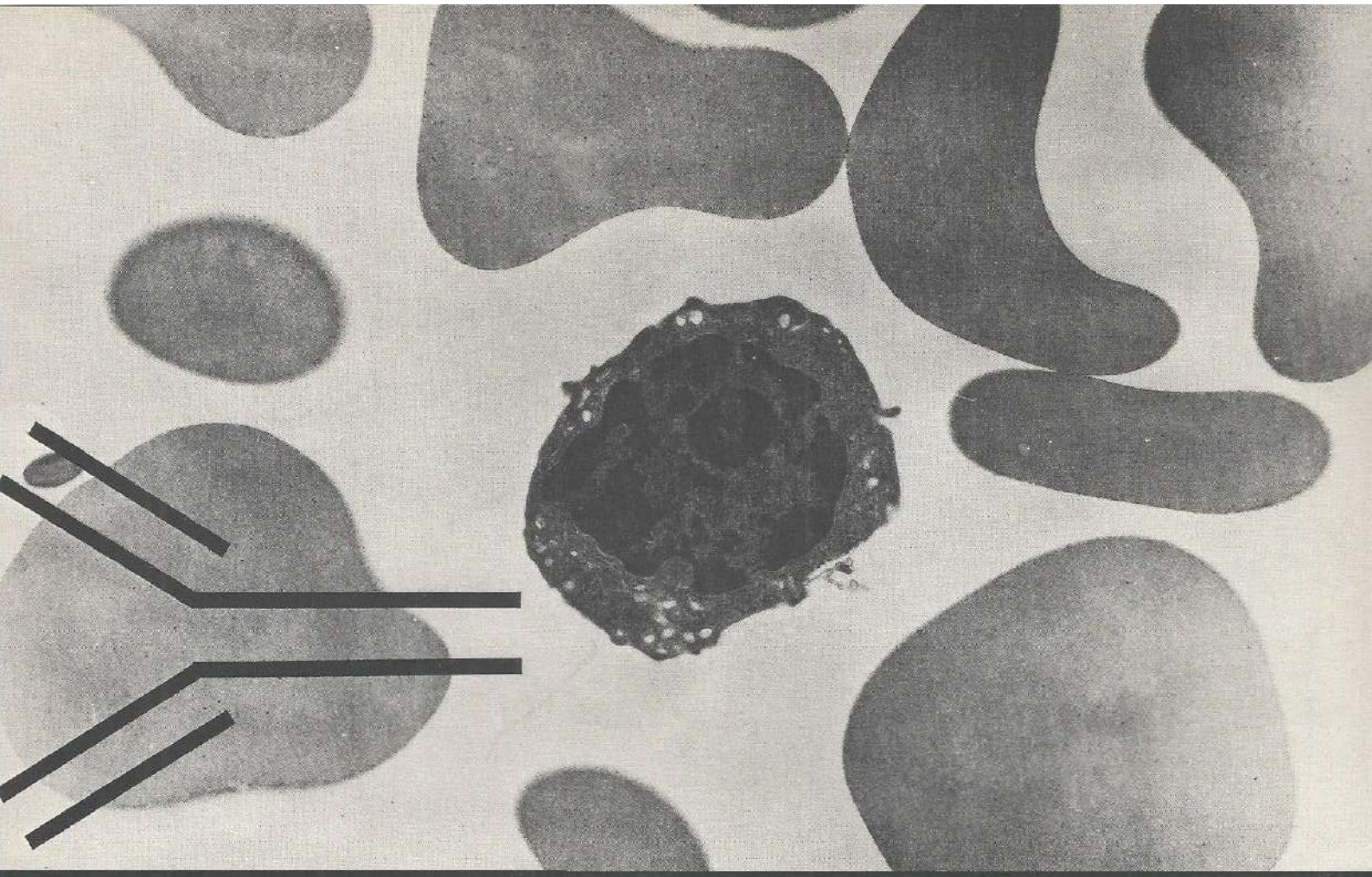
subclass of the IgG autoantibodies is also of importance in vivo. Initially, the patient suffered from AIHA with IgG warm autoantibodies. During prednisone therapy the severe haemolytic anaemia vanished completely. However, strong erythrocyte autoantibodies remained present. The direct antiglobulin test on the patient's erythrocytes was still strongly positive and free IgG warm autoantibodies remained present in the serum. This remained so also after prednisone therapy was stopped. At this stage, the erythrocyte survival time was only slightly decreased, the ^{51}Cr $T_{\frac{1}{2}}$ of his cells was 21 d. This was not due to a dysfunction of the patient's spleen; labelled donor erythrocytes, sensitized with IgG anti-D, or damaged by heating, were eliminated normally. With antisera specific for the various IgG subclasses, it was found that the IgG autoantibodies, present on the patient's red cells, were mainly IgG4. In fact, IgG4 was detectable with the immunoprecipitation technique in ether eluates from the red cells. Apart from IgG4, some IgG1 and a trace of IgG3 was demonstrable on the red cells with the antiglobulin test. Both light chain types were detectable. This immunochemical pattern of the autoantibodies remained remarkably stable during a period of 7 years and during this time the patient remained in a steady clinical state without therapy. He eventually died of a myocardial infarction.

The monocyte adherence test, performed with red cell samples from the patient taken over a period of several years and stored in liquid nitrogen, repeatedly gave weakly positive results. Since there appears to exist a very good correlation between the results of the monocyte adherence test and the occurrence of signs of increased haemolysis (van der Meulen et al., 1978), the above findings are in accordance with the slightly increased red cell turnover. Therefore, it seems probable that the IgG1 autoantibodies and/or the weak IgG3 autoantibodies, which were also present, were responsible for both the slightly increased red cell destruction and the weakly-positive adherence test.

The course of the haemolytic disease in this patient may be explained by a switch in the synthesis of the subclass of the autoantibodies. Probably in the first phase, when haemolysis was manifest, 'active'

IgG autoantibodies were produced, which led to adherence of red cells to macrophages in the spleen and to red cell destruction. A switch to the production of 'inactive' IgG4 autoantibodies drastically changed this situation. Whether this switch occurred spontaneously or was induced by prednisone therapy is not clear.

notes



chapter III
destruction of IgG-sensitized erythrocytes by human blood monocytes. modulation of inhibition by IgG

A. Fleer, F.W. van der Meulen, Els Linthout, A.E.G. Kr. von dem Borne and C.P. Engelfriet

Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands

British Journal of Haematology, 1978, 39,

summary

The in vitro interaction between monocytes and erythrocytes sensitized with non-complement binding IgG antibodies (i.e. the Rh antibody anti-D:EAIgG anti-D) is completely inhibited by low concentrations of IgG (e.g. 30-100 µg/ml). However, the interaction between monocytes and erythrocytes sensitized with IgG anti-A (EAIgG anti-A) is not inhibited by IgG. The findings presented in this paper indicate that this difference is probably due to the difference in the number of IgG antibody molecules per EAIgG. Thus, the higher the number of IgG antibody molecules per EAIgG, the less the interaction between EAIgG and monocytes is inhibited by IgG.

A second factor which proved to have a strong influence on inhibition by IgG was the number of EAIgG per monocyte. When the number of EAIgG per monocyte was increased from 1 to 32, the percentage of inhibition by a fixed amount of IgG (50 µg/ml) decreased significantly. This in vitro effect is only evident when relatively weakly-sensitized erythrocytes are used and, in vivo, destruction of these weakly-sensitized red cells (e.g. EAIgG anti-D) is confined to the spleen. Since a considerable haemoconcentration occurs in this organ, it is conceivable that a high EAIgG:macrophage ratio is accomplished. The latter data are an indication that this high ratio may allow interaction between weakly-sensitized erythrocytes and splenic macrophages despite the presence, in vivo, of a high concentration of IgG, and that, in this way, in the spleen, the inhibitory effect of IgG is overcome.

introduction

Mononuclear phagocytes (monocytes and macrophages) may be responsible for the in vivo destruction of erythrocytes sensitized with incomplete, non-complement binding IgG allo- and autoantibodies in man. Arguments in favour of this assumption have been presented in recent reviews (Brown, 1974; Engelfriet et al., 1974; Logue & Rosse, 1976). However, it was also emphasized in these paper that in vitro the interaction between monocytes and IgG-sensitized red cells (EAIgG) (adherence as well as cytotoxicity and phagocytosis) is easily inhibited by low concentrations of IgG. Thus, it was not easy to understand how the postulated monocyte-mediated mechanism could operate in vivo in the presence of far higher amounts of IgG.

It is well known that in vivo destruction of erythrocytes sensitized with non-complement binding IgG antibodies is nearly always confined to the spleen (Jandl & Kaplan, 1960; Mollison et al., 1965). Jandl et al. (1957) observed that a considerable haemoconcentration occurs in the spleen. This haemoconcentration is probably a decisive factor in making destruction of red cells possible, since LoBuglio et al. (1967) reported that serum no longer inhibited the adherence of EAIgG to monocytes in vitro when the haematocrit of the red cell suspension exceeded 75%. Secondly, Mollison et al. (1965) and Jandl & Kaplan (1960) observed that erythrocytes very heavily sensitized with non-complement binding IgG antibodies could also be destroyed outside the spleen (notably in the liver).

Considering these findings, we decided to study in detail the following variables in vitro: (1) the influence of the number of EAIgG per monocyte on the interaction between monocytes and EAIgG in the presence of a fixed amount of IgG; (2) the effect of the degree of sensitization of red cells on monocyte-EAIgG interaction and the inhibition of this latter process by IgG.

The interaction between monocytes and EAIgG was studied by two assays: adherence of EAIgG to monocytes as well as monocyte-mediated damage of EAIgG (lysis as well as an increase in the osmotic fragility of the red cells).

materials and methods

Sera and immunoglobulin preparations

A rabbit antiserum against human IgG (RAHI) was prepared according to the method described by Van der Giessen et al. (1974). An IgG fraction of this antiserum was produced by DEAE-Sephadex column chromatography. From this IgG fraction $F(ab')_2$ fragments were prepared by pepsin digestion according to the method of Nisonoff et al. (1960). The purity of the $F(ab')_2$ preparation was estimated by means of SDS-polyacrylamide gel electrophoresis and no undigested IgG was detected. Fab fragments were prepared by the papain digestion method of Porter (1959) with the modifications described by Van der Giessen et al. (1974). After separation of the Fab fragments from undigested IgG (Van der Giessen et al., 1974), the former were freed from Fc fragments by carboxymethyl-cellulose-Sephadex chromatography at pH 5.5. The precipitation of human IgG by the starting material (IgG fraction of rabbit anti-human IgG serum) was only inhibited by the material recovered in the first peak. We, therefore, concluded that this peak contained Fab fragments

Human IgG was prepared from normal human plasma by Cohn fractionation. Further purification was carried out by DEAE-Sephadex column chromatography.

Fetal calf serum (FCS) was obtained from Gibco Bio-Cult, Glasgow, Scotland. Anti-D sera were obtained from donors who had been immunized against the Rh (D) antigen by repeated intravenous injections with OR_2r red cells. Anti-A sera were obtained from females who had formed IgG anti-A antibodies during pregnancy. The specificity and titre of the antisera were determined by standard methods. All sera were heat-inactivated for 1 h at 56°C. Purified IgG fractions were prepared from anti-A sera by Sephadex G-200 gel filtration. These fractions were subsequently dialyzed overnight against cold phosphate-buffered saline (PBS).

Preparation of monocytes

50 ml of blood were defibrinated with glass beads and subsequently spun over Ficoll-Isopaque (density = 1.077 g/cm^3 at 20°C). The interface layer which con-

tained only mononuclear cells was washed three times in minimal essential medium (MEM), obtained from Gibco, Grand Island, N.Y., USA, supplemented with 10% (v/v) FCS (MEM 10% FCS). After washing, the mononuclear cells were resuspended in MEM 20% FCS to a final concentration of 10^7 cells/ml. This suspension was layered on plastic Petri dishes (Optilux, Falcon Plastics, Oxnard, Cal., USA), 2.5 ml per dish. After incubation for 1½ h at 37°C, the plates were vigorously washed to remove the non-adherent cells and then the adherent cells were carefully scraped off with a piece of silicone rubber and resuspended in MEM 10% FCS.

Finally, the cells were washed once and the cell concentration was adjusted to 10^6 monocytes/ml. The average percentage of monocytes in the preparations was 71 ± 13 (mean ± SD of 20 donors), as judged by morphology and size distribution (Loos et al., 1976).

Preparation, labelling and sensitization of erythrocytes

A 5% suspension of A_1 or 0 Rhesus-positive (type R_2R_2) erythrocytes from freshly drawn heparinized venous blood was prepared in a storage medium containing 2% BSA. The red cell suspensions were used for 1-2 weeks. Prior to use the red cells were washed three times in PBS and resuspended in MEM 10% FCS to a concentration of $2x10^9$/ml.

For labelling, equal volumes of red cells (10^9/ml) and a $Na_2{}^{51}CrO_4$ solution (Radiochemical Centre, Amersham, U.K., specific activity of 50-400 mCi/mg Cr) were mixed and incubated for 1-2 h at 37°C. The erythrocytes were then washed three times in MEM 10% FCS and subsequently resuspended to a concentration of 1-$32x10^6$ cells/ml).

Red cells were sensitized by mixing them ($2x10^9$/ml) with an equal volume of undiluted anti-D serum. Subsequently, they were incubated with ^{51}Cr and treated exactly as described above. OR_2R_2 red cells already sensitized with anti-D, were further treated with the IgG fraction of rabbit anti-human IgG serum after labelling of the red cells with ^{51}Cr. Cells that had been both anti-D-sensitized and ^{51}Cr-labelled red cells (10^9/ml) were incubated with dilutions of RAHI (in MEM 10% FCS) for 30 min at 37°C. Subsequently, the cells were washed three times in MEM 10% FCS and adjusted to a concentration of 1-$32x10^6$ cells/ml.

Red cells were treated with papain by incubating packed cells with an 1% solution (Sigma Chem. Co., St. Louis, Mo., USA) for 10 min at 37°C. The cells were washed three times in saline and the red cell concentration was made up to $2x10^9$ cells/ml. Subsequently, sensitization with anti-D and labelling of the red cells was performed as previously described.

Cytotoxicity assay

This was performed as described by Zeijlemaker et al. (1975). Briefly, 50 µl of monocyte suspension (10^6/ml), 50 µl of erythrocyte suspension (1-$32x10^6$/ml) and 50 µl of antiserum dilution or 50 µl of MEM 10% FCS, were added to wells of Cooke round-bottom microtitre plates. After incubation for 16 h at 37°C, the plates were centrifuged at 500 x g for 10 min at room temperature. A sample of 100 µl was taken from the supernatant of each well to determine the percentage of ^{51}Cr-release. Specific cytotoxicity (cytotoxic lysis or lysis are used as synonyms) was calculated as follows:

$$\text{Specific cytotoxicity} = \frac{E - S}{\text{total cmp}} \times 100\%$$

in which E is experimental release (monocytes with sensitized erythrocytes), S is spontaneous release (monocytes with non-sensitized erythrocytes) and total cpm (counts per minute) the total amount of radioactivity per 50 µl red cell suspension. Each experiment was performed in triplicate or quadruplicate. From the percentage of specific cytotoxicity the absolute number of erythrocytes lysed was calculated by multiplying this percentage with the number of erythrocytes per well.

Determination of monocyte-induced increase in the osmotic fragility of red cells

An increase in the osmotic fragility of the red cells was determined by the following procedure: 50 µl of monocyte suspension (10^6 cells/ml), 50 µl of anti-D-sensitized red cells (10^6 to $32x10^6$ cells/ml) and 50

µl of MEM 10% FCS were added to wells of Cooke round-bottom microtitre plates. After incubation for 16 h at 37°C, the plates were centrifuged and a 100 µl sample was taken from the supernatant to determine ^{51}Cr-release. From this, the percentage of specific cytotoxicity was calculated (see Cytotoxicity assay). Subsequently, the remaining supernatant was carefully removed and 200 µl of a buffered 0.5% NaCl solution was added to the wells. The cell pellets were stirred and incubated for 30 min at room temperature. Thereafter, the plates were centrifuged again and a 100 µl sample was taken from the supernatant to determine the ^{51}Cr-release induced by this hypotonic shock. The percentage increase in the osmotic fragility due to the incubation with monocytes was calculated as follows: percentage ^{51}Cr-release by 0.5% NaCl from EAIgG anti-D incubated with monocytes minus percentage ^{51}Cr-release of EAIgG incubated without monocytes. The number of red cells specifically lysed by 0.5% NaCl was calculated by multiplying this figure with the number of red cells added to the wells. The total number of red cells damaged by monocytes was defined as the sum of the number of EAIgG lysed by monocytes (cytotoxic lysis) plus the number of EAIgG which had led to the increase in the osmotic fragility.

Adherence assay

To determine the number of monocytes that bound sensitized erythrocytes, 100 µl of monocyte suspension (2.5×10^6/ml) were mixed with 50 µl of anti-D-sensitized erythrocyte suspension (concentration: 2×10^7 to 10^8/ml). 150 µl MEM 10% FCS were added to the monocyte-erythrocyte mixture and after mixing the suspension was centrifuged at 150 x g for 10 min at room temperature. The pellet was carefully resuspended and incubated for 1 min at room temperature with 25 µl of a solution containing 4 µg/ml of acridine orange. The percentage of rosette-forming monocytes (those which had bound three or more erythrocytes) was determined by fluorescent microscopy.

Determination of the amount of IgG on sensitized red cells by radioactive protein A binding assay

Protein A was purchased from Pharmacia, Uppsala, Sweden. It was labelled with ^{125}I by chloramine T method described by Dorval et al. (1974). The resultant specific activity was 75.000 cpm/µg protein A. 10-50 µl of the ^{125}I-labelled protein A solution (concentration of protein A: 1.2 µg/µl) were incubated with 50 µl of red cell suspensions (5×10^6 erythrocytes) for 20 min at room temperature. After incubation, the red cells were washed three times in PBS + 2% (v/v) BSA. Three washings were found to be sufficient to remove unbound radioactivity. The red cell pellets were subsequently resuspended in 50 µl PBS + 2% BSA and transferred to fresh tubes. The radioactivity and the red cell numbers were counted and expressed as cpm/10^6 erythrocytes. The tests were performed in duplicate.

results

Inhibition by IgG of lysis of EAIgG by monocytes

From Fig. 1 it can be seen that the lysis of EAIgG anti-D by monocytes was strongly inhibited by IgG. However, Fig. 2 shows that IgG hardly inhibited the lysis of human red cells sensitized with IgG anti-A by monocytes except when IgG anti-A was used in a very low concentration (12.5 µg/ml). Even then, a high concentration of IgG (about 1 mg/ml) was necessary to achieve 50% inhibition. When anti-D-sensitized red cells were used as target cells, 50% inhibition was obtained with 0.1-30 µg/ml of IgG (range of experiments with monocytes from 20 donors).

These findings suggested that the number of IgG antibody molecules per red cell was probably important in determining the degree of inhibition by IgG. Group A_1 red cells have about 10^6 A_1 antigen sites (Cartron et al., 1974; Economidou et al., 1967; Williams & Voak, 1972) and these can bind more antibody than homozygous Rh(D)-positive erythrocytes which have only 30.000 D antigen sites (Masouredis et al., 1976; Rochna & Hughes-Jones, 1965). To test this hypothesis, we increased the number of IgG antibody molecules on anti-D-sensitized red cells by

further treatment with rabbit anti-human IgG.

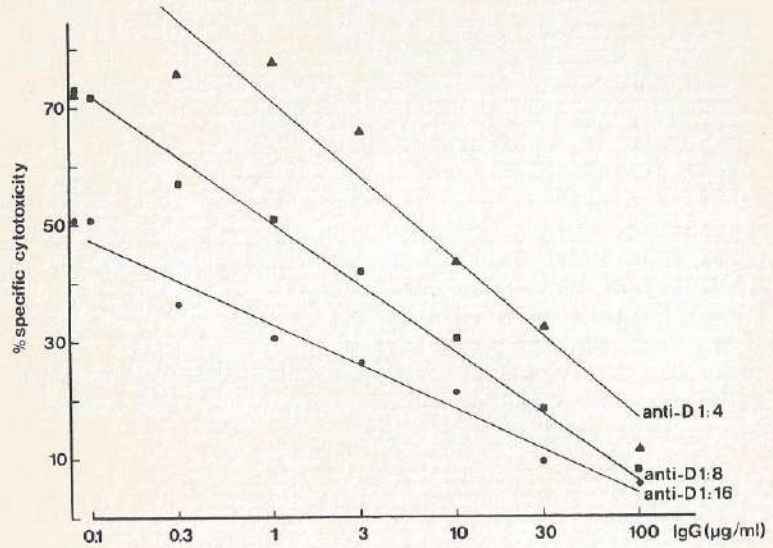


Fig. 1 - Inhibition by IgG of lysis of EAIgG anti-D by monocytes. Ordinate: % specific cytotoxic lysis. Abscissa: IgG concentration. Control values (lysis in the absence of IgG) are shown directly adjacent to the ordinate. Results of a representative experiment are shown. ▲, EAIgG anti-D 1:4; ■, EAIgG anti-D 1:8; ●, EAIgG anti-D 1:16.

Effect of number of IgG antibody molecules per EAIgG on inhibition by IgG of lysis of EAIgG by monocytes

In order to quantitate the increase in the number of IgG molecules after red cells sensitized with anti-D were treated with rabbit anti-human IgG (RAHI), we used a radiolabelled protein A binding assay, recently described by Dorval et al. (1974). The binding of the protein to various types of EAIgG anti-D used in the experiments described in this paper is shown in Table I. From this, it can be seen that treatment of EAIgG anti-D with RAHI IgG led to in a significant enhancement of protein A binding ($0.05>p>0.025$ for RAHI 1 µg/ml; $0.005>p>0.001$ for RAHI 10 µg/ml; t-test for paired observations, one-tailed). Moreover, EAIgG anti-D-treated with RAHI in a final concentration of 10 µg/ml bind significantly more protein A than those treated with RAHI in a final concentration of 1 µg/ml ($0.025>p>0.01$; t-test for paired observations, one-tailed).

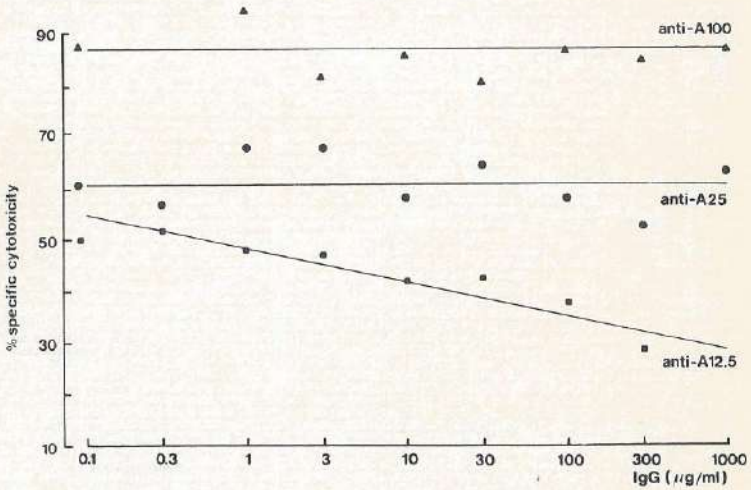


Fig. 2 - Effect of IgG on lysis of EAIgG anti-A by monocytes. Ordinate: % specific cytotoxic lysis. Abscissa: IgG concentration. Lysis in the absence of IgG is shown directly adjacent to the ordinate. Results of a representative experiment are shown. ▲, Anti-A 100 µg/ml; ●, anti-A 25 µg/ml; ■, anti-A 12.5 µg/ml.

TABLE I

The specific binding of ^{125}I-labelled protein A to red cells sensitized either with anti-D alone (EAIgG anti-D) or with anti-D and rabbit anti-human IgG (RAHI)*:

EAIgG anti-D	700 ± 145	(n=10)
EAIgG anti-D + RAHI (1 µg/ml)	1523 ± 517	(n=10)
EAIgG anti-D + RAHI (10 µg/ml)	3280 ± 800	(n=10)

*Assessed by subtracting the radioactivity bound by non-sensitized red cells from the radioactivity bound by sensitized red cells and expressed as cpm/10^6 red cells (mean ± SEM).

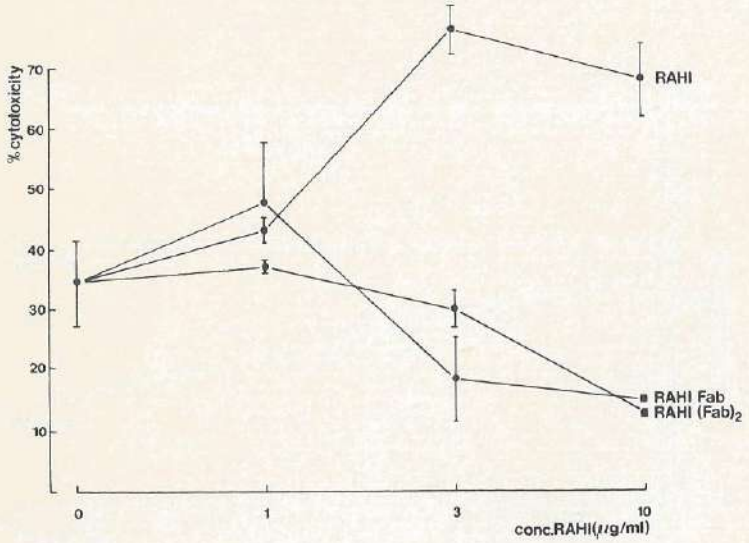


Fig. 3 - Effect of treatment of EAIgG anti-D with the IgG fraction of rabbit anti-human IgG (RAHI) or its $F(ab')_2$ and Fab fragments on lysis of EAIgG by monocytes. EAIgG anti-D were sensitized with RAHI IgG or its $F(ab')_2$ or Fab fragments in final concentrations indicated on the abscissa. Ordinate: % specific cytotoxic lysis. Mean ± SEM of experiments with monocytes from five donors.

The effect of treatment of EAIgG anti-D with RAHI IgG or its $F(ab')_2$ and Fab fragments on the lysis of these red cells by monocytes is shown in Fig. 3. From this, it is evident that, although RAHI IgG treatment enhances monocyte-mediated lysis of these red cells, the Fab and $F(ab')_2$ fragments have just the opposite effect, presumably because they cover the Fc parts of the IgG anti-D molecules on the red cell without adding their own Fc fragment. Support for the latter comes from the experiments with protein A, because the Fab and $F(ab')_2$ fragments of RAHI IgG in final concentrations of 10 µg/ml inhibited the binding of protein A by anti-D-sensitized red cells (data not shown).

The effect of treatment of EAIgG anti-D with RAHI IgG on the inhibition of monocyte-mediated lysis of these red cells by IgG is shown in Fig. 4. As already mentioned, only low concentrations of IgG were needed to achieve 50% inhibition of lysis of EAIgG anti-D by monocytes. However, when EAIgG were treated with 1 µg/

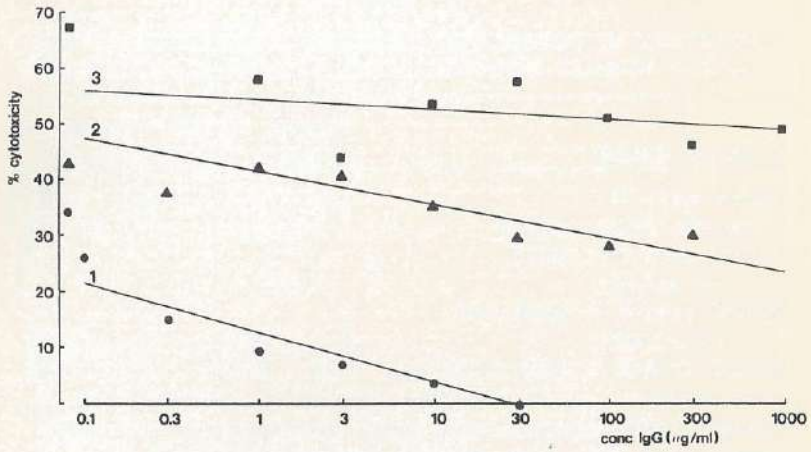


Fig. 4 - Inhibition by IgG of monocyte-mediated lysis of EAIgG anti-D. Effect of treatment of EAIgG anti-D with rabbit anti-human IgG (RAHI). Ordinate: % specific cytotoxic lysis. Abscissa: IgG concentration. Specific cytotoxic lysis in absence of IgG is shown directly adjacent to the ordinate. Results of a representative experiment are shown. ■, EAIgG anti-D + RAHI (10 µg/ml); ▲, EAIgG anti-D + RAHI (1 µg/ml); ●, EAIgG anti-D.

ml RAHI IgG, more than 1 mg/ml of IgG was required to achieve the same inhibition and when 10 µg/ml RAHI IgG was used, the inhibition was virtually abolished.

Apart from increasing the number of IgG antibody molecules on EAIgG anti-D, RAHI has also been reported to induce clustering of the Rh(D) antigens which are normally homogeneously dispersed over the red cell membrane (Masouredis et al., 1976; Victoria et al., (1975). To investigate whether this was primarily responsible for the observed effects of RAHI on IgG inhibition, we studied the effect of papain treatment of red cells because such treatment also leads to clustering of the Rh(D) antigens by anti-D (Victoria et al., 1975) without an appreciable increase in the number of IgG antibody molecules bound to the erythrocyte (Hughes-Jones et al., 1964; Masouredis, 1962; Victoria et al., 1975), at least not to the same extent as treatment with RAHI IgG. The results of these experiments are shown in Table II.

TABLE II

Inhibition by normal IgG of monocyte-mediated cytotoxicity towards IgG-sensitized erythrocytes (EAIgG)

Type of EAIgG	Amount of IgG needed to achieve 50% inhibition	
EAIgG anti-D	1.0 ± 0.5	(0.1-2.8)*
E papain AIgG anti-D	2.4 ± 1.4	(0.2-6.0)
EAIgG anti-D + RAHI (10 µg/ml)	>1000	
EAIgG anti-D + RAHI Fab (10 µg/ml)	0.3 ± 0.2	(0.1-0.8)
EAIgG anti-D + RAHI $F(ab')_2$	0.7 ± 0.3	(0.2-1.1)

*Mean ± SEM (µg/ml), and range of experiments with monocytes from five donors (in parentheses).

They clearly indicate that the effect of RAHI on IgG inhibition was very much stronger than that of papain treatment of red cells. The results of treating EAIgG anti-D with the Fab and $F(ab')_2$ fragments of RAHI IgG are also shown. These fragments had no effect on, or even enhanced the ability of, IgG to inhibit the lysis of anti-D-sensitized red cells by monocytes.

Effect of number of EAIgG per monocyte on inhibition by IgG of lysis of EAIgG by monocytes

A fixed number of monocytes ($5x10^4$ per well) were added to increasing numbers of EAIgG anti-D so that EAIgG:monocyte ratios of 1:1 to 32:1 resulted. The left-hand figure in Fig. 5 shows that although lysis is inhibited in the presence of IgG (50 µg/ml), it is less at higher EAIgG:monocyte ratios, since a significant negative correlation was found between the percentage of inhibition of lysis by IgG and the EAIgG:monocyte ratio ($r = -0.983$; $p<0.001$).

The same phenomenon (decrease in inhibition by

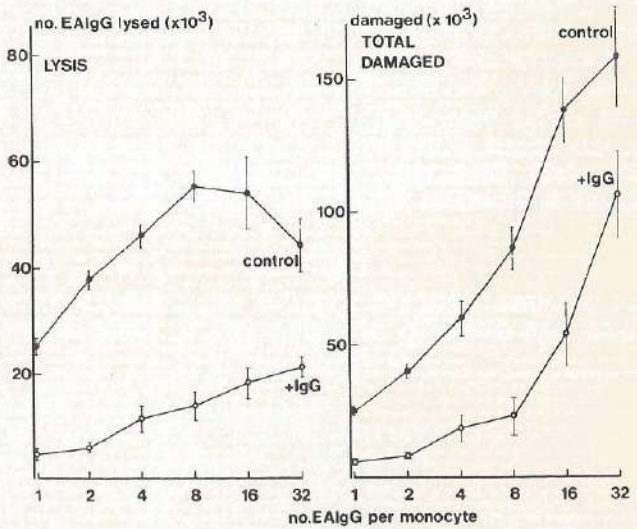


Fig. 5 - Inhibition by IgG of monocyte-mediated lysis of EAIgG anti-D. Effect of variation in the EAIgG: monocyte ratio. Abscissa: EAIgG:monocyte ratio. Ordinate: number of EAIgG specifically lysed ($x10^3$, left) or damaged ($x10^3$, right). Mean ± SEM of experiments with monocytes from five donors.

IgG with increasing number of EAIgG per monocyte) was seen when the total number of EAIgG damaged by monocytes was studied (by applying a hypotonic shock to

the red cells after incubation with or without monocytes; see Matrials and Methods). This procedure gives a better estimate of the total number of EAIgG processed by monocytes than cytotoxic lysis, since it also includes those red cells which have an increased osmotic fragility (manuscript in preparation). The right-hand figure in Fig. 5 shows that there was a significant negative correlation between inhibition by IgG and the number of EAIgG per monocyte ($r = -0.974$; $p = 0.001$).

A similar competition between EAIgG and IgG was seen in the first step of the interaction between EAIgG and monocyte, namely the adherence of EAIgG to monocytes. Table III shows that the percentage of rosette-forming monocytes decreases in the presence of IgG (20 µg/ml) while the percentage of the same cells increased with increasing red cell concentration, both in the presence and in the absence of IgG. Table III also shows that, in the absence of IgG, 50% of the monocytes would bind EAIgG when the red cell concentration was about 20×10^6/ml, while in the presence of IgG (20 µg/ml) this level was reached at a red cell concentration between 50×10^6 and 100×10^6/ml.

TABLE III

Relation between concentration of sensitized red cells (EAIgG) and monocyte Fc adherence in presence or absence of IgG

	Concentration of EAIgG		
	20×10^6/ml (4:1)*	50×10^6/ml (10:1)	100×10^6/ml (20:1)
% Rosette-forming monocytes in the absence of IgG	47 ± 2†	74 ± 4	87 ± 2
% Rosette-forming monocytes in the presence of IgG (20 µg/ml)	26 ± 2	42 ± 3	54 ± 3

* EAIgG:monocyte ratio
† Mean ± SEM (experiments with monocytes from 5 donors)

discussion

The findings presented in this paper show that there are at least two factors that determine the degree of inhibition by IgG on the interaction between human mononuclear phagocytes and IgG-sensitized red cells. The first is the number of IgG antibody molecules per erythrocyte. The importance of this factor was first suggested by the fact that the lysis of EAIgG anti-A by monocytes was not inhibited by IgG (except at low concentration of IgG anti-A), while the lysis of EAIgG anti-D was easily inhibited by IgG, even when red cells were optimally sensitized. It has been amply demonstrated that suitable red cells can bind considerably more IgG anti-A antibody molecules than Rh antibodies. Additional evidence for this was obtained by using red cells that had been treated with rabbit anti-human IgG after sensitization with anti-D. According to the literature, one molecule of human IgG binds 7 to 9 molecules of rabbit anti-human IgG (Constantoulakis et al., 1963; Costea et al., 1962; Kabat & Mayer, 1961; Rochna & Hugh-Jones, 1965). Our own data, obtained from the ^{125}I-labelled protein-A binding assay (Dorval et al., 1974), are somewhat lower than this figure. Treatment of anti-D-sensitized erythrocytes with RAHI IgG increased the binding of protein A to these red cells from 2.2 (RAHI concentration: 1 µg/ml) to 4.7 times (RAHI concentration: 10 µg/ml). However, these figures probably underestimated the real increase for a number of reasons. Ghetie et al. (1974) reported that the protein A:IgG combining ratio varied from 2:1 to 1:2. With the D-antigen on untreated red cells, it is unlikely that the small protein A molecule could bridge the relatively large distance between two anti-D molecules but when the antigen-site is clustered by treatment with RAHI, it is possible that the same A molecule could attach to two (rabbit) IgG molecules. In other words, it is probable that the increase in protein A molecules would be less than the increase in IgG antibody molecules. Furthermore, there are two papers in which it was reported that rabbit IgG binds less protein A than human IgG (Biberfeld et al., 1975; Kronvall, 1973).

In view of these findings, it seems reasonable to assume that treatment of anti-D-sensitized red cells

with RAHI results, in our hands, in an increase in the number of IgG antibody molecules per red cell of at least 5 times (RAHI concentration: 1 µg/ml) to 10 times (RAHI concentration: 10 µg/ml) which figures are comparable to the 7-9 times increase mentioned above. The treatment of anti-D-sensitized red cells with RAHI IgG considerably reduced the effect of IgG on the lysis of EAIgG by monocytes. Treatment of EAIgG anti-D with RAHI in a final concentration of 10 µg/ml completely abolished the inhibitory effect of IgG and was up to 1 mg/ml not able to prevent the interaction between monocytes and strongly sensitized erythrocytes, presumably because these red cells could compete more effectively with free IgG molecules for the IgG binding sites on the monocyte membrane (the so-called Fc receptors).

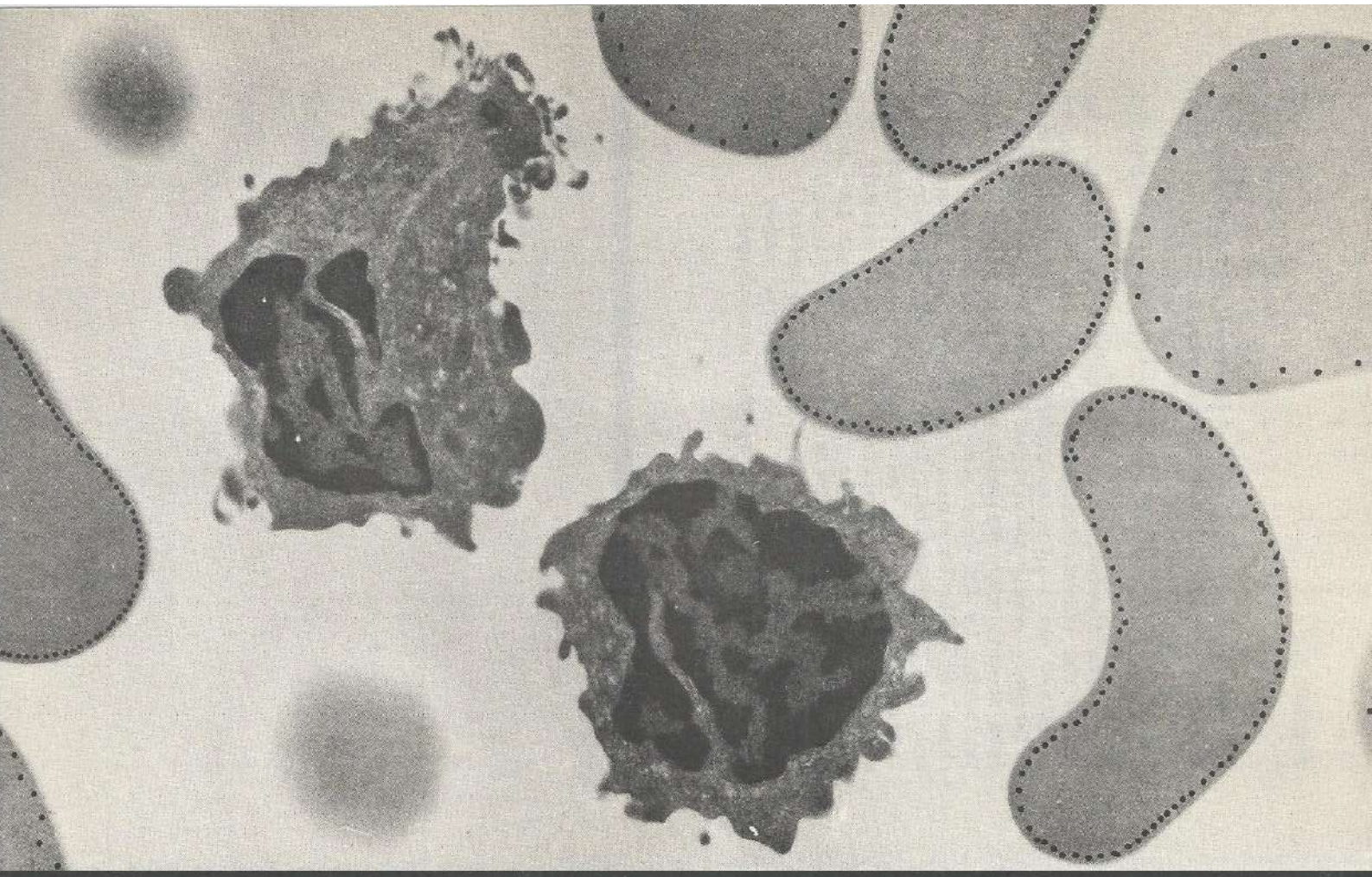
The importance of the number of IgG molecules per cell possibly explains why in vivo destruction of strongly sensitized red cells is not confined to the spleen, while the destruction of weakly sensitized red cells is (Constantoulakis et al., 1963; Jandl & Kaplan, 1960; Mollison et al., 1965). However, in theory, mononuclear phagocytes should be incapable of destroying weakly sensitized red cells at all, because the IgG levels of the plasma and extracellular fluid should completely inhibit the interaction between them. If the destruction is due to this cause, the effect of free IgG is somewhat neutralized.

One of the most important factors which makes this destruction possible in the spleen, is probably the marked haemoconcentration which occurs in this organ (Jandl et al., 1957). The findings of LoBuglio et al. (1967) are in favour of this theory. They found that when monocytes were incubated in vitro with mixtures of anti-D-sensitized red cells and serum, inhibition by serum of the binding of these cells to monocytes was decreased when the ratio of red cells to serum was increased. From their experiments, it was not clear, however, whether this decrease was solely due to the increase in the haematocrit or whether the concomitant decrease in the serum content of the mixture was the most important factor. For this reason, we performed experiments in which the concentration of monocytes and IgG was kept constant and the concentration of erythrocytes was varied. The findings confirmed those of LoBuglio et al. (1967) and again emphasize the competition that occurs between free IgG molecules and EAIgG

for the IgG binding sites on the monocyte membrane. When the proportion of EAIgG per monocyte is increased, binding and destruction of EAIgG are both increased, even in the presence of free IgG molecules, and inhibition by IgG is decreased.

In conclusion, it can be stated that inhibition by IgG of the interaction between monocytes and IgG-sensitized red cells in vitro occurs when the red cells are relatively weakly sensitized (such as with Rh antibodies or with non-complement binding IgG autoantibodies). Inhibition by IgG decreases when the concentration of these red cells increases relative to that of monocytes and free IgG molecules. Therefore, the haemoconcentration, which occurs in the spleen, may promote binding and destruction of sensitized red cells by splenic mononuclear phagocytes in two ways (although IgG is present in a high concentration): (1) it decreases the amount of plasma, thus lowering the concentration of free IgG molecules; (2) it increases the number of red cells relative to that of splenic macrophages.

notes



chapter IV
quantitative aspects of the destruction of red cells sensitized with IgG1 auto antibodies. an application of flow cytofluorometry

F.W. van der Meulen, H.G. de Bruin, P.C.M. Goosen, E.C.E. Bruynes, C.J. Joustra-Maas, H.G. Telkamp, A.E.G. Kr. von dem Borne and C.P. Engelfriet

Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, the Laboratory of Experimental and Clinical Immunology, and the Department of Haematology, University Clinic of Internal Medicine, Binnengasthuis, University of Amsterdam, Amsterdam, The Netherlands.

summary

The purpose of this study was to determine whether quantitative or qualitative factors are of major importance in the destruction of red cells sensitized with incomplete warm autoantibodies of subclass IgG1.

To that end, the relative amount of IgG1 antibody present on the red cells of patients with autoantibodies of this subclass only, was measured by means of continuous flow cytofluorometry. This technique appeared to be suitable to compare amounts of antibody on red cells and gave reproducible results. The fluorescence intensity of the patients' red cells, obtained after incubation with an FITC-labelled anti-IgG1 serum, was studied in relation to the presence or absence of signs of increased haemolysis in vivo and in relation to the cytotoxic activity of normal monocytes towards these red cells in vitro.

It appeared that it was predominantly the amount of IgG1 autoantibody rather than its quality, that determined whether or not these antibodies induced haemolysis in vivo or cytotoxic activity of monocytes in vitro. This also was true with regard to methyldopa-induced IgG1 autoantibodies.

introduction

Strong evidence is available that red cells are destroyed in vivo under the influence of non-complement binding IgG antibodies as a result of adherence to 'Fc receptors' on phagocytic cells (Brown, 1974; Engelfriet et al., 1974; Logue & Rosse, 1976, Borne et al., 1977a; Meulen et al., 1978). Thus, since only IgG1 and IgG3 are capable of adherence to mononuclear phagocytes, the subclass composition of IgG non-complement binding autoantibodies is of great importance in relation to their clinical significance.

Previously, we have studied the relation between the subclass of IgG incomplete warm autoantibodies and the presence or absence of increased red cell destruction in vivo (Engelfriet et al., 1974; Borne et al., 1977b; Meulen et al., 1978). We found that IgG2 or IgG4 autoantibodies are indeed not associated with haemolytic anaemia, whereas evidence of a shortened red cell lifespan is present in nearly all patients on whose red cells IgG3 is detectable.

Sensizitization with IgG1 autoantibodies alone -which occurs in the vast majority of patients- leads to increased destruction of red cells in some of these patients, but not in others. No clear relation was found between the titre of the direct antiglobulin test (DAGT) with anti-IgG1 serum and the presence or absence of signs of increased haemolysis in vivo. We have interpreted this -incorrectly as will be shown in this paper- as being due to a qualitative difference between IgG1 molecules, either capable or not of reacting with the 'Fc receptor'.

Since the titre of the antiglobulin test is fully inadequate for the quantification of antibody (Chaplin, 1973) and since a much better method for determining the number of antibodies bound on a red cell has recently become available, we have re-examined this problem. The relative number of antibody molecules bound on cells can be accurately measured by means of continuous flow cytofluorometry with fluorescein isothiocyanate (FITC)-labelled antiglobulin reagents (Herzenberg as quoted by Greaves, 1975; Borne et al., 1978; Verheugt et al., in press; Aaij et al., 1978). Thus, the relative number of IgG1 antibodies on the red cells of a number of patients with IgG1 incomplete warm autoantibodies only, could be established by determining the intensity of the fluorescence obtained with an FITC-labelled anti-IgG1 serum. The relation between the intensity of the fluorescence and the presence or absence of overt haemolytic anaemia can be studied. The intensity of the fluorescence obtained with patients' red cells can also be studied in relation to in vitro damage of these cells by adherence to peripheral blood monocytes of healthy volunteers (Fleer et al., in press[b]).

In this paper, it will be shown that the actual amount of IgG1 autoantibody is a critically important factor, which determines whether increased haemolysis in vivo as well as damage of red cells by monocytes in vitro occurs.

materials and methods

Erythrocytes

Erythrocytes were studied from patients with exclusively IgG1 incomplete, non-complement binding warm autoantibodies. None of the patients received corticosteroid therapy at the time of their blood donation. The subclass of the IgG antibodies was determined in the DAGT with subclass-specific antisera.

The red cells of 30% of the patients were also agglutinated by anti-complement serum. Using specific reagents, it could be established that C3d was present on these cells, but not C4c or C3c. No specific anti-C4d serum was available for the detection of C4d. Red cells were stored in liquid nitrogen. After thawing, the erythrocytes were washed in phosphate (5.8 mM)-buffered saline (140 mM NaCl) (PBS) and suspended in the required medium (vide infra).

Serological techniques

The DAGT was performed as previously described (Engelfriet et al., 1968).

The titration score was calculated by evaluation of the degree of agglutination, which was expressed as a number, and by adding up the figures for each tube (Dacie, 1956).

Preparation of FITC-labelled antiserum

The IgG fraction of a specific rabbit-anti-human IgG serum was isolated by Sephadex DEAE-A50 chromatography after precipitation with 40% ammonium sulphate. This fraction was then digested with papain (1% w/w). The F(ab) fragments were purified by CM-Sephadex column-chromatography and labelled with FITC (The, 1970). A fluorescein/protein (F/P) ratio of 1 was obtained. F(ab) fragments were used instead of total IgG to avoid agglutination of red cells in the cuvette of the cytofluorograph. However, it appeared later on that the agglutinates could be easily disrupted by resuspension and, therefore, no difficulties were encountered when papain treatment was omitted. For this reason the anti-IgG1 serum (see below) was not hydrolysed.

An anti-IgG1 serum was prepared in rabbits by immunization with H-chains of purified IgG1 myeloma proteins and it was made subclass-specific by absorption with IgG2, IgG3 and IgG4 myeloma proteins (van der Giessen, 1974). The anti-IgG1 antibodies were isolated by binding to, and elution from, a Sepharose 4B column, which carried IgG1 myeloma proteins. The antibodies thus obtained were conjugated to FITC with a final F/P ratio of 2.1.

Preparation of red cells for cytofluorometric measurement

Erythrocytes were washed three times in PBS. Thereafter, 10^6 red cells were suspended in 0.075 ml of a ten-fold dilution in PBS of anti-IgG serum or of anti-IgG1 serum. After 30 min incubation at room temperature, the cells were washed twice, resuspended in PBS to a final concentration of 10^6/ml and transferred to the cytofluorograph.

The fluorescence of rh D-positive erythrocytes (OR_2R_2), sensitized in vitro (1 h, 37°C, final serum dilution 1:4) with IgG anti-D alloantibodies (EAIgG) from donor G, who was immunized with OR_2r red cells, served as a positive control. This anti-D serum (titre 1:2000 in the indirect antiglobulin test with anti-IgG serum) was selected for this study because of its high content of IgG1 antibodies.

Cytofluorometric measurement

The fluorescence of the red cells was determined in a Biophysics Cytofluorograph, model 4180A-FC200. This continuous flow microfluorometer, in conjunction with a Biophysics 2100 Distribution Analyzer (100 channels), recorded both pulse height, i.e. the fluorescence intensity per cell (as a channel-index value) and the number of cells causing a certain pulse height. From these results, the relative fluorescence intensity (RFI) of the whole cell population could be calculated by dividing the sum of the products of the cells per channel times the channel-index number by the total number of cells registered (10,000 cells) (Verheugt et al., in press; Borne et al., 1978; Aaij et al., 1978).

Every fourth measurement was followed by the recording of the standard sensitized erythrocyte preparation (the positive control) to account for the drift in the apparatus. As a negative control, the RFI of the same non-sensitized red cells (OR_2R_2) was determined. The results of the measurements presented in this paper are expressed as percentage fluorescence (= relative fluorescence) and calculated as follows:

$$\text{percentage fluorescence} = \frac{RFI_{\text{patients' red cells}} - RFI_{\text{neg.control}}}{RFI_{\text{positive control EAIgG}} - RFI_{\text{neg.control}}} \times 100\%$$

In this way, the relative amount of antibody present on red cells could be compared. The storage of red cells in liquid nitrogen caused no detectable change in the cytofluorometrically determined amount of cell-bound antibody.

Preparation of monocytes

Cryopreserved monocytes of two healthy human volunteers were used. These cells were isolated from the buffy coat of 500 ml citrated venous blood. From the mononuclear leukocyte suspension, obtained by Ficoll-Isopaque density gradient centrifugation (d=1.077 g/cm^3) monocytes were further purified by adherence to glass petri dishes at 37°C for 90 min. The monolayer was washed twice and scraped off with a piece of silicone

rubber. The suspension thus obtained contained 70-80% monocytes, the remainder being lymphocytes and less than 3% granulocytes as determined by morphologic differentiation and electronic sizing with a Coulter counter, model ZF, supplemented with a pulse height analyzer (Channelyzer, model C-1000) (Loos et al., 1976).

Monocytes were frozen in minimal essential medium (Gibco, Paisley, Scotland), buffered with Tris 0.025 M (pH 7.4) (MEM-Tris), which contained 20% fetal calf serum (FCS) (Gibco, Biocult, Paisley, Scotland) and 10% dimethyl sulfoxide (DMSO) (Baker Chemicals B.V., Deventer, The Netherlands) as cryoprotectant. Cooling was performed by means of a controlled rate cooling programme as described elsewhere (du Bois et al., 1976) and the cells were stored in liquid nitrogen. After thawing, the monocytes were washed and resuspended in the required media.

The capacity of monocytes to lyse red cells sensitized with incomplete anti-D alloantibodies was unchanged by this procedure (van der Meulen, 1977).

Cytotoxic assay

Lysis of sensitized red cells by monocytes was determined as described by Fleer et al. (in press[a]). Briefly, patients' red cells were labelled with $Na_2Cr^{51}O_4$ (Radiochemical Centre, Amersham, UK) and incubated with monocytes in wells of microtiter plates (Greiner, Alphen a/d Rijn, The Netherlands). After incubation for 16 h at 37°C, the plates were centrifuged and the percentage of chromium^{51} release was determined in a sample of the supernatant. After correction for the spontaneous Cr^{51}-release the number of red cells lysed by the monocytes could be calculated.

The cytotoxicity of monocytes towards red cells sensitized in vitro (1 h, 37°C, final serum dilution 1:4) with IgG anti-D alloantibodies (EAIgG) of donor A served as a positive control. This anti-D serum (titre 1:2000 in the indirect antiglobulin test with anti-IgG serum) was selected for its induction of a strong cytotoxic activity.

The percentages of cytotoxicity were calculated as follows:

percentage cytotoxicity =

$$\frac{\text{number of patients' red cells lysed}}{\text{number of EAIgG lysed}} \times 100\%$$

Non-sensitized red cells were not lysed by monocytes. All measurements were performed in duplicate. The storage of red cells in liquid nitrogen did not alter the cytotoxic action of the monocytes towards these cells.

Criteria for haemolysis in vivo

At least four of the following parameters were used: blood haemoglobin level, or haematocrit or erythrocyte count (as a measure for the degree of anaemia); erythrocyte osmotic fragility (as a measure for red cell damage); serum haptoglobin level, serum LDH activity, serum bilirubin level, or the half life, determined by using ^{51}Cr-labelled red cells ($^{51}Cr\ T_{\frac{1}{2}}$) (as a measure for increased red cell destruction), and reticulocyte count, or erythrocyte G-6-PD activity (as measures for compensatory red cell production). Anaemia and signs of increased red cell production alone were insufficient for the diagnosis. Other causes for haemolytic anaemia than red cell autoimmunity were excluded as far as possible. Normal values used were those of Williams et al. (1972).

results

Specificity of the FITC-labelled anti-IgG1 serum

In agglutination techniques, the anti-IgG1 serum reacted specifically with IgG1 myeloma proteins coupled to glutaraldehyde-fixed red cells by means of chromium chloride. The use of these glutaraldehyde-fixed red cells in the cytofluorograph to check the specificity of the antiserum after FITC-labelling led to non-specific reactions probably due to aspecific binding of FITC-labelled globulins to glutaraldehyde. However, with red cells not treated with glutaraldehyde, the FITC-labelled anti-IgG1 serum retained its specificity (Table I).

TABLE I

IgG subclass of the coating myeloma protein:	% fluorescence
IgG1	15
IgG2	1.2
IgG3	0.6
IgG4	0.8

Specificity of the FITC-labelled anti-IgG1 serum. Red cells were coated with myeloma proteins of different IgG subclasses by means of chromium chloride. The relative fluorescence obtained with red cells not treated with glutaraldehyde is given. The reaction is expressed as percentage fluorescence (see Methods).

Reproducibility of the measurement

The reproducibility of this comparative cytofluorometric method is indicated by the mean ± S.D. of 7 determinations, performed on the red cells of one patient, on different days over a period of two months. The percentage fluorescence was: 17.7 ± 1.9.

Relation between fluorescence, agglutination titre and score

Rh D-positive red cells (OR_2R_2) were sensitized with two-fold dilutions of the anti-D serum of donor G, resulting in final dilutions ranging from 1:4 to 1:2000.

By means of the AGT, the agglutination titres and scores of the differently sensitized cells were determined with an anti-IgG serum (Fig. 1). The degree of fluorescence, resulting from the incubation with the same, FITC-labelled, anti-IgG serum was also determined and expressed as percentage fluorescence of the control. It appeared that over a wide range of measurement, there was an excellent linear correlation (r^2=0.998) between the dilution of the anti-D serum used for the sensitization and the determined fluorescence (Fig. 1). Thus, cytofluorometry seemed to be a suitable method for the comparison of the relative amount of IgG antibody in an antiserum as well as on sensitized red cells from pa-

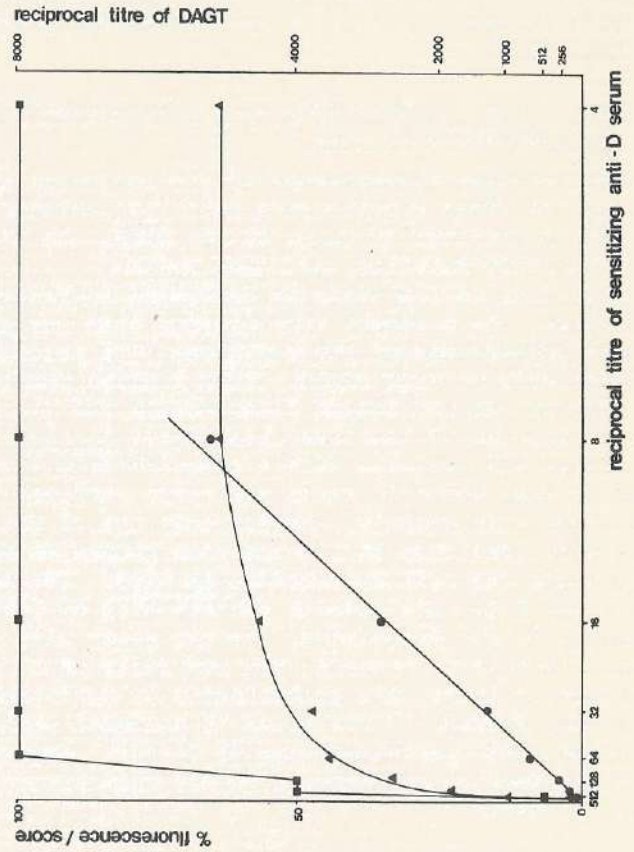


Fig. 1 - Relation between fluorescence, agglutination titre and agglutination score determined with red cells sensitized with dilutions of an anti-D serum. Red cells (OR_2R_2) were sensitized with different dilutions of an anti-D serum (abscissa) and the agglutination titre and score were determined by means of the DAGT. The relative fluorescence (expressed as percentage fluorescence) was measured cytofluorometrically. Left ordinate: agglutination score and percentage fluorescence. Right ordinate: reciprocal titre of DAGT. For cytofluorometry and the antiglobulin test the same anti-IgG serum was used. ●—●, percentage fluorescence; ■—■, agglutination titre; ▲—▲, agglutination score.

tients with AIHA due to incomplete warm IgG autoantibodies. It is clear from the figure that the correlation with either the titre of the AGT or the score was much less good.

Relation between fluorescence with FITC-labelled anti-IgG1 and haemolysis in vivo

The relative fluorescence of the red cells of 29 patients, on whose erythrocytes only IgG1 autoantibodies were detectable by means of the DAGT, was determined with FITC-labelled anti-IgG1 serum.

According to the criteria mentioned in Materials and Methods, the patients were divided into two groups: one with indications of increased red cell destruction, the other with no such signs. Fig. 2 shows the relation between the percentage fluorescence and the presence or absence of increased haemolysis in vivo.

Almost all patients with a comparatively large amount of IgG1 bound to their red cells suffered from overt haemolytic anaemia, whereas only one of the patients with less than 3% fluorescence showed evidence of increased red cell destruction in vivo. Two exceptions A and B to this general pattern will be further considered in the Discussion. In the group with signs of overt haemolytic anaemia, the red cells of 35% (6/17) of the patients were agglutinated by anti-complement serum, whereas in the group of patients without such signs, this percentage was 8% (1/12). However, this difference in percentages is not significant ($p>0.1$, χ^2-test).

Relation between fluorescence with FITC-labelled anti-IgG1 and cytotoxicity in vitro

The cytotoxic action of peripheral blood monocytes of healthy volunteers towards the red cells from the patients mentioned in the preceding paragraph was measured and expressed as percentage of cytotoxicity of the control. In Fig. 3, this percentage is plotted against the percentage of fluorescence, obtained with the same erythrocytes.

There is a direct relation between the cytotoxicity by monocytes in vitro and the amount of IgG1, expressed as fluorescence intensity (Spearman's rank correlation coefficient r=0.89).

The two patients A and B, mentioned in the prece-

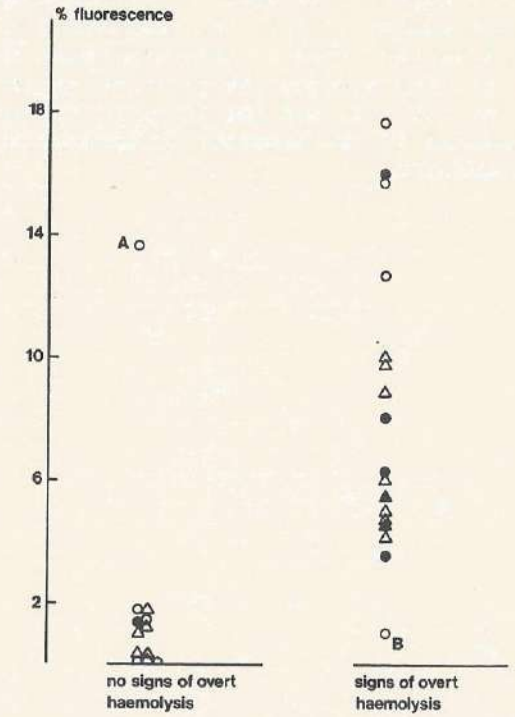


Fig. 2 - Relation between fluorescence of red cells with FITC-labelled anti-IgG1 and haemolysis in vivo. Ordinate: percentage fluorescence of the patients' red cells determined by cytofluorometry. The patients are divided into two groups according to the criteria for haemolysis in vivo. Exceptions are marked A and B (see Discussion).
▲, methyldopa-associated cases
●, idiopathic and symptomatic cases
closed symbols, complement components detectable on the red cells;
open symbols, no complement components detectable on the red cells.

ding paragraph, are also marked in Fig. 3. On the red cells of 25% (7/29) of the patients, C3d was detectable. However, there was no significant difference between the slopes when the populations with or without complement components were tested (t-test for comparison of slopes, $p>0.1$, Dixon & Massey, 1969). This indicates that the established relation between the relative amount of antibody on the red cell and the in vitro cytotoxicity was not influenced by the presence or absence of this complement component.

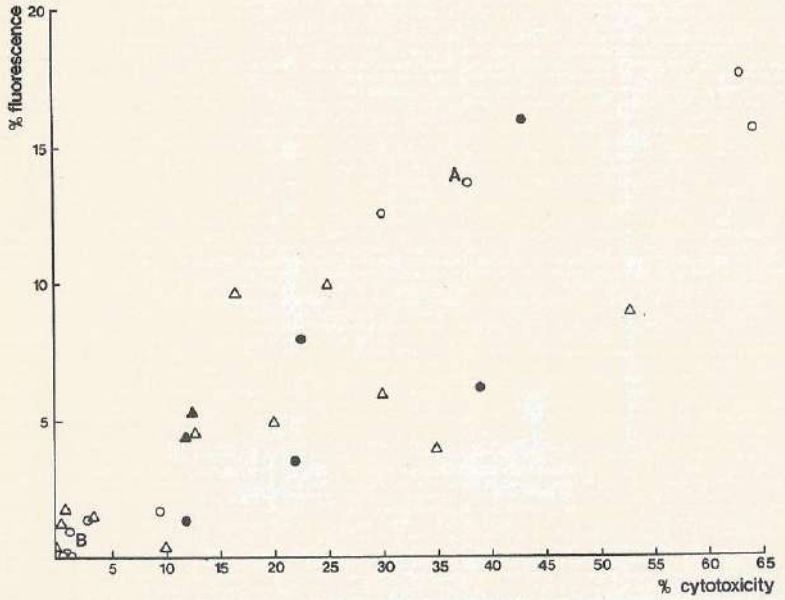


Fig. 3 - Relation between fluorescence and cytotoxicity in vitro. Abscissa: percentage of cytotoxic lysis in vitro of patients' red cells by monocytes of healthy volunteers. Ordinate: percentage fluorescence of the same red cells with FITC-labelled anti-IgG1 serum. A and B represent the same patients as marked in Fig. 2.
▲, methyldopa-associated cases
●, idiopathic and symptomatic cases
closed symbols, complement components detectable on the red cells;
open symbols, no complement components detectable on the red cells.

discussion

The results presented in this paper show that continuous flow cytofluorometry is a suitable technique for the measurement of red cell antibodies free in serum or bound to cells. Cytofluorometric measurements decreased linearly with the dilution of the antiserum in which the rh D-positive erythrocytes had been incubated (Fig. 1). Doubling the serum dilution resulted in half the percentage of fluorescence, thus suggesting that -in this case- also half the amount of antibody was bound to the red cell surface.

To correct for variations in circumstances of measurement occurring during the period in which the experiments were performed, the fluorescence of the same batch of rh D-positive red cells, sensitized under standard conditions with a standard anti-D serum, was regularly determined. After correction for background fluorescence, the results obtained with the patients' erythrocytes were expressed as percentage of fluorescence of the corrected control value. This procedure led to a satisfactory reproducibility.

The results of cytofluorometric measurement of the patients' red cells showed a distinct difference in the number of antibody molecules on the red cells of patients with and without signs of increased haemolysis. There appears to be a 'threshold', i.e. a critical degree of sensitization above which increased red cell destruction in vivo becomes apparent. This indicates that our previous interpretation that two kinds of -qualitatively different- IgG1 autoantibodies are present, is incorrect (Engelfriet et al., 1974; Meulen et al., 1978).

Our results are in agreement with the finding of Mollison (1967) that a critical amount of cell-bound IgG is necessary to induce measurable destruction of red cells in vivo.

Since antibody-dependent cytotoxicity exerted by mononuclear phagocytes in the spleen is probably an important mechanism of red cell destruction in AIHA (Fleer et al., in press[b]), we have investigated the relation between the intensity of fluorescence and the degree of cytotoxic lysis by monocytes in vitro. The establishment of a direct correlation between

these two parameters suggests that stronger sensitization with IgG1 antibodies above a certain threshold leads to a higher degree of haemolysis in vivo. This concept is compatible with the results of many investigators who studied both allo- and autoantibodies (Jandl & Kaplan, 1960; Mollison et al., 1965; Gilliland et al., 1970; Rosse, 1971) and found that the amount of antibody on the red cell surface was generally proportional to the rate of haemolysis in vivo.

From our study, two discrepancies to the above-mentioned pattern emerge . In case A, signs of overt haemolytic anaemia were absent and the ^{51}Cr $T_{\frac{1}{2}}$ was 22 days (normal value above 23.5 days), whereas much IgG1 was detectable on the red cells. The spleen of this patient functioned normally since red cells sensitized in vitro with anti-D alloantibodies and labelled with ^{51}Cr were immediately removed by this organ. The patient's erythrocytes, however, were strongly lysed in vitro by monocytes of healthy volunteers, in accordance with the amount of IgG1 on the erythrocytes (Fig. 3). This indicated that these antibodies as such could induce haemolysis. Finally, we tested the patient's own monocytes. These cells showed a normal cytotoxic and erythrophagocytic behaviour towards the patient's own red cells from the previous haemolytical episode, as well as towards red cells sensitized with IgG alloantibodies. For the observed discrepancy we have, so far, no explanation.

In case B, fluorescence as well as cytotoxicity were low whereas overt haemolysis was present, a finding for which also no other cause could be detected.

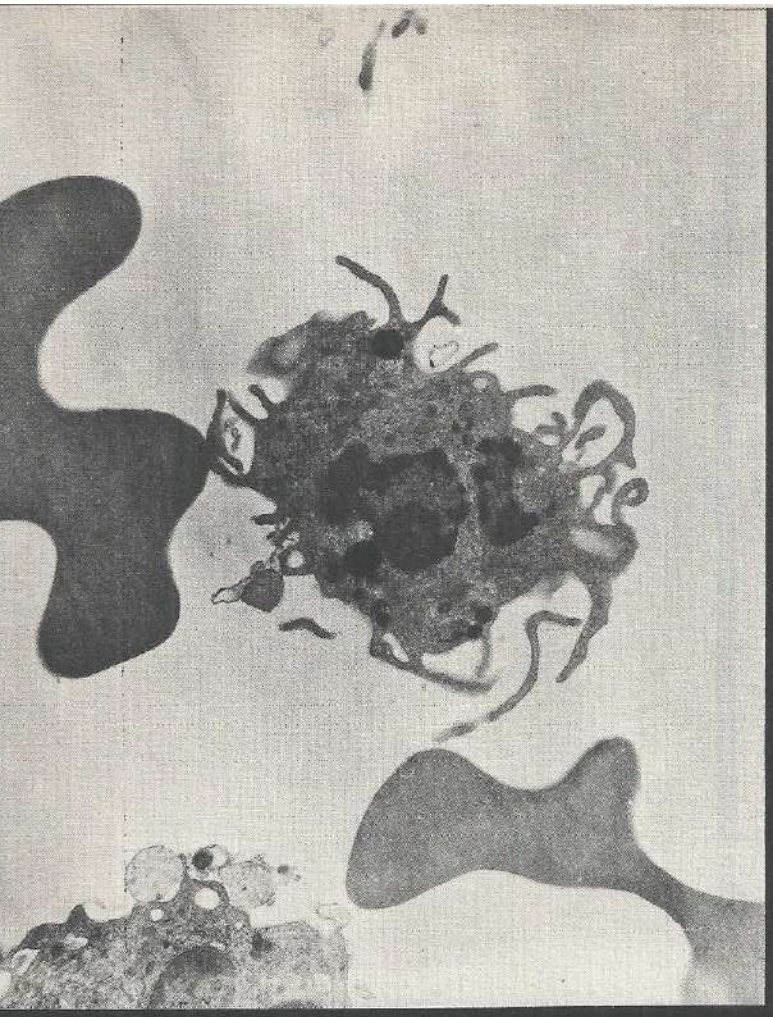
As stated by Worlledge (1973), 15% of the patients receiving methyldopa develop IgG incomplete warm autoantibodies against red cells, but less than 1% of the patients show signs of increased red cell destruction. In our series, 52% (15/29) of the patients were under methyldopa therapy. Nine of them suffered from haemolytic anaemia and six did not. Without exception, the percentage fluorescence was well above the 'threshold' in the cases of increased haemolysis, whereas the fluorescent signal was weak in those cases in which no evidence of increased red cell destruction was found. This suggests that in case of IgG1 autoantibody development under methyldopa treatment, the occurrence of haemolysis depends on the amount of antibody on the

red cell surface, which is in agreement with recent findings of Worlledge et al. (personal communication) who used a semiquantitative antiglobulin test.

In the present study, the erythrocytes from 25% (7/29) of the patients were also agglutinated by anti-complement serum. Using specific reagents, it could be shown that C3d was present on these cells, but not C4c or C3c. An anti-C4d serum was not available. There was no significant difference in the presence of C3d in the group of patients with or without haemolytic anaemia.

Although Kurlander et al. (1978) reported that C3d enhanced the cytotoxic activity of monocytes in vitro towards IgG-sensitized red cells, in the underlying study the relation between the amount of IgG1 autoantibody and the cytotoxic activity of monocytes in vitro was not influenced by the presence or absence of C3d. In the former experiments, though, C3d was fixed to the red cells in vitro, whereas in our study this component of complement had been bound to the cells in vivo.

notes



chapter V
longitudinal studies of red cell destruction in auto immune haemolytic anaemia. application of in vitro assays

F.W. van der Meulen, L.I. Oei-Tan, A.E.G. Kr. von dem Borne & C.P. Engelfriet

Department of Immunohaematology, Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, and the University Clinic of Internal Medicine, Department of Haematology, Binnengasthuis, Amsterdam, The Netherlands.

summary

In a longitudinal study of patients with autoimmune haemolytic anaemia with IgG non-complement binding warm autoantibodies, the course of the disease and the effect of treatment were studied in relation to the subclass composition and the relative amount of IgG antibody detectable on the patients' red cells, obtained from consecutively collected blood samples.

The adherence of the patients' red cells to peripheral blood monocytes (PBM) of healthy volunteers and the subsequent cytotoxic lysis of these erythrocytes by the phagocytes in vitro were also determined.

On the red cells of the patients, who responded well to corticosteroid therapy, a significant decrease of the amount of IgG antibody was detectable. This was associated with a diminished adherence and cytotoxicity in vitro. In some cases, the IgG subclass composition was also altered.

In the patients with IgG3 autoantibodies, improvement coincided with the disappearance of this subclass. The adherence and cytotoxic lysis in vitro decreased concomitantly.

Recovery or improvement of the patients with IgG1 autoantibodies was accompanied by a decrease of the number of IgG1 molecules as well as by a decreased interaction between the patients' erythrocytes and the mononuclear phagocytes in vitro. The presence of IgG2 and IgG4 autoantibodies was not associated with increased red cell destruction in vivo, nor did it lead to binding and lysis of these erythrocytes by PBM in vitro.

On the basis of several cases, the different reactions to therapy are discussed.

introduction

Much evidence has recently accumulated which indicates that adherence of red cells, sensitized with non-complement binding IgG antibodies to 'Fc receptors' on mononuclear phagocytes, is a crucial step in the destruction of such cells in vivo (Brown, 1974; Engelfriet et al., 1974; Logue & Rosse, 1976; Borne et al., 1977a; Meulen et al., 1978). Thus, in our own investigations (Engelfriet, 1974; Borne et al., 1977b; Meulen et al., 1978), we found that the presence of IgG3 autoantibodies as detected by means of the DAGT is nearly always accompanied by increased red cell destruction in vivo and adherence to PBM in vitro.

Patients with only IgG2 and IgG4 autoantibodies do not suffer from autoimmune haemolytic anaemia (AIHA), and the red cells of such patients are not bound to PBM in vitro; IgG1 autoantibodies only caused increased haemolysis when present above a critical level, while above this threshold there seemed to be a relation between the degree of sensitization of the red cells and the rate of erythrocyte destruction (data submitted for publication). From their experiments with IgG alloantibodies, Jandl & Kaplan (1960) and Mollison et al. (1965) concluded that the amount of non-complement binding IgG antibodies determined the rate of destruction of red cells under the influence of these antibodies. Thus, it is clear that IgG subclass composition and the amount of IgG antibody on the patient's red cells are of great significance in the diagnosis, course and treatment of AIHA.

The initial process of adherence of IgG-sensitized erythrocytes to phagocytes in vitro either leads to phagocytosis or to cytotoxic damage outside the phagocyte (LoBuglio et al., 1967; Huber & Fudenberg, 1968; Abramson et al., 1970b; Holm et al., 1974). Fleer et al. (in press, a,b,c) found indications that particularly the latter process is of major importance in vivo.

Until now, most of our investigations with the patients' red cells were performed only once, i.e. when the patient's blood was first sent to the laboratory. To investigate the course of AIHA in time and the influence of therapy, we collected blood samples during the course of the disease and studied the

IgG subclass composition and the relative amount of IgG antibody on the patients' red cells in relation to the degree of haemolysis. Furthermore, adherence of the patient's erythrocytes to mononuclear phagocytes in vitro and the subsequent cytotoxic lysis of these cells by the phagocytes, which both most probably reflect processes operating in vivo, were studied.

materials and methods

Erythrocytes

Experiments were performed with erythrocytes from patients with IgG non-complement binding warm autoantibodies only. The group of patients was heterogeneous with regard to the accompanying disease, therapy and duration of the disease. From 16 patients with evidence of increased haemolysis, consecutive blood samples, collected during the course of the illness and treatment, were stored in liquid nitrogen. None of the patients received corticosteroid therapy at the time when the first blood sample was taken.

The subclass of the IgG antibodies was determined by the direct antiglobulin test (DAGT) using subclass-specific antisera. After thawing, the erythrocytes were washed in phosphate-buffered saline (PBS) and suspended in the required media (see below). Thus, the various blood samples from the same patient could be tested on the same day.

The cryopreservation of red cells did not affect the results of subsequent measurements, such as the titre in the DAGT or the relative fluorescence. Neither did it alter the adherence of the red cells to monocytes or the cytotoxic activity of the phagocytes towards these cells in vitro (see below).

Cytofluorometric measurements

Measurements of the relative amount of antibody on the red cells from the various blood samples were performed by means of cytofluorometry, as was described elsewhere in detail (Chapter IV). In short, patients' red cells were incubated with fluorescein isothiocyanate (FITC)-labelled anti-IgG serum or

anti-IgG1 serum for 30 min at room temperature. Fluorescence of the red cells was determined in a Biophysics Cytofluorograph, model 4180 A-FC 200, and the relative fluorescence intensity (RFI) of the cell population calculated as described (Verheugt et al., in press; Borne et al., 1978).

A standard erythrocyte preparation (Rh D-positive red cells (OR_2R_2), sensitized with an anti-D (EAIgG anti-D)) served as the positive control. To correct for the background fluorescence, the RFI of the same non-sensitized Rh D-positive red cells (OR_2R_2) was determined. The results of the measurements, presented in this paper, are expressed as percentage fluorescence (= relative fluorescence) and calculated as follows:

$$\text{percentage fluorescence} = \frac{\text{RFI patient's red cells} - \text{RFI } OR_2R_2}{\text{RFI EAIgG anti-D} - \text{RFI } OR_2R_2} \times 100\%$$

In this way, the relative amount of antibody present on the various red cells could be compared.

Preparation of monocytes

Cryopreserved as well as freshly prepared monocytes of healthy volunteers were used. The cryopreservation was performed as described elsewhere in detail (Chapter VII). In short, monocytes were isolated from the buffy coat of citrated venous blood by means of Ficoll-Isopaque density gradient centrifugation and adherence to glass petri dishes. After suspension in a medium containing fetal calf serum (FCS) and dimethyl sulfoxide (DMSO) as cryoprotectant, the cells were frozen according to a controlled rate cooling programme (du Bois et al., 1976) and stored in liquid nitrogen. The capacity of monocytes to bind and lyse red cells, sensitized with incomplete anti-D alloantibodies, was unchanged by this procedure (Meulen et al., 1977).

All experiments were also performed with freshly prepared monocytes. These cells were also isolated by means of Ficoll-Isopaque density gradient centrifugation and adherence to plastic (Falcon plastic tissue culture dishes, 3003, Oxnard, Cal., USA), as previously described (Meulen et al., 1978).

Cytotoxicity assay

Lysis of sensitized red cells by monocytes was determined as described by Fleer et al. (in press,a). Briefly, patients' red cells were labelled with $Na_2Cr^{51}O_4$ and incubated with monocytes in wells of microtitre plates. After incubation for 16 h at 37°C, the plates were centrifuged and the percentage of ^{51}Cr-release was determined in a sample of the supernatant. After correction for the spontaneous ^{51}Cr-release, the number of red cells lysed by the monocytes could be calculated. The cytotoxicity of monocytes towards EAIgG anti-D served as a positive control. Percentage cytotoxicity was calculated as follows:

$$\text{percentage cytotoxicity} = \frac{\text{number of patient's red cells lysed}}{\text{number of EAIgG anti-D lysed}} \times 100\%$$

Non-sensitized red cells were not lysed by monocytes.

Adherence assay

The rosette test was performed as described elsewhere in detail (Chapter VII). In short, a suspension of monocytes was mixed with the patient's red cell suspension, centrifuged and subsequently incubated at room temperature. After the addition of acridine orange, the cells were carefully resuspended and the percentage of rosette-forming cells determined with a Leitz Orthoplan fluorescence microscope, according to Brostoff (1974).

EA-rosette formation with EAIgG anti-D served as a positive control. The percentage adherence was calculated as follows:

$$\text{percentage adherence} = \frac{\%\text{ rosette-forming cells with patient's red cells}}{\%\text{ rosette-forming cells with EAIgG anti-D}} \times 100\%$$

Non-sensitized red cells (OR_2R_2) were not bound by monocytes.

Criteria for haemolysis in vivo

As is indicated in Chapter IV, at least four of

the following parameters were used: blood haemoglobin level, haematocrit or erythrocyte count, erythrocyte osmotic fragility, serum haptoglobin level, serum LDH activity or serum bilirubin level , erythrocyte half life (determined by using ^{51}Cr-labelled red cells) (^{51}Cr $T_{\frac{1}{2}}$), and reticulocyte count or erythrocyte G6PD-activity.

Anaemia and signs of increased red cell production alone were insufficient for the diagnosis. Normal values used were those of Williams et al. (1972).

results

Relation between haemolysis in vivo and cytotoxicity in vitro

In order to study this relation, we first examined the red cells of 37 untreated patients with non-complement binding IgG autoantibodies. They were incubated in vitro with PBM of healthy volunteers and the presence or absence of signs of increased red cell destruction in vivo was correlated to the cytotoxic lysis of these cells in vitro.

It was found that in nearly all cases in which there were no signs of haemolytic anaemia, the relative cytotoxicity was below 10%, whereas in nearly all cases of overt haemolysis, the percentage cytotoxicity was above this critical level (Fig. 1). Thus, the determination of cytotoxic lysis of patients' red cells in vitro by mononuclear phagocytes seems to be a suitable parameter to discriminate between a state with and one without signs of increased red cell destruction in patients with IgG non-complement autoantibodies.

Survey of patients included in the longitudinal study

Table I gives a survey of the 16 patients suffering from autoimmune haemolytic anaemia (AIHA) included in this study and shows the various parameters of the disease in the course of time.

Seven patients developed overt haemolysis during a-methyldopa therapy, one other patient during treatment with L-dopa. In five of the eight remaining cases, other autoimmune diseases or chronic lymphatic

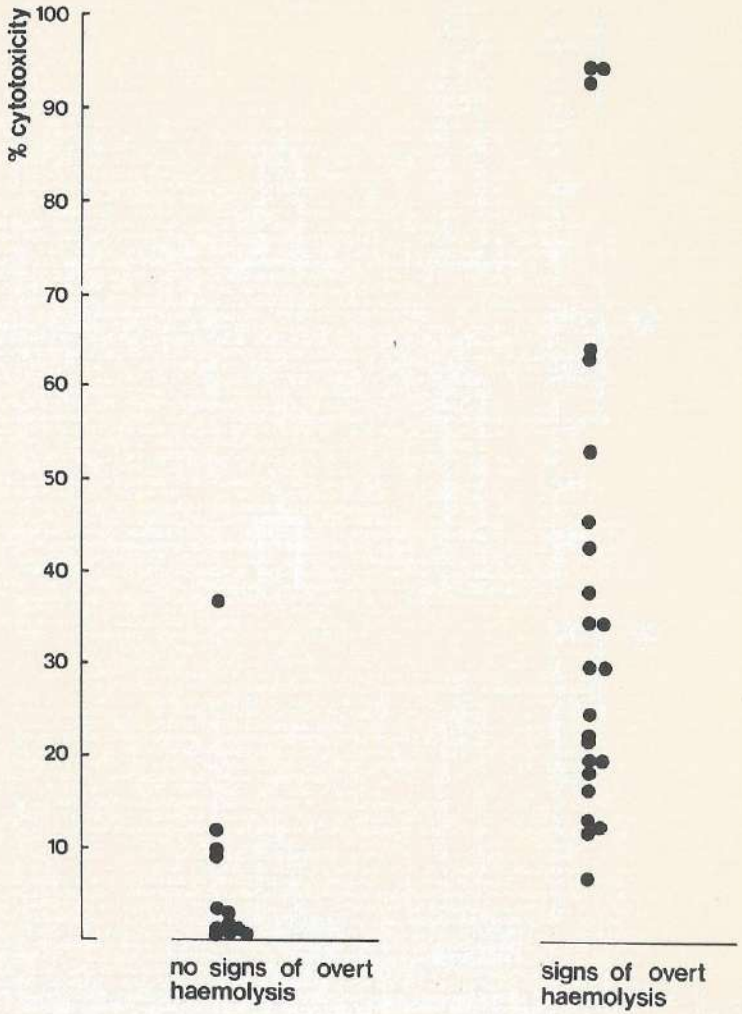


Fig. 1 - Relation between haemolysis in vivo and cytotoxicity in vitro. Ordinate: percentage cytotoxicity obtained by incubating the patients' red cells with PBM in vitro. The patients are divided into two groups according to the criteria for haemolysis in vivo (see Methods).

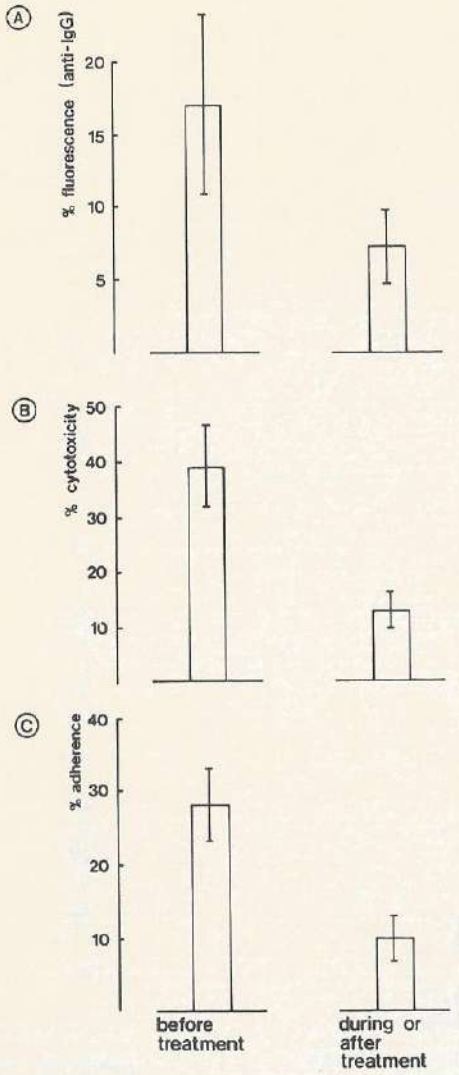


Fig. 2 - Relative fluorescence, cytotoxicity and adherence determined with the patients' red cells collected before and after a period of treatment. Ordinate: A., percentage fluorescence with FITC-labelled anti-IgG serum; B., percentage cytotoxic lysis of patients' red cells by PBM in vitro; C., percentage adherence of patients' red cells to PBM in vitro. Given are the mean ± SEM, calculated from determinations with red cells of 15 patients.

leukaemia were present. Three patients died during the course of the study: one had a fatal myocardial infarction, the two other patients died of a pneumonia.

At the time the first blood sample was taken, none of the patients received corticosteroid therapy; when the last blood sample was taken, all but one patients were treated with this drug. One patient recovered by the mere withdrawal of a-methyldopa.

Fluorescence, adherence and cytotoxicity in the course of time

In 15 of the 16 patients the degree of haemolysis decreased during treatment. In this group, a significantly larger amount of antibody, as determined by cytofluorometric measurement, was detectable on the patients' erythrocytes before than after the period of treatment. (Student's t-test for paired observations, $p<0.02$) (Fig. 2A).

The percentage of adherence and cytotoxicity, obtained with red cells from the first blood samples, were also significantly higher than those of later samples ($p<0.0001$) (Figs. 2B and C). However, in one patient (v.D.), the signs of increased haemolysis persisted during prednisone therapy. This situation was reflected in vitro by the relative fluorescence, adherence and cytotoxicity, which remained at very high levels throughout the time of investigation.

Since individual patients showed different reactions to therapy, some cases will be considered in more detail.

LEGEND TO TABLE I

Survey of laboratory, serological and experimental data of the patients included in the longitudinal study. Given are the results of the examinations of blood samples collected before (upper line) and after (lower line) a period of treatment.
a: p.c.: packed red cell transfusion;
b: the survival time of normal erythrocytes is noted in brackets;
c: normal values of serum LDH activity.

TABLE I

Patient Age, Sex	Concom. disease	Medication	Hb gr%	ret ‰	Hp mgr%	LDH U/L	^{51}Cr $T^1/_2$ b	reciprc. IgG_1	agglut. titre $IgG_{2,3,4}$	%fluorescence IgG	%fluorescence IgG_1	%Cytotoxicity	%Adherence
			Laboratory examinations						*Serology*			*In vitro tests*	
A-v.D, 78 female		a-m. dopa	7.2	225	0	468		128		3	4	30	17
		predn.	11	25	90	96c		±		2	2.5	10	6
D, 83 female			8	145	20	150c		512		7.7	6.5	38	64
		predn.	12	7	170	168c		1000		6.1	5.7	24	38
E-d.H, 77 female		a-m. dopa	7.8	274	15	363		1000		17	5	21	1
		predn.	12.8	20	105			±		3	1	1	0
H. -N, 76 female		a-m. dopa	7	276	0	628		4000	IgG_3: 16	13	4	63	71
		predn.	12.3	84	15	330		256	–	9	1	21	22
H.-E, 79 female	Autoimmune thyroiditis	p.c.[a]	4.6	390	0	545		4	IgG_2: 256 3: 32 4: 128	90	3	19	17
		predn.	14.2	60	15	300		–	IgG_2: 128 3: – 4: 8	24	1	0	0
			15.2	10		200c							
v.H-v.G female, 70			7	76	15	458		512		49	6	93	18
		predn.	11.8	30	60	158c		128		33	4	36	16
B., 81 male	Chron Lymph Leukaemia	p.c.[a]	12		0	774		64		5	2.5	20	40
		predn.	6.2			465							
	Myocard. inf.		6.9		80	320		16		4	2	6	19
W.-K, 69 female		a-m. dopa	10.7	80		330							
			13.9	35	130	250c		256		3	4.1	35	21
			13.8	40	150	200c		±		2.3	3.7	6	10
v.D., 65 male	Chron. Lymph. Leukaemia		9.7	80	0	490	10 (23)	4000	IgG_2: 64 3: 64 4: 256	277	127	94	65
	Pneumonia	p.c.[a] predn.	8.7	130	0	420		4000	idem	268	149	111	64
K.-B, 67 female		a-m. dopa predn.	7.9	196	10	476	11 (30)	1000		13.1	5	25	27
		splenect	13.7	6	230	172c		256		5.5	2	5	1
K.-V, 60 female	Diab. Mell. M. Parkinson	L. dopa predn.	7.7	50	0	609	6 (40)	512	IgG_3: ±	13	11	94	24
			11.7	12	60	220c		128	–	6	7	31	4
S., 78 male	Pariet. cell antibodies		7.4	216	10	680	6	256		7.8	4.4	38	16
		predn.	11.5	70	20	556	(28)	256		5.7	3	23	5
V.-W, 72 female		a-m. dopa	9.9	90	0	360	14	2000		6	3	15	20
		predn.	12.8	10	150	225c	(23)	64		1	0.5	0	0
W-d. B, 69 female	Rheum. Arthr. Diab. Mell.		8.9	124	0	592	8	256		2.5	1.4	6	24
		predn.	13.3	25	90	385c	(23)	256		0	1.2	3	7
W., 87 male			6.7	220	20	790		1000		15.1	15.8	64	42
		predn.	12.8	25	10	380c		1000		4.8	2.7	16	18
Y-v.G, 81 female	Diab. Mell. Bronchopn.	a-m. dopa	9.6	500	0	430		512		9	10	25	20
		predn.	12	66	110	251c		1000		4	6	10	2

Case reports

Case 1

The various data of patient V.-W. (a woman aged 71) obtained in the course of time are summarized in Fig. 3. She had been treated with a-methyldopa for 5 years and had developed AIHA. On her red cells only IgG1 autoantibodies were detectable. When the drug was discontinued, she showed only little improvement. Therefore, prednisone therapy was started and this resulted in a normalization of the haematological parameters in about 3 weeks. Thus, the blood haemoglobin level steadily rose, while the percentage reticulocytes declined and the serum LDH activity returned to a normal level. The rise in serum haptoglobin reflected the decrease of red cell destruction. Concomitantly, the amount of IgG1 antibody on the patient's red cells decreased as did the percentages of adherence and cytotoxicity in vitro. The patient was kept on a low dose of prednisone for one year.

Three weeks before the last measurement, this treatment was stopped. No signs of haemolytic anaemia recurred, whereas the DAGT was still positive with anti-IgG serum and anti-IgG1 serum. The relative fluorescence with FITC-labelled anti-IgG1 serum was low and adherence and cytotoxicity were negative.

Case 2

On admission to the hospital, the red cells of patient v.H.-v.G. (aged 70) were heavily sensitized with IgG1 antibodies, resulting in a strong binding to, and lysis by, monocytes in vitro. While the patient improved under prednisone treatment, the percentages adherence and cytotoxicity steadily decreased, as did the amount of antibody on the erythrocytes. Remission of the anaemia, however, was complete, while much IgG was still detectable on her red cells and the adherence and cytotoxic test remained positive (Fig. 4).

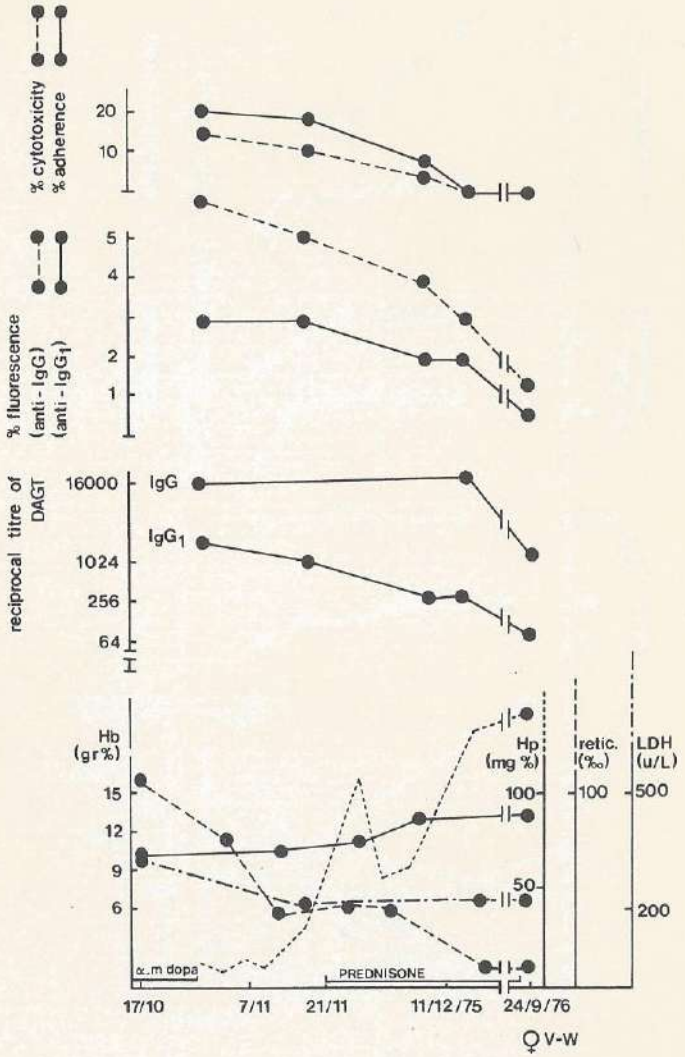


Fig. 3 - Laboratory, serological and experimental data of case 1 in the course of time.

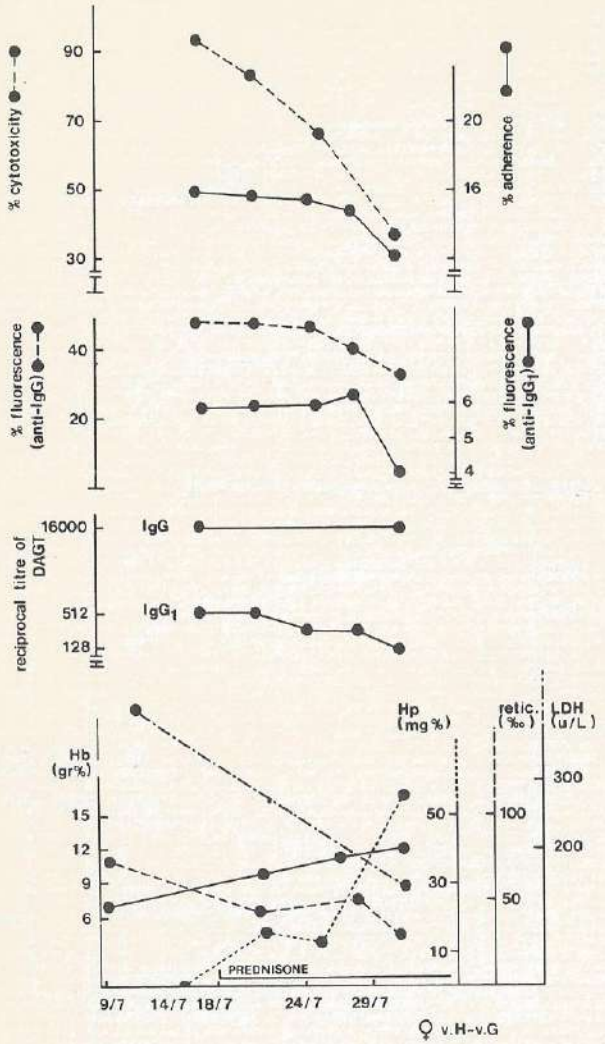


Fig. 4 - Laboratory, serological and experimental data of case 2 in the course of time.

Case 3

Patient H.-N. (aged 76) used a-methyldopa and was admitted to the hospital with anaemia, jaundice and splenomegaly. On her red cells, a large amount of IgG antibody of the subclasses IgG1 and IgG3 was detectable. Adherence and cytotoxicity were high and fell immediately to lower levels once IgG3 antibodies were no longer detectable, after discontinuation of the drug. However, from then on recovery was slow and corticosteroid therapy was instituted. In the last period of investigation, she still showed signs of increased haemolysis and IgG antibodies were found on her erythrocytes in a more or less constant quantity. The percentages adherence and cytotoxicity also remained largely unchanged. Fig. 5 shows the results of the examinations performed in this case.

Case 4

In the blood of the patient H.-E., autoantibodies against thyroid tissue were present, apart from autoantibodies against red cells. At the onset of the AIHA, all four IgG subclasses could be detected on the patient's erythrocytes, which were readily bound and lysed by PBM in vitro. The patient received 4 units of packed red blood cells under intravenous administration of corticosteroids after which she was treated with 60 mg prednisone daily.

In parallel with the disappearance of IgG3 autoantibodies, the adherence and cytotoxicity tests became negative. However, a rather heavy load of IgG antibodies of the subclasses 2 and 4 remained detectable. IgG1 antibodies were present in only a small amount. The patient gradually improved and finally recovered completely (Fig. 6).

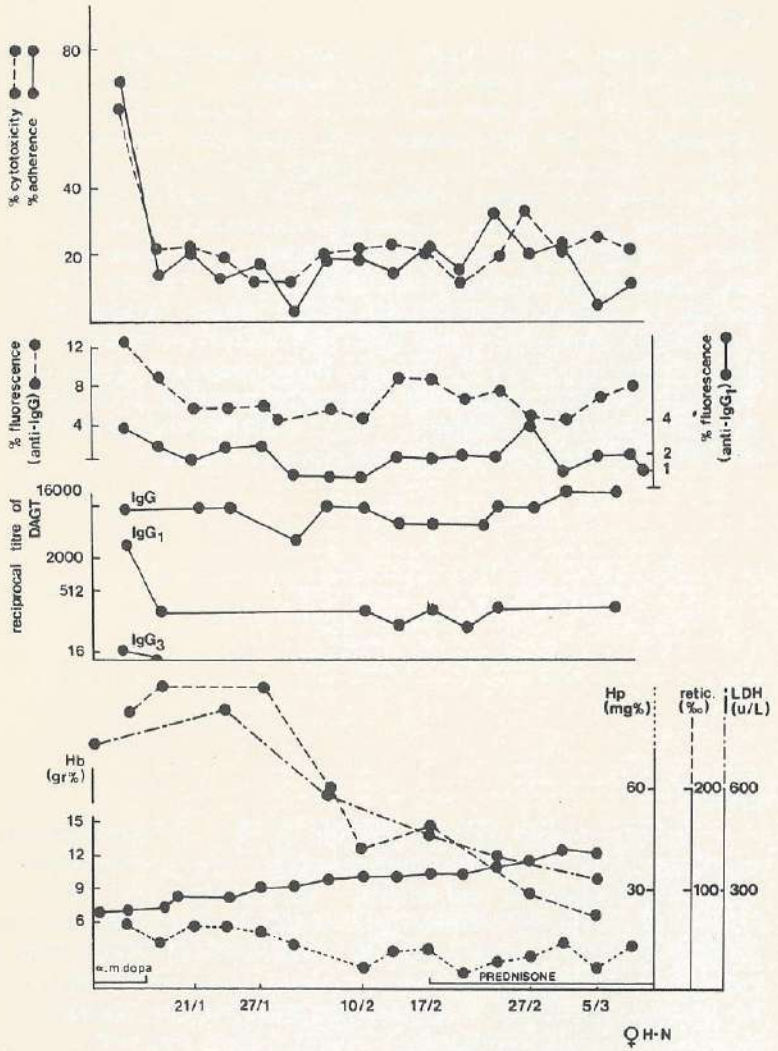


Fig. 5 - Laboratory, serological and experimental data of case 3 in the course of time.

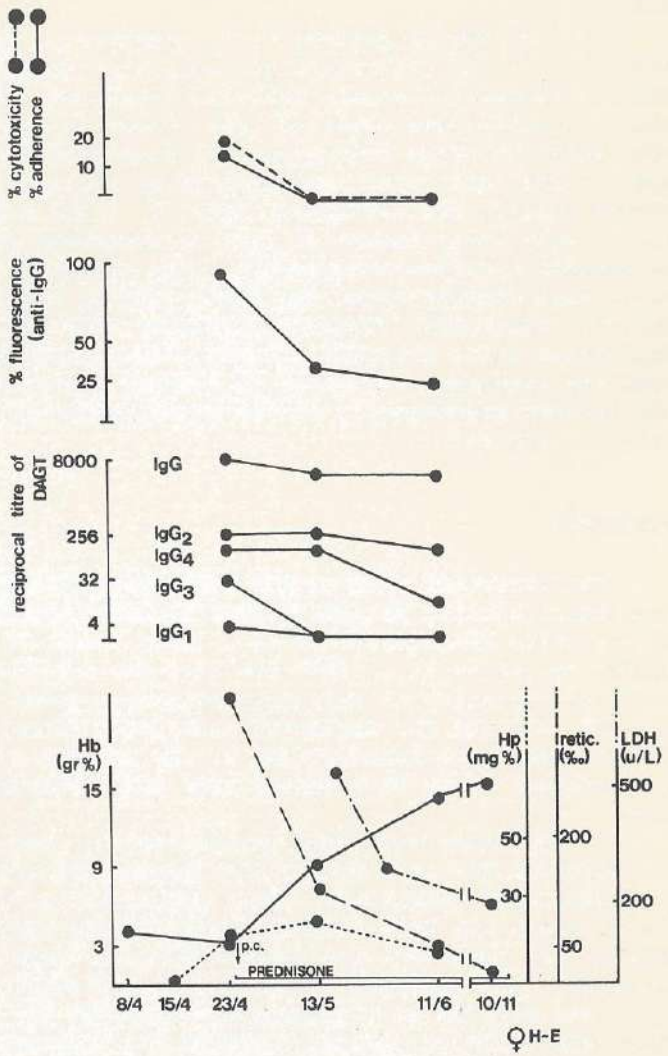


Fig. 6 - Laboratory, serological and experimental data of case 4. ↓ p.c.: packed red cell transfusion.

Case 5

In the case of patient v.D., admitted to the hospital for the evaluation of haemolytic anaemia, a chronic lymphatic anaemia was also diagnosed. Laboratory examinations revealed high titres in the DAGT with anti-IgG serum throughout his hospitalization and his red cells were agglutinated by antisera directed against the subclasses IgG1, 2, 3 and 4 (Fig. 7). A very large amount of IgG1 autoantibody was determined cytofluorometrically. The patient's red cells were readily bound to, and lysed by, PBM of healthy volunteers. Despite treatment with 60 mg prednisone daily for over 4 weeks, this situation remained unchanged and until he died of pneumonia, the patient persistently showed severe signs of haemolytic anaemia.

discussion

The important role of the subclasses of IgG non-complement binding autoantibodies in the destruction of red cells (Engelfriet et al., 1974; Borne et al., 1977b; Meulen et al., 1978) and also the relation between the quantity of IgG1 autoantibodies bound on the red cell and the degree of haemolysis (Chapter IV) are once more demonstrated by the results of this longitudinal study in patients with such autoantibodies.

In all cases with IgG3 autoantibodies, the signs of increased red cell destruction diminished strongly, or disappeared completely, when, during the course of the disease, the IgG3 autoantibodies became undetectable on the patients' red cells (patients 3 and 4 as examples). At the same time that the increased haemolysis was reduced or disappeared, adherence to, and cytotoxic lysis by, PBM in vitro of the patients' red cells was markedly reduced or became negative. That IgG2 and IgG4 autoantibodies do not affect red cell survival is again demonstrated in the fourth patient, where red cells after recovery from the haemolytic episode, were still strongly sensitized by IgG2 and IgG4 autoantibodies. The presence of these antibodies also did not provoke cytotoxic damage by PBM in vitro. In the patients with IgG1 autoantibodies alone, the

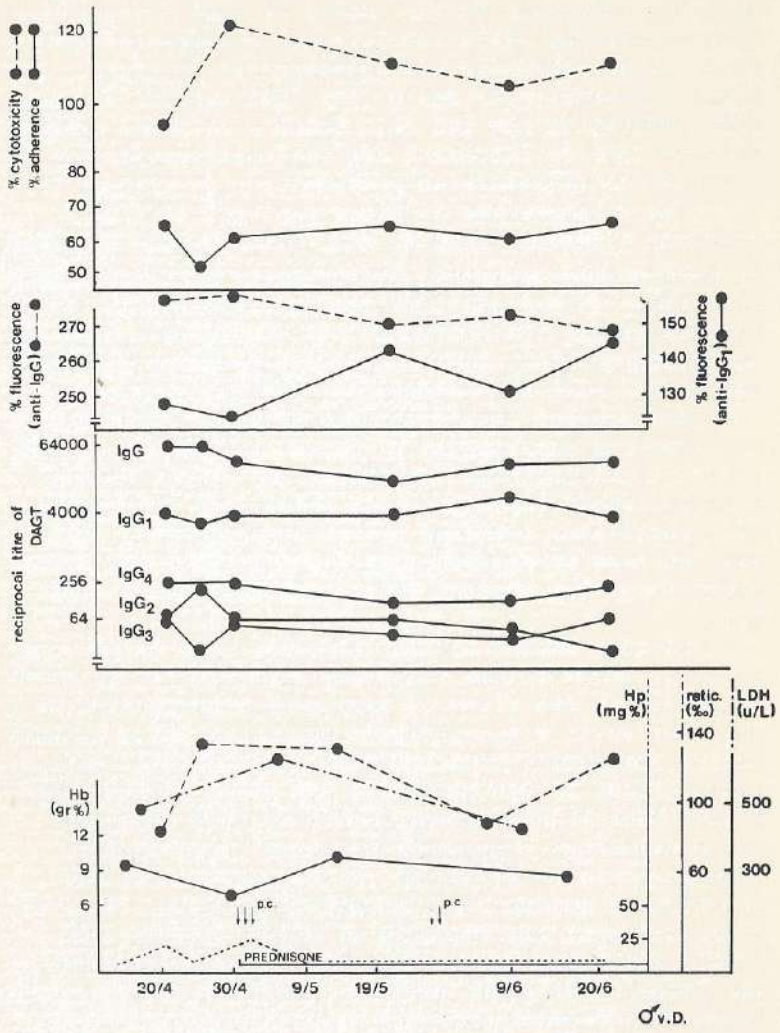


Fig. 7 - Laboratory, serological and experimental data of case 5. ↓ p.c.: packed red cell transfusion.

signs of increased in vivo haemolysis abated along with the reduction in the number of IgG1 autoantibodies on the red cells and this was accompanied by a decrease in adherence and cytotoxicity in vitro. Patient 1 demonstrates again that IgG1 autoantibodies when present on the red cell below a critical number, neither lead to increased red cell destruction in vivo nor to adherence or cytotoxicity in vitro.

The observations in these patients very well show the different results of corticosteroid therapy. In many of the patients, the quantity of autoantibody detectable on the patients' red cells gradually decreased during the course of the treatment. This is in agreement with results reported by Rosse (1971), who showed that corticosteroids had this effect.

The results in some of our patients show that the subclass composition of the autoantibodies detectable on the patients' red cells may change during corticosteroid therapy in the sense that antibodies of a particular subclass become undetectable. This was the case in the patients 3 and 4 where red cells after some time stopped being agglutinated by anti-IgG3.

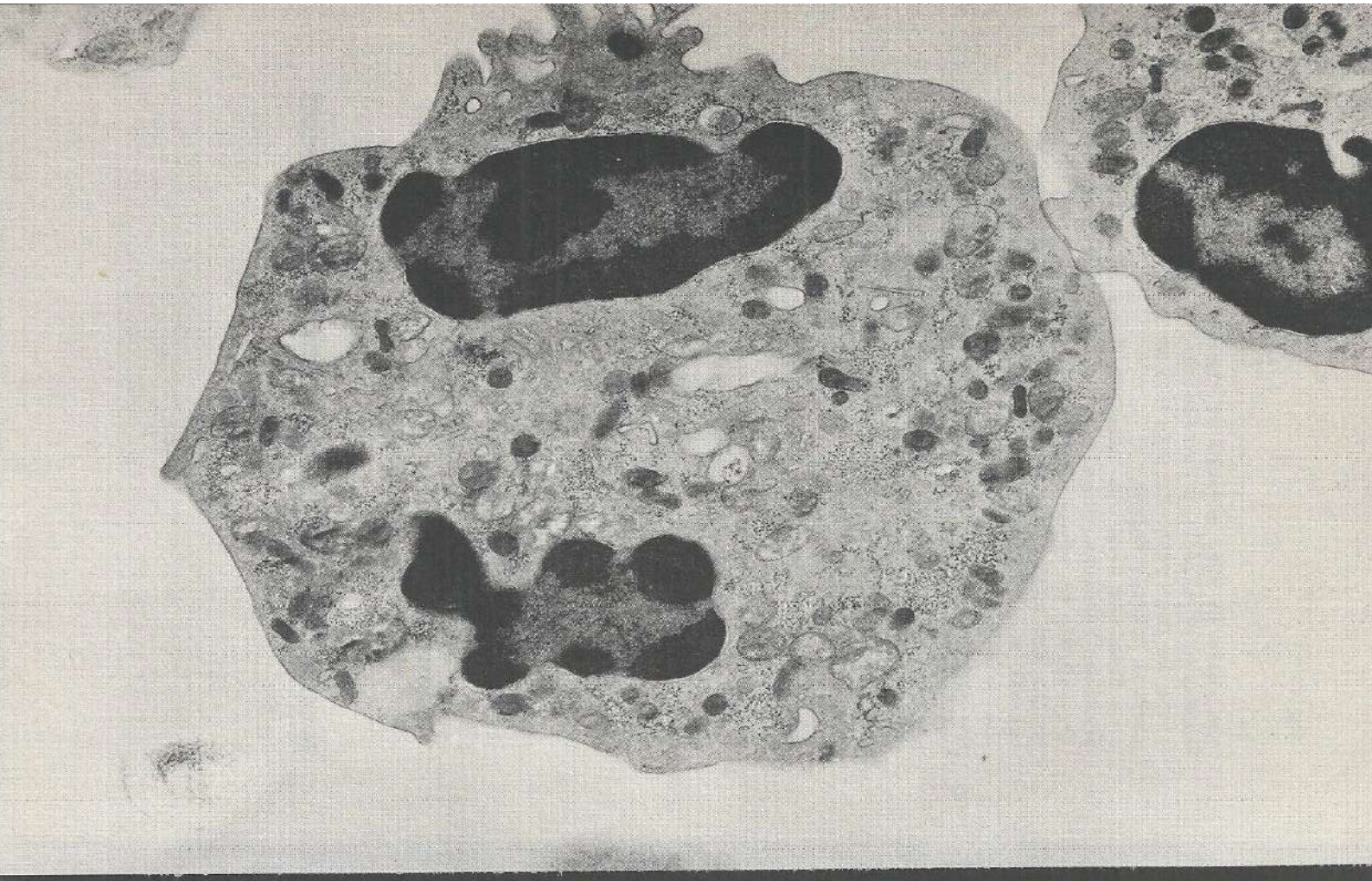
Another effect of the treatment with corticosteroids is shown by the following observation: in several patients the signs of increased haemolysis disappeared while a considerable amount of IgG autoantibodies were still detectable on the patients' red cells (Table I) and while the red cells of these patients were still strongly bound to, and lysed by, the mononuclear phagocytes of normal individuals. This suggests an effect of corticosteroids on the function of mononuclear phagocytes in vivo. This is in accord with the findings of others (Kaplan and Jandl, 1961; Mollison, 1962; Rosse, 1971; Atkinson and Frank, 1974), who noticed a decrease in the clearance of IgG-sensitized red cells in vivo under the influence of corticosteroids. In one patient, increased haemolysis in vivo continued in spite of the treatment indicating that the above effect of corticosteroids is not always such that the rate of red cell destruction returns to normal. Fleer et al. (in press, c) showed that in vitro hydrocortisone totally inhibits or only decreases the cytotoxic activity of PBM towards EAIgG anti-D. This reflects

the above in vivo effects of the drug and thus one therapeutic effect of corticosteroids may be a decline in the cytotoxic activity of mononuclear phagocytes towards EAIgG.

From the findings reported above as well as from previous findings (Engelfriet et al., 1974; Meulen et al., 1978), it appears that when IgG3 is detectable on the red cells of a patient, there is always increased red cell destruction in vivo. This means that when the number of IgG3 antibodies per red cell is large enough to allow detection by the antiglobulin test, there is always also an interaction with mononuclear phagocytes in vivo, although nearly always only in the spleen. In the case of IgG1, however, no increased haemolysis occurs when the number of antibody molecules per red cell remains below a minimum, indicating that in the case of IgG1 more antibody molecules per cell are necessary for interaction with mononuclear phagocytes than is necessary for detection in the antiglobulin test. We can as yet not offer a definite explanation for this difference between IgG1 and IgG3. We think that it may be due to a difference in affinity for the Fc receptor and further investigations will be undertaken to examine this possibility. Application of cytofluorometry will then be necessary to assess the exact amount of antibody of the various IgG subclasses present on the red cells.

Finally, since there are indications (Mackenzie, 1975; Kay & Douglas, 1977) that the activity of PBM, isolated from patients with AIHA towards EAIgG, in vitro is increased compared to that of PBM of normal controls, it will be worth while to perform similar longitudinal studies with the patients' red cells and their own mononuclear phagocytes.

notes



chapter VI
destruction of IgG-sensitized erythrocytes by granulocytes of patients suffering from acute infectious diseases

F.W. van der Meulen, G. Frijstein, E.C.E. Bruynes,
A.E.G. Kr. von dem Borne and C.P. Engelfriet

Department of Immunohaematology, Central Laboratory of the Netherlands Red Cross Blood Transfusion Service and the University Clinic of Internal Medicine, Dept. of Haematology, Binnengasthuis, Amsterdam, The Netherlands.

summary

The cytotoxic activity towards IgG anti-D-sensized red cells in vitro was investigated of granulocytes, monocytes and lymphocytes from patients with acute infectious diseases.

The cytotoxic activity of normal granulocytes was 10-15 times less than that of monocytes from the same donors; During infection, granulocytes showed a significantly enhanced cytotoxic capacity, which normalized after recovery of the patients to the level of activity exhibited by control granulocytes.

The cytotoxic lysis of sensitized red cells by monocytes remained unchanged during the inflammatory state. Lymphocytes did not show any cytotoxic activity towards anti-D-sensitized erythrocytes in our test system, neither during nor after the time of infection.

The results suggest that granulocytes may contribute to the increased red cell destruction observed in patients with autoimmune haemolytic anaemia during infections.

introduction

In patients with autoimmune haemolytic anaemia (AIHA) due to IgG non-complement binding autoantibodies, infectious disease may be associated with an exacerbation of the haemolytic process (Dacie, 1962; Leddy & Swisher, 1971). The increased haemolysis can be partly attributed to a rise in the titre of the autoantibodies, observed under these circumstances. On the other hand, 'activation' of the mononuclear phagocytic system, which may occur during infection (Kay & Douglas, 1977), might also be of importance, since evidence has accumulated that mononuclear phagocytes play an essential role in the destruction of red cells sensitized with non-complement binding IgG antibodies (EAIgG) (Brown, 1974; Engelfriet et al., 1974; Logue & Rosse, 1976; Meulen et al., 1978).

Adherence of the red cells to phagocytes by the Fc moiety of the sensitizing antibodies results in vitro in cytotoxic damage and lysis of the erythrocytes outside the phagocytic membrane. Most likely, this is an important mechanism of destruction of red cells sensitized with IgG antibodies in vivo (Fleer et al., in press,a,b,c). To study whether 'activation' of mononuclear phagocytes could be a contributory factor in the increased destruction of EAIgG during infection, the cytotoxic activity towards anti-D-sensitized red cells was determined of peripheral blood monocytes (PBM) isolated from the blood of patients with an acute infectious illness. The cytotoxic activity of granulocytes and lymphocytes was also investigated.

A very important aspect of the process of destruction of EAIgG anti-D in vitro is the inhibition by free IgG of the adherence to, and therefore the cytotoxic damage by, the phagocytes. This inhibition undoubtedly also influences this process in vivo. We therefore studied the inhibitory effect of free IgG on the cytotoxic lysis by free IgG of EAIgG by mononuclear phagocytes and granulocytes taken from patients during and after recovery of an acute infectious disease. It appeared that the cytotoxic activity of granulocytes towards EAIgG anti-D was significantly enhanced and less easily inhibited by IgG during the acute stage of infection. The activity of monocytes in this test system remained unchanged.

There was no indication at any stage that lymphocytes display cytotoxic activity towards red cells sensitized with IgG antibodies.

materials and methods

Selection of patients

Leukocytes were studied from patients suffering from an infectious disease as indicated by clinical signs and symptoms as well as by the results of laboratory examinations (Williams et al., 1972). Only those patients were included in this study in whom the aetiologic agent was identified by means of direct microscopy after staining, culture of the micro-organism or a rise in antibody titre. Blood samples were taken soon after admission to the hospital, during the acute stage of the disease and after complete clinical recovery.

White blood cells were isolated simultaneously from blood samples of the patients and from the blood of healthy volunteers, matched for age and sex. For a particular patient, the same volunteer served always as a control. In the blood samples from patients and controls, the total white cell count was determined as well as the differential cell count in May-Grünwald-Giemsa stained blood smears.

Preparation of leukocyte suspensions

Heparinized peripheral blood was mixed with dextran (5% w/v, M.W. 180,000; N.V. Poviet, Amsterdam, The Netherlands) and incubated for 45 min at 37°C to facilitate red cell sedimentation. The supernatant was removed, diluted with phosphate-buffered saline (PBS) and subjected to Ficoll-Isopaque density gradient centrifugation (d=1.077 g/cm^3, 20°C), followed by the isolation of the various cell types.

Isolation of monocytes

Part of the mononuclear cells collected from the interface layer of the Ficoll-Isopaque centrifugation were further purified by adherence to plastic petri dishes, as described in detail before (Meulen et al.,

1978). After incubation for 90 min at 37°C, the non-adherent cells were removed by two washings and the monolayer gently scraped off with a piece of silicone rubber. The suspension thus obtained contained 70-80% monocytes, the remainder being lymphocytes and less than 3% granulocytes, as determined by morphological differentiation and electronic sizing with a Coulter counter, model ZF, supplemented with a pulse height analyzer (Channelyzer, model C-1000) (Loos et al., 1976).

Isolation of lymphocytes

Another part of the mononuclear leukocytes obtained from the interface layer was treated with carbonyl iron to remove the monocytes. The suspension, thus prepared, contained less than 7% monocytes and about 1% granulocytes, as assessed by the above-mentioned methods.

Isolation of granulocytes

Granulocytes were recovered from the pellet after Ficoll-Isopaque centrifugation, and washed twice. Cytocentrifuged cell preparations were made with a Shandon Elliot apparatus (Shandon Southers Products Ltd., Runcorn, Cheshire, UK) (1000 rpm, 5 min), and according to microscopical examination (May-Grünwald-Giemsa staining) over 95% of the cells were granulocytes. The percentage stabs was also determined.

All leukocytes were suspended in Minimal Essential Medium (MEM) (Gibco, Paisley, Scotland), buffered with 25 mM Tris-HCl (pH 7.4 at 37°C) and supplemented with 10% (v/v) fetal calf serum (FCS) (Gibco, Biocult, Paisley, Scotland), penicillin (100 IE/ml) and streptomycin (100 µg/ml) (P/S).

Cytotoxicity assay

The cytotoxicity assay was performed as described by Fleer et al. (in press,a). Briefly, Rh D-positive red cells (OR_2R_2) were sensitized with an anti-D serum (titre in the indirect antiglobulin test 1:2000 with an anti-IgG serum) and labelled with $Na_2Cr^{51}O_4$ (Radiochemical Centre, Amersham, UK). The sensitized erythrocytes (EAIgG anti-D) were suspended in MEM-Tris containing 10% FCS and P/S, and adjusted to various

concentrations (1, 4 and 20×10^6/ml). Next, 50 µl of the leukocyte suspensions (10^6 leukocytes/ml) and 50 µl of the red cell suspension (1, 4 and 20×10^6 EAIgG/ml) were added to the wells of round-bottom microtiter plates (Greiner, Alphen a/d Rijn, The Netherlands) in combination with 100 µl MEM-Tris with 10% (v/v) FCS and P/S. The plates were incubated for 16 h at 37°C and then centrifuged. A sample of 100 µl of the supernatant was taken, its radioactivity was counted in a Packard Gamma spectrometer, and the percentage of ^{51}Cr-release determined. After correction for the spontaneous ^{51}Cr-release from labelled EAIgG incubated without leukocytes, the number of red cells specifically lysed by the leukocytes was calculated. For statistical reasons, the cytotoxic activity of the leukocytes is expressed in this paper as the sum of the number of red cells lysed at the various EAIgG:monocyte ratios, i.e. 1:1, 4:1 and 20:1. Inhibition by free IgG (Cohn fraction II; Cohn et al., 1940) was studied at an EAIgG: leukocyte ratio of 1:1.

In some experiments, cytochalasin B (ICI, Alderley Park, Cheshire, UK) dissolved in dimethyl sulfoxide (DMSO) was added to the wells in a final concentration of 1 µg/ml, and hydrocortisone sodium succinate (Organon, Oss, The Netherlands) was used in a final concentration of 5 mM (Fleer et al., in press,c).

results

A survey of the patients included in this study is given in Table I. In all cases but one, the infection was bacterial. The total number of white blood cells and the differential cell count of the blood of all healthy controls were within the normal limits defined by Williams et al. (1972).

Cytotoxic activity of monocytes

Monocytes isolated from patients in the acute stage of the infection had no significantly increased cytotoxic activity towards IgG-sensitized red cells, as compared to the activity of monocytes from the same individuals obtained after recovery ($p>0.05$, Student's t-test for unpaired observations) or obtained from normal

TABLE I

Survey of the patients included in the study and results of laboratory examinations

Age	Sex	Diagnosis	Total white cells $x10^9/l$	Differential cell count (%) stabs	PMN	lympho	mono	eosinoph. and basoph.	
36	male	campylobacterenteritis	9.5	9	43	29	5	4	acute stage
			6.3	2	50	42	4	2	recovery
48	male	influenza pneumonia	10.7	1	70	26		3	
			3.8		71	27	2		
20	female	Hinfluenza pneumonia	10.2	8	75	10	5	2	
			6.2	4	57	41	3	4	
59	female	salmonella paratyphi enteritis	7.8	2	68	28	2		
			5.7	2	54	40	3	1	
73	male	Klebsiella pneumonia	13.6	27	52	17		4	
			4	3	72	23		2	
42	male	pneumococcus meningitis	16.3	18	61	18		3	
			4.6	2	57	39		2	
52	female	E.coli septicaemia	18	43	47	9		2	
			5.5	4	68	27		1	
43	female	E.coli septicaemia	6.7	1	52	42		5	
			4.8		78	26		6	
68	male	pneumococcus bronchopneumonia	6.0	18	62	16		4	
			5.2	3	72	25		0	

Of each patient, values of examinations performed in the acute stage (upper line) and after recovery (lower line) are given.

controls ($p>0.05$, Student's t-test for paired observations (Fig. 1). Neither was there a difference in the inhibition by free IgG in the acute stage ($p>0.05$, Student's t-test for paired observations) (Table II).

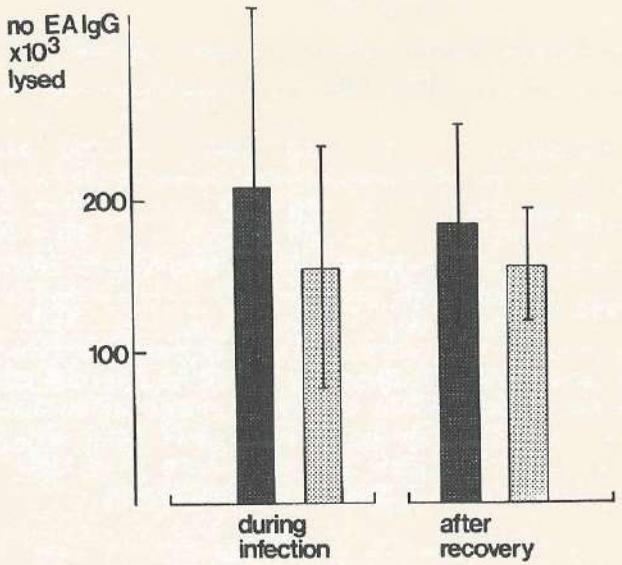


Fig. 1 - Lysis of EAIgG by monocytes of patients during infection and after recovery, in comparison to the number of red cells, lysed by monocytes of healthy volunteers. Ordinate: sum of the number of EAIgG anti-D lysed at EAIgG:monocyte ratios of 1:1, 4:1 and 20:1. Mean ± SEM of 7 experiments.

■: patients;

▒: controls.

Cytotoxic activity of granulocytes

Normal granulocytes showed about 10-15 times less activity towards EAIgG anti-D than did monocytes from the same individuals. During the acute infectious period, a significantly higher number of sensitized red cells were lysed by the patients' granulocytes when compared with the period after recovery ($p<0.05$, Student's t-test for unpaired observations).

TABLE II

Percentage inhibition of cytotoxic lysis caused by free IgG

IgG concentration		10 µg/ml	100 µg/ml
Monocytes of	patients	58 ± 23	76 ± 19
	controls	70 ± 30	88 ± 28
Granulocytes of	patients	79 ± 32	n.d.
	controls	100	n.d.

The cells were obtained from the blood of healthy controls and from patients in the acute stage of an infection. The inhibition by IgG is expressed as a percentage of the lysis of EAIgG anti-D without free IgG. n.d. = not done. Mean ± SEM of 6 experiments.

This enhanced activity was also statistically significant in comparison to the cytotoxic capacity of normal volunteers ($p<0.05$, Student's t-test for paired observations (Fig. 2). In the acute inflammatory state, the susceptibility of the granulocytes to inhibition by IgG was significantly diminished ($p<0.05$, Student's t-test for paired observations) (Table II).

Cytotoxic activity of lymphocytes

When lymphocyte-rich suspensions of patients and of healthy individuals were incubated with IgG-sensitized red cells, this led to a very low level of cytotoxicity in comparison with the monocyte-rich suspensions (Table III). Thus, lymphocytes from patients and from healthy donors, if they have cytotoxic activity towards EAIgG at all, are far less active than mononuclear phagocytic cells.

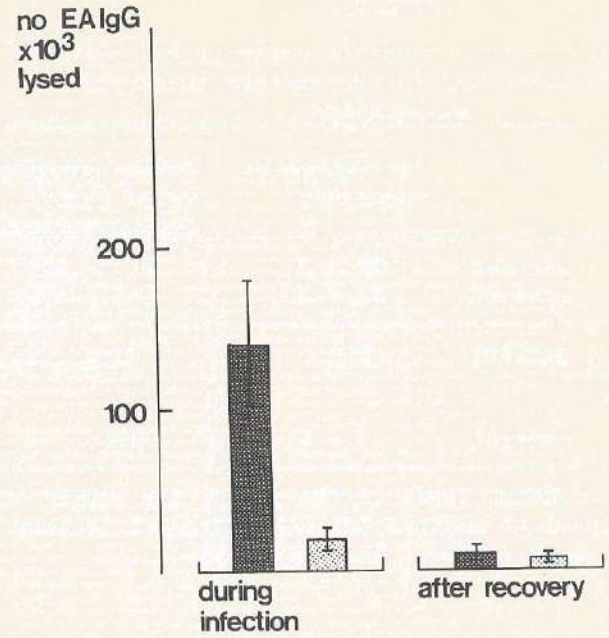


Fig. 2 - Lysis of EAIgG by granulocytes of patients during infection and after recovery, in comparison to the number of red cells lysed by granulocytes of healthy volunteers. Ordinate: sum of the number of EAIgG anti-D lysed at EAIgG:leukocyte ratios of 1:1, 4:1 and 20:1. Mean ± SEM of 9 experiments.

▓: patients;

▒: controls.

Effects of cytochalasin B and hydrocortisone on granulocyte cytotoxicity

In three patients, the influence of cytochalasin B (1 µg/ml) and hydrocortisone (5 mM) on the cytotoxic activity of granulocytes was tested in the acute stage

TABLE III

Relation between the cytotoxic activity and the percentage of monocytes in mononuclear leukocyte suspensions

Mononuclear leukocytes		Percentage of monocytes	Number of EAIgG lysed ($x10^3$) during infection
Adherent	patient	69 ± 5	211 ± 122
	control	71 ± 5	144 ± 85
Iron-treated mononuclear leukocytes	patient	5 ± 1	23 ± 3
	control	5 ± 2	20 ± 3

The number of EAIgG lysed is the sum of the number of red cells lysed at various EAIgG:mononuclear leukocyte ratios. Mean ± SEM of 5 experiments.

of the disease. As shown in Fig. 3, cytochalasin B clearly enhanced the cytotoxic capacity of the patients' granulocytes, whereas hydrocortisone markedly decreased the red cell lysis.

The influence of these drugs on the cytotoxic activity of granulocytes from healthy donors is depicted in the same figure. Hydrocortisone abolished the cytotoxic lysis, whereas cytochalasin B had no significant effect.

Relation between the number of stabs and cytotoxic activity

Since many stab forms were present in the granulocyte suspensions of the patients, obtained in the acute inflammatory stage, and since the leukocytes in these suspensions showed a marked cytotoxic activity, the relation between the percentage of stabs and the number of lysed red cells was studied. It follows

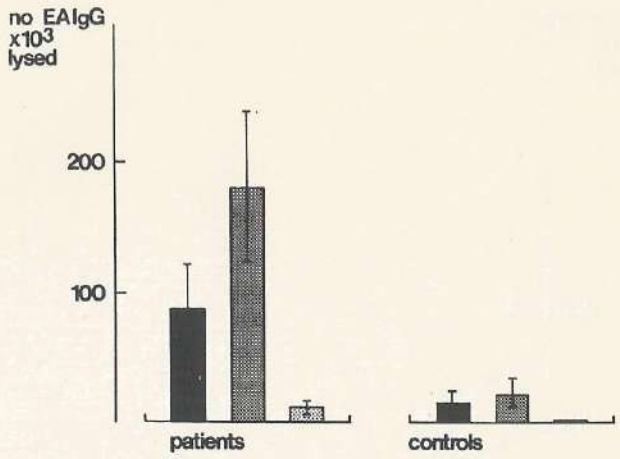


Fig. 3 - Influence of cytochalasin B and hydrocortisone on cytotoxic lysis of EAIgG by granulocytes of patients (left) and of healthy volunteers (right). Ordinate: sum of the number of EAIgG anti-D lysed at EAIgG:leukocyte ratios of 1:1, 4:1 and 20:1. Mean ± SEM of 3 experiments.

■: no drugs added;

▒: cytochalasin B added (1 µg/ml);

▒: hydrocortisone added (5 mM).

from Fig. 4 that the two parameters do not show a significant correlation (correlation coefficient: r^2 = 0.13).

discussion

The capacity of leukocytes of patients suffering from acute infectious diseases to lyse EAIgG in vitro was investigated in order to determine whether these cells show an enhanced cytotoxic activity towards IgG anti-D-sensitized red cells.

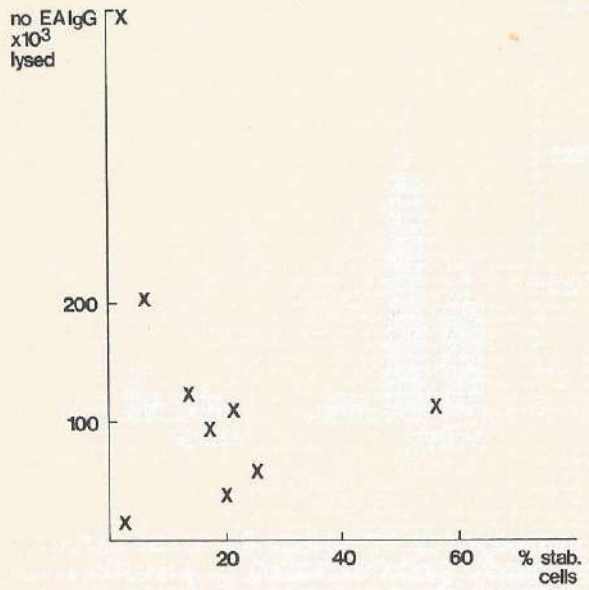


Fig. 4 - Relation between percentage stab forms and number of EAIgG anti-D lysed. Ordinate: sum of the number of EAIgG anti-D lysed at EAIgG:leukocyte ratios of 1:1, 4:1 and 20:1; abscissa: percentage stab forms present in granulocyte suspensions incubated with EAIgG anti-D.

The findings of others (Holm, 1972; MacDonald et al., 1975; Gill et al., 1977) that granulocytes of healthy individuals show cytotoxic activity towards IgG-sensitized erythrocytes, was confirmed by our study, although anti-D-sensitized red cells were not lysed very effectively by granulocytes of normal persons. However, we measured a marked increase in the lytic activity of granulocytes isolated from patients with an acute infectious disease. Moreover, the granulocytes were less susceptible to inhibition by free IgG in the 'activated state than after recovery. This may implicate that the number of 'Fc receptors' on

the granulocyte membrane increases during an infectious disease. Possibly, there is a relation between this finding and that of Verheugt et al. (in press), who found that the direct antiglobulin test (DAGT) with anti-immunoglobulin serum often is positive in patients suffering from an infection. Together, this suggests that during an acute inflammatory illness, granulocytes may contribute to the destruction of red cells sensitized with non-complement binding IgG autoantibodies in vivo.

Whether the enhanced activity of granulocytes can be explained as being due to increased binding of EAIgG to the granulocytes in vitro, or whether it is due to a more severe damage of the adherent red cells, has not yet been investigated. The increase of cytotoxic activity of granulocytes during infection contrasts with the decreased bactericidal capacity of such cells (McCall et al., 1971; Soelberg and Hellum, 1972), indicating that the two cell functions depend on different metabolic processes.

The cytotoxic activity was stimulated by cytochalasin B and depressed by hydrocortisone. These drugs respectively enhance (Hawkins, 1973; Goldstein et al., 1975) and decrease (Wright & Malawista, 1973; Hawkins, 1974) lysosomal enzyme release. Our results lead to the hypothesis that lysosomal enzyme release is an important event in extracellular cytotoxic lysis by granulocytes, as has also been shown for the destruction of EAIgG by monocytes (Fleer et al., in press,c). To find further support for this assumption, studies are required on lysosomal enzyme release provoked by the incubation of granulocytes with EAIgG anti-D in vitro.

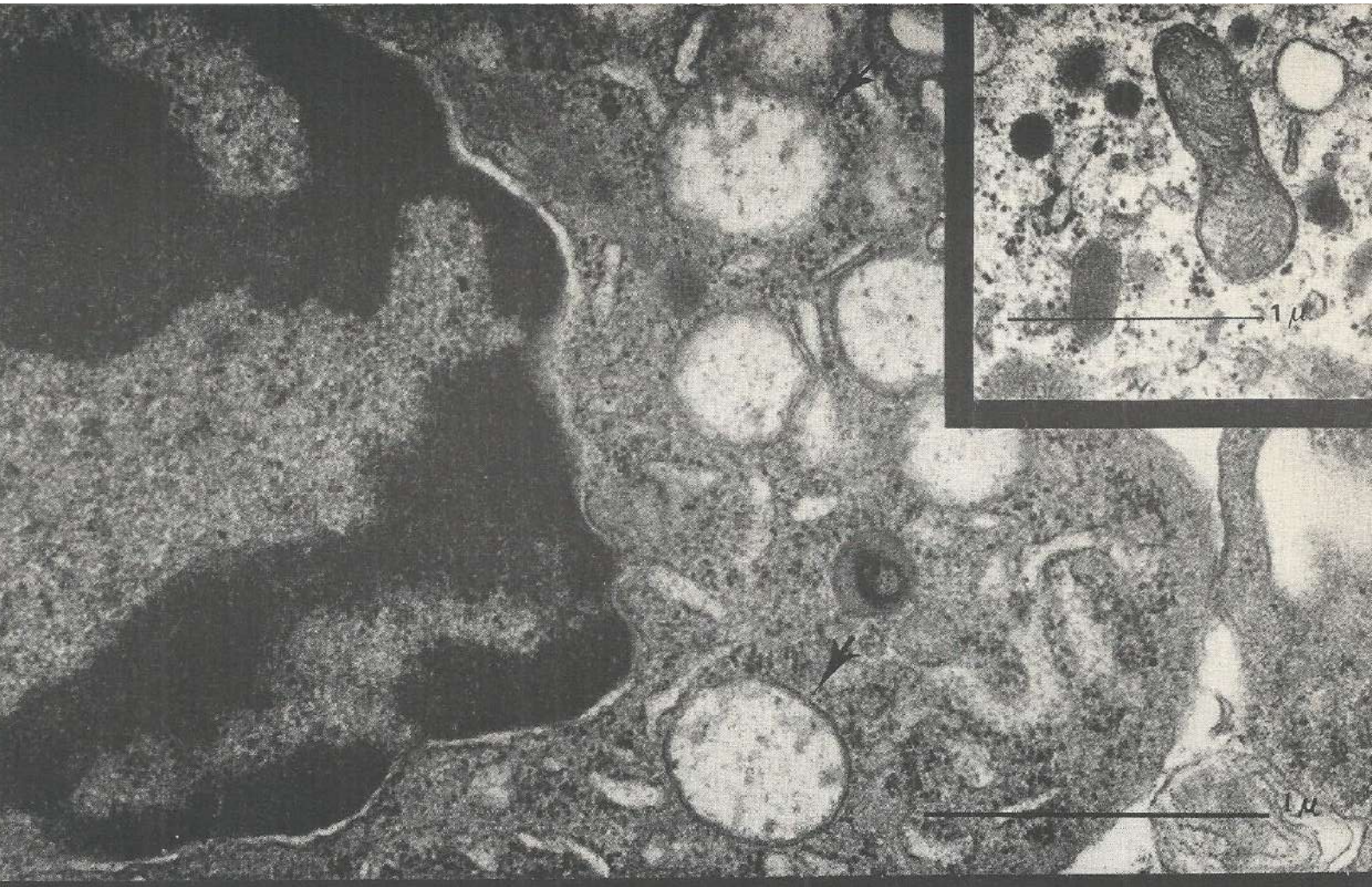
Macrophages can be activated by various bacterial stimuli (Kay & Douglas, 1977) and in that state show an enhanced capacity to interact with IgG-sensitized red cells in vivo (Atkinson & Frank, 1974) and in vitro (Rhodes, 1975; Mørland & Kaplan, 1977). Instead of macrophages, we investigated monocytes, which may be considered as relatively immature mononuclear phagocytes moving to tissues and inflammatory sites (Meuret & Hoffmann, 1973; Meuret et al., 1975).

Neither the cytotoxic activity of the monocytes nor its inhibition by free IgG was changed during infection. Possibly, this is due to the functional

immatureness of these cells.

Like others (Holm & Hammarström, 1973; MacDonald et al., 1975; Zeijlemaker et al., 1975; Fleer et al., in press,c), we found no indication that lymphocytes from normal individuals or from patients with infectious diseases are capable of lysing human red cells sensitized with IgG non-complement binding antibodies although this may depend on the test system used. The cytotoxic activity of the lymphocyte preparations we measured may be attributed most probably to the contaminating monocytes.

notes



chapter VII
cryopreservation of human monocytes

F.W. van der Meulen, M. Reiss, E.A.M. Stricker, E. van Elven and A.E.G. Kr. von dem Borne

Central Laboratory of the Netherlands Red Cross Blood Transfusion Service and Laboratory of Experimental and Clinical Immunology, University of Amsterdam, Amsterdam, The Netherlands

summary

Monocytes were isolated from human peripheral blood by means of Ficoll-Isopaque density gradient centrifugation and adherence to glass. These cells were then frozen according to an automatically controlled cooling programme and stored in liquid nitrogen.

After freezing, thawing and washing, 63% of the cells present before cryopreservation were recovered. Over 95% of the recovered cells excluded trypan blue. Storage at -196°C did not alter the percentage of monocytes (70-80%) in the supensions.

Although the percentage of cells forming rosettes with erythrocytes sensitized with IgG antibodies (EAIgG) was unaltered after freezing, EA-rosette formation was more readily inhibited by free IgG. The capacity of monocytes to lyse EAIgG was not influenced by cryopreservation, in contrast to their potency to phagocytize zymosan particles, which was decreased. The chemotactic response towards casein was also diminished after freezing. There was no significant difference in reactivity between monocytes frozen for a short time (2-15 h) and those frozen for a longer period (more than 3 months).

Electron microscopical pictures showed alterations in mitochondrial structure of the frozen cells.

introduction

Mononuclear phagocytes play a substantial role in host defense and appear to take part in many immunological processes. Therefore, monocytes are intensively studied and the development of monocyte function tests is in full progress (van Furth, 1975; Cline, 1978).

Since monocytes of different normal individuals show a great variation in activity in several test systems, it would be advantageous to have a fixed and well-defined test batch of these cells at one's disposal. Cryopreservation -with conservation of monocyte functional properties- could provide this facility.

Also, when variations in the function of the monocytes of one individual are the subject of investigation, e.g. in longitudinal studies, long term storage of monocytes can be equally important. In that way, one would be able to test on one day samples taken at various times.

Finally, the possibility to preserve monocytes in large quantities would mean a gain of time: the thawing of monocytes takes considerably less time than repeatedly isolating these cells from small amounts of peripheral blood.

In our laboratory a method of programmed freezing and storage in liquid nitrogen was tested on lymphocytes by Eijsvoogel et al. (1973). In the present preliminary study we tested the effect of cryopreservation, according to this programme, on several human monocyte functions in vitro.

materials and methods

Isolation of monocytes

Citrated blood (500 ml) of healthy volunteers was collected. After centrifugation, (5 min, 1000 g at 20°C) plasma and cells were separated; 50 ml of the upper layer (buffy coat) of the packed blood cells was collected. Mononuclear leukocytes were isolated by centrifugation of the buffy coat on a Ficoll-Isopaque layer (d=1.077 g/cm^3 at 20°C). The cells collected from the interface layer were washed and resuspended to a concentration of 10^7 cells/ml in MEM (Gibco,

Paisley, Scotland) buffered with 25 mM Tris-HCl (pH 7.4 at 37°C) and supplemented with 20% (v/v) heat-inactivated fetal calf serum (FCS) (Gibco, Bio-Cult, Paisley, Scotland), penicillin (100 IE/ml) and streptomycin (100 µg/ml) (P/S). The cells were layered on glass Petri dishes ($6x10^5$ cells/cm^2) and incubated at 37°C for 90 min. Non-adherent cells were removed by two washings. Adherent cells were scraped off with a piece of silicone rubber, washed and resuspended in MEM-Tris, containing 10% (v/v) FCS and P/S.

The final suspensions contained 70-80% monocytes, the remainder being lymphocytes and less than 3% granulocytes as determined by morphologic differentiation and electronic sizing with a Coulter Counter, model ZF, supplemented with a pulse height analyzer (Channelyzer, model C-1000) (Loos et al., 1976). Cell counting was performed with a Coulter Counter.

Preparation of monocytes for freezing

The cells were resuspended in MEM-Tris, containing 20% (v/v) FCS and P/S. The cell suspension was kept on melting ice and slowly diluted with an equal volume of ice-cold MEM-Tris, containing 20% (v/v) dimethyl sulfoxide (DMSO) (Baker Chemicals, B.V., Deventer, The Netherlands), 20% (v/v) FCS and P/S, leading to a final concentration of the cryoprotectant of 10% (v/v). The final cell concentration varied from $5-10x10^6$ cells/ml. The monocyte suspension was put into 2 ml glass ampoules (2 ml per ampoule) and kept on ice. The ampoules were then flame-sealed and transferred to the freezer.

Cooling and storage equipment

As described earlier in detail (du Bois et al., 1976), we used a Cryoson BV 4 automatic biological freezer in conjunction with a Cryo Diffusion RBP 200 VLR liquid nitrogen pressure vessel.

Frozen cells were stored in a liquid nitrogen storage vessel (Union Carbide Refrigerator LNR-250 or Cryo Diffusion CF-400), in which the ampoules were kept under the liquid nitrogen level (-196°C).

Cooling procedure

The cell suspensions were cooled at a rate of 1.4°C/min -as measured within the ampoules- until a

temperature of -30°C was reached. In the ampoules a linear cooling rate was obtained by compensating the heat of crystallization by a transiently increased cooling rate in the freezer. From -30°C until -100°C cooling was continued at a rate of 6-7°C/min. Thereafter, the ampoules were transferred to the storage vessel.

Thawing

Thawing was performed by immediate immersion of the ampoules in a waterbath at 37°C under continuous shaking. Once the ice nucleus had disappeared, the suspension was slowly diluted tenfold with cold MEM-Tris, containing 20% (v/v) FCS and P/S. The cells were then washed, resuspended in MEM-Tris containing 10% (v/v) FCS and P/S, and counted.

EA-rosette formation and inhibition

Sensitized erythrocytes (EAIgG) were prepared by incubating rh D-positive red cells (OR_2R_2) with an incomplete anti-D serum. After an incubation for 60 min at 37°C the cells were washed three times and resuspended in MEM-Tris.

For the rosette tests, 150 µl of MEM-Tris containing 10% (v/v) FCS and P/S, 100 µl of the monocyte suspension (concentration 2.5×10^6 monocytes/ml) and 50 µl of the red cell suspension (concentration 10^8 EAIgG/ml) were mixed, centrifuged at 160 g during 8 min and incubated for 15 min at room temperature. Thereafter, 25 µl of a solution, containing 4 µg of acridine orange/ml (E. Gurr Ltd., London, UK) in phosphate (5.8 mM)-buffered saline (140 mM NaCl)) (PBS), was added and the cells were carefully resuspended. The percentage of rosette-forming cells was determined with a Leitz Orthoplan fluorescence microscope, according to Brostoff (1974).

Inhibition of EA-rosette formation (Huber and Fudenberg, 1968) was established by adding increasing amounts of plasma IgG Cohn fraction II (Cohn et al., 1940) to the suspensions.

Cytotoxic lysis of EAIgG

The capacity of monocytes to damage sensitized red cells was measured as described by Fleer et al. (in press[a]).

In short, EAIgG were prepared as described above and labelled with $Na_2{}^{51}CrO_4$ (Radiochemical Centre, Amersham, UK). The cells were washed and resuspended at various concentrations in MEM-Tris with 10% FCS and P/S. In wells of round-bottom microtitre plates (Greiner, Alphen a/d Rijn, The Netherlands), 50 µl of the monocyte suspension (10^6 monocytes/ml) and 50 µl of the red cell suspension (0.5 to 16×10^6 EAIgG/ml) were added to 50 µl MEM-Tris containing 10% (v/v) FCS and P/S. The plates were incubated for 16 h at 37°C and then centrifuged. A sample of 100 µl of the supernatant was taken; the radioactivity was counted in a Packard gamma spectrometer and the percentage of ^{51}Cr-release determined. After correction for the spontaneous ^{51}Cr-release from labelled EAIgG's, incubated without monocytes, the number of red cells specifically lysed by the monocytes was calculated.

This antibody-mediated cytotoxic lysis could be inhibited by small amounts of plasma IgG. ^{51}Cr-release in the presence of different concentrations of IgG was determined.

Phagocytosis of serum-treated zymosan (STZ)

Zymosan particles were labelled with ^{125}I (lactoperoxidase method) and opsonized by serum treatment as described before (Reiss and Roos, 1978).

For the determination of adherence plus ingestion, monocytes suspended in MEM-Tris containing 5% (v/v) FCS and P/S (2×10^6 monocytes/ml) were incubated with the ^{125}I-STZ in a particle-to-cell ratio of about 6. At intervals, samples of 0.3 ml of the incubation mixture were added to 1 ml of ice-cold PBS, containing sodium fluoride (10 mM) and human serum albumin (0.5 g/100 ml). The mixture was then centrifuged on an ice-cold Ficoll-Isopaque layer of high density to separate the free particles from the cells. The radioactivity in the interface (expressed as a percentage of the total radioactivity) represented cell-associated STZ, i.e. adherent plus ingested particles. In order to correct for the elution of radioactivity from particles, this percentage was also determined in blank incubations without cells, treated in exactly the same way (Reiss and Roos, 1978).

Chemotactic response

For the determination of the chemotactic response a modification of the method described by Zigmond and Hirsch (1973) was applied. Monocytes in Earle's salt solution, buffered with Hepes (25 mM) (200 µl of 10^6 monocytes/ml), were layered on a Sartorius cellulose nitrate filter, with a pore size of 12 µm (Sartorius Membranfilter G.m.b.H., Göttingen, W-Germany). Upon stimulation by casein (5 mg/ml), dissolved in Earle's salt solution beneath the filter, the monocytes migrated into the filter. After 70 min of incubation at 37°C the filters were fixed with alcohol, washed and stained. 'Random' migration in the absence of casein was measured as a control.

The chemotactic response was expressed as the microscopically determined distance over which the leading cell front had migrated into the filter. Each cell suspension was measured with 5 filters and 5 readings were taken per filter. The mean of these 25 values was taken as the chemotactic response.

Electron microscopy

Freshly prepared and cryopreserved monocytes were fixed in a paraformaldehyde-glutaraldehyde fixative according to Karnovsky (1965) and then postfixed in osmium tetroxide (Millonig, 1961). After dehydration, the cells were embedded in Epon. Ultrathin sections were cut with a Reichert Ultramicrotome. The sections were stained with uranyl acetate and lead citrate and examined with a transmission electron microscope (Philips EM-300). Photographs were taken on an electron microscope film (Kodak, 3¼ x 4").

results

Influence of cryopreservation on cell counts and viability

After freezing, thawing and washing, 62-64% of the cells present before cryopreservation were recovered (Table I). Long term storage (over 3 months) gave the same results as short time cryopreservation (2-15 h).

Over 95% of the cells, either fresh or frozen and thawed excluded trypan blue. The mean percentage of monocytes in the suspensions was not changed by the preservation procedure.

TABLE I

Influence of cryopreservation on cell counts, EA-rosette formation and chemotactic response

	fresh cells[a]	frozen[a] 2-15 h	frozen[a] >3 months
percentage of cells recovered (n=12)	'100'	62±5	64±5
percentage of cells excluding trypan blue (n=12)	97±3	94±4	95±3
percentage of monocytes in suspension (size distribution) (n=12)	74±5	75±5	73±4
percentage of EA-rosette forming cells (n=12)	72±5	76±5	74±3
migration towards casein[b] (n=6)	73±5	51±3	52±4
'random' migration[b] (n=6)	28±2	25±2	28±2

[a] mean ± SEM

[b] migration distance in µm

Influence of cryopreservation on EA-rosette formation and inhibition

The mean percentages of rosette-forming cells are given in Table I. When fresh and frozen samples were compared, no differences in rosette-forming capacity were noted.

As an alternative, we also tested the influence of cryopreservation on the inhibition of EA-rosette formation by free IgG. Fig. 1 shows the dose-response curves obtained with fresh and frozen monocytes of 6 different donors. Regression analysis (Dixon and Massey, 1969) showed that EA-rosette formation was more easily inhibited by IgG after cryopreservation than before ($p<0.05$). There was no significant difference between short and long term frozen samples.

Influence of cryopreservation on cytotoxic lysis of EAIgG

Fig. 2 shows the effect of cryopreservation on the cytotoxic action of monocytes towards red cells sensitized with IgG antibodies. With the method for unpaired observations, no significant difference between fresh and frozen samples was found ($p>0.1$, $n=12$).

In 7 experiments the inhibition of this cytotoxic lysis by IgG was investigated before and after freezing. In contrast to the findings with the EA-rosette inhibition, we did not find a significant difference in inhibition by IgG after cryopreservation (Fig. 3) (methods for unpaired observations, $p>0.1$).

Influence of cryopreservation on phagocytosis of serum-treated zymosan

Fig. 4 shows the influence of the preservation procedure on the capacity of monocytes to bind and ingest STZ particles. A significant decrease in uptake of STZ by cryopreserved monocytes was found with the Student's t-test for paired observations ($p<0.05$, $n=10$). Incubation of monocytes at 37°C in MEM-Tris, with 20% (v/v) FCS and P/S, up to 24 h did not restore the phagocytic capacity to the level of fresh cells.

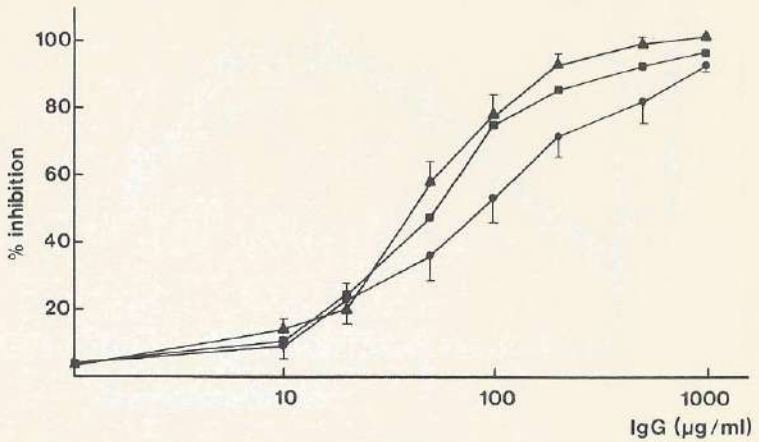


Fig. 1 - Influence of cryopreservation on IgG-induced inhibition of EA-rosette formation. Ordinate: percentage inhibition of adherence of EAIgG to monocytes in vitro, calculated in relation to the non-inhibited control. Abscissa: concentration of free IgG in the medium. Results are the mean ± SEM of 6 experiments. ●—●, fresh cells; ■—■, frozen cells (2-15 h); ▲—▲, frozen cells (>3 months).

Influence of cryopreservation on the chemotactic response

Table I shows that the directed migration of monocytes was influenced by cryopreservation in a different way than was the 'random' non-directed migration. Frozen monocytes of 6 donors migrated significantly less towards casein than did unpreserved cells. In contrast, 'random' migration remained unaltered after freezing. After prolonged cryopreservation (3 months), the monocytes behaved similarly to those thawed after 2-15 h.

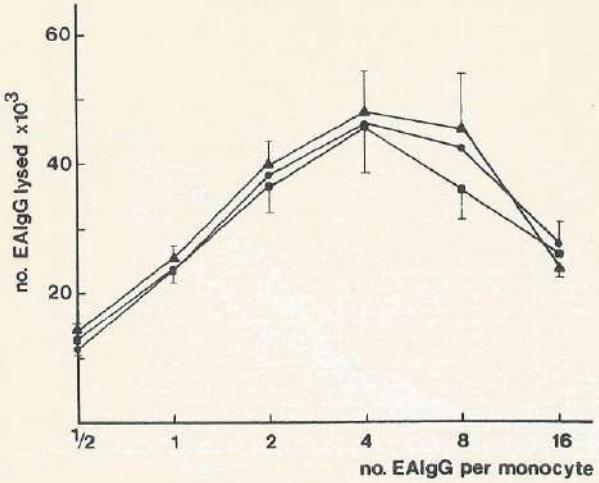


Fig. 2 - Influence of cryopreservation on cytotoxic lysis of EAIgG. To $5x10^4$ monocytes, varying numbers of EAIgG were added resulting in EAIgG:monocyte ratios of 0.5:1 to 16:1 (abscissa). Ordinate: number of EAIgG lysed ($x10^3$). The data presented are the mean ± SEM of experiments with monocytes from 12 donors.
●—●, fresh cells; ■—■, frozen cells (2-15 h); ▲—▲, frozen cells (>3 months).

Influence of cryopreservation on electron microscopical characteristics

The ultrastructure of freshly isolated monocytes was comparable to that described by Tanaka and Goodman (1972).

After freezing and thawing some monocytes were damaged; these cells showed disrupted plasma membranes and loss of cytoplasmic organelles. In the intact cells, some mitochondria were slightly swollen, as indicated by the arrow in the picture opposite the front page of this chapter. In the inset, an intact mitochondrion of a fresh monocyte is shown. The cytoplasm of the frozen cells was less electron-dense than that of the control cells and in the cytoplasm of the cryopreserved monocytes electron-dense particles were present.

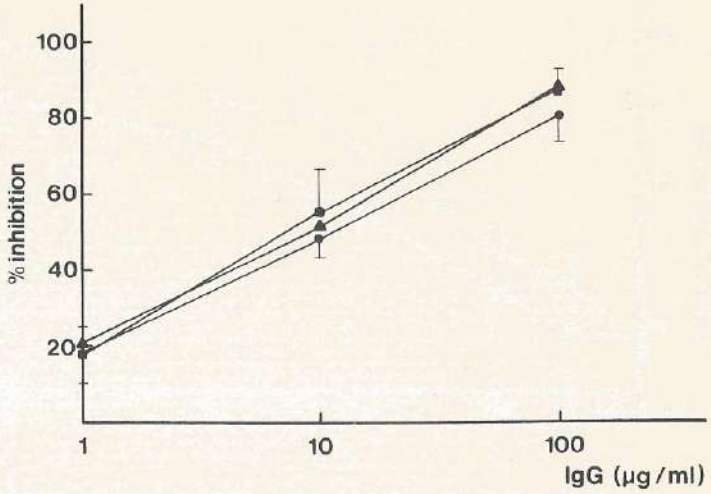


Fig. 3 - Influence of cryopreservation on IgG-induced inhibition of cytotoxic lysis of EAIgG. Abscissa: final concentration of IgG; ordinate: percentage inhibition of cytotoxic lysis at an EAIgG:monocyte ratio of 1:1. The dose-response curves from the mean ± SEM of 7 experiments are given.
●—●, fresh cells; ■—■, frozen cells (2-15 h); ▲—▲, frozen cells (>3 months).

discussion

In this study we have investigated the effect of an automatically controlled freezing programme on human blood monocytes. After freezing, 63% of the original cells were recovered. Trypan blue exclusion was equal to that of fresh monocytes, but this test only excludes severe membrane damage (Malanin, 1972; Harris and Griffiths, 1974). The unchanged capacity to form EA-rosettes also roughly indicates an intact membrane function. As an alternative measurement, we also determined the influence of cryopreservation on

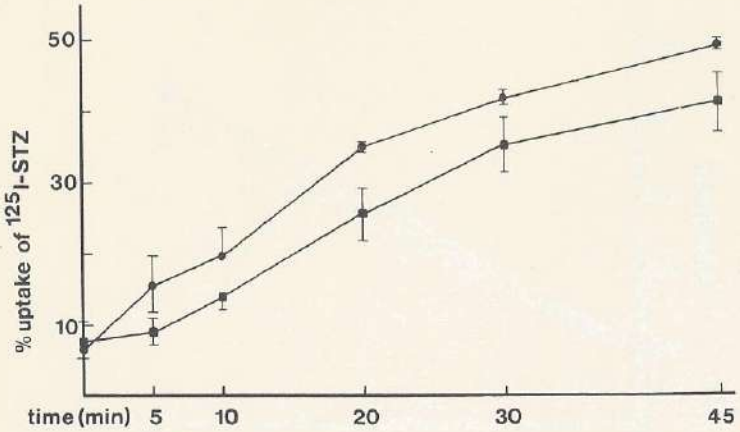


Fig. 4 - Influence of cryopreservation on adherence plus phagocytosis of ^{125}I-STZ. Ordinate: cell-associated radioactivity expressed as percentage of the total radioactivity per sample.
●—●, fresh cells; ■—■, frozen cells (2-15 h).

the inhibition of EA-rosette formation by free IgG. The dose-response curves showed that less free IgG was needed to inhibit red cell adherence to monocytes after cryopreservation than before. This indicates an alteration of membrane-binding characteristics and, although in our study no defects could be observed at the submicroscopical level, it is known that cryopreservation can cause membrane damage (Meryman et al., 1977).

No decrease in the cytotoxic activity towards EAIgG could be detected, nor was the IgG-induced inhibition of this process significantly altered. Since the cytotoxic activity of the monocytes is presented here as the number of red cells lysed and EA-rosette formation is expressed as the percentage of monocytes binding sensitized erythrocytes, the effects of IgG inhibition on the two assays are hardly comparable. Moreover, test variables (temperature, duration of the experiment) differed widely in both experimental systems. This antibody-mediated cytotoxicity is most

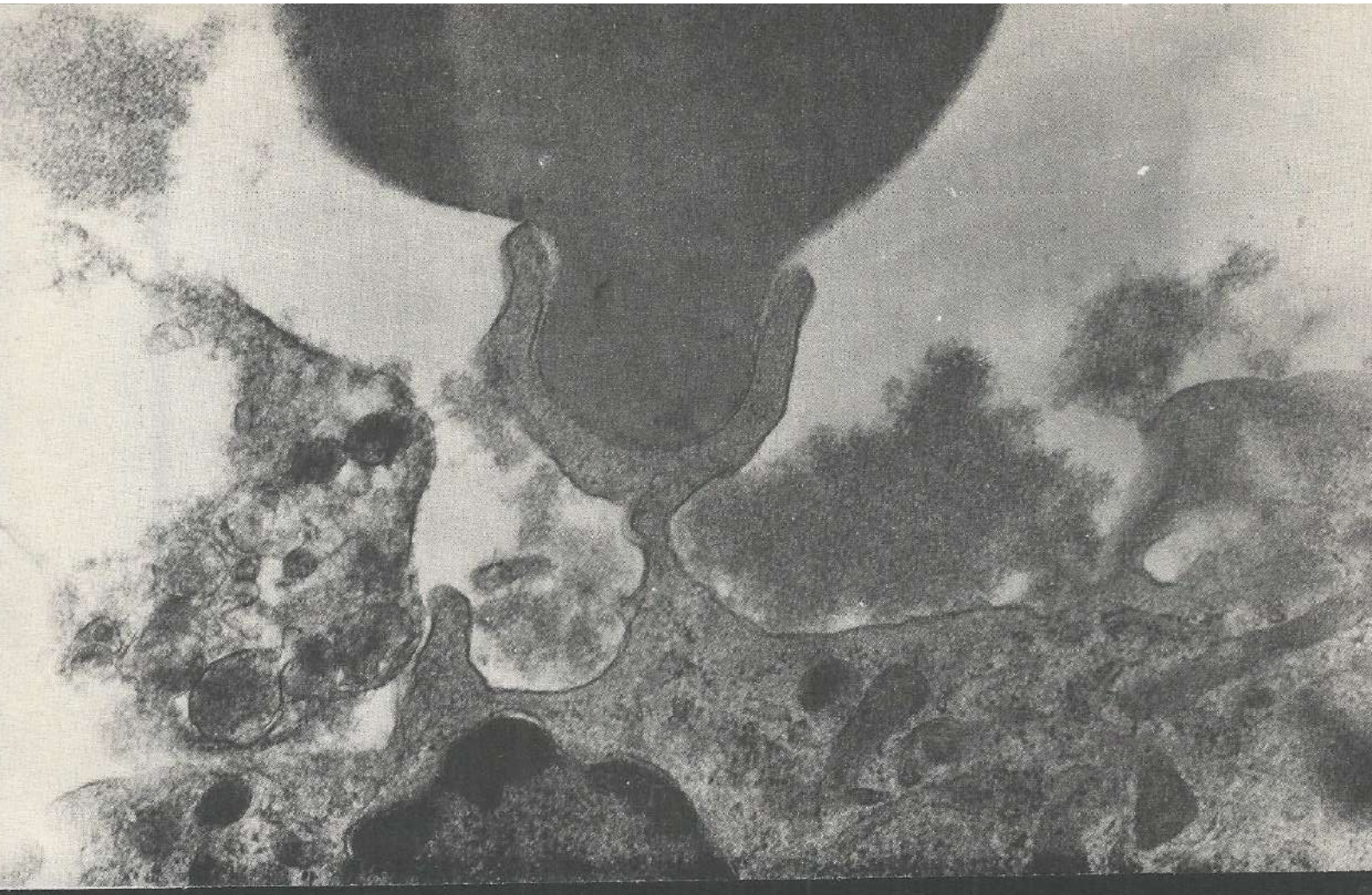
probably effectuated by lysosomal enzyme release (Fleer et al., in press[b]). Although cryopreservation may cause damage of lysosomes (Persidsky and Ellett, 1971), we did not detect abnormalities in these cell organelles in our electron microscopical observations.

We found a statistically significant decrease in the capacity to bind and phagocytize opsonized STZ particles. Since mitochondria are probably of major importance in the energy supply for the latter process (Reiss and Roos, 1978), this decrease might correspond with our electron microscopical findings that some mitochondria were slightly swollen and, therefore, perhaps invalidated. Since we found that the only assay involving prolonged incubation at 37°C (cytotoxicity towards EAIgG) was unaltered, we tried to restore the phagocytic capacity to pre-freezing levels by incubating the monocytes up to 24 h at 37°C after thawing. However, no functional recovery was achieved.

The chemotactic response was significantly reduced by our cryopreservation procedure, whereas 'random' migration remained unaltered. This is in contrast with the findings of Dean and Strong (1977), who reported a decrease in non-directed migration of human mononuclear cells after freezing. However, comparison of results published by various authors is hazardous since by many of them cells of different species were used, frozen according to different procedures and tested in different assays. Changes in freezing programmes may, for instance, influence various populations of cells in different ways (Farrant et al., 1972; Leibo and Mazur, 1971).

The results presented here were obtained by using one fixed freezing programme. To optimize recovery and monocytic function after cryopreservation, we are at present testing the effect of several variables, such as the isolation procedure of monocytes, the concentration of DMSO and of serum, the concentration of cells in the vials and the cooling rate.

notes



summary

This thesis deals with the question which factors determine whether or not increased destruction of red cells will occur in patients with IgG non-complement binding autoantibodies and secondly -in the case of increased red cell destruction due to these antibodies- by which mechanism the patients' red cells are destroyed in vivo.

As described in Chapter I, the subclass composition of IgG on red cells of patients with IgG non-complement autoantibodies was studied in relation to the occurrence of increased haemolysis in vivo and the adherence of these cells to peripheral blood monocytes (PBM) from healthy volunteers in vitro.

The presence of IgG3 autoantibodies was almost always accompanied by haemolytic anaemia, whereas only part of the patients with IgG1 autoantibodies showed signs of increased haemolysis. The presence of IgG2 and IgG4 autoantibodies was not associated with increased red cell destruction. An identical relation between subclass composition and increased haemolysis was found between subclass composition and adherence of the patients' erythrocytes to PBM; thus, a strong correlation appeared to exist between positive adherence in vitro and increased red cell destruction in vivo.

The above results strongly support the assumption that adherence of red cells sensitized with IgG non-complement binding autoantibodies to mononuclear phagocytes plays an important role in the destruction of these cells in vivo.

It was suggested -incorrectly as will be shown in Chapter IV- that IgG1 autoantibodies are of two kinds, only one of which causes adherence to phagocytes and thus increased red cell destruction.

In Chapter II, the importance of the subclass composition with regard to the occurrence of haemolysis once more is illustrated.

A patient is described who, notwithstanding a strongly positive direct antiglobulin test with anti-IgG serum, did not suffer from haemolytic anaemia. The survival of the patient's red cells, measured with ^{51}Cr, was only slightly decreased.

In vitro, the sensitized cells of the patient

showed only a weak adherence to monocytes. The patient's spleen functioned normally, since ^{51}Cr-labelled donor erythrocytes, either sensitized with IgG anti-D or damaged by heating, were eliminated from the circulation and sequestered in the spleen.

It appeared that the patient's red cells were sensitized mainly by IgG4, whereas only weak IgG1 and IgG3 autoantibodies were detectable.

In Chapter III the results are presented of the study, which factors influence the inhibition by free IgG of the interaction between red cells, sensitized with IgG non-complement binding anti-D alloantibodies (EAIgG anti-D) and PBM in vitro.

From the results, it became clear that at least two factors, i.e. the number of sensitized red cells per monocyte as well as the amount of antibody sensitizing the erythrocytes influence the susceptibility to inhibition by IgG. Thus, an increase in the EAIgG anti-D:monocyte ratio and an increase of the number of IgG antibody molecules on the red cell surface led to a decrease in inhibition of adherence and cytotoxicity in vitro.

In the spleen, the circumstances are such that the above factors are operative in vivo.

In Chapter IV, the finding that some patients with IgG1 autoantibodies do show signs of increased haemolysis in vivo, whereas other do not, is reconsidered. In particular, it was investigated whether this observation had to be explained by qualitative or quantitative differences of the sensitizing IgG1 autoantibodies. To that end, the relative amount of IgG1 antibody, present on the red cells of patients with autoantibodies of this subclass only, was measured by means of continuous flow cytofluorometry. This technique appeared to be suitable to compare the amount of antibody on red cells and it yielded reproducible results. The fluorescence intensity of the patients' red cells, obtained after incubation with a fluorescein isothiocyanate (FITC)-labelled anti-IgG1 serum, was studied in relation to the presence or absence of signs of increased haemolysis in vivo and in relation to the cytotoxic activity of normal monocytes towards these cells in vitro.

It appeared that predominantly the amount of IgG1

autoantibody rather than its quality determined whether or not these antibodies induced haemolysis in vivo or cytotoxicity of monocytes in vitro. This also was true with regard to IgG1 autoantibodies induced by methyldopa therapy.

Chapter V contains the results of a longitudinal study of patients with autoimmune haemolytic anaemia (AIHA) with non-complement binding warm autoantibodies, in which the course of the disease and the effect of treatment were studied in relation to the subclass composition and the relative amount of IgG antibody detectable on the patients' red cells, obtained from consecutively collected blood samples.

The adherence of the patients' red cells to PBM of healthy volunteers and the subsequent cytotoxic lysis of these erythrocytes by the phagocytes in vitro were also determined.

To determine the relevance of the cytotoxicity test as a parameter in AIHA, first of all the relation between the occurrence of increased haemolysis in vivo and the degree of cytotoxicity in vitro was studied.

The finding that red cells from patients with signs of increased red cell destruction in vivo were lysed in vitro more effectively than those obtained from patients without such signs strongly support the assumption that in AIHA extracellular cytotoxic lysis exerted by mononuclear phagocytes is an important mechanism of destruction of red cells sensitized with IgG non-complement binding autoantibodies in vivo.

On the red cells of the patients, who responded well to corticosteroid therapy, a significant decrease of the amount of IgG antibody was detectable. This was associated with a diminished adherence and cytotoxicity in vitro.

In some cases, the IgG subclass composition was also altered in the sense that antibodies of certain subclasses became undetectable. In the patients with IgG3 autoantibodies, improvement coincided with the disappearance of this subclass. The adherence and cytotoxic lysis in vitro decreased concomitantly. Recovery or improvement of the patients with IgG1 autoantibodies was accompanied by a decrease of the number of IgG1 molecules as well as by a decreased interaction between the patients' erythrocytes and the

mononuclear phagocytes in vitro. The presence of IgG2 and IgG4 autoantibodies was not associated with increased red cell destruction in vivo, nor did it lead to binding and lysis of these erythrocytes by PBM in vitro.

Moreover, our results indicated that corticosteroids probably also affect the function of mononuclear phagocytes in vivo. On the basis of several cases, different reactions to therapy are discussed.

In Chapter VI, data are presented concerning the cytotoxic activity in vitro towards IgG-sensitized red cells exerted by granulocytes, monocytes and lymphocytes from patients suffering from an acute infectious disease.

During the infection, granulocytes showed a significantly enhanced cytotoxic capacity, which after recovery became equal to the rather low level of activity exhibited by granulocytes from normal controls. The cytotoxic lysis of sensitized red cells by monocytes remained unchanged during the inflammatory state; lymphocytes did not show any cytotoxic activity towards anti-D-sensitized erythrocytes in our test system.

The results suggest that granulocytes may contribute to the increased red cell destruction observed in patients with AIHA during infections.

Chapter VII, finally, presents the results of the study in which the influence of cryopreservation of PBM on recovery and function of these cells was determined.

PBM were frozen according to an automatically controlled cooling programme and stored in liquid nitrogen. After freezing, thawing and washing, 63% of the cells present before cryopreservation were recovered. Over 95% of the recovered cells excluded trypan blue. Storage at $-196^{\circ}C$ did not alter the percentage of monocytes (70-80%) in the suspensions.

Although the percentage of cells forming rosettes with EAIgG anti-D was unaltered after freezing, EA-rosette formation was more readily inhibited by free IgG. The capacity of monocytes to lyse EAIgG anti-D was not influenced by cryopreservation, in contrast to their potency to phagocytose zymosan particles, which was decreased. The chemotactic response towards ca-

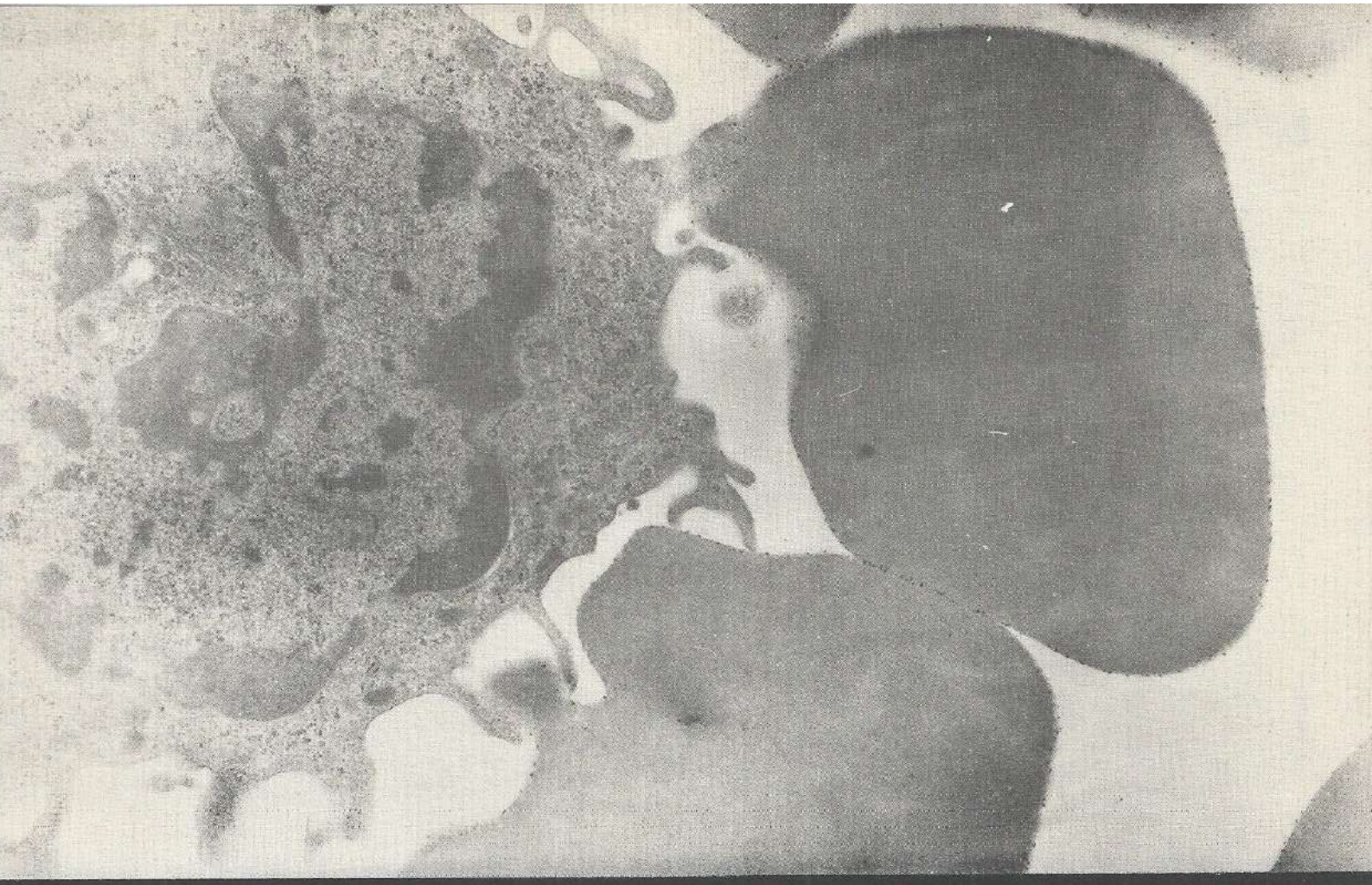
sein was also diminished after freezing. There was no significant difference in reactivity between monocytes frozen for a short time (2-15 h) and those frozen for a longer period (more than 3 months). Electron microscopical pictures showed alterations in mitochondrial structure of the frozen cells.

The above results indicated that cryopreservation of PBM, albeit with some changes in function, allows long-term storage of these cells. In part of the investigations, described in Chapters IV and V, cryopreserved PBM were used.

The results of the studies described in this thesis strongly suggest that in AIHA red cells sensitized with IgG non-complement binding autoantibodies are destroyed as a result of adherence of these cells to and subsequent lysis by mononuclear phagocytes in vivo.

Furthermore, the subclass composition as well as the amount of IgG autoantibody present on the patients red cells seems to determine whether or not the inhibition by free IgG in vivo can be overcome and thus whether or not increased haemolysis will occur. From this point of view, the finding that the presence of IgG3 autoantibodies nearly always is associated with haemolytic anaemia suggests that IgG antibodies of this subclass, when bound to the red cells, prevent rather easily the inhibition, whereas IgG2 and IgG4 autoantibodies do not seem to take actively part in this process. The effect of IgG1 autoantibodies clearly depends on the amount of antibody on the red cells.

Since the destruction of erythrocytes mainly takes place in the spleen, the special environment in this organ favours this process especially with regard to the phenomenon of inhibition by free IgG.



samenvatting

In dit proefschrift wordt de vraagstelling behandeld welke faktoren bepalend zijn voor het al dan niet optreden van verhoogde bloedafbraak in patiënten met IgG niet-complement bindende auto-antistoffen en -zo er sprake is van verhoogde bloedafbraak door deze antistoffen teweeggebracht- via welk mechanisme deze versterkte haemolyse wordt geëffektueerd.

Zoals uiteengezet in Hoofdstuk I, werd de relatie bestudeerd tussen de aanwezigheid van de verschillende IgG subklassen op de erytrocyten van patiënten met bovengenoemde auto-antistoffen en het al of niet optreden van verhoogde bloedafbraak in vivo. Bovendien werd onderzocht of the patiënte-erytrocyten in vitro gebonden werden door monocyten, welke geïsoleerd waren uit het bloed van gezonde donoren.

Het bleek, dat de aanwezigheid van IgG3 auto-antistoffen vrijwel altijd gepaard ging met het bestaan van een haemolytische anaemie, terwijl de aanwezigheid van IgG1 auto-antistoffen in sommige patiënten wel, maar in andere niet was geassocieerd met symptomen, veroorzaakt door een verkorting van de overlevingsduur van erytrocyten. Patiënten met IgG2 en IgG4 auto-antistoffen vertoonden geen tekenen van verhoogde bloedafbraak en hun erytrocyten werden in vitro niet gebonden aan monocyten.

Het verband, dat gevonden werd tussen de adherentie van patiënte-erytrocyten aan monocyten en de aanwezigheid van de onderscheiden subklassen, bleek identiek te zijn aan het verband tussen de subklasse samenstelling en het voorkomen van haemolytische anaemie. Dit impliceerde, dat er een sterke relatie werd gevonden tussen de aanwezigheid van verhoogde bloedafbraak in de patiënt en de binding van patiënte-erytrocyten aan monocyten in vitro, terwijl bij afwezigheid van verhoogde bloedafbraak ook geen binding in vitro waarneembaar was. Deze resultaten ondersteunen de veronderstelling, dat adherentie van met inkomplete IgG auto-antistoffen beladen erytrocyten aan mononucleaire fagocyten een belangrijke rol speelt in de afbraak van deze rode bloedcellen in patiënten met AIHA.

Voorts werd gesuggereerd, dat IgG1 auto-antistoffen verder onder te verdelen zouden zijn in 2 typen, waarvan slechts één binding aan mononucleaire fagocyten en daarmee verhoogde bloedafbraak zou kunnen bewerkstelligen. In Hoofdstuk IV wordt de onjuistheid van deze gedachte aangetoond.

In Hoofdstuk II wordt een patiënt beschreven op wiens erytrocyten IgG4 auto-antistoffen aantoonbaar waren in hoge titer, terwijl deze zelfde rode cellen slechts zwak werden geagglutineerd door anti-IgG1 en anti-IgG3 serum in de direkte antiglobuline test. Aangezien de overlevingsduur van de patiënte-erytrocyten, bepaald met ^{51}Cr, slechts gering verkort was en in vitro maar een zeer zwakke binding aan monocyten werd waargenomen vormt dit andermaal een bevestiging van het belang van de subklassen in verband met het al of niet optreden van verhoogde bloedafbraak. De milt van deze patiënt funktioneerde normaal, aangezien donor-erytrocyten, gesensibiliseerd met IgG anti-D allo-antistoffen, in dit orgaan werden gesequestreerd.

In Hoofdstuk III worden de faktoren belicht, die belangrijk zijn in verband met de remming van de interaktie gesensibiliseerde erytrocyt-monocyt in vitro.

De verkregen resultaten maken duidelijk, dat tenminste twee faktoren de remming door IgG kunnen beïnvloeden. In de eerste plaats bleek, dat verhoging van het aantal erytrocyten per monocyt leidde tot een verminderde remming door IgG. Verhoging van het aantal sensibiliserende IgG antistoffen per erytrocyt had een soortgelijk effekt. In het milieu van de milt kunnen deze twee faktoren hun invloed op de remming door IgG doen gelden.

In Hoofdstuk IV wordt teruggekomen op de in Hoofdstuk I vermelde bevinding, dat sommige patiënten met uitsluitend IgG1 auto-antistoffen lijden aan AIHA terwijl in andere patiënten met deze antistoffen geen tekenen van versterkte haemolyse aantoonbaar zijn. Duidelijker gezegd, het is onderzocht of deze waarneming berust op het bestaan van kwalitatieve verschillen binnen de IgG1 antistoffen, of dat juist kwantitatieve faktoren, de hoeveelheid IgG1 auto-antistoffen, van invloed zijn. De relatieve hoeveelheid IgG1 op de erytrocyten werd bepaald met behulp

van een fluorescentie techniek, die geschikt was gebleken om de hoeveelheden IgG antistof, aanwezig op in verschillende mate gesensibiliseerde erytrocyten met elkaar te vergelijken. Na inkubatie met een anti-IgG1 serum, waaraan een fluorescerend label gekoppeld was, werd de sterkte van de fluorescentie bepaald. Op deze manier werd de hoeveelheid IgG1 antistoffen op erytrocyten van patiënten met AIHA vergeleken met die aanwezig op erytrocyten van patiënten die géén symptomen van verhoogde bloedafbraak vertoonden.

Tevens werd de mate, waarin de verschillende patiëntecellen werden gelyseerd door monocyten in vitro onderzocht. De resultaten wezen uit, dat voornamelijk de hoeveelheid IgG1 op de erytrocyten, en niet de kwaliteit van deze antistoffen, bepaalde of er versterkte haemolyse optrad. Bovendien werd een fraai verband tussen de op de erytrocyt aanwezige hoeveelheid IgG1 en de afbraak van deze patiënte-erytrocyten door monocyten in vitro aangetoond.

Het bovenvermelde gold ook voor die gevallen, waarin de auto-antistoffen waren ontstaan tijdens het gebruik van a-methyldopa.

In Hoofdstuk V worden de resultaten beschreven van een longitudinaal onderzoek, verricht in patiënten met autoimmuun haemolytische anaemie (AIHA), waarbij het verband werd onderzocht tussen het verloop van de ziekte en het effekt van de therapie enerzijds en het voorkomen van de verschillende IgG subklassen en de relatieve hoeveelheid antistof op de erytrocyten anderzijds. Hiertoe werden in de loop van de tijd regelmatig bloedmonsters van de patiënt afgenomen. Tevens werd de binding van de patiënte-erytrocyten aan monocyten en de daaruit voortvloeiende afbraak van deze cellen in vitro bestudeerd.

Om de betekenis van de cytotoxiciteits-test als parameter in AIHA te bepalen, werd allereerst het verband onderzocht tussen de aanwezigheid van tekenen van verhoogde bloedafbraak in vivo en de cytotoxie in vitro. Het bleek, dat de erytrocyten van de patiënten met een manifeste haemolyse sterker werden afgebroken door de monocyten dan die van patiënten zonder symptomen van verhoogde bloedafbraak. Dit versterkte andermaal de veronderstelling, dat cytotoxische lysis van erytrocyten een belangrijk destruktie mechanisme is in de afbraak van erytrocyten in AIHA.

In de patiënten, die goed op de therapie reageerden, kon een significante daling worden aangetoond van de hoeveelheid antistoffen op de rode bloed cellen. Deze daling ging gepaard met een afnemende binding en lysis van deze cellen in vitro. In sommige gevallen veranderde gedurende de behandeling met corticosteroïden de samenstelling van de subklassen op de erytrocyten. Zo trad bij patiënten met IgG3 auto-antistoffen een duidelijke verbetering van de haemolytische anaemie op met het verdwijnen van deze subklasse. Te zelfder tijd verminderde de adherentie van de patiënte-erytrocyten aan monocyten. Ook het aantal erytrocyten, dat door de fagocyten in vitro werd gelyseerd, nam af.

Samen met de vermindering van de hoeveelheid IgG1 auto-antistoffen verbeterden de patiënten, op wier erytrocyten deze subklasse aantoonbaar was. Ook in deze gevallen werd de interaktie tussen de rode cellen en de monocyten in vitro zwakker. De aanwezigheid van IgG2 en IgG4 auto-antistoffen op de patiënte-erytrocyten leidde niet tot verhoogde bloedafbraak in vivo noch tot adherentie en cytotoxie in vitro.

Naast de bovengenoemde effekten leek de therapie met corticosteroïden ook de werking van de mononucleaire fagocyten in vivo te beïnvloeden. Aan de hand van enkele gevallen worden de verschillende reakties op de behandeling besproken.

In Hoofdstuk VI wordt de cytotoxische aktiviteit van granulocyten, monocyten en lymfocyten, geïsoleerd uit het bloed van patiënten lijdende aan een akute infektieziekte, in vitro bestudeerd.

Tijdens de ziekte bleken de granulocyten van deze patiënten significant meer met anti-D gesensibiliseerde erytrocyten te lyseren dan na genezing, terwijl de cytotoxische aktiviteit van monocyten onveranderd bleef gedurende de periode van de infektie. Lymfocyten waren in deze proefopstelling niet in staat gesensibiliseerde erytrocyten te lyseren.

Bovenstaande resultaten suggereren, dat granulocyten een bijdrage zouden kunnen leveren aan de verhoogde erytrocytenafbraak, welke af en toe wordt waargenomen bij patiënten met AIHA als zij een infektie doormaken.

In hoofdstuk VII tenslotte, worden de experimenten vermeld, die verricht werden om de invloed van

cryopreservatie -het 'diepvriezen'- van monocyten op de funktie van deze cellen vast te stellen.

De monocyten werden ingevroren volgens een automatisch gekontroleerd koelingsprogramma en daarna opgeslagen in vloeibare stikstof. Na vriezen, dooien en wassen werd 63% van het oorspronkelijk aanwezige aantal cellen teruggewonnen. Gemeten met de trypaanblauw exclusie test, was 95% van deze cellen vitaal. Het percentage monocyten in de celsuspensies (70-80%) veranderde niet door de opslag bij -196°C. Alhoewel het percentage cellen, dat erytrocyten bond niet was veranderd na bevriezen en weer ontdooien, bleek dat deze rozetvorming makkelijker geremd werd door toevoeging van IgG.

De cytotoxische aktiviteit van monocyten jegens erytrocyten, die met IgG allo-antistoffen beladen zijn, was door de vries- en dooiprocedure niet veranderd, in tegenstelling tot hun vermogen om zymosan deeltjes te fagocyteren, dat was verminderd. Ook de chemotaktische respons na stimulatie door caseïne was na het vriezen lager dan daarvoor.

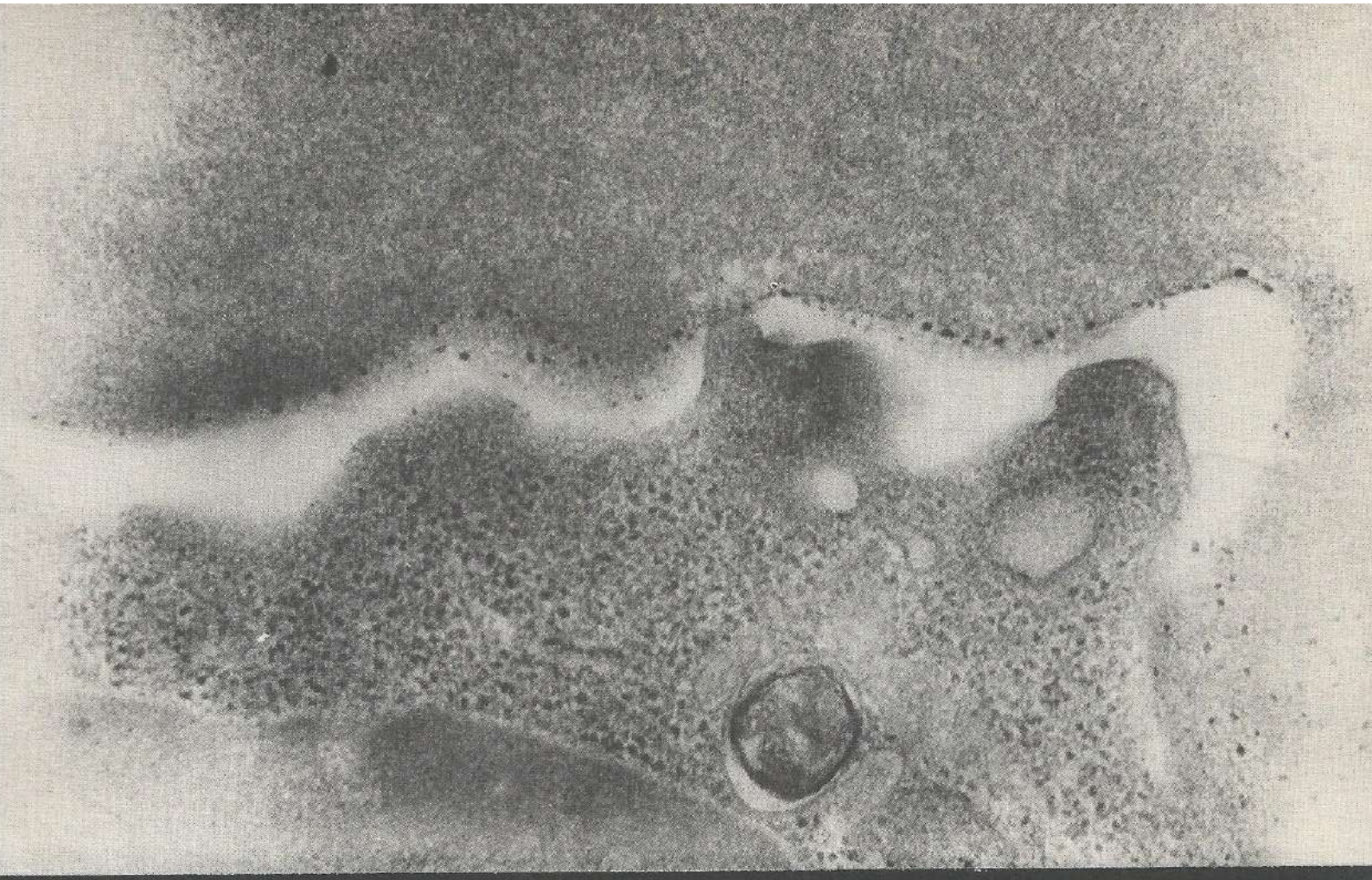
De reaktiviteit in de verschillende testsystemen van monocyten, die slechts voor korte duur (2-15 uur) waren opgeslagen in vloeibare stikstof, verschilde niet van die van monocyten, die langer dan 3 maanden bewaard werden.

Bovengenoemde resultaten impliceren, dat het mogelijk is monocyten bij -196°C te bewaren, zij het dan met een enigszins veranderde reaktiviteit in sommige testsystemen als gevolg. In de experimenten, beschreven in de Hoofdstukken IV en V, is gedeeltelijk gebruik gemaakt van monocyten, die op deze wijze zijn bewaard.

De resultaten van de onderzoekingen, die zijn neergelegd in dit proefschrift, ondersteunen ten sterkste de veronderstelling, dat in patiënten met AIHA, erytrocyten worden afgebroken, doordat zij gebonden en daarna beschadigd of vernietigd worden door mononucleaire fagocyten in vivo.

De samenstelling van de IgG subklassen, alsmede de hoeveelheid antistoffen op de patiënte-erytrocyten lijken (mede) te bepalen of de remming in vivo door IgG al dan niet overwonnen kan worden en daarmee of er wel of geen manifeste haemolyse optreedt. Gerede-

neerd vanuit dit gezichtspunt, suggereert de waarneming, dat de aanwezigheid van IgG3 auto-antistoffen bijna altijd gepaard gaat met verhoogde bloedafbraak, dat antistoffen van deze subklasse, gebonden aan de erytrocyt, betrekkelijk makkelijk de remming door IgG kunnen overwinnen. IgG2 en IgG4 auto-antistoffen lijken niet aktief deel te nemen aan bovengenoemde processen, terwijl het effekt van de IgG1 auto-antistoffen duidelijk afhangt van de hoeveelheid van deze subklasse, die aan het erytrocytenoppervlak is gebonden. Aangezien de afbraak van erytrocyten in patiënten met AIHA hoofdzakelijk in de milt is gelokaliseerd, lijkt het speciale milieu in dit orgaan van groot belang met betrekking tot het verschijnsel van de remming door IgG.



samenvatting voor de leek

Rode bloedlichaampjes transporteren zuurstof van de longen naar alle organen en weefsels en zijn dus voor een goed funktioneren van het lichaam van levensbelang. Sommige mensen echter breken hun eigen rode bloedlichaampjes af, doordat ze antistoffen gaan produceren tegen deze bloedcellen.

In de normale situatie worden antistoffen gevormd tegen schadelijke bakteriën en virussen, die het lichaam zijn binnengedrongen. Zij worden aangemaakt door het afweerapparaat -ook wel 'immuunapparaat' genoemd- en dragen bij tot een snelle vernietiging van de ziektekiemen. De werking van het afweerapparaat is nu zo, dat het antistoffen maakt die gericht zijn tegen produkten waar het lichaam nog niet mee in aanraking is geweest, die m.a.w. dus 'vreemd' zijn voor het lichaam. Eigen rode bloedcellen zijn natuurlijk niet 'vreemd' voor het afweerapparaat en als dus patiënten antistoffen gaan maken tegen hun eigen bloedlichaampjes is er kennelijk iets mis met het gezond funktioneren van het immuunapparaat.

In de patiënt hechten deze antistoffen zich aan het oppervlak van de rode bloedcellen, die dan in de milt door bepaalde witte bloedcellen worden vernietigd. Met een Latijnse term wordt deze verhoogde afbraak van rode bloedlichaampjes 'haemolyse' genoemd (haem = rood bloed, lyse = afbraak). Het gevolg van de haemolyse is, dat de patiënt een tekort krijgt aan rode bloedcellen: hij of zij lijdt aan een bloedarmoede (Latijn: anaemie). Hiermee is dan de naam van de ziekte, die in dit proefschrift centraal staat meteen verklaard: Auto (= zelf, eigen) Immuun Haemolytische Anaemie: Bloedarmoede veroorzaakt door een verhoogde afbraak van rode bloedlichaampjes onder invloed van auto-antistoffen (= antistoffen, die de patiënt tegen zijn eigen cellen maakt).

Nu zijn er ook patiënten, die weliswaar auto-antistoffen op hun rode bloedcellen hebben, maar die geen tekenen van verhoogde afbraak van rode bloedcellen vertonen.

In dit proefschrift wordt de vraagstelling behandeld, welke faktoren bepalen of er wel of geen verhoogde afbraak optreedt en in die gevallen, waarin dat wel zo is, hóe die rode bloedcellen dan worden afgebroken in het lichaam.

Voordat ik hier verder op inga, wil ik eerst twee zaken nog wat verder toelichten.

In de eerste plaats is er nog iets meer over de antistoffen te vertellen. Antistoffen zijn eiwitten en hebben allerlei eigenschappen, die scheikundig bepaald kunnen worden. Op grond daarvan worden ze ingedeeld in verschillende soorten of klassen. De bovengenoemde auto-antistoffen behoren tot de zgn. IgG klasse, eenvoudig gezegd tot de antistof-eiwit klasse G. Deze IgG antistoffen zijn weer verder onder te verdelen in 4 zgn. subklassen, die alle een nummer hebben gekregen. Zo kennen we de subklassen IgG1, IgG2, IgG3 en IgG4. Deze onderverdeling is van belang voor de bespreking van het tweede punt.

Het is uit proeven, die andere onderzoekers gedaan hebben, duidelijk geworden, dat bepaalde witte bloedcellen, de zgn. mononucleaire fagocyten (mono = één, nucleus = kern, fago = eten en cyt = cel, dus cellen met één kern, die kunnen 'eten') op hun celoppervlak plaatsen hebben waar IgG antistoffen kunnen aanhechten. Zo is het dan voorstelbaar, dat een rode bloedcel, beladen met IgG antistoffen, wordt gebonden aan deze witte bloedcellen. Dit kontakt kan leiden tot de afbraak van het rode bloedlichaampje.

In het laboratorium kunnen we het samenspel tussen de rode en witte bloedlichaampjes in een reageerbuisje bestuderen. Wij hebben in onze proeven veel gebruik gemaakt van monocyten, een mononucleaire fagocyt in een jong stadium, die in het bloed circuleren en via allerlei laboratorium technieken te isoleren zijn van de overige bloedcellen. Als deze monocyten in een reageerbuis samengebracht worden met rode bloedcellen waar IgG antistoffen opzitten, kunnen de rode bloedcellen als een kransje om de monocyt heen gaan zitten; ze vormen dan a.h.w. een 'rozet'. Op de voorkant van het proefschrift is een zeer sterke vergroting van zo'n rozet afgebeeld.

Nu komt de rol van de IgG subklassen ter sprake. Alleen de subklassen IgG1 en IgG3 zijn in staat om binding van rode bloedcellen aan monocyten te bewerkstelligen. Als de subklassen IgG2 en/of IgG4 op het celoppervlak van de rode bloedcel aanwezig zijn, treedt er geen 'rozetvorming' op.

De bevinding, dat in het reageerbuisje rode bloedcellen, beladen met IgG antistoffen, worden ge-

bonden aan monocyten en vervolgens afgebroken worden, leidde tot de veronderstelling, dat in het lichaam een soortgelijk mechanisme werkzaam zou kunnen zijn bij de afbraak van rode bloedlichaampjes beladen met IgG autoantistoffen. Dit temeer, daar deze rode cellen vooral worden afgebroken in de milt, waar juist veel mononucleaire fagocyten aanwezig zijn. Hiermee zijn we terug bij de bovengenoemde vraagstelling, waarop we in dit proefschrift een antwoord proberen te vinden door gebruik te maken van de volgende onderzoekstechnieken.

Rode bloedcellen, beladen met IgG auto-antistoffen, d.w.z. rode bloedlichaampjes afgenomen van patiënten met deze antistoffen, werden in een reageerbuisje samengebracht met monocyten van gezonde personen. In sommige proeven werd gebruik gemaakt van rode bloedcellen, die in het reageerbuisje beladen werden met IgG antistoffen (geen auto-antistoffen). Het samenspel tussen rode en witte bloedlichaampjes werd bestudeerd door (a) te letten op het al dan niet optreden van 'rozetvorming'. Onder de mikroskoop werd dus gekeken of de rode bloedcellen gebonden werden door de monocyten, en (b) te meten hoeveel rode bloedlichaampjes er afgebroken werden door de monocyten. Hiertoe werden de rode bloedcellen voorzien van een radioaktief label, m.a.w. radioaktief 'gemerkt'. Als een rode bloedcel wordt afgebroken komt het radioaktieve label vrij en door de hoeveelheid vrijgekomen radioaktiviteit te meten met een soort Geigerteller, kunnen we berekenen hoeveel rode bloedcellen er zijn vernietigd.

Om verdere aanwijzingen te hebben, dat de binding van rode bloedcellen aan mononucleaire fagocyten een belangrijke rol speelt in de afbraak in het lichaam, wordt in Hoofdstuk I bestudeerd, welke IgG subklassen aanwezig waren in patiënten die bloedarmoede hadden en welke subklassen aanwezig waren in patiënten die géén tekenen van verhoogde afbraak vertoonden. Het bleek, dat vrijwel alle patiënten met subklasse IgG3 tekenen van verhoogde afbraak van rode bloedcellen vertoonden, terwijl deze tekenen ontbraken bij de patiënten wier rode bloedcellen IgG2 en IgG4 op hun celoppervlak hadden. Van de patiënten met IgG1 auto-antistoffen vertoonden sommigen wel, maar anderen niet, verhoogde afbraak. Verder bleek, dat de rode bloedlichaampjes van alle patiënten die een tekort aan rode bloed-

lichaampjes hadden, werden gebonden aan monocyten, terwijl deze binding niet optrad met de rode bloedcellen van de patiënten zonder verhoogde bloedafbraak. Dit wijst erop, dat binding aan mononucleaire fagocyten belangrijk kan zijn voor de afbraak van rode cellen in het lichaam: geen binding, geen afbraak; wel binding, wel afbraak.

In Hoofdstuk II wordt een patiënt beschreven, die wel veel IgG auto-antistoffen op zijn rode bloedcellen had, maar die geen tekenen van verhoogde afbraak vertoonde. Ook in het reageerbuisje werden zijn rode cellen nauwelijks door monocyten gebonden. Deze patiënt bleek veel IgG4 auto-antistoffen te hebben en zijn geval ondersteunt dus de bovengenoemde veronderstelling. Toch zijn er resultaten van proeven beschreven, die het erg moeilijk maken om aan te nemen, dat in het lichaam de binding van de rode bloedcel, beladen met IgG antistoffen, de eerste stap is in de afbraak van deze cellen. Het bleek nl., dat de binding van rode cellen, beladen met IgG antistoffen, aan monocyten in het reageerbuisje sterk belemmerd kon worden door vrij IgG toe te voegen. Dit vrij IgG heeft dezelfde struktuur als het IgG van de antistoffen en kan dus ook de plaats op het celoppervlak van de monocyt bezetten, die ook ingenomen kan worden door de IgG antistoffen aanwezig op de rode bloedcel. Er ontstaat dus a.h.w. een strijd tussen het vrije IgG en het antistof-IgG op de rode bloedcel om de beschikbare aanhechtingsplaatsen op het oppervlak van de monocyt. Veel vrij IgG in het reageerbuisje betekende, dat minder rode bloedcellen konden worden gebonden aan de monocyten en bijgevolg verminderde het aantal rode cellen, dat werd afgebroken.

Deze waarneming is belangrijk, omdat in het lichaam nog veel meer vrij IgG in het bloed aanwezig is dan nodig was om in het reageerbuisje de binding van de rode cel aan de monocyt te voorkomen. Hoe moeten we ons dan voorstellen, dat toch de met IgG-antistof beladen rode bloedcel in het lichaam wordt afgebroken? Bekend is, dat rode bloedcellen, beladen met IgG antistoffen in de milt en niet elders in het lichaam worden afgebroken. Kennelijk zijn er in dit orgaan bepaalde omstandigheden, die ertoe leiden, dat het vrije IgG daar niet de binding van rode bloedlichaampjes aan de mononucleaire fagocyt remt. In de milt nu kunnen

de rode cellen heel dicht bij de mononucleaire fagocyten komen en hopen zich veel rode bloedcellen op rond deze witte bloedcellen. Bovendien is de antistof belading van de rode bloedcellen hier extra groot, waarschijnlijk omdat de auto-antistoffen geproduceerd worden in de milt. Wij hebben het belang van deze twee faktoren willen onderzoeken met proeven in het laboratorium. Daartoe (Hoofdstuk III) hebben we in het reageerbuisje aan monocyten en rode bloedcellen, beladen met IgG antistoffen, vrij IgG toegevoegd en bestudeerd hoe de remmende werking van dit vrij IgG werd beïnvloed door nabootsing van de bovengenoemde twee faktoren. Het bleek, dat als wij maar heel veel rode cellen aan de monocyt aanboden, de remming verminderde. Ook was dit het geval als we de rode cel met heel veel antistoffen beladen. Op deze manier verkregen we een aanwijzing hoe, speciaal in de milt, toch de binding tussen de met IgG auto-antistof beladen rode bloedcel en de mononucleaire fagocyt kan plaats vinden. De mogelijkheden, die dit orgaan biedt om de remmende werking van het vrije IgG in het bloed te overwinnen, verklaren waarom juist de milt de belangrijke plaats is waar de afbraak van de rode cellen van de patiënten met haemolytische anaemie is gelokaliseerd. Als de verschillende medicijnen die er zijn om deze ziekte te bestrijden niet helpen, wordt bij deze patiënten dan ook de milt operatief verwijderd.

In Hoofdstuk IV komen we terug op de in Hoofdstuk I vermelde waarneming dat sommige patiënten met IgG1 auto-antistoffen wel, en anderen geen, tekenen van verhoogde afbraak van rode bloedcellen vertoonden. Inmiddels hadden we nl. een techniek tot onze beschikking gekregen om te bepalen hoeveel antistoffen er op de rode bloedlichaampjes aanwezig zijn. Hierbij wordt gebruik gemaakt van antistoffen, die in konijnen opgewekt zijn tegen de IgG antistoffen. M.a.w. deze konijne-antistoffen zullen zich gaan binden aan de IgG antistoffen, die al op de rode cel zitten en er zullen meer konijne-antistoffen gaan zitten op die rode bloedcellen waar meer IgG antistoffen op aanwezig zijn. Aan de konijne-antistoffen is een stofje gebonden, dat gaat fluoresceren als er een laserstraal op valt. De situatie is een beetje te vergelijken met het lichtknopje, dat een groen licht uitstraalt in het donker als er overdag zonlicht op geschenen heeft.

Kort en goed, hoe meer IgG antistoffen op de rode cel, hoe meer konijne-antistof gebonden wordt, hoe sterker de fluorescentie wordt. Met een apparaat is de sterkte van de fluorescentie te meten en op deze manier is dus de hoeveelheid IgG1 antistof op de rode bloedcellen van de ene patiënt te vergelijken met die op de rode cellen van een ander. Uit ons onderzoek bleek, dat die patiënten met IgG1 antistoffen, die geen tekenen van verhoogde afbraak vertoonden, duidelijk minder antistof op het oppervlak van hun rode cellen hadden dan die patiënten, die wel een haemolytische anaemie hadden. Het werd duidelijk, dat er een bepaalde minimale hoeveelheid IgG1 nodig was om in het lichaam verhoogde bloedafbraak te kunnen veroorzaken. Waarschijnlijk is deze minimale belading dan nodig om de remmende werking van het vrije IgG te kunnen overwinnen.

Hoofdstuk V beschrijft het verloop van de afbraak van rode bloedcellen in patiënten, die worden behandeld met corticosteroïden, het geneesmiddel bij uitstek om de verhoogde afbraak te remmen. Het bleek, dat in de loop van de behandeling met deze bijnierschorshormonen de hoeveelheid IgG auto-antistoffen op de rode bloedcellen sterk terugliep. In het reageerbuisje namen dan ook de rozetvorming en de afbraak van de rode bloedcellen van de patiënt sterk af. Bovendien werden aanwijzingen verkregen, dat deze medicijnen ook een werking hadden op de mononucleaire fagocyten in het lichaam, zodat ze minder goed in staat zijn om de rode cellen af te breken.

Het is reeds lang bekend, dat patiënten met IgG auto-antistoffen een verhoging van de rode bloedcel afbraak kunnen vertonen als zij een infektieziekte doormaken, zoals bv. een longontsteking. Dit kan misschien gedeeltelijk worden toegeschreven aan het feit, dat de hoeveelheid IgG auto-antistof gaat stijgen, maar de mogelijkheid zou kunnen bestaan, dat de witte bloedcellen -die ook belangrijk zijn in het opruimen van de ziektekiemen- op de een of andere manier extra aktief worden en daardoor de rode cellen, beladen met de auto-antistof, extra fel afbreken.

In Hoofdstuk VI hebben wij deze laatste gedachte willen testen door te onderzoeken of monocyten van patiënten met een akute infektieziekte inderdaad in het reageerbuisje meer rode bloedcellen, beladen met

IgG antistoffen, afbraken dan de monocyten van gezonde kontrôle personen. Daarbij hebben we ook deze aktiviteit bestudeerd van andere witte bloedcellen, die normaal weinig reaktief zijn tegen met IgG-antistof beladen rode bloedlichaampjes. Tot onze verrassing bleek, dat niet de monocyt maar wel de zgn. granulocyt (een cel met een meervormige kern, die ook kan 'eten') een grote stijging in aktiviteit jegens de rode cellen ging vertonen.

Het is dus mogelijk, dat de verhevigde afbraak van rode cellen, die kan worden waargenomen bij patiënten met IgG auto-antistoffen wanneer zij een infektie doormaken, mede veroorzaakt wordt door een witte bloedcel die in de normale toestand niet aktief lijkt deel te nemen aan het afbraakproces.

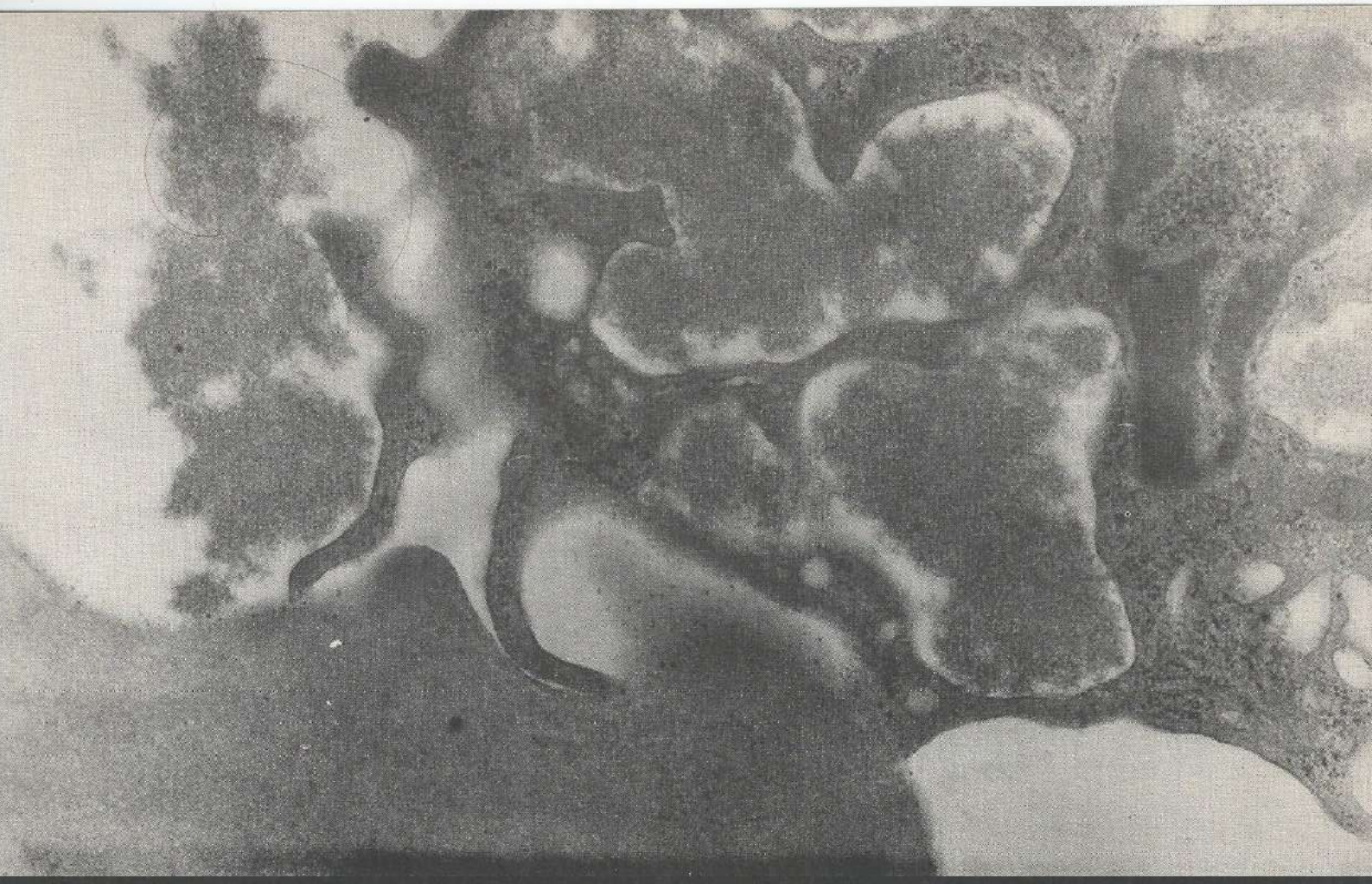
In Hoofdstuk VII tenslotte wordt een bijkomend onderzoek beschreven, dat met de bovenbehandelde ziekte niet direkt iets te maken heeft. In de loop van de onderzoekingen werd het duidelijk, dat het iedere dag opnieuw moeten isoleren van monocyten uit het bloed van donoren een tijdrovende zaak was. Bovendien liep de aktiviteit van de monocyten van de verschillende donoren nogal uiteen. Het zou daarom heel prettig zijn om te kunnen beschikken over een grote voorraad monocyten waar men steeds een klein beetje af zou kunnen nemen. Omdat bekend was, dat andere witte bloedcellen heel goed bewaard kunnen worden bij een hele lage temperatuur, nl. -196°C, de temperatuur van vloeibare stikstof- hebben wij de invloed van het diepvriezen op de funktie van monocyten onderzocht in een aantal testsystemen. Het bleek, dat het vermogen om rozetten te vormen met rode bloedcellen, beladen met IgG antistoffen, en het vermogen om deze cellen af te breken in het reageerbuisje, door deze bewaarprocedure niet noemenswaard waren veranderd. In een aantal proeven, beschreven in de vorige hoofdstukken, hebben wij dan ook zonder bezwaar gebruik kunnen maken van deze 'diepvries' monocyten.

Terugkomend op de vragen, waarop wij door onze onderzoekingen een antwoord hoopten te vinden, kunnen wij het volgende beeld schetsen: Het is zeer waarschijnlijk, dat bij de afbraak van rode bloedcellen, beladen met IgG auto-antistoffen, de mononucleaire fagocyten een belangrijke rol spelen. De vraag of er

verhoogde bloedafbraak zal optreden in patiënten met deze IgG auto-antistoffen wordt ten dele bepaald door de IgG subklassen, die in deze IgG antistoffen vertegenwoordigd zijn: Is de subklasse IgG3 aanwezig, dan is er vrijwel altijd ernstige afbraak. Zijn er alleen IgG2 en/of IgG4 auto-antistoffen, dan zijn er geen tekenen van een haemolytische anaemie. In het geval van IgG1 auto-antistoffen zal het van de hoeveelheid IgG1 afhangen of er wel of geen afbraak op gaat treden. Waarschijnlijk staat in alle gevallen centraal in hoeverre de antistoffen in staat zijn de in het lichaam bestaande remmende werking van het vrije IgG te doorbreken. De rol van de milt is bijzonder belangrijk in dit proces.

De afbraak vindt waarschijnlijk plaats, doordat de rode bloedcellen worden gebonden aan de mononucleaire fagocyten in het lichaam, die vervolgens deze cellen afbreken. Hoe meer antistoffen zich op de rode bloedcellen bevinden, hoe sterker de afbraak is. De behandeling met de bijnierschorshormonen -de corticosteroïden- leidt enerzijds tot een vermindering van de hoeveelheid antistoffen op de rode bloedcel, die gepaard kan gaan met een verandering van de IgG subklassen samenstelling; anderszijds lijkt er een effekt te zijn van de corticosteroïden op het vermogen van de mononucleaire fagocyten om de met antistoffen beladen rode bloedcellen af te breken.

Tot slot is gesteld, dat de toegenomen afbraak van rode bloedcellen, die waargenomen werd bij patiënten met IgG auto-antistoffen tijdens een akute infektieziekte, mede veroorzaakt lijkt te worden door deelname van granulocyten aan het afbraakproces.



references

introduction

Abramson, N., LoBuglio, A.F., Jandl, J.H. and Cotran, R.S. (1970a) The interaction between human monocytes and red cells. Binding characteristics. Journal of Experimental Medicine, 132, 1191.

Abramson, N., Gelfand, E.W., Jandl, J.H. and Rosen, F.S. (1970b) The interaction between human monocytes and red cells. Specificity for IgG subclasses and IgG fragments. Journal of Experimental Medicine, 132, 1207.

Archer, G.T. (1965) Phagocytosis by human monocytes of red cells coated with Rh antibodies. Vox Sanguinis, 10, 590.

Borne, A.E.G. Kr. von dem (1971a) Autoimmuun Haemolytische Anaemie. Academisch Proefschrift. Drukkerij "Aemstelstad", Amsterdam.

Borne, A.E.G. Kr. von dem, Engelfriet, C.P., Beckers, D. and Loghem, J.J. van (1971b) Autoimmune haemolytic anaemias. IV. Biochemical studies of red cells from patients with autoimmune anaemia with incomplete warm autoantibodies. Clinical and Experimental Immunology 8, 377.

Borne, A.E.G. Kr. von dem, Beckers, D., Meulen, F.W. van der and Engelfriet, C.P. (1977a) IgG4 autoantibodies against erythrocytes without increased haemolysis. A case report. British Journal of Haematology, 37, 137.

Borne, A.E.G. Kr. von dem, Beckers, D. and Engelfriet, C.P. (1977b) Mechanisms of red cell destruction mediated by non-complement binding IgG antibodies: the essential role in vivo of the Fc part of IgG. British Journal of Haematology, 36, 485.

Coombs, R.R.A., Mourant, A.E. and Race, R.R. (1945) A new test for the detection of weak and 'incomplete' Rh agglutinins. British Journal of Experimental Pathology, 26, 255.

Dacie, J.V. (1962) The Haemolytic Anaemias: Congenital and acquired. Part II. In: The Autoimmune Haemolytic Anaemias, 2nd edition. J. & A. Churchill, Ltd. London.

Engelfriet, C.P., Borne, A.E.G. Kr. von dem, Beckers, D. and Loghem, J.J. van (1974) Autoimmune Haemolytic Anaemias: serological and immunochemical characteristics of the antibodies; mechanisms of cell destruction. Series Haematologica, 7, 328.

Fleer, A. (1978) Destruction of sensitized erythrocytes by human monocytes in vitro. Academisch Proefschrift, Rodopi, Amsterdam.

Fleer, A., Schaik, M.L.J. van, Borne, A.E.G. Kr. von dem and Engelfriet, C.P. Destruction of sensitized erythrocytes by human monocytes in vitro. Effects of cytochalasin B, hydrocortisone and colchicine. Scandinavian Journal of Immunology, in press (a).

Fleer, A., Koopman, M.G., Borne, A.E.G. Kr. von dem and Engelfriet, C.P. Monocyte-induced increase in osmotic fragility of human red cells sensitized with anti-D alloantibodies. British Journal of Haematology, in press (b).

Furth, R. van and Cohn, Z.A. (1968) The origin and kinetics of mononuclear phagocytes. Journal of Experimental Medicine, 128, 415.

Holm, G. (1972) Lysis of antibody-treated human erythrocytes by human leucocytes and macrophages in tissue culture. International Archives of Applied Allergy, 43, 671.

Holm, G., Engwall, E., Hammarström, S. and Natvig, J.B. (1974) Antibody-induced hemolytic activity of human blood monocytes. The role of antibody class and subclass. Scandinavian Journal of Immunology, 3, 173.

Huber, H. and Fudenberg, H.H. (1968) Receptor sites of human monocytes for IgG. International Archives of Applied Allergy, 34, 18.

Huber, H., Douglas, S.D. and Fudenberg, H.H. (1969) The IgG receptor: an immunological marker for the characterization of mononuclear cells. Immunology, 17, 7.

Issitt, P.D., Pavone, B.G., Goldfinger, D., Zwicker, H., Issitt, C.H., Tessel, J.A., Kroovand, S.W. and Bell, C.A. (1976) Anti-Wr^b and other autoantibodies responsible for positive direct antiglobulin test in 150 individuals. British Journal of Haematology, 34, 5.

Jandl, J.H. and Kaplan, M.E. (1960) The destruction of red cells by antibodies in man. III. Quantitative factors influencing the pattern of haemolysis. in vivo. Journal of Clinical Investigation, 39, 1145.

Jandl, J.H., Richardson-Jones, A. and Castle, W.B. (1957) The destruction of red cells by antibodies

in man. I. Observations on the sequestration and lysis of red cells altered by immune mechanisms. Journal of Clinical Investigations, 36, 1428.
Leddy, J.P. and Swisher, S.N. (1971) Acquired immune hemolytic disorders. In: Immunological Diseases. M. Santer (ed.), Little, Brown & Co., Boston, p. 1083.
LoBuglio, A.F., Cotran, R.S. and Jandl, J.H. (1967) Red cells coated with immunoglobulin G: binding and sphering by mononuclear cells in man. Science, 158, 1582.
Loutit, J.F. and Mollison, P.L. (1946) Haemolytic icterus (Acholuric jaundice): congenital and acquired. Journal of Pathological Bacteriology, 58, 711.
Messner, R.P. and Jelinek, J. (1970) Receptors for human globulin on human neutrophils. Journal of Clinical Investigation, 49, 2165.
Mollison, P.L., Crome, P., Hughes-Jones, N.C. and Rochna, E. (1965) Rate of removal from the circulation of red cells sensitized with different amounts of antibody. British Journal of Haematology, 11, 461.
Rosse, W.F. (1971) Quantitative immunology of immune hemolytic anemia. II. The relationship of cell-bound antibody to hemolysis and the effect of treatment. Journal of Clinical Investigation, 50, 734.
Wagley, P.F., Shu Chu Shen, Frank, H.G. and Castle, W.B. (1948) Studies on the destruction of red blood cells. VI. The spleen as a source of a substance causing agglutination of the red blood cells of certain patients with acquired hemolytic jaundice by an anti-human serum rabbit serum. Journal of Laboratory and Clinical Medicine, 33, 1197.
Weiner, H. and Vos, G.H. (1963) Serology of acquired hemolytic anemias. Blood, 22, 606.
Zinkham, W.H. and Diamond, L.K. (1952) In vitro erythrophagocytosis in acquired hemolytic anemia. Blood, 7, 592.

chapter I

Abramson, N., LoBuglio, A.F., Jandl, J.H. & Cotran, R.S. (1970a) The interaction between human monocytes and red cells. Binding characteristics. Journal of Experimental Medicine, 132, 1191.
Abramson, N., Gelfand, E.W., Jandl, J.H. & Rosen, F.S. (1970b) The interaction between human monocytes and red cells. Specificity for IgG subclasses and IgG fragments. Journal of Experimental Medicine, 132, 1207.
Atkinson, J.P. & Frank, M.M. (1974) Studies of the in vivo effects of antibody. Interaction of IgM antibody and complement in the immune clearance and destruction of erythrocytes in man. Journal of Clinical Investigation, 54, 339.
Borne, A.E.G. Kr. von dem, Engelfriet, C.P., Beckers, Do & Loghem, J.J. van (1971) Autoimmune haemolytic anaemias. IV. Biochemical studies of red cells from patients with autoimmune anaemia with incomplete warm autoantibodies. Clinical and Experimental Immunology, 8, 377.
Borne, A.E.G. Kr. von dem, Engelfriet, C.P. Reynierse, E., Beckers, Do & Loghem, J.J. van (1973) Autoimmune haemolytic anaemias. VI. ^{51}Chromium survival studies in patients with different kinds of warm autoantibodies. Clinical and Experimental Immunology, 13, 561.
Borne, A.E.G. Kr. von dem, Beckers, Do & Engelfriet, C.P. (1977a) Mechanisms of red cell destruction mediated by non-complement binding IgG antibodies. The essential role in vivo of the Fc part of IgG. British Journal of Haematology, 36, 485.
Borne, A.E.G Kr. von dem, Beckers, Do, Meulen, F.W. van der & Engelfriet, C.P. (1977b) IgG4 autoantibodies against erythrocytes without increased haemolysis. British Journal of Haematology, 37, 137.
Bøyum, A. (1968) Isolation of mononuclear cells and granulocytes from human blood. Scandinavian Journal of Laboratory and Clinical Investigation, 2, suppl. 77.
Brown, D.L. (1973) Annotation: The immune interaction between red cells and leucocytes and the pathogenesis of spherocytosis. British Journal of Haematology, 25, 691.
Dacie, J.V. (1962) The Haemolytic Anaemias. Part II: The autoimmune haemolytic anaemias, 2nd edition, J. & A. Churchill Ltd., London.

Engelfriet, C.P., Borne, A.E.G. Kr. von dem, Beckers, Do & Loghem, J.J. van (1974) Autoimmune haemolytic anaemias: Serological and immunochemical characteristics of the autoantibodies; mechanisms of cell destruction. Series Haematologica, 7, 328.

Evans, R.S., Turner, E., Bingham, M. & Woods, R. (1968) Chronic hemolytic anemia due to cold agglutinins. II. The role of C' in red cell destruction. Journal of Clinical Investigation, 47, 691.

Holm, G., Engwall, E., Hammarström, S. & Natvig, J.B. (1974) Antibody-induced haemolytic activity of human blood monocytes. The role of antibody class and subclass. Scandinavian Journal of Immunology, 3, 173.

Huber, H. & Fudenberg, H.H. (1968) Receptor sites of human monocytes for IgG. International Archives of Allergy and Applied Immunology, 34, 18.

Jaffé, C.J., Atkinson, J.P. & Frank, M.M. (1976) The role of complement in the clearance of cold agglutinin-sensitized erythrocytes in man. Journal of Clinical Investigation, 58, 942.

Jandl, J.H., Richardson Jones, A. & Castle, W.B. (1957) The destruction of red cells by antibodies in man. I. Observations on the sequestration and lysis of red cells altered by immune mechanisms. Journal of Clinical Investigation, 36, 1428.

Jandl, J.H. & Kaplan, M. (1960) The destruction of red cells by antibodies in man. III. Quantitative factors influencing the pattern of hemolysis in vivo. Journal of Clinical Investigation, 39, 1145.

LoBuglio, A.F., Cotran, R.S. & Jandl, J.H. (1967) Red cells coated with immunoglobulin G: Binding and sphering by mononuclear cells in man. Science, 158, 1582.

Mollison, P.L., Crome, P. Hughes-Jones, N.C. & Rochna, E. (1965) Rate of removal from the circulation of red cells sensitized with different amounts of antibody. British Journal of Haematology, 11, 461.

Prankerd, T.A.J. (1963) The spleen and anemia. British Medical Journal, 2[1], 517.

Reynolds, H.Y., Atkinson, J.P., Newball, H.H. & Frank, M.M. (1975) Receptors for immunoglobulin and complement on human alveolar macrophages. Journal of Immunology, 114/6, 1813.

Rinehart, J.J., Balcerzak, S.P., Sagone, A.L. & LoBuglio, A.F. (1974) Effects of corticosteroids on human monocyte function. Journal of Clinical Investigation, 54, 1337.

Schreiber, A.D., Parsons, J., McDermott, P. & Cooper, R.A. (1975) Effects of corticosteroids on the human monocyte IgG and complement receptors. Journal of Clinical Investigation, 56, 1189.

Wagley, P.F., Shu Chu Shen, Frank, H.G. & Castle, W.B. (1948) Studies on the destruction of red blood cells VI. The spleen as a source of a substance causing agglutination of the red blood cells of certain patients with acquired hemolytic jaundice by an anti-human serum rabbit serum. Journal of Laboratory and Clinical Medicine, 33, 1197.

Williams, E.J. (1959) Regression Analysis. J. Wiley & Sons, Inc., New York.

Williams, W.J., Beutler, E., Erslev, A.J. & Wayne Rundles, R. (1972) Hematology. McGraw-Hill Book Company, New York.

Worlledge, S.M. (1973) Immune drug-induced hemolytic anemias. Seminars in Hematology, 10/4, 327.

chapter II

Abramson, N., Gelfand, E.W., Jandl, J.H. & Rosen, F.S. (1970) The interaction between human monocytes and red cells. Specificity for IgG subclasses and IgG fragments. Journal of Experimental Medicine, 132, 1207.

Abramson, N. & Schur, P.H. (1972) The IgG subclasses of red cell antibodies and relationship to monocyte binding. Blood, 40, 500.

Borne, E.A.G. Kr. von dem, Engelfriet, C.P., Beckers, Do & Loghem, J.J. van (1971) Autoimmune haemolytic anaemias. IV. Biochemical studies of red cells from patients with autoimmune haemolytic anaemia with incomplete warm autoantibodies. Clinical and Experimental Immunology, 8, 377.

Borne, A.E.G. Kr. von dem, Beckers, Do & Engelfriet, C.P. (1977) Mechanisms of red cell destruction mediated by non-complement binding IgG antibodies: the essential role in vivo of the Fc part of IgG. British Journal of Haematology, 36, 485.

Dacie, J.V. & Worlledge, S.M. (1969) Autoimmune hemolytic anemias. Progress in Hematology, 6, 82.

Engelfriet, C.P., Borne, A.E.G. Kr. von dem, Giessen, M. van der, Beckers, D. & Loghem, J.J. van (1968) Autoimmune haemolytic anaemias. I. Serological studies with pure anti-immunoglobulin reagents. Clinical and Experimental Immunology, 3, 605.

Engelfriet C.P., Borne, A.E.G. Kr. von dem, Beckers, D. & Loghem, J.J. van (1974) Autoimmune haemolytic anaemia: serological and immunochemical characteristics of the autoantibodies; mechanisms of cell destruction. Series Haematologica, 7, no. 3, 328.

Giessen, M. van der, Lange, B. de & Lee, B. van der (1974) The production of precipitating antiglobulin reagents specific for the subclasses of human IgG. Immunology, 27, 655.

Hay, F.C., Torrigiani, G. & Roitt, I.M. (1972) The binding of human IgG subclasses to human monocytes. European Journal of Immunology, 2, 257.

Holm, G. (1972) Lysis of antibody-treated human erythrocytes by human leukocytes and macrophages in tissue culture. International Archives of Allergy and Applied Immunology, 43, 671.

Holm, G. & Hammerström, S. (1973) Haemolytic activity of human blood monocytes. Lysis of human erythrocytes treated with anti-A serum. Clinical and Experimental Immunology, 13, 29.

Holm, G., Engwall, E., Hammerström, S. & Natvig, J.B. (1974) Antibody induced hemolytic activity of human blood monocytes. The role of antibody class and subclass. Scandinavian Journal of Immunology, 3, 173.

Huber, H. & Fudenberg, H.H. (1968) Receptor sites of human monocytes for IgG. International Archives of Allergy and Applied Immunology, 34, 18.

Huber, H. & Fudenberg, H.H. (1969) Die Phagocytose von Erythrocyten-Antikörper-Komplexen in vitro. Blut, 19, 357.

Hughes Jones, N.C. & Szur, L. (1957) Determination of the sites of red-cell destruction using ^{51}Cr-labelled cells. British Journal of Haematology, 3, 320.

Jandl, J.H., Greenberg, M.S., Yonomoto, R.H. & Castle, W.B. (1956) Clinical determination of the sites of red cell sequestration in hemolytic anemias. Journal of Clinical Investigation, 35, 842.

LoBuglio, A.F., Cotran, R.S. & Jandl, J.H. (1967) Red cells coated with immunoglobulin G: binding and sphering by mononuclear cells in man. Science, 158, 1582.

Meulen, F.W. van der, Hart, Mia van der, Fleer, A., Borne, A.E.G. Kr. von dem, Engelfriet, C.P. & Loghem, J.J. van (1978) The role of adherence to human mononuclear phagocytes in the destruction of red cells sensitized with non-complement binding IgG antibodies. British Journal of Haematology, 38, 541.

Mollison, P.L. & Veall, N. (1955) The use of the isotope ^{51}Cr as a label for red cells. British Journal of Haematology, 1, 62.

Rubin, H. (1963) Antibody elution from red blood cells. Journal of Clinical Pathology, 16, 70.

Biberfeld, P., Ghetie, V. & Sjöquist, J. (1975) Demonstration and assaying of IgG antibodies in tissues and on cells by labelled staphylococcal protein A. Journal of Immunological Methods, 6, 249.

Brown, D.L. (1974) The behaviour of phagocytic cell receptors in relation to allergic red cell destruction. Series Haematologica, 7, 348.

Cartron, J.P., Gerbal, A., Hughes-Jones, N.C. & Salmon, C. (1974) 'Weak A' phenotypes: relationship between red cell agglutinability and antigen site density. Immunology, 27, 723.

Constantoulakis, M., Costea, N., Schwartz, R.S. & Damenshek, W. (1963) Quantitative studies of the effect of red-blood-cell sensitization on in vivo hemolysis. Journal of Clinical Investigation, 42, 1790.

Costea, N., Schwartz, R., Constantoulakis, M. & Damenshek, W. (1962) The use of radioactive antiglobulin for the detection of erythrocyte sensitization. Blood, 20, 214.

Dorval, G., Welsh, K.I. & Wigzell, H. (1974) Labeled staphylococcal protein A as an immunological probe in the analysis of cell surface marker. Scandinavian Journal of Immunology, 3, 405.

Economidou, J., Hughes-Jones, N.C. & Gardner, B. (1967) Quantitative measurements concerning A and B antigen sites. Vox Sanguinis, 12, 321.

Engelfriet, C.P., Borne, A.E.G. Kr. von dem, Beckers, D. & Loghem, J.J. van (1974) Autoimmune haemolytic anaemia: serological and immunochemical characteristics of the autoantibodies; mechanisms of cell destruction. Series Haematologica, 7, 328.

Ghetie, V., Nilsson, K. & Sjöquist, J. (1974) Detection and quantitation of IgG on the surface of human lymphoid cells by rosette formation with protein A-coated sheep red blood cells. European Journal of Immunology, 4, 500.

Giessen, M. van der, Lange, B. de & Lee, B. van der (1974) The production of precipitating antiglobulin reagents specific for the subclasses of human IgG. Immunology, 27, 655.

Hughes-Jones, N.C., Gardner, B. & Telford, R. (1964) The effect of ficin on the reaction between anti-D and red cells. Vox Sanguinis, 9, 175.

Jandl, J.H., Richardson Jones, A. & Castle, W.B. (1957) The destruction of red cells by antibodies in man. I. Observations on the sequestration and lysis of red cells altered by immune mechanisms. Journal of Clinical Investigation, 36, 1428.

Jandl, J.H. & Kaplan, M.E. (1960) The destruction of red cells by antibodies in man. III. Quantitative factors influencing the patterns of hemolysis in vivo. Journal of Clinical Investigation, 39, 1145.

Kabat, E.A. & Mayer, M.M. (1961) Experimental Immunochemistry, 2nd edn., p. 26. Charles C. Thomas, Springfield, Ill., USA.

Kronvall, G. (1973) A surface component in group A, C, and G streptococci with non-immune reactivity for immunoglobulin G. Journal of Immunology, 111, 1401.

LoBuglio, A.F., Cotran, R.S. & Jandl, J.H. (1967) Red cells coated with immunoglobulin G: binding and sphering by mononuclear cells in man. Science, 158, 1582.

Logue, G. & Rosse, W. (1976) Immunologic mechanisms in autoimmune hemolytic disease. Seminars in Haematology, 13, 277.

Loos, H., Blok-Schut, B., Kipp, B., Doorn, R. van & Meerhof, L. (1976) Size distribution, electronic recognition, and counting of human blood monocytes. Blood, 48, 743.

Masouredis, S.P. (1962) Reaction of I^{131} anti-Rh_o(D) with enzyme-treated cells. Transfusion 2, 363.

Masouredis, S.P., Sudora, E.J., Mahan, L. & Victoria, E.J. (1976) Antigen site densities and ultrastructural distribution patterns of red cell Rh antigens. Transfusion, 16, 94.

Mollison, P.L., Crome, P., Hughes-Jones, N.C. & Rochna, E. (1965) Rate of removal from the circulation of red cells sensitized with different amounts of antibody. British Journal of Haematology, 11, 461.

Nisonoff, A., Wissler, F.C. & Lipman, L.N. (1960) Properties of the major component of a peptic digest of rabbit antibody. Science, 132, 1770.

Porter, R.R. (1959) The hydrolysis of rabbit γ-globulin and antibodies with crystalline papain. Biochemical Journal, 73, 119.

Rochna, E. & Hughes-Jones, N.C. (1965) The use of purified ^{125}I-labelled anti-γ globulin in the determination of the number of D antigen sites on red cells of different phenotypes. Vox Sanguinis, 10, 675.

Victoria, E.J., Muchmore, E.A., Sudora, E.J. & Masouredis, S.P. (1975) The role of antigen mobility in anti-Rh_o(D)-induced agglutination. Journal of Clinical Investigation, 56, 292.

Williams, M.A. & Voak, D. (1972) Studies with ferritin-labelled Dolichos biflorus lectin on the numbers and distribution of A sites on A_1 and A_2 erythrocytes, and on the nature of its specificity and enhancement by enzymes. British Journal of Haematology, 23, 427.

Zeijlemaker, W.P., Roos, M.Th.L., Schellekens, P.Th.A. & Eijsvoogel, V.P. (1975) Antibody-dependent human lymphocytotoxicity: a micro assay system. European Journal of Immunology, 5, 579.

chapter IV

Aaij, C., Borne, A.E.G. Kr. von dem, Verheugt, F.W.A. & Engelfriet, C.P. (1978) The use of continuous flow cytofluorometry in immunohaematology. Proc. of the VIth Int. Conf. on Immunofluorescence and related staining techniques. Knapp, W. (Ed.). North-Holland Biomedical Press, Amsterdam, p. 137.

Bois, M.J.G.J. du, Schellekens, P.Th.A., Wit, J.J.F.M. de & Eijsvoogel, V.P. (1976) In vitro reactivity of human lymphocytes after cryopreservation, using a programmed cooling device. Scandinavian Journal of Immunology, 5, suppl. 5, 17.

Borne, A.E.G. Kr. von dem, Beckers, Do & Engelfriet, C.P. (1977a) Mechanisms of red cell destruction mediated by non-complement binding IgG antibodies. The essential role in vivo of the Fc part of IgG. British Journal of Haematology, 36, 485.

Borne, A.E.G. Kr. von dem, Beckers, Do., Meulen, F.W. van der & Engelfriet, C.P. (1977b) IgG4 autoantibodies against erythrocytes without increased haemolysis British Journal of Haematology, 37, 137.

Borne, A.E.G. Kr. von dem, Verheugt, F.W.A., Oosterhof, F., Riesz, L.E. von, Brutel de la Rivière, A. & Engelfriet, C.P. (1978) A simple immunofluorescence test for the detection of platelet antibodies. British Journal of Haematology, 39, 195.

Brown, D.L. (1974) The behaviour of phagocytic cell receptors in relation to allergic red cell destruction. Series Haematologica, 7, 348.

Chaplin, H., Jr. (1973) Clinical usefulness of specific antiglobulin reagents in Autoimmune Hemolytic Anemias. In: Progress in Hematology VIII, 25. Grune and Stratton, New York.

Dacie, J.V. (1956) Practical Haematology, 2nd edition. J. & A. Churchill, Ltd., London.

Dixon, W.J. & Massey, Jr., F.J. (1969) Introduction to statistical analysis, 3rd ed. McGraw-Hill Book Company, New York.

Engelfriet, C.P., Borne, A.E.G. Kr. von dem, Giessen, M. van der, Beckers, D. & Loghem, J.J. van (1968) Autoimmune haemolytic anaemias. I. Serological studies with pure anti-immunoglobulin reagents. Clinical and Experimental Immunology, 3, 605.

Engelfriet, C.P., Borne, A.E.G. Kr. von dem, Beckers, D. & Loghem, J.J. van (1974) Autoimmune haemolytic anaemias. Serological and immunochemical characteristics of the autoantibodies: mechanisms of cell destruction. Series Haematologica, VII, 328.

Fleer, A., Meulen, F.W. van der, Linthout, E., Borne, A.E.G. Kr. von dem & Engelfriet, C.P. Destruction of IgG-sensitized erythrocytes by human blood monocytes. Modulation of inhibition by IgG. British Journal of Haematology, in press[a].

Fleer, A., Schaik, J.L. van, Borne, A.E.G. Kr. von dem & Engelfriet, C.P. Destruction of sensitized erythrocytes by human monocytes in vitro. Effects of cytochalasin B, hydrocortisone and colchicine. Scandinavian Journal of Immunology, in press[b].

Giessen, M. van der, Lange, B. de & Lee, B. van der (1974) The production of precipitating antiglobulin reagents specific for the subclasses of human IgG. Immunology, 27, 655.

Gilliland, B.C., Leddy, J.P. & Vaughan, J.H. (1970) The detection of cell-bound antibody on complement-coated human red cells. Journal of Clinical Investigation 49, 898.

Greaves, M.F. (1975) Clinical applications of cell surface markers. In: Brown, E.B. (ed.) Progress in Hematology, IX, 255. Grune and Stratton, New York.

Huber, H. & Fudenberg, H.H. (1968) Receptor sites of human monocytes for IgG. International Archives of Allergy and Applied Immunology, 34, 18.

Holm, G., Engwall, E., Hammarström, S. & Natvig, J.B. (1974) Antibody-induced hemolytic activity of human blood monocytes. The role of antibody class and subclass. Scandinavian Journal of Immunology, 3, 173.

Jandl, J.H. & Kaplan, M. (1960) The destruction of red cells by antibodies in man. III. Quantitative factors influencing the pattern of hemolysis in vivo. Journal of Clinical Investigation, 39, 1145.

Kurlander, R.J., Rosse, W.F. & Logue, G.L. (1978) Quantitative influence of antibody and complement coating of red cells on monocyte-mediated cell lysis. Journal of Clinical Investigation, 61, 1309.

Logue, G. & Rosse, W.F. (1976) Immunologic mechanisms in autoimmune hemolytic disease. In: Miescher, P.A. and Jaffé, E.R. (eds.) Seminars in Hematology Vol. XIII, no. 4, 277. Grune and Stratton, New York.

Loos, J.A., Blok-Schut, B., Kipp, B., Doorn, R. van & Meerhof, L. (1976) Size distribution, electronic recognition, and counting of human blood monocytes. Blood, 48, 743.

Meulen, F.W. van der, Borne, A.E.G. Kr. von dem & Engelfriet, C.P. (1977) Storage of monocytes in liquid nitrogen. In: Perspectives in Inflammation. Willoughby, D.A., Giroud, J.P. and Velo, G.P. (eds.) MTP Press, Ltd., Lancaster, p. 635.

Meulen, F.W. van der, Hart, M. van der, Fleer, A., Borne, A.E.G. Kr. von dem, Engelfriet, C.P. & Loghem, J.J. van (1978) The role of adherence to human mononuclear phagocytes in the destruction of red cells sensitized with non-complement binding IgG antibodies British Journal of Haematology, 38, 541.

Mollison, P.L., Crome, P., Hughes-Jones, N.C. & Rochna, E. (1965) Rate of removal from the circulation of red cells sensitized with different amounts of antibody. British Journal of Haematology, 11, 461.

Mollison, P.L. (1967) Blood Transfusion in Clinical Medicine, 4th ed. Blackwell Scientific Publication, Oxford, U.K.

Rosse, W.F. (1971) Quantitative immunology of immune hemolytic anemia. II. The relationship of cell-bound antibody to hemolysis and the effect of treatment. Journal of Clinical Investigation, 50, 734.

The, T.H. & Feltkamp, T.E.W. (1970) Conjugation of fluorescein isothiocyanate to antibodies. Immunology, 18, 875.

Verheugt, F.W.A., Borne, A.E.G. Kr. von dem, Noord-Bokhorst, J.C. van & Engelfriet, C.P. Auto-immune granulocytopenia: the detection of granulocyte autoantibodies with the immunofluorescence test. British Journal of Haematology, in press.

Williams, W.J., Beutler, E., Eslen, A.J. & Wayne Rundles, R. (1972) Hematology. McGraw-Hill Book Company, New York.

Worlledge, S.M. (1973) Immune drug-induced hemolytic anemias. Seminars in Hematology, 10, 327.

chapter V

Abramson, N., Gelfand, E.W., Jandl, J.H. & Rosen, F.S. (1970a) The interaction between human monocytes and red cells. Specificity for IgG subclasses and IgG fragments. Journal of Experimental Medicine 132, 1207.

Abramson, N., LoBuglio, A.F., Jandl, J.F. & Cotran, R.S. (1970b) The interaction between human monocytes and red cells. Binding characteristics. Journal of Experimental Medicine 132, 1191.

Atkinson, J.P. & Frank, M.M. (1974) Complement-independent clearance of IgG-sensitized erythrocytes: inhibition by cortisone. Blood 44/5, 629.

Bois, M.J.G.J. du, Schellekens, P.Th.A., Wit, J.J.F.M. de & Eijsvoogel, V.P. (1976) In vitro reactivity of human lymphocytes after cryopreservation using a programmed cooling device. Scandinavian Journal of Immunology 5, suppl. 5, 17.

Borne, A.E.G. Kr. von dem, Beckers, D. & Engelfriet, C.P. (1977a) Mechanisms of red cell destruction mediated by non-complement binding IgG antibodies. The essential role in vivo of the Fc part of IgG. British Journal of Haematology 36, 485.

Borne, A.E.G. Kr. von dem, Beckers, D., Meulen, F.W. van der & Engelfriet, C.P. (1977b) IgG4 autoantibodies against erythrocytes without increased haemolysis: a case report. British Journal of Haematology 37, 137

Borne, A.E.G. Kr. von dem, Verheugt, F.W.A., Oosterhof, F., Riesz, L.E. von, Brutel de la Rivière, A. & Engelfriet, C.P. (1978) A simple immunofluorescence test for the detection of platelet antibodies. British Journal of Haematology 39, 195.

Brostoff, J. (1974) A simple technique for counting rosettes using acridine orange. Journal of Immunological Methods 5, 303.

Brown, D.L. (1974) The behaviour of phagocytic cell receptors in relation to allergic red cell destruction. Series Haematologica 7, 348.

Engelfriet, C.P., Borne, A.E.G. Kr. von dem, Beckers, D. & Loghem, J.J. van (1974) Autoimmune Haemolytic Anaemias. Serological and immunochemical characteristics of the autoantibodies: mechanisms of cell destruction. Series Haematologica 7, 328.

Fleer, A., Meulen, F.W. van der, Linthout, E., Borne, A.E.G. Kr. von dem & Engelfriet, C.P. Destruction of IgG-sensitized erythrocytes by human blood monocytes. Modulation of inhibition by IgG. British Journal of Haematology, in press,a.

Fleer, A., Koopman, M.G., Borne, A.E.G. Kr. von dem & Engelfriet, C.P. Monocyte-induced increase in osmotic fragility of human red cells sensitized with anti-D alloantibodies. British Journal of Haematology, in press,b.

Fleer, A., Schaik, J.L. van, Borne, A.E.G. Kr. von dem & Engelfriet, C.P. Destruction of sensitized erythrocytes by human monocytes in vitro. Effects of cytochalasin B, hydrocortisone and colchicine. Scandinavian Journal of Immunology, in press,c.

Hay, F.C., Torrigiani, G. & Roitt, I.M. (1972) The binding of human IgG subclasses to human monocytes. European Journal of Immunology 2, 257.

Holm, G., Engwall, E., Hammarström, S. & Natvig, J.B. (1974) Antibody-induced haemolytic activity of human blood monocytes. The role of antibody class and subclass. Scandinavian Journal of Immunology 3, 173.

Huber, H. & Fudenberg, H.H. (1968) Receptor sites of human monocytes for IgG. International Archives of Allergy and Applied Immunology 34, 18.

Huber, H., Douglas, S.D., Nusbacher, J., Kochwa, S. & Rosenfield, R.E. (1971) IgG subclass specificity of human monocyte receptor sites. Nature 229, 419.

Jandl, J.H. & Kaplan, M.E. (1960) The destruction of red cells by antibodies in man. III. Quantitative factors influencing the patterns of haemolysis in vivo. Journal of Clinical Investigation 39, 1145.

Kaplan, M.E. & Jandl, J.H. (1961) Inhibition of red cell sequestration by cortisone. Journal of Experimental Medicine 114, 921.

Kay, N.E. & Douglas, S.D. (1977) Monocyte-erythrocyte interaction in vitro in immune hemolytic anemias. Blood 50/5, 889.

LoBuglio, A.F., Cotran, R.S. & Jandl, J.H. (1967) Red cells coated with immunoglobulin G: binding and sphering by mononuclear cells in man. Science 158, 1582.

Logue, G. & Rosse, W.F. (1976) Immunologic mechanisms in autoimmune hemolytic disease. Seminars in Haematology 13/4, 277.

Loos, J.A., Blok-Schut, B., Kipp, B., Doorn, R. van & Meerhof, L. (1976) Size distribution, electronic recognition and counting of human blood monocytes. Blood 48, 743.

Mackenzie, M.R. (1975) Monocyte sensitization in autoimmune hemolytic anemia. Clinical Research 23/III, 278A.

Meulen, F.W. van der, Borne, A.E.G. Kr. von dem & Engelfriet, C.P. (1977) Storage of monocytes in liquid nitrogen. In: Perspectives in Inflammation. Willoughby, D.A., Giroud, J.P. & Velo, G.P. (eds.). MTP Press, Ltd., Lancaster, p. 635.

Meulen, F.W. van der, Hart, M. van der, Fleer, A., Borne, A.E.G. Kr. von dem, Engelfriet, C.P. & Loghem, J.J. van (1978) The role of adherence to human mononuclear phagocytes in the destruction of red cells sensitized with non-complement binding IgG antibodies. British Journal of Haematology 38, 541.

Mollison, P.L. (1962) The reticulo-endothelial system and red cell destruction. Proceedings of the Royal Society of Medicine 55, 915.

Mollison, P.L., Crome, P., Hughes-Jones, N.C. & Rochna, E. (1965) Rate of removal from the circulation of red cells sensitized with different amounts of antibody. British Journal of Haematology 11, 461.

Rosse, W.F. (1971) Quantitative immunology of immune hemolytic anemia. II. The relationship of cell-bound antibody to hemolysis and the effect of treatment. Journal of Clinical Investigation 50, 734.

Verheugt, F.W.A., Borne, A.E.G. Kr. von dem, Noord-Bokhorst, J.C. van & Engelfriet, C.P. Autoimmune granulocytopenia: the detection of granulocyte autoantibodies with the immunofluorescence test. British Journal of Haematology, in press.

Williams, W.J., Beutler, E., Eslen, A.J. & Wayne Rundles, R. (1972) Hematology. McGraw-Hill Book Company, New York.

chapter VI

Atkinson, J.P. & Frank, M.M. (1974) The effect of Bacillus Calmette-Guerin-induced macrophage activation on the in vivo clearance of sensitized erythrocytes. Journal of Clinical Investigation 53, 1742.

Brown, D.L. (1974) The behaviour of phagocytic cell receptors in relation to allergic red cell destruction. Series Haematologica, 7, 348.

Cohn, E.J., Meckin, T.L., Oneley, J.L. & Hughes, W.L. (1940) Preparations and properties of serum and plasma proteins. I. Size and charge of proteins separating upon equilibration across membranes with ammonium sulphate solutions of controlled pH, ionic strength and temperature. Journal of American Chemical Society, 62, 3386.

Dacie, J.V. (1962) The Haemolytic Anaemias. Part II: The autoimmune haemolytic anaemias, 2nd edition. J. & A. Churchill, Ltd., London.

Engelfriet, C.P., Borne, A.E.G. Kr. von dem, Beckers, D. & Loghem, J.J. van (1974) Autoimmune haemolytic anaemia: Serological and immunochemical characteristics of the autoantibodies; mechanisms of cell destruction. Series Haematologica, 7, 328.

Fleer, A., Meulen, F.W. van der, Linthout, E., Borne, A.E.G. Kr. von dem & Engelfriet, C.P. Destruction of IgG-sensitized erythrocytes by human blood monocytes. Modulation of inhibition by IgG. British Journal of Haematology, in press[a].

Fleer, A., Koopman, M.G., Borne, A.E.G. Kr. von dem & Engelfriet, C.P. Monocyte-induced increase in osmotic fragility of human red cells sensitized with anti-D alloantibodies. British Journal of Haematology, in press[b].

Fleer, A., Schaik, M.L.J. van, Borne, A.E.G. Kr. von dem & Engelfriet,C.P. Destruction of sensitized erythrocytes by human monocytes in vitro. Effects of cytochalasin B, hydrocortisone and colchicine. Scandinavian Journal of Immunology, in press[c].

Gill, P.G., Waller, C.A. & MacLennan, I.C.M. (1977) Relationships between different functional properties of human monocytes. Immunology 33, 873.

Goldstein, I.M., Roos, D., Kaplan, H.B. & Weissmann, G. (1975) Complement and immunoglobulins stimulate superoxide production by human leucocytes independently of phagocytosis. Journal of Clinical Investigation 56, 1155.

Hansen, N.E., Karle, H., Andersen, V., Malmquist, J. & Hoff, G.E. (1976) Neutrophilic granulocytes in acute bacterial infection. Sequential studies on lysozyme, myeloperoxidase and lactoferrin. Clinical and Experimental Immunology, 26, 463.

Hawkins, D. (1973) Neutrophilic leucocytes in immunologic reactions in vitro: I. Effect of cytochalasin B. Journal of Immunology, 110, 294.

Hawkins, D. (1974) Neutrophilic leucocytes in immunologic reactions in vitro: III. Pharmacologic modulation of lysosomal constituent release. Clinical Immunology and Immunopathology, 2, 141.

Holm, G. (1972) Lysis of antibody-treated human erythrocytes by human leucocytes and macrophages in tissue culture. International Archives of Applied Allergy, 43, 671.

Holm, G. & Hammarström, S. (1973) Haemolytic activity of human blood monocytes. Lysis of human erythrocytes treated with anti-D serum. Clinical and Experimental Immunology, 13, 29.

Kay, N.E. & Douglas, S.D. (1977) Mononuclear phagocyte. Development, structure, function and involvement in immune response. New York State Journal of Medicine, 327.

Leddy, J.P. & Swisher, S.N. (1971) Acquired immune hemolytic disorders. In: Immunological Diseases. M. Santer (ed.). Little, Brown & Co., Boston, 1083.

Logue, G. & Rosse, W. (1976) Immunologic mechanisms in autoimmune hemolytic disease. Seminars in Haematologica, 13, 277.

Loos, H., Blok-Schut, B., Kipp, B., Doorn, R. van & Meerhof, L. (1976) Size distribution, electronic recognition and counting of human blood monocytes. Blood, 48, 743.

MacDonald, H.R., Bonnard, G.D., Sordat, B. & Zawodnik, S.A. (1975) Antibody-dependent cell-mediated cytotoxicity: heterogeneity of effector cells in human peripheral blood. Scandinavian Journal of Immunology, 4, 487.

McCall, C.E., Caves, J., Cooper, R. & DeChatelet, L. (1971) Functional characteristics of human toxic neutrophils. Journal of Infectious Diseases, 124, 68.

Meulen, F.W. van der, Hart, M. van der, Fleer, A., Borne, A.E.G. Kr. von dem, Engelfriet, C.P. & Loghem, J.J. van (1978) The role of adherence to human mononuclear phagocytes in the destruction of red cells sensitized with non-complement binding IgG antibodies. British Journal of Haematology, 38, 541.

Meuret, G. & Hoffmann, G. (1973) Monocyte kinetic studies in normal and disease states. British Journal of Haematology, 24, 275.

Meuret, G., Detel, U., Kilz, H.P., Senn, H.J. & Lessen, H. von (1975) Human monocytopoiesis in acute and chronic inflammation. Acta Haematologica, 54, 328.

Mørland, B. & Kaplan, G. (1977) Macrophage activation in vivo and in vitro. Experimental Cell Research, 108, 279.

Rhodes, J. (1975) Macrophage heterogeneity in receptor activity: The activation of macrophage Fc receptor function in vivo and in vitro. Journal of Immunology, 114, 976.

Soelberg, C.O. & Hellum, K.B. (1972) Neutrophil granulocyte function in bacterial infections. Lancet ii, 727.

Verheugt, F.W.A., Borne, A.E.G. Kr. von dem, Noord-Bokhorst, J.C. van & Engelfriet C.P. Auto-immune granulocytopenia: the detection of granulocyte auto-antibodies with the immunofluorescence test. British Journal of Haematology, in press.

Williams, W.J., Beutler, E., Erslev, A.J. & Wayne Rundles, R. (1972) Hematology. McGraw-Hill Book Company, New York.

Wright, D.G. & Malawista, S.E. (1973) Mobilization and extracellular release of granular enzymes from human leucocytes during phagocytosis: inhibition by colchicine and cortisol but not by salicylate. Arthritis and Rheumatism, 16, 749.

Zeijlemaker, W.P., Roos, M.Th.L., Schellekens, P.Th.A. & Eijsvoogel, V.P. (1975) Antibody-dependent human lymphocytotoxicity: a micro assay system. European Journal of Immunology, 5, 579.

chapter VII

Bois, M.J.G.J. du, Schellekens, P.Th.A., Wit, J.J.F.M. de, Eijsvoogel, V.P. In vitro reactivity of human lymphocytes after cryopreservation using a programmed cooling device. Scand. J. Immunol. 5, suppl. 5: 17, 1976.

Brostoff, J. A simple technique for counting rosettes using acridine orange. J. immunol. Meth. 5: 303, 1974.

Cline, M.J. Monocytes and macrophages: Functions and Diseases. Ann. Int. Med. 88: 78, 1978.

Cohn, E.J., Meckin, T.L., Oneley, J.L., Hughes, W.L. Preparations and properties of serum and plasma proteins. I. Size and charge of proteins separating upon equilibration across membranes with ammonium sulphate solutions of controlled pH, ionic strength and temperature. J. Amer. Chem. Soc. 62: 3386, 1940.

Dean, D.A., Strong, D.M. Improved assay for monocyte chemotaxis using frozen stored responder cells. J. immunol. Meth. 14: 65, 1977.

Dixon, W.J., Massey Jr., F.J. Introduction to statistical analysis, 3rd ed, p. 162 and 193. McGraw-Hill Book Company, New York, 1969.

Eijsvoogel, V.P., du Bois, M.J.G.J., Wal, R. v.d., Huisman, D.R., Raat-Koning, L. Cryopreserved lymphocytes, functional properties in vitro. In: The Cryopreservation of Normal and Neoplastic Cells. Eds. Weiner, Oldham and Schwarzenberg, Inserm, Paris, p. 101, 1973.

Farrant, J., Knight, S.C., Morris, G.J. Use of different cooling rates during freezing to separate populations of human peripheral blood lymphocytes. Cryobiology 9: 516, 1972.

Fleer, A., Meulen, F.W. v.d., Linthout, E., Borne, A.E.G. Kr. von dem, Engelfriet, C.P. Destruction of IgG-sensitized erythrocytes by human blood monocytes. Modulation of inhibition by IgG. Brit. J. Haemat., in press[a].

Fleer, A., Schaik, M.L.J. van, Borne, A.E.G. Kr. von dem, Engelfriet, C.P. Destruction of sensitized erythrocytes by human monocytes in vitro. Effects of cytochalasin B, hydrocortisone and colchicine. Scand. J. Immunol., in press[b].

Furth, R. van (ed.) Mononuclear phagocytes in immunity, infection and pathology. Blackwell Scientific Publications, London, 1975.

Harris, L.W., Griffiths, J.B. An assessment of methods used for measuring the recovery of mammalian cells from freezing and thawing. Cryobiology 11: 80, 1974.

Huber, H., Fudenberg, H.H. Receptor sites of human monocytes for IgG. Int. Arch. Allergy 34: 18, 1968.

Karnovsky, M.J. A formaldehyde-glutaraldehyde fixative of high osmolality for use in electron microscopy. J. Cell Biol. 27: 137[a], 1965.

Leibo, S.P., Mazur, P. The role of cooling rates in low temperature preservation. Cryobiology 8: 447, 1971.

Loos, J.A., Blok-Schut, B., Kipp, B., Doorn, R. van, Meerhof, L. Size distribution, electronic recognition, and counting of human blood monocytes. Blood 48, no. 5: 743, 1976.

Malanin, Th.I. Injury of human polymorphonuclear granulocytes frozen in the presence of cryoprotective agents. Cryobiology 9: 123, 1972.

Meryman, H.T., Williams, R.J., Douglas, M.St.J. Freezing injury from 'solution effects' and its prevention by natural or artificial cryoprotection. Cryobiology 14: 287, 1977.

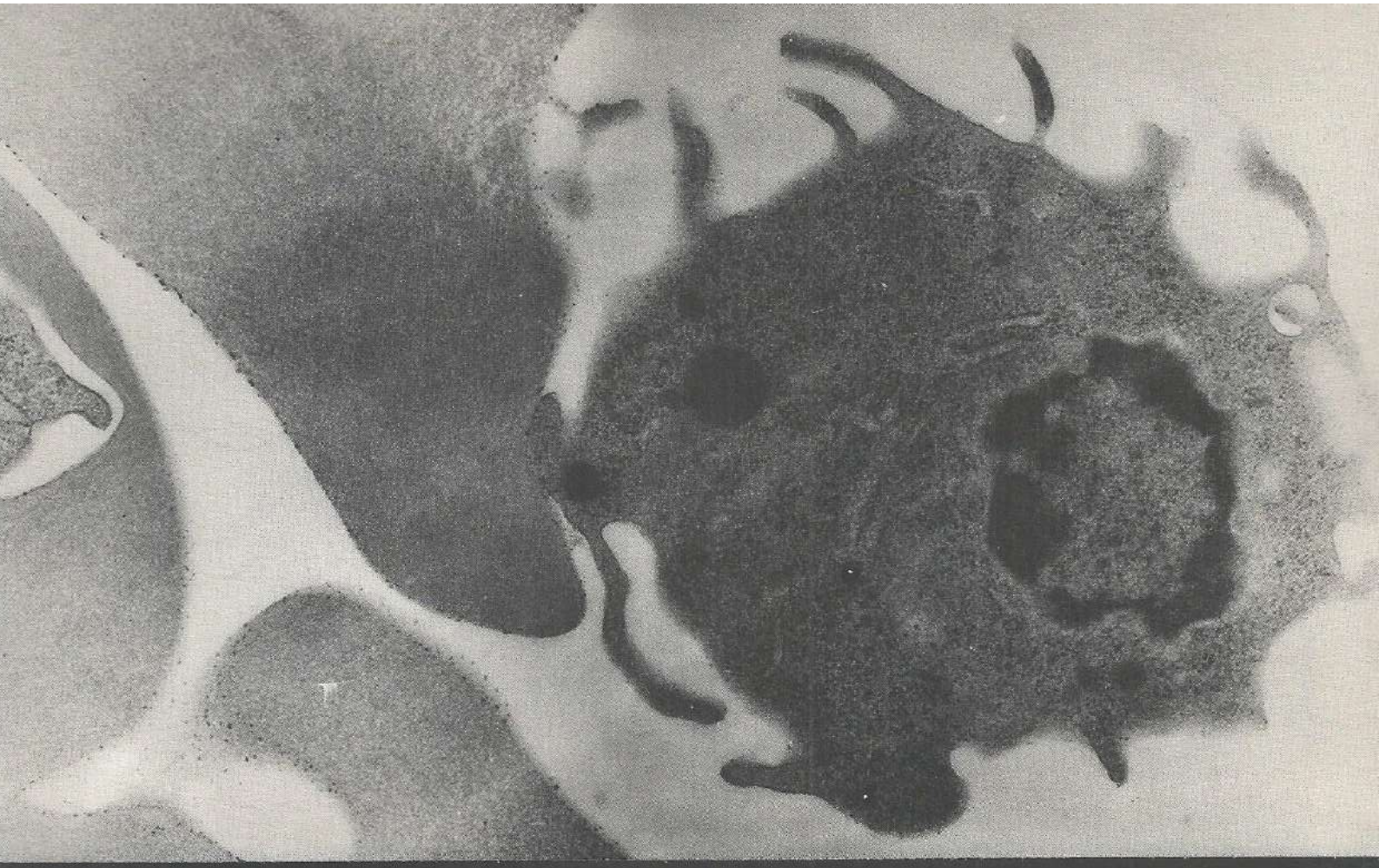
Millonig, G. Advantages of a phosphate buffer for OsO_4 solutions in fixation. J. appl. Physics 32: 1637, 1961.

Persidsky, M.D., Ellett, M.H. Lysosomes and cell cryoinjury. Cryobiology 8: 345, 1971.

Reiss, M., Roos, D. Differences in oxygen metabolism of phagocytozing monocytes and neutrophils. J. clin. Invest., 61: 480, 1978.

Tanaka, Y., Goodman, J.R. Electron microscopy of human blood cells. Harper and Row, publishers, New York, 1972.

Zigmond, S.H. and Hirsch, J.G. Leucocyte locomotion and chemotaxis. New methods for evaluation and demonstration of cell-derived chemotactic factor. J. exp. Med. 137: 387, 1973.



glossary

ABBREVIATIONS

AGT	–	*antiglobulin test*
AIHA	–	*autoimmune haemolytic anaemia*
BSA	–	*bovine serum albumin*
^{51}Cr $T_{\frac{1}{2}}$	–	*^{51}Chromium half life*
DAGT	–	*direct antiglobulin test*
DMSO	–	*dimethyl sulfoxide*
EA	–	*erythrocyte-antibody complex*
EAIgG	–	*IgG-sensitized red cells*
EAIgG anti-A	–	*IgG anti-A-sensitized red cells*
EAIgG anti-D	–	*IgG anti-D-sensitized red cells*
FCS	–	*fetal calf serum*
FITC	–	*fluorescein isothiocyanate*
F/P ratio	–	*fluorescein/protein ratio*
G6PD	–	*glucose-6-phosphate dehydrogenase*
^{125}I	–	*^{125}Iodide*
IgA	–	*immunoglobulin A*
IgG	–	*immunoglobulin G*
IgM	–	*immunoglobulin M*
LDH	–	*lactate dehydrogenase*
MEM	–	*minimal essential medium*
MEM-Tris	–	*minimal essential medium buffered with Tris-HCl*
PBM	–	*peripheral blood monocyte*
PBS	–	*phosphate-buffered saline*
P/S	–	*penicillin/streptomycin*
RAHI	–	*rabbit anti-human IgG*
RFI	–	*relative fluorescence intensity*
Rh D	–	*rhesus D-positive*
SEM	–	*standard error of the mean*
STZ	–	*serum-treated zymosan*

ADHERENCE – A. Binding of red cells to leukocytes. B. Binding of monocytes to glass or plastic surfaces. Since lymphocytes do not adhere to these surfaces, this property of the mononuclear phagocytes provides a possibility to separate the two cell populations. After adherence of the monocytes, the lymphocytes can be washed away.

AGGLUTINATION – In this thesis: Clumping of red cells induced by the reaction of antibodies with antigens present on the red cell surface. For example, agglutination of red cells by an antiserum containing antibodies directed against IgG, indicates the presence of IgG antibodies on these cells.

AGGLUTINATION TITRE – The highest dilution of serum which still causes clumping of red cells.

ALLOANTIBODY – Antibody produced by one individual that reacts with antigens of another individual of the same species.

ANTIBODY-MEDIATED CYTOTOXIC LYSIS – In this thesis: Lysis of red cells adhering to phagocytes by antibodies directed against antigens present on the red cell.

ANTI-D SERUM – In this thesis: Serum containing IgG antibodies directed against the D antigen, present on Rhesus-positive red cells. When Rhesus-positive red cells are incubated with an anti-D serum, the IgG antibodies bind strongly to the red cell surface.

ANTIGLOBULIN TEST – Test in which dilutions of an antiserum containing antibodies directed against human immunoglobulins (i.e. antiglobulins) are added to red cells in vitro. The occurrence of red cell clumping is determined. If, for example, immunoglobulin G (IgG) is present on the red cell surface, erythrocytes will clump together and the antiglobulin test with anti-IgG serum is positive. Antiglobulin sera are raised in rabbits. The animals are repeatedly injected with immunoglobulins to stimulate the production of antibodies.

ANTI-IgG SERUM - Antiserum containing antibodies directed against immunoglobulin G. Agglutinates red cells coated with IgG antibodies.

AUTOIMMUNE HAEMOLYTIC ANAEMIA - Haemolytic anaemia due to autoantibodies directed against red cells.

AUTOANTIBODY - Antibody reacting with an antigen which is a normal constituent of the body of the individual by whom the antibody is produced.

BUFFY COAT - The layer of white cells that forms between the red cell layer and the plasma when anticoagulated blood is centrifuged.

CASEIN - Major milk protein stimulating directed migration of phagocytes.

CHEMOTACTIC RESPONSE - Reaction in which the directed locomotion of cells, induced by chemical substances, is determined. The cells move towards the source of a concentration gradient of the substance.

^{51}CHROMIUM $T_{\frac{1}{2}}$ - In this thesis: Method to estimate the red cell life-span in vivo. Erythrocytes are radioactively labelled with ^{51}Chromium and (re) injected into the circulation. In blood samples, collected in the course of time, the radioactivity is determined. In this way, the red cell half life ($T_{\frac{1}{2}}$) can be calculated.

COMPLEMENT - A system of serum enzymes that is mainly activated by antigen-antibody reactions. Complement is made up of many components.

CRYOPRESERVATION - In this thesis: Preservation by storage at -196°C. This is the boiling temperature of nitrogen at one atmosphere. In vessels filled with liquid nitrogen red and white blood cells and tissues can be stored for long periods.

CRYOPROTECTANT - Agents which protect biological material from damage during freezing and thawing. Examples: dimethyl sulfoxide and glycerol.

CYTOCHALASIN B - Substance which belongs to a group of metabolites derived from moulds. These agents have a variety of effects on cells in vitro. Biological effects include among others: inhibition of phagocytosis and cell mobility and stimulation of lysosomal enzyme release.

CYTOFLUOROGRAPH® - Apparatus to measure the fluorescence intensity of individual cells in a continuous flow system.

CYTOFLUOROMETRY - Method to measure the fluorescence intensity of individual cells.

CYTOTOXIC ACTIVITY - In this thesis: Activity of one cell population (effector cells) leading to the destruction of another cell population (target cells). For example: the cytotoxic activity of monocytes towards red cells sensitized with IgG antibodies.

DIRECT ANTIGLOBULIN TEST - An application of the antiglobulin test in which red cells from a patient are washed and tested with an antiglobulin serum. Agglutination indicates that the red cells are sensitized in vivo with an incomplete antibody.

DIMETHYL SULFOXIDE - Powerful solvent dissolving many organic compounds. It has the ability to penetrate plant and animal tissues and to protect cells during freezing.

ELUATE - In this thesis: Material containing antibodies liberated from the red cell surface.

EXTRACELLULAR LYSIS - Cytotoxic activity effected outside the effector cell (see Cytotoxic Activity).

F(ab) FRAGMENT - Fragment of immunoglobulin G molecules, obtained by enzymatic digestion. It contains one site to combine with an antigen, but is unable to cause agglutination.

$F(ab')_2$ FRAGMENT - Fragment of immunoglobulin G molecules, obtained by enzymatic digestion. The enzyme cleaves the IgG molecules into $F(ab')_2$ and Fc fragments.

Fc FRAGMENT - Fragment of immunoglobulin G molecules, obtained by enzymatic digestion. This fragment is essential for the adherence of red cells sensitized with IgG antibodies to leukocytes.

"Fc RECEPTOR" - Not well-defined site on the surface of monocytes, granulocytes and some lymphocytes. This site combines with the Fc fragment of immunoglobulins.

FICOLL-ISOPAQUE DENSITY GRADIENT CENTRIFUGATION - Method for the separation of cells based on their difference in specific gravity. Peripheral blood is layered on a mixture of Ficoll (a polysucrose) and Isopaque (a heavy chemical) with a defined density. After centrifugation cells of a higher density than the gradient can be recovered from the bottom of the tube whereas cells with a lower density float on the gradient, in the so-called interface between plasma and gradient material.

FLOW CYTOFLUOROMETRY - Cytofluorometry by means of a flow system. In this thesis, for example, red cells with IgG antibodies on their surface were incubated with a fluorescent anti-IgG serum. In a continuous flow these cells pass a laserbeam, which excites the fluorescent label. The subsequent emission of fluorescence is recorded.

FLUORESCEIN - Substance which emits visible light of a characteristic wave length when irradiated with a certain (shorter) wave length.

FLUORESCEIN ISOTHIOCYANATE - A reactive fluorescein-derivative which can combine with proteins, for example with antibodies.

FLUORESCEIN PROTEIN RATIO - Number of fluorescein isothiocyanate molecules per molecule of protein.

HAEMAGGLUTINATION - Agglutination of erythrocytes.

HAEMOCONCENTRATION - Decrease of the fluid content of the blood with resulting increase in, for example, erythrocyte concentration.

HAEMOLYSIS - Destruction of red cells.

IMMUNOGLOBULINS - Serum proteins that may carry antibody activity. In human, 5 major structural classes can be distinguished: immunoglobulin G, A, M, D and E.

IMMUNOGLOBULIN G - The major immunoglobulin in the serum of man. In humans, it can be divided into 4 subclasses, IgG1, IgG2, IgG3 and IgG4 on the basis of structural differences.

INCOMPLETE ANTIBODIES - Antibodies that coat erythrocytes but do not agglutinate them. Their presence can be detected by the antiglobulin test.

INCUBATION - Application of environmental conditions (position, temperature, humidity, etc.) in vitro, which influences the development of a process.

INDIRECT ANTIGLOBULIN TEST - Application of the antiglobulin test for the detection of free incomplete antibodies in serum.

IN VITRO - In glass; in tube; in the laboratory.

IN VIVO - In the body.

LYSOSOMAL ENZYMES - Enzymes present in the granules within certain white blood cells. These enzymes play an important role in intracellular digestion and are involved in many types of cell injury. They may also be released from the cell.

MICROTITRE PLATE - In this thesis: Plastic plate containing 96 wells, each with a capacity of 0.25 ml.

MIGRATION - In this thesis: Movement of cells; directed or indirected ('random').

MINIMAL ESSENTIAL MEDIUM - Salt solution of controlled pH and osmolarity, containing essential nutrients for cell survival.

MITOCHONDRIA - Corpuscles which are the principal sites of the generation of energy within the cell.

MONOLAYER - In this thesis: Single layer of monocytes attached to plastic or glass.

MONONUCLEAR PHAGOCYTE SYSTEM - System of phagocytic cells considered to share a common origin from the bone marrow. These cells circulate for 1½-4 days in the blood as monocytes. Thereafter they move to tissues (spleen, liver and lymph nodes) or sites of inflammation and are then called macrophages.

OPSONIZATION - Coating of micro-organisms and other particles with factors present a.o. in plasma, leading to an increased susceptibility to phagocytosis.

OR_2R_2 - Certain Rhesus D-positive red cells.

NON-COMPLEMENT BINDING ANTIBODIES - Antibodies not able to activate the complement system or to bind complement components when present on the red cell surface.

RABBIT ANTI-HUMAN IgG - Antibodies raised in rabbits against human immunoglobulin G.

ROSETTE - In this thesis: Monocyte with three or more IgG-sensitized red cells bound to its surface.

SENSITIZATION - In this thesis: Coating with antibody.

SEROLOGY - The study of antigen-antibody reactions in vitro.

SPHEROCYTE - Small globular erythrocyte without the usual central pallor.

SUBCLASS - See immunoglobulin G.

SUPERNATANT - In this thesis: Fluid above cells.

SUSPENSION - In this thesis: A mixture of cells and medium.

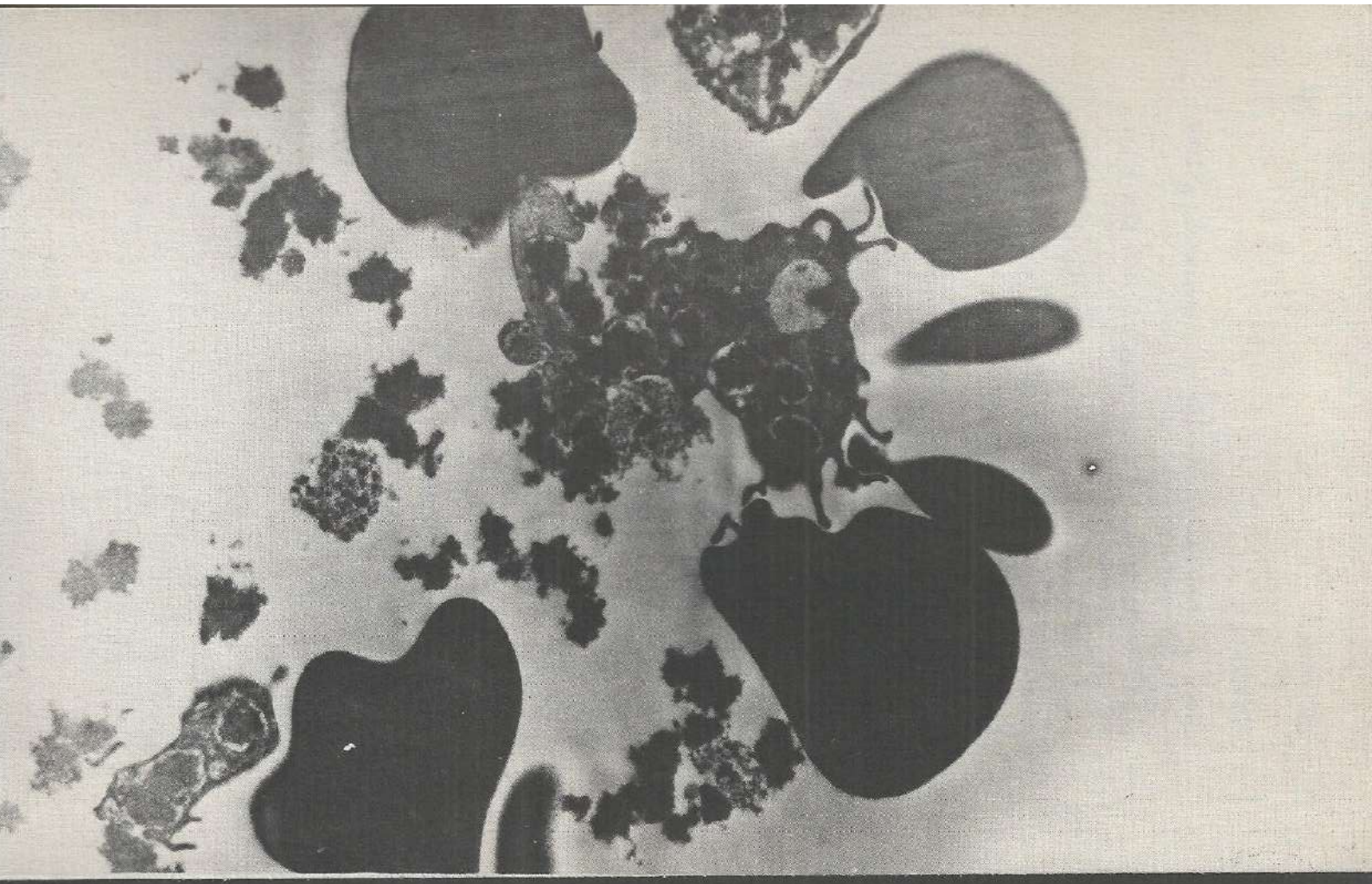
TRYPAN BLUE - Stain used to assess the viability of cells. Cells with a normal membrane permeability do not take up trypan blue. Dead and dying cells stain blue.

WASHING - A term used in serology to indicate the method of removal of all traces of an unwanted soluble substance from a suspension of cells. Accomplished by centrifugation, removal of supernatant and resuspension in fresh medium. Repeated three times.

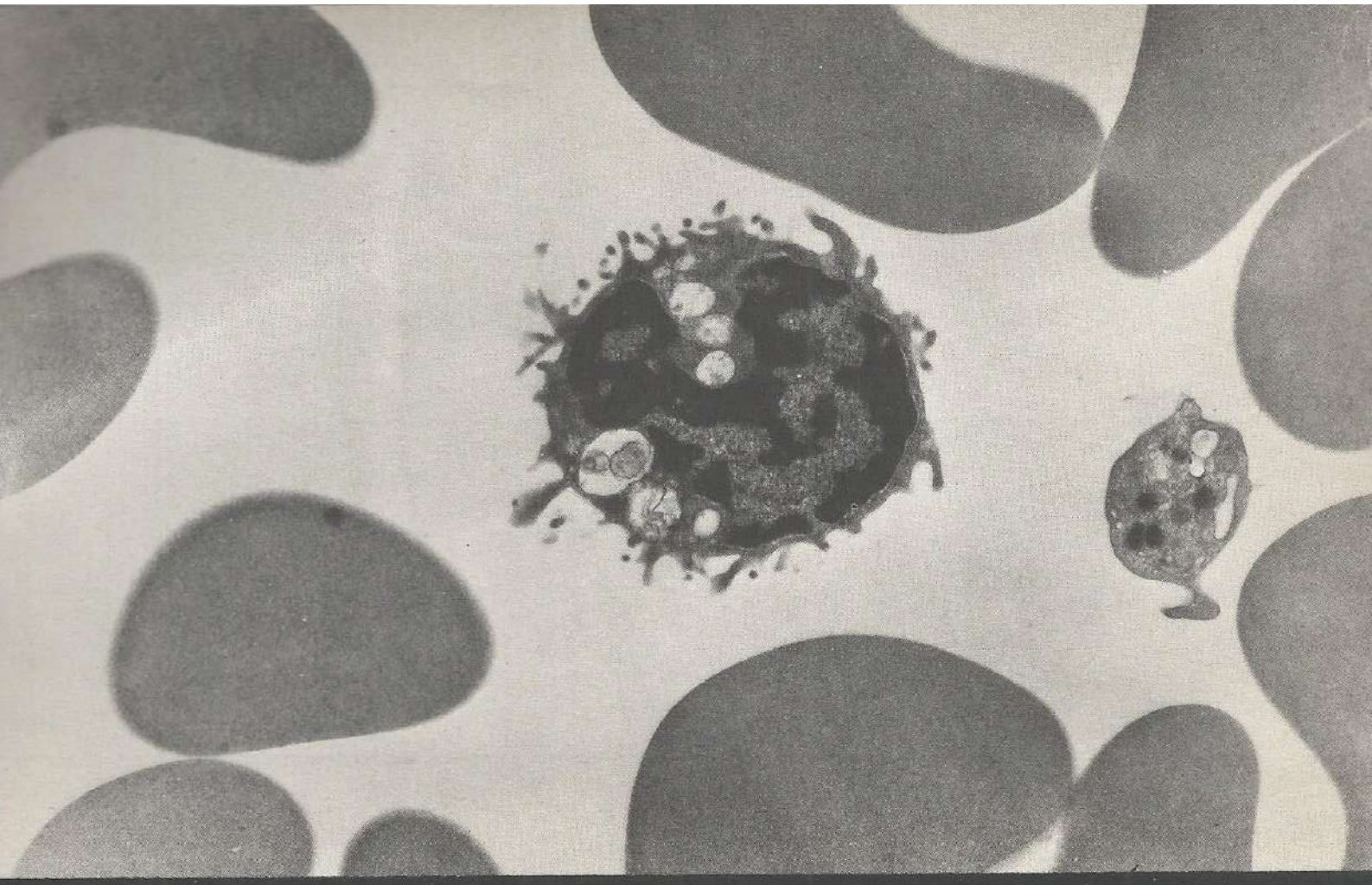
WASHING OF MONOLAYERS - Removal of non-adherent cells.

ZYMOSAN - Acetone-extracted yeast membranes.

PICTURES



index



colofon

this thesis was prepared in the department of immunohaematology, central laboratory of the netherlands red cross blood transfusion service and the laboratory of experimental and clinical immunology of the university of amsterdam, amsterdam, the netherlands.

the studies presented in this thesis were financially supported by a grant from the foundation for medical research (FUNGO).

dr. a.e.g. kr. von dem borne, dr. d. roos and dr. w.p. zeijlemaker advised the author in the preparation of the manuscript.

statistical advices: mr. m.f.m. janssen.

electron microscopical pictures: mr. j. agterberg and mr. h.l. mollevanger.

figures and photography: audiovisual department of the netherlands cancer institute.

typing of the manuscript: ms. jetty gerritsen.

jacket and interior design: hans kroeze.

in addition to those mentioned above, the author wishes to thank all who have contributed to this thesis for their pleasant cooperation.

published by rodopi, amsterdam, the netherlands.