Studies on the In Vivo Effects of Antibody

INTERACTION OF IgM ANTIBODY AND COMPLEMENT IN THE IMMUNE CLEARANCE AND DESTRUCTION OF ERYTHROCYTES IN MAN

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ABSTRACT Purified human IgM isoagglutinins were utilized to sensitize ^{51}Cr-labeled erythrocytes so as to produce a known number of complement-fixing sites. These cells were then reinfused into the erythrocyte donor. A minimum of 20 C1-fixing sites/erythrocyte were required for decreased survival. As the amount of antibody coating the erythrocytes was increased, a larger percentage was sequestered. With 80 C1-fixing sites, more than 75% of the injected erythrocytes were removed from the circulation within 10 min. In each case, the clearance pattern consisted of rapid hepatic sequestration followed by a gradual return of a portion of the erythrocytes into the circulation where they survived normally.

Clearance was shown to be dependent upon activation of the classical complement pathway, since sensitized cells survived normally in hereditary angioedema patients with low levels of C4 and no detectable C2. Exposure of sensitized cells to fresh serum for 15 min led to the deposition of 550–800 C3 molecules/C1-fixing site. Such cells were immune adherence positive, were agglutinated by anti-C3b, formed rosettes with human alveolar macrophages, and were sequestered in vivo, presumably because of the interaction of cell-bound C3b with the C3b receptor on hepatic macrophages. After exposure to heated serum as a source of the C3b inactivator, the cells were immune adherence negative, were agglutinated only by anti-C3d, did not form rosettes with macrophages, and survived normally in vivo despite, being Coombs positive. Cleavage of cell-bound C3b to C3d may explain the release phase of the IgM clearance pattern. Whereas erythrocytes coated with IgM antibody and complement were previously thought to be sequestered in the liver because of extensive membrane damage, these experiments suggest that clearance is determined by the interaction of erythrocyte-bound complement fragments with specific receptors on hepatic macrophages.

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INTRODUCTION

Since the late 1940's when it first became possible to radiolabel erythrocytes and study their fate in man, there have been many investigations of autoimmune hemolytic anemia (1–3). Interest in this condition has focused upon its importance as a model of autoimmune disease in man. Furthermore, it provides a model for the direct exploration of the consequences of cell sensitization by antibody and the mechanisms of cell destruction (2, 4–7). Early studies demonstrated that erythrocyte sensitization often leads to decreased survival and suggested that sensitization of these cells by antibody in the absence of complement activation results in splenic sequestration due to subtle antibody-mediated membrane damage (4, 5, 8). In contrast, antibodies which mediated the deposition of complement on the cell surface appeared to produce major membrane damage, and such cells were sequestered by reticuloendothelial elements of the liver (4, 6–9).

A number of clinical observations, however, questioned this simple formulation. For example, Evans et al. demonstrated that Coombs-positive erythrocytes from patients with cold agglutinin-mediated hemolytic anemia were more resistant to intravascular destruction when transfused into these patients than were normal erythrocytes (10, 11). Furthermore, patients in clinical remission from hemolytic anemia often could be shown to have antibody and complement on their cells, as judged by tests performed with gamma and nongamma (anticomplement) Coombs sera, although their cells underwent normal survival (1, 3, 12).

During the course of studies of the interactions of IgG and IgM antibodies and complement, it became

clear that these two classes of antibody interacted with complement by different molecular mechanisms, and thus might be expected to produce different biological effects (13–15). Therefore, a model for immune clearance[1] and destruction of erythrocytes was established in the guinea pig to allow the function of these separate immunoglobulin classes to be assessed in molecular terms (16, 17). In the case of IgG, there was progressive clearing of the cells from the circulation, primarily by the spleen at low levels of sensitization. Complement activation played a major role in augmenting clearance. With IgM, the pattern of clearance was entirely different. Cells coated with sufficient numbers of IgM molecules to effect clearance were rapidly sequestered by the liver. However, a major proportion of the cells were not destroyed but were returned to the circulation where they survived normally, despite the presence of antibody and complement on their surface. In this case, clearance was entirely complement dependent, and IgM could not mediate clearance in the absence of an intact classical complement pathway.

The clearance curves generated with IgM-sensitized erythrocytes in the guinea pig model did not resemble those typically noted in clearance of sensitized cells in man. However, most studies of the clearance of sensitized erythrocytes in man did not utilize highly purified immunoglobulin fractions, control for the number of sensitizing antibody molecules, nor examine the possibility of antibody elution. Thus, it was of importance to determine whether the findings in the guinea pig model were peculiar to that species or whether these findings might prove true in humans and serve to explain many of the aforementioned clinical observations.

METHODS

Study subjects. All subjects used in these studies were of blood type A or B. 23 normal volunteers between 18 and 24 yr of age, of either sex, and taking no systemic medications were utilized in in vivo studies. All were hospitalized at the National Institutes of Health and had normal or negative evaluations of the following parameters: physical examination, complete blood count, direct and indirect Coombs' tests, cold agglutinins, Australia antigen, liver enzymes, and direct and total bilirubin. Informed consent was obtained, and volunteers were used only once for an in vivo clearance study. Volunteers received only autologous erythrocytes, which in certain experiments had been previously exposed to autologous serum.

Two patients with low serum complement secondary to hereditary angioneurotic edema (HANE)[2] were also studied. One patient, a 62-yr-old male, had frequent determinations demonstrating less than 0.05% of the normal level of C4 and no detectable C2. At the time of study, this patient was taking no medications and was free of clinical symptoms. The second patient, a 58-yr-old female, had been followed for a 6-mo period with less than 0.05% of the normal level of C4 and no detectable C2. At the time of the study, this patient was receiving only antihypertensive medications and was free of clinical symptoms.

Complement studies. Hemolytic titrations of human C4 and C2 and immunochemical analysis of the C1 inhibitor were performed as previously reported (18–20). Human C3 was quantitated by utilizing commercial plates (Hyland Div., Travenol Laboratories, Inc., Costa Mesa, Calif.). Cell-bound C3 was measured by a previously published modification of the method of Borsos and Leonard (21, 22).

IgM isoagglutinins. Anti-A and anti-B complement-fixing IgM isoagglutinins were used in this study. Australia antigen-negative sera (as tested by counter immunoelectrophoresis and radioimmunoassay) with high titer isoagglutinins were obtained from donors to the NIH blood bank program or from Ortho Diagnostics, Raritan, N. J. The donors were individuals who had given blood previously and had been shown not to be carriers of hepatitis. Hemagglutinin and hemolysin activity were measured as previously reported for each antisera, before and after 2-mercaptoethanol treatment (16, 23). The sera with highest agglutinating activity which was sensitive to mercaptoethanol were chosen for further purification. The techniques of immunoglobulin purification were identical to those previously reported for rabbit IgM (16). Highly purified IgM from individual antisera was obtained by the following sequential steps: (*a*) precipitation with 5.4 molal ammonium sulfate at 0°C and centrifugation for 15 min at 900 *g* at 0°C; (*b*) separation of the immunoglobulin precipitate into 19S and 7S fractions by Sephadex G200 gel filtration; (*c*) concentration of the column fractions by ultrafiltration; and (*d*) sucrose density ultracentrifugation of the 19S pool. Portions of the final highly purified IgM preparations were passed through a 0.45-μ Millipore filter (Millipore Corp., Bedford, Mass.). The filtrate was shown to be free of Australia antigen by radioimmunoassay in the NIH blood bank and was shown to be sterile and pyrogen free by the NIH pharmaceutical development service.

Quantitation of the number of antibody molecules per erythrocyte. The C1a fixation and transfer test was utilized to quantitate in absolute molecular terms the average number of complement-fixing IgM antibody sites per erythrocyte in the sensitized population. The method employed was outlined previously (16, 24, 25). Human IgM anti-A and -B isoagglutinins generated a typical dose-response curve with a slope of approximately 1.0 (Fig. 1). The slope of 1.0 has been taken to indicate that one antibody molecule is capable of fixing one molecule of C1 (24). Radioiodination of a representative portion of an IgM pool was performed as previously reported (17, 26). The number of radio-labeled IgM molecules absorbed per erythrocyte was calculated and compared with the number obtained utilizing the C1a fixation and transfer test. 100 C1-fixing sites/cell were generated with 120–180 molecules of IgM by direct comparison with binding by antibody in the iodinated pool. However, only 0.4% of the pool showed specific binding, and the number of bound IgM molecules by the radio-label method was therefore less precise than the C1 fixing data. Others have shown that every IgM anti-A molecule fixes C1 (24).

Antiglobulin reagents. Antihuman C3 and IgM were obtained commercially from Hyland, Meloy Laboratories, Springfield, Va., and from Behring Diagnostics, Inc., Woodbury, N. Y. The preparation and specificity of the anti-

[1] Clearance refers to the accelerated removal of erythrocytes from the circulation by an abnormal mechanism.

[2] *Abbreviations used in this paper:* HANE, hereditary angioneurotic edema; HBSS, Hanks' balanced salt solution; PNH, paroxysmal noctural hemoglobinuria.

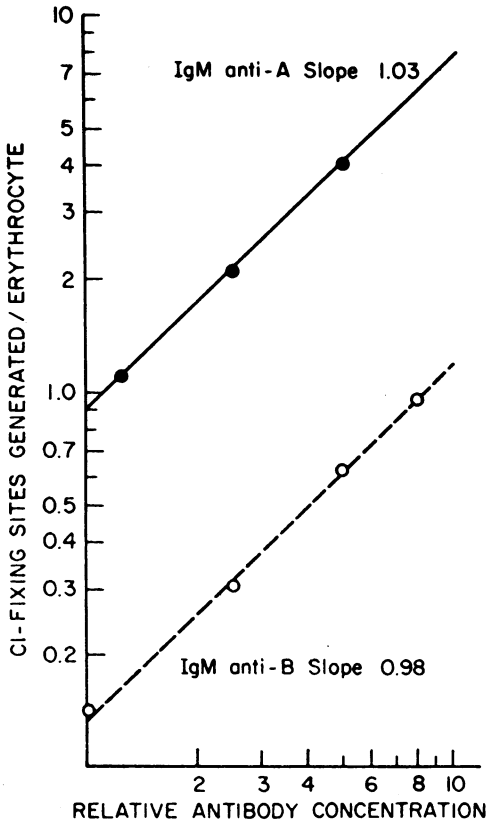

FIGURE 1 C1a fixation and transfer: human IgM, anti-A, and anti-B isoagglutinins. Numbers of cell-bound C1-fixing sites were determined on an absolute molecular basis as a function of the concentration of antibody used to sensitize the erythrocyte population. The slope of 1.0 is taken as an indication of the fact that one IgM molecule is capable of fixing one molecule of C1.

human C3b and C3d have been previously described (27). After absorption with human type A erythrocytes, these regents were shown to have the proper specificity. The anti-IgM sera and anti-C3 did not agglutinate erythrocytes sensitized with IgG or IgA, and the preparations did not agglutinate sensitized erythrocytes coated with the first, fourth, and second complement components. Antiglobulin (Coombs) titers were obtained with a microtiter method as previously reported (17).

Clearance studies. This technique was performed by utilizing sterile pyrogen-free equipment and buffers. On the day of the study, 5 ml blood was drawn by venipuncture and placed in a test tube containing 0.75 ml of acid-citrate-dextrose solution (USP Formula A) (Fenwal Laboratories, Inc., Morton Grove, Illinois). The cells were sedimented at 1000 rpm for 5 min at 4°C and the serum and buffy coat removed. Erythrocytes were washed three times in Hanks' balanced salt solution (HBSS) and standardized spectrophotometrically to a concentration of 6.6 $\times 10^8$ cells/ml. Ten μCi of sterile sodium chromate with an average sp act of 4.9 μg/ml (Amersham/Searle Corp., Arlington Heights, Ill.) was added dropwise with constant mixing per milliliter of standardized erythrocyte suspension and the mixture incubated for 30 min at 37°C with frequent mixing. The ^{51}Cr-labeled erythrocytes were washed three times in HBSS and once in 0.9% saline (Abbott Laboratories, North Chicago, Ill.) before being resuspended (3.3×10^8 cells/ml) in physiologic saline. A portion of these cells were sensitized by adding dropwise with constant mixing an equal volume of the highly purified appropriate IgM antibody preparation diluted in physiologic saline. The mixture was incubated for 30 min at 37°C with frequent mixing, then washed and resuspended in normal saline (3.3×10^7 cells/ml). A 10-ml portion of this material was injected into an anticubital vein, and erythrocyte survival was determined by timed serial bleedings from the opposite arm. 11-ml samples were obtained by withdrawing blood through a 19-gauge small vein infusion set (Abbott Laboratories) into a plastic syringe lightly coated with sodium heparin (The Upjohn Co., Kalamazoo, Mich.). Exactly 10 ml was transferred to test tubes and the erythrocytes packed by centrifugation at 3,000 rpm for 10 min. The number of counts in the plasma and packed erythrocytes was obtained by utilizing a gamma scintillation counter (Packard Instrument Co., Inc., Downers Grove, Ill.). Multiple samples were obtained during the initial 7 h of the study and daily thereafter until the rate of erythrocyte survival was determined.

Localization of the site of sequestration of IgM-sensitized erythrocytes was established by using an organic plastic localizing whole body counter in a lead room (28).

Calculation of erythrocyte survival. The erythrocyte survival data was analyzed, as recommended by the International Committee for Standardization in Haematology (29). All ^{51}Cr measurements were corrected for elution and physical decay. The recipients erythrocyte volume was calculated from the patient's weight, height, and corrected venous hematocrit, as described by Nadler, Hidalgo, and Bloch and modified by Mollison (3, 30). Using standard dilution formulae, the zero time value was determined by dividing the total number of injected counts per minute by the calculated erythrocyte volume. The numbered counts per minute per milliliter erythrocytes was corrected to an initial value of unity, so that the data from individuals of differing size could be compared and statistically analyzed. In the control studies with labeled but unsensitized erythrocytes, the experimentally determined erythrocyte volume was within 10% of the calculated volume, and the 60- and 120-min samples were 98±6% of the 6- and 10-min samples. Data was plotted on semilog paper and, when appropriate, a straight line was calculated by the method of least squares in order to determine the best fitting decay curve.

Effect of in vitro incubation of IgM-sensitized erythrocytes in serum on cell survival. IgM-sensitized erythrocytes were prepared as described up to the point of injection. The cells were then sedimented and resuspended in 2 ml of fresh autologous serum/ml of the original erythrocyte solution. In some studies, the cells were incubated at 37°C for 1–2 h in the autologous fresh serum. In other studies, the cells were incubated in the fresh serum for 15 min. After 15 min, portions of erythrocytes were removed, washed once in 0.9% saline, and resuspended in 2 vol of fresh heated (56°C for 30 min) serum to 1 vol of cells for 1 or 2 h. Heating serum at 56°C destroys C1 and C2 but does not destroy the C3 inactivator (31–33). Portions of these samples were reinfused into the normal cell donors and the survival measured. Other portions were tested for antibody and C3 fragments coating the erythrocyte surface with commercial anti-IgM, anti-C3 reagents, and specific antisera against C3b and C3d fragments of C3. These samples were also tested for the ability to mediate immune

adherence reactions. Immune adherence assays were performed by utilizing a microtiter technique. 5 μl of 3.3×10^7 sensitized and serum-treated human erythrocytes were placed in 25 μl of isotonic veronal-buffered saline. 50 μl of 5×10^7 washed human erythrocytes, known to react positively in immune adherence, were added to each well. The preparation was mixed and then incubated for 30 min at 37°C, and immune adherence reactivity was checked at 15 and 30 min.

Studies of antibody transfer. It was of importance to determine whether antibody might transfer to unlabeled cells during the course of the clearance studies. This possibility was examined in three separate ways. (*a*) Erythrocytes were sensitized with radio-labeled antibody and counts per milliliter determined. After counting, these cells were washed multiple times and incubated for 1, 2, or 3 h at 37°C in fresh heated and 0.01 M EDTA-treated serum (1 part 0.1 M EDTA to 9 parts serum incubated for 30 min at 37°C). The mixtures were then washed and the cell buttons recounted. (*b*) Cells from patients with paroxysmal nocturnal hemoglobinuria (PNH) were sensitized with the IgM antibody preparation over a wide range of antibody dilutions. PNH erythrocytes are very susceptible to antibody and complement-mediated lysis (34). The percent of erythrocytes lysed at varying antibody concentrations was determined in the presence of a relative excess of serum complement, and a dose-response curve was constructed. The experiment was then repeated after incubating the sensitized erythrocytes for 30 min at 37°C with a three-fold excess of unsensitized donor erythrocytes. Transfer of antibody to unsensitized cells would have been reflected in a shift in the dose-response curve. (*c*) Radio-labeled sensitized erythrocytes (40 IgM C1-fixing sites/erythrocyte) were incubated for 15 min at 37°C with a 10-fold excess of unlabeled, unsensitized, autologous erythrocytes to act as receptor for low avidity antibody. These cells were then examined in clearance studies and compared with erythrocytes incubated at 37°C for 15 min in physiological saline in the absence of unlabeled erythrocytes acting as a receptor.

RESULTS

Characterization and quantitation of antibody preparations. Two serum sources with high titer IgM anti-A and one with high titer anti-B were purified and subsequently used to sensitize the appropriate erythrocytes. There were no detectable differences in the in vitro or in vivo behavior of these antibody preparations at similar levels of erythrocyte sensitization. The C1a fixation and transfer test (Fig. 1) was used to quantitate in absolute molecular terms the number of antibody molecules coating the erythrocytes at varying dilutions of the antibody preparations. At the highest antibody concentrations utilized in the clearance studies (80 IgM C1-fixing sites/erythrocyte), there was less than 10% hemolysis after 2 h incubation in an excess of fresh compatible human serum. Erythrocytes (3.3×10^8/ml) sensitized with one of the anti-A preparations to produce 20, 40, and 80 IgM C1-fixing sites/cell were exposed to an equal volume of compatible fresh serum for 15 min at 37°C. After exposure to fresh serum, the cells were washed, and the number of membrane-bound C3 molecules was determined on portions of the cell preparations. These three preparations had 16,600, 24,800, and 46,800 C3 molecules/cell, respectively. Unsensitized erythrocytes exposed to fresh serum had less than 1,500 molecules of C3/cell. Thus, under these conditions, 550–800 C3 molecules were deposited on the erythrocyte membrane at each C1-fixing site. This method of analysis of cell-bound C3 detects both C3b and its major degradation product C3d. After a 15-min period incubation, one would expect most of the cell-bound product to be in the form of C3b.

Between 44 and 62 (mean 53) molecules of IgM antibody/cell were sufficient to cause detectable direct agglutination. 20 and 40 IgM C1-fixing sites could not be detected by antiglobulin reagents but were detected by anti-C3 reagents after exposure to fresh serum.

Normal erythrocyte survival. In five separate studies, the mean $t_{\frac{1}{2}}$ of ^{51}Cr-labeled human erythrocytes was 55.3±9.1 days, with a range of 44–71 days. Studies performed with unsensitized ^{51}Cr-labeled erythrocytes incubated in fresh normal serum, serum heated at 56°C for 30 min, and 0.01 M EDTA-treated serum demonstrated comparable survival. This mean life span of 110.6 days is within the normal range and indicates normal erythrocyte survival for the labeled but unsensitized erythrocytes utilized in this study (3).

Survival of IgM-sensitized erythrocytes in normal man. Clearance curves at 20, 40, and 80 IgM C1-fixing sites/erythrocyte are shown in Fig. 2. The general clearance pattern is one of initial rapid sequestration ($t_{\frac{1}{2}}$ of between 3 and 4 min) over a 10–12-min period and then a slower period lasting several hours, during which a variable percentage of the cells returned to the circulation. At least 20 IgM C1-fixing sites/cell were required to detect initial sequestration. Most of the cleared erythrocytes were returned to the circulation over a 3-hr period at this low level of sensitization. Increasing the level of sensitization to 40 IgM C1-fixing sites led to the initial sequestration of between 45 and 70% (mean of five studies 56.2±5.1% [SE]). 20–50% of these cells subsequently returned to the circulation. At 80 IgM C1-fixing sites/erythrocyte, greater than 75% of the cells were removed initially. No cells returned to the circulation over the first 3 h post-injection, although between 5 and 10% reappeared in the circulation by 24 h (Fig. 2). At all levels of sensitization studied, once the period of release was complete, the erythrocytes showed normal survival. Representative studies at 20 and 40 IgM C1-fixing sites/erythrocyte are shown in Fig. 3.

Studies with 40 IgM C1-fixing sites/erythrocyte and with unsensitized cells were conducted in the whole body counter (Fig. 4). After injection of the radio-labeled but unsensitized cells, there was equilibration of counts throughout the body within 3 min. However, if

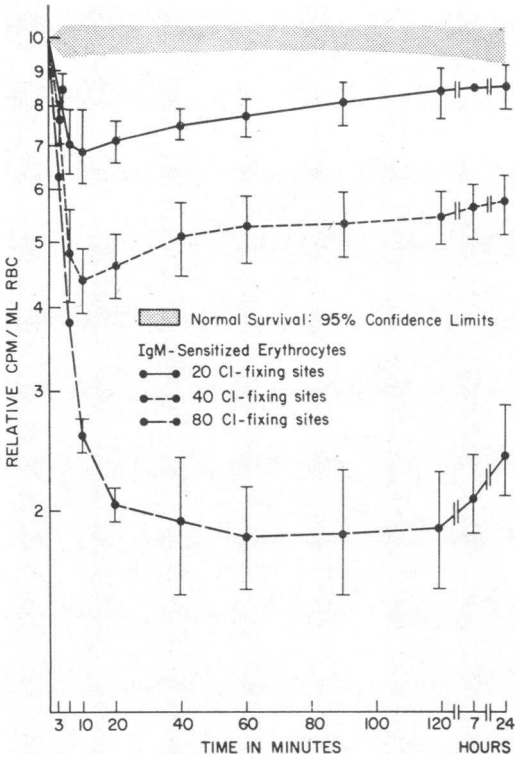

FIGURE 2 Survival of ^{51}Cr-labeled human erythrocytes: shown are clearance patterns at three levels of sensitization with anti-A IgM isoagglutinin. These data represent the mean±1.0 SE for three, five, and three normal subjects respectively at 20, 40, and 80 IgM C1-fixing sites/erythrocyte. The shaded area in this and subsequent figures represents the 95% confidence limits for survival of radio-labeled unsensitized cells in five normal individuals. Shown on the ordinate are relative cpm per milliliter red blood cells. Subjects received a total dose of about 5 μCi ^{51}Cr, and counts were corrected to a zero time figure of 1,000 cpm.

the cells were coated with IgM antibodies, counts progressively increased over the liver for 12 min after injection. During the subsequent 60 min, a significant percentage of the sequestered cells returned to circulation, as evidenced by the gradual decrease in counts over the liver and a corresponding increase in counts in the circulation.

The possibility that antibody elution could account for the sequestration and release pattern seen with IgM-coated erythrocytes was examined by three different methods. Radio-labeled antibody remained attached to erythrocytes, despite multiple washings and incubation for 1, 2, or 3 h in fresh, heated, or EDTA-treated serum. After these procedures, 100±5% of the counts remained in the cell button. Incubating IgM-sensitized PNH erythrocytes in an excess of unsensitized erythrocytes did not result in a decreased percentage of lysis after the addition of complement, when compared to a portion of these same cells incubated in veronal-buffered saline. The hemolysin curves with these two cell preparations were identical. Finally, in one experiment, erythrocytes sensitized with 40 IgM C1-fixing sites were incubated in a 10-fold excess of autologous unlabeled and unsensitized cells before injection. The clearance curve in this situation fell within 1 SE of five individuals in whom the erythrocytes were injected immediately after sensitization.

Clearance of IgM-sensitized erythrocytes in patients with depressed levels of complement components C4 and C2. On the day of the study, these patients with hereditary angioedma were receiving no medications and had a serum C4 titer of less than 50 and no detectable C2. The patients' type A erythrocytes were sensitized with 40 IgM C1-fixing sites/erythrocyte. The cells were reinfused, and survival followed exactly as in other studies. Fig. 5 compares these patients' clearance curves with five normals at the same level of sensitization. It is apparent that these patients were unable to clear erythrocytes sensitized with 40 IgM C1-fixing sites/erythrocyte and, in fact, these cells had normal survival. Incubation of a portion of these sensitized cells in a 10-fold excess of autologous fresh serum

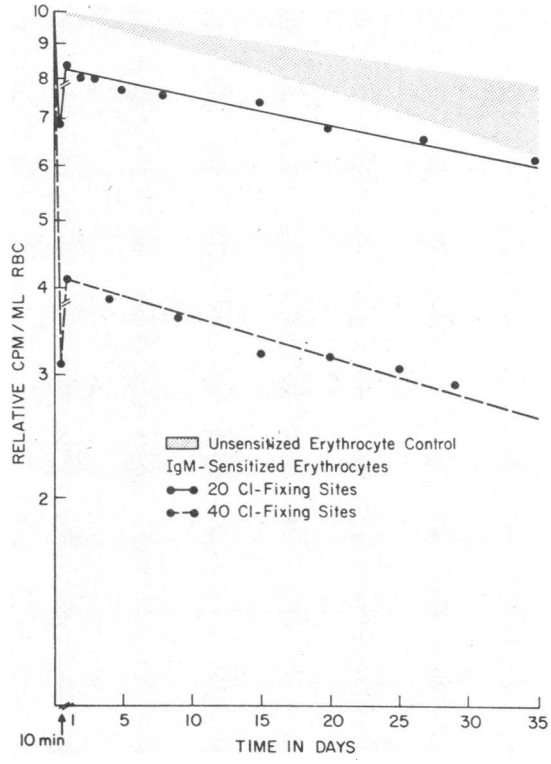

FIGURE 3 Representative study of the survival of erythrocytes sensitized with 20 (●——●) and 40 (● - - - ●) IgM C1-fixing site/cell. After the initial period of sequestration and release, the cells survived normally in these studies as well as in 12 other experiments in which the survival was followed for at least 20 days.

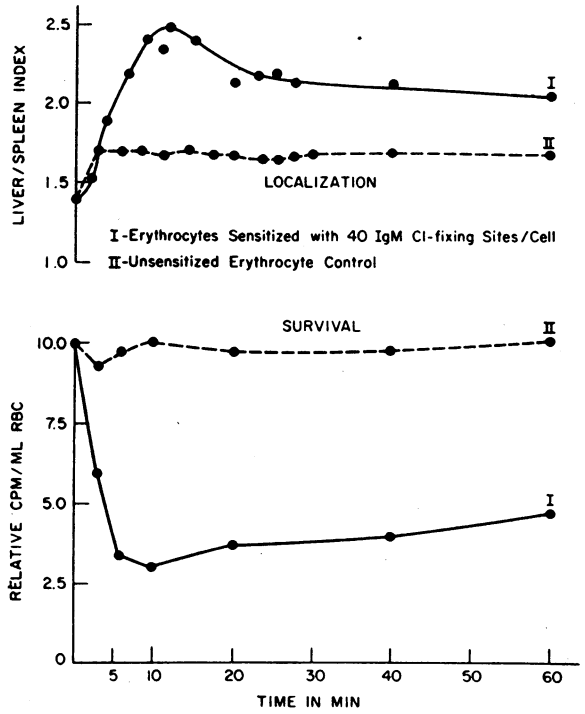

FIGURE 4 Localization and survival of sensitized and unsensitized chromated erythrocytes in normal subjects. Chromated cells were injected at zero time and localization of the injected erythrocytes was determined in the whole body counter. Simultaneous samples were obtained for determination of cell clearance. Individual I, who received sensitized cells, showed the typical clearance pattern and an associated increase in the liver/spleen index. (Liver/spleen = net counts collected by liver detector/net counts collected by spleen detector) This was not seen in individual II, who received unsensitized erythrocytes.

did not lead to detectable C3 deposition, as evidenced by negative immune adherence assays and by failure of anti-C3 to cause agglutination. A second portion of these sensitized cells incubated in fresh serum obtained from a normal individual were positive for immune adherence reactivity and agglutinated by anti-C3.

Effects of the prior incubation in serum on the in vivo and in vitro behavior of IgM-sensitized erythrocytes. Erythrocytes sensitized with 40 IgM C1-fixing sites were incubated for 1 or 2 h at 37°C in autologous fresh serum. In Table I, the extent of clearance of such erythrocytes is compared to that of cells with the same level of sensitization but without prior exposure to fresh serum. Fresh serum partially protected the sensitized erythrocytes from clearance. In these studies, sensitized erythrocytes were also incubated in fresh serum for 15 min at 37°C. The configuration and magnitude of the clearance curve was identical to that seen with sensitized erythrocytes in which there was no prior serum incubation. The data suggests that the rate of hepatic blood flow, rather than that of complement activation, determines the rate of removal of IgM-sensitized erythrocytes.

If erythrocytes were first exposed to fresh serum for 15 min, washed in 0.9% saline, and then resuspended in heated serum for 60 or 120 min, the sensitized erythrocytes either survived normally or showed minimally increased clearance (Table I). Incubation of sensitized erythrocytes for 60 or 120 min in heated serum or EDTA-treated serum without prior exposure to fresh unheated serum led to no alteration in the clearance pattern.

A partial explanation for these findings was obtained by determining the nature of the complement fragment on the sensitized erythrocytes (Table I). After 15, 60, or 120 min incubation in fresh serum, sensitized cells remained immune adherence positive and were agglutinated by anti-C3b and -C3d. However, when sensitized erythrocytes were first exposed to fresh serum for 15 min at 37°C and then to heated serum for 60 min, the erythrocytes became immune adherence negative. These cells were strongly agglutinated by anti-C3d but weakly or not at all by anti-C3b.

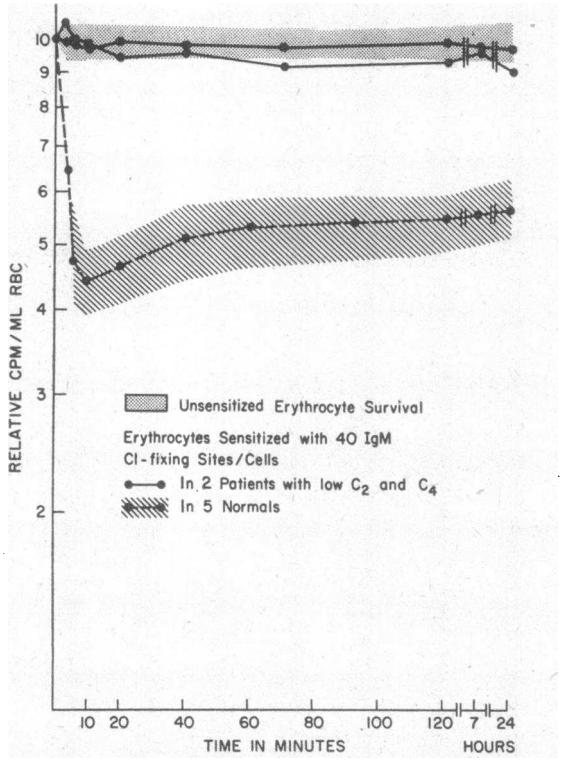

FIGURE 5 A comparison of survival of IgM-sensitized erythrocytes in normals and patients with no detectable C2 and low C4 levels. The area enclosed by the hatching represents the mean±SE for five normals at 40 IgM C1-fixing sites/erythrocyte.

TABLE I

Correlation of Cell-Bound C3 Fragments with Clearance and Immune Adherence Reactivity: Red Cells Sensitized with 40 IgM Cl-Fixing Sites/Cell

Experimental groups	Serum incubation		Agglutination of anti-C3 fragments		Immune adherence	Clearance at 10 min (mean ±1 SE)
	Fresh	Heated*	C3b	C3d		
	min					%
1	0	0	− −	− −	− −	45–62‡
2	15	0	+ + + +	+ + + +	+ + + +	45–62
3	60 or 120	0	+ + +	+ + + +	+ + +	30–40
4	15	60	±	+ + + +	− −	0–10

* Serum was heated at 56°C for 30 min to destroy complement but leave the C3 inactivator activity intact.

‡ Complement components are bound upon injection into normal subjects and then their reactivity would be identical to that of group 2.

DISCUSSION

In these experiments, chromated erythrocytes were sensitized with highly purified IgM antibody directed at ABO blood group antigens and reinjected into the erythrocyte donor. The level of sensitization was controlled by use of the Cla fixation and transfer test which allowed for the determination of the absolute number of Cl-fixing (IgM) sites/cell. The survival of the sensitized cells was followed, and sites of cell sequestration were determined in the whole body counter. In addition, the role of complement was investigated by studying clearance in patients with depressed levels of the components C4 and C2.

Cells sensitized with as few as 20 IgM Cl-fixing sites/cell showed evidence of clearance, and 45–62% of the cells were regularly cleared when 40 Cl-fixing sites were present on the sensitized cells. This quantity of antibody was undetectable by direct agglutination techniques, as well as by an indirect Coombs technique. Cells coated with these low levels of IgM antibody were rapidly cleared by the liver upon injection, and the rate of clearance of these cells appeared to be only limited by liver blood flow. After initial sequestration, a portion of the cells was destroyed, and a portion was returned to the circulation where they survived normally. Transfer experiments demonstrated that the antibody was of high avidity, and elution of antibody within the limited period of study could not have accounted for the experimental findings.

This pattern of IgM clearance has generally not been appreciated in man. However, Lewis et al. noted in one patient that cells coated with cold agglutinin showed a pattern of hepatic sequestration followed by release into the circulation (34*a*). Evans et al. noted that erythrocytes coated with cold agglutinin would take hours to finally equilibrate after initial rapid sequestration of a large percentage of the injected cells (10, 11). Jandl, Jones, and Castle noted a pattern of hepatic sequestration and release on one individual who received erythrocytes coated with anti-B isoagglutinins, although this was thought to be due to the fact that the cells were agglutinated at the time of injection (4). In other studies, injection of erythrocytes coated in vitro with isoagglutinins or injection of type A or B cells into individuals with circulating isoagglutinins to the infused cell type showed a two-part clearance curve (4, 6–9). There was rapid initial hepatic sequestration or lysis of a large percentage of the injected cells followed by a period of normal or slightly decreased cell survival. The high level of erythrocyte sensitization obtained in the latter studies may account for the failure to observe the pattern of sequestration and release reported here.

Recently, utilizing animal models of immune hemolytic anemia, two groups reported a clearance pattern similar to that demonstrated herein. Brown, Lachmann and Dacie injected human cold agglutinins into rabbits and described sequestration via immune adherence to hepatic macrophages and subsequent release (35). Schreiber and Frank injected radio-labeled erythrocytes coated with highly purified heterologous IgM antibodies into guinea pigs and showed a similar sequestration and release pattern (16). In the guinea pig model, 60 IgM complement fixing sites were required for clearance rather than 20 sites, as observed in these studies, and the release of sequestered cells was more extensive and more rapid. Nevertheless, the similarities between the results reported here and those observed in the guinea pig model are striking. It should be emphasized that liver sequestration was not simply a result of complement activation in the guinea pig model, since erythrocytes sensitized by complement-fixing IgG antibody were cleared by the spleen via a complement-dependent mechanism. It will be of importance to determine whether complement-fixing IgG antibody leads to splenic sequestration in human beings.

At the levels of sensitization studied, accelerated clearance of IgM-coated cells was entirely complement dependent, since normal survival of the sensitized cells was observed in two angioedema patients with markedly depressed levels of C4 and C2. Patients with HANE have an intact alternate complement pathway (36), and this finding indicates that this pathway in humans does not mediate clearance of IgM-sensitized cells. These data are in agreement with the recent work in animals, suggesting that antibodies directed at normal mammalian cell membranes are inefficient at activating the alternate complement pathway (37, 38).

The normal survival of IgM-sensitized cells, in a setting where the classical complement pathway cannot be activated thru C3, is also in keeping with animal models of immune hemolytic anemia. Guinea pigs lacking C4 or depleted of late components (C_{8-9}) and rabbits depleted of C_{8-9} were unable to clear cells heavily sensitized with IgM complement-fixing antibodies (17, 35). That C3 was the critical component was suggested by the normal clearance of IgM-coated erythrocytes in rabbits with a total deficiency of C6 and the lack of clearance of EAC142 cells in C_{8-9}-depleted rabbits (35).

The failure of clearance of IgM-coated cells suggests that receptors for human IgM are either absent or of such low avidity that 40 sites is not sufficient to effect clearance. Although hepatic macrophages have not been specifically examined, in studies not reported here, we were unable to demonstrate either an heterologous or homologous IgM receptor on human alveolar macrophages and peripheral blood monocytes.[3] Others have also been unable to demonstrate either a homologous or heterologous IgM receptor on human peripheral blood monocytes (39–42).

These clearance data support the concept advanced in our earlier studies (17). IgM antibody to the ABO blood group antigens activates complement via the classical complement pathway, which results in the sequential deposition of C1, C4, C2, and C3 on the cell surface. At least 20 IgM molecules/cell are required to activate the clearance mechanism, and this level of sensitization leads to the deposition of about 16,000 C3 molecules on the erythrocyte surface. Fixed macrophages of the reticuloendothelial system, particularly Kupffer cells, have a surface receptor which recognizes the deposition product of activated C3, presumably C3b. Erythrocytes with C3b on their surface are immune adherence positive (33, 43). Circulating erythrocytes with C3b on their surface may bind to the receptor on hepatic macrophages through an immune adherence-like reaction. IgM and C3b are relatively poor stimuli for ingestion, and many of the erythrocytes adhere to the macrophage surface but are not phagocytized (44). These adherent cells may be continually exposed to complement and to the C3 inactivator (C3INA). Thus, a portion of the adherent cells may have C3b destroyed by the C3INA and escape back into the circulation. We have previously demonstrated that such cells have IgM and C3 on their surface and survive normally as Coombs-positive erythrocytes (17).

The molecular form of C3 remaining on the surface of the erythrocytes after cleavage is termed C3d. Cells with membrane-bound C3d are immune adherence negative, although they remain Coombs positive. In the in vivo clearance studies, erythrocytes prepared as described in Methods, with C3d on their surface and with no detectable C3b, had normal survival. These same cells were bound poorly or not at all to human alveolar macrophages.[3] The data are entirely in keeping with the findings that Coombs-positive erythrocytes from patients with cold agglutinin (IgM) autoimmune hemolytic anemia have C3d but not C3b on their surface and survive better when reinjected into the donors than do normal erythrocytes (10, 11, 45, 46). These circulating cells would be expected to have had C3b deposited on their surface, to have had the C3b cleaved to C3d, and therefore, to be less susceptible to destruction.

These studies in man point out a clearance pattern of IgM-coated erythrocytes consisting of rapid hepatic sequestration followed by release of a significant portion of these cells into the circulation. Such cells survive normally thereafter, probably as Coomb's-positive erythrocytes. Complement is required for the clearance of IgM-sensitized erythrocytes, and the reactivity of sensitized erythrocyte population is largely dependent on the nature of the C3 fragment bound to the erythrocyte membrane.

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