

## Role of Antibody and Complement in the Immune Clearance and Destruction of Erythrocytes I. IN VIVO EFFECTS OF IgG AND IgM COMPLEMENT-FIXING SITES

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### Research Article

A model which permits evaluation in molecular terms of the role of antibody and of complement in the immune destruction of erythrocytes was established in the guinea pig. IgM and IgG immunoglobulins were isolated from rabbit anti-guinea pig erythrocyte antisera and were used to sensitize <sup>51</sup>Cr-labeled guinea pig erythrocytes. The average number of complement-fixing sites per erythrocyte formed by antibody was determined for each of the various preparations by the Cla fixation and transfer test. The rate of clearance and of organ localization was determined for cells sensitized with either IgM or IgG antibodies, and dose-response curves were established in normal guinea pigs and guinea pigs with a genetically controlled, complete absence of the fourth component of complement (C4).

At least 60 complement-fixing sites per cell were required for accelerated clearance of IgM-sensitized erythrocytes. The bulk of cells with IgM sites were cleared by the liver within 5 min after injection and were then slowly returned to the circulation where they survived normally. There was no accelerated clearance whatsoever of IgM-sensitized erythrocytes in C4-deficient guinea pigs.

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# Role of Antibody and Complement in the Immune Clearance and Destruction of Erythrocytes

## I. IN VIVO EFFECTS OF IgG AND IgM COMPLEMENT-FIXING SITES

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**ABSTRACT** A model which permits evaluation in molecular terms of the role of antibody and of complement in the immune destruction of erythrocytes was established in the guinea pig. IgM and IgG immunoglobulins were isolated from rabbit anti-guinea pig erythrocyte antisera and were used to sensitize <sup>51</sup>Cr-labeled guinea pig erythrocytes. The average number of complement-fixing sites per erythrocyte formed by antibody was determined for each of the various preparations by the C1a fixation and transfer test. The rate of clearance and of organ localization was determined for cells sensitized with either IgM or IgG antibodies, and dose-response curves were established in normal guinea pigs and guinea pigs with a genetically controlled, complete absence of the fourth component of complement (C4).

At least 60 complement-fixing sites per cell were required for accelerated clearance of IgM-sensitized erythrocytes. The bulk of cells with IgM sites were cleared by the liver within 5 min after injection and were then slowly returned to the circulation where they survived normally. There was no accelerated clearance whatsoever of IgM-sensitized erythrocytes in C4-deficient guinea pigs.

As few as 1.4 IgG complement-fixing sites per cell resulted in decreased erythrocyte survival. There was no evidence of immediate tissue sequestration and release. Progressive trapping and destruction of erythrocytes by the spleen was responsible for most of the clearance of IgG-sensitized cells. Clearance of IgG-sen-

sitized cells was markedly impaired in guinea pigs with C4 deficiency; however, there was some decrease over normal survival.

The data indicate that IgG and IgM antibodies interact with complement in vivo by mechanisms which are qualitatively or quantitatively different and produce different biologic effects.

### INTRODUCTION

Investigation into the pathophysiology of autoimmune hemolytic anemia has focused primarily upon the differences between complement-fixing and noncomplement-fixing antibodies (1, 2). It has been suggested that cells sensitized with complement-fixing antibodies are cleared from the circulation primarily by the liver and those sensitized with noncomplement-fixing antibodies are cleared predominantly by the spleen (1-3). These previous pathophysiologic studies are limited by the fact that they are semiquantitative and do not examine the role of antibody and complement in molecular terms. Recent advances in complement research now permit a quantitative appraisal of the action of antibody and complement in the clearance of sensitized erythrocytes (4). It has become clear that such an appraisal must take into account the differing biologic activity of the various classes of antibody (5).

In this and subsequent papers, utilizing a guinea pig model, we will attempt to define in molecular terms the effects of antibody class and the various complement components in experimental immune hemolytic anemia.

### METHODS

*Buffers and complement reagents.* Isotonic veronal-buffered saline pH 7.4 containing 0.1% gelatin and optimal

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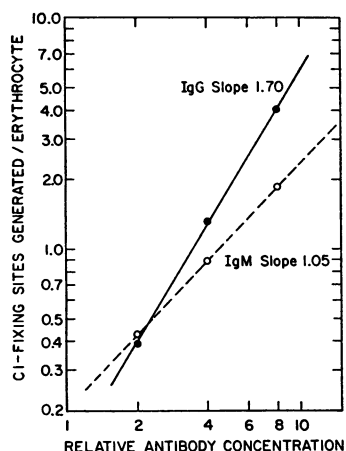


FIGURE 1 C1 fixation and transfer test: IgM and IgG anti-guinea pig erythrocyte antibodies.

amounts of  $\text{Ca}^{++}$  and  $\text{Mg}^{++}$  (VBS)<sup>1</sup> and veronal-buffered saline containing 0.01 M ethylenedinitrietetraacetic acid (EDTA buffer) were prepared as previously described (7). Low ionic strength VBS-dextrose buffer ( $\mu=0.065$ ) containing gelatin,  $\text{Ca}^{++}$ , and  $\text{Mg}^{++}$  was also prepared as previously reported (6).

Fresh frozen guinea pig serum was obtained from Suburban Serum Laboratories, Silver Spring, Md. In experiments utilizing sheep erythrocyte intermediates in the complement sequence, the fresh serum was absorbed twice with packed sheep erythrocytes at 0.0°C (7). Partially purified C2 and C1 were prepared by modifications of established methods (7, 8) as were cells coated with hemolytic antibody and the fourth component of complement (EAC4), (9).

**Guinea pigs.** All studies utilized outbred guinea pigs from a closed colony designated 'NIH multipurpose.' A subline of these guinea pigs has recently been developed with a genetically controlled, total deficiency of the fourth component of complement (6). This represents the only known genetic defect early in the classical complement sequence before the formation of biologically active products of complement interaction.

**Erythrocytes and anti-erythrocyte antisera.** Erythrocytes, obtained from donors by intracardiac puncture, were washed three times in EDTA buffer, with removal of the buffy coat, and twice with VBS before use. Rabbit anti-guinea pig erythrocyte antisera were prepared utilizing two different methods. One group of New Zealand White rabbits were immunized in the hind footpads with a total of 1 ml each of a 10% suspension of guinea pig erythrocytes in VBS emulsified in an equal volume of Freund's complement adjuvant (Difco Laboratories, Detroit, Mich.). These animals received a booster injection of the 10% erythrocyte suspension emulsified in Freund's incomplete adjuvant (Difco Laboratories) 2-4 wk after primary immunization. They were exsanguinated 2 wk after the second immunization. The second group of rabbits was immunized by the intravenous administration of 1 ml of progressively

<sup>1</sup> Abbreviations used in this paper: C4, fourth component of complement; DVBS, low ionic strength buffer; RBC, red blood cells, VBS, veronal-buffered saline.

increasing concentrations of guinea pig erythrocytes ( $1 \times 10^8$  to  $3 \times 10^8$  RBC) in VBS given three to four times per week over the course of 3 wk. Bleedings were done 5-7 days after the last injection. Hemagglutinin and hemolysin activity was measured for each individual antiserum and those with the highest titer of antibody activity were chosen for further purification.

**Purification of immunoglobulins.** The globulin fraction of serum from individual rabbits was prepared by precipitation with 5.4 molal ammonium sulfate at 0°C and centrifugation for 15 min at 900 g at 0°C. The precipitate was suspended in a few drops of saline and applied to a  $60 \times 5$  cm column of Sephadex G 200 in VBS (10). The flow rate was 35 ml/hr. 7-ml samples were collected and the optical density at 280 nm determined spectrophotometrically. The first protein peak containing the bulk of the macroglobulins and the second peak containing most of the  $\gamma$ G immunoglobulins were each pooled and concentrated by ultrafiltration (Schleicher & Schuell, Inc., Keene, N. H.). Portions of the concentrated material were further purified by ultracentrifugation at 45,000 g in a 10-30% w/v sucrose gradient (11). These highly purified 19S and 7S fractions from individual antisera were utilized for further studies.

**Quantitative antibody and complement studies.** Hemolytic antibody titers of the various fractions were determined using a 15 min kinetic titration in the small volume method (7). Titers were expressed as the dilution of each fraction that produced 50% hemolysis of  $5 \times 10^8$  guinea pig erythrocytes in exactly 15 min at 37.0°C in the presence of about 20 CH<sub>50</sub> of guinea pig complement.

Hemagglutination titers of the fractions were also obtained as a noncomplement-dependent means of quantitating antibody present. These were performed utilizing a micro-titer technique.  $3.4 \times 10^8$  washed guinea pig erythrocytes in a volume of 25  $\lambda$  were added to 25  $\lambda$  of serial twofold falling dilutions of each fraction in VBS. The tubes were mixed and incubated at 37°C for 2 hr and the degree of agglutination scored from 1+ to 4+. The titer was scored as the reciprocal of the dilution which produced 1+ agglutination.

**Quantitation of the number of complement-fixing sites per cell.** The C1 fixation and transfer method was used to determine (in absolute molecular terms) the average number of complement-fixing antibody sites per erythrocyte in the sensitized erythrocyte population (12). The test was performed as previously described (12) except that dextrose was substituted for sucrose in the low ionic strength buffer. Guinea pig erythrocytes were sensitized with several dilutions of the various immunoglobulin fractions exactly as noted in the section on erythrocyte survival and then exposed to excess activated C1 in the presence of low ionic strength buffer (DVBS). After cell-bound antibody was allowed to complex with C1, the free C1 was removed from the reaction mixture, and the bound C1 allowed to transfer from the original sensitized cells to an acceptor cell—the cellular intermediate in the complement sequence EAC4. This intermediate was then specifically lysed by the addition of excess C2 and the late components of complement. The degree of lysis of the cellular complement intermediate accurately reflects the number of C1 bound in the original sensitized cell population and thus the number of complement-fixing sites on the original cells.

Typical dose-response curves in the C1 fixation and transfer test were obtained with the IgG and IgM fractions (Fig. 1). The IgM fraction generated a curve with a slope of 1.05 and the IgG fraction showed a slope of 1.7. The

fact that IgM and IgG antibodies generate dose-response curves of this general type in C1 fixation and transfer tests has been taken to support the concept that a single molecule of IgM is sufficient to fix C1 and sensitize a cell for lysis and that an IgG doublet is required for C1 fixation (13).

**Erythrocyte survival and tissue sequestration studies.** Fresh guinea pig erythrocytes were obtained by cardiac puncture and washed with EDTA buffer and VBS as previously noted. They were standardized spectrophotometrically to a concentration of  $5.4 \times 10^8$  cells/ml and incubated with sodium dichromate (Amersham/Searle Corp., Arlington Heights, Ill.), ( $25 \mu\text{Ci/ml}$  of RBC suspension) with frequent mixing in a water bath at  $37^\circ\text{C}$  for 30 min. The chromated cells were washed three times in VBS and resuspended in VBS to a final concentration of  $2.7 \times 10^8$  RBC/ml. A portion of the cells was sensitized by adding an equal volume of a dilution of the IgG or IgM preparation with constant mixing and then incubating the mixture at  $37^\circ\text{C}$  for 30 min with frequent mixing. The mixtures were sedimented, the supernatant fluids discarded, and the cells resuspended in VBS to a final concentration of  $2.7 \times 10^8$  RBC/ml. In none of these suspensions was significant microscopic agglutination noted. A volume of 1 ml was injected into the hind foot vein of the guinea pigs and erythrocyte survival was determined by serial 0.1 ml bleedings from the retroorbital sinus of the guinea pigs with a calibrated bleeding pipette. The portions of blood were suspended in 1 ml EDTA buffer and the number of counts/min per milliliter of blood obtained utilizing a gamma scintillation counter (Packard Instrument Co., Downers Grove, Ill.). At the levels of sensitization utilized, guinea pig erythrocytes were markedly resistant to lysis by guinea pig complement both in vivo and in vitro. Nevertheless, controls were performed at the various levels of sensitization to establish the fact that less than 5% of the radiolabel was found free in the plasma fraction and the remainder of the label was associated with the erythrocyte pellet.

Data was plotted on semilog paper and where appropriate, a straight line was calculated by the method of least squares in order to determine the best fitting decay curve (14). 95% confidence limits for decay curve slopes were established for normal erythrocyte survival with data from 20 normal guinea pigs. In no case did the survival of a preparation of chromated erythrocytes fall outside of these confidence limits.

Studies were performed to determine whether transfer of antibody from sensitized cells might occur during the period of clearance. As a model for what might occur in vivo, cells were incubated in vitro with a 10-fold excess of unsensitized erythrocytes which might act as a receptor for any antibody molecules which might dissociate from the radiolabeled cells. Preparations of sensitized, radiolabeled erythrocytes were incubated in vitro for 1 hr at  $37^\circ\text{C}$  with a 10-fold excess of unsensitized, unlabeled erythrocytes to act as a receptor for low avidity antibody molecules. The survival characteristics of these cells after antibody transfer were examined as described above and compared with radiolabeled RBC sensitized with antibody and incubated at  $37^\circ\text{C}$  in the absence of unlabeled erythrocytes acting as an antibody receptor.

At each level of sensitization of erythrocytes with IgG and IgM antibodies, groups of animals were sacrificed at various time points. Liver, lungs, spleen, and kidneys were removed. The radioactivity of the entire organ was either determined directly, or the entire organ was dissolved in

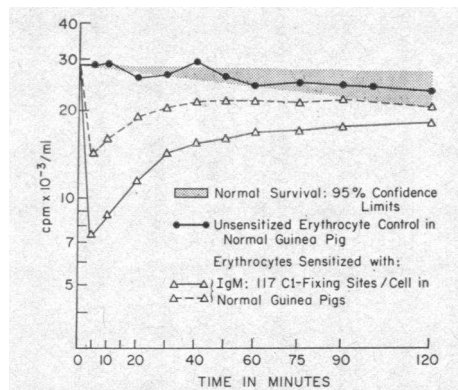


FIGURE 2 Survival of  $^{51}\text{Cr}$ -labeled guinea pig erythrocytes: 117 IgM C1-fixing sites/erythrocyte in two normal guinea pigs.

4 N NaOH and radioactivity of the entire organ determined by counting a portion of the dissolved organ. Parallel studies were performed in control animals who received radiolabeled, unsensitized erythrocytes.

## RESULTS

**Normal erythrocyte survival.** The mean half-time of survival ( $t_{1/2}$ ) of  $^{51}\text{Cr}$ -labeled guinea pig erythrocytes was 7.5 days after reinjection into the guinea pig. The mean slope calculated by a linear regression equation, and the 95% confidence limits of the slope are shown as the shaded area in each survival study.

**Survival of IgM-sensitized erythrocytes.** At least 60 complement-fixing IgM sites per erythrocyte were required for accelerated clearance. With increasing numbers of IgM C1-fixing sites per erythrocyte the extent of sequestration became more marked. Shown in Fig. 2 is the pattern obtained in two different animals when injected cells were sensitized with 117 IgM C1-fixing sites per erythrocyte. Between 50 and 75% of the administered cells were removed from the circulation within 5 min after injection. Most of these cells were returned to the circulation over a period of 2 hr and then showed normal survival. Shown also in Fig. 2 is the survival of erythrocytes from this pool of cells when injected without sensitization. The extent of normal variation from animal to animal can also be seen in this and subsequent figures.

Since elution of low affinity IgM antibody might in part explain the release of the cells after sequestration, an antibody transfer study was performed. As seen in Fig. 3, the sequestration and release pattern was the same for cells after incubation with a large excess of unlabeled erythrocytes which might act as a receptor for low avidity antibody. Thus, there was no evidence for antibody transfer. As a further control, a pool of low avidity antibody was studied by this same technique. Here the data indicate that transfer of antibody has

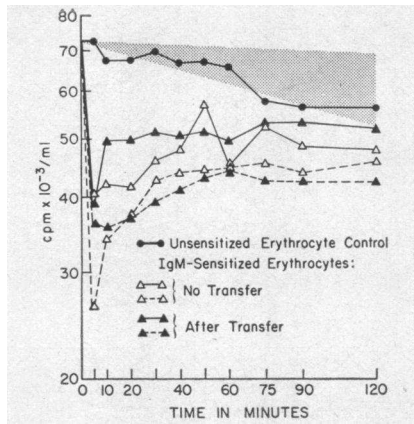


FIGURE 3 Antibody transfer study: survival of  $^{51}\text{Cr}$ -labeled erythrocytes after in vitro antibody transfer, 117 IgM C1-fixing sites/erythrocyte.

occurred; thus, decreased sequestration of the cells after a period of antibody transfer was observed (Fig. 4).

When the number of IgM C1-fixing sites per erythrocyte was increased to 234 and 468 sites per cell, the percentage of cells sequestered increased and the number of cells returned to the circulation fell. The pattern of erythrocyte sequestration seen with 234 IgM C1-fixing sites per erythrocyte is shown in Fig. 5. About 80% of the cells were sequestered within 5 min and about 50% of the cells were returned to the circulation over a period of 3 hr. Subsequently the erythrocyte survival paralleled that of the controls.

It was possible in these studies to differentiate the effects of antibody from those of complement by use of the C4-deficient strain of guinea pigs. When IgM-sensitized erythrocytes were injected into these guinea pigs, there was no sequestration whatsoever. The high-

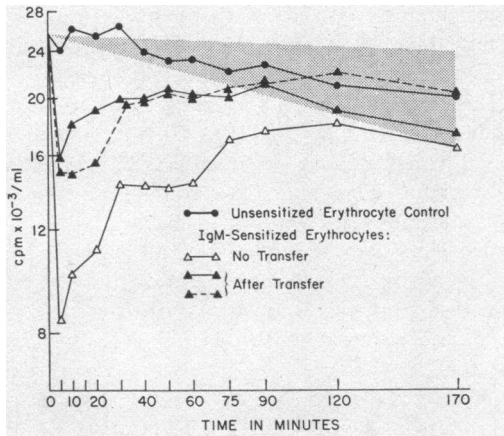


FIGURE 4 Antibody transfer study: survival of  $^{51}\text{Cr}$ -labeled guinea pig erythrocytes sensitized with low avidity antibody, 117 IgM C1-fixing sites/erythrocyte.

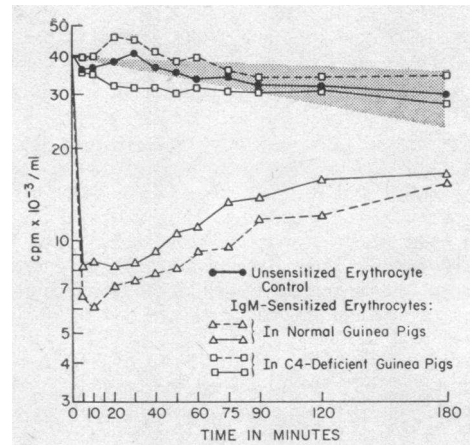


FIGURE 5 Survival of  $^{51}\text{Cr}$ -labeled erythrocytes with 234 IgM C1-fixing sites/erythrocyte in two normal and two C4-deficient guinea pigs.

est level of sensitization studied in the C4-deficient animals was 234 IgM C1-fixing sites per erythrocyte and the survival of these cells was completely normal (Fig. 5).

It should be noted that erythrocyte survival was followed for 14 days at every level of sensitization and was normal after the initial period of sequestration and release (Fig. 6). Data, presented in the accompanying paper, demonstrate that these sensitized cells having normal survival remain Coombs positive after release back into the circulation (15).

*Survival of IgG-sensitized erythrocytes.* In the case of IgG-sensitized erythrocytes, the survival curves were entirely different from those of the IgM-sensitized red cells discussed in the previous section. As few as 1.4 IgG C1-fixing sites per erythrocyte resulted in decreased erythrocyte survival. There was no evidence of immediate sequestration of the sensitized cells from the circulation with subsequent release, as was noted with IgM-sensitized cells. As shown in Fig. 7, IgG-sensitized erythrocytes were progressively removed from the circulation over the first 2-3 hr of study. With 17 IgG C1-fixing sites per cell about 90% of cells were removed from the circulation within 2 hr. The few surviving cells were subsequently cleared at a normal rate (Fig. 8). The clearance pattern in C4-deficient guinea pigs will be discussed below.

Studies were performed to determine whether transfer of antibody might account for these survival characteristics. As shown in Fig. 9, there was no evidence for transfer of high avidity IgG antibody. A low avidity antibody fraction showed the typical pattern of IgG-sensitized erythrocyte survival; however, after a period of in vitro transfer, the cells appeared to have fewer IgG sites.

With increasing numbers of IgG C1-fixing sites/cell the IgG pattern of clearance remains characteristic; however, the extent of clearance becomes more marked (Fig. 10).

The survival of IgG-sensitized erythrocytes in C4-deficient guinea pigs is illustrated in Figs. 7 and 10. The C4-deficient guinea pigs have a marked impairment in their ability to clear the sensitized cells. Nevertheless, survival is decreased over control values. One can overcome the deficit in sequestration in C4-deficient guinea pigs by use of large amounts of IgG antibody; erythrocytes with 511 IgG C1-fixing sites per cell showed markedly decreased survival.

*Sites of erythrocyte sequestration and destruction.* Erythrocytes with 60 and 117 IgM C1-fixing sites per cell were injected intravenously into guinea pigs, and animals were sacrificed at 5 min, 1 hr, 2 hr, and 24 hr. As seen in Table I, IgM antibody led to liver sequestration 5 min after injection. The subsequent fall in hepatic radioactivity with time indicates release of the radiolabeled cells into the circulation.

Animals that received erythrocytes with 1.4 and 117 IgG C1-fixing sites per cell were sacrificed at the same time intervals. IgG sensitization led to progressive splenic clearance with some liver sequestration. Shown in Table I are the results obtained at 17 sites per cell. The results obtained with 1.4 sites per cell were similar except that fewer cells were cleared. Not shown in the

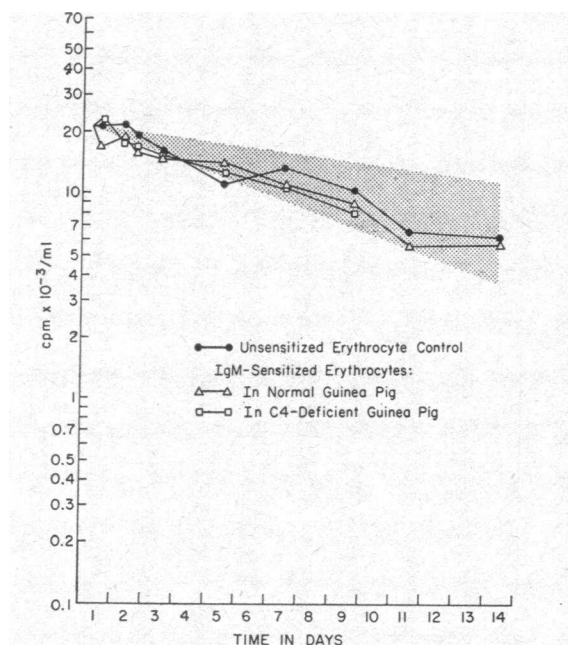


FIGURE 6 Survival of  $^{51}\text{Cr}$ -labeled guinea pig erythrocytes with 117 IgM C1-fixing sites/erythrocyte from 2 hr to 14 days after injection.

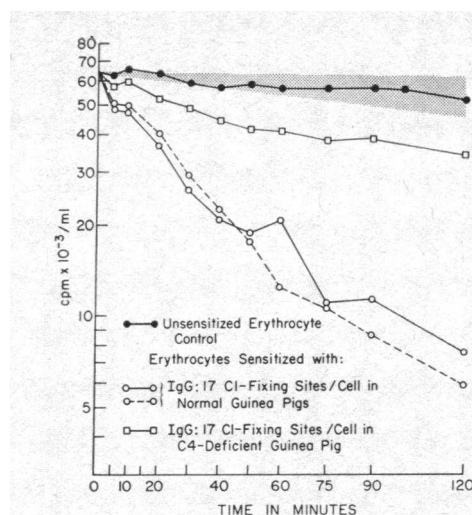


FIGURE 7 Survival of  $^{51}\text{Cr}$ -labeled erythrocytes with 17 IgG C1-fixing sites/erythrocyte in two normal and one C4-deficient guinea pig.

table is the fact that the minimal clearance of  $\gamma\text{G}$ -sensitized erythrocytes in C4-deficient guinea pigs can be accounted for by splenic uptake. Interestingly, in normal guinea pigs clearance by the liver became predominant with levels of sensitization greater than 90 IgG C1-fixing sites per cell. The pattern was typical of IgG (Fig. 10), however, and never resembled that seen with IgM antibody in that clearance was slower

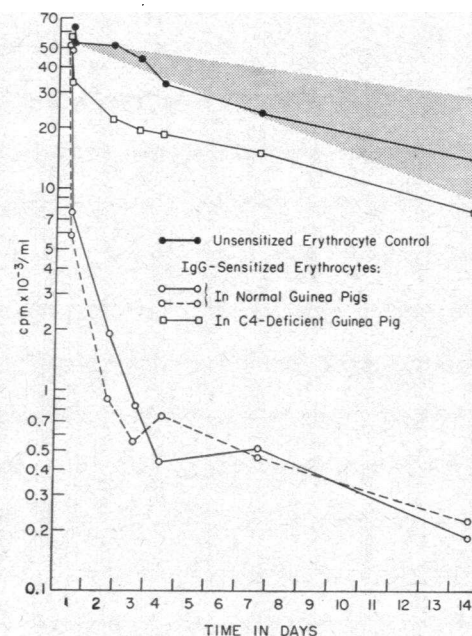


FIGURE 8 Survival of  $^{51}\text{Cr}$ -labeled guinea pig erythrocytes with 17 IgG C1-fixing sites/erythrocyte from 2 hr to 14 days after injection.

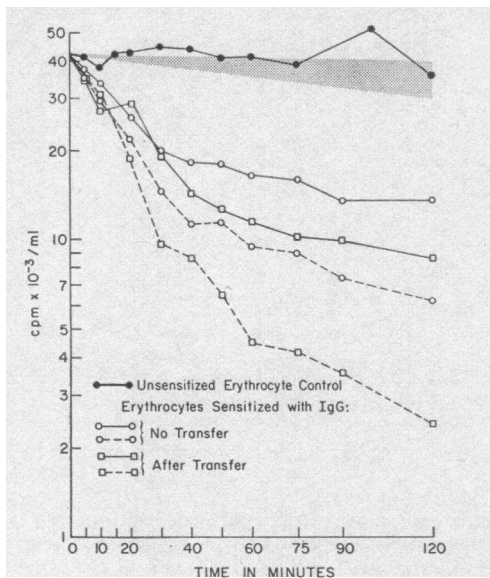


FIGURE 9 Survival of  $^{51}\text{Cr}$ -labeled erythrocytes after in vitro antibody transfer, 17 IgG C1-fixing sites/erythrocyte.

and there was no release of cleared cells. At 90 IgG C1-fixing units per erythrocyte, the C4-deficient guinea pig still showed predominant splenic sequestration. At

TABLE I  
Organ Sequestration of Radiolabeled Erythrocytes $\ddagger$

	Control $\S$	117 IgM C1-fixing sites/cell	17 IgG C1-fixing sites/cell
		%	
5 min after injection			
Clearance from circulation over control values	—	55	27.5
In circulation	84.7	42.3	58.8
Liver	6.6	45.0	29.1
Spleen	0.4	8.3	9.7
Lung	7.0	3.7	—
Kidney	1.2	1.8	2.3
1 hr after injection			
Clearance from circulation over control values	—	23.0	87.7
In circulation	72.0*	51.9	9.9
Liver	20.4	35.4	27.5
Spleen	3.7	5.1	61.3
Lung	—	—	—
Kidney	3.2	3.8	1.1

\* Control points taken 2 hr after injection.

$\ddagger$  Mean per cent sequestration of at least two animals at every point. No attempt has been made to correct for radioactivity of blood within organs.

$\S$  Radiolabeled unsensitized cells.

511 sites per cell these animals also showed liver sequestration.

## DISCUSSION

In the present experiments, a guinea pig model of in vivo clearance of erythrocytes has been established which allows for the quantitative estimation of the role of complement-fixing IgG and IgM antibodies and of complement in immune clearance. Several lines of in vitro investigation have already suggested that IgM and IgG antibodies interact with complement by different molecular mechanisms (16-18). For example, it has been shown that a single molecule of IgM on a cell surface is sufficient to initiate complement fixation, and that a doublet (two molecules side by side) of the usual IgG in serum is most likely required to produce a complement-fixing site (13). Nevertheless, electron microscopic and molecular studies have suggested that IgG antibody, although inefficient at initiating complement fixation, may produce more membrane damage than does IgM (5, 19).

The in vivo data presented here support that concept. Clearly, a complement-fixing site formed by the interaction of IgM antibody with the cell surface reacts very differently from a complement-fixing site formed by the interaction of IgG antibody and the cell. Thus, we find that 60 or more IgM C1-fixing sites are required to initiate in vivo clearance of sensitized erythrocytes. On the other hand, as few as 1.4 complement-fixing IgG sites on an erythrocyte surface are sufficient to induce increased rates of erythrocyte clearance. Fewer than 1.4 complement-fixing sites per cell were not associated with increased clearance. The patterns of clearance and the sites of organ sequestration differ for IgG and IgM antibodies. IgM antibody on a cell surface leads to rapid liver sequestration of the sensitized cells. These cells may be released back into the circulation where they undergo normal survival. Data detailed in the accompanying paper demonstrate that these cells remain Coombs positive when they reappear in the circulation (15).

IgG-coated cells have an entirely different pattern of clearance. They are progressively removed from the circulation by the spleen and do not reappear in the circulation. If a sufficiently large number of IgG complement-fixing antibody sites are present, cells may be cleared by the liver; however, they still show the typical "IgG pattern" (Fig. 10) of clearance.

The experimental model described in this paper has the major advantage of being able to separate the effects of complement-fixing antibody on the cell surface from the effects of complement itself. We have used a recently developed subline of guinea pigs with a genetically controlled, total deficiency of C4 to examine



the effects of antibody in the absence of normal complement function. It has been shown that the C4-deficient guinea pigs do possess an alternate pathway into the complement sequence which allows them to bypass C4 (20); however, data from our laboratory indicate that antibodies on red cell surfaces are extremely inefficient in activating this alternate pathway *in vitro*.<sup>2</sup> Thus, in C4-deficient guinea pigs, IgM-sensitized cells have a normal survival with as many as 234 C1-fixing antibody sites per cell. On the other hand, cells sensitized with IgG show slightly increased rates of clearance in C4-deficient guinea pigs. It has been well demonstrated that macrophages have an IgG receptor (21, 22) and this receptor may facilitate clearance of IgG-coated cells in the absence of active complement. The data indicate that erythrocyte clearance dependent upon the IgG receptor is quantitatively much less important than that dependent on complement. Many IgG molecules are required to form a complement-fixing site, but cells sensitized with fewer than the number required to form one complement-fixing site have a normal survival. It is quite possible that both IgG and complement molecules are required on an erythrocyte surface to obtain effective adherence to macrophages and subsequent phagocytosis. This would explain the complement requirement of IgG-sensitized cells and some of the differences between the activities of IgG and IgM antibodies.

The data presented here are compatible with the bulk of the extensive experimental data which has accumulated on the pathophysiology of hemolytic anemia in man and animals. As early as 1949 it was suggested that cells coated with cold agglutinin (IgM) and transfused into normal subjects might take hours to equilibrate or to reach their maximal concentration in the circulation (23). The cells were subsequently removed from the circulation at a normal rate. Similar observations were made by Jandl, Jones, and Castle (24) and Lewis, Dacie, and Szur (25). The latter authors, utilizing cold agglutinin sensitized erythrocytes, suggested that sequestration and release was responsible for the clearance pattern obtained.

Recently, Brown, Lachmann, and Dacie reported that a human IgM cold agglutinin when injected into rabbits produced a similar pattern of liver sequestration and release to that reported here for IgM-coated erythrocytes (26). The authors prepared liver imprints which showed that immune adherence was responsible for the liver sequestration. They did not attempt, however, to estimate the number of IgM molecules per cell required to produce this pattern.

<sup>2</sup> Unpublished observations.

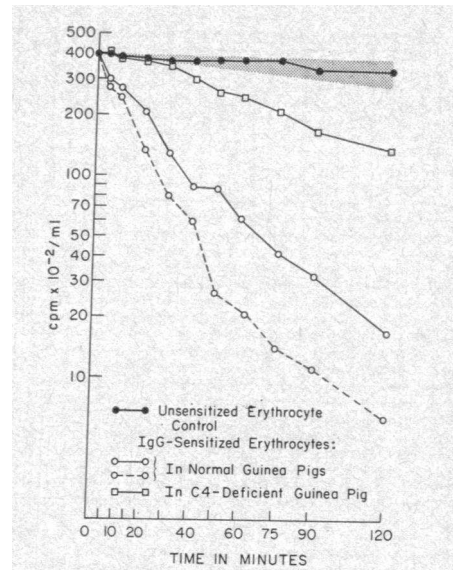


FIGURE 10 Survival <sup>51</sup>Cr-labeled erythrocytes with 90 IgG C1-fixing sites/erythrocyte in C4-deficient and two normal guinea pigs.

The literature is unclear with regard to the effect of IgG and complement on erythrocyte clearance. A number of investigators have sensitized erythrocytes with hyperimmune sera which presumably contain mostly IgG antibody (3, 24, 27, 28). They noted that rates of clearance increased progressively with increasing levels of sensitization and that the pattern was one of progressive destruction of erythrocytes, rather than sequestration and release. Progressive complement-dependent destruction was also noted in studies of the clearance of IgG-sensitized rat erythrocytes from the circulation of the mouse (30). The two patterns of clearance, sequestration, and release in the case of IgM and progressive destruction in the case of IgG and hyperimmune sera, which have been reported in animals and man, suggest that our findings are not unique to the guinea pig.

Whether noncomplement-fixing antibodies will affect erythrocyte survival in a manner similar to that of complement-fixing IgG and IgM in the C4-deficient guinea pig, remains to be determined. Many *in vivo* studies have utilized erythrocytes sensitized with antibodies felt to be noncomplement fixing (1, 24, 28, 29). These cells were cleared primarily by the spleen. Clarification of the biologic effects of IgM and IgG antibody on erythrocyte survival is essential to understanding the pathophysiology of immune hemolytic anemia in man. Such clarification will require further definition of the numbers and types of molecules on the cell membrane.



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