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

II. MOLECULAR NATURE OF IgG AND IgM COMPLEMENT-FIXING SITES AND EFFECTS OF THEIR INTERACTION WITH SERUM

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Abstract A model for the immune clearance and destruction of homologous erythrocytes has been further explored. In this model, every IgM anti-erythrocyte antibody molecule in an antibody preparation was shown to fix C1. About 2000 IgG antibody molecules were required to form a C1-fixing site on the guinea pig erythrocyte surface. 60 IgM complement-fixing sites per erythrocyte were required for the immune clearance of IgM-sensitized erythrocytes. This number of sites could be detected by a direct agglutination test. 1.4 complement-fixing sites were required for immune clearance of IgG-sensitized cells, a number of molecules which could not be detected by direct agglutination. This number could, however, be detected with the use of a Coombs antiglobulin reagent.

Depletion of the late components of complement (C3-9) with cobra venom was associated with the loss of ability to clear IgM-sensitized cells and a marked deficit in the ability to clear IgG-coated cells. Thus, late (C3-9) components of complement as well as an early component (C4) were required for normal clearance of sensitized erythrocytes. There was no evidence that activation of the alternate pathway of complement action could lead to accelerated erythrocyte clearance.

In vitro incubation of IgG and IgM-sensitized erythrocytes in fresh serum led to deposition of C3 and C4 on the erythrocyte surface. IgM-sensitized cells treated in this way had a normal survival. IgM-sensitized cells also were shown to remain Coombs positive after their release from the liver. The evidence suggests that the interaction of an IgM site with fresh serum in vivo and in vivo leads to formation of a site which allows for sequestration of cells in the liver. With continued exposure to serum components, this site is destroyed or inactivated. This serum-dependent inactivation is complement-dependent as shown by the use of EDTA-treated and C4-deficient serum. IgG complement-fixing sites are only partially inactivated by incubation in fresh serum, further emphasizing the differences in the biologic activity of IgM and IgG antibodies.

Introduction

In the accompanying paper we described an experimental model designed to examine the function of complement-fixing IgG and IgM antibody and of complement in the immune clearance and destruction of erythrocytes (1). We demonstrated that complement-fixing sites formed by IgM antibody behave differently from those formed by IgG, and that the patterns of clearance and the organ localization of cleared cells differ with cells sensitized with each of the two classes of antibody. In this paper we explore the molecular events which accompany the formation of a complement-fixing site and the evolution of a complement-fixing site in the presence of fresh serum in order to obtain an in vitro correlate of our in vivo findings. We attempt to relate routine clinical tests, such as the direct agglutination test and the Coombs antiglobulin test, to these molecular events. Finally, by use of guinea pigs with serum complement components (C3-9) depleted

1 Abbreviations used in this paper: C3-9, late components of complement; C4, fourth component of complement; CoF, cobra venom factor; RBC, red blood cells; VBS, veronal-buffered saline.

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by the injection of purified cobra venom factor, we attempt to determine whether these later steps in the complement sequence are required for the clearance of IgG and IgM-sensitized cells.

METHODS

Buffers and complement reagents, guinea pigs, guinea pig erythrocytes, and rabbit anti-guinea pig erythrocyte antisera were prepared as noted in the preceding paper (1). Immunoglobulin purification, erythrocyte survival studies, and quantitative antibody and complement studies were also performed as in the preceding paper (1). Serum titers of C1, C4, C2, and of the classical C3-9 complex were performed by modifications of established methods (2, 3).

Antiglobulin reagents. A guinea pig antisera to rabbit immunoglobulin was prepared by immunizing NIH multipurpose guinea pigs with guinea pig erythrocytes coated with rabbit antibody. Washed guinea pig erythrocytes were sensitized with rabbit anti-guinea pig erythrocyte antibody in the presence of EDTA. The erythrocytes were emulsified in an equal volume of Freund’s complete adjuvant (Difco Laboratories, Detroit, Mich.) and guinea pigs were injected with 1 ml each of a 10% suspension of these erythrocytes. The animals received a booster injection of the 10% antibody-coated erythrocyte suspension emulsified in Freund’s incomplete adjuvant (Difco Laboratories) 4 wk after primary immunization and were exsanguinated 2 wk after the second immunization. This antisera recognized both rabbit IgM and IgG on immunoelectrophoretic analysis. In unpublished studies, attempts to develop blood group-specific antisera were unsuccessful and these antisera did not distinguish guinea pig, blood group specificities.

Guinea pig γ2 anti-rabbit immunoglobulin was prepared by dialyzing guinea pig anti-rabbit immunoglobulin antisera against 0.01 m potassium phosphate buffer, pH 8.25, at 0.0°C. 2 cc of the antisera was then applied to a DEAE column equilibrated in 0.01 m potassium phosphate buffer, pH 8.25. The γ2 peak was eluted in 0.01 m phosphate buffer, and the effluent was brought to an ionic strength of 0.15 m by the addition of 3.0 m NaCl. Both the original antisera and the γ2 fraction were employed as Coombs antiglobulin reagents in the subsequent experiments.

Guinea pig anti-guinea pig C4 was prepared as in the experiments of Elinman, Green, and Frank (3). Rabbit anti-guinea pig C3 was a gift of Dr. H. Shin.

Antiglobulin (Coombs) titers were obtained utilizing a microtiter technique. 3.4 × 10^9 washed guinea pig erythrocytes in a volume of 25 μl VBS were added to 25 μl of serial twofold falling dilutions of each immunoglobulin in VBS. The tubes were mixed and incubated at 37°C for 30 min, and 25 μl of an optimal concentration of antirabbit immunoglobulin reagent in VBS was added. The mixtures were reincubated for 1–2 hr at 37°C and the degree of agglutination scored from 1+ to 4+. The titer was scored as the reciprocal of the dilution which produced 1+ agglutination.

Radioiodination of IgG and IgM fractions. Radiiodination of a representative IgG and IgM pool was accomplished by the method of McFarlane (4). The IgG fraction was iodinated to the extent of 1.8 iodine molecules per molecule of IgG and the macroglobulin pool to the extent of 0.7 iodine molecules per molecule of IgM. Portions of guinea pig erythrocytes were sensitized with dilutions of the radiolabeled fractions at 37°C for 30 min. The sensitized cells were washed thoroughly with VBS and the amount of radiolabel absorbed determined with a gamma scintillation counter (Packard Instrument Co., Inc., Downers Grove, Ill.). The number of IgG and IgM molecules absorbed per erythrocyte was calculated from the protein content of the antibody fraction and the per cent protein absorbed as measured by radioactive uptake and compared with the number of Cl-fixing sites per erythrocyte which were determined as discussed in the previous paper. Controls for specificity of uptake of radiolabeled rabbit anti-guinea pig erythrocyte IgG and IgM antibodies indicated that less than 0.1% of the radiolabeled proteins bound nonspecifically to rabbit erythrocytes.

Purification of cobra venom factor. The purification procedure was a modification of the method of Nelson (5). 500 mg of Naja-naja cobra venom (Ross Allen Reptile Institute, Inc., Silver Springs, Fla.) was dissolved in 25 ml of 0.007 M phosphate-buffered saline, pH 7.5, and applied to a 21 × 25 cm DEAE-cellulose column equilibrated with 0.007 M phosphate-buffered saline. The column was eluted with increasing concentrations of NaCl with a straight line gradient increasing to 0.3 M. The second protein peak, eluted in about 0.2 M NaCl, was pooled and dialyzed against 0.02 M phosphate-buffered saline. The pH was adjusted to 5.0 and the fraction was applied to a CM-cellulose column which was eluted with a straight line gradient up to 0.5 M. There was no protein peak noted when the optical density was determined at 280 nm; however, an anticomplementary peak was noted at 0.25 M NaCl. This anticomplementary peak was concentrated by ultrafiltration (Schleicher & Schuell, Inc., Keene, N. H.). The concentrated fraction was free of the cobra venom neurotoxin and was quite stable. 3–4 cc portions of the purified cobra venom fraction were administered intravenously to NIH multipurpose guinea pigs 5 hr later, at a time of marked depression of serum complement titer (CH50), IgG and IgM-sensitized 51Cr erythrocytes were injected, and RBC survival curves determined as in (1). Immediately before injection of the labeled cells, blood was obtained for C1, C4, C2, and C3-9 titrations.

The effect of in vitro incubation of IgG and IgM-sensitized erythrocytes with guinea pig serum on their in vitro survival. A series of experiments was performed with sensitized cells which were incubated in normal guinea pig serum to permit in vitro attachment and activation of complement components. IgG and IgM-sensitized erythrocytes (2.7 × 10^8 cells per ml) prepared as previously described (1), with 17 and 117 complement-fixing sites of antibody, respectively, were sedimented and suspended in 2 ml of normal guinea pig serum, C4-deficient guinea pig serum (3), or guinea pig serum to which disodium EDTA buffer was added to a final concentration of 0.02 m. The mixtures were each incubated at 37°C for 1 hr, sedimented, resuspended to a final concentration of 2.7 × 10^8 RBC per ml in VBS, and erythrocyte survival curves determined. Under these conditions of incubation, IgG-sensitized cells showed virtually no hemolysis in normal guinea pig serum, in C4-deficient serum, or in EDTA serum. IgM-sensitized cells with 117 or more Cl-fixing sites showed minimal hemolysis in normal serum (1-10%) and none in C4-deficient serum or EDTA serum.

IgG and IgM-sensitized erythrocytes which had been incubated in fresh guinea pig serum were tested by the Coombs test for the presence of immunoglobulin, C3, and C4 on their surface. The cells were suspended in VBS at a concentration of 3.4 × 10^6 cell per 25 ml. 25 μl portions were added to 25 μl of serial twofold falling dilution of

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either guinea pig anti-rabbit immunoglobulin, guinea pig anti-guinea pig C4, or rabbit anti-guinea pig C3. The degree of agglutination was scored from 1+ to 4+.

A method was devised to determine whether the injected radiolabeled IgM-sensitized cells remain Coombs positive when they are returned to the circulation after sequestration in vivo. Guinea pigs were injected with 1 ml of $2.7 \times 10^6$ radiolabeled RBC sensitized with 60 and 117 complement-fixing units of IgM antibody, and erythrocyte survival curves determined. After the characteristic pattern of initial sequestration and release was documented, the guinea pigs were exsanguinated (2 hr after injection). The erythrocytes were washed and resuspended in VBS to a concentration of $2.7 \times 10^6$ RBC per ml and incubated with 0.25 ml of guinea pig anti-rabbit immunoglobulin (1:10 in VBS) for 30 min at 37°C. The cells were washed, resuspended in 1 ml VBS, and incubated for 60 min at 37°C with 0.5 ml of 1:5 dilution in VBS of fresh human serum absorbed twice with guinea pig erythrocytes at 0.6°C. After the period of incubation, the cells were sedimented, the radioactivity in the cell button and supernatant fluids determined, and the optical density (OD) of the supernatant measured spectrophotometrically. The degree of hemolysis of the sensitized radiolabeled cells, comprising less than 0.1% of the total red cell mass, was compared with the degree of lysis of the unlabeled unsensitized cells of the guinea pig. Two controls for specificity were performed. Portions of the pool of cells were incubated in human complement in the absence of guinea pig anti-rabbit immunoglobulin antibody and the degree of hemolysis of the radiolabeled cells determined. Also, cells were obtained from a guinea pig injected with unsensitized radiolabeled erythrocytes and studied as above. The rational for this technique was based on our observation that, although guinea pig erythrocytes sensitized with IgG and IgM antibody are resistant to lysis in the presence of guinea pig serum, they are readily lysed in the presence of human serum.

RESULTS

The absolute number of antibody molecules required to form a C1-fixing site with IgG and IgM antibodies was determined. Studies relating the per cent uptake of radiolabeled immunoglobulin from the IgG and IgM pools (1.5 and 5.2% respectively) to the protein content of the IgG and IgM pools indicated that 103 molecules of IgM by uptake of radiolabel formed 117 C1-fixing sites per cell. 2012 IgG molecules per cell were required to form 1.4 complement-fixing sites per cell. Both figures are consistent with those published for the sheep erythrocyte (6). As calculated from radioactive uptake, these figures suggest that every IgM antibody molecule in this preparation had complement-fixing activity whereas many IgG molecules were required to form C1-complement fixing sites. It has been suggested that a doublet of IgG, two molecules side by side, are required to initiate complement fixation and that many IgG molecules are required before, by chance, a doublet is formed (6, 7).

An attempt was made to estimate the sensitivity of the semi-quantitative agglutination test in absolute terms by relating the direct agglutinin titer to the absolute number of antibody molecules absorbed. About 12,000 IgG molecules per erythrocyte were necessary for 1+ direct agglutination. When guinea pig anti-rabbit immunoglobulin was added (direct Coombs test) agglutination of IgG-sensitized cells could be detected with about 1570 molecules of IgG antibody per erythrocyte. Since 1.4 complement-fixing sites (2012 molecules) of IgG per red blood cell were necessary for accelerated clearance, the minimal number of IgG antibody molecules per erythrocyte needed to effect an increased red cell clearance could be detected with antoglobulin reagents. However, without the assistance of an anti-globulin reagent, one could not detect direct agglutination of erythrocytes sensitized with 17 complement-fixing sites (10,060 IgG molecules) of IgG antibody per RBC, a concentration of antibody which results in approximately 90% of the cells being destroyed within 2 hr (1).

In the case of IgM-sensitized erythrocytes, at least 50 molecules of antibody per RBC were necessary for detectable direct agglutination. There was no increase in sensitivity with the antglobulin reagent. Since 60 complement-fixing sites of IgM antibody per erythrocyte were necessary for altered clearance (1), it was possible to detect the minimal number of IgM sites required for increased clearance utilizing an agglutination test without a facilitating antglobulin reagent.

Cobra factor-treated guinea pigs. A series of experiments was performed to determine whether pre-treatment of guinea pigs with cobra venom factor would alter the survival of sensitized cells. Cobra factor has been shown to specifically deplete animals of the late-acting complement components (C3-9). As shown in the preceding paper of this series, C4, an early component in the sequence of complement component interactions, is essential to the normal clearance of sensitized erythrocytes. 4-5 hr after the intravenous administration of purified cobra venom factor (CoF), guinea pigs had normal serum titers of C1, C4, and C2. Assays for the C3-9 complex, however, revealed a greater than 95% fall in activity.

When IgM-sensitized erythrocytes (117 C1-fixing sites per cell) were injected into the CoF-treated guinea pigs, the pattern of sequestration and release was abolished and the IgM-sensitized erythrocytes survived normally (Fig. 1). The clearance of IgG-sensitized erythrocytes (17 C1-fixing sites per cell) was also markedly impaired in CoF-treated guinea pigs, but the rate of clearance was more rapid than normal (Fig. 2). The clearance patterns in CoF-treated animals resembled that previously described in C4-deficient guinea pigs (1).
Effect of in vitro preincubation of IgG and IgM-sensitized erythrocytes in fresh guinea pig serum on clearance. After incubation of IgM-sensitized erythrocytes with normal guinea pig serum in vitro, the characteristic pattern of sequestration and release was not observed (Fig. 3). Thus, preincubation protected the IgM-coated cells from liver sequestration. The protective effect of guinea pig serum was not seen with either EDTA-treated guinea pig serum or C4-deficient guinea pig serum. In the case of IgG-sensitized erythrocytes, incubation with normal guinea pig serum was only moderately protective and the cells continued to have abnormal survival (Fig. 4). Under the conditions of preincubation, sensitized guinea pig erythrocytes were resistant to lysis by guinea pig complement and in this resemble human erythrocytes which are also resistant to hemolysis by complement. This resistance was seen in the markedly low hemolytic antibody titer (<1/5) for both the IgG and IgM fractions and by the resistance of sensitized cells to lysis when incubated in undiluted normal guinea pig serum.

After 1 hr incubation with guinea pig serum, both IgM and IgG-sensitized cells were still strongly agglutinated by anti-C3, and anti-C4, and anti-immunoglobulin, suggesting that incubation of sensitized cells with normal serum did not lead to loss of antibody or complement component reactivity.

In vivo studies demonstrating that erythrocytes remain Coombs positive after sequestration and release. When guinea pigs were exsanguinated 2 hr after the injection of radiolabeled IgM-sensitized red blood cells and their erythrocytes incubated with absorbed fresh human serum, little lysis was observed indicating that the period of in vivo circulation had led to resistance to lysis. When, however, anti-rabbit immunoglobulin was added before absorbed human serum, greater than 90% of the radiolabeled cells were lysed indicating that the radiolabeled cells still contain IgM immunoglobulin on their surface after their return to the circulation. This compared with only 14% lysis of the erythrocyte pool determined spectrophotometrically. Although radiolabeled unsensitized cells were slightly more susceptible to lysis than unlabeled erythrocytes, the degree of lysis was not in the range observed for the radiolabeled sensitized cells.

**DISCUSSION**

In the first paper of this series, a model of immune clearance was established which demonstrated that IgG and IgM anti-erythrocyte antibodies differ in their ability to induce immune destruction of erythrocytes. Different patterns of both clearance and organ localization with IgG and IgM-sensitized erythrocytes were obtained, suggesting that the interaction of IgG and IgM antibody with complement leads to the formation of different active sites on the erythrocyte surface. In this paper we have attempted to further quantify and characterize the interaction of these antibodies with serum components and to define the steps required for generation of these active products.

Utilizing radiolabeled, purified immunoglobulin fractions, we have determined the number of molecules required to form a C1-fixing site with IgG and IgM antibody. 103 molecules of IgM antibody were required to form 117 IgM complement-fixing sites, suggesting that every IgM antibody molecule was able to fix C1. This finding is in accord with published values for IgM anti-Forssman antibody. An average of 2012 IgG anti-
body molecules were required to form 1.4 IgG complement-fixing sites on an erythrocyte surface. This value is also in accord with published values in the anti-Forsman sheep cell system.

The antiglobulin or Coombs test, utilizing potent anti-immunoglobulin reagents, was found sufficiently sensitive to detect the minimum number of antibody molecules or complement-fixing sites necessary to alter the erythrocyte clearance with either IgG or IgM. This is in contrast to a direct agglutination test which could not detect a concentration of IgG antibody per erythrocyte which resulted in approximately 90% of the cells being cleared within 2 hr. The number of molecules of IgG and of IgM found necessary to give positive agglutination (12,000 and 50 respectively) correspond to those obtained with human red cells (8). The value for IgG is slightly higher than that reported for the antiglobulin test in studies performed utilizing what is perhaps a more indirect method (9).

The first paper in this series demonstrated the fact that C4, an early component in the complement sequence, was necessary for clearance of IgM-sensitized cells and for optimal clearance of IgG-sensitized cells. Since C4-deficient guinea pigs possess an alternate pathway into the complement sequence which bypasses C4 (10), the faster than normal clearance of IgG-coated cells in C4-deficient guinea pigs might be explained by activation of the bypass complement pathway. This bypass pathway enters the complement sequence at C3 (11), and animals depleted of C3 should have the classic pathway and the alternate pathway blocked. Studies were therefore performed in cobra venom-treated animals depleted of C3 and late components in the sequence of complement reactions, but shown to have normal levels of C1, C4, and C2. In these animals, the pattern of rapid sequestration of cells within the liver with subsequent slow return to the circulation, characteristic of IgM-sensitized erythrocytes, was abolished. The clearance of IgG-sensitized cells was also markedly impaired in the CoF-treated guinea pigs, emphasizing the importance of components beyond C2 in the complement pathway in the removal of IgG-sensitized erythrocytes from the circulation. The pattern of clearance of IgG-sensitized erythrocytes was strikingly similar in C4-deficient and C3-9-depleted animals. Thus, the evidence suggests that neither the classical nor alternate complement pathway contributed to the clearance of these cells. It seems probable that the IgG receptor on macrophages facilitated the clearance of IgG-sensitized erythrocytes in the absence of active complement; however, in this case, the clearance was markedly suboptimal. Presumably, in the guinea pig, IgG receptor-dependent clearance requires many thousands of IgG molecules per cell.

Studies presented here indicate that IgM-sensitized cells have antibody on their surface after sequestration by the liver and return to the circulation, demonstrating that dissociation or destruction of antibody is not responsible for release of cells from the liver. This was shown utilizing a sensitive hemolytic assay capable of detecting the few circulating radiolabeled Coombs-positive cells. These data are in keeping with the in vitro studies which show that incubation with whole guinea

![Figure 3](image3.png)  
**Figure 3** Survival of \(^{3}Cr\) guinea pig erythrocytes with 17 IgM Cl-fixing sites/erythrocyte after incubation in vitro in normal guinea pig serum or in C4-deficient guinea pig serum. Incubation in EDTA serum led to a clearance pattern which was similar to that noted after incubation in C4-deficient serum.

![Figure 4](image4.png)  
**Figure 4** Survival of \(^{3}Cr\) guinea pig erythrocytes with 17 IgG Cl-complement-fixing sites/erythrocyte after incubation in vitro in normal guinea pig serum or in EDTA serum. Incubation with serum from C4-deficient guinea pigs led to partial protection in some but not all cases.
pig serum protects IgM-sensitized cells from being sequestered in the liver. This property of serum appears to be dependent upon activation of the complement sequence, as it is absent from EDTA-treated and C4-deficient serum. After incubation in fresh serum, the sensitized cells have antibody, C3, and C4 on their surface; nevertheless they have a normal survival. Thus, a positive antiglobulin test for both antibody and complement is compatible with normal erythrocyte survival.

Observations on the effect of serum on survival of erythrocytes sensitized with antibody have been made in a number of animal models and in a number of clinical studies. As early as 1946, Loutit and Mollison (12) observed that red cells sensitized with anti-A antibody and incubated in vitro with serum had normal in vivo survival. Subsequently, Evans, Turner, Bingham, and Woods were able to demonstrate in patients the increased resistance to in vitro lysis and in vivo clearance of human erythrocytes sensitized with human cold agglutinin. Resistance to lysis could be correlated with the accumulation of C3 and C4 on the red cell membranes (13, 14). The authors felt that the increased resistance might in part be explained by the accumulation of complement proteins on the cell surface sterically inhibiting antibody action. Möller in 1964, reported that mouse erythrocytes sensitized with isoantibody and incubated in fresh serum, were resistant to in vitro hemolysis. He noted that antibody conferred resistance to in vivo immune clearance and, furthermore, that this resistance to lysis and clearance was more marked with 19s than with 7s mouse isoantibody (15-17). He recognized the difficulty of comparing IgM and IgG on the basis of agglutinating units, considering the vast difference in agglutinating activity of the two classes of antibody, and he could not be certain whether the observed differences were quantitative or qualitative.

Our findings are similar in a model which allows for comparison of the two classes of antibodies on the basis of complement-fixing sites generated. The protective activity in serum was shown to be complement-related by use of C4-deficient serum and EDTA-treated serum. It seems likely that protection of sensitized cells against destruction in vivo by in vitro incubation in serum, reflects the sequence of events initiated by the in vivo injection of sensitized cells. The data is most compatible with the concept that an active site is generated on injection of IgM-sensitized cells into the guinea pig which leads to liver sequestration, probably through a mechanism of immune adherence to cells within the liver (24, 18). On continued exposure to serum, this site is inactivated or destroyed, although antigenically detectable complement fragments remain on the erythrocyte surface. The RBC is therefore released from the liver into the circulation where it undergoes normal survival. The serum factor responsible for the release of erythrocytes coated with antibody and complement is still unknown, but may well be the C3 inactivator.

This study did not attempt to determine the nature of the active site on the erythrocyte surface which leads to sequestration. The site formed by IgM must differ either quantitatively or qualitatively from that formed by IgG. A series of cell surface receptors have recently been described which promote the adherence of antibody and complement-coated erythrocytes to macrophages and lymphocytes. Receptors have been described with specificity for IgM, IgG, and complement on macrophages and for complement on lymphocytes (19-23). It seems likely that these cellular receptors play a major role in both sequestration and clearance.

In vitro incubation with whole guinea pig serum only partially protected IgG-sensitized erythrocytes from being cleared by the spleen, even though the IgG-sensitized cells had one-seventh the number of C1-fixing sites available for inactivation as compared with the IgM-sensitized cells. In this report IgG complement-fixing sites again are shown to react differently from IgM complement-fixing sites. The relative inability of in vitro incubation in serum of IgG-sensitized cells to protect the cells from in vivo clearance in part explains the continued clearance of these cells from the circulation. Sites which can interact with the clearance mechanism are not inactivated by serum and are available for long periods of time. These data still do not explain the basic difference in the mechanism by which IgM and IgG-sensitized cells are cleared. Nevertheless, they readily suggest the nature of the in vitro models which would provide an explanation for these findings. Construction of such models is now in progress.

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