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PARTIAL IMMUNE ELIMINATION OF HOMOLOGOUS RED BLOOD CELLS IN MICE[1]

ROBERT P. ERICKSON, LEONARD A. HERZENBERG AND RONALD GOOR

Department of Genetics, Stanford University School of Medicine, Palo Alto, California

SUMMARY

Cr^{51}-labelled homologous red blood cells are only partially eliminated in passively or actively immune mice. Host factors do not seem to be involved, as animals that contain uneliminated cells are capable of eliminating a portion of freshly added cells. (The quantity and components of antibody, homologous and heterologous complement, and the possibility of transfer of label were looked at specifically.) Of cell-specific factors, studies with Fe^{59}-labelling and phenylhydrazine indicate that the age of the erythrocytes does not explain the variable elimination.

INTRODUCTION

Goodman and Smith (*6*) found that hyperimmunization of mice with bone marrow of another strain had no marked effect on the life span of subsequently transfused erythrocytes from the donor strain. Similarly, in his studies on parbiosis in the mouse, Tokuda (*19*) observed no immune destruction of erythrocytes even at the height of parabiotic intoxication, under conditions where immunization of one partner was expected. N. A. Mitchison (personal communication) has also noticed and commented on the surprising resistance of mouse erythrocytes to isoimmune elimination.

Differing from these workers, we find that homologous mouse erythrocytes are subject to immune elimination. However, the proportion of red cells destroyed is small and quite variable. The studies reported here were undertaken to document and to attempt to understand this variability in the hope of improving the efficiency of immune elimination.

Mouse strains and sources. A/Jax (*H-2*^{a}), C57BL/10SnHz (*H-2*^{b}), C57BL/10-*H-2*^{d}/SnHz (*H-2*^{d}), C3H/SnHz (*H-2*^{k}), C3H.SW/SnHz (C3H-*H-2*^{b}), supplied by Dr. G. D. Snell in 1961 and bred at Stanford; C57BL/6Jax, retired breeder females from The Jackson Laboratory, Bar Harbor, Maine; DBA/2Jax, retired breeders via Simonsen Laboratories, Gilroy, California; BALB/cTu, from the colony of the late Dr. F. C. Turner.

Solution. Acid citrate dextrose (ACD) as described by Gibson et al. (*5*), containing merthiolate 1/10,000.

Cr^{51} labelling of erythrocytes in vitro. Blood was collected from the tail artery into ACD. Erythrocytes were sedimented (1000 *g*, 5 min) and the supernatant

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and buffy coat discarded. They were then washed once in saline and suspended in 1.5 ml ACD/ml packed cells. Approximately 0.2 mc of Cr^{51} (sodium radiochromate (Abbott) made isotonic), was added per 2.5 ml red cells suspension and the mixture was incubated at room temperature for 45 min with mixing. Ascorbate was sometimes added to destroy any residual $Cr^{51}O_4$ but was not found essential as the cells were washed once in normal saline before being made up to 10% (v/v) for injection. Very short incubation periods which left free Cr^{51} in the supernatant were used to demonstrate the effectiveness of the washing procedures. Less than 3% labelling of host cells was observed. Labelling efficiency varied from 60–90% with a mean of about 75%. There was little increase with further incubation. Each animal received 0.5 ml of the 10% suspension. The route of administration was usually i.p. but in some experiments i.v. injections were used.

Labelling of erythrocytes in vivo with Fe^{59}. One-tenth mc Fe^{59} (in about 0.5 ml radioferrous citrate (Abbott) neutralized with NaOH) was injected per animal. Erythrocytes were collected at 5 days, when specific activity was maximal, and prepared for injection as above. To prevent reutilization of Fe^{59} by hosts eliminating Fe^{59} red cells, all animals receiving these cells were given 2 mg Proferrin (saccharated iron oxide, Merck, Sharp and Dohme) several hours prior to injection and at 2 day intervals during the experiment.

Sampling and counting. Ten μl of blood was expressed from the tip of the cut tail of each mouse. The samples were counted in a deep-well scintillation counter (Packard, Gamma Spectrometer, counting efficiency about 2%).

Antiserum production (*8*) and serological techniques (*14*) have been described previously.

RESULTS

Figures 1 and 2 indicate a considerable variation both in rate and extent of elimination among animals within a given test group and frequently there is some overlap between immune and nonimmune groups. Nevertheless a rapid initial elimination of 20–80% of the labelled cells in passively immune animals is usually observed, followed after about 20 hours by elimination at a rate comparable to that in the control nonimmune animals.

Figure 1 shows elimination curves for labelled C57BL/10-*H-2*^{d} erythrocytes in passively immune C57BL/10 animals. At 20 hours, counts in the control group (injected with 0.2 ml normal serum) have decreased relatively little while counts in the passively immune group (0.2 ml hyperimmune C57BL/10 anti-C57BL/10-*H-2*^{d} serum per animal) have dropped precipitously by that time, although they then remain constant. Injection of additional immune serum at 40 hours does not cause a further decrease in the counts.

Actively immunized animals also eliminate only a fraction of the Cr^{51}. After i.p. injection of hyperimmune animals (6 or more weekly spleen injections) with labelled cells, the counts in the circulation gradually rise to about 20% of total counts injected at 24 hours, and then decrease slowly as in the nonimmune control. Thus, in the immune animal only a proportion of the cells reaches the circulation but those that do are not eliminated any differently from nonimmune controls.

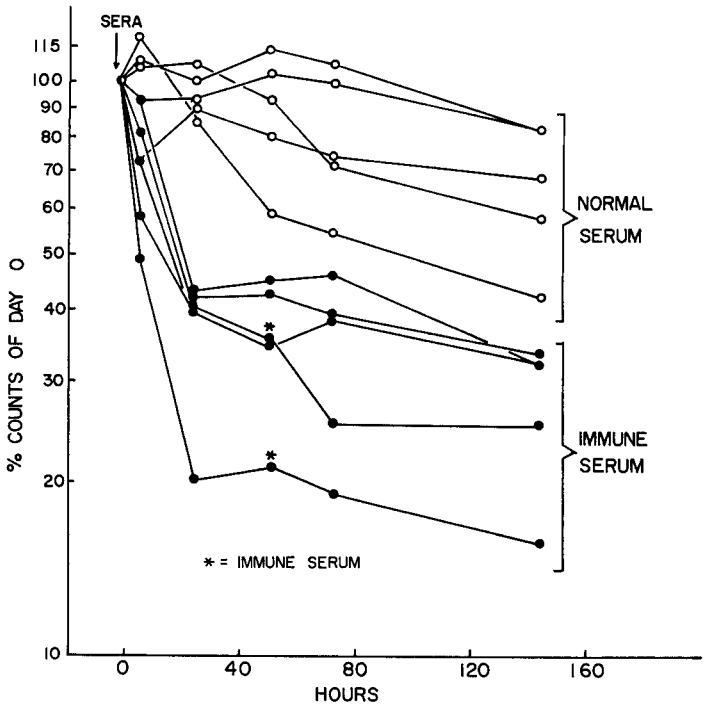

FIGURE 1. Elimination of Cr^{51} labelled erythrocytes in individual mice compared to control mice. Ten C57BL/10 (*H-2*^{b}) mice were injected with Cr^{51}-labelled C57BL/10 (*H-2*^{d}) erythrocytes. Samples were taken 1 day later (time 0). The animals were then injected with 0.2 ml sera (1 ml of a 1/5 dilution). Five received normal serum while the other 5 received *H-2*^{b} anti-*H-2*^{d}. At 48 hours, 2 of the latter 5 animals received another injection of antiserum.

While the reasons for the persistence of such a high proportion of the injected Cr^{51} and for the recurrent observation of only partial elimination in immunized animals remain obscure, the following possibilities have been investigated and found inadequate to fully explain the phenomenon. Because of the statistical variability, groups of five animals were used for an experimental or control condition and most experiments were repeated.The amount of variability is shown in Figures 1 and 2. Figure 1 shows elimination curves for individual animals in a Cr^{51}-labelled erythrocyte elimination experiment. Figure 2 shows standard deviations for groups of five animals in a Fe^{59}-labelled erythrocyte elimination experiment.

Transfer of label to host cells. (a) Fe^{59}-labelled erythrocytes in animals saturated with Fe^{55} were eliminated with the same pattern as Cr^{51}-labelled cells, demonstrating that Cr^{51} labelling: (1) is as stable as Fe^{59} labelling; (2) was not preferential or selective for persistent erythrocytes; (3) did not cause resistance to elimination (see Fig. 2 for elimination curves with Fe^{59}-labelled erythrocytes). (b) To test further the possibility that the host erythrocytes became Cr^{51}-labelled during immune elimination, five C57BL/10 animals were given C57BL/10-*H-2*^{d} erythrocytes and C57BL/10 anti-*H-2*^{d} antiserum. Elimination was

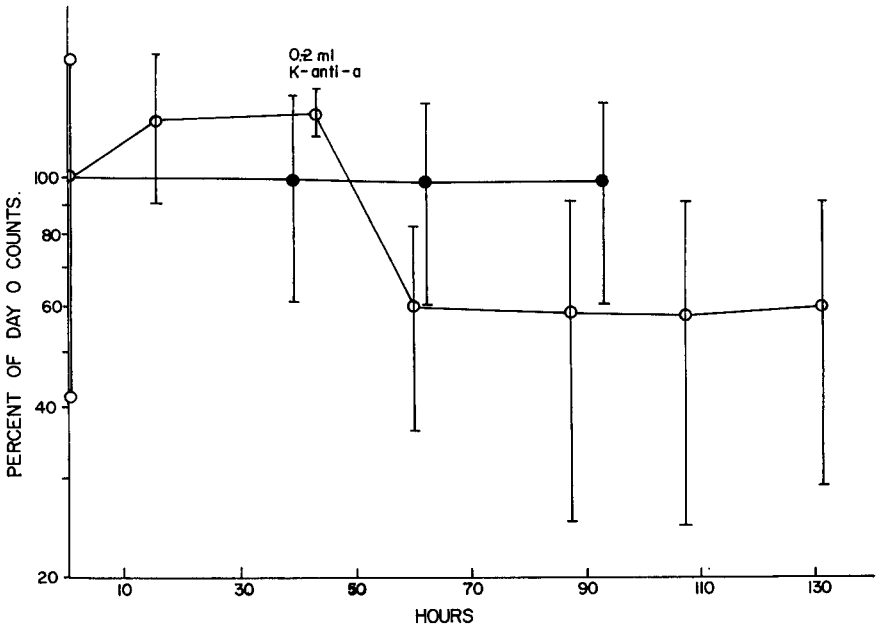

FIGURE 2. Elimination of Fe^{59}-labelled erythrocytes in groups of 5 homologous and isologous mice. Ten C3H (*H-2*^{k}) mice were injected i.v. and i.p. (due to partial failure i.v.) with A strain, Fe^{59}-labelled erythrocytes. Samples were taken within 10 minutes (time 0) and at later intervals. One group of 5 animals (0) received 0.2 ml H-2^k anti-H-2^a serum at 43 hours. Standard deviations are shown as bars around the points. They were calculated on percentages of time 0 taken for each animal.

followed until the curves had leveled. At this point, the animals were bled, erythrocytes collected, washed, and reinjected into C57BL/10-*H-2*^{d} hosts. *H-2*^{d} anti-*H-2*^{b} antiserum, which would have caused immune elimination of any Cr^{51} if it had been on labelled *H-2*^{b} (C57BL/10) erythrocytes was then injected. There was no decrease in circulating counts indicating that the label was still present on *H-2*^{d} cells.

Depletion of recipient's ability to eliminate foreign erythrocytes. At 107 hours, when their elimination curves had leveled, five recipients were given more labelled erythrocytes from the donor strain. Eighty percent of the newly added counts were eliminated during the next 40 hours and then the curves leveled, demonstrating the ability of the recipients to further eliminate cells. Therefore it is unlikely that any serum factor, such as hemolytic complement, could have been depleted in the host animals.

Lack of complement. Assays in vitro have shown mouse sera to be low in hemolytic complement activity (*10*). Addition of complement by injection of 0.4 ml guinea pig serum[2] (2 × 0.2 ml) at an interval of several days had no detectable effect on elimination. Some of the recipient strains have been found to be geneti-

[2] Obtained from Travenol Laboratories, Morton, Illinois. The lyophilized sera was reconstituted and frozen once.

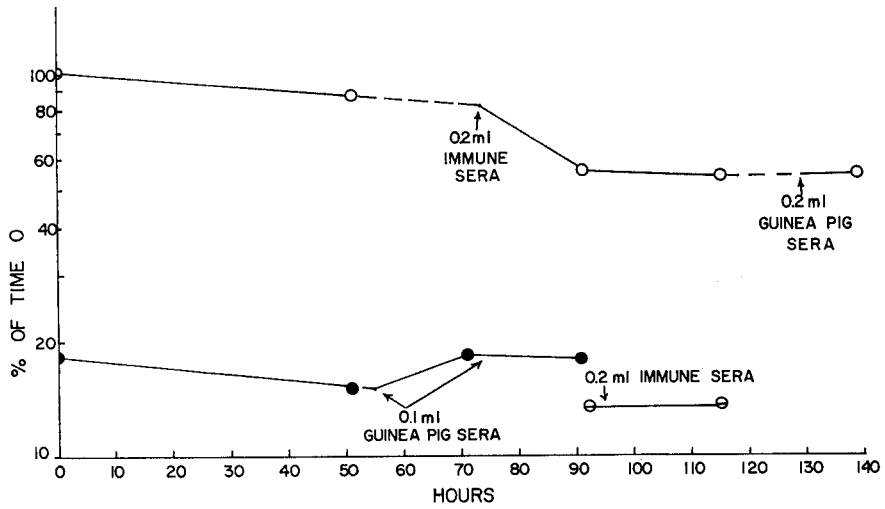

FIGURE 3. Transfer of partially eliminated erythrocytes to a second host without further elimination. Ten C3H ($H\text{-}2^k$) mice, 5 normal (0) and 5 hyperimmune (●) against A ($H\text{-}2^a$), were injected with Cr^{51}-labelled A ($H\text{-}2^a$), rbc's. Samples were taken 1 day later (time 0) and various treatments given: the normal animals received 0.2 ml $H\text{-}2^k$ anti-$H\text{-}2^a$ sera (1 ml of a 1/5 dilution) and 0.2 ml of guinea pig complement while the actively immune animals received only complement (2 × 0.1 ml). At 91 hours the 5 hyperimmune mice were exsanguinated into ACD, the 2 ml of packed rbc's were made up to 40% (v/v) in 0.9% NaCl and 1 ml of the suspension was injected into each of 5 normal C3H ($H\text{-}2^k$) mice (○) which had first been bled (to reduce blood volume). Samples were taken and 0.2 ml of antisera injected. The gap in the curve indicates 70% recovery of the labelled cells. When treatments were performed but a sample was not taken at that time, a dotted line is used to extend the curve along its predicted course.

cally deficient for hemolytic complement activity (*15*). Nevertheless, immune elimination was not less efficient in these strains than in others.

Depletion of antibody. As seen in Figure 1, injection of additional isoantisera of the same type does not increase elimination. Hemagglutination titers were obtained and those of the passively immune animals remained high through at least the first 72 hours after injection of antiserum and cells. One-fourth as much serum (50 μl) gave essentially as much elimination, again indicating that antibody was not limiting.

Excess of antibody. The possibility that excess antibody inhibited immune elimination was ruled out as doses of antibody smaller than 50 μl showed less immune elimination and the injection of additional donor erythrocytes (non-labelled) to recipients that had partially eliminated cells did not cause further elimination of the labelled cells.

Antisera to an insufficient number of H-2 components. Möller (*12*) has shown that tumor cells resistant to a single cytotoxic antibody can be killed by a mixture of antisera from different strains directed against the H-2 components of the tumor-bearing strain. A combination of two sera which should contain antibodies directed at most of the known antigenic components of the $H\text{-}2^a$ phenotype, i.e., C3H($H\text{-}2^k$) anti-A($H\text{-}2^a$) and C57BL/10-$H\text{-}2^d$ anti-A($H\text{-}2^a$) (*7*), were injected within 3 days of one another into animals that had received Cr^{51}-labelled $H\text{-}2^a$

erythrocytes. Although the antibodies were circulating simultaneously (see above), there was no more elimination than where one kind of antiserum was injected.

If the incomplete elimination of transferred erythrocytes is neither an artifact of measurement nor the result of deficiency or exhaustion of systemic factors, then the question is raised as to whether only a portion of the donor strain erythrocytes is sensitive to isoimmune elimination, the rest, in fact, being resistant to this kind of attack. To test for this, Cr^{51}-labelled A strain cells were injected into actively immunized C3H mice (anti-*H-2*^{a}). When 20% of the counts had reached the circulation and the elimination curve was flat, the mice were bled from the tail arteries into citrate-saline. The packed erythrocytes were washed and resuspended in saline and injected into five more C3H mice, and 24 hours later, *H-2*^{k} anti-*H-2*^{a} serum was given as usual (Fig. 3). This transferred population, which had been thus depleted of its "sensitive" fraction of erythrocytes, showed no evidence of immune elimination.

The reason for the heterogeneity of erythrocytes again remains obscure. It seems unlikely that erythrocytes of differing ages show differential sensitivity as is demonstrated by the following. (a) Iron uptake occurs during erythropoesis and Fe^{59} labels only young cells but the young, Fe^{59}-labelled cells prepared in this fashion are eliminated similarly to those labelled with Cr^{51} (Fig. 2). (b) Phenylhydrazine has a selective toxic effect upon mature erythrocytes and the net result of its use is a great increase in the number of reticulocytes in the circulating blood (*4*). Hosts injected with a "young" population of erythrocytes prepared from mice treated with phenylhydrazine (2 × 0.06 mg/g body wt.) still had 20% of counts left when the rapid phase of elimination tapered off. While there was more elimination of counts here than in most other experiments (about 50% elimination was average), this is within the variation observed.

Returning to Figure 1, it is evident that mouse erythrocytes do not induce their own elimination. Thus they can serve as an index for the onset of an immune response to antigens on themselves (e.g. H-2 antigens). An experiment to exploit this property was set up as follows: Non-immune C57BL/6 animals were injected with $\frac{1}{10}$ of a BALB/c spleen at the same time as they received labelled BALB/c erythrocytes. The rate of elimination in these mice was at first not significantly different from that in a control group of nonimmunized animals. On day 7 a rapid elimination of Cr^{51} was observed in the spleen-injected animals. This lasted for about 2 days, and then leveled off again to the rate shown in the controls leaving, on the average, 50% of the Cr^{51}.

DISCUSSION

The definite but only partial immune elimination of homologous erythrocytes in the mouse is probably at least in part due to heterogeneity of the erythrocyte population with respect to susceptibility to immune destruction in vivo. This is consistent with the observation of Owen (*13*) that a fraction of normal mouse red cells is resistant to immune hemolysis with mouse antisera and rabbit complement in vitro. Owen found that donor type erythrocytes from mice irradiated

and then given homologous bone marrow were more susceptible to hemolysis than the donors' red blood cells were originally.

Our observations are also consistent with those reported by Winn (*20*) on incomplete hemolysis of mouse erythrocytes in vitro. He used isoantisera and guinea pig complement in testing the red cells of A strain mice and found that hemolysis varied from 45 to 75% among littermates. Cells of interstrain hybrid mice showed greater variability in the degree of hemolysis with antisera against the hybrid tissue and greater resistance to antisera against the parental tissue than the parental erythrocytes.

Although the differences between susceptible and nonsusceptible cells are not known, they may lie in the amount or distribution of surface isoantigens or in the degree of "physiologic" resistance to immune destruction. Sinclair has shown that the variably incomplete lysis of cattle red blood cells by sera (isologous and heterologous) directed against the H-antigen of the A system is probably due to a partial deficiency of antigenic sites (*17*). In addition to this cell heterogeneity, the variability of rates of elimination from animal to animal with a single suspension of labelled cells should not be overlooked. Speculation on the causes of this variability is not profitable at this point.

Immune elimination has been tested in several mouse strain combinations with both actively and passively immunized animals. No consistent differences have been observed in the completeness of immune elimination. A major nonspecific factor which might have been limiting immune destruction in vivo, hemolytic complement, is an unlikely explanation of the results. Although complement can only be detected in some strains of mice and then by a special test, host factors seem not to be involved as an animal that has partially eliminated some homologous cells can eliminate a proportion of fresh homologous cells.

It has been known for some time that mouse red blood cells are poor sources of isoantigens (*3, 9, 11, 18*). In other species, man and pigeon, the demonstration of immunological nonreactivity on some cells has been thought to be due to somatic mosaicism, although evidence speaks against mutation or cross-over as possible explanations (*1, 2, 16*). The results here reported might suggest that such explanations have to be taken with caution until the immunological basis of the phenomenon is better understood.

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