Blood Clearance of Sheep Erythrocytes in Mice Measured Immunologically and by Visual Count* (34106)

PETER G. SOHNLE AND DIETER H. SUSSDORF
(Introduced by A. A. Hirata)
Department of Microbiology, Cornell University Medical College, New York, New York 10021

There are several possible methods for determining the clearance rate of foreign erythrocytes from the bloodstream of a laboratory animal. Perhaps the most direct way is differential counting if the erythrocytes involved are physically distinguishable as is the case when nucleated avian erythrocytes are followed in a mammalian species (1). Radioactive labels have been employed to determine clearance rates of mammalian erythrocytes in other mammalian species. Ingraham (2) used the external ^{35}S sulfanilic acid label on sheep erythrocyte stroma (SRBC) and Wust (3) traced SRBC marked with the internal ^{75}Se label. These methods are very sensitive, but have the major disadvantage of following the label and not the antigen itself.

In the present work an antibody-inhibition assay was used in an attempt to make a more direct, immunologic estimate of the clearance rate of foreign mammalian erythrocytes. By measuring uptake of specific hemolytic antibody, SRBC were followed in the circulation of the mouse. The results were compared to those obtained with a second method involving hemocytometer counts of SRBC in mouse blood. The SRBC became morphologically readily distinguishable from mouse cells when both cell types were suspended in hypotonic blood-diluting fluid. The blood clearance curves obtained by both methods were compared and found to be in good agreement.

Besides being more direct, these methods have certain other advantages over radioactive labeling. Neither method requires any prior treatment of the SRBC; at least some types of radioactive labeling have been shown to affect the clearance rate of the antigen used (4). Also, both methods are relatively rapid and require low-cost equipment.

Materials and Methods. The mice used were 8-week-old females of the ICR strain obtained from Camm Research Institute, Wayne, New Jersey. Their weights averaged 32 g, and they were given food and water *ad libitum*. These mice carry the Forssman antigen in the spleen and consequently are free of circulating, natural anti-Forssman hemolysin. Since they also served as the source for the SRBC-antiserum used in the inhibition assay, the specificity of this serum was directed predominantly against the isophile antigens of the sheep cells.

SRBC were obtained in Alsever's solution, washed, and suspended in saline to 50% by photometric standardization. To determine clearance, each mouse was injected intravenously with 0.2 ml of the 50% suspension. The dose, which corresponded to 1.6×10^9 cells, was not adjusted to the individual mouse weights because they varied over a very narrow range.

Thirty seconds, 15, 30, 60, and 120 min after injection, the mice were killed by cervical fracture. This procedure also served to rupture the great vessels allowing blood to collect in the thoracic cavity from which it was aspirated with a heparinized capillary pipette. The mouse blood was washed twice in veronal buffer (5) (centrifugation at 700 *g*) and the cells were suspended in a volume twice that of the original blood volume. Blood samples from three mice were pooled for each time point and separate groups were used for each method.

* This work was supported by Training Grant 1-T1-AI-281-01 from the National Institute of Allergy and Infectious Diseases and Grant GB-7351 from the National Science Foundation.

For the inhibition assay, the suspensions of washed blood cells were diluted 10^{-1}, 10^{-2}, and 10^{-3}. To 1.8 ml of each dilution, 0.2 ml of a mouse antiserum against SRBC containing 20 units of 50% hemolysin was added (see below for method of titration). The suspensions were mixed and incubated at 37° for 2 hr. After centrifugation of the suspensions at room temperature, the supernatant fractions were titrated for remaining antibody according to the method described by Chapman and Sussdorf (6). According to this assay, a hemolysin unit is defined as the reciprocal of that fraction of a milliliter of sample which lyses 50% of 1.7×10^7 SRBC in the presence of four 50% units of guinea pig complement, in 30 min at 37° and in a reaction volume of 2.5 ml.

Control preparations for the inhibition assay consisted of erythrocytes from uninjected mice plus antiserum, and antiserum alone. Mouse cells did not significantly affect the hemolytic activity of the anti-SRBC serum.

Results. Figure 1 shows the percentage of hemolysin units remaining after the reaction of anti-SRBC serum with various dilutions of

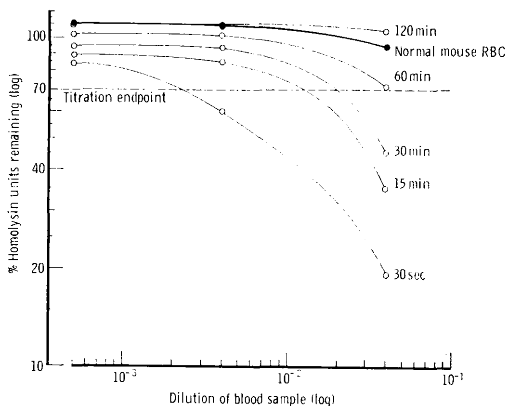

FIG. 1. Immune assay for sheep erythrocytes circulating in the mouse. Samples of mouse blood taken at various times after injection of sheep erthrocytes were serially diluted and combined with a standard amount of anti-sheep erythrocyte serum. Shown is the percentage of antibody units remaining after absorption in relation to sample dilution. Sheep erythrocyte concentration was calculated from the sample dilution required to leave 70% of the added hemolysin unabsorbed (see calibration curve in Fig. 2).

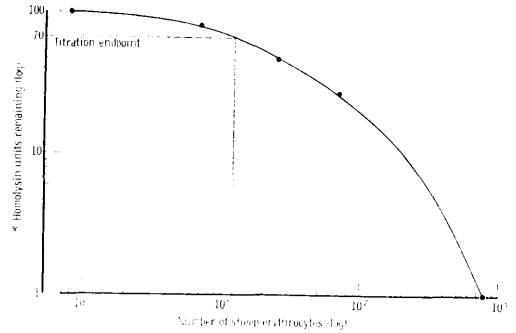

FIG. 2. Calibration curve correlating percentage of hemolysin units remaining after absorption of a standard amount of antiserum by increasing numbers of sheep erythrocytes. A 30% hemolysin uptake (70% remaining) in the immune assay for circulatory clearance of sheep erythrocytes corresponded to 1.23×10^6 cells.

mouse blood samples. The times indicated are the intervals between injection of SRBC into the mice and collection of the blood samples. From each of these plots the dilution of mouse blood that would result in 30% antibody uptake (70% remaining) was determined. The number of sheep erythrocytes corresponding to a 30% uptake of the antibody activity added to the assay system was read from a calibration curve of antibody uptake vs. number of erythrocytes (Fig. 2).

The accuracy of the immunologically determined sheep cell clearance was checked by a method permitting visual differentiation between mouse and sheep erythrocytes. When a mixture of mouse and sheep erythrocytes are suspended in Gower's solution diluted 1:4 with distilled water, the sheep cells appear wrinkled while the mouse cells look normal (Fig. 3). To check the reliability of this distinction mixtures were made of varying dilutions of washed sheep erythrocytes and a standard volume of whole mouse blood. The mixtures were incubated at 37° for 2 hr to simulate conditions within the animals. Differential hemocytometer counts were made in diluted Gower's solution to determine the number of SRBC present per milliliter of cell mixture. These counts are compared with the counts of SRBC diluted identically but mixed with saline instead of mouse blood. Table I shows that the counts

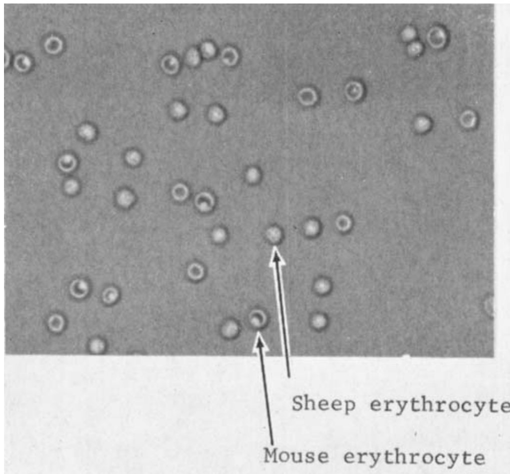

FIG. 3. Sheep and mouse erythrocytes (× 500) suspended in Gower's solution diluted 1:4 with distilled water. The mouse cells retain a normal configuration while the sheep cells are smaller, lack refractility at the edge, and appear wrinkled.

are in agreement within 10%.

Figure 4 represents the blood clearance curves obtained by the two methods. They are in good agreement even though they were determined in different groups of mice. Within 1 hr over 95% of the foreign erythrocytes were cleared, and at 2 hr the numbers remaining were undetectable by these methods. This rate of clearance is markedly greater than that reported by Wust (3). He followed the same dose of internally (^{75}Se) labeled SRBC in mice and found 40–60% of the label still present at 2 hr. The faster clearance observed by us could not be at-

TABLE I. Hemocytometer Counts of SRBC Diluted in Mouse Blood.[a]

Dilution of SRBC	Counts (× 10^7) in: Mouse blood	Saline
1:10	198 ± 17.8	212 ± 7.1
1:100	20 ± 7.1	22 ± 2.8
1:1000	2.3 ± 0.9	2.3 ± 0.4

[a] The counts were made on the basis of the changed morphology of SRBC and unaltered appearance of mouse RBC in hypotonic Gower's solution. These counts are compared with those of SRBC diluted in saline instead of mouse blood. Mean and standard deviation of three dilutions.

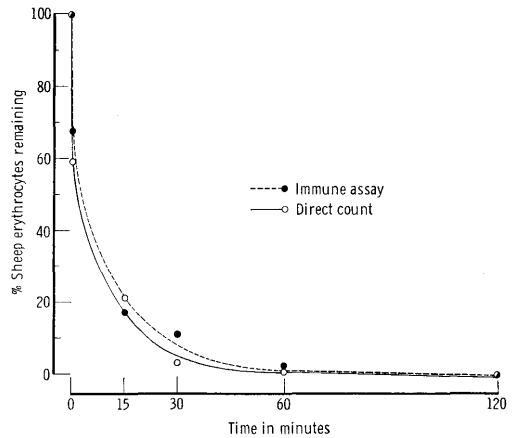

FIG. 4. Blood clearance of sheep erythrocytes in the mouse as measured immunologically and by direct hemocytometer count.

tributed to the presence of natural, circulating anti-Forssman hemolysin. The apparent persistence of antigen in Wust's experiments was probably due to the inclusion of substantial amounts of soluble label in the blood clearance determinations as indicated by the high levels of label present in the plasma of these animals.

Summary. Immunological and visual counting methods are described for following the circulatory clearance of sheep erythrocytes in mice. In the immunological assay the uptake of anti-sheep erythrocyte hemolysin by mouse blood was measured. Visual counts were based on the finding that, contrary to mouse erythrocytes, sheep erythrocytes are morphologically altered by hypotonic Gower's solution. The clearance rates observed by the two methods were in close agreement.

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Received April 25, 1969. P.S.E.B.M., 1969, Vol. 131.