central pool. For this reason it was expected that after the introduction of 10 mM xylocaine in the distal pool the downward peak would become a large downward phase (1*d*, Fig. 3, *13–24*). However, the secondary action of the anesthetic developed so rapidly that only the deflection resulting from decremental conduction in the first gap remained (Fig. 3, *16*; cf. ref. 1*d*). For the same reason only trapezoidal action potentials appear in Figure 3, *17–20*.

The presentation of the theory of the isolated fiber will be continued.

* This work has been supported in part by a grant (NB 02650) from the USPHS.

† Present address: Department of Otolaryngology, Vanderbilt University, Nashville, Tennessee.

[1] (*a*) Lorente de Nó, R., and V. Honrubia, these PROCEEDINGS, **53**, 757 (1965); (*b*) *ibid.*, p. 938; (*c*) *ibid.*, p. 1384; (*d*) *ibid.*, **54**, 82 (1965); (*e*) *ibid.*, p. 388; (*f*) *ibid.*, p. 770; (*g*) *ibid.*, p. 1061.

[2] Lorente de Nó, R., and Y. Laporte, *J. Cell. Comp. Physiol.*, **35**, suppl. 2 (1950).

[3] Lorente de Nó, R., in *The Neuron*, Cold Spring Harbor Symposia on Quantitative Biology, vol. 17 (1952), p. 299.

---

*FACILITATION OF IMMUNE HEMOLYSIS BY AN INTERACTION BETWEEN RED CELL-SENSITIZING ANTIBODY AND γ-GLOBULIN ALLOTYPE ANTIBODY**

BY EBERHARDT WEILER, ELSA W. MELLETZ, AND EVELYN BREUNINGER-PECK

THE INSTITUTE FOR CANCER RESEARCH, PHILADELPHIA

Communicated by Thomas F. Anderson, August 30, 1965

When a complex forms between red blood cells and antibodies against red cells, sensitization occurs: subsequent exposure to complement will cause lysis of the red cells (immune hemolysis).[1] It has been found[2] that hemolysis can be inhibited, when the antibodies prepared in one species to sensitize red cells are exposed to "heterotype" antibodies against them prepared in another species. This "neutralization" of an antibody by heterotype antibody is analogous to virus neutralization, or enzyme inhibition by antibody.

In this paper a special interaction between red cell antibodies and their anti-antibodies will be considered, which involves mouse γ-globulin allotypes.

In mice, γ-globulins (of the 7Sγ2*a* class)[3] occur in several genetic variants (allotypes), each determined by one of a series of alleles at the Ig-1 locus.[4, 5] γ-Globulin variants can be recognized by using allotype antibodies as specific reagents. These are produced in mice homozygous for one of the Ig-1 alleles, by injecting γ-globulin from mice carrying another allele. Such allotype antibodies react with allotypic γ-globulin, including red cell-sensitizing antibodies (see Fig. 1). (In this system, the red cell-sensitizing antibodies and the allotype antibodies are different allotypic variants of mouse γ-globulin.)

It will be shown that γ-globulin allotype antibodies, in contrast to heterotype antibodies, do not neutralize red cell-sensitizing antibodies. On the contrary, they facilitate hemolysis, for the interaction between red cell-sensitizing antibody and allotype antibody caused a marked increase in the sensitizing efficiency of antisera to red cells. Facilitation of hemolysis was also studied in single cell assays, invented by Jerne[6] and Ingraham;[7] among populations of antibody-forming cells subpopulations

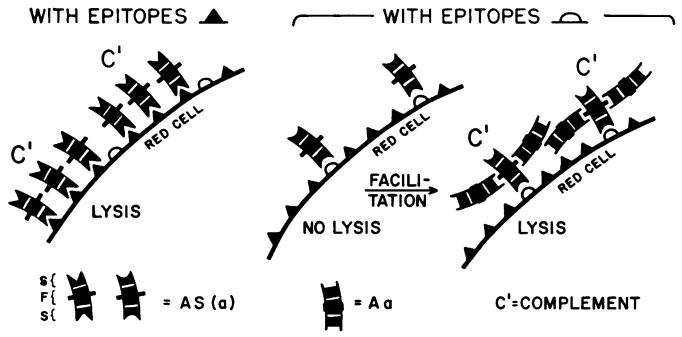

FIG. 1.—Diagram illustrating the system and interpreting the results. Sheep red cell antibodies carrying allotype *a* [*AS*(*a*)] are attached to two different red cell antigenic groups (epitopes). On the right, allotype antibodies against *a* (= *Aa*) have complexed with "lonely" *AS*(*a*). Note sites combining with antigen on the *S* piece, and allotype structures on the *F* piece of antibody molecules. (*Aa* carries allotype *b* on its *F*-piece.)

could be recognized, differing in their response to allotype antibody. The facilitation effect introduces a new aspect of the mechanism of immune hemolysis.

In a companion paper experiments will be reported[8] in which the facilitation effect served as a tool to study the question of whether in individual heterozygous cells both parental allotypes are phenotypically expressed, or only one.

Materials and Methods.—Mouse allotypes: The mouse strains C3H/HeNICR and C57Bl/6NICR were used in this work. According to one of several nomenclatures proposed (literature in ref. 3), the C3H strain carries the allele Ig-1^a, and its 7Sγ2a globulin has the corresponding phenotype Ig-1a. The allelic gene of the C57Bl strain is Ig-1^b, and the corresponding phenotype is Ig-1b.

Abbreviations: (See Fig. 1.) The genotypes will be designated *a/a* for homozygous C3H mice or cells, and *b/b* for homozygous C57Bl. The γ-globulin allotypes will be referred to as *a* for Ig-1a, and *b* for Ig-1b. *A* denotes antiserum; thus the allotype antiserum against Ig-1a is called *Aa*, and that against Ig-1b is called *Ab*. Mouse antisera against sheep red cells are *AS*(*a*), when they were produced in mice homozygous for the Ig-1^a allele, and *AS*(*b*), when they were produced in Ig-1^b homozygous mice. Similarly, *N*(*a*) is normal serum from *a/a* mice, and *N*(*b*) from *b/b* mice.

Allotype antisera were prepared in *a/a* and *b/b* female mice. The immunizing antigen for both strains was mouse antibody against rabbit red cells, obtained as the ascites fluid[9] in female *a/b* F_1 hybrid mice. (In their γ-globulin these carry both parental allotypes.) It was injected as an agglutinate with rabbit red cell stromata. The first injection was in Freund's adjuvant. Subsequent injections were given intraperitoneally once a week for 7–8 weeks. Additional courses of 3 or 4 injections each were given with intervals of 1–3 months between courses. After each course the mice were bled repeatedly from the retroorbital sinus. Serum giving strong precipitation bands in micro-Ouchterlony tests, with undiluted normal serum as the antigen, were frozen in small portions in dry ice and stored at −50°C. Four individual anti-Ig-1b sera were used, which did not differ in their ability to cause facilitation. Anti-Ig-1a serum from a single individual was used for all tests.

Antisera against sheep red cells were prepared by one primary intravenous injection of stromata, corresponding to about 10^9 cells, followed by three secondary injections, 2 days apart, after a rest period of 5 weeks. The mice were bled on days 5, 7, and 9 after the last injection.

Sheep cell antisera were assayed in an agar diffusion test. Assemblies consisting of a microscope slide and a Plexiglas matrix containing conical holes, as described by Crowle,[10] were charged with a portion of the following mixture: 2 ml liquefied agar (Difco, purified), at a concentration of 1.2% in water; 0.5 ml of 5 × concentrated veronal buffer;[11] and 0.25 ml washed sheep red cells in veronal buffer so as to give 5×10^7 cells/ml in the final mixture. The holes ("wells") in the Plexiglas matrix were filled with 7–8 μl of serially diluted antiserum, and the assemblies kept for 18 hr at 2°C; the matrices were then removed, and the slides washed in a large volume of veronal

buffer for 3 hr. Allotype antiserum (or normal mouse serum) was pipetted onto the slides, and left for 1 hr at room temperature. The serum was then drained off, and the slides were flooded with complement ($^1/_3$ or $^1/_4$ reconstituted lyophilized guinea pig serum, BBL) incubated for 1 hr at 37°C, rinsed in buffer, fixed in mercuric chloride (Hayem's solution), washed in water, and dried. The diameters of concentric areas of hemolysis around the wells were measured with a reticle in a hand comparator. Hemolysis did not occur when the complement had been heated to 56°C before use.

Spleen cell donors for the single cell assays were injected twice intravenously with approximately 2×10^9 sheep red cell stromata. The first injection was at about 8 weeks of age, the second 8–13 weeks later. Five or six days after the second injection the spleen cells were prepared for assay by raking with a stainless steel wire mesh; clumps were eliminated by sedimentation.

The hemolytic plaque assay technique of Jerne[6] had to be modified, to permit the sequential application of different allotype antisera, and to allow staining by fluorescent antibodies. Therefore, a different technique was devised (Fig. 4). The mixture of spleen cells, target red cells, and culture medium in agar was not poured onto hard agar plates, but spread thinly on 25 × 75-mm microscope slides. The following mixture was prepared in 10 × 75-mm tubes standing in 45°C waterbath: 0.205 ml Eagle's minimum essential medium (MEM) with 2 times the prescribed amino acid concentration; 0.10 ml of 2.4% hot agar (Difco, purified) in saline; 0.15 ml of allotype antiserum (or normal mouse serum); 0.04 ml of sheep red blood cells, 3.5×10^9/ml in MEM; and

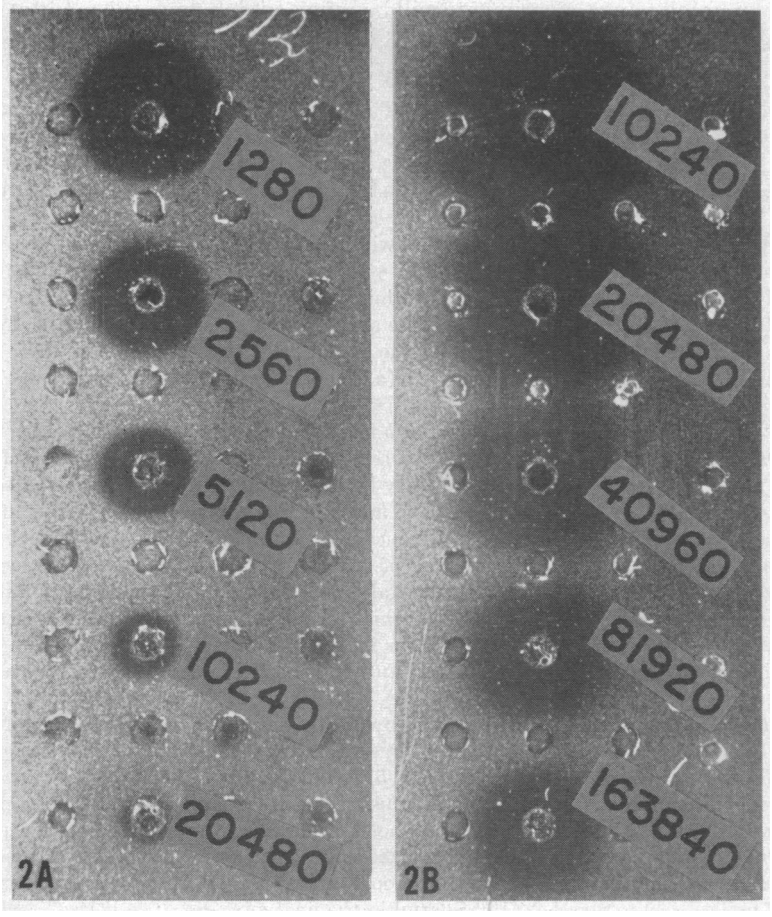

FIG. 2.—Disks of hemolysis produced by C3H anti-sheep red cell serum, *AS(a)*, diffusing into agar containing the target red cells. (*A*) Control with normal serum; (*B*) facilitated by allotype antiserum *Aa*. Dilutions are given in the photograph. Dark field illumination from one side. Magnification 4 ×.

0.04 ml spleen cells, 40 × 10^6/ml in MEM. Components were added in that order. The final number of nucleated spleen cells was 3.6 × 10^5/slide. (DEAE-Dextran, recommended by Jerne,[6] was not found to increase the number of plaques with this technique, and therefore it was omitted.) Onto agar-coated slides held at 45°C, 0.09 ml of this mixture was spread, as evenly as possible. An area of 6–8 cm^2 was covered. The slides were then quickly transferred to an ice-cooled aluminum plate, where the agar gelled within a few seconds. They were then immersed in liquid paraffin in plastic Petri dishes. The paraffin had been equilibrated with Eagle's medium, and with CO_2 to the desired pH. The preparations were incubated for 105 min at 37°C in an atmosphere of 5% CO_2 in air. The slides were then subjected to the following series of operations: washing in veronal buffer to remove the paraffin; flooding with complement (diluted 1/4) and incubation in a moist chamber, 70 min at 37°C; washing in veronal buffer 15–25 min; photography on 35-mm film (first-round picture); incubation with the second allotype antiserum (diluted 1/20) 60 min at room temperature; rinsing in buffer; second incubation with complement; rinsing in buffer; photography (second-round picture); fixation in Hayem's solution; washing in water; drying. For analysis, photographic prints providing about 4 × magnification were made, and the scoring of plaques was aided by a hand comparator.

Results.—(1) *Hemolytic disk assays:* Sheep cell antisera, diffusing from the Plexiglas wells into the agar layer, combine with sheep red cells in areas concentric with the wells. Disks of hemolysis developed when the slides were incubated with complement (Fig. 2). When disk areas (minus the area directly underneath the lower well opening, 0.8 mm^2) are plotted against the logarithm of the relative serum concentration, most points lie on a straight line (Fig. 3). Deviations were observed with very small disks and sometimes with very large disks (>30 mm^2), which tended to cause a slight downward bend of the curve. The slope of the curve showed only a slight dependence on the red cell concentration between 5 × 10^7 and 5 × 10^8 cells/-ml. The disks were considerably larger, at given serum concentrations, when the preparations had been treated with allotype antiserum, instead of normal mouse serum (Fig. 2). The efficiency of red cell sensitization by red cell antiserum has thus been increased. This is the effect referred to as hemolysis facilitation. It is obtained only when the allotype antiserum was directed against the allotype of the sheep cell antibody: *Aa* facilitated sensitization by *AS(a)*, but not by *AS(b)*, and *Ab* caused facilitation only in concert with *AS(b)*. Facilitation is allotype-specific. (19*S* antibodies, obtained after a primary injection of red cell stromata, could not be facilitated. They do not carry the Ig-1 allotype structure.[3])

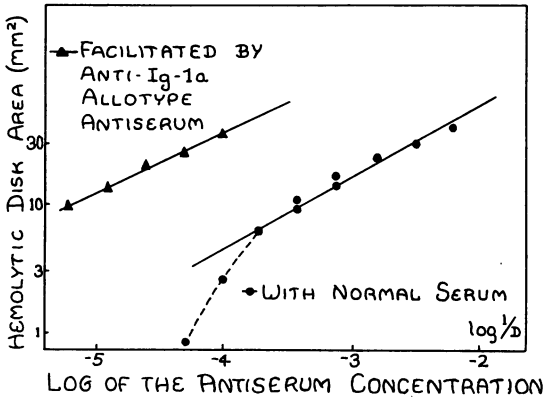

FIG. 3.—Disks of hemolysis produced by serial 2-fold dilutions of a C3H anti-sheep red cell antiserum, *AS(a)*, without facilitation, and after facilitation by allotype antiserum *Aa*.

Facilitation causes the curve relating disk areas to antiserum concentrations to be shifted toward lower serum concentrations (Fig. 3). Although slopes varied with different antisera (between the extreme values of 0.47 and 0.84), and although the slope of the facilitation curves tended to be somewhat less than that of control curves, the mean distance between the two, taken parallel with the abscissa, could

be used as an approximate measure of the factor by which the sensitizing efficiency of a given antiserum was increased as compared to the control. Three different $AS(b)$ sera were facilitated by Ab by factors of 6.4, 9, and 12, respectively. Two $AS(a)$ sera were facilitated by factors of 33 and 49, respectively, when incubated with Aa. Aa serum appears to have the higher efficiency of facilitation, as compared to Ab.

Since facilitation occurs on agar slides after they had been washed in buffer for 3 hr, allotype antibody must combine with sheep red cell antibody already attached to its target antigen. When slides were washed for 18 hr in a large volume of buffer after treatment with allotype antiserum, but before incubation with complement, the disks were only slightly smaller. It appears that the ternary complex of sheep red cells, anti-sheep cell antibody, and allotype anti-antibody is relatively stable.

Facilitation could also be demonstrated in conventional test tube assays.

(2) *Hemolysis facilitation in single cell experiments* (see diagram in Fig. 4): When individual spleen cells in an agar layer secrete antibody, surrounding sheep red cells become sensitized, and plaques of hemolysis develop during a subsequent incubation with complement.[6, 7] The appearance of plaques after normal mouse serum had been present during the secretion phase (first round) is shown in Figure 5*A*. Allotype antiserum was then applied to the same preparations and they were again incubated with complement (second round). As shown in Figure 2*B*, additional plaques became visible, and some small plaques became enlarged. Other plaques were not affected. The increase in the size of some plaques and the appearance of new plaques occurred only when the allotype antiserum was directed against the allotype characteristic of the spleen cells used: Aa produced the effect with C3H spleen cells (a/a) and Ab with C57Bl spleen cells (b/b). This shows again that facilitation of hemolysis is allotype-specific.

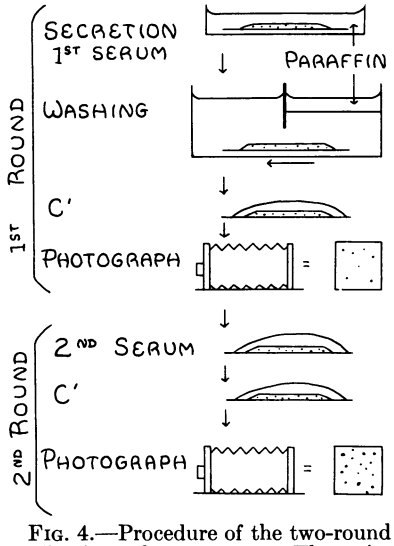

FIG. 4.—Procedure of the two-round facilitation plaque assay. The mixture of antibody-producing spleen cells and target red cells is in a thin agar layer on a microslide, and is incubated under liquid paraffin. This and subsequent applications of complement (C') and allotype antiserum are indicated in the diagram. Examples of photographs taken after each round are given in Figs. 2*A* and *B*. For the various incubation times, as well as washings and rinsings between different reagents, see *Materials and Methods*.

In comparing photographs taken after the first, and again after the second round (Figs. 5*A* and *B*), individual plaques were classified as to the degree to which they were affected by the second-round allotype antiserum (Table 1). A sample experiment with b/b spleen cells is presented in Table 1. When Ab was present in the second round, following $N(b)$ in the first (sequence $N(b)$, Ab), nearly half of the plaques originally present became significantly larger; new plaques were 4–6 times as numerous as those that remained the same. Only "larger" and "new, large" plaques were scored as definitely facilitated, because some of the "new, small" plaques might have been elevated in size from just below to just above

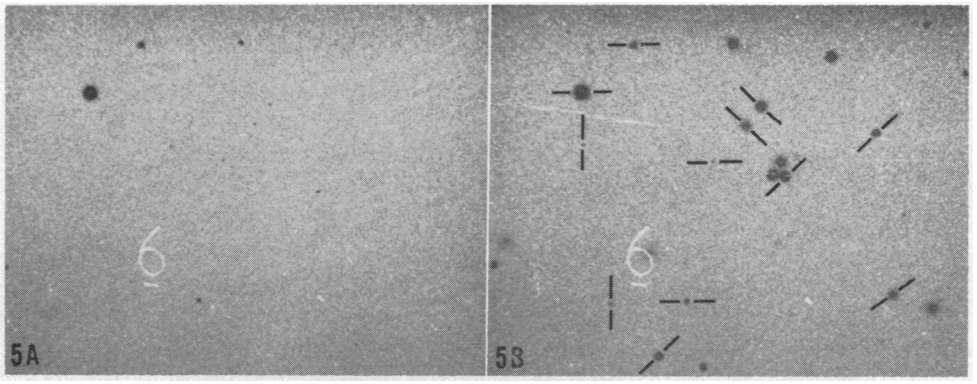

FIG. 5.—(*A*) Plaques as they appeared after the first round, with normal serum present. The cells were of *b/b* genotype. (*B*) Plaques after the second round; *Ab* allotype antiserum had caused facilitation. Examples of plaques after the second round are marked according to the classifications given in Table 1: ——, "same"; \, "larger"; |, "new, small"; /, "new, large." About 1/3 of the area of a preparation is shown. Dark field illumination from one side. Magnification 4 ×.

background grain merely by the additional action of complement (see the *N(b)*, *N(a)* control).

The facilitation effect could also be demonstrated when allotype antiserum was present during the secretory phase, in the first round (see Fig. 4). The number of plaques visible after the first round is the sum of "same" and "larger" plaques. Examination of Table 1 reveals that approximately five times as many plaques had developed after the first round, with *Ab* present, as compared to normal serum [*N(a)*]. This result introduces a new aspect to the facilitation effect. In contrast to second-round facilitation, and to the agar diffusion experiments with red cell antisera, allotype antisera present in the first round could react with red cell antibodies *before* their attachment to red cells. Nevertheless, facilitation occurred, and not neutralization. (In seven other experiments with *b/b* cells, there were, on the average, 3.8 times as many plaques present after *Ab* in the first round, as after

TABLE 1

PLAQUES AFTER THE SECOND ROUND COMPARED TO PLAQUES AFTER THE FIRST ROUND

Serum*		No. of Plaques after Second Round†				Second-round facilitation‡
First round	Second round	Same	Larger	New, small	New, large	
			b/b Cells			
N(b)	*Ab*	9	7	18	36	43
Aa	*Ab*	13	10	22	32	42
Ab	*Aa*	99	0.7	2	0	0.7
N(b)	*N(a)*	19	1	4	0.7	2
Ab	*Ab*	101	0.3	3	0.3	0.7
			a/a Cells			
N(a)	*Aa*	15	5	15	21	26
Ab	*Aa*	15	7	8	13	19§
Aa	*Ab*	56	0	0.7	0	0
N(a)	*N(b)*	22	0	5	0.3	0.3
Aa	*Aa*	67	0	2	1	1

* For abbreviations see *Materials and Methods.*

† Individual plaques after the second round were classified in comparison to their first-round image: "same," plaques unchanged, or increased in diameter by less than 2.5 ×; "larger," increase in diameter after second round 2.5 × or more; "new, small," plaques were new after second round, with a diameter of less than 0.16 mm; "new, large," new after second round, diameter 0.16 mm or larger (cf. Fig. 2). All numbers are averages of triplicate determinations.

‡ Sum of "larger" and "new, large" plaques.

§ In this case, deviation of facilitation value from sum of "larger" and "new, large" is due to averaging and rounding off to whole numbers.

normal serum.) Table 1 also shows that Aa did not cause facilitation with b/b cells, whether it was present in the first round or in the second. In fact, when it was present in the first round, instead of $N(b)$, it did essentially not affect second-round facilitation by $A(b)$: second-round facilitation in the Aa, Ab sequence was 98 per cent of that in the $N(b)$, Ab sequence. A control Ab, Ab was included, to show that facilitation in the first round was essentially complete: the repeated application of the same allotype antiserum in the second round produced negligible additional facilitation. (In another experiment, decreasing the concentration of allotype antiserum to $^1/_3$, or increasing it 3 times, did not affect the degree of facilitation.)

An analogous experiment with a/a cells is also given in Table 1. The result was the reciprocal of that with b/b cells: Aa caused facilitation, while Ab was ineffective. In contrast to the C57Bl experiment, the degree of facilitation by the active allotype serum Aa present in the second round was somewhat smaller, when the inactive Ab had been present in the first round (Ab, Aa), than after normal serum ($N(a)$, Aa). Expressed in per cent, second-round facilitation in Ab, Aa was 73 per cent of that in $N(a)$, Aa. In another experiment with C3H cells, the corresponding value was 52 per cent. This deviation from the theoretical 100 per cent is interpreted as being due to a small remnant of allotype antiserum carried over from the first round into the second, which decreases somewhat the efficiency of the counter-specific second-round allotype antiserum. The effect could be alleviated by prolonged washing after the first round, but (with the exception of one experiment with b/b cells, Table 1) could not be quite eliminated.

First-round facilitation by Aa caused an increase in the number of plaques by a factor of 3, as compared to the normal serum controls. (The corresponding factor in four other experiments was 4.5, 4.3, 3.8, and 3.6, respectively. The average of all experiments was 3.8.)

Discussion (see Fig. 1).—Why does allotype antibody not inhibit sensitization, by interacting with sensitizing antibody? A reasonable answer may come from a consideration of the topology of antibody molecules.[12, 13] Two sites are involved: one, which combines with antigen, and another, which carries the allotype structure, and can be occupied by allotype antibody. In mouse antibodies, these two sites are on different parts of the molecule. The combining sites specific for red cell antigen are located in the (electrophoretically) "slow" papain split product, while the allotype structure is in the "fast" piece.[14] Thus the allotype antibody attaches at a point too far away to inhibit, by steric hindrance, the site combining with antigen. A contrasting situation is found with rabbit allotypes A4 and A5. These structures are in the L-chains,[15] and thus located in the "slow" pieces together with the combining site for antigen. Here, allotype antibody has indeed been found to inhibit red cell sensitization.[16]

Why does allotype antibody cause facilitation? In the following, a possible mechanism will be proposed (see Fig. 1). The point of departure is the finding that γ-globulin molecules bind complement only when they are brought into an aggregated state, by immunological or physical means; dimerization may be sufficient.[17] In analogy it is proposed that antibodies on the surface of red cells bind complement and cause lysis only when they are attached in clusters, and not when they are single. Sheep red cells carry on their surface antigenic groups with different specificities (epitopes);[18] some may be in close proximity, and others widely spaced, as

has been illustrated electronmicroscopically for human red cells.[19] Antibodies against the closely spaced epitopes attach in clusters, bind complement, and cause lysis; antibodies against the widely spaced epitopes are alone and ineffective. When the allotype antibody against such "lonely" antibodies on the red cell surface is allowed to combine with them, γ-globulin aggregates form as antigen-antibody complexes. According to the proposal, the formation of such aggregates, or clusters, leads to the binding of complement, and thus to cell lysis.

According to this view, facilitation is not equally effective with different epitopes on the red cell surface. It has been shown that most[20] or almost all[21] individual cells produce antibody against only one antigen or epitope. Consequently, in the single cell facilitation experiments, individual plaques should differ from each other in their response to facilitation. This is what has been found.

Summary.— A specific interaction between mouse antibodies against sheep red cells and Ig-1 allotype antibody is described, which causes an increase in the efficiency of sensitization by sheep red cell antisera. This effect, called facilitation of hemolysis, leads also to an increase in the number and size of plaques in Jerne-type single cell assays. It can be used to determine the allotype of antibodies produced by single cells.

Valuable discussions with Drs. Jack Schultz, Thomas F. Anderson, and Ivan Jeanne Weiler and their helpful criticisms of this manuscript are gratefully acknowledged.

* This investigation was supported by U.S. Public Health Service research grants CA 04946 and CA 06927.

[1] Ehrlich, P., and J. Morgenroth, *Berlin. Klin. Wochschr.*, **36**, 481 (1899).

[2] Romeyn, J. A., and E. Onysko, *Immunology*, **7**, 217 (1964).

[3] Fahey, J. L., J. Wunderlich, and R. Mishell, *J. Exptl. Med.*, **120**, 243 (1964).

[4] Lieberman, R., and S. Dray, *J. Immunol.*, **93**, 584 (1964).

[5] Herzenberg, L. A., N. L. Warner, and L. A. Herzenberg, *J. Exptl. Med.*, **121**, 415 (1965).

[6] Jerne, N. K., A. A. Nordin, and C. Henry, in *Conference on Cell-Bound Antibodies*, NAS-NRC, ed. B. Amos and H. Koprowski (The Wistar Institute Press, 1963), p. 109.

[7] Ingraham, J. S., and A. Bussard, *J. Exptl. Med.*, **119**, 667 (1964).

[8] Weiler, E., these PROCEEDINGS, in press.

[9] Weiler, E., *Immunology*, **7**, 197 (1964).

[10] Crowle, A. J., *J. Lab. Clin. Med.*, **55**, 593 (1960).

[11] Kabat, E. A., and M. M. Mayer, *Experimental Immunochemistry* (Springfield: C C Thomas, 1964), 2nd ed., p. 149.

[12] Fleischman, J. B., R. R. Porter, and E. M. Press, *Biochem. J.*, **88**, 220 (1963).

[13] Edelman, G. M., and J. A. Gally, these PROCEEDINGS, **51**, 846 (1964).

[14] Mishell, R., and J. L. Fahey, *Science*, **143**, 1440 (1964).

[15] Kelus, A. S., *Biochem. J.*, **88**, 4P (1963).

[16] Ingraham, J. S., A. A. Biegel, and C. W. Todd, *Federation Proc.*, **24**, 180 (1965).

[17] Ishizaka, K., T. Ishizaka, and J. Banovitz, *J. Immunol.*, **94**, 824 (1965).

[18] Jerne, N. K., *Ann. Rev. Microbiol.*, **14**, 341 (1960).

[19] Lee, R. E., and J. D. Feldman, *J. Cell Biol.*, **23**, 396 (1964).

[20] Attardi, G., M. Cohn, K. Horibata, and E. S. Lennox, *J. Immunol.*, **92**, 335 (1964).

[21] Nossal, G. J. V., *Advan. Immunol.*, **2**, 163 (1962).