ROBERT A. NELSON, JR.*
DAVID S. NELSON**

Department of Microbiology, Yale University School of Medicine, and The Howard Hughes Medical Institute

ON THE MECHANISM OF IMMUNE-ADHERENCE

II. Analogy to Mixed Aggregation of Sensitized Antigens in the Presence of Complement; Immune-Adherence with Animal Platelets

The results in the preceding paper provide strong presumptive evidence that the union of primate erythrocytes (E) to particulate antigens sensitized with both antibody (Ab) and complement (C′) is not caused by simple electrostatic bonds. Further definition of the nature of the attachment has been complicated by the fact that immune-adherence (IA) is strictly dependent upon C′, which is not well defined from a physico-chemical standpoint, and upon primate erythrocytes, the surface of which represents an exceedingly complex substrate.

The need for definitive experimentation is emphasized by the reports of Lamanna and Hollander[20] and of Lamanna[19] in which a number of apparently unrelated particulate substances were purported to substitute for the primate erythrocyte as a substrate or indicator particle for combination with antigens sensitized with Ab and C′. The term serologic adhesion was employed for these various reactions. If substantiated, these observations would simplify considerably the problem of defining mechanism and would indicate that immunologically nonspecific factors were involved. Our experiences over the past four years are not in accord with this concept. Repeated assays with a wide variety of substances consistently yielded negative results.

As a result of a critical examination of our methods and of those cited in reports on serologic adhesion, we were struck by one major divergence in technique. Except in reaction mixtures containing Ab, C′, and erythrocytes from the same individual donor, i.e., an "autologous system," our custom invariably consisted of either: (a) the preliminary absorption of sera used as Ab or as C′ for 30 to 60 minutes with indicator erythrocytes or other in-

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* Associate Professor of Microbiology, Yale University School of Medicine.

** Present address: Department of Bacteriology, Medical School, University of Sydney, Sydney, Australia.

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dicator particles; or (b), a washing of the antigen-Ab-C′ complex before mixture with the indicator erythrocytes. Both procedures were designed primarily to avoid blood group antibodies which might react with the indicator erythrocytes, particularly when serum from a heterologous species was employed either as Ab or as C′. Since these precautions were not cited by earlier workers or by Lamanna we developed the hypothesis that certain of their results might be a manifestation of a mixed aggregation of two sensitized particulate antigens joined together by C′. Such a hypothesis would necessitate two assumptions. First, the source of the second Ab, i.e., Ab capable of sensitizing the indicator particle, was presumed to be natural Ab either in the antiserum to the antigen being tested or in fresh serum used as C′. Second, it was postulated that C′ or some component of C′ was polyvalent, i.e., a single molecule or group of molecules could simultaneously attach to sensitized sites on two separate antigens, perhaps in a manner analagous to that involved in systems wherein C′ has been found to influence precipitin reactions.[21, 23, 24]

The experiments cited herein provide initial evidence to support this hypothesis. They clearly demonstrate mixed aggregation which occurs only in the presence of Ab reactive with the indicator particle and in the presence of C′. Similarities of this phenomenon to immune-adherence and to conglutination are cited below. In addition, data are presented which suggest that platelets from certain animals react in immune-adherence similarly to human erythrocytes. As mentioned above, our original studies[28] demonstrated only that human platelets do not substitute for human erythrocytes.

EXPERIMENTAL RESULTS

A. COMPLEMENT AND MIXED AGGREGATION

1. *The requirement for antibody to sheep erythrocytes for reactivity either with particles of an immune precipitate or with sensitized* T. PALLIDUM. Exper. 050457 and 050857. A precipitate of bovine serum albumin and rabbit Ab formed in the equivalence zone was used. The precipitate was washed and resuspended in veronal buffer containing Ca^{++} and Mg^{++} (VB^{++}) so that 1 ml. of the suspension contained about 8.8μg. Ab nitrogen. A 5 per cent suspension of sheep erythrocytes (E) was prepared in VB^{++} to give an O.D. of .680 when diluted 1/15 with water. A portion was diluted ¼ in VB^{++}. A second portion was sensitized with a quantity of rabbit hemolysin estimated to yield about 1000 Ab molecules per cell, washed, and resuspended in VB^{++} to the original concentration, then also diluted ¼ in VB^{++}. Separate samples of both the sensitized (EA) and the unsensitized (E) cells were centrifuged, and the packed cells were lysed by

the addition of distilled water. The stromata were brought back to the original concentration by the addition of a volume of 1.7 per cent saline equal to that of the distilled water used for lysis. These suspensions were also diluted ¼ in VB++. Two aliquots of a pool of guinea pig C′ were used, the first was untreated, and the second was absorbed twice at 0° C. with packed sheep red cells in order to remove the natural sheep hemolysin. The

TABLE 1. THE ATTACHMENT OF PARTICLES OF AN IMMUNE PRECIPITATE (SERUM ALBUMIN-ANTI-ALBUMIN) TO SENSITIZED SHEEP ERYTHROCYTES (EA) IN THE PRESENCE OF DILUTIONS OF FRESH GUINEA PIG SERUM USED AS C′

Assay 1	PERCENTAGE OF PARTICLES ADHERENT *C′ dilutions—unabsorbed serum*						
Indicator particle	*1/1*	*1/2*	*1/4*	*1/8*	*None*	*1/1 heated*	*1/1 with EDTA*
E	36**	34**	4**	0**	0	0	0
EA	84*	84*	20*	6*	2	0	0
E, lysed	36	22	4	0	0	0	0
EA, lysed	88	80	36	0	0	0	0
Human RBC	..	..	..	82	0	0	0

Assay 2	PERCENTAGE OF PARTICLES ADHERENT *C′ dilutions—absorbed serum*					
Indicator particle	*1/1*	*1/2*	*1/4*	*1/8*	*1/16*	*None*
E	0	0	0	0	0	0
EA	74*	98*	36*	10*	4*	0
E, lysed	4	0	0	0	0	0
EA, lysed	96	92	42	14	0	0
Human RBC	..	..	..	..	82	0

* Complete lysis of Ab-sensitized cells by C′.
** Complete or partial lysis by "natural" Ab in unabsorbed serum used as C′.

C′ titers were respectively 208 and 186 $C'H_{50}$ units per ml. The reaction mixtures shown in Table 1 consisted of 0.5 ml. of immune precipitate; 0.5 ml. of C′ dilutions in VB++; and, 0.5 ml. of sheep erythrocytes, i.e., intact or lysed E or EA. Controls were prepared containing heated guinea pig serum and unheated serum plus .01 *M* EDTA. Another set of controls contained 0.1 ml. of washed human erythrocytes in place of the sheep cells. The mixtures were shaken periodically by hand during incubation at 37° C. for 45 minutes. They were then examined by darkfield microscopy and the percentage of precipitate-particles adherent to erythrocytes was counted.

From the results in Table 1 it can be seen: (a) that the adherence of precipitate-particles to sheep cells occurred only in the presence of Ab to the erythrocytes. From the results with unabsorbed versus absorbed C′ it was obvious that the natural blood group Ab in normal guinea pig serum served as a sensitizing substance for the sheep E. The C′ in the mixtures containing EA caused lysis of the sheep cells so that stromata really acted as the indicator particles. In contrast with reactivity in IA with the human erythrocytes, but, for as yet undetermined reasons, only low dilutions of serum as C′ were effective. In contrast to the report of Lamanna and Hol-

TABLE 2. PERCENTAGE OF *T. pallida* ATTACHED TO SHEEP ERYTHROCYTES IN THE PRESENCE OF UNTREATED GUINEA PIG SERUM AND OF GUINEA PIG SERUM ABSORBED WITH SHEEP ERYTHROCYTES

Guinea pig C′	*Human syphilis serum* as Ab (Absorbed with sheep E)*	
	None	*0.1 ml.*
Unabsorbed	2%	66%
Absorbed	0%	0%
Absorbed, plus hemolysin	1%	70%

* The serum was from a patient with latent syphilis diagnosed on the basis of clinical history and a positive treponemal immobilization test.

lander[20] it should be noted that no difference in reactivity occurred between intact and lysed erythrocytes nor between intact and lysed sensitized erythrocytes.

Exper. 060457. As outlined in Table 2, reaction mixtures were prepared containing 0.2 ml. of a suspension of *T. pallidum* in an albumin-phosphate medium; 0.1 ml. of heated serum from a patient with latent syphilis; 0.1 ml. of standardized sheep E; 0.1 ml. of rabbit hemolysin diluted 1/800; and, 0.25 ml. of guinea pig C′. The human syphilis serum was absorbed twice at 0° C. with sheep erythrocytes in order to remove blood group antibody.

After incubation at 37° C. for 60 minutes samples of the various mixtures were examined by darkfield microscopy. Significant adherence of treponemes to the sheep erythrocytes occurred only in the presence of syphilis serum and C′. However, and more significant, no adherence occurred in the mixtures containing about 46 $C'H_{50}$ units of guinea pig C′ which had been absorbed with sheep erythrocytes. Definitive evidence that Ab to sheep erythrocytes is of prime importance in this type of adherence was supplied by the restoration of reactivity upon the addition of rabbit hemolysin to mixtures containing absorbed C′.

2. *The requirement for C′ in mixed aggregation of sensitized* S. TYPHOSA *and sensitized sheep erythrocytes.* Exper. 060557. A suspension of *S. typhosa* 0901 was killed by heating at 65° for 30 minutes, washed 3 times, and resuspended in SAVB++. The suspension was adjusted to an O.D. of .260 at 400 mμ. on the Beckman DU spectrophotometer and then diluted 1/10 for assay. A rabbit antiserum against *S. typhosa* (agglutination titer: 12,800 units per ml.) was diluted 1/1000 in SAVB++. Sheep erythrocytes were prepared as above and one portion was sensitized with an equal

TABLE 3. THE REQUIREMENT FOR COMPLEMENT IN MIXED AGGREGATION OF SENSITIZED SHEEP ERYTHROCYTES (EA) WITH SENSITIZED *S. typhosa*

Erythrocytes	*Guinea pig serum absorbed twice with erythrocytes (E)*	*Percentage of* S. typhosa *adherent to erythrocytes with dilutions of guinea pig serum*						
		1/1	*1/2*	*1/4*	*1/8*	*1/16*	*1/32*	*None*
Intact EA	Untreated*	80	86	84	46	10	0	0
Intact EA	Untreated, plus EDTA†	0	0	0	0	0	0	0
Intact EA	Decomplemented	0	0	0	0	0	0	0
Lysed EA	Untreated, plus EDTA†	0	0	0	0	0	0	0
Intact E	Untreated‡	0	0	0	0	0	0	0
Lysed E	Untreated	0	0	0	0	0	0	0

* Complete lysis of the sensitized erythrocytes occurred in all mixtures containing dilutions of C′.
† No lysis of EA occurred in the presence of EDTA.
‡ No lysis of E occurred in any reaction mixture.

portion of rabbit hemolysin diluted 1/800. A sample of the nonsensitized cells was mixed with distilled water so that about 90 per cent of the cells were lysed. Guinea pig C′ which had been absorbed twice with sheep erythrocytes as above was diluted in SAVB++. Dilutions were also prepared of one sample of C′ which had been "decomplemented" by treatment at 0°C. for 24 hours with the washed precipitate from a mixture of bovine serum albumin and rabbit antibody. This treated serum was not reactive in the usual hemolytic assay.

Reaction mixtures containing 0.2 ml. bacteria; 0.2 ml. of rabbit antityphoid serum; 0.1 ml. sheep E; 0.1 ml. rabbit hemolysin; and 0.2 ml. of C′ dilutions were prepared as in Table 3. By darkfield microscopy the percentage of bacteria adherent to erythrocytes was measured after incubation for 60 minutes at 37° C.

The identification of C′ as an essential substance for mixed aggregation in these reaction mixtures was strongly implicated. No mixed aggregation

occurred with the guinea pig serum which was "decomplemented" by treatment with an immune precipitate. Similarly no aggregation occurred in the mixtures containing .001 *M* EDTA which binds Ca^{++} and Mg^{++} and hence inhibits $C'_{1,4}$ and C'_2.

Furthermore, no attachment of sensitized bacteria to either intact or lysed sheep erythrocytes occurred in the presence of guinea pig C′ which had been absorbed to remove naturally occurring agglutinins for sheep erythrocytes. This result agrees with the preceding observations with washed immune precipitates and with *T. pallidum* (Tables 1 and 2), and with our previously published results.

3. *A role of auto-antibody in IA.* In view of the fact that mixed aggregation of two sensitized antigens requires C′, we have considered the possibility that immune-adherence could be explained on the assumption that human erythrocytes have small amounts of auto-antibody on their surface. Attempts to identify Ab on the surface of human red cells as the "receptor site" have been unsuccessful to date.

First, attempts were made to dissociate the hypothetical Ab. Six-tenths ml. of packed normal human erythrocytes was washed three times with saline and resuspended in 30 ml. of distilled water. Two portions of 15 ml. were washed twice with distilled water. After high-speed centrifugation, the deposit from one batch was resuspended with 10 ml. of 15 per cent NaCl. The deposit from the second batch was resuspended with 10 ml. of acetate buffer, pH 3.0. Both samples were incubated for 60 minutes at 37°, washed, and brought to 10 ml. with $SAVB^{++}$. These preparations were divided and mixed with a sample of washed presensitized yeast cells and with a sample of yeast cells mixed with serum as Ab and C′. While readings were difficult because the stromata were badly distorted, about 40 per cent of red cells pretreated either with hypertonic NaCl or at low pH, were observed to be attached to the sensitized yeast. No difference was noted between the washed and unwashed antigen.

Second, in view of the possibility that auto-antibody might develop late in humans, erythrocytes from young children were examined. Blood was collected in modified Alsever's solution from 34 children between the ages of 8 days and 10 years. Greater than 78 per cent of the washed erythrocytes from each individual reacted in immune-adherence with washed typhoid bacilli presensitized with Ab and C′.

B. THE REACTIVITY OF NONPRIMATE PLATELETS IN IMMUNE-ADHERENCE

Exper. 071157. The reactivity of platelets as indicator particles in IA was examined using sensitized sheep erythrocytes as the particulate antigen. Platelets were isolated from fresh blood of human beings and guinea

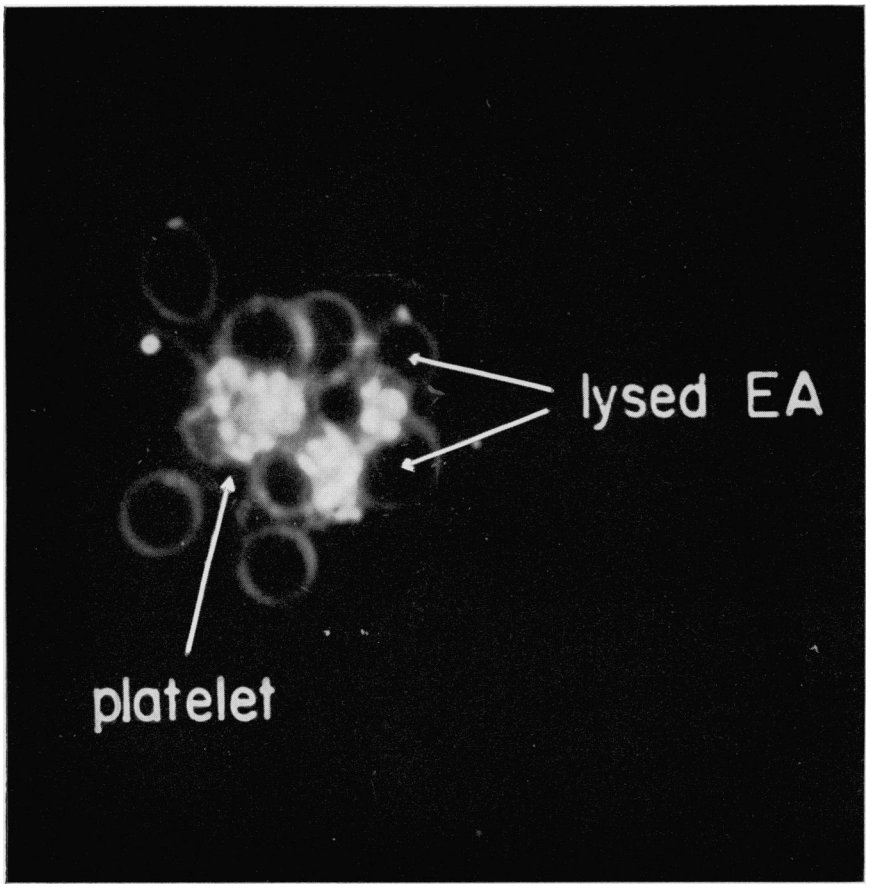

FIG. 1. Photomicrograph of immune-adherence between sensitized sheep erythrocytes (EA) and washed guinea pig platelets. The sheep erythrocytes are lysed due to reactivity with C′. Three platelets are visible in the photograph.

pigs. Five ml. of whole blood were mixed respectively with 5 ml. of cold Alsever's solution, 5 ml. of cold .006 *M* EDTA in veronal buffer, and with approximately 2 ml. of washed resin (IRC-50 in the Na^+cycle). All mixtures were centrifuged at 0° C. for 30 minutes at 600 rpm. The supernatant fluids contained platelets and plasma were diluted with 5 ml. of Alsever, EDTA, and veronal respectively and were washed three times by centrifugation at 2000 rpm for 20 minutes. The final deposit was re-

TABLE 4. ADHERENCE OF GUINEA PIG PLATELETS TO SENSITIZED SHEEP ERYTHROCYTES IN THE PRESENCE OF DILUTIONS OF FRESH HUMAN AND GUINEA PIG SERUM AS C'

Platelets	*Method of isolation*	*Serum used as C'**	*Percentage of platelets adherent with dilutions of C'*				
			1/1	*1/4*	*1/16*	*1/64*	*None*
Human	Alsevers	Human	0	0	0	0	0
	.003 *M* EDTA	Human	0	0	0	0	0
	IRC-50 resin	Human	0	0	0	0	0
Human	Alsevers	Guinea pig	0	0	0	0	0
	.003 *M* EDTA	Guinea pig	0	0	0	0	0
Guinea pig	Alsevers	Human	20†	58	92	86‡	0
	Alsevers	Guinea pig	95	95	95	95	0

* Complete lysis of EA with human C' 1/1 and 1/4; partial lysis with 1/16. Complete lysis of EA with guinea pig C' 1/1, 1/4, 1/16; partial lysis with 1/64.

† Distortion of platelets was noted microscopically.

‡ Only about 5 per cent of EA was lysed with 1/64 human C'. The majority of platelets were attached to intact erythrocytes indicating that lysis is not a prerequisite for adherence.

suspended in $SAVB^{++}$ and centrifuged at 400 rpm for 5 minutes to remove the few erythrocytes and the rare clumps of platelets. The supernatant fluids containing only platelets were standardized to 50 per cent transmittance on the Beckman DU spectrophotometer at a wave length of 400 mμ. Reaction mixtures were prepared as follows: 0.2 ml. of $SAVB^{++}$; 0.1 ml. of EA washed and standardized as usual to an O.D. of .340 at 541 mμ; 0.2 ml. of standardized platelets in $SAVB^{++}$; and, 0.1 ml. of dilutions of fresh autologous serum as C'. The latter serums were obtained from clotted blood drawn at the same time as the blood used to isolate platelets.

All mixtures were shaken intermittently during incubation at 37° C. for 45 to 60 minutes. Samples were withdrawn and examined by darkfield

microscopy for the percentage of platelets attached to the sensitized particulate antigen EA. The lysis of EA by C′ was recorded.

Certain typical results are shown in Table 4, and an example of adherence of platelets and EA is shown in Figure 1. In the presence of fresh serum, the sensitized particulate antigen, EA, became attached to about 95 per cent of the guinea pig platelets. No adherence of human platelets occurred despite the proved combination of the human C′ with the particulate antigen as measured by lysis of the EA. Nor did adherence occur with human platelets in the presence of guinea pig C′. An inhibition of reactivity of guinea pig platelets occurred with low dilutions of human serum. The mechanism of this inhibition is not clear.

Because of the striking difference of reactivity of guinea pig and human platelets this type of assay has been repeated. To date, platelets have been isolated as outlined above from 25 human beings. In reaction mixtures containing either human C′ or guinea pig C′ all samples were nonreactive with sensitized sheep erythrocytes, sensitized starch granules, and sensitized typhoid bacilli. This lack of reactivity agrees with our original observation that human platelets do not react in IA, and stands in contrast with a recent report that human platelets react equally well with sensitized *T. pallidum* in what was termed serologic adhesion.[19,20] Of greater interest, however, are the results with guinea pig platelets. The reaction between EA and guinea pig platelets is particularly striking even with 0.1 ml. of C′ diluted 1/64, which is about the same level of reactivity of C′ in immune-adherence involving human erythrocytes as indicator particles. In order to classify this reaction as IA or C′-dependent mixed aggregation and to exclude a possible role of blood-coagulation factors in this reaction, additional experiments were performed.

1. Platelets from 12 monkeys *(M. rhesus)* failed to react with a variety of sensitized antigens in the presence of either autologous serum or pooled homologous serum as C′. Erythrocytes from all 12 monkeys reacted in IA with sensitized typhoid bacilli with autologous serum as C′.

2. Rabbit platelets reacted with EA as the particulate antigen in the presence of autologous C′.

3. Guinea pig platelets were incubated with autologous serum for 20 minutes at 37°, washed, and then mixed with EA. No mixed aggregations resulted.

4. Suspensions of rice starch and of *S. typhosa* were sensitized with Ab only and with Ab and C′. The suspensions were washed three times in SAVB++ and samples were mixed with washed human erythrocytes and washed guinea pig platelets. Approximately 70 per cent of both the starch granules and the typhoid bacilli which had been sensitized with Ab and

C′ became attached to the human erythrocytes and to the guinea pig platelets. No attachment to the indicator particles occurred with untreated starch or typhoid bacilli, or with these antigens when sensitized only with Ab. Since no free serum was present in the reaction mixtures containing the sensitized and washed antigens and the platelets, these results are interpreted as being due to immune-adherence, and not as a reflection of some factor involved in the blood coagulation system, or of some factor liberated in serum as a result of reaction of the antigen-antibody complex with C′.

5. The products of lysis of sheep E and EA by water did not induce adherence of EA with guinea pig platelets in mixtures lacking C′.

6. The adherence of platelets to some unlysed EA at high C′ dilutions (Table 4) was confirmed in two additional trials.

7. Recent assays performed in collaboration with Dr. Maria Siqueira have demonstrated that the soluble O antigen of *S. typhosa,* anti-typhoid serum, and guinea pig C′ induce striking agglutination of guinea pig platelets. This agglutination response appears to be analogous to hemagglutination of human erythrocytes caused by IA with either particulate or soluble antigens.[20] No agglutination has occurred in reaction mixtures containing human platelets.

DISCUSSION

The results obtained herein are interpreted to mean that mixed aggregation of two sensitized particulate antigens may occur in mixtures containing complement. At least three factors of major consequence have evolved.

First, insight has developed into the mechanism of serologic adhesion as reviewed by Lamanna.[19] It seems likely that certain of the experiments described by this investigator were misinterpreted as immune-adherence due to an oversight of natural antibody to the "indicator particle" in the serum used as a source of C′ or in the serum used as Ab to *T. pallidum.* The presence of natural Ab capable of inducing immune-hemolysis in the presence of C′ would of course explain the peculiar circumstance wherein only lysed animal erythrocytes were stated to be reactive in adhesion with sensitized *T. pallidum.* Therefore, we are left with the conclusion that C′-dependent mixed aggregation and immune-adherence are probably separate phenomena and that both are different from acid-adhesion as outlined in the preceding paper. The differentiation of IA from mixed aggregation is not definitive, however, in that the bond in IA remains obscure. It is theoretically possible that primate erythrocytes are sensitized *in vivo* with blood group Ab, which is not removed by repeated washing *in vitro.*

Second, platelets from certain animal species react with sensitized particulate antigens in the presence of C'. Because of the similarities of this reactivity of nonprimate platelets to immune-adherence with primate erythrocytes, it is postulated that the two reactions represent similar mechanisms, and, for the present, both are termed immune-adherence. It is of considerable interest that a sharp differentiation in behavior of erythrocytes versus platelets occurs in different animal species. Primate platelets have been consistently nonreactive while among erythrocytes, those from primates have been the only reactors of any significance. It seems clear that immune-adherence with human erythrocytes as here defined is identical with the

TABLE 5. SCHEMATIC REPRESENTATION OF THE BOND INVOLVED IN C'-DEPENDENT MIXED AGGREGATION

$$G_1 \cdot A_1 - C' - A_2 : G_2$$
or
$$G_1 \cdot A_1 \cdot C' - Cg - C' \cdot A_2 \cdot G_2$$

$G_1 \cdot A_1$ represents a particulate antigen (G) sensitized with its specific antibody (A).
$G_2 : A_2$ represents a second particulate antigen, e.g. the indicator particle, sensitized with its specific antibody or with natural antibody.
Cg conglutinin

"red cell adhesion" described by Duke and Wallace[10] in 1930. Similarly, certain examples of the Rieckenberg adhesion phenomenon between sensitized leptospira or trypanosomes and rat platelets may well represent IA, but there is serious doubt that adhesion to other substrates such as lysed non-primate erythrocytes, primate platelets, bacteria, etc., represents the same phenomenon. Instead, our results would suggest that reactions with the latter substances are due either to C'-dependent mixed aggregation, or under certain circumstances, to acid-adhesion.

Third, the necessity for a clear interpretation of the mechanism of C'-dependent mixed aggregation is emphasized by the possible role of conglutinin in the experiments herein. Conflicting concepts of conglutinin have been offered by different investigators,[12, 14, 40] but perhaps the most comprehensive description of this substance and its role in immunological phenomena may be found in the reports of Coombs and associates.[1, 2, 3, 14] Using the terminology of the latter, it is conceivable that conglutinin is the agent in guinea pig serum which causes mixed aggregation in the presence of C' by a mechanism schematically outlined in Table 5.

Regardless of the interpretation as to mechanism it seems obvious that mixed aggregation might well serve as a method of detection of Ab to a

particular microbe or tissue particle. However, it seems equally obvious that precautions should be taken to use optimally sensitized "indicator particles" rather than to depend upon often variable (and occasionally unrecognized!) natural Ab in the serum used as C′. There are, however, certain theoretical disadvantages, particularly in the fact that only low dilutions of guinea pig or human serum suffice as a source of C′ or of conglutinin.

Considerable emphasis is given to the necessity for additional definitive experimentation upon the substances responsible for the reactivity of normal sera with a variety of bacteria and other particulate antigens. As outlined herein the responsible factors are serum proteins which require complement for their reactivity. Hence they are termed "natural" antibody. In addition to their role in the above experiments their potential importance in other biological systems has been indicated in parallel experiments[27] which have resulted in the concept that the phenomena ascribed to properdin in normal serum are not due to a single entity but instead are caused by natural antibody acting in conjunction with three of the components of complement, i.e., C'_1, C'_4, and C'_2. While there is ample evidence that some natural antibodies show rather broad cross-reactivity, associated experiments in this laboratory have demonstrated an immunological specificity of the natural antibodies involved in neutralization of different bacteriophages (T series) of *E. coli*,[4] and of the natural antibodies which react with starch granules and with zymosan (yeast cell wall) particles.[41]

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