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

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

ON THE MECHANISM OF IMMUNE-ADHERENCE

I. Differentiation from Acid-Adhesion of Bacteria to Erythrocytes

INTRODUCTION

Immune-adherence (IA) has been described as a specific immunological reaction wherein microorganisms or other particulate antigens sensitized with antibody (Ab) and complement (C′) become attached to the surface of human or monkey erythrocytes.[26, 28] Certain practical advantages of IA noted in recent studies have emphasized the necessity for an understanding of the fundamental nature of the reaction. For example, the marked sensitivity of IA permits the detection of Ab concentrations as low as .01 to .005 μg. Ab nitrogen (N) per ml. of serum[30]; a wide range of bacteria,[15-18, 29] viruses,[20, 26] and soluble antigens,[38] react in IA; and some applications of IA as a diagnostic test for syphilis[5, 6, 28] have been reported. The technique for measuring IA has been simplified considerably by the development of a hemagglutination assay.[30] Also, there is evidence that the attachment of bacteria to erythrocytes enhances phagocytosis of the microbes *in vitro*. This latter observation, coupled with the demonstration that IA occurs *in vivo*, has suggested a possible role of erythrocytes in defense of the host against infectious agents.[26] A final feature of some practical importance concerns the observation that C′ in human serum is as active as guinea-pig C′, e.g., 1 ml. of normal human serum diluted to 1/400 ± 10 per cent provides adequate C′ for IA with a variety of antigens. Reproducible reactivity at such high serum dilutions provides a latitude for measurement of decline in disease states which often was difficult to detect by the classical hemolytic assay using sheep erythrocytes and rabbit antiserum wherein C′ reactivity was lost at dilutions of human serum exceeding 1/40 or 1/60. The use of IA in studies of human C′ has demonstrated a definitive requirement for both Ca^{++} and Mg^{++}.[41]

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* Present address: Department of Bacteriology, Medical School, University of Sydney, Sydney, Australia.
** Associate Professor of Microbiology, Yale University School of Medicine.
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These observations have stimulated our efforts to comprehend the quantitative aspects of IA and in particular the nature of the bond established between the sensitized particulate antigen and the normal erythrocyte. The latter has proved elusive to date but during the course of our investigation conditions have been defined under which two different reactions occur which mimic IA in the final result. These two phenomena tentatively have been termed "acid-adhesion" and "C'-dependent mixed aggregation" and are described in this and the following paper, respectively. With attention to detail, it is simple to differentiate these three reactions, all of which are influenced by both Ab and C'. The importance of such differentiation is emphasized by the recent report of Lamanna and Hollander[20] which casts misleading doubts on the specificity of the primate erythrocyte in IA. Further, an extensive review of the literature on "serological adhesion" by Lamanna[19] demonstrates the confused and often contradictory information which exists in this potentially useful immunological area.

MATERIALS AND METHODS

Bacteria. Various organisms were harvested from broth cultures after 6 to 8 hours' incubation at 37° C. The bacteria were killed either by heat (65° C. for 20 min.) or by formalin added to a final concentration of 0.5 per cent, or by both methods. They were washed twice in sterile saline which had been boiled to remove dissolved CO_2 or in saline containing 0.5 per cent formalin, and stored as a concentrated suspension in the same fluid in a refrigerator at 0-4° C. (hereafter referred to as 2° C.). Assays for IA and for acid-adhesion gave quantitatively similar results with washed living cultures and with heat- or formalin-killed cultures.

(a) *Diplococcus pneumoniae,* Types III, VIII, XIII, and XXXI (hereafter referred to as Pn III, Pn VIII, etc.) were passed through mice and grown in brain heart infusion broth containing 0.5 per cent glucose, 0.1 per cent $NaHCO_3$, and 20 mg. per cent glutamine.

(b) *Escherichia coli,* strain K12, and *Micrococcus pyogenes,* var. albus, were grown in beef heart infusion broth supplemented with .002 per cent para-amino-benzoic acid.

(c) *Salmonella typhosa,* strain 0901, was grown on Difco nutrient agar in Blake bottles. The bacteria were washed off the surface of the agar with sterile saline and the resulting suspension killed, washed, and resuspended in sterile saline.

Rickettsia. R. burneti (egg-adapted, American 9 Mile strain) generously was supplied by Dr. Herald Cox of Lederle Laboratories. This concentrated suspension was diluted 1/200 and washed in SAVB++ by high speed centrifugation.

Spirochetes. Four batches of *Treponema pallidum* were isolated from testicular syphilomata of rabbits at a maximum of 8 days after inoculation. Three batches were from rabbits which had been exposed to 600 r total body X-irradiation before inoculation. X-ray treatment was given to inhibit Ab formation and so avoid the risk of isolating organisms already sensitized with Ab *in vivo.* These treponemal suspensions were generously supplied by Sylvana Chemical Co., Orange, New Jersey.

Starch granules. Suspensions of washed granules of rice starch (Fisher) and of *Amaranthus cruentus* starch (U. S. Department of Agriculture, Peoria, Illinois) were prepared at a concentration of 20 mg. per ml. in sterile saline and stored at 2° C. These varieties were selected because of their small and uniform sizes (average diameters 5 mμ. and 1 mμ. respectively) and because it has been postulated that Ab in normal serum will sensitize the granules so that they react in a variety of immunological phenomena.[7, 29, 33]

Antibody. (a) Antisera to Pn XIII, Pn XXXI, and *S. typhosa* were prepared by injection of formalinized intact bacteria into rabbits. Pooled sera were heated at 56° C. for 60 min. and stored at —38° C. The Ab N contents of these sera were determined by micro-Kjeldahl analysis of the precipitates from mixtures of the soluble polysaccharides with the anti-pneumococcal sera and of the agglutinated bacteria from mixtures of *S. typhosa* with its antiserum.

(b) Human syphilitic sera were obtained through the courtesy of Dr. C. R. Rein. These were from patients with proved syphilis and had been found reactive in the treponemal immobilization test.

(c) In several instances sera from adult normal individuals were used as a source of "natural" Ab. Because heating often resulted in a decrease of Ab activity, these sera could not be heated to provide antibody free from C′. Therefore, when indicated, the particulate antigen was treated with fresh serum for a short time, usually 30 min., at exactly 0° C. in the presence of 0.01 *M* EDTA (trisodium ethylene diamine tetra-acetate) and then washed in buffer before assay. We have been unable to demonstrate any significant uptake of C′ on the resultant antigen-antibody complex under these circumstances.

Throughout this work it has been necessary to avoid marked agglutination of bacteria. This was usually done by using dilute suspensions of antigens and relatively high dilutions of antisera. The IA_{50} dose of Ab was determined as the amount of antiserum required to induce specific adherence of 50 per cent of a standardized suspension of bacteria to human E in the presence of "excess" C′ as outlined below. For optimal sensitization, 3 IA_{50} doses of Ab were used. With bacteria this usually represented a one plus or a trace agglutinating dose.

Complement. Pooled normal guinea pig serum (Carworth Farms) was used as a source of C′. The hemolytic titer was between 200 and 210 $C'H_{50}$ units per ml. Normal human sera also were used as C′, as indicated in specific protocols. Sera used as sources of C′ were stored in an electric refrigerator at —38° C. and handled at 0° C. after thawing.

The $C'IA_{50}$ titer was determined as that amount of serum which induced specific adherence of 50 per cent of a standardized suspension of bacteria or starch granules to human red cells when the antigens were sensitized with about 3 IA_{50} doses of Ab. Under these circumstances both human and guinea pig were ordinarily reactive at dilutions of approximately 1/400 and 1/300, respectively. In assays in which "excess" C′ was desired for sensitization of the antigen-Ab complex, we generally employed 1 ml. of a 1/20 or 1/40 dilution of serum or its equvialent, i.e., 10 to 20 $C'IA_{50}$ doses.

Erythrocytes (E). Human, sheep, rabbit, guinea pig, and chicken cells were used. Blood was drawn aseptically from either a vein (human, sheep, chicken) or the heart (rabbit, guinea pig) and stored in modified Alsever's solution at 2° C. Prolonged storage (about one month) did not influence the reactivity of the cells in either IA or

acid-adhesion. Before use, the cells were washed three times and made up to a 2 per cent suspension either in saline or in an appropriate buffer; the buffy coat was removed after each centrifugation. For IA the suspension was adjusted so that exactly 1 ml. diluted to 10 ml. with water gave an optical density (O.D.) of .395 when examined at a wave length of 541 on the Beckman DU spectrophotometer. In experiments on acid-adhesion, chicken cells were used most frequently, since they were less subject to gross distortion at low pH than were the E of other species.

Buffers. A stock of 5 times isotonic veronal buffer was prepared by dissolving 4.6 gm. 5,5 diethyl barbituric acid in 500 ml. of hot distilled water and then adding this solution to a second solution containing 83.8 gm. sodium chloride, 2.52 gm. sodium bicarbonate, and 3 gm. 5,5 diethyl barbital. After cooling to room temperature, the total volume was adjusted to 2,000 ml. with distilled water. For sensitization of antigens and for IA, isotonic veronal buffer (pH 7.3 to 7.6) containing 0.0005 *M* Mg++, 0.00015 *M* Ca++, and 0.1 per cent bovine serum albumin was used (SAVB++). In some instances, the albumin was omitted (VB++). To cover a wide range of pH values a glycine-acetate-phosphate buffer series was used.[31]

Sensitization of organisms. The stock suspension of bacteria or starch granules was centrifuged, washed, and resuspended in SAVB++ to a suitable concentration. Dilutions of Ab and of C' were prepared in SAVB++. Equal volumes of organisms, Ab, and C' were incubated at 37° C. for 60 min. If the volume of the reaction mixture was less than 1 ml., this was done in a water bath with periodic shaking by hand. Larger volumes were incubated in a 37°-oven with continuous mechanical rotation (about 6 rpm). After incubation the mixtures were diluted in ice-cold saline, centrifuged at 0° C., washed, and resuspended to their original concentration in either SAVB++ (for IA) or saline (for acid-adhesion). The washing removed traces of serum proteins which might interfere with the buffer system at low pH. The removal of natural hemagglutinins from guinea pig serum was essential when IA assays were made by the hemagglutination pattern technique.[30] With nearly all the bacteria some slight to moderate clumping occurred as a result of sensitization with Ab and rapid centrifugation.

Reaction of antigens and indicator particles. (a) Immune-adherence: 0.5 ml. of SAVB++, 0.1 ml. of spectrophotometrically standardized human E, and 0.1 ml. of washed sensitized particulate antigen were incubated at 37° C. for 30 to 40 min. Unsensitized particles and particles sensitized with Ab only were included as controls in all cases. The mixtures were shaken periodically in the first 10 min., and then the erythrocytes were permitted to settle at 37° C. Adherence was detected either by the production of a hemagglutination pattern, or by counting the percentage of particles adherent to E by means of darkfield microscopic examination. At least 50 organisms were counted.

(b) Acid-adhesion: 0.5 ml. of glycine-acetate buffer and 0.1 ml. of a suspension of the indicator particle were incubated at 37° C. for 10 to 20 min. Erythrocytes and platelets of some species agglutinated at low pH and if antigens were added immediately, nonspecific trapping in these clumps occurred. It was also found that the change in red cells, induced by acid, took an appreciable time to occur and was most rapid at 37° C. After this pre-incubation 0.1 ml. of antigen was added and the mixture incubated at 37° C. or at room temperature for 30 min. The percentage of antigen particles adherent was determined by darkfield microscopic examination. In all experiments in which the

percentage of antigen particles adherent was counted, erythrocytes or other indicator particles were present in excess.

Precision of counting. When duplicate counts of the percentage of adherent particles were made on several samples of the same reaction mixture by two observers, the error was found to be ± 6 per cent.

Alsever's solution (modified). Distilled water was added to 2.05 gm. glucose, 0.8 gm. trisodium citrate, and 0.42 gm. sodium chloride, to make a total volume of 100 ml. The pH of the solution was adjusted to 6.1 with 5 per cent citric acid.

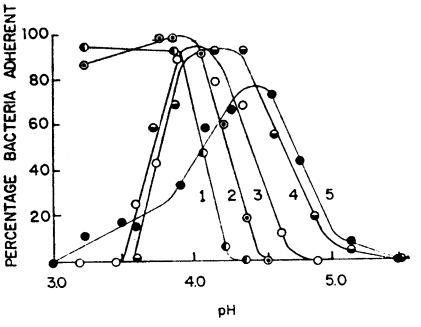

FIG. 1. The percentage of bacteria attached to chicken erythrocytes at varying pH.

Curve	*Bacteria*
1	Pneumococcus, type XIII
2	Pneumococcus, type VIII
3	Pneumococcus, type III
4	*M. albus*
5	*E. coli*

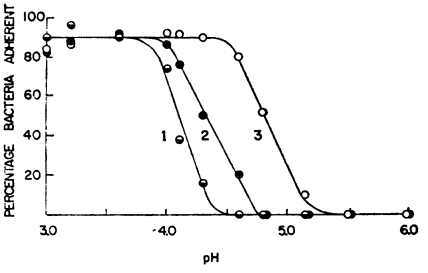

FIG. 2. The adhesion of *D. pneumoniae*, type XXXI to chicken erythrocytes at varying pH.

Curve	*Treatment of pneumococci*
1	None
2	Antibody only
3	Antibody and complement

EXPERIMENTAL RESULTS

A. ATTACHMENT OF BACTERIA TO INDICATOR PARTICLES AT LOW pH

1. *Adhesion of unsensitized antigens to chicken E at low pH.* Saline suspensions of unsensitized antigens were incubated with chicken red cells as described above. The following bacteria were used: Pn III, Pn VIII, Pn XIII, Pn XXXI, *E. coli, M. albus,* and *S. typhosa.*

The percentage of organisms adherent is plotted against pH in Figure 1. Pn XXXI showed essentially the same type curve as Pn XIII and is not plotted since its characteristics are equally well shown in Figure 2. *S. typhosa* did not adhere over the pH range tested. No adherence was seen at any time with any bacteria above pH 5.5. Higher pH values are therefore omitted from all graphs. It is clear that the curves differ markedly from one

another and that for each of the bacteria tested the curve was fairly characteristic. With all the bacteria maximum adherence occurred at or below pH 4.5. The behavior below this point varied.

Below pH 5.5 certain changes were seen in the red cells. These were: (a) lysis, (b) spontaneous agglutination, and (c) a change in the color of the suspension from red to brown, probably indicating the formation of acid hematin.

2. *Adhesion of sensitized antigens to chicken red cells at low pH.* Exper. 041657: Washed suspensions of Pn XXXI (unsensitized, sensitized with rabbit Ab, and sensitized with both Ab and guinea pig C′) were incubated with chicken E at various pH's (Fig. 2). Sensitization with Ab alone shifted the curve significantly to the right, i.e., adhesion occurred at a higher pH than when unsensitized organisms were used. This effect was more marked when the organisms were sensitized with both Ab and C′. Similar results were obtained with Pn XIII. A number of other bacteria were tested, as well as *A. cruentus* starch. All were sensitized with sufficient Ab and C′ to allow about 70 per cent of the particles to react in IA with human E. With the exception of *E. coli,* for which normal rabbit serum provided the Ab, and *A. cruentus* starch, for which normal human serum was used as Ab, immune rabbit sera were used. Guinea pig C′ was used in all cases. The data on adhesion at various pH levels are collected from several separate assays and plotted in Figure 3.

When these curves are compared, a remarkable degree of uniformity is apparent as regards (a) the pH above which adhesion decreases markedly (4.5 in all cases) and (b) the pH above this at which adhesion is approximately half maximal (4.8 in all cases). This is in sharp contrast with the widely differing behavior of the various antigens when unsensitized (Fig. 1). Similar uniformity was not apparent below pH 4.5, where the behavior tended to return to that of the unsensitized antigen. Nor were similar results obtained with organisms sensitized with Abalone: Pn XIII and Pn XXXI behaved similarly when sensitized with Ab, but neither *S. typhosa* nor *A. cruentus* starch adhered unless exposed to C′ as well. *E. coli* behaved almost identically no matter what the extent of sensitization, the adhesion of Ab-C′ sensitized organisms at pH's above 4.5 being only slightly greater than that of unsensitized, while treatment with Ab alone did not change the curve at all.

The behavior of all the Ab-C′ sensitized antigens suggests that sensitization with Ab and C′ makes their surfaces similar from the point of view of electrostatic charge, at least at pH of 4.5 and above. If this be so, then the differences in behavior below pH 4.5 might be due to dissociation of Ab and

C′ from the antigen. The irregular results with Ab-sensitized antigens could well be due to dissociation, as Singer and Campbell[85] have evidence that other antigen-Ab precipitates dissociate below pH 4.5. The original surface could thus be uncovered resulting in a reactivity similar to that of the unsensitized particle. In all instances, however, it should be noted that Ab and C′ failed to induce attachment at pH 6.5 to 7.5, thus supporting our original contention that IA does not occur with nonprimate erythrocytes.

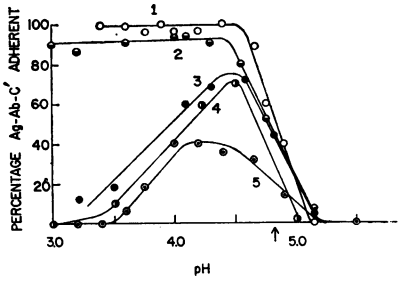

FIG. 3. The attachment to chicken erythrocytes of bacteria pretreated with antibody and guinea pig complement.

Curve	*Bacteria*
1	*D. pneumoniae,* type XIII
2	*D. pneumoniae,* type XXXI
3	*E. coli*
4	*A. cruentus* starch
5	*S. typhosa*

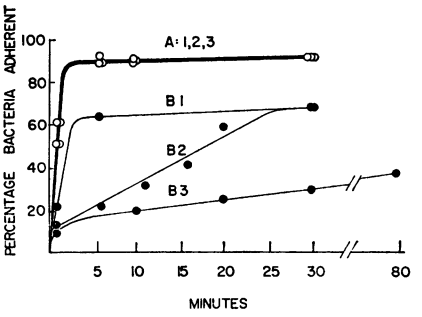

FIG. 4. The rate of attachment of pneumococci to erythrocytes via immune-adherence (series B) and acid-adhesion (series A).

Temperature	*Curves*	
37° C.	A1	B1
24°	A2	B2
0°	A3	B3

3. *Adhesion to erythrocytes of other species.* A series of experiments similar to those with chicken E was performed using Pn XIII mixed with rabbit, sheep, or guinea pig E as the indicator particle. Similar though not identical results were obtained. The red cells underwent the same changes as did those of the chicken, except that the ghosts formed by lysis at low pH were much more distorted, making microscopic counts more difficult. Rabbit and guinea pig cells behaved similarly to chicken cells, but the highest pH values for 100 per cent and 50 per cent adherence to sheep cells were slightly lower than the corresponding values for the cells of other species.

The adhesion of unsensitized or Ab-sensitized Pn XIII to human E also was similar to that seen with red cells of chicken, guinea pig, and rabbit. Ab-C′ sensitized organisms adhered to human E above pH 5.5, presumably due to true IA. However, at lower pH's the curve followed that of acid-

adhesion and it was not possible to separate the two phenomena. When sensitized *A. cruentus* starch was used, the degree of adherence to human erythrocytes was approximately constant from pH 4 to pH 9.

In another series of experiments red cells were lysed in distilled water before exposure to low pH or before reaction with sensitized antigens in IA. The curves of acid-adhesion were found to be shifted to the right. For example, 50 per cent of Ab-C′ sensitized Pn XIII were adherent to intact guinea pig red cells at pH 4.6, while the same percentage was adherent to lysed guinea pig cells at pH 4.9. With sensitized Pn XXXI and chicken E the corresponding figures were 4.8 and 5.0. Lysed human cells were less reactive in IA than were intact cells.

4. *Adhesion to particles other than erythrocytes.* (a) Immune-adherence: Other blood and tissue cells have been assayed from time to time for their reactivity in IA. *T. pallidum* sensitized with human Ab and C′, washed sensitized Pn XIII, and washed sensitized rice starch granules all gave negative results with human platelets at neutral pH. Monkey kidney cells from tissue cultures did not react in IA with either sensitized Pn XIII or with sensitized rice starch. Negative results were obtained when *E. coli* was used as the indicator particle in IA with a variety of washed sensitized antigens.

(b) Acid-adhesion: At low pH, monkey kidney cells, human platelets, and *E. coli* were all capable of combining with Pn XIII and with rice starch granules. For example, 44 per cent of the monkey kidney cells in a suspension at pH 3.9 became attached to unsensitized starch. In unbuffered saline through which CO_2 had been bubbled, lowering the pH to about 5.0, 20 per cent of Ab-C′ sensitized Pn XIII were attached to washed human platelets, thereby simulating the response shown in Figure 3 (curve 1) for sensitized Pn XIII reacting with chicken E, while in control mixtures only 3 per cent of Ab-sensitized and 1 per cent of unsensitized organisms were adherent. In buffer at pH 3.5, 100 per cent attachment of unsensitized organisms to washed human platelets was observed.

Reactivity of sensitized bacteria or starch granules with these indicator particles has not been seen above pH 5.5 using either washed sensitized antigens or reaction mixtures containing serum and cells from the same species.

B. FURTHER DIFFERENTIATION OF IA AND ACID-ADHESION: QUANTITATIVE STUDIES

1. *Effect of time and temperature of incubation on acid-adhesion and on IA.* Exper. 042057. A suspension of Pn XXXI was sensitized with rabbit Ab and guinea pig C′, washed, and resuspended in saline as described above.

The components of the reaction mixtures for IA and for acid-adhesion were brought to the desired temperature. For acid-adhesion (series A), 0.1 ml. of 2 per cent chicken E in saline were pre-incubated at 37° C. for 20 min. in buffer at pH 3.6 and then brought to the desired temperature. Two-tenths ml. of washed sensitized organisms were then added to the mixtures. Small samples were taken at intervals by capillary pipette and immediately examined by darkfield microscopy. A similar procedure was followed for IA (series B) except that the mixture of human E and SAVB++ was not pre-incubated.

The results are presented in Figure 4. The acid-induced adhesion of sensitized Pn XXXI to chicken red cells was independent of temperature, proceeding very rapidly at 0°, 24°, and 37° C. Identical results were obtained when unsensitized Pn XXXI and chicken cells were incubated together at pH's of 3.0, 3.6, and 4.1.

It was noticed that, if chicken cells and buffer of low pH were mixed at 0° C. without previous incubation at 37° C., the changes usually seen in the cells did not occur and the organisms would not adhere to the unchanged cells over a period of 75 min. After overnight incubation of the whole mixture both the usual changes and the expected degree of adherence were seen. Similar pre-incubation of human E at 37° C. before cooling to 0° and adding antigen did not alter the rate of IA at 0° C. It appears that these changes are necessary for adhesion to occur at low pH, although, as pointed out below, their persistence on raising the pH is not accompanied by adherence. The reason for this is not clear. The essential point is that once they have occurred, the acid-adhesion which follows differs greatly in its kinetics from IA.

IA, on the other hand, is a temperature-dependent process. Adherence occurs rapidly at 37° C. and more slowly at 24° C. At 0° C. the reaction is not only slow but also, over the time range studied, incomplete. These results agree with those previously published[26] and with those of Taverne,[36] who used bacteriophage T2 sensitized with Ab and C′. Taverne suggested that the slower reaction at 0° might be due to a decreased rate of collision between sensitized antigen and red cells. If this were so, it would be expected that the reaction would proceed faster when more concentrated E were used in the reaction mixture, since there would be a greater chance of collision between the bacteria and E. However, in another assay (Exper. 043057), almost identical rates of IA occurred at 0° in reaction mixtures containing 0.1 ml. of 2, 5, 10, and 25 per cent suspensions of erythrocytes. This indicates that the slower reaction at 0° C. is not due merely to mechanical factors.

2. The effect of salt concentration on IA and on acid-adhesion. It was thought that the temperature independent acid-adhesion might be due to the formation of an electrostatic bond between oppositely charged particles or sites on the surfaces of the particles, while IA was due to the formation of some other type of bond not primarily electrostatic in nature. Therefore the effect of increasing concentrations of sodium, calcium, and aluminum salts on the formation of the complex and on dissociation of the formed complex was examined in each case. In experiments on IA, however, aluminum could not be used as it caused too much agglutination of human E for microscopic counts to be possible.

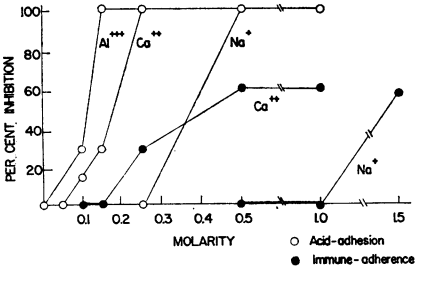

FIG. 5. The inhibitory action of salt concentration on immune-adherence and on acid-adhesion.

Exper. 050657 and 051157. For acid-adhesion two sets of reaction mixtures were made up. In both, 0.5 ml. of buffer, pH 3.2, and 0.2 ml. of 2 per cent chicken cells in saline were incubated together at 37° C. for 20 min. To one was added 0.5 ml. of the salt and 0.2 ml. of a suspension of Pn XXXI; to the other, the bacterial suspension alone. Both were incubated at 37° C. for 30 min.; the second set then received 0.5 ml. of the salt. A similar procedure was followed for IA, except that SAVB++ was used instead of glycine-acetate buffer and saline, and the organisms were sensitized with amounts of Ab and C′ estimated to give 70 per cent adherence to human E.

The results obtained are presented in Figure 5. Moderate concentrations of salts completely inhibited the formation of the bond in acid-adhesion. The degree of inhibition produced by a given salt was in proportion to the charge on the cation. Because of gross damage to chicken cells, sodium and calcium were not tested above 1.0 *M*, but up to this point neither salt dissociated the preformed bacterium-erythrocyte complexes. 0.15 *M* or more concentrated aluminum chloride produced about 25 per cent dissociation. The effect of sodium and calcium chlorides on acid-adhesion of sensitized Pn XXXI to chicken cells at pH 4.1 was similar although a little more marked. The adhesion of unsensitized Pn XXXI was also more sensitive to salt concentration at pH 3.9 than at pH 3.6 or 3.2.

In contrast with the inhibition of acid-adhesion by salts at moderate concentrations, only at relatively high concentrations did sodium and calcium have any effect on the formation of the bond in IA. 1.5 *M* sodium chloride

was required to produce detectable inhibition, while acid-adhesion was completely inhibited by 0.5 *M* salt. Again, 1.0 *M* calcium chloride was only half as effective in inhibiting IA as was 0.25 *M* salt in inhibiting acid-adhesion. Complete inhibition of IA was not produced by any salt concentration, although considerable distortion of cells was caused by the higher concentrations. It is possible that at these concentrations of added salt (e.g. 1.5 *M* or 8.5 per cent sodium chloride) there is some dissociation of Ab and/or C′

TABLE 1. REVERSIBILITY OF THE CHANGE INDUCED IN CHICKEN ERYTHROCYTES (E) BY LOW PH

E treated at pH:	*E incubated with Pn XIII at pH*	*Percentage of organisms adherent*	
		Sensitized	*Unsensitized*
3.5	3.5	100	98
	7.5	16	0
4.0	4.0	94	34
	7.5	8	0
4.55	4.55	100	0
	7.5	0	0
4.9	4.9	28	0
	7.5	2	0
7.5	7.5	0	0

from the antigen. As with acid-adhesion, the bond once formed was not broken at high salt concentrations.

The results of these experiments are considered to support the idea that acid-adhesion is primarily due to the formation of an electrostatic bond, while IA is primarily due to the formation of a bond of some other type.

3. *Reversibility of acid-induced changes in red cells and antigen, and of acid-adhesion.* Exper. 022657. Suspensions of chicken cells were treated with buffers of various pH's for 20 min. at 37° C., washed, and resuspended in buffer of either the original pH or pH 7.5. The cells were then incubated for 30 min. at 37° C. with a suspension in the appropriate buffer of Pn XIII, either sensitized with Ab and C′, or unsensitized. The percentages of bacteria adherent under the various conditions are shown in Table 1.

The change in the red cells induced by low pH and which resulted in adhesion at that pH was reversed by raising the pH. Similarly, exposure of unsensitized Pn XIII to pH 3.5 for 30 min. at 37° C., followed by washing

and resuspension in buffer of neutral pH and incubation with untreated red cells, did not result in adherence.

In another experiment a complex was formed between unsensitized Pn XXXI and chicken erythrocytes at pH 3.2 in which 80 per cent of the bacteria became adherent to cells. The mixture was centrifuged at 2700 rpm for 15 min. (sufficient to spin down all the bacteria as well as the E), washed, and resuspended in VB^{++} (pH 7.4), and again examined microscopically. Eighty per cent of the bacteria remained adherent during incubation for several hours.

TABLE 2. EFFECT OF TRYPSIN AND OF TANNIC ACID TREATMENT OF ERYTHROCYTES ON IMMUNE-ADHERENCE AND ACID-ADHESION

	Percentage of antigen particles adherent						
Treatment of erythrocytes	*Immune-adherence: Rice starch and human E*	*Acid-adhesion: Pn XXXI and chicken E*					
		pH 3.0	*3.35*	*3.9*	*4.1*	*4.3*	*4.6*
None	88	76	84	72	52	8	0
Trypsin, 0.5%	0	82	84	68	56	16	0
Tannic acid 1:10,000	0	82	80	72	40	12	0

Although the bond once formed persists when the pH is raised, the changes in the erythrocytes and antigen are reversed when treated similarly but separately. The visible structural changes in the erythrocytes do persist, so that these alone are not invariably accompanied by adherence. A reversible change at the sub-microscopic, probably molecular, level, seems indicated.

The complex in IA is not dissociated by quite marked changes in pH since the adherence of washed sensitized *A. cruentus* starch granules is constant from pH 4 to pH 9.

4. *Effect of trypsin and of tannic acid treatment of E on IA and acid-adhesion.* Exper. 042957 and 050157. (a) Trypsin treatment: 1 ml. of a 10 per cent suspension of chicken or human red cells in phosphate-buffered saline, pH 7, was mixed with 4 ml. of 0.5 per cent trypsin (Difco) in the same medium and incubated at 37° C. for 60 min. The cells were then washed three times and made up to a 2 per cent suspension in normal saline. Chicken E were agglutinated moderately by trypsin, but the clumps broke up after the first washing.

(b) Tannic acid treatment: A 2 per cent suspension of chicken or human cells in saline was incubated with an equal volume of 1 : 10,000 tannic acid in saline for 30 min. at 37° C., washed three times, and resuspended in saline.

Assays for IA and for acid-adhesion were carried out in the usual manner with normal and with treated cells. Rice starch granules and *Rickettsia burneti* both sensitized with human "natural" Ab and with human C′ were used in IA assays. Unsensitized Pn XXXI was used in assays of acid-adhesion.

As shown in Table 2, neither treatment had any effect on the range or degree of acid-adhesion. In agreement with our previous results in 1954, the reactivity of human E in IA was abolished both by tanning and by trypsinization. The inhibition of IA by trypsin treatment of the E might thus be due either to the destruction of a receptor or to its masking by the protein enzyme. Whichever occurred, it had no effect on the reactivity of chicken cells in acid-adhesion. Tannic acid treatment is commonly used to render cells capable of absorbing antigens. The reverse effect occurred with IA: the cells became incapable of adhering to sensitized particulate antigens. Since the mechanism of its action is unknown, very little can be deduced from the fact that it inhibits IA. The point to be noted is that these two inhibitors of IA have no effect on acid-adhesion and thus serve to differentiate between the two phenomena.

These results also may be useful in identification of the reactive sites on the surface of primate erythrocytes. Recent assays by Mr. Shaun Ruddy in this laboratory have shown that low concentrations of crystalline chymotrypsin (3 μg.) and of formaldehyde (0.12 per cent) will inactivate or block the reactivity of human E in IA. These collected data are suggestive that the "receptor site" for IA is protein in nature.

DISCUSSION

The experimental results provide several criteria for the differentiation of acid-adhesion of bacteria to other particles from immune-adherence of particulate antigens to primate erythrocytes. These are summarized in Table 3. The effects of changes in salt concentration and temperature on acid-adhesion are indicative that the bond between the particles is electrostatic. As would be expected, the electrostatic attraction varies with the pH and with the species and strain of microorganisms used. Acid-adhesion is also influenced by the presence of Ab and C′ on the surface of the antigen, a point of more practical importance. With the various bacteria studied sensitization with Ab and C′ caused a "shift to the right," i.e., adhesion to

erythrocytes occurred at a higher pH when sensitized bacteria were used than when the bacteria were unsensitized. With Ab and/or C′ from other species of animal it is conceivable that at certain pH's attachment of sensitized bacteria to red cells could occur as a result of either acid-adhesion or IA. Differentiation, as outlined in Table 3, is simple.

TABLE 3. COMPARISON BETWEEN IMMUNE-ADHERENCE AND ACID-ADHESION

Factor	*Acid-adhesion*	*Immune-adherence*
pH	Occurs only below pH 5	Constant over wide pH range (4-9)
Ab and C′	Not essential, but range of adherence is different for sensitized organisms	Essential
Species of erythrocytes	Occurs with all species tested (human, chicken, sheep, rabbit, guina pig)	Occurs with human and monkey erythrocytes only
Other substrates	Platelets (human and rabbit); monkey kidney cells; *E. coli*	Occurs with certain nonprimate platelets only
Time and temperature	Rate of adherence is rapid and constant from 0° to 37° C.	Rate of adherence varies with temperature: rapid at 37°; less rapid at 24°; and slow at 0° C.
Ionic strength	Inhibited by high salt concentration	Not inhibited by high salt concentration
Trypsinizing erythrocytes	No effect	Complete inhibition
Tanning erythrocytes	No effect	Complete inhibition
Previous lysis of erythrocytes	Slight increase in reactivity at high pH's	Human ghosts less reactive than intact human cells. No reaction with lysed nonprimate red cells.

An analogous situation exists with respect to the agglutination of microorganisms by Ab and by auto-agglutination at low pH. The latter phenomenon, acid-agglutination, has been studied by several investigators since 1922.[8, 9, 25, 31, 32] de Kruif[8] found that the optimal pH for auto-agglutination was characteristic not only of the species of bacterium used but often of an individual strain. The addition of immune serum broadened the zone of acid-agglutination.[32] Organisms heavily sensitized with Ab showed a cataphoretic isoelectric point similar to that of γ-globulin. This work suggested

an explanation of our results on acid-adhesion in terms of differences in charge between indicator particle and antigen.

Ponder and Ponder[84] have shown that the isoelectric point of human red cell ghosts is below pH 2.0. The similar ranges of adhesion of sensitized and unsensitized Pn XIII to erythrocytes of all the other species tested indicates that the isoelectric points of these cells are close to that of human erythrocytes. Above this point (pH 2) the surfaces are negatively charged. Below their isolectric points the antigens are positively charged and an electrostatic bond may result. The upper pH limits for adhesion of unsensitized organisms agree quite well with the cataphoretic isoelectric points measured by other workers.[11, 18, 37, 39] Some bacteria do not show reversal of charge below the iso-electric point, e.g., some strains of staphylococci[39] and *S. pullorum.*[18] The failure of *S. typhosa* to adhere when unsensitized may be due to the lack of an amphoteric component on its surface.

That the situation is a little more complex than this is indicated by the decline in adhesion with a further decrease in pH as observed herein with *E. coli, M. albus,* and Pn III (Fig. 1). There are three possible explanations: (a) The positive charge on the bacterium is not very great, and the attraction between bacterium and red cell, initially not strong, declines as the negative charge on the red cell diminishes, until bonds can no longer persist; (b) The bacterium becomes less positive at pH's far below its isoelectric point, as has been described for *B. cereus,*[11] and the attraction becomes too weak for adhesion to occur; (c) It is possible that in all cases the initial attraction is not between oppositely charged particles but between oppositely charged parts of two surfaces with a low net charge of the same sign. Acid-adhesion could thus occur both above and below the isoelectric point of either particle and would depend on the presence of highly but oppositely charged sites on the two particles, each of which had a sufficiently low net charge to allow initial contact.

The shift of the curves in the direction of higher pH when the antigens were sensitized with Ab or with Ab and C′ is probably due to at least partial covering of the surface with protein of higher isoelectric point. The curves did not move as far in the direction of higher pH as might have been anticipated from the isoelectric point of rabbit γ-globulin, pH 5.8 and β-globulin, pH 6.3. Two explanations may be offered for this. First, only a limited amount of Ab was used in sensitizing the bacteria, because agglutination during washing had to be avoided. It is known that very large doses of Ab are required before the maximum acid-agglutination occurs at the isoelectric point of γ-globulin. If the surface is only partly covered with Ab, added C′ seems unlikely to cover much more of the surface, and a similar argument

may be applied. Second, the charge on rabbit γ-globulin is low on either side of the isoelectric point[85] and the positive charge on Ab-sensitized organisms may be too low for attachment to red cells to occur until the pH is well below the isoelectric point of the Ab.

The results of the present study provide little insight into the nature of the attachment of erythrocyte to sensitized bacterium via immune-adherence. The available data indicate that the bond is not electrostatic, although attachment of sensitized bacteria to erythrocytes due to an electrostatic bond can, in the circumstances defined herein, occur. The lack of influence of salt concentration or pH, the influence of temperature on kinetics, and the specific nature of the primate erythrocyte all indicate an immunological reaction based on a complex mechanism the nature of which still remains obscure.

CORRECTION

In the paper entitled "The possible relationship of etiocholanolone to periodic fever," published in *The Yale Journal of Biology and Medicine* (1958, *30,* 395), an error has been found in the chromatograms on page 400. The standard compound D, called "Dehydroisoandrosterone" in Systems 1 and 3 is too nonpolar to be that compound. Subsequent paper chromatography with true dehydroisoandrosterone whose structure has been verified by infra-red spectrum revealed its mobility to be much more polar than the standard etiocholanolone and the original standard "dehydroisoandrosterone."

This error in paper chromatography does not alter the specificity of the identification of etiocholanolone in plasma.

Unfortunately too little of this original standard nonpolar C-19 compound was available to confirm its real chemical structure.

PHILIP K. BONDY, GEORGE L. COHN,
WALTER HERRMANN, AND KENNETH R. CRISPELL