IMMUNE-ADHERENCE OF BACTERIOPHAGE T2

JANICE TAVERNE

*From the Department of Bacteriology, London School of Hygiene and Tropical Medicine, London, W.C.*1

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BACTERIA sensitized with specific antibody and all four components of complement will attach themselves to human red blood cells at 37°. This phenomenon was described by Nelson in 1953 and called the immune-adherence phenomenon (I-A). Nelson (1953, 1956) demonstrated the occurrence of immune-adherence with many different species of bacteria, with rickettsiae, with particulate polysaccharide antigens (*e.g.*, starch granules) and with one virus, vaccinia. The degree of immune-adherence was measured either by counting adherent antigen particles or organisms directly under the microscope, or by determining the percentage of organisms removed from a suspension after incubation with human erythrocytes for a period at 37°, which were then deposited by light centrifugation. Subsequently, he found that immune-adherence could be measured by the formation of agglutination patterns by the clumped red cells settling in the bottom of a tube. It was thus possible to study immune-adherence of the smaller viruses, such as poliovirus, which Nelson and Woodworth (to be published) have shown to produce haemagglutination patterns with human erythrocytes in conditions suggesting strongly an immune-adherence mechanism. Confirmation of immune-adherence by small viruses is difficult because the number of particles adherent to red cells cannot be counted visually, and neither adherent virus nor residual virus in the supernatant fluid can be assayed by ordinary methods because the virus has been exposed to antibody. This difficulty could be overcome by labelling the virus with a radioactive isotope, and for this purpose the most suitable virus seemed to be bacteriophage. This paper describes experiments with bacteriophage T2 labelled with a radioactive isotope, which demonstrated that the fully sensitized virus adhered to the red cell surface, a result conforming exactly with Nelson's immune-adherence phenomenon. From this one concludes that haemagglutination obtained with phage T2 and other small viruses sensitized with antibody and complement is probably also an expression of immune-adherence.

MATERIALS AND METHODS

Virus.—T2 (wild type) stocks were prepared in bacteria growing in M9 synthetic medium (Delbrück, 1940) with *Bacterium coli* strain B as host. Phage was purified by three cycles of differential centrifugation at 4000 r.p.m. on an MSE angle centrifuge for 15 min. and at 14–20,000 ***g*** for 1 hr. on the Spinco centrifuge. It was re-suspended in phosphate buffer and had a titre of 1–5 $\times$ 10^{11} plaque-forming particles per ml. titrated on *Bact. coli* B.

^{35}S-labelled phage was prepared in bacteria growing in M9 synthetic medium with $MgCl_2$ substituted for $MgSO_4$ and 50 μc per ml. of carrier-free ^{35}S added. It was also purified by three cycles of differential centrifugation. The stock was absorbed with a variant of *Bact. coli* B resistant to phage T2 (B/2), and with washed red cells.

Radioactivity was measured by counting disintegrations per minute on an Ekco scaler, type N529, using dried samples sufficiently small to avoid loss by self-absorption.

Antiserum.—Antiserum was prepared by Dr. E. W. McCloy in a rabbit by repeated intravenous injections of broth culture T2 lysate over a period of six weeks. The antiserum had a K value of 2000 per min., measured at a serum dilution of 2×10^{-4} (Adams, 1950). Before use in I-A experiments, serum, usually diluted 1/4, was absorbed twice for 30 min. at 37° with 10^9 *Bact. coli* per ml. of serum. Serum was inactivated at 56° for 30 min.

Diluent.—Veronal buffered saline (Fulton and Dumbell, 1949) containing 0·5 per cent crystalline bovine plasma albumin was used in all I-A experiments.

Complement.—Guinea-pigs were bled by cardiac puncture and the fresh serum was pooled and stored at − 70°. Before use, complement diluted 1/4 was absorbed with 10^9 *Bact. coli* B per ml. and when necessary the complement was also absorbed with packed human red cells. Complement absorptions were done at 0°.

Red blood cells.—Group O Rh+ human blood in acid-citrate-dextrose solution was obtained from the National Blood Transfusion Service. Blood was stored at 4° and discarded a month after it was drawn. Red cells were washed three times in veronal buffer before use.

Glassware.—All glassware was acid cleaned. Kahn tubes were sterilized covered with aluminium caps.

I-A tests by haemagglutination patterns.—Antigen suspension in volumes of 0·5 or 1 ml. were mixed with 0·2 ml. volumes of antiserum dilutions in Kahn tubes. Volumes of 0·1 ml. of complement, usually diluted 1/16, were added and the tubes shaken and placed in a 37° water-bath. After 10 min., 0·1 ml. of a 1·5 per cent suspension of red cells was added to each tube. The tubes were shaken and incubated for 45–60 min. It was important to take readings at this time when the cells would have settled to the bottom and agglutinated cells would not have begun to slip. I-A haemagglutination is not as strong as that caused by influenza virus, for example ; the pattern is easily disrupted and little or no clumping of the cells is visible under the microscope.

Controls.—The following controls, which must be negative, were included in every test : antigen, antiserum and complement each alone with red cells, and red cells alone in diluent. As a control of complement activity, a tube was also included containing a standard suspension of a particulate antigen, usually *Amaranthus cruentus* starch, sensitized with antibody and known to undergo I-A. A dilution of complement was chosen which would give maximum I-A but which did not itself agglutinate red cells. It was often necessary to absorb human red cell agglutinins from guinea-pig serum to obtain such a dilution.

I-A tests with labelled T2.—These experiments were made by incubating reaction mixtures which included dilutions of labelled T2 suspension, antibody, complement and red cells, the quantities depending on the experiment, for 1 hr. at 37° and then centrifuging the mixture to deposit the red cells. The supernate was withdrawn ; the red cell pellet was washed once and re-suspended in 0·5 ml. of diluent. Samples from the supernate and pellet were dried and radioactivity assayed. The numbers of disintegrations in the pellet were expressed as percentages of total disintegrations in the reaction mixture.

RESULTS

Haemagglutinating properties of complement-phage mixtures

Neither T2 nor antiserum nor a mixture of them agglutinated human red cells. Complement, which was always first absorbed with human red cells, did not by itself agglutinate human red cells either, but when 0·1 ml. of it was mixed with 0·5 ml. of T2 at 10^8 particles/ml., haemagglutination was produced in the absence of antiphage serum. As the complement had already been absorbed with human red cells, this effect was not due to antibody in the guinea-pig serum against human red cells. It was more probably due in part to normal antibody against *Bact. coli* because a further absorption with *Bact. coli* rendered the complement usable at dilutions greater than 1/4. It is known that bacterial antigens in solution can undergo immune-adherence (Turk, to be published) ; it is very probable that dissolved *Bact. coli* antigens in the phage lysate would

react with any " normal " antibody there might be in the complement, to give immune-adherence, and hence haemagglutination, not due to the phage.

Lysates containing 10^{11} and 10^{10} particles per ml. gave haemagglutination in absence of antiserum with some samples of complement, even after two absorptions with *Bact. coli*. Some antibody to *Bact. coli* may have remained in the complement even then, since it is notoriously difficult to remove normal antibody by absorption ; but some of the haemagglutinating activity of complement and T2 mixtures may have been due to normal antibody to the phage rather than to the bacterium.

Evidence that low dilutions of complement may induce immune-adherence of T2 by virtue of normal anti-phage antibody in it came from the following experiments : Serial twofold dilutions of fresh guinea-pig serum in 0·1 ml. amounts were mixed with 0·4 ml. of labelled T2 at 1×10^9 particles/ml., 0·5 ml. of diluent and 0·5 ml. of a 10 per cent suspension of red cells. The mixtures were incubated for 1 hr. at 37° and the radioactivity in the supernate and pellet assayed in the usual way. Table I gives the result of this titration.

TABLE I.—*Immune-adherence of Labelled T2 in the Presence of Low Dilutions of Complement and the Absence of Antiserum*

Reaction mixture:
- T2 : 4×10^8 plaque-forming particles in 0·1 ml.
- Complement dilutions : 0·1 ml.
- Diluent : 0·5 ml.
- R.B.C. $4{\cdot}5 \times 10^8$ in 0·5 ml.

Complement : final dilution	Percentage of total activity in pellet
—	1
1/15	70
1/30	58
1/60	28
1/120	11
1/240	2
1/480	<1

Thus normal guinea-pig serum induced adherence of T2, and a 50 per cent end-point was obtained between 1/2 and 1/4 initial serum dilution and between 1/30 and 1/60 final dilution. Fresh human serum was found to have an end-point at a similar dilution. In addition, both normal guinea-pig and human sera inactivate phage T2.

In spite of the anomalies introduced by presumed normal antibodies in the complement, it always proved possible by suitable absorption with human red cells and with *Bact. coli* to produce a complement giving satisfactory negative controls when used at initial dilutions greater than 1/4 and having a satisfactory immune-adherence titre (32–64) against known antigen/antibody systems.

I-A haemagglutination with T2

Table II illustrates a representative I-A chessboard titration of T2 against its antiserum. Controls of all reagents alone and in pairs were negative. Haemagglutination only occurred in tubes which contained T2, antibody and complement, and at the dilutions shown in the Table. No haemagglutination occurred with rabbit or sheep red cells.

TABLE II.—*Chessboard Titration of Phage T2 Against T2 Antiserum by I-A Haemagglutination Patterns*

Antiserum : final dilution	Plaque-forming particles of T2 per ml. 10^9	10^8	10^7	10^6	0
1/90	4	3	2	0	0
1/180	4	3	1	0	0
1/360	4	3	1	0	0
1/720	4	3	0	0	0
1/1440	4	2	0	0	0
1/2880	4	0	0	0	0
1/5760	0	0	0	0	0
0	0	0	0	0	0

Each tube contained 0·5 ml. of T2 dilution or diluent, 0·2 ml. of antiserum dilution, 0·1 ml. of complement diluted 1/16, and 0·1 ml. of 1·5 per cent suspension of R.B.C.
4 = very strong haemagglutination.
3 = strong haemagglutination.
2 = definite haemagglutination.
1 = weak haemagglutination.
0 = no haemagglutination.

Similar results of titrations were obtained on many different occasions using two different antisera, many samples of complement and different batches of red cells. The kind of haemagglutination and general results of these titrations were the same as those observed by Nelson with other antigens. Haemagglutination was obtained in experiments in which a total of $1{\cdot}5 \times 10^7$ red cells were used when more than 5×10^6 total phage particles were present. It therefore appears that haemagglutination could only be obtained when the ratio of plaque-forming particles to red cells was of the order 1 : 1. The ratio of plaque-forming particles to antigenic particles was not known ; it seems probable that haemagglutination requires more than one sensitized antigen particle adherent to each red cell.

I-A with phage labelled with ^{35}S

In order to show that labelled T2 particles immune-adhered and were carried down with the red cells, a difficulty had to be overcome. This was, that phage at a final concentration of 10^9 particles per ml., when incubated with antiserum at the concentration necessary for I-A, but without complement, would not remain suspended during centrifugation, whether red cells were present or not. In such experiments, there was no haemagglutination, but 40–60 per cent of the radioactive phage was found in the very slight deposit after centrifugation, even after very light centrifugation at 540 r.p.m. for 10 min. on an MSE angle centrifuge. The most probable explanation is that phage at this high concentration was being agglutinated by the antibody, although no flocculation could be seen. Concentrations of phage less than about 10^8 particles per ml. could not be used, because the proportion of labelled particles was inevitably small, and more dilute phage gave insufficient " counts " to permit a valid reading. For instance, a phage stock containing $6{\cdot}5 \times 10^{10}$ plaque-forming particles per ml. gave only $1{\cdot}7 \times 10^5$ counts per min. per ml., or $2{\cdot}6 \times 10^{-6}$ counts per plaque-forming particle.

However, it was possible to find a concentration of phage low enough to avoid deposition due to agglutination yet still high enough for assay by radioactivity. Lanni and Lanni (1953) stated that collisions between phage particles can only

be ignored at concentrations of 10^7 particles per ml. or less. For the purpose of experiments like that shown in Table III, collisions did not matter so long as, in the time occupied by the experiment, they were insufficient to cause deposition on centrifugation : controls showed that this condition was satisfied.

TABLE III.—*Demonstration of Adherence of Labelled Phage Particles to Red Cells in the Presence of Antibody and Complement*

Plaque-forming particles of T2 per ml.	Antiserum*	Complement*	R.B.C. per ml.	Percentage of total activity in deposit
$2{\cdot}5\times10^9$	1/2100	—	—	42
$2{\cdot}5\times10^9$	1/2100	1/224	—	19
$2{\cdot}5\times10^9$	1/2100	—	1×10^9	61
$2{\cdot}5\times10^9$	1/2100	1/224	1×10^9	90
$3{\cdot}5\times10^9$	—	1/128	1×10^9	3
$2{\cdot}5\times10^8$	1/1200	—	—	<1
$2{\cdot}5\times10^8$	1/1200	1/224	—	<1
$2{\cdot}5\times10^8$	1/1200	—	1×10^8	10
$2{\cdot}5\times10^8$	1/1200	1/224	1×10^8	93

* Final dilution.

As can be seen from the Table, at a final concentration of $2{\cdot}5 \times 10^8$ phage particles per ml. in the absence of complement and in the presence of antibody at a concentration which would give I-A, 10 per cent and under of the phage was deposited.

Both antibody and complement were necessary at the higher and at the lower concentration of phage shown in order to bring down 90 per cent of the phage. This experiment was repeated using different complements, human and guinea-pig, and different batches of red cells and gave similar results each time.

Rate of I-A reaction measured with labelled phage

The rate at which the pre-formed phage-antibody-complement complex was adsorbed to red cells was measured by incubating 1 ml. of labelled T2 suspension at 4×10^9/ml., 0·8 ml. of 1/300 antiserum, 0·4 ml. of 1/16 complement and 1·4 ml. of diluent for 1 hr. at 37°. Of 50 per cent red cells 0·4 ml. was then added, and samples removed from the mixture at intervals, pipetted into an ice-cold tube and centrifuged to stop the reaction. The distribution of radio-activity in supernate and pellet was determined. This reaction happened very quickly ; 50 per cent of the phage was adherent in 4 min. and the reaction was complete in 25 min. When the rate of union between phage, antibody, complement and red cells was measured from the time of mixing of all four reagents to complete immune-adherence, a much longer time was required, *i.e.*, 18 min. for 50 per cent adherence and 55 min. for 100 per cent adherence. Presumably the time difference was required for the formation of the phage-antibody-complement complex. From neutralization experiments it was found that 95 per cent of the phage was inactivated in 3 min., so that we conclude that the slowest part of the I-A reaction must be the fixation of complement.

The results of these experiments are plotted in Fig. 1 and 2. Also plotted in Fig. 2 is the course of inactivation of the phage in the complete reaction mixture.

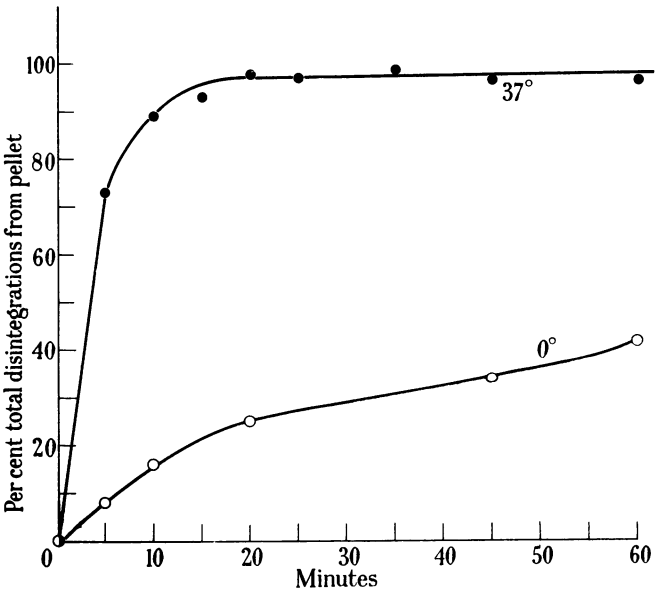

FIG. 1.—The rate of adherence of pre-formed phage-antibody-complement complex to red cells at 37° and at 0°.

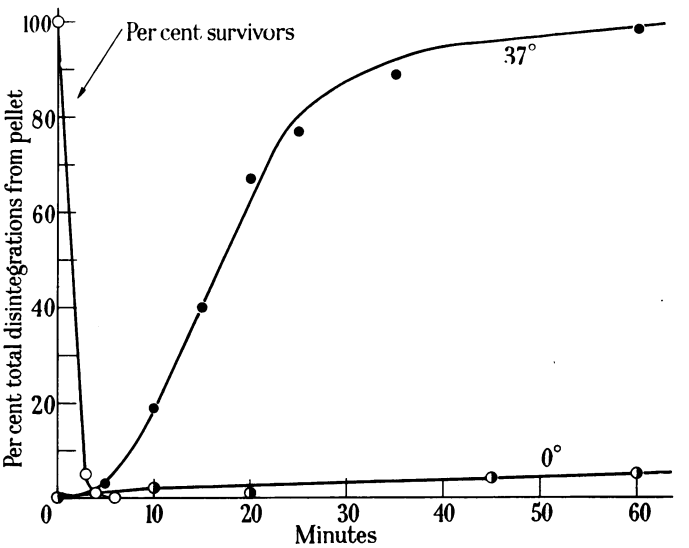

FIG. 2.—The rate of union of phage, antibody, complement and red cells at 37° and at 0°. Also shown is the rate of inactivation of T2.

Measurements of rate of reaction and rate of adherence were repeated in the same way but using ice-cold reagents and keeping the reaction mixtures, one of which had been previously incubated for 1 hr. at 37° before addition of red cells, in an ice-bath. Negligible fixation of complement occurred at 0°, which is consistent with the fact that the third component of complement, C'3, is only fixed at 37° (Mayer, Levine, Rapp and Marrucci, 1954). It also appears that the rate of adherence, as opposed to the rate of reaction, is very much slower at 0°, perhaps because of the reduced rate of collision at the lower temperature.

The rates of reaction obtained with the bacteriophage system are similar to those obtained by Nelson with *Staphylococcus aureus* as the antigen.

DISCUSSION

Immune-adherence has been described for a number of different antigen systems. Nelson has described the reaction with bacteria, with starch and zymosan particles, with rickettsiae and with a large virus, vaccinia. Turk (to be published) has demonstrated that soluble polysaccharide antigens from a number of bacteria and soluble protein antigens undergo immune-adherence. The different nature of these antigens emphasizes that immune-adherence is a general immunological phenomenon.

The haemagglutination technique (Nelson and Woodworth, to be published) for measuring immune-adherence appears to be a valuable one for investigating the behaviour in this reaction of small antigens which cannot be counted under the microscope. It was important therefore to check with one small antigen system that immune-adherence could be demonstrated by another technique as well as by haemagglutination. In the two kinds of experiment just described, although close quantitative comparison is difficult, it would appear that the general proportions in which the reagents acted in haemagglutination and in labelled phage experiments are of the same order. The results suggest that haemagglutination was caused by adherent sensitized phage particles. It can be concluded that with small, as with large, particulate antigens the haemagglutination technique is a valid one for investigating immune-adherence.

Finally, it should be stressed that in experiments in which complement is used in high concentrations, particular attention must be paid to the natural antibodies it contains.

SUMMARY

Bacteriophage T2, in the presence of anti-phage serum and complement, agglutinates human red cells by immune-adherence. Both phage and antiserum can thus be titrated by a haemagglutination pattern test.

Phage T2 in the presence of complement alone will agglutinate red cells. Absorption of complement with *Bact. coli* prevents haemagglutination in the absence of antiserum if complement is used at an initial dilution greater than 1/4.

Experiments with phage labelled with ^{35}S suggest that both normal guinea-pig and human sera at high concentration contain small amounts of phage antibody.

In the presence of immune serum and complement over 90 per cent of the phage particles adhered to human red cells at 37°.

The rate of adherence of the pre-incubated phage-antibody-complement complex to the red cells and the rate of union of all four reagents from the time

of mixing were measured at 37° and 0°. Fixation of complement appears to be the slowest part of the reaction.

It is concluded from a comparison between haemagglutination experiments and experiments with phage labelled with ^{35}S that haemagglutination is probably caused by the adherence of the phage particle itself to the surface of the red cell and that the haemagglutination technique is therefore a valid one for the investigation of immune-adherence with small antigens.

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