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Antigenic Relationship Between Pseudomonas Bal-31 Membranes and Bacteriophage PM2

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Summary. Antisera against bacteriophage PM2 and against membranes of its host cell, Pseudomonas BAL-31, were prepared. Cross-reactivity between these two antigens and both antisera was found by immunodiffusion, complement fixation and viral neutralization experiments. Anti-membrane sera up to a dilution of 1/100 were able to neutralize 60% of the infective capacity of PM2. This neutralizing capacity was partially abolished by the presence of Pseudomonas BAL-31 membranes. It is concluded that similar antigenic determinants are present in the PM2 phage and in the host membrane.

Bacteriophage PM2 is a unique phage having a membranelike structure as an outer coat. PM2 was originally isolated by Espejo and Canelo (1968*a*) who observed that this phage was extremely sensitive to organic solvents.

The presence of phospholipids in the virus particle as well as the appearance of negatively stained PM2 under the electron-microscope led them to postulate that PM2 would have a nucleoprotein core surrounded by a lipid-containing membrane (Espejo & Canelo, 1968*b*).

Recent X-ray diffraction studies of PM2 indicate that the bacteriophage lipid is organized in a bilayer structure presumably surrounded by an external protein coat (Harrison, Casper, Camerini-Otero & Franklin, 1971).

The phospholipid content of PM2 is qualitively similar to that present in the host Pseudomonas BAL-31, but the relative amounts of phosphatidylethanolamine and phosphatidylglycerol are different in both (Braunstein & Franklin, 1971). It has been demonstrated that the viral phosphatide is not synthesized de novo after infection, since the specific activity of the phos-

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phatide phosphorus contained in the prelabeled bacteria and in the virus progeny is approximately the same (Espejo & Canelo, 1968*b*).

Under the electron-microscope complete viral particles are visualized in the bacterial cytoplasm near the cytoplasmic membrane, before cell lysis (Cota-Robles, Espejo & Haywood, 1968; Dahlberg & Franklin, 1970). This observation suggests that the process of acquisition of the phage membrane does not take place by budding of the bacterial membrane, as has been demonstrated in animal cells infected by many enveloped viruses (Acheson & Tamm, 1970). Furthermore, Pseudomonas has a complex rigid wall common to gram negative bacteria surrounding the cytoplasmic membrane, which would not allow budding of the membrane (Espejo & Canelo, 1968*c*).

It was important to know if any component of the bacterial membrane was incorporated into the phage membrane, as it has been reported to occur in enveloped animal viruses (Cartwright, Smale & Brown, 1969). In an attempt to look for this possibility, immunological studies were done using purified bacterial membranes as well as purified PM2 as antigens. The antibodies thus obtained were tested against both antigens to analyze the possible cross-reactivity between them.

Materials and Methods

Conditions for growth of Pseudomonas BAL-31 are those described by Espejo and Canelo (1968*a*).

Purification of Bacteriophage PM2

According to Espejo and Canelo (1968*a*), the bacterial lysates were extracted with polyethylene glycol 4000 and sodium dextran sulphate 500, concentrating the phage and bacterial debris in the interphase. The dextran sulphate 500 was precipitated using cold saturated KCl; the supernatant containing the bacteriophage and bacterial debris was dialyzed extensively against buffer, and centrifuged at $60{,}000 \times g$. The pellet was resuspended in a small volume of buffer and layered on a CsCl solution (0.45 g/ml) and centrifuged for 15 hr in an SW39 rotor at 37,000 rpm in an ultracentrifuge Spinco Model L (Fig. 1*A*). This phage preparation will be called CsCl-PM2.

Layering of the phage suspension was found to be essential since mixing of the phage with the CsCl solution caused substantial phage denaturation, resulting in the appearance of other components with different and heterogeneous bouyant density.

A further purification step was performed by centrifuging CsCl-PM2 after dialysis against buffer, through an 8 to 22% sucrose gradient in an SW39 rotor at $35{,}000 \times g$ for 20 min in a Spinco Model L (Fig. 1*B*). This preparation will be called sucrose-PM2.

Polyacrylamide Gel Electrophoresis

The procedure was similar to that described by Summers, Maizel and Darnell (1965). The gels contained 7.5% (w/v) acrylamide, 0.2% (w/v) N,N′-methylene *bis* acrylamide,

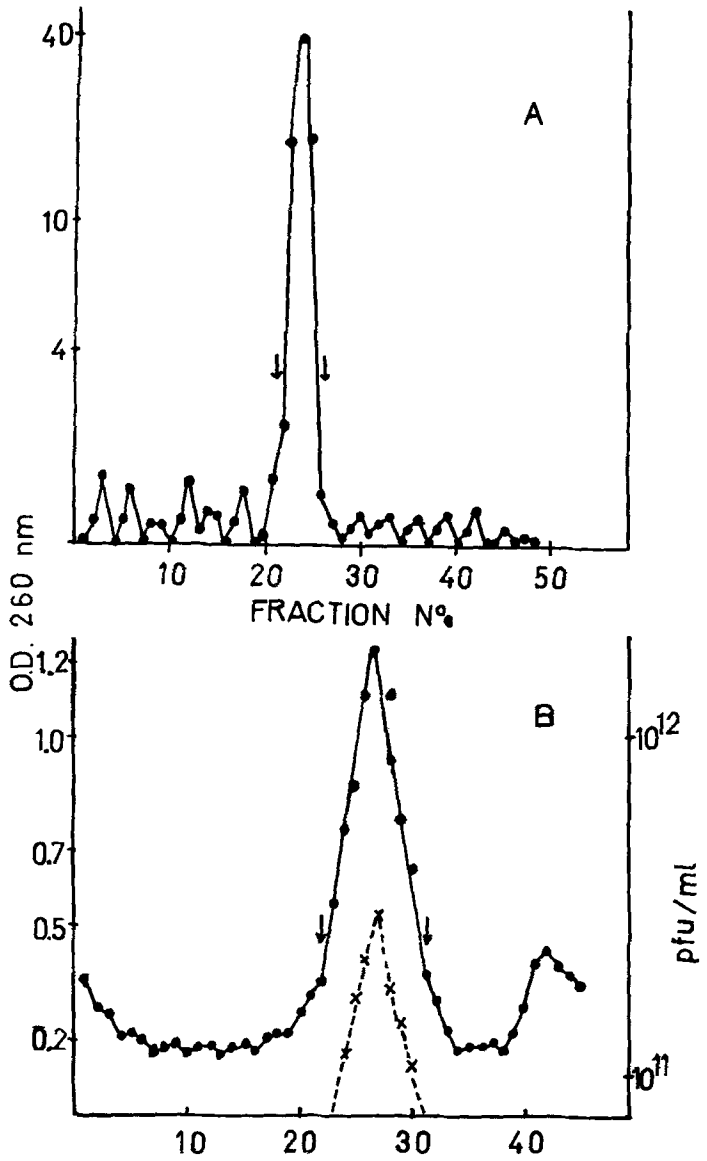

Fig. 1. (*A*) Absorbance at 260 nm profile of PM2 preparation after centrifugation for 15 hr in CsCl 0.4 g/ml of 1 M NaCl, Tris 0.02 M, pH 8.1. (*B*) Absorbance at 260 nm profile (•) and distribution of infectivity (×) of PM2 preparation after 20-min centrifugation on a gradient 8 to 22% sucrose in 1 M NaCl, Tris 0.02 M, pH 8.1

0.06% (w/v) tetramethylenediamide, 0.1% (w/v) sodiumdodecyl sulphate, 0.1 M sodium phosphate, pH 7.2, and 0.08% (w/v) ammonium persulphate. The buffer used for electrophoresis was 0.1 M sodium phosphate, pH 7.2, with 0.1% SDS (Viñuela, Algranati & Ochoa, 1967). After the electrophoretic fractionation, the polyacrylamide gels were suspended in a 50-fold volume of 12.5% TCA, as described by Chrambach, Reisfeld,

Wyckoff and Zaccari (1967) but at room temperature overnight. Next day the gels were immersed in staining solution freshly prepared by a 1:20 dilution in 12.5% TCA of a 1% aqueous solution of Coomassie blue. After 5 hr of staining, the gels were transferred to 10% TCA to be discolored and afterwards photographed.

Preparation of Bacterial Membranes

To obtain spheroplasts, Pseudomonas BAL-31 were treated with lysozyme and EDTA as described by Birdsell and Cota-Robles (1967). The buffer used was 1 M NaCl, 0.02 M Tris, pH 8.1, (BD); all other conditions remaining the same. The spheroplasts thus obtained were centrifuged at $5{,}000 \times g$ at room temperature for 15 min. The pellet was resuspended in 0.01 M NaCl, 0.02 M Tris, pH 8.1, for producing the osmotic lysis. For membrane preparation, the procedure described by Kaback and Stadtman (1966) was followed. Subsequently, the membranes were resuspended in 20% sucrose-BD, 0.01 M $MgSO_4$, layered on 60% sucrose-BD, 0.01 M $MgSO_4$ and centrifuged at $64{,}000 \times g$ in an SW 39 rotor for 90 min at 4 °C in a model L Spinco ultracentrifuge. The membranes were collected at the interphase, and dialyzed extensively against a large volume of BD. Electron-microscopy of the preparation revealed predominantly empty vesicles (Fig. 2).

Labeling of Pseudomonas BAL-31

BAL-31 cells were grown in synthetic medium (Franklin, Salditt & Silbert, 1969) and extensively labeled with 0.25 mC of ^{3}H-leucine (Schwarz-Mann, 15 C/mole) for

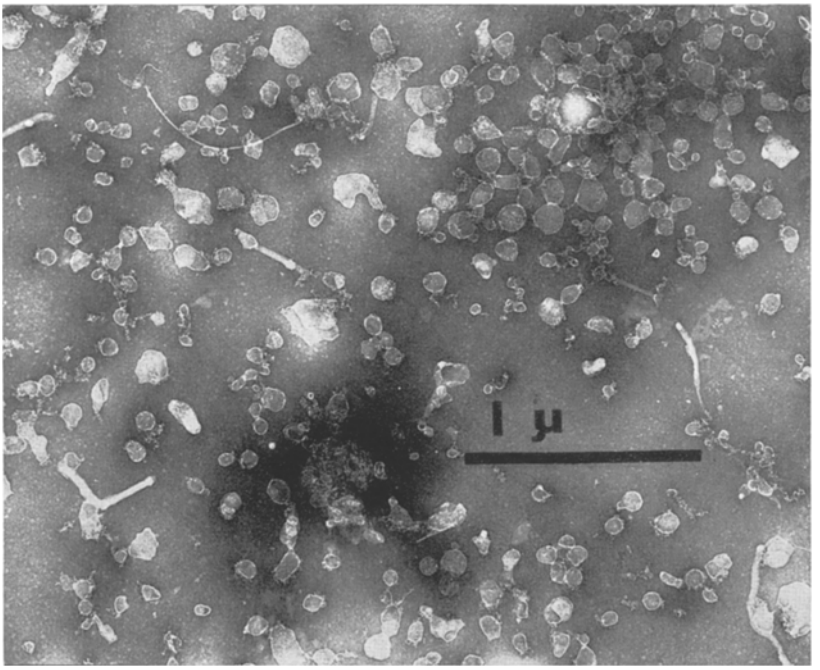

Fig. 2. Electron-microscopy of Pseudomonas BAL-31 membranes obtained as described in Materials and Methods. The preparation was negatively stained with 2% phosphotungstic acid

two generations. The cells were centrifuged and washed two times with the same volume of buffer (1 M NaCl, 0.02 M Tris, pH 8.1), resuspended in the same buffer and extensively sonicated in a Raytheon Sonic Oscillator Model DF 101 at 10 kc/sec.

Electron-microscopy

The membrane preparations were negatively stained with 2% phosphotungstic acid, pH 7.4, and observed in a Phillips EM 300, operated at 80 kW.

Protein Determinations

Protein concentrations were routinely determined by the Lowry method, modified for microscale (Lowry, Rosebrough, Farr & Randall, 1951), or by ninhydrin determination of total nitrogen (Schiffman, Kabat & Leskowitz, 1962).

Preparation of Anti-Membrane Serum

Membrane preparations containing 4 mg of total N per ml (0.1 ml) were inoculated serially every other day by intracardiac puncture of adult male guinea pigs, totaling 10 injections. The animals were bled 10 days after the last injection. The serum was filtered through a Millipore filter HAWP, and kept at 4 °C. Another way of preparing anti-membrane serum, was to inoculate BAL-31 purified membranes mixed with complete Freund's adjuvant, by intramuscular injection in the rear legs of adult male guinea pigs. Four injections, each containing 1 mg of membrane protein, were performed every 20 days. One month after the last injection, the guinea pigs were bled. Very poor antisera were obtained by this method and all the experiments described here were done using the anti-membrane serum obtained by the first method.

Preparation of Anti-PM2 Serum

A virus suspension containing 100 μg of viral protein (0.7 ml) was thoroughly mixed with 0.5 ml of complete Freund's adjuvant; 0.6 ml of the mixture was inoculated in each rear leg of adult male guinea pigs. After one month the same dose of virus was inoculated intracardially; 15 days later the animals were bled.

Complement Fixation

The method of Wasserman and Levine (1961) was used. Incubation time for fixation was 60 min at 37 °C and overnight at 4 °C. Complement fixation was performed in the presence of an equal volume of complement inactivated at 56 °C for 1 hr.

Immunodiffusion

The amounts of the antigens used were 8 μg of membrane protein, and 70 μg of viral protein (sucrose-PM2). They were sonicated in a Raytheon Sonic Oscillator Model DF 101 for 45 sec at 0 °C, at 10 kc/sec. The Ouchterlony plate for double diffusion was prepared on a microscope slide with 3.5 ml of 0.75% agarose in buffered saline.

Ten microliters of antigens and antibodies, respectively, were located into the wells of the Ouchterlony plate, and were allowed to diffuse for 24 hr. After staining with 1% Amido black, pictures were taken.

Neutralization Tests

The method described by Adams (1959) was followed for phage neutralization in BD buffer, using the antisera diluted 1/100. When membranes were added to the reaction mixture, they were preincubated in the presence of PM2 for 10 min at 28 °C. The reaction was started with the addition of antiserum and the incubation was continued at the same temperature. Plaque-forming units (PFU) determinations were done in triplicate.

Complement. A pool of lyophilized sera from guinea pigs was kept under vacuum at −20 °C.

Hemolysin. This was prepared according to Kabat and Mayer (1961), lyophilized, and kept at −20 °C.

Results

Controls on Phage Purity

The question of whether this lipid-containing phage has host cell antigens is a difficult one. To answer it properly it is necessary to check the purity of the viral preparation in quantitative terms. For this purpose BAL-31 cells were labeled and treated as described in Materials and Methods. The labeled cell fragments (8.3×10^6 cpm/mg of bacterial protein) were mixed with the phage lysate and the phage purification was continued as described in Materials and Methods. The sedimentation profile of PM2 in CsCl showed a 0.019% contamination with labeled fragments but the velocity sedimentation pattern in sucrose gradient presented 17 cpm/ml at the peak of PM2 (Fig. 3). In quantitative terms it means, that from 1.25×10^7 total cpm added as disrupted cells to the phage lysate only 0.0014% of the bacterial protein was present in the purified PM2.

To check qualitatively the purity of this phage preparation, a sample of CsCl-PM2 was extensively dialyzed against buffer BD and an aliquot of 10 µliters was put onto acrylamide gels. Electrophoresis was carried out at room temperature at 9 mAmp/gel for 2 hr; Fig. 4 is a photograph of the electrophoresis in which four protein bands are seen. This result is in agreement with the data published by Datta, Camerini-Otera, Braunstein and Franklin (1971), employing a different virus purification.

Complement Fixation Tests

Anti-membrane BAL-31 with Purified Membranes. The anti-membrane serum was able to fix complement at a dilution of 1/600, reaching a maximum

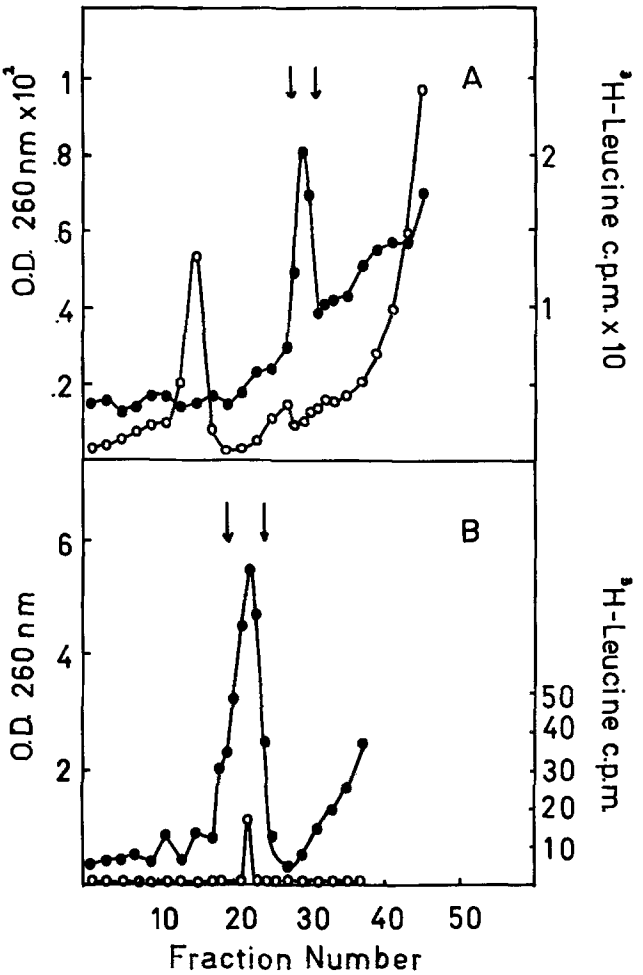

Fig. 3. (*A*) Absorbance at 260 nm of PM2 after CsCl gradient (•). Labeled cell fragments distribution in CsCl (○). (*B*) Label distribution (○) and absorbance at 260 nm profile (•) of PM2 in 8 to 22% sucrose gradient

value of 95% fixation with 0.5 μg of membrane protein (Fig. 5, dashed lines).

Anti-membrane BAL-31 with Purified PM2. Anti-membrane serum at the same dilution of 1/600 was also able to fix complement with CsCl-PM2 as antigen. A 95% complement fixation was achieved with 8 μg of viral protein (Fig. 6, dashed lines). In this experiment, 16 times more viral protein was required to obtain the same amount of complement fixation than was necessary with bacterial membranes. Both antigens, when present in higher amounts than those shown in the figures (10 and 15 μg of protein,

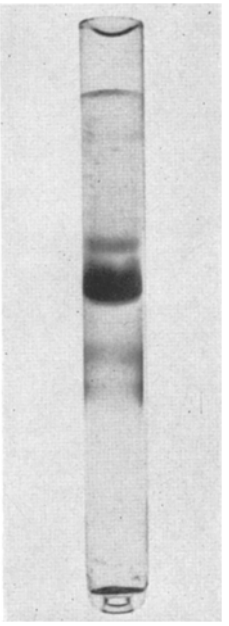

Fig. 4

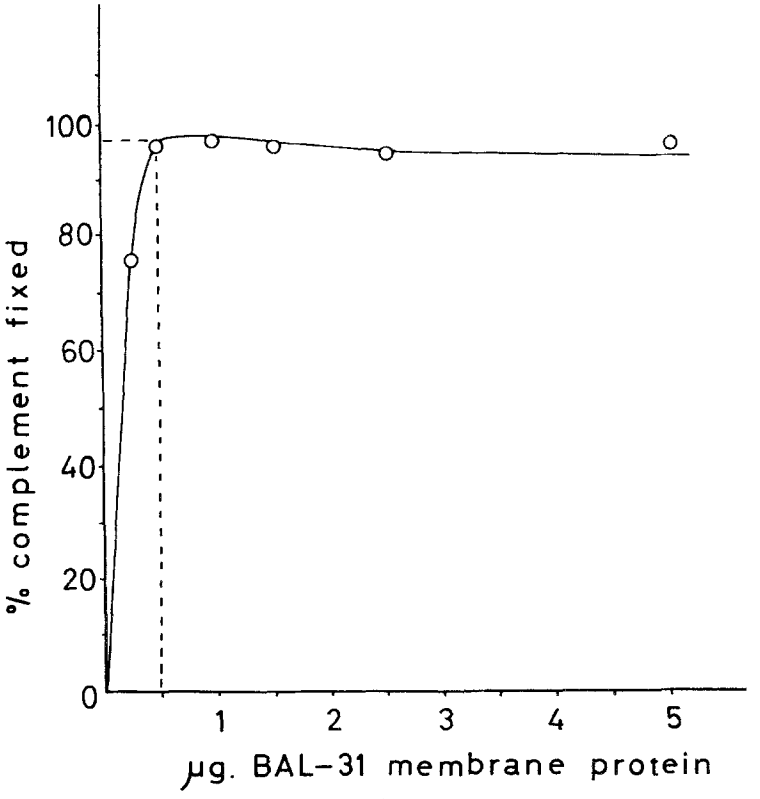
100
80
60
40
20
0
% complement fixed
1
2
3
4
5
µg. BAL-31 membrane protein

Fig. 5

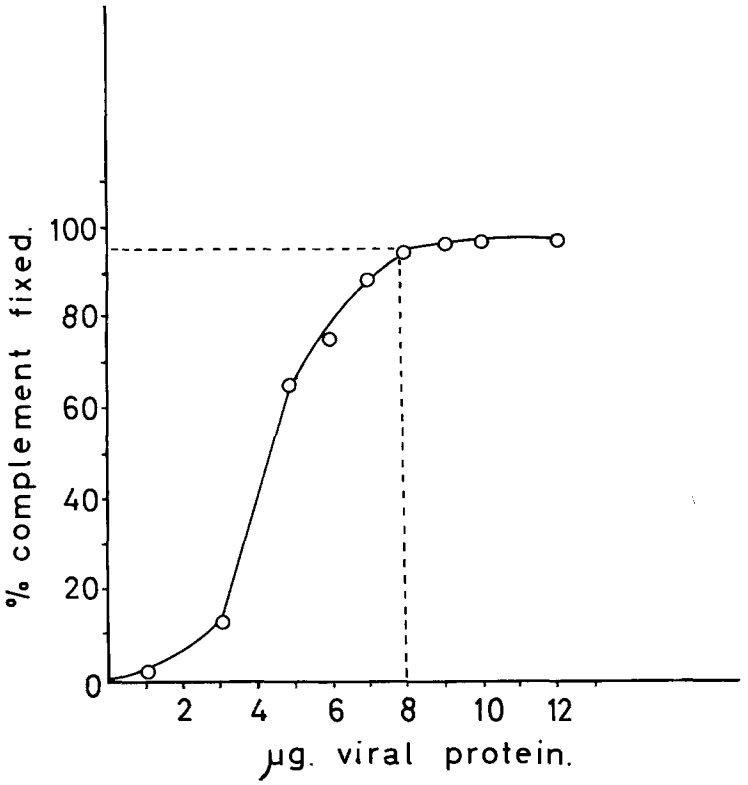

Fig. 6. Complement fixation test of anti-membrane of BAL-31 serum and bacteriophage PM2. The antibody was diluted 1/600. All other conditions were the same as in Fig. 5

respectively), produced nonspecific complement fixation in the presence of normal serum obtained by preimmunization bleeding or by pooled inactivated sera from nonimmunized animals.

Anti-PM2 with PM2. The anti-PM2 serum was able to fix complement at a dilution of 1/1000 in the presence of CsCl-PM2, reaching a maximum of 95% fixation with 0.5 µg of viral protein (Fig. 7).

Fig. 4. Electrophoresis of CsCl-PM2 structural proteins in 7.5% polyacrylamide gels Purified virus was treated with 1% sodium dodecyl sulfate and 1% 2-mercaptoethano and then subjected to electrophoresis for 2 hr at 9 mAmp/gel

Fig. 5. Complement fixation test of anti-membrane of Pseudomonas BAL-31 serum and purified membranes. The antibody was diluted 1/600. Incubation was performed at 37 °C for 1 hr and overnight at 4 °C

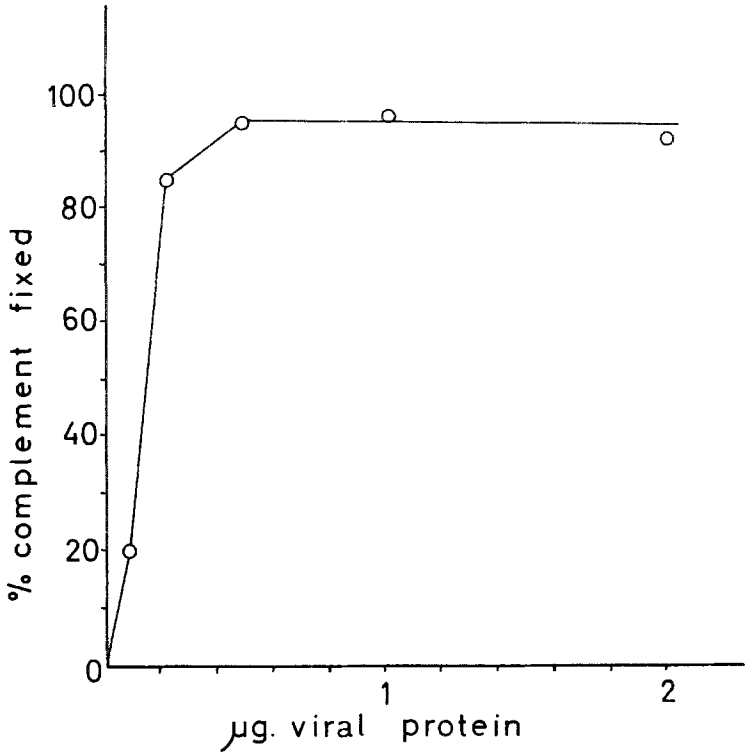

Fig. 7. Complement fixation test of anti-PM2 serum and bacteriophage PM2. The antibody was diluted 1/1000. All other conditions were the same as in Fig. 5

Immunodiffusion Experiments

Fig. 8 shows the immunoprecipitation bands that were obtained with anti-membrane serum using BAL-31 membranes and sucrose-PM2 as antigens. At least four precipitation bands are observed between membranes and anti-membrane serum, one of them showing complete fusion with the band obtained with PM2 and membrane antibody. Under these conditions no line of precipitation was obtained either with sera from preimmunization bleeding or with pooled normal guinea pig sera.

When membrane antisera were obtained by intramuscular inoculation of purified membranes mixed with complete Freund's adjuvant, no cross-reaction could be detected with purified phage.

No cross-reaction was observed when anti-PM2 was tested against both antigens, in agreement with the results of viral neutralization using this same antiserum.

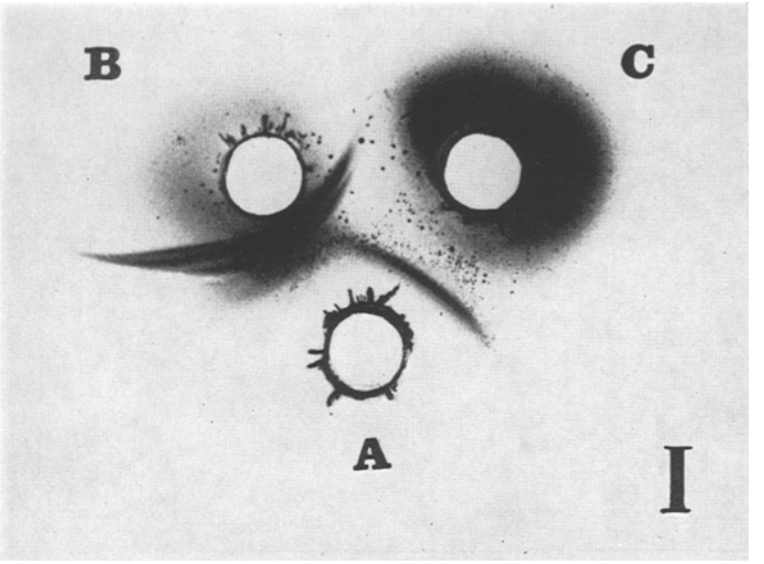

Fig. 8. Immunodiffusion. Wells: (*A*) Anti-membrane antibodies reacting with (*B*) Pseudomonas BAL-31 membranes (*C*) Bacteriophage PM2. The antigens were sonicated and located into the well. The plates were stained with 1% amido black and the picture taken

Neutralization Experiments

Although cross-reactivity between purified bacterial membranes and purified PM2 was detected by the two different methods described above, the possibility that this was due to the presence of contaminating membrane material in our bacteriophage preparation was not excluded. Therefore, we examined the capacity of anti-membrane antisera to neutralize the infectivity of PM2. The neutralizing capacity of anti-membrane sera was tested in the presence of varying amounts of membrane to see whether they compete with the phage for the neutralizing antibodies.

Anti-PM2 with CsCl-PM2. The kinetics of neutralization was studied in the absence and in the presence of a fixed amount of membrane from BAL-31. In Fig. 9, it is shown that in the absence of membrane, the amount of PM2 neutralized increased with time; after 30 min 0.8% of the input phage remained as infective bacteriophage. In the presence of 1 μg of membrane protein, the amount of free PM2 from 10 min on was approximately 2.0% higher than the control without membrane.

Anti-PM2 with CsCl-PM2 in the presence of variable amounts of membrane protein. Less neutralization of the input phage by anti-PM2 serum

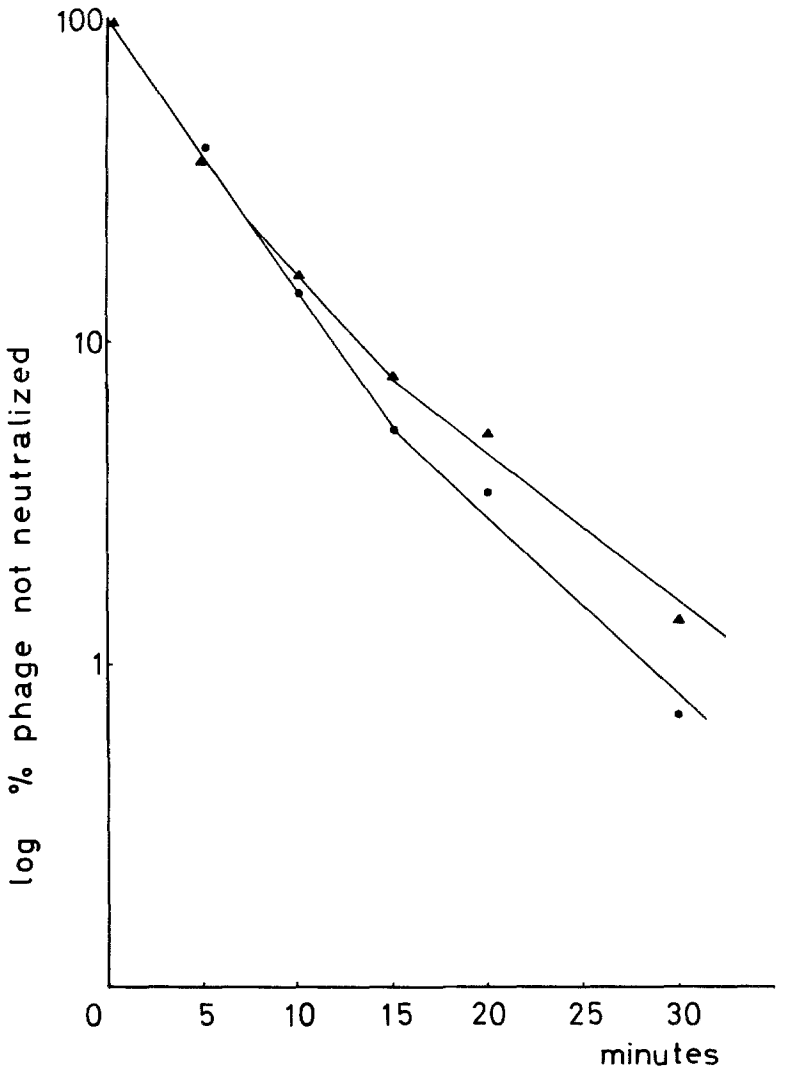

Fig. 9. Neutralization kinetics of PM2 by anti-PM2 serum in the absence (●) and in the presence of 1 μg of membrane protein of Pseudomonas BAL-31 (▲). The antibody was diluted 1/100. The incubation was performed at 28 °C; at different times an aliquot was withdrawn and treated as described in Materials and Methods to determine infective PM2

was achieved when increasing amounts of membrane were added to the incubation medium. Fig. 10 shows that in the presence of 1 μg of membrane protein the amount of remaining infectious PM2 increased 1.6% with respect to the control without membrane. Although these small differences were reproducible, they cannot be considered as significant because they are within the statistical error of the plaque-forming unit determination.

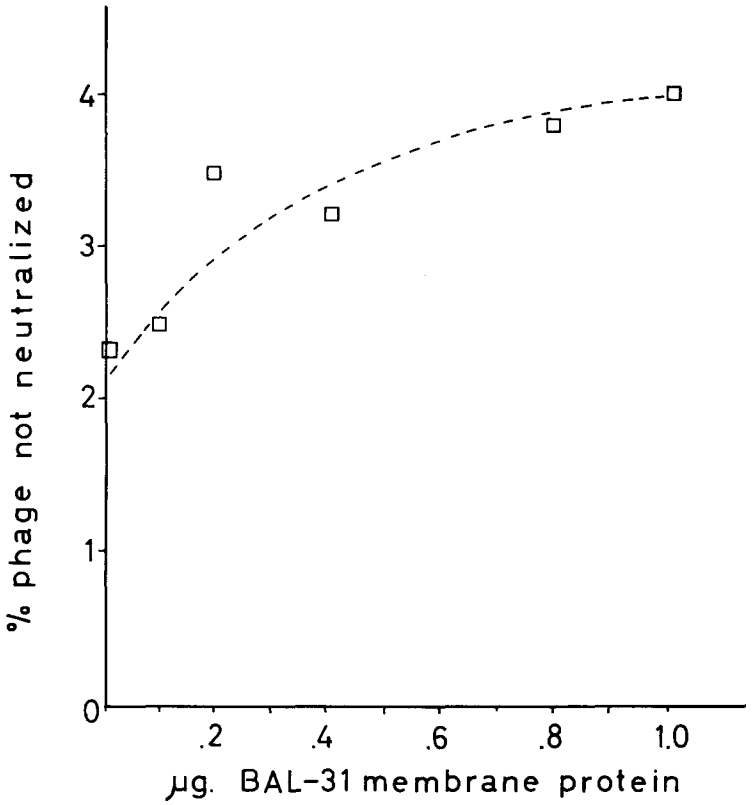

Fig. 10. Neutralization of PM2 by anti-PM2 serum in the presence of variable amounts of host cell membrane. The antibody was diluted 1/100. The mixtures were incubated for 30 min at 28 °C; the aliquots were treated as in Fig. 9

Anti-membrane serum with PM2 in the presence of variable amounts of membrane protein. In the absence of added bacterial membranes a fixed amount of anti-membrane serum neutralized 63% of the bacteriophage in 30 min. As seen in Fig. 11, when membranes were added to this incubation mixture the amount of neutralized PM2 began to decrease reaching a minimal value of about 20%, after 1.2 μg of membrane protein have been added. The addition of more membrane protein reversed the neutralization of PM2 by the anti-membrane serum, causing an increase in the amount of neutralized phage.

The same pattern was observed in all the four experiments carried out using different membrane and PM2 preparations. At each membrane concentration a control assay was performed using normal serum; no neutralization of PM2 was detected. These experiments were done using the

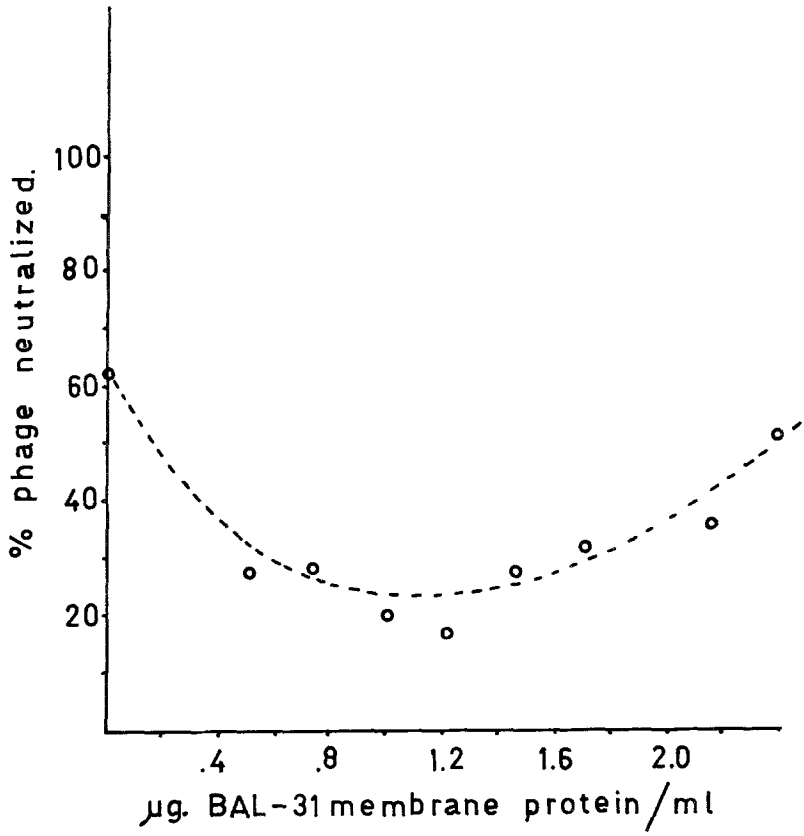

Fig. 11. Neutralization of PM2 by anti-membrane serum in the presence of variable amounts of membrane protein. The antibody was diluted 1/100. The samples were preincubated for 10 min at 28 °C. The reaction was started by the addition of the antibody and incubated for 30 min at the same temperature. An aliquot was taken from every test tube and treated as in Fig. 9

CsCl-PM2 preparation. The same results were obtained when antisera were tested against sucrose-PM2 preparations.

When anti-membrane serum and membranes were preincubated for 10 min and the reaction started by the addition of PM2, no difference was found in comparison to the experiments just described.

To examine the possible interaction between PM2 and membranes in the absence of any antibody, varying amounts of membranes and phage were incubated at 28 °C for times ranging from 10 to 40 min; no change in the number of infective units was detected. Therefore, the decrease in the neutralizing effect, observed in Fig. 10, cannot be ascribed to an interaction between membranes and bacteriophage.

Discussion

The complement fixation experiments utilizing anti-membrane serum with host cell membrane and bacteriophage as antigens, indicate that there are antigenically related structures present in phage PM2 and in the membrane of Pseudomonas BAL-31. The same conclusion can be made from the immunodiffusion experiments since there is cross-reactivity between both antigens and anti-membrane antibody.

In these two experiments a doubt about the purity of the bacteriophage preparation could be raised, but considering the fact from the isotopic dilution experiments, that a maximum of 0.019% of bacterial protein is present in the purified phage, this possibility is very unlikely. Such a small amount of contaminants would not give a detectable reaction with either one of the immunological techniques. On the other hand, the level of contamination would have been too high, in order to account for the quantitative data obtained by complement fixation (16 times more viral protein, in order to obtain the same complement fixation as with membrane protein). Such an amount of contaminants would have been easily detected by the procedures employed to study the phage purity.

If bacterial membranes are contaminating the viral preparation, as fragments independent from the virus, these could not account for the neutralization of PM2 by anti-membrane antibodies. Experiments in Fig. 11 demonstrate that the anti-membrane antibody is reacting specifically with a bacteriophage component, since up to 60% of the infective capacity of PM2 is neutralized. These data indicate that there is a common antigen in both, bacteriophage and the Ps BAL-31 membrane. The presence of bacterial membranes decreased the amount of phage neutralized by the antibody, achieving a minimum with 1.2 μg of protein membrane, as seen in the same figure. It is clear that the neutralizing capacity of anti-membrane serum increases when membranes are added in amounts higher than 1.2 μg of membrane protein.

Among the several explanations that can be advanced, we favor the idea of a spontaneous association of the phage with membranes or membrane fragments; this could mean that new antigenic-determinants are now present on the surface of the viral particle. The anti-membrane serum will show then a corresponding increase in neutralizing capacity since additional antibody of different specificity could react with the phage-membrane complex. The neutralization of PM2 by anti-membrane was not complete, and did not follow first-order kinetics, suggesting that the cross-reacting material in PM2 could be an outer component which is not directly in-

volved in the infective process and consequently is not the same antigen that reacts with the viral antiserum.

The results presented here are in contradiction with an earlier report (Truden& Franklin, 1971), in which it was concluded that no antigenic relationship existed between PM2 and its host bacterium. In those experiments antibodies were prepared by intramuscular injection of membranes; this is a crucial issue since when membranes were inoculated in this way, the antiserum obtained did not show cross-reaction by immunodiffusion or neutralization determinations, as was pointed out in Materials and Methods.

The neutralization of PM2 using anti-PM2, both in the presence and in the absence of membrane, indicated that the majority of the specific neutralizing antibodies are not responsible for the cross-reaction between virus and bacterial membrane, since the membranes can not compete with PM2 for anti-PM2. These findings could also be explained by the same arguments given above and are in agreement with the limited neutralizing capacity of anti-membrane serum and also with the immunodiffusion.

The phage spikes, which could be playing an important role in the absorption process, would be coded by the bacteriophage genome. The process of neutralization by anti-membrane serum could be due to an outer component, different from the spikes, which would be reacting with the anti-membrane serum producing a steric hindrance of the structures responsible for the infection. An alternative explanation is that anti-membrane antibodies could provoke aggregation of the bacteriophage, decreasing the actual number of infective particles.

It seems rather improbable that the phospholipid component of the bacteriophage coat could react with anti-membrane antibodies, since phospholipids are known to be poor antigens. But it could be possible to think that complement fixation and immunodiffusion reactions were due to the common phospholipids present in both bacterial membranes and bacteriophage (Braunstein & Franklin, 1971), since in the first case the reaction mixture does not have high salt concentration, which is necessary for keeping the phage structure and consequently its biological activity. The second reaction needs both antigens to be disrupted, in order to diffuse into the agarose. The neutralization reaction, on the other hand is more difficult to explain in this way, since it has been demonstrated by Harrison *et al.* (1971), that in the case of PM2 they form an inner layer, tightly packed and surrounded by the outer proteins, and the reaction was held in a medium appropriate to keep PM2 intact.

The data presented here indicate that there are common antigenic structures in the PM2 coat and in the host membrane; these results must

be corroborated by other methods. It has been reported that membrane-bound proteins in Pseudomonas BAL-31, are actively synthetized after PM2 infection (Datta, Braunstein & Franklin, 1971). Under these conditions, the authors detected new, different protein fractions; however, it remains to be established if these proteins are incorporated into the bacteriophage structure. The way by which the proteins are acquired by PM2 is still a problem that remains to be clarified in the future.

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