

Postadsorption Neutralization of Poliovirus

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Nineteen neutralizing murine monoclonal antibodies against poliovirus type 1, including representatives reacting with each of the antigenic sites on the virion, were tested for their abilities to neutralize the virus either before or after attachment to susceptible cells. All antibodies neutralized unattached virus; six had reasonable titers of postadsorption neutralization (PAN). Experiments with antibodies lacking PAN activity showed that Fc-specific rabbit anti-mouse antibodies could confer PAN activity. PAN was shown to involve the prevention of the cell-mediated conversion of virus to 135S and 80S particles. Evidence that one of the PAN-positive antibodies probably bound bivalently to preadsorbed virions, whereas a PAN-negative antibody bound monovalently, is presented. Two PAN-positive antibodies were added to an excess of virus in suspension, and only one antibody caused the virus to aggregate.

The neutralization of picornaviruses involves different mechanisms, depending on the nature and concentration of the antibody, the relative amounts of virus and antibody, and the composition of the medium. For most monoclonal antibodies (MAbs), aggregation was recognized as a major mechanism; presumably, aggregative neutralization is accomplished by the simple reduction of the number of infectious units. Most MAbs raised against poliovirus 1 (22) or human rhinovirus 14 (4) are capable of aggregation. However, aggregating antibodies may shift to a nonaggregating mechanism of neutralization when conditions are changed. For instance, when used in great excess, the antibody may cover the virions, thereby impeding cell attachment without causing aggregation (22), and at low ionic strength, the mechanism may switch to a disruptive one by which virions are reduced to empty capsids (2, 6).

Some neutralizing immunoglobulin G's (IgGs) have been shown to form stable complexes with single poliovirions (9, 11). It has been speculated that these antibodies were capable of monogamous bivalency (i.e., binding of both paratopes to a single virion), but it has not been shown at what stage of infection such antibodies were active. They might block one or more of the viral functions of attachment to cells, penetration or uncoating.

It has been recognized by Mandel (13, 14) that a reduction in the number of infective centers could still be achieved by allowing antibody to act on virus that was already attached to cells but not yet internalized (postadsorption neutralization [PAN]). In the present paper, the abilities of antisera and MAbs to effect PAN and standard neutralization (STAN) in suspension will be compared.

It will also be shown that PAN involves the inhibition of eclipse product formation, i.e., the modification of input virions to RNA-containing, but structurally and antigenically altered, 135S particles and to 80S empty capsids (7).

Neutralization of virus in suspension can be enhanced by rabbit anti-mouse IgG (sensitization; for a review, see reference 12). As will be shown, a similar effect is observed in PAN. This and other observations to be reported are compatible with the possibility that PAN requires monogamous bivalency.

MATERIALS AND METHODS

Poliovirus. Two poliovirus type 1 strains were used. Sabin-1 (LSc-2ab) was kindly provided by J. Peetermans (SmithKline Beecham Biologicals, Rixensart, Belgium), and Mahoney was provided by E. Wimmer (Department of Microbiology, State University of New York at Stony Brook). Virus labelling and purification were as previously described (7).

PAN. HEK cell monolayers (20) were grown in 55-mm petri dishes. Poliovirus (45 to 70 PFU) was added in 0.2 ml of Tris buffer (137 mM NaCl, 25 mM Tris, 5 mM KCl, 1 mM phosphate, 1 mM MgSO₄, 1 mM CaCl₂ [pH 7.2]). After 1 h at 4°C, the cells were washed three times with 5 ml of buffer to remove unadsorbed virus; after adding 0.2 ml of a 10-fold dilution of antiserum or ascitic fluid in buffer, the cells were allowed to stand for 1.5 h at 4°C and were again washed three times to remove unbound antibody. Five milliliters of the first overlay, which consisted of modified Eagle's medium, 1.1% agar, and 10% calf serum, was added. After the petri dishes were incubated for 2 days at 37°C, 5 ml of a second overlay (with 0.006% neutral red) was added. The plaques were counted the next day. The number of plaques was 45 to 70 in the controls without antibody.

PAN in the presence of anti-immunoglobulin. PAN in the presence of immunoglobulin was as described above except that after unbound antibody was washed away, 0.2 ml of rabbit anti-mouse immunoglobulin (Miles Laboratories) was added in 10-fold dilutions, and the cells were again incubated for 1 h at 4°C before the first overlay was added.

STAN. Equal volumes of virus and 10-fold dilutions of an antibody in Tris buffer were mixed and incubated for 1.5 h at 4°C. HEK cell monolayers were infected with 0.4 ml of the mixtures and incubated for 1 h at 4°C. The cells were washed three times with buffer to remove unbound virus-antibody complexes. Further treatment (overlays and plaque counting) was as described above. The number of plaques was 45 to 70 in the controls without antibody.

Postadsorptive antibody treatment and detergent lysis. To HeLa suspension cells (10⁶ cells per ml) in modified Eagle's medium, ³⁵S-labelled, purified Mahoney virus (10,000 virions per cell) was added. After 1 h at 18°C, the cells were washed twice with ice-cold medium to remove unbound virus and resuspended in the original volume (about 10% of

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TABLE 1. STAN and PAN titers of antisera against the Mahoney strain of poliovirus

Antiserum ^a	Titer (log ₁₀)	
	STAN	PAN
Rabbit	5.0	4.5
Preimmune rabbit	<1.0	<1.0
Mouse	4.0	3.0
Preimmune mouse	<1.0	<1.0
Guinea pig	3.5	2.5
Human	2.5	1.5

^a Hyperimmunized animals. Human, vaccinated adult.

the input virus adsorbed to the cells). Next, 10-fold dilutions of antibody in modified Eagle's medium were added. After 1.5 h at 4°C, the cells were again washed with medium to remove unbound antibody, resuspended in the original volume, and divided into two portions. One was incubated at 4°C and the other was incubated at 37°C for 0.5 h. The cells were again washed with ice-cold medium to remove eluted virus particles (elution amounted to less than 10% of the adsorbed virus when the cells were incubated at 4°C and to roughly 50% after incubation at 37°C). The cells were resuspended (5×10^7 cells per ml) in ice-cold phosphate-buffered saline (PBS; 0.14 M NaCl, 2.7 mM KCl, 10 mM phosphate [pH 8.0]). Nonidet P-40 was added to a final concentration of 1%, and after 10 min on ice, the cell lysate was clarified by 3 min of centrifugation at $12,000 \times g$ (only about 5% of the radioactivity was lost to the pellet).

Percentage of virus radioactivity removable from cell lysate by protein A-bearing staphylococci. To 0.08 ml of cell lysate, 0.04 ml of a 10% suspension of formaldehyde-fixed, protein A-bearing staphylococci or 0.04 ml of buffer (control) was

added, and the mixture was incubated for 1 h at 4°C. After removing the bacteria by centrifugation (2 min, $12,000 \times g$), 0.08 ml of supernatant was used for radioactivity assay. The results are expressed as percentages of the controls.

Sucrose gradient centrifugation of cell-associated virus particles after antibody treatment. The cell lysate was layered on a 15 to 30% sucrose gradient in PBS, with a 70% Nycodenz (Nycomed) cushion at the bottom of the tube. Centrifugation was for 2.5 h at $170,000 \times g_{av}$ in a MSE SW 40.6 rotor. Fractions of 0.4 ml were collected, and 0.15 ml of each was used for total radioactivity assay; to a second 0.15-ml portion, a 0.05-ml suspension of protein A-bearing staphylococci was added. After standing for 1 h at 4°C, the bacteria were pelleted (2 min, $12,000 \times g$), and 0.15 ml of the supernatant was used to determine the viral radioactivity not associated with antibodies. The amount of antibody-associated radioactivity was determined by subtraction.

RESULTS

Relative efficiency of antibodies in STAN and PAN. Polyclonal antisera (Table 1) and murine MABs from several laboratories (Table 2) were tested for their abilities to effect PAN, i.e., to reduce the number of plaques when added to cells that had already adsorbed, but not yet internalized, the virus. To this end, monolayer cells were infected, washed, and treated in the cold with serial 10-fold dilutions of the antibody. After removing unbound antibody, the cells were further treated as usual for plaque titration (see Materials and Methods). The dilution that caused 50% reduction in the number of plaques (rounded to the nearest half-log unit) was considered to represent the PAN titer of the antibody. The same antibodies were also titrated in STAN. Each antibody dilution was incubated with virus, and then plated (see Materials and Methods). Again, the dilution causing 50%

TABLE 2. STAN and PAN titers of 19 MABs

Characteristics of antibodies					Titer (log ₁₀)				PAN capacity
Antigenic site	Designation		Isotype	Immunogen	Mahoney		Sabin-1		
	No.	Reference			STAN	PAN	STAN	PAN	
1+H ^a	95	25	IgG	Synthetic peptide ^b	3.0	<1.0			—
2	35-1f4	3, 19	IgG2a(κ)	1a/S3 ^c	3.5	<1.0	3.5	<1.0	—
2+H ^a	35-1c3	3, 19	IgG2b(κ)	1a/S3	6.0	1.5	5.0	<1.0	—
2	36-5h2	3, 19	IgG3(κ)	1a/S3	2.0	<1.0			—
2	271	16	IgG	Mahoney	5.0	<1.0	3.5	<1.0	—
2	646	16	IgM	Sabin-1	4.0	<1.0	4.5	<1.0	—
2	427	16	IgM	Sabin-1	4.5	<1.0	4.5	<1.0	—
2	429	16	IgM	Sabin-1	4.5	<1.0	4.5	<1.0	—
2	4	18	IgG2b	Sabin-1	4.5	3.5	4.5	3.5	+
3A	424	16	IgG	Sabin-1	1.5	<1.0	6.0	2.5	—
3A	423	16	IgG	Sabin-1	3.0	<1.0	>5.0	2.0	—
3A	1	18	IgG2a	Sabin-1	4.5	<1.0	6.0	4.5	+
3A	9	18	IgG3	Sabin-1	<1.0	<1.0	4.5	3.5	+
3A	11	18	IgG2a	Sabin-1	3.0	<1.0	4.5	3.0	+
3A	12	18	IgG2a	Sabin-1	3.5	<1.0	4.5	3.0	+
3B	35-1g10	3, 19	IgG2a(κ)	1a/S3	3.5	<1.0			—
3B	33-5b5	3, 19	IgG2b(κ)	1a/S3	3.5	<1.0	3.5	<1.0	—
3B	35-1h2	3, 19	IgG2a(κ)	1a/S3	3.5	<1.0	3.5	<1.0	—
3B	15	18	IgG2a	Sabin-1	4.5	3.0	4.5	3.0	+

^a These antibodies bind to both H and N antigen.

^b The mice were primed with a synthetic peptide comprising amino acids 93 to 104 of capsid protein VP1 and boosted with Mahoney.

^c 1a/S3 is a Brunhilde-derived type 1 strain.

plaque reduction was considered to represent the antibody's STAN titer.

As shown in Table 1, the PAN titers of polyclonal antisera, as determined with the Mahoney strain of poliovirus, varied from $10^{1.5}$ to $10^{4.5}$. All PAN titers of sera were within 1 \log_{10} unit of their STAN titers. The results remained unchanged after decomplexation (not shown).

Table 2 lists the results obtained with 19 neutralizing MABs directed against all four antigenic sites of poliovirus and belonging to different isotypes: IgM, IgG2a, IgG2b, and IgG3. By using the Mahoney strain, 16 of the 19 MABs were unable to cause PAN (titer, <1.0). The three exceptions were the MABs 4, 15, and 35-1c3. The latter scored exceptionally high (10^6) in STAN, and its PAN titer, though measurable, was 4.5 \log_{10} units lower. On the other hand, MABs 4 and 15 had STAN and PAN titers of $10^{4.5}$ and $10^{3.5}$, respectively. These were the only MABs which resembled the polyclonal sera in having PAN titers only 1.0 to 1.5 \log_{10} units lower than their STAN titers.

Seventeen MABs were also tested for STAN and PAN titers against the Sabin strain of poliovirus type 1 (Table 2). Six MABs (MABs 1, 4, 9, 11, 12, and 15) had PAN titers comparable to their STAN titers (1.0 to 1.5 \log_{10} units lower). Two of these, MABs 4 and 15, which recognized antigenic sites 2 and 3B, respectively, neutralized the Mahoney and Sabin viruses to the same extent. The four other PAN-positive MABs (MABs 1, 9, 11, and 12), all of which recognized antigenic site 3A, caused PAN of the homologous Sabin strain only; the Mahoney strain was not neutralized under these conditions, even though it was efficiently neutralized in suspension.

The PAN-negative MABs were retested with a Brunhilde-derived 1a/S3 strain as the antigen, since some MABs were raised to this immunogen (Table 2). None was able to cause PAN, although all neutralized 1a/S3 virus efficiently in STAN (not shown).

Since all polyclonal antisera were PAN positive, and most MABs were negative, it was thought possible that the efficiency of polyclonal sera resulted either from cooperation of antibodies directed against different antigenic sites or from nonimmunoglobulin serum components. However, when mixtures of up to four MABs, representing all antigenic sites, were tested for PAN against the Mahoney virus, they yielded uniformly negative results, even when preimmune mouse serum was added (not shown).

In summary, all polyclonal sera tested were PAN positive. With MABs, three different situations were encountered: (i) the PAN activity of 13 of the 19 MABs against Mahoney and Sabin-1 virus was feeble or undetectable; (ii) four MABs, all derived from Sabin-1 and belonging to antigenic site 3A, efficiently neutralized the homologous strain, but not Mahoney; and (iii) two MABs, also Sabin-1 derived but belonging to sites 2 and 3B, neutralized the Mahoney and Sabin-1 viruses equally well.

Enhancement of PAN by anti-immunoglobulin antisera. The effect of anti-immunoglobulin antibodies on PAN was investigated with Mahoney virus and two well-characterized IgG2a(κ) MABs which lacked PAN activity. The usual postadsorption incubation with the MAB was itself followed by incubation with the anti-immunoglobulin antisera (see Materials and Methods).

The IgG2a-specific antiserum caused a dose-dependent enhancement of PAN (Table 3). At the 1:10 dilution, it raised the PAN titer of both MABs to $10^{3.5}$ or $10^{4.0}$, i.e., to near their STAN titers (Table 2). The anti-IgG2b, anti- κ , and anti- λ sera failed to enhance PAN, and none of the anti-

TABLE 3. Enhancement of PAN titer by anti-immunoglobulin antisera

Primary antibody		Anti-immunoglobulin antiserum		PAN titer of MAB (\log_{10})
MAB	Isotype	Specificity	Dilution factor (\log_{10})	
35-1f4	IgG2a(κ)	Anti-IgG2a	1	3.5
		Anti-IgG2a	2	3.0
		Anti-IgG2a	3	1.5
		Anti-IgG2a	4	1.5
		Anti-IgG2b	1	1.5
		Anti- κ	1	<1.0
		Anti- λ	1	<1.0
		None		<1.0
35-1h2	IgG2a(κ)	Anti-IgG2a	1	4.0
		Anti-IgG2a	2	1.5
		Anti-IgG2a	3	1.0
		Anti-IgG2a	4	<1.0
		None		<1.0
None		Anti-IgG2a	1	<1.0
		Anti-IgG2b	1	<1.0
		Anti- κ	1	<1.0
		Anti- λ	1	<1.0

immunoglobulin antisera caused PAN by itself. In conclusion, both PAN-negative MABs became efficient neutralizers when aided by anti-immunoglobulin antisera directed at their Fc part (the anti- κ antiserum had no effect, possibly because the epitopes on the antibody's light chains were inaccessible in virus-bound antibody). These results prove that the PAN-negative MABs had attached to the virus, further suggesting that the inability of most MABs to effect PAN was probably not for lack of binding to preadsorbed virus.

Negative correlation between the PAN capacity of antibodies and their release as a result of virus modification. HeLa suspension cells were incubated with ^{35}S -labelled Mahoney virus at 18°C . At this temperature, the virus is adsorbed, but not internalized, so that it remains accessible to antibody (10). After washing, antibody was allowed to attach to the preadsorbed virus at 4°C ; the cells were further incubated for 0.5 h, either at 4 or 37°C , and finally lysed with detergent. The percentage of antibody-carrying particles was then determined with protein A-bearing staphylococci (see Materials and Methods). The results are shown in Table 4.

When the final incubation of the cells was at 4°C (i.e., in the absence of any virus modification), essentially all the radioactivity was removable by the protein A-bearing organisms in an antibody-mediated process, showing that all particles had picked up antibody.

When the final incubation was at 37°C , the results depended on the PAN capacity of the antibody. With PAN-negative MABs, only about 30% of the radioactivity was still removable, meaning that the remainder had lost the antibody. Since the MABs used in these experiments were N specific, this result suggested that 70% of the preadsorbed viral particles had lost their N antigenicity during the incubation at 37°C , consistent with their modification to eclipse particles (7). When PAN-positive antibodies were used, essentially all the viral particles still carried antibody after incubation at 37°C , suggesting that their modification had been blocked by the antibody.

In agreement with literature data (14), elution was not

TABLE 4. Negative correlation between the PAN capacities of antibodies and their release from preadsorbed Mahoney virus

Antibody ^a	PAN capacity ^b	% of particles carrying antibody ^c after final incubation of cells at:	
		4°C	37°C
MAb 35-1f4	—	90	28
MAb mixture ^d	—	95	33
MAb 4	+	89	88
Rabbit antiserum	+	95	89
Mouse antiserum	+	92	82
None		8	0

^a A total of 0.005 ml of ascitic fluid or antiserum per 10⁶ cells.^b Against Mahoney virus (see Tables 1 and 2).^c Viral radioactivity removable from cell lysate by protein A-bearing staphylococci.^d MAbs 95, 35-1f4, 423, and 33-5b5 plus preimmune mouse serum.

enhanced by the antibody (results not shown); thus, PAN cannot be explained by the loss of virus-antibody complexes from the cells. It was also observed that the virions penetrated normally into the cells, even though laden with PAN-positive antibody (unpublished data).

Binding of MAb 4 to virions and eclipse products. The results exposed above suggested a link between PAN and virion modification. However, when experiments were carried out to test this hypothesis, the interpretation of the results was complicated by the slower sedimentation of virions with attached antibody. This retardation sometimes caused intact virions to co-sediment with 135S eclipse particles; therefore, a method had to be devised to distinguish between native virions and eclipse products sedimenting at similar velocities.

In one experiment, virus was adsorbed to HeLa cells for 0.5 h at 37°C to allow formation of 135S and 80S eclipse particles (7), and the cell extract was analyzed by sucrose gradient centrifugation. To a portion of each gradient fraction MAb 4 was added, and then protein A-bearing bacteria were added. The profiles in Fig. 1 show the particles that could be immunoprecipitated with MAb 4 (open circles) and those that couldn't (closed circles). As expected, 135S particles were not recognized by the antibody, which is strictly N specific. In this and all similar experiments, a small amount of viral material collected on the high-density cushion (fraction 28), probably because of incomplete extraction of the viral particles from the cells.

In another experiment, ³⁵S-labelled virus was incubated *in vitro* in the cold, either with or without a 1,600-fold molar excess of MAb 4 (Fig. 2). In the antibody-free control (Fig. 2A), the virus peak reached the expected 160S location, and none was removable by protein A-bearing organisms. When the virus had been reacted with MAb 4 (Fig. 2B), all virions had taken up antibody (open circles), and the peak had shifted from fraction 20 to 17.

As this result shows, particles that have been in contact with MAb 4 and sediment in the 135 to 160S region may be interpreted as virions, provided they can be removed by protein A-bearing staphylococci; on the other hand, particles which sediment at the 135S position and are not so removable may be interpreted as the 135S eclipse product.

Inhibition of virion modification. To examine the link between PAN and the inhibition of virion modification, MAb 4 (PAN positive) was tested for its effect on the formation of

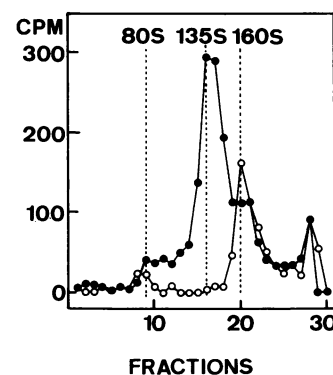


FIG. 1. Lack of binding of MAb 4 to 135S eclipse particles. HeLa cells were incubated for 0.5 h at 37°C with ³⁵S-labelled Mahoney virus, washed, and detergent lysed. The lysates were analyzed by sucrose gradient centrifugation. A portion of each gradient fraction was used for total radioactivity assay. To a second 0.15-ml portion, 0.02 ml of the 10⁻¹ dilution of MAb 4 was added for 1 h at 4°C and then protein A-bearing staphylococci for 0.5 h at 4°C. The bacteria were pelleted, and the amount of radioactivity thus removed was computed by subtraction. Open circles, particles immunoprecipitable by MAb 4; closed circles, not precipitable.

eclipse products. Virus was preadsorbed to HeLa cells at 18°C, and the cell suspension was divided into two portions. One received MAb 4, and the other served as a control without antibody. After 1.5 h at 4°C and washing, the cells

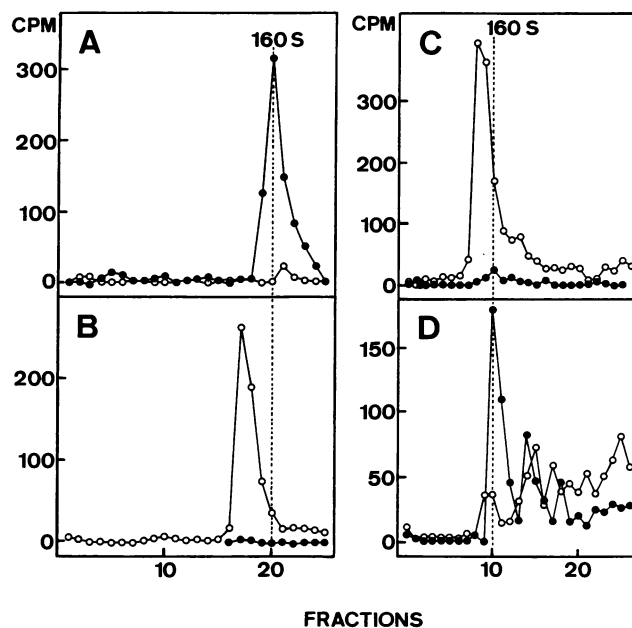


FIG. 2. Sedimentation profiles of virus-antibody complexes. In the first experiment, 50 pM ³⁵S-labelled Mahoney virus was incubated for 1.5 h at 4°C without antibody (A) or with 80 nM MAb 4 (B) and then subjected to sucrose gradient centrifugation for 2.5 h at 170,000 × g_{av}. In a second, independent experiment, 0.12 pM ³⁵S-labelled virus (C) or a mixture of 0.12 pM ³⁵S-labelled plus 0.96 pM unlabelled Mahoney virus was incubated for 1.5 h at 4°C with 2 nM MAb 15 and then subjected to sucrose gradient centrifugation for 1.25 h at 170,000 × g_{av}. For each gradient fraction, the amount of radioactivity of the particles with (open circles) and without (closed circles) antibody was determined with protein A-bearing bacteria.

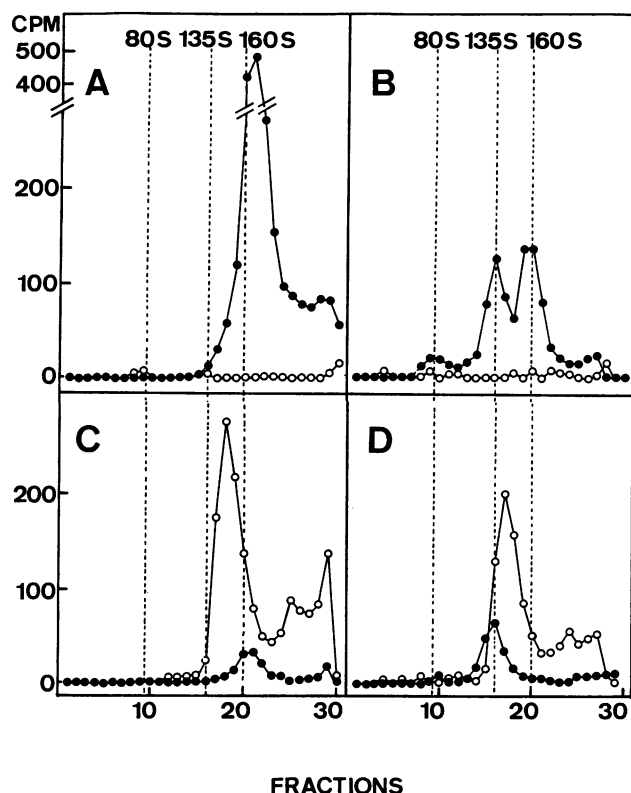


FIG. 3. Effects of MAb 4 (PAN positive) on the formation of eclipse particles. HeLa cells preadsorbed for 1 h at 18°C with ^{35}S -labelled Mahoney virus were further incubated for 1.5 h at 4°C without antibody (A and B) or with 0.37 pmol of MAb 4 (0.005 ml of ascitic fluid) per 10^6 cells (C and D). The cells were washed and further incubated for 0.5 h at 4°C (A and C) or at 37°C (B and D). After again washing the cells, lysates were analyzed by sucrose gradient centrifugation. For each gradient fraction the amount of radioactivity bound (open circles) or unbound (closed circles) to antibody was determined with protein A-bearing staphylococci.

were further incubated, either at 4 or 37°C, and their lysates were analyzed by sucrose gradient centrifugation.

The results without antibody are shown in Fig. 3A and B. No eclipse products were formed when the final incubation of the cells was at 4°C; the radioactivity formed a single peak of unmodified 160S virions with some aggregates (Fig. 3A). When the cells had been incubated at 37°C (Fig. 3B), roughly half of the virions were modified to 135S and a few 80S particles (the total radioactivity was also reduced owing to elution).

Figures 3C and D show the result of treating the preadsorbed virus with MAb 4. After incubation at 4°C (Fig. 3C), only a tiny amount of radioactivity consisted of 160S virions without antibody (closed circles); the bulk of the virions now carried antibody and peaked at fraction 18 instead of 20.

Our interpretation of Fig. 3D is as follows: the peak at fraction 17 (open circles) consisted of antibody-carrying, unmodified virions, and the smaller peak of antibody-free particles at fraction 16 (closed circles) consisted of 135S particles. Comparison with Fig. 3B shows that MAb 4 inhibited the formation of 135S particles.

Three antisera (mouse, rabbit, and human [Table 1]) and the PAN-positive MAb 15 (Table 2) caused a similar shift in the sedimentation rate of the virions and a similar reduction

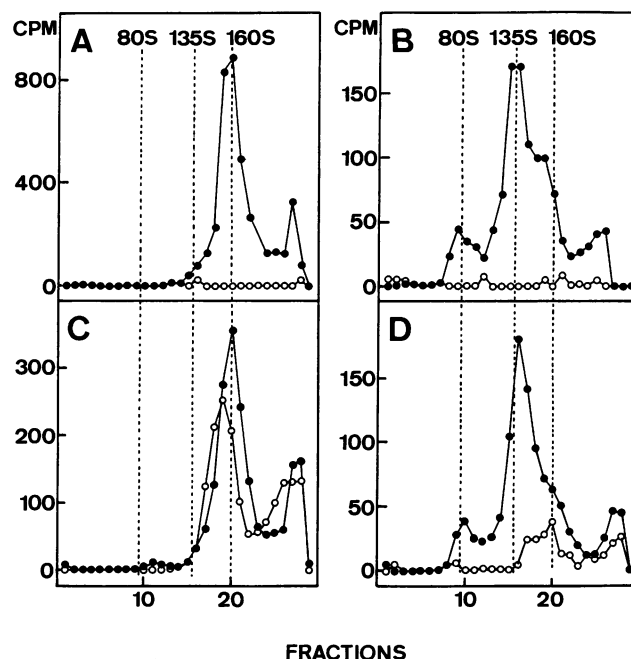


FIG. 4. Effects of MAb 35-1f4 (PAN negative) on the formation of eclipse particles. Except for the choice of antibody, all procedures were as described in the legend to Fig. 3. A total of 0.05 ml of ascitic fluid per 10^6 cells was used. (A) No antibody, final incubation at 4°C; (B) no antibody, final incubation at 37°C; (C) MAb 35-1f4, final incubation at 4°C; (D) MAb 35-1f4, final incubation at 37°C. Open circles, particles carrying antibody; closed circles, particles devoid of antibody.

in the formation of 135S material (not shown). It should be noted that MAb 15 differs from MAb 4 by its ability to cause aggregation. When in excess, MAb 15 binds to single virions (Fig. 2C), but when the virus supply is increased, it causes the formation of virion oligo- and polymers (Fig. 2D).

The experiment was repeated with the PAN-negative MAb 35-1f4 at the same neutralization capacity (10 times more ascitic fluid was used, since the STAN titer of 35-1f4 was 1 log₁₀ unit lower than that of MAb 4 [Table 2]). The results are shown in Fig. 4. In the absence of antibody, about 65% of the virions were modified to 135S and 80S particles during the 37°C incubation (Fig. 4B). Following treatment with MAb 35-1f4 and incubation at 4°C (Fig. 4C), approximately half of the virions carried antibody, and the peak of these antibody-carrying particles was shifted from fraction 20 to 19. During incubation at 37°C, there was extensive virion modification to 135S and 80S (Fig. 4D), as in the control without antibody (Fig. 4B), showing that the PAN-negative MAb 35-1f4 failed to inhibit the formation of eclipse products. Similar results were obtained with three other PAN-negative MAbs (MAbs 95, 423, and 33-5b5) and with a mixture of four PAN-negative MAbs (MAbs 95, 35-1f4, 423, and 33-5b5) and preimmune mouse serum (results not shown).

To summarize, all polyclonal sera and the two PAN-positive MAbs tested (MAbs 4 and 15) inhibited virion modification to the 135S particles, whereas PAN-negative MAbs lacked this effect.

Relationship between the amount of antibody bound to preadsorbed virions and the inhibition of their modification. The large shift in the sedimentation velocity of virions with

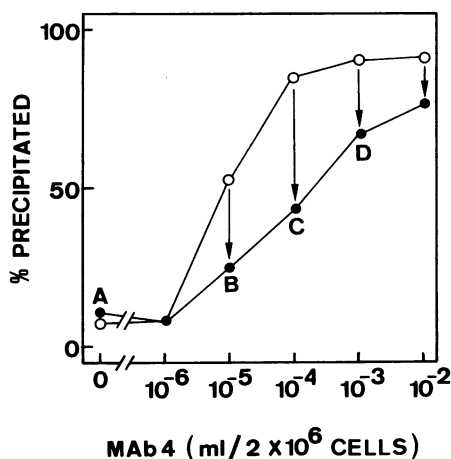


FIG. 5. Immunoprecipitation of infected cell lysates by MAb 4. HeLa cells preadsorbed for 1 h at 18°C with ³⁵S-labelled Mahoney virus were incubated for 1.5 h at 4°C with 10-fold dilutions of MAb 4 or without antibody. The cells were washed and further incubated for 0.5 h at 4°C (open circles) or at 37°C (closed circles). After the cells were washed again, lysates were prepared, and a sample was submitted to protein A-aided immunoprecipitation. The amount of antigen precipitated was expressed as percent input radioactivity. The concentration of MAb 4 in undiluted ascitic fluid was 7.3 μM. A to D, lysates selected for further analysis (see legend to Fig. 6). Arrows, loss of antibody-carrying particles due to modification at 37°C.

attached PAN-positive antibodies suggested postadsorption binding of many antibody molecules per virion. It was questioned whether this abundance was required to inhibit virion modification. Tenfold dilutions of MAb 4 were allowed to attach to preadsorbed virus; after removal of unbound antibody, the cells were further incubated for 0.5 h, either at 4 or 37°C, and the percentage of antibody-carrying particles was determined (Fig. 5). When the cells had been incubated at 4°C (open circles) with 10⁻⁵ ml of ascitic fluid per 2 × 10⁶ cells (corresponding to 30 pM specific antibody and to 7.5 molecules per preadsorbed virion), about half of the virions carried antibody; this proportion rose to 90% when 10 times more antibody was given.

When the final incubation of the cells had been at 37°C, the proportion of antibody-carrying particles was reduced because of virion modification (Fig. 5, arrows). At the lower antibody concentrations (points B and C), roughly 50% of the virions was modified; but with 10⁻³ ml of ascitic fluid (point D), this proportion was reduced to 25%. It is concluded that 100 times more antibody was needed to block conversion to eclipse products at 37°C than to render virions removable by protein A-bearing bacteria. Assuming that a single bound antibody molecule makes a virion removable, then a greater number of antibody molecules appears to be required to prevent its modification.

Selected lysates were analyzed by sucrose gradient centrifugation (Fig. 6). In the control without antibody, roughly 60% of the virions were modified to 135S and 80S eclipse particles (Fig. 6A). This proportion did not change much when 10⁻⁵ or 10⁻⁴ ml of MAb 4 was used (Fig. 6B and C), but was severely reduced by 10⁻³ ml (Fig. 6D), in agreement with the results presented in Fig. 5. Note that the peak position of the antibody-laden native virions shifted from fraction 19 to 16 as the amount of input antibody was increased; this supports our interpretation that these peaks

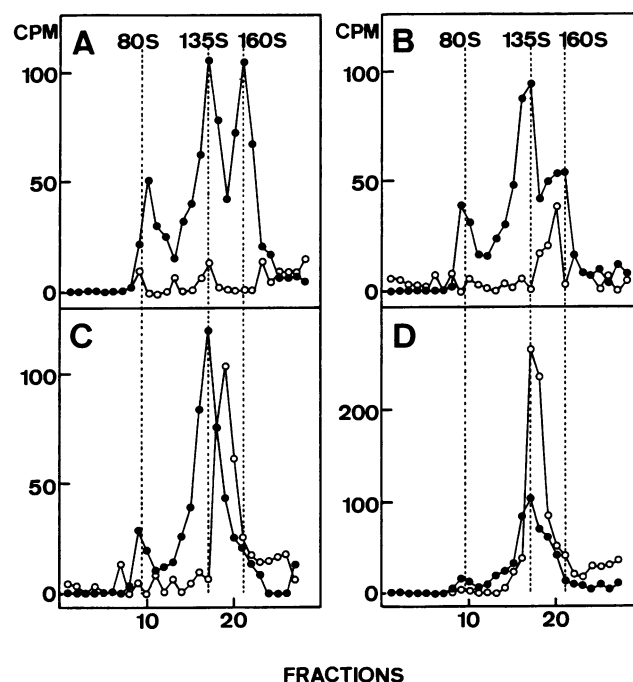


FIG. 6. Dose dependence of the inhibition of eclipse particles formation by MAb 4. Lysates were prepared from cells incubated at 37°C (A to D in Fig. 5) and analyzed by sucrose gradient centrifugation. (A) no antibody; (B) 5 × 10⁻⁶ ml of MAb 4 as ascitic fluid per 10⁶ cells; (C) 5 × 10⁻⁵ ml/10⁶ cells; (D) 5 × 10⁻⁴ ml/10⁶ cells. Open circles, particles carrying antibody; closed circles, particles devoid of antibody.

represented native virus with increasing amounts of antibody molecules attached.

Monovalent and apparent bivalent binding of MAbs to preadsorbed virus. The well-known preference of antibody 35-1f4 for bigamous binding (1, 21) suggests that this antibody might attach itself to preadsorbed virions by only one paratope, leaving the second available to catch a second virion. On the other hand, it was expected that the nonaggregating MAb 4 (11) would be unable to catch a second virion. The following experiment was designed to test the ability of the two antibodies to catch challenge virus when attached to preadsorbed virions.

After ³⁵S-labelled virus was preadsorbed to HeLa cells, one portion of the cells received MAb 35-1f4 and the other received MAb 4. After incubation at 4°C and removal of unbound antibody, ³H-labelled challenge virus was added. The cells were washed, and their lysates were analyzed. The results obtained with MAbs 35-1f4 and 4 are shown in Fig. 7A and B, respectively. In both panels, the profile of the challenge ³H-virus comprised a peak at the 160 S location of native virus. This peak presumably represented virus that had adsorbed to unoccupied cellular receptors. The preadsorbed ³⁵S-virus also showed a peak corresponding to monomeric virions. With MAb 35-1f4 (Fig. 7A), this peak was retarded by only half a fraction, but with MAb 4 the displacement was by two fractions, consistent with the more abundant attachment of MAb 4.

A large amount of doubly labelled aggregates was found only with MAb 35-1f4 (Fig. 7A, fractions 23 to 28). Since these aggregates were doubly labelled, they must have been formed by joining preadsorbed ³⁵S- and challenge ³H-viri-

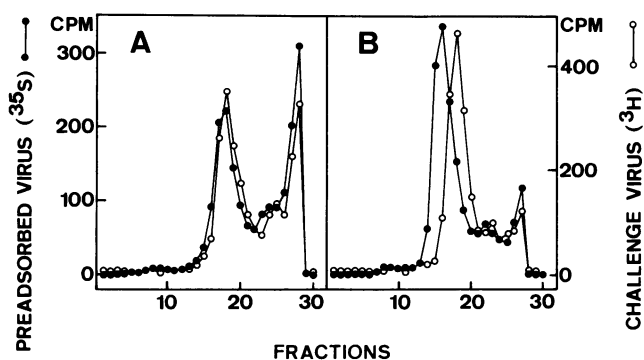


FIG. 7. Binding of challenge virus by MAbs attached to preadsorbed virus. HeLa cells preadsorbed for 1 h at 18°C with ^{35}S -labelled Mahoney virus were further incubated for 1.5 h at 4°C with MAb 35-1f4 (A; 0.05 ml/10⁷ cells) or with MAb 4 (B; 0.005 ml/10⁷ cells). The cells were washed, and 30 pM [^3H]uridine-labelled Mahoney virus was added for 1.5 h at 4°C. After the cells were washed again, lysates were analyzed by sucrose gradient centrifugation. Closed circles, ^{35}S radioactivity of preadsorbed virus; open circles, ^3H radioactivity of bound challenge virus.

ons. Other interpretations are possible, but unlikely. The presence of ^3H in the aggregates may be explained by the hypothetical release of antibody previously attached to preadsorbed virus, which then caused aggregation of challenge virus. However, since the aggregated material was doubly labelled, the formation of ^3H aggregates must have been accompanied by the formation of a similar amount of ^{35}S aggregates, and this can be excluded; the formation of ^{35}S aggregates would indeed require the release of a sizeable amount of preadsorbed virus, and at 4°C the release of cell-associated virus is negligible.

In conclusion, after attaching to preadsorbed virus, MAb 35-1f4 retained the ability to bind challenge virus, which implies at least transient monovalent binding. The contrasting result obtained with MAb 4 probably means that monogamous bivalency was permanent with this antibody.

DISCUSSION

Four polyclonal antisera (Table 1) neutralized cell-adsorbed virus, in agreement with Mandel's (13) original observations; 6 of 19 MAbs also demonstrated reasonable PAN titers (Table 2). These PAN-positive antibodies recognized three of the four antigenic sites and belonged to three different isotypes (IgG2a, IgG2b, and IgG3). On the other hand, all six PAN-positive antibodies were raised in the same laboratory with Sabin-1 as the immunogen. More extensive analysis will show whether the observed distribution is due to chance or whether the production of PAN-positive MAbs depends on some as-yet-unidentified factors.

When cells with preadsorbed virions are incubated at 37°C, the 160S virions are converted to 135S and 80S particles (7). All PAN-positive antibodies blocked this cell-mediated modification. At least with MAb 4 this inhibition was dose dependent (Fig. 6).

Three factors appear to be important in PAN: (i) the concentration of the antibody, (ii) the strength of the bond between paratope and epitope, and (iii) whether the binding of the antibody to the virion is mono- or bivalent.

The concentration effect is illustrated by the fact that even three antibodies which were classified as PAN negative (35-1c3, 423, and 424) effected PAN when used in vast

excess; these three antibodies had extremely high STAN titers, and their PAN titers were 3.5 log₁₀ units or more lower (Table 2).

The importance of the strength of the bond between paratope and epitope is demonstrated by Sabin-1-derived antibodies belonging to antigenic site 3A (MAbs 1, 9, 11, and 12 [Table 2]). There is an amino acid difference between the Sabin-1 and the Mahoney strains in this antigenic site (18). One of the Sabin-1-derived MAbs (MAb 9) was so strain specific that it failed to neutralize Mahoney in STAN. With the three other MAbs (MAbs 1, 11, and 12), the lower bond strength to Mahoney was reflected in a lowering of their STAN titers by 1.0 to 1.5 log₁₀ units, but all three had PAN activity only against the Sabin strain. Therefore, the PAN titer of an antibody may be more strongly affected by bond strength than its STAN titer.

The requirements for PAN were further studied mainly with three MAbs (MAbs 35-1f4, 4, and 15) with different characteristics. Antibody 35-1f4 is exclusively an aggregator; when used at normal ionic strength it causes virion aggregation without intrinsic damage, and even a vast excess of antibody (10⁴ molecules per virion) fails to suppress aggregation, as it does with most other MAbs (21, 22). Antibody 35-1f4 thus derives its STAN capacity from bigamous binding.

In contrast, MAb 4 binds abundantly to monomeric Mahoney virus; it is probably incapable of bigamous binding, since it did not cause virion aggregation at any molar ratio (11). MAb 15 combines the abilities to bind abundantly to single virions and to cause aggregation, depending on the antibody/virion ratio (Fig. 2C and D) (8). A similar behavior was reported for MAb 1c (22).

When attached to preadsorbed virus, MAb 35-1f4 retained the ability to bind challenge virus (Fig. 7). Thus, when this antibody was denied the possibility of bigamous binding, as in PAN, one paratope remained free. In contrast, the PAN-positive MAb 4 did not bind challenge virus. This observation must be interpreted jointly with this antibody's inability to induce aggregation. Both features can be explained in two different ways: (i) this antibody's preference for monogamous, bivalent binding, i.e., its ability to exploit bivalent binding to its full energetic advantage (5) and (ii) the MAb's incapacity for bigamous binding. For sterical reasons, bigamous binding to large spherical objects, such as poliovirus virions, requires that the antibody's Fab segments form a rather large angle (at least 120°, according to a rough calculation, assuming that the Fab segments bind normally to the virion's surface). It is theoretically possible that the antibody's hinge region lacks the required flexibility for bigamous binding, but since recent crystallographic evidence suggests great freedom of movement at the hinges (8), this explanation seems improbable.

Monogamous bivalency thus remains the least contrived explanation of our observation that MAb 4, once attached to a preadsorbed virion, fails to bind challenge virus. This mode of binding should ensure strong interaction with the virion (5), and such interaction may, in turn, be required for PAN.

The observation that MAb 35-1f4 achieves PAN when helped by anti-immunoglobulin antibody directed at its Fc segment may also be tentatively interpreted as a requirement for bivalent binding.

The PAN-positive MAbs do not appear to act by reducing virus uptake or by increasing elution; they may act by stabilizing the viral capsid or by obstructing some essential interaction with a cellular structure. According to our find-

ings with MAb 4, the chance that a virion's modification to 135S will abort depends on the amount of attached antibody, though no precise figures can be given. Interestingly, STAN experiments with MAb 4 showed that 6 to 10 molecules of antibody per virion were capable of neutralization to 1/e in the absence of aggregation (11). These facts suggest that a similar mechanism may underlie STAN and PAN by nonaggregating antibodies.

Wetz (24) showed that antisera against poliovirus type 1 were capable of protecting the virus against the effects of incubation at 37°C in hypotonic buffer and proposed that antibody-mediated stabilization of virion structure might be an important mechanism of neutralization. This paper extends these results in a biologically relevant situation with individual MAbs.

At least three different mechanisms are known for neutralization of picornaviruses before contact with cells. The first is aggregation, a nondestructive bundling mechanism known to operate at poliovirus concentrations of less than 10^3 PFU/ml (21); the second is virion disruption, a mechanism that may be triggered by subnormal ionic strength (2, 6) or supranormal temperature (5a); and the third is the covering of virions with a layer of antibody, so as to prevent their adsorption to host cells (4, 14). These mechanisms require only the antibodies' Fab functions, but their antiviral role in vivo is complemented by the Fc-dependent removal of immune complexes. The enhancement of protection by Fc-dependent functions has been particularly well studied in foot-and-mouth disease (15).

A virion that succeeds in penetrating the first line of humoral defenses, but happens to carry PAN-positive antibody, even though it attaches to and enters a host cell, may prove unable to uncoat; thus, PAN may be seen as a late intervention of humoral immunity to prevent the establishment of infection. Finally, even after the onset of poliovirus infection, antibodies may act to prevent cell death (23).

The contribution of PAN to antiviral defense is unknown, but the presence of high PAN titers in all polyclonal poliovirus antisera suggests that it may be important. PAN probably operates with many other viruses, even though it has been little studied. A recent study with human cytomegalovirus also demonstrated the possibility to neutralize preadsorbed virus and the existence of both PAN-positive and PAN-negative MAbs (17).

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