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Virus Aggregation as the Cause of the Non-neutralizable Persistent Fraction

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The non-neutralizable or persistent fraction of virus populations has been found to be caused by aggregated virus. Detailed investigation was performed with the prototype strain of echovirus type 4 (Pesascek), as this virus is notorious for its large non-neutralizable fraction. When Pesascek virus was clarified by low-speed centrifugation, homologous antiserum hardly neutralized the virus. However, when the virus was filtered through membranes having a porosity only twice the diameter of the virus, monodispersed virus was obtained which was efficiently neutralized. Serum titers were up to 1,000 times higher if the neutralization test was carried out with monodispersed virus. Virus in non-neutralizable aggregates was found to constitute 30% of the infective units of unfiltered Pesascek virus but only 0.1% of the antigenically related DuToit strain. This explains why DuToit strain has been a more satisfactory indicator strain for detecting type 4 antibodies, regardless of the echo 4 strain used for inducing the antibodies. Clarified suspensions and ultrafiltrates of viruses belonging to the picorna-, reo-, myxo-, adeno-, herpes-, and poxvirus groups were studied. Clarified suspensions yielded persistent fractions of 0.005% for poliovirus, of 0.1% for reovirus, of 0.6% for influenza virus, of <0.001% for adenovirus, of 0.06% for herpesvirus, and of 10 to 30% for vaccinia virus. In all cases the persistent fractions were removed by membrane filters which had a pore diameter no larger than twice that of the virus under test, and the high concentration of virus in each ultrafiltrate was completely neutralized by antiserum.

Thirty years ago, Burnet and his colleagues (5) showed that a significant fraction of vaccinia and myxomatosis virus could not be neutralized by antiserum. In 1953, working with poliovirus, Ledinko and Melnick (14) described the "breakthrough" phenomenon as the delayed appearance of virus infectivity in cultures that had received virus and an equivalent amount of antibody. Subsequently, a number of investigators reported persistent fractions for a variety of viruses, and attributed this insensitivity to antibodies to: (i) a distinct, nonsusceptible viral population (7, 16, 20), (ii) dissociation of the virus-antibody complex (8), and (iii) a nonavid antibody (13).

Viruses of several different groups were reinvestigated, but, because the non-neutralizable fraction is reported to be greater with strains of echovirus type 4 than with other viruses, such strains were investigated in greater detail. Ever since it was first isolated (17), Pesascek virus was found to be difficult to neutralize. However, antisera that yielded either low or negative titers by tube neutralization tests were found to yield titers about 100-fold higher by tests involving 80 to 90% plaque reduction (10). Even with only 50 to 100 plaque-forming units (PFU) as challenge virus, complete plaque reduction was almost never achieved. In tube neutralization tests, Pesascek antiserum that failed to neutralize homologous virus readily neutralized a related strain (DuToit), indicating that the antiserum was potent (2, 10, 12, 15, 25, 30).

Attempts to explain the echovirus type 4 breakthrough phenomenon have been made. The possibility of dissociation of the virus-antibody complex was investigated, but Pesascek serum was found to be bound to Pesascek virus to the same degree as it was to DuToit (25). Even though antibody was bound, scarcely any of the virus was neutralized. Barron and Karzon (2) suggested that the neutralization breakthrough occurred because echovirus 4 was present as two antigenic variants, only one of which could be neutralized. However, from the studies to be reported, it now appears that the persistent fraction in populations of echovirus consists of virus aggregates which are non-neutralizable. Similar observations were made with poliovirus, reovirus, herpesvirus, influenza virus, and vaccinia virus.

MATERIALS AND METHODS

Monkey kidney (MK) cells. Kidneys from immature rhesus and green monkeys were trypsinized. Kidney cells were grown in Melnick's medium A and maintained in medium B (18).

Viruses and their assays. Two plaque-purified strains of echovirus type 4 were used: prototype Pesascek and the related DuToit strain. A stock of each virus was grown in a single lot of rhesus MK cells maintained in medium B. Viruses were harvested when 50% of the cells in the infected cultures showed cytopathic changes. The harvest was centrifuged at 3,000 rev/min for 20 min, and the clear supernatant fluid was frozen at −40 C until used. The titers of stocks used in these studies were 10^6 PFU/ml for both strains.

Other viruses were plaque-purified strains of virulent poliovirus: type 1, Mahoney; type 2, MEF 1; and type 3, P24. Attenuated strains were Sabin's plaque-purified lines as used in the oral poliovaccine (type 1, LSc; type 2, P712; and type 3, Leon). Other virus groups were represented by plaque-purified stocks of vaccinia virus (WR strain), herpesvirus (KOS strain), reovirus 1 (strain 716), adenovirus (SV15), and a plaque-producing strain of influenza virus type B (23). These viruses were grown and assayed in green MK cells, with the exception of the reovirus, which, like the echoviruses, was grown and assayed in rhesus MK cultures.

All viruses were assayed by the PFU method. Overlay medium consisted of Earle's salt solution, 0.4% $NaHCO_3$, 0.1% skim milk, 1:60,000 neutral red, and 1.5% agar (Difco). To enhance plaque formation of Pesascek virus, 1 mM cysteine was used in the overlay (28). For other enteroviruses, 25 mM $MgCl_2$ was included in overlay medium (28); for reovirus, 1:60 Oxoid pancreatin (27); and for adenovirus, influenza virus, and herpesvirus, 400 μg of protamine sulfate per ml (23, 24).

Neutralization tests. After 1 hr of incubation at 37 C, the virus-serum mixtures were placed in an ice-water bath (0 C) until they were assayed (within 30 min). Subsequent treatments of the mixtures are described under Results.

Filtration through membranes (26). To filter echoviruses through 50-mμ membranes, virus adsorption had to be avoided; this was achieved by pretreating the membranes with nutrient medium. However, the medium contains components which coat membranes and prevent subsequent virus adsorption, and it must first be clarified through other membranes to prevent clogging of the virus-filtering membranes. To prevent adsorption of the membrane-coating components to the clarifying membranes, fetal calf serum was diluted 10-fold in distilled water (as in hypotonic solution the essential components do not adsorb to membranes) before it was used for pretreatment of the membranes.

In practice, 100 ml of 10% fetal calf serum in distilled water was filtered at 25 psi through a 90-mm AP 20 clarifying pad and then in series through 0.3-, 0.22-, 0.1-, and 0.05-μ membranes (Millipore Corp., Bedford, Mass.). To the final filtrate, 10× Earle's saline was added in sufficient volume to restore isotonicity. This filtrate was then capable of coating but not clogging membranes to be used for virus filtration. Three membranes with pore sizes of 0.22, 0.1, and 0.05 μ were placed in a single 25-mm holder and treated with 10 ml of the coating solution. After the solution passed through the membranes, the membrane was washed with 5 ml of tris(hydroxymethyl) aminomethane (Tris) buffer to remove residual serum. Virus was then passed through the membrane. The void volume was colorless, so that collection was started only when the phenol red of the virus suspension was first observed in the filtrate.

RESULTS

Neutralization tests with unfiltered and filtered echovirus type 4 strains. Based on previous titrations of the stocks, Pesascek and DuToit strains were diluted to contain 200 PFU per 0.1 ml and mixed with equal volumes of hyperimmune horse serum (9) that had been serially diluted in Tris buffer. After 1 hr of incubation at 37 C, samples were assayed for residual virus by the plaque method. Pesascek and DuToit viruses were passed through a series of membranes to yield 50-mμ filtrates, which were mixed with serum and assayed as described above. In a duplicate experiment, the same dilutions of virus and serum were inoculated into tube cultures to determine cytopathic end points in fluid medium. When a picornavirus with diameters of 25 to 30 mμ is filtered through a membrane with a 50-mμ pore diameter, only those virions in a monodispersed state can pass. Thus, the ultrafiltrate will be referred to as monodispersed virus.

From the results of these experiments (Tables 1 and 2), it is evident that the non-neutralizable fraction of Pesascek virus is in the form of aggregated units of virus. Unfiltered Pesascek virus in the small dose of 120 PFU was not completely neutralized by homologous serum in the plaque reduction tests at a dilution as low as 1:10, whereas the monodispersed virus was completely neutralized at a dilution of 1:640. When an 80% plaque reduction was used as the end point, the serum had a titer of 640 against unfiltered virus, but a titer of 10,240 with monodispersed virus. If monodispersed virus preparations are used for comparison, Pesascek and DuToit viruses behave in precisely the same fashion. The results of the tube neutralization tests presented a similar picture. The reference hyperimmune horse serum had been reported as failing to neutralize the Pesascek virus which had been used as immunizing antigen (9). For this reason, the DuToit strain had to be employed as an indicator strain in measuring type 4 antibodies. However, once the aggregates had been removed from the Pesascek stock, it could be used as effectively as DuToit for measuring type 4 antibodies.

Effects of residual serum on neutralization tests. It would appear that virus in aggregates can

replicate even if antibody is bound to the aggregate. If the cell infected with such an antibody-virus aggregate releases virus particles into a cellular environment containing residual free antibodies, the progeny virus would be largely neutralized. Ledinko and Melnick (14) showed that the breakthrough phenomenon occurred with poliovirus only after cultures were changed to fresh medium, thus reducing the concentration of residual serum. Clark and Tyrrell (6) reported that high dilutions of serum gave neutralization end points similar to those of low dilutions (when both concentrations were in excess), if the cultures were washed after the virus-antibody adsorption period. If the cultures were not washed, the lower dilutions of serum gave higher neutralization end points, because the residual free antibodies prevented progeny virus from spreading the infection in the culture.

To explore the effect of residual serum, the following experiment was carried out. A neutralization test was performed with Pesascek virus as described for Table 1 above. After adsorption of the virus-serum mixtures on monolayers, half of the cultures were washed three times with nutrient medium, and the agar overlay was added to all the cultures, washed and unwashed. Readings were made on the 4th and 6th days. The results of this test are shown in Table 3. With unfiltered virus, when residual serum was removed from cultures after adsorption of the virus-antibody complex, the previ-

TABLE 1. *Cross-neutralization tests with monodispersed virus*[a]

Virus	Serum	Type of virus	Virus activity (PFU/0.1 ml)							Plaque reduction		
			Tris buffer control	In presence of serum dilutions								
				10	40	160	640	2,560	10,240	49,960	100%	90%	80%
Pesascek	Pesascek	Unfiltered	120	7	12	21	24	62	79	99	<10[b]	40	640
		Monodispersed	92	0	0	0	0	2	10	44	640	2,560	10,240
	DuToit	Unfiltered	120	14	6	19	28	37	92	100	<10	40	160
		Monodispersed	92	0	0	0	0	12	29	75	640	640	10,240
DuToit	Pesascek	Unfiltered	101	0	0	0	0	8	39	90	640	2,560	2,560
		Monodispersed	111	0	0	0	0	3	49	65	640	2,560	2,560
	DuToit	Unfiltered	101	0	0	0	0	14	17	55	640	640	640
		Monodispersed	111	0	0	0	0	0	17	31	2,560	2,560	10,240

[a] Test read on 6th day.
[b] Reciprocal of serum end point.

TABLE 2. *Cross-neutralization with monodispersed virus*[a]

Virus	Serum	Type of virus	TCD_{50} in tests	Serum titer
Pesascek	Pesascek	Unfiltered	750	<10
			75	80
		Monodispersed	600	640
			60	10,240
	DuToit	Unfiltered	750	<10
			75	40
		Monodispersed	600	640
			60	2,560
DuToit	Pesascek	Unfiltered	500	640
			50	2,560
		Monodispersed	520	640
			52	2,560
	DuToit	Unfiltered	500	640
			50	2,560
		Monodispersed	520	2,560
			52	10,240

[a] Test read on 6th day.

ously recognized breakthrough of Pesascek virus (top line) was very pronounced. With monodispersed virus, there was no difference between the results in washed and unwashed cultures, indicating total neutralization of the original challenge virus. In fact, no neutralization was evident in the washed cultures even on the 4th-day reading, with unfiltered virus.

Measurement of persistent fraction. The following experiment was performed to determine the quantity of non-neutralizable aggregates that were present in the stock preparation. Undiluted Pesascek and DuToit viruses, both unfiltered and monodispersed preparations, were each mixed with an equal volume of undiluted homologous serum. After incubation at 37 C for 1 hr, the virus-serum mixtures were assayed. Cultures were washed three times with nutrient medium to remove residual serum just prior to overlay, so as to favor the detection of progeny virions. The results of this experiment are shown in Table 4. Stock Pesascek virus was decreased in titer in the presence of homologous serum by 0.6 log, indicating that about 25% of the virus population was non-neutralizable, whereas 5.6 log units of dispersed virus were completely neutralized. Stock DuToit virus was decreased in titer 3.0 log by homologous serum, and again the monodispersed virus was completely neutralized. Thus, unfiltered DuToit virus contains only 0.1% non-neutralizable virus. This would not be detected when 100 PFU or 100 TCD_{50} are used in neutralization tests.

Estimation of size and number of non-neutralizable Pesascek aggregates. The following experiments were performed to determine the size of non-neutralizable aggregated virus. They also provided data on the level of the persistent fraction after the virus was passed through a series of membranes of different porosities.

The virus was diluted to contain 200 PFU/0.1 ml in Tris buffer. Part was mixed with an equal volume of Tris buffer and another part with 1:1,000 Pesascek serum. After 1 hr of incubation at 37 C, the samples were plated. A duplicate sample of 200 PFU/0.1 ml of virus in Tris was filtered through a pretreated 0.45-μ membrane; a part of the 0.45-μ filtrate was mixed with the antiserum and another part with Tris as described above. The remainder of the 0.45-μ filtrate was passed through a pretreated 0.3-μ membrane, and the 0.3-μ filtrate was treated with antiserum and Tris. Serial filtrations were carried out through pretreated 0.22-, 0.1-, and 0.05-μ membranes.

To determine whether the decrease in PFU by each filtration was due to adsorption of virus to the pretreated membranes, the 0.05-μ filtrate was serially passed through two more freshly pretreated membranes and treated with antiserum or Tris. The results of the assays are shown in Table 5. The unfiltered stock contained 36% of its activity in the non-neutralizable fraction. After filtration through the membranes, virus was decreased linearly with a cumulative loss of 32% of the virus after filtration through the 0.05-μ membranes. The filtrates showed that, as aggregates were removed, the persistent fraction was gradually decreased. The total amount of virus that could be removed by filtration was 32%, and the original level of the persistent fraction divided by the unfiltered stock ranged from 25% (Table 4) to 36% (Table 5).

TABLE 3. *Effects of residual serum on plaque reduction tests*

Pesascek virus	Type of virus	Reciprocal of serum end point at 90% plaque reduction	
		4th day	6th day
Unwashed cultures	Unfiltered	160	<10
	Monodispersed	2,560	2,560
Washed cultures	Unfiltered	<10	<10
	Monodispersed	2,560	2,560

Recovery of non-neutralizable aggregates from membranes. The next experiment was performed in an attempt to recover the non-neutralizable aggregates from the membranes. Undiluted Pesascek virus was filtered through treated membranes of 0.22-, 0.1-, and 0.05-μ porosities to yield monodispersed virus. The membranes were washed with 5 ml of Tris buffer, and this washing was assayed. The membranes were carefully removed from the filter holder, inverted, and placed in a fresh filter holder. The membranes were back-washed by forcing 5 ml of Tris buffer through the inverted membranes (in 0.05-, 0.1-, and 0.22-μ order) to recover virus that had been retained on them. Samples of the unfiltered virus, the filtrates, the straightforward washing, and fluid from the back-washing of the filtrates were treated with Tris buffer or 1:10 antiserum. After 1 hr of incubation at 37 C, the samples were plated. Before overlay, cultures were rinsed three times with nutrient medium to remove residual antibodies. The results are shown in Table 6.

Unfiltered virus was only reduced by 0.4 log with 1:10 antiserum, but monodispersed virus was completely neutralized. Virus recovered by back-washing the inverted membranes again contained a large persistent fraction, for it was only decreased in titer by 0.6 log in the presence of antiserum.

TABLE 4. *Measurement of persistent fraction*

Virus	Type of virus	Treatment[a]	Log PFU/ml	Non-neutralizable fraction
Pesascek	Unfiltered	Tris	5.9	
		Pesascek serum	5.3	0.25
	Monodispersed	Tris	5.6	
		Pesascek serum	0.0	0.0000
DuToit	Unfiltered	Tris	6.2	
		DuToit serum	3.2	0.001
	Monodispersed	Tris	6.1	
		DuToit serum	0.0	0.0000

[a] Virus plus equal volume of Tris buffer (control) or undiluted antiserum. Held 1 hr at 37 C and then assayed.

TABLE 5. *Estimation of size and number of non-neutralizable Pesascek aggregates*

Virus	PFU/0.1 ml		Fraction PFU retained by filter (cumulative)[a]	Non-neutralizable fraction in each filtrate
	In Tris buffer	In 1:1,000 serum		
Unfiltered	117	42		(0.36)
Filtrates				
0.45 μ	104	20	0.09	0.19
0.3 μ	95	16	0.19	0.17
0.22 μ	91	10	0.22	0.11
0.1 μ	84	2	0.28	0.02
Filtrate, 0.05 μ				
1st	82	0	0.30	0.00
2nd	82	0	0.30	0.00
3rd	79	0	0.32	0.00

[a] Obtained from column of data in assay of filtrates of virus in Tris buffer without serum.

TABLE 6. *Recovery of non-neutralizable aggregates by back-washing membranes*[a]

Pesascek virus	Log PFU/ml	
	Tris	Pesascek serum, 1:10
Unfiltered	6.3	5.9
Filtrate of 0.22-, 0.1-, and 0.05-μ membranes	6.0	0.0
Washing of membranes with buffer	4.7	0.0
Back-washing of 0.05-, 0.1-, and 0.22-μ membranes	5.6	5.0

[a] In 0.22-, 0.1-, and 0.05-μ series.

Adsorption of antibody to non-neutralizable aggregates. The following experiment was performed to determine whether aggregated virus binds antibody, even though the virus is not neutralized in the process. Virus-antibody mixtures were filtered through a 50-mμ membrane, and were assayed for free (excess) antibody in the filtrates. Stock Pesascek virus was diluted threefold in Tris buffer and mixed with an equal volume of Pesascek serum used in excess at a dilution of 1:10. Controls without serum were included. After 1 hr of incubation at 37 C, part of each sample was assayed. As shown in Fig. 1, the remainder of each of the two samples was filtered through a 50-mμ membrane, and the filtrates were assayed. To test for free antibody, the filtrate of the stock virus-serum mixture was then serially diluted in Tris buffer, and each dilution was mixed with an equal volume of monodispersed virus to yield a final concentration of 50 PFU/0.1 ml. To demonstrate that the aggregate had remained in the filter, the 50-mμ membrane used to filter the stock virus-serum mixture was removed from the filter holder, placed in a mortar, and homogenized with alundum. The homogenate was taken up in 5 ml of 10% bovine fetal serum in medium B and assayed.

A similar experiment was carried out with monodispersed virus (left part of Fig. 1). Monodispersed virus (50-mμ filtrate) was mixed with an equal volume of Pesascek serum diluted 1:10; the virus was incubated for 1 hr at 37 C and then assayed. A control without serum was included. The virus-serum mixture was filtered through a freshly prepared 50-mμ membrane, and the filtrate was assayed. As above, this virus-serum filtrate was then serially diluted in Tris, mixed with monodispersed virus, and assayed to measure free antibody. Again, the 50-mμ membrane used for filtration of the virus-serum mixture was obtained, homogenized, and assayed. The amount of excess antibody in the stock virus-serum mixture was one-fourth the amount of free antibody in the filtrate of the monodispersed virus-serum mixtures. This is shown by the fact that the 100% plaque reduction end point of the stock virus-

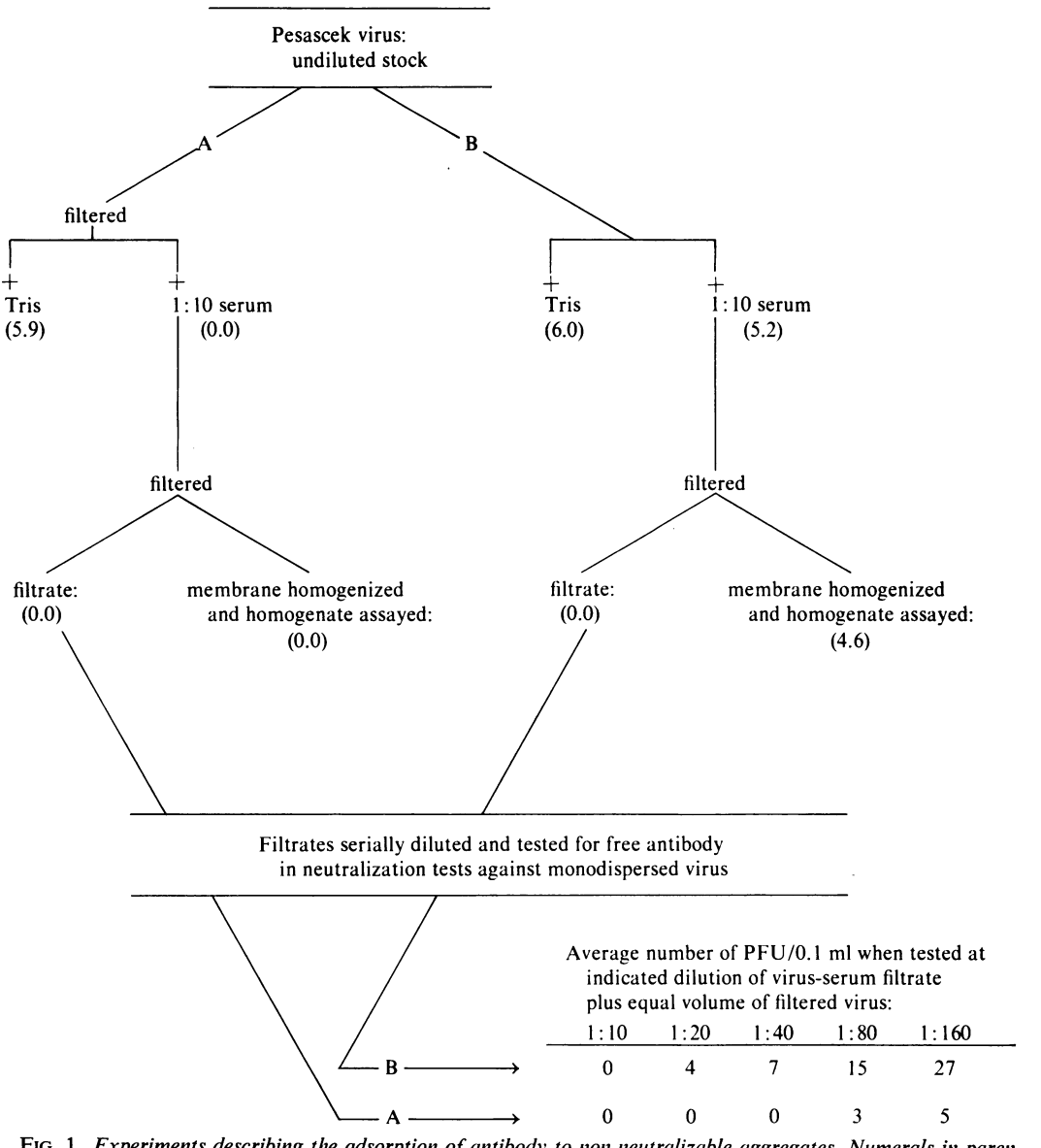

FIG. 1. *Experiments describing the adsorption of antibody to non-neutralizable aggregates. Numerals in parentheses indicate* $\log_{10}$ *titer (PFU/ml). Filtered virus was used in the neutralization tests (calculated dose, 50 PFU/0.1 ml; dose found in test, 46 PFU/0.1 ml), in tests A and B at the bottom of the figure.*

serum filtrates yielded a value of 1:10, as compared with 1:40 for the monodispersed virus-serum filtrate.

Genetic nature of Pesascek virus. The following experiment was performed to determine whether monodispersed virus particles bred progeny that, like DuToit, was neutralizable without filtration. Undiluted virus and an 0.05-μ filtrate were each inoculated into MK cultures, and after 48 hr (75% cytopathic effects) the progeny were harvested and neutralization tests were performed. Progeny virus derived from the unfiltered parent and from the monodispersed seed behaved alike. Both harvests were non-neutralizable unless they were filtered through 0.05-μ membranes. However, monodispersed virus of both harvests (100 PFU) was readily neutralized to the same degree by Pesascek serum (end point about 1:10,000).

Persistent fraction of polioviruses. Another subgroup of the picornaviruses was investigated in view of the thorough studies that had been carried out with them by Dulbecco et al. (7) and by

Mandel (16). Undiluted, unfiltered type 1 poliovirus (Mahoney) and 10-fold dilutions of the virus were mixed with equal volumes of undiluted or diluted hyperimmune horse serum (9), or with Tris buffer. Undiluted virus was filtered through an 0.05-μ membrane and also treated with antisera. Neutralization tests were carried out as described above. The results of such a series of tests are shown in Fig. 2.

Neutralization with monodispersed virus yielded a first-order inactivation curve, whereas the unfiltered virus manifested 3 to 4 logs of persistent virus. In this curve, undiluted monodispersed virus was compared to 10^{-1} unfiltered virus in the various dilutions of immune serum, since filtration of the virus caused the titer to decrease to one-third of its value with unfiltered virus. Experiments with other polioviruses (type 1, LSc; type 2, P712 and MEF 1; and type 3, Saukett and Leon) gave similar results; however, only those for Saukett are tabulated in Table 7, where the results on viruses belonging to different groups are listed.

Persistent fraction of other viruses. Similar neutralization tests were carried out with vaccinia virus, herpesvirus, reovirus, influenza virus, and adenovirus. The chief variation in the experiments with these larger viruses was that membranes of larger porosity were used to permit the viruses to pass through the pores (pore sizes are listed in the footnotes of Table 7).

The filtered sample of vaccinia virus was completely neutralized by 1:10 antiserum, whereas the unfiltered virus persisted with 4.2 log virus even in the presence of undiluted serum. The persistent fraction of vaccinia virus was reported as early as 1937 (5), and the tendency of vaccinia virus to aggregate has also been well documented (22).

Similarly, reovirus could not be completely neutralized unless passed through a proper-sized membrane. Reovirus has also been examined in an electron microscope and shown to contain a significant fraction of aggregates (29). Similarly, herpesvirus and influenza virus could be completely neutralized only if the viruses were filtered through proper-sized membranes. In contrast to the other viruses, adenovirus was completely neutralized without prior filtration of the virus. Thus, it is likely this virus strain is not significantly aggregated, or that aggregates of this virus are inactivated by antibody.

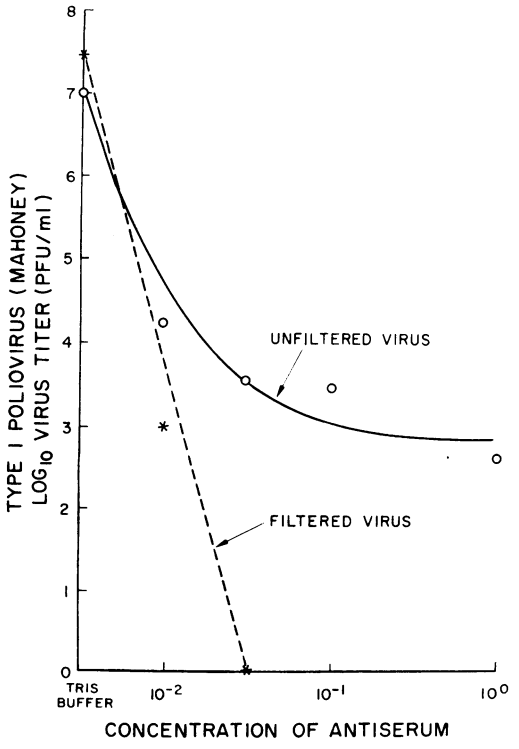

FIG. 2. *Removal of the persistent fraction of poliovirus by filtration through a membrane of 0.05 μ average pore diameter.*

Separation of mixed virus suspensions. In view of the results presented above, the separation of mixtures of viruses by neutralization was reinvestigated. Echovirus (type 4 Pesascek) and poliovirus (type 1 Mahoney) were mixed together in amounts of 10^6 and 10^4 PFU/ml, respectively, since these concentrations, when inoculated into cultures individually, produced 50% cytopathic effect on the 2nd day. Passage of the echovirus, the poliovirus, and the mixture were made, and first harvests were examined. Tenfold dilutions of filtered and of unfiltered virus were mixed with 1:10 homotypic serum, 1:10 heterotypic serum, and a mixture in which each serum was present at a final concentration of 1:10. Tris buffer was used as a control.

As shown in Fig. 3, effective neutralization occurred only when the viruses had been filtered through 0.05-μ membranes. Serial passage of the first-harvest filtered samples treated with both echovirus and poliovirus serum failed to produce cytopathic effects even after blind passage. First harvest of the mixed virus suspension, when filtered and treated with only poliovirus serum, was serially transferred two times, and the third passage upon filtration was completely neutralized by echovirus serum. Similarly, the first-harvest mixture that was filtered and treated with only echovirus serum yielded a third harvest which, after filtration, was completely neutralized by polioserum. When the tests were carried out with

TABLE 7. *Persistent fractions of other viruses*

Viruses	Concn of virus used	Log virus titer (PFU/ml) after incubation with Tris buffer or antiserum				Non-neutralizable fractions based on undiluted serum
		Tris	Antiserum			
			Undiluted	1:10	1:100	
Vaccinia						
Unfiltered	Undiluted	6.0	5.0	5.1	5.7	0.10
	10^{-1}	5.0	4.2	4.0	4.7	0.16
Filtered[a]	Undiluted	4.9	0.0	0.0	3.1	0.00000
Reovirus						
Unfiltered	Undiluted	7.2	5.1	5.3	6.8	0.01
	10^{-1}	6.2	3.9	3.8	5.6	0.005
Filtered[b]	Undiluted	6.7	0.0	2.0	3.7	0.000000
Influenza						
Unfiltered	Undiluted	5.1	2.5	2.7	3.1	0.003
Filtered[c]	Undiluted	5.0	0.0	0.0	0.0	0.00000
Adenovirus						
Unfiltered	Undiluted	6.7	0.0	2.1	3.5	0.000000
	10^{-1}	5.7	0.0	0.0	0.0	0.000000
Filtered[b]	Undiluted	6.0	0.0	0.0	0.0	0.000000
Type 3 poliovirus						
Unfiltered	Undiluted	8.0	3.7	3.6	4.5	0.00005
	10^{-1}	7.0	3.0	3.7	4.2	0.0001
Filtered[d]	Undiluted	7.8	0.0	2.0	2.5	0.0000000
Herpesvirus						
Unfiltered	Undiluted	7.1	3.7	3.5	5.2	0.0004
Filtered[e]	Undiluted	7.0	0.0	0.0	2.1	0.0000000

[a] Filtrate, 0.65 μ.
[b] Filtrate, 0.22 μ.
[c] Filtrate, 0.3 μ.
[d] Filtrate, 0.05 μ.
[e] Filtrate, 0.45 μ.

unfiltered virus, the results were in doubt, for even the mixture of both antisera failed to neutralize the harvest; even when a single serum was used, one could not be certain that the recovered virus was not a mixture containing heterotypic virus and a persistent fraction of the homotypic virus.

Deaggregation and loss of the persistent fraction. Methods known to break up virus aggregates were tested to determine whether such treatments would decrease the non-neutralizable fraction. Stock Pesascek virus was treated as follows: (i) filtered through an 0.05-μ membrane as described above, (ii) treated in a Raytheon sonic oscillator at 20 kc/sec for 5 min, or (iii) treated with 1% trypsin for 2 hr at 37 C. A portion of the suspension was also centrifuged at 12,000 $\times$ *g* for 2 hr—a force that is not high enough to sediment monodispersed virus, but one that should cause large aggregates to be sedimented. Test material was taken from the top third of the tube. Each sample, including an untreated control, was then diluted 1:3 in Tris buffer and mixed with an equal volume of Tris buffer or 1:10 Pesascek serum. After an incubation period of 1 hr at 37 C, the samples were assayed for infectious units. The results of this test are shown in Table 8.

TABLE 8. *Persistent fraction of Pesascek strain of echovirus 4 after various treatments*

Virus treatment	Log virus titer (PFU/ml) after incubation with Tris or antiserum		Non-neutralizable fraction
	Tris buffer	1:10 Pesascek serum	
Unfiltered stock virus	6.0	5.5	0.30
Plus sonic treatment	6.0	5.4	0.25
Plus centrifugation	6.2	3.5	0.002
Plus trypsin digestion	5.7	3.4	0.005
Plus filtration through 0.05-μ membrane	6.1	0.0	0.000000

As described above, the stock virus had a high

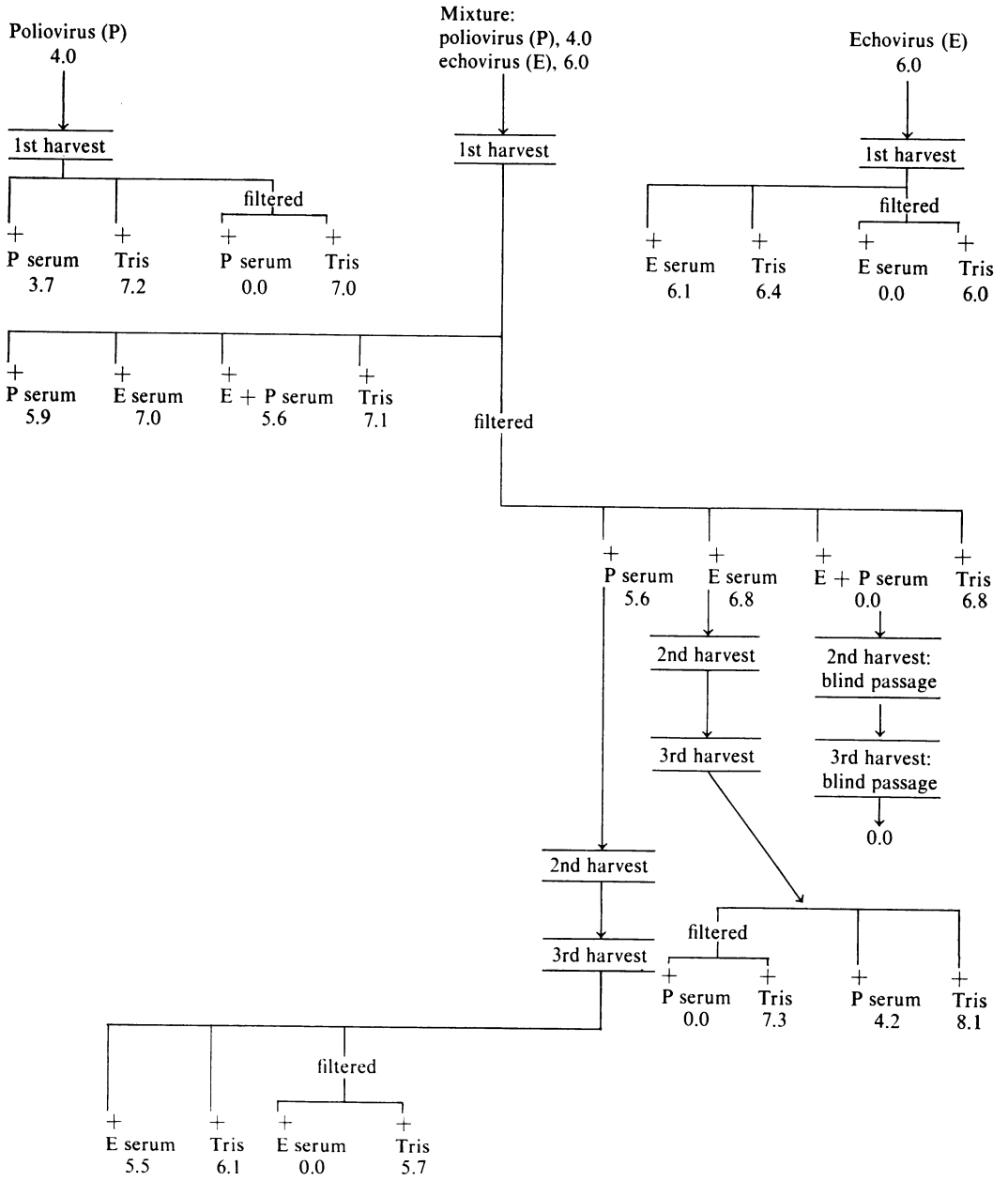

FIG. 3. *Flow diagram of the separation of a mixed virus suspension containing echovirus type 4 (Pesascek) and poliovirus type 1 (Mahoney). Numerals indicate* log_{10} *titer (PFU/ml).*

persistent fraction of 0.30, which was completely removed by filtration through an 0.05-μ membrane. Centrifugation or trypsin treatment decreased the persistent fraction from 0.30 to 0.002 or 0.005, respectively. These results are consistent with the removal or dispersion of large aggregates. However, sonic treatment did not influence the persistent fraction.

DISCUSSION

The breakthrough phenomenon and the non-neutralizable or persistent fraction of viruses has not heretofore been satisfactorily explained. Dulbecco et al. (7) demonstrated a persistent fraction with poliovirus and western equine encephalitis virus, and considered the non-neutralizable fractions to be distinct, nonsusceptible

populations. This theory has been supported by others (16, 20). Fazekas de St. Groth and his associates (8) attributed such phenomena to the dissociation of the virus-antibody complex, and Lafferty (13) suggested that nonavid antibody was the key factor. Bradish et al. (4) suggested that antibodies themselves aggregate virus, resulting in virus persistence. Ashe and Notkins (1) neutralized a major portion of the persistent fraction of herpesvirus with anti-γ-globulin, and they believed that added globulin acted by attaching to and forming a bridge between the antiviral molecules bound to the viral surface, thus blocking critical sites. However, the effects they reported for added anti-γ-globulin might well have been in reducing the number of infectious aggregates by forming a smaller number of aggregates of larger size, with specific antibodies combined on different aggregates by means of a bridge of anti-γ-globulin.

In the current report, we have shown that the persistent fraction of a number of viruses tested is due to aggregated virus. Large aggregates of virus are known to exist for a number of enteroviruses (3, 11, 19), vaccinia virus (22), and reovirus (29). The non-neutralizable aggregates may contain virions with some neutralizable sites that are not available to antibody molecules. Neutralized virus is known to adsorb to cells (16, 20). Even if the surface of the aggregate were neutralized, the aggregate might become activated upon cellular contact. The failure of aggregates to be neutralized is reminiscent of the failure of poliovirus aggregates to be completely inactivated by formaldehyde (21).

Of the 13 viruses tested in this study, only one failed to manifest a persistent fraction. In a number of cases, the persistent fraction of the virus tested was a small proportion of the virus population, and would not affect routine neutralization tests with only 100 TCD_{50} or PFU. However, in the case of viruses like echo 4 and vaccinia, the large fraction of persistent virus markedly influences such tests, unless monodispersed virus is used.

The separation of mixtures of virus by neutralization is difficult if the viruses include a significant non-neutralizable fraction. If monodispersed virus is used, viruses of similar size can be readily separated by neutralization even when—as in the case of echovirus type 4 Pesascek strain—the non-neutralizable fraction contains 30% of the entire activity of the stock containing aggregates.

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LITERATURE CITED

1. ASHE, W. K., AND A. L. NOTKINS. 1966. Neutralization of an infectious herpes simplex virus-antibody complex by anti-γ-globulin. Proc. Natl. Acad. Sci. U.S. **56**:447–451.
2. BARRON, A., AND D. T. KARZON. 1961. Characteristics of echo 4 virus (Shropshire) isolated during epidemic aseptic meningitis. J. Immunol. **87**:608–615.
3. BENGTSSON, S., V. HANZON, L. PHILIPSON, AND A. WESTMAN. 1960. Echo virus particles in sprayed preparations from purified suspensions. J. Ultrastruct. Res. **4**:482–486.
4. BRADISH, C. J., J. O. FARLEY, AND H. E. N. FERRIER. 1962. Studies on the nature of the neutralization reaction and the competition for neutralizing antibody between components of the virus system of foot-and-mouth disease. Virology **18**:378–400.
5. BURNET, F. M., E. V. KEOGH, AND D. LUSH. 1937. The immunological reactions of filterable viruses. Australian J. Exptl. Biol. Med. Sci. **15**: 227–247.
6. CLARKE, S. K. R., AND D. A. J. TYRRELL. 1958. The neutralization of influenza and other viruses by homologous immune serum. Studies in roller tube cultures. Arch. Virusforsch. **8**: 453–468.
7. DULBECCO, R., M. VOGT, AND A. STRICKLAND. 1956. A study of the basic aspects of neutralization with two animal viruses, Western Equine Encephalitis virus and poliomyelitis virus. Virology **2**:162–205.
8. FAZEKAS DE ST. GROTH, S., G. S. WATSON, AND A. F. REID. 1958. The neutralization of animal viruses. I. A model of virus-antibody interaction. J. Immunol. **80**:215–224.
9. HAMPIL, B., J. L. MELNICK, C. WALLIS, R. W. BROWN, E. T. BRAYE, AND R. R. ADAMS, JR. 1965. Preparation of antiserum to enteroviruses in large animals. J. Immunol. **95**:895–908.
10. ITOH, H., AND J. L. MELNICK. 1957. The infection of chimpanzees with echoviruses. J. Exptl. Med. **106**:677–688.
11. JAMISON, R. M., AND H. D. MAYOR. 1966. Comparative study of seven picornaviruses of man. J. Bacteriol. **91**:1971–1976.
12. JAMISON, R. M., H. D. MAYOR, AND J. L. MELNICK. 1963. Studies on echo 4 virus (picornavirus group) and its intracellular development. Exptl. Mol. Pathol. **2**:188–202.
13. LAFFERTY, J. J. 1963. The interaction between virus and antibody. II. Mechanism of the reaction. Virology **21**:76–90.
14. LEDINKO, N., AND J. L. MELNICK. 1953. Poliomyelitis viruses in tissue culture. V. Reaction of virus and antibody; variables of the quantitative neutralization test. Am. J. Hyg. **58**:223–247.

15. MALHERBE, H., R. HARWIN, AND A. H. SMITH. 1957. An outbreak of aseptic meningitis associated with echo virus type 4. S. African Med. J. **31**:1261–1264.
16. MANDEL, B. 1962. Early stages of virus-cell interaction as studied by using antibody. Cold Spring Harbor Symp. Quant. Biol. **27**:123–136.
17. MELNICK, J. L. 1954. Application of tissue culture methods to epidemiological studies of poliomyelitis. Am. J. Public Health **44**:571–580.
18. MELNICK, J. L., H. A. WENNER, AND L. ROSEN. 1964. The enteroviruses, p. 194–242. *In* E. H. Lennette and N. J. Schmidt [ed.], Diagnostic procedures for viral and rickettsial diseases, 3rd ed. American Public Health Association, Inc., New York.
19. RIFKIND, R. A., G. GODMAN, C. HOWE, C. MORGAN, AND H. ROSE. 1961. Structure and development of viruses as observed in the electron microscope. VI. Echovirus type 9. J. Exptl. Med. **114**:1–12.
20. RUBIN, H., AND R. M. FRANKLIN. 1957. On the mechanism of Newcastle disease virus neutralization by immune serum. Virology **3**:84–95.
21. SALK, J. E. 1955. Poliomyelitis vaccine in the fall of 1955. Am. J. Public Health **46**:1–14.
22. SHARP, D. G., AND K. S. KIM. 1966. Multiplicity reactivation and radiation survival of aggregated vaccinia virus. Virology **29**:359–366.
23. TAKEMOTO, K. K., AND P. FABISCH. 1963. Influence of acid polysaccharides on plaque formation by influenza A2 and B viruses. Proc. Soc. Exptl. Biol. Med. **114**:811–814.
24. TYTELL, A. A., H. A. TOROP, AND F. J. MCCARTHY. 1962. Adenovirus plaque formation in grivet monkey kidney cells. Proc. Soc. Exptl. Biol. Med. **109**:916–918.
25. WALLIS, C., AND J. L. MELNICK. 1965. Infectivity of type 4 echovirus-antibody complex. Virology **26**:175–179.
26. WALLIS, C., AND J. L. MELNICK. 1967. Concentration of enteroviruses on membrane filters. J. Virol. **1**:472–477.
27. WALLIS, C., J. L. MELNICK, AND F. RAPP. 1966. Effects of pancreatin on the growth of reovirus. J. Bacteriol. **92**:155–160.
28. WALLIS, C., F. MORALES, J. POWELL, AND J. L. MELNICK. 1966. Plaque enhancement of enteroviruses by magnesium chloride, cysteine, and pancreatin. J. Bacteriol. **91**:1932–1935.
29. WALLIS, C., K. O. SMITH, AND J. L. MELNICK. 1964. Reovirus activation by heating and inactivation by cooling in $MgCl_2$ solutions. Virology **22**:608–619.
30. YOHN, D. S., AND W. MCD. HAMMON. 1960. ECHO 4 viruses: improved methods and strain selection for identification and serodiagnosis. Proc. Soc. Exptl. Biol. Med. **105**:55–60.