ANTIGENIC VARIANTS OF INFLUENZA A VIRUS (PR8 STRAIN)

III. Serological Relationships of a Line of Variants Derived in Sequence in Mice Given Homologous Vaccine*

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During the past three years experimental studies to determine the role of the immune state of the host (the mouse) in the development of antigenic variants of influenza virus type A have been made. At the present time, seven successive generations of antigenic variants of influenza A PR8-S virus, each derived from the previous one by serial passage in the lungs of mice immunized with the homologous agent, have been produced. These variants show no change when passed in non-immune animals. The details of the methods employed, the serological and immunological characteristics of the first four variants compared with the parent PR8-S strain have been published (1, 2).

As previously reported the first four variants showed a progressive serological deviation from the parent PR8-S virus. However, while showing progressively less ability to react with antisera of the preceding variants and the PR8-S virus they retained the capacity to provoke antibody to the preceding variants and the parent virus (2). The three most recently derived variants show essentially no reaction with PR8 antiserum while continuing to elicit considerable amounts of antibody which react with the preceding variants and the original PR8-S virus. This report gives (*a*) the serological relationships of the seven variants to one another and to the original parent PR8 virus and (*b*) the results of cross-hemagglutinin inhibition tests with the certain epidemic A and A′ strains and the variant viruses.

Materials and Methods

Production of Variant Viruses.—The first four and the sixth variants were passed serially without difficulty in the lungs of mice vaccinated with the homologous virus. During the development of the fifth variant it was necessary to pass the virus alternately in immunized

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and non-immunized animals to maintain the strain. In the development of all variants, however, it was necessary to begin the homologous passage of the virus in animals with low H.I. antibody titers of 40 to 80. The mice were immunized by the intraperitoneal inoculation of 0.5 ml. of formalin-inactivated allantoic fluid influenza virus vaccine. They were then bled from the tail vein 9 to 12 days later for determination of individual antibody titers. For the first few passages three mice were selected which had H.I. titers of 40 to 80. They as well as a group of six unvaccinated mice were then inoculated intranasally with a 10 per cent mouse lung suspension of homologous virus. At 48 hours the three vaccinated mice and three mice from the unvaccinated group were sacrificed and the lungs from each group pooled.

The EID_{50} titers of both normal and vaccinated mouse lung virus suspensions were determined. If the EID_{50} titer of the lung virus suspensions from vaccinated mice was $10^{-6.0}$ or above, it was employed for the subsequent passage. If the EID_{50} virus titer was below $10^{-6.0}$, the lung virus suspension from the unvaccinated mice was used to inoculate the next group of vaccinated mice. Once the virus grew in high titer in the lungs of immunized mice with a given antibody titer, animals with higher antibody levels were then employed for subsequent passages. To achieve such antibody titers mice were given two or three intraperitoneal doses of vaccine at weekly intervals. Final passages were carried out in animals with homologous antibody titers of 1:240 to 1:960 depending upon the variant strain under study.

When virus was found to grow in high titer in the lungs of mice with high homologous antibody titer it was tested for antigenic variation. H.I. tests employing the original and final passage strains as antigens were set up with ferret antisera prepared with the original strain. If the H.I. titer against the final passage strain was significantly (fourfold or greater) lower than that observed with the original homologous virus, a new antigenic variant was considered to have been produced. After five or more serial passages in the lungs of normal mice a stock lung virus suspension was prepared and frozen for subsequent tests.

Antiserum for each variant was prepared by inoculating two ferrets intranasally with the stock lung virus suspension. The animals were bled twice at 14 and 28 days. The antisera were then pooled and frozen in small aliquots.

Mouse Lung Suspensions.—All mouse lung virus suspensions were prepared by grinding the lungs in a sterile mortar with alundum and beef heart infusion broth containing 1000 units of penicillin and 1 mg. of dihydrostreptomycin per ml. The supernatant fluid was removed by centrifuging the suspension at 1500 R.P.M. for 8 minutes and divided into small aliquots and frozen at −40°C.

Virus for Serological Tests and Vaccines.—In all serological tests and for all vaccines, infected allantoic fluids from the first egg passage were used. Ten to eleven day old chick embryos were inoculated intraallantoically with 0.2 ml. of 10^{-4} dilution of mouse lung virus suspension in antibiotic broth. After 48 hours incubation at 37°C. and chilling overnight at 4°C., the allantoic fluid was harvested, pooled, centrifuged lightly, and dialyzed overnight at 4°C. against 10 volumes of 0.85 per cent NaCl containing 0.01 M phosphate buffer at pH 7.0. Small aliquots of dialyzed fluid were quickly frozen and stored at −40°C. in an electric deep freeze.

Preparation of Vaccines.—Formalin to give a final concentration of 1:4000 was added to dialyzed allantoic fluid which was then incubated at 37°C. for 2 hours and kept at 4°C. for at least 2 days before use. Vaccine was not kept longer than 1 month at 4°C.

Serological Tests.—Hemagglutination (HA) and hemagglutinin-inhibition (H.I.) tests were carried out by the method recommended for influenza studies by the Committee on Standard Serological Procedures (3). In this test 0.2 ml. each of virus and serum and 0.4 ml. of 0.5 per cent washed chicken red blood cells were employed instead of 0.25 and 0.5 ml. respectively. Chicken blood cells were kept at 4° in modified Alsever's solution no longer than 7 days. For each day's tests the cells were washed at least three times in 0.85 per cent buffered saline.

Blood for serum antibody titers in vaccinated mice was obtained by snipping off the tip of

the tail after heating with an infrared lamp to dilate the vein. The blood was then collected in a white blood cell counting pipette previously rinsed in heparin solution (2 mg. per ml.) and dried. Blood was drawn to the 1.0 mark and diluted with buffered saline to the 11 mark above the bulb. After mixing well, the diluted blood was centrifuged in a small test tube at 2000 R.P.M. for 15 minutes and the supernatant fluid withdrawn. Since the dilution of blood in the pipette is 1:10 and the average hematocrit value of mouse blood is about 50, the supernatant fluid was considered to represent a 1:20 dilution of plasma. Therefore, the lowest antibody titer that could be determined with the H.I. test was 1:40. All titers are expressed as the reciprocal of the initial dilution of serum giving about 50 per cent H.I. with 4 HA units of virus.

Although the viruses used in this study were not inhibited by normal ferret serum all ferret antisera were treated with receptor-destroying enzyme, RDE (4).

Neutralization tests were carried out in eggs by the method of Hilleman and Horsfall (5). The antibody titers are expressed as the reciprocal of the initial dilution of serum protecting 50 per cent of the eggs, when approximately 1000 EID_{50} of virus are used.

Because the homologous titers of the antisera used in these tests varied considerably, titer ratios, that is $\frac{\text{heterologous titer}}{\text{homologous titer}} \times (100)$, were computed in order to facilitate comparison of serological results (6).

Antibody Absorption Tests.—A modification of the method described by Walker and Horsfall (7) was used. Ferret antiserum was diluted so that the H.I. titer was about 64 per 0.2 ml. Two ml. of this diluted serum was used to resuspend a pellet of virus spun down from 2 ml. of concentrated virus having an HA titer of 4096 to 8192 per ml. After overnight incubation at 4°C., the mixture was centrifuged at 49,000 *g* for 45 minutes in a model L Spinco. The supernatant fluid was again used to resuspend another pellet of the same virus after which it was allowed to stand overnight at 4°C. After centrifuging at 49,000 *g* in the Spinco, excess virus was removed from the supernatant fluid by the addition of 0.1 ml. packed washed chicken red blood cells (CRC). After 90 minutes at 4°C. and centrifugation at 2000 R.P.M. for 10 minutes to remove the CRC, the supernatant serum was tested for residual virus. It was found that the treatment with chicken red cells always removed all residual virus. Antibody titers were then determined by the H.I. test.

Virus Titrations in Eggs.—Determination of EID_{50} virus titration in eggs was carried out by intraallantoic inoculation of 0.1 ml. of falling tenfold dilutions into each of four 10 to 11 day old chick embryos. After 48 hours' incubation at 37°C. and chilling overnight, individual allantoic fluids were tested for hemagglutination on a spot plate (8). The highest dilution of virus infecting 50 per cent of the eggs was calculated by the method of Reid and Muench (9).

Limiting Dilutions in Eggs.—Twofold dilutions of mouse lung virus suspension were made beyond the EID_{50} titer and 0.1 ml. of each dilution inoculated intraallantoically into each of fifteen 10 to 11 day old embryonated eggs. After incubating for 96 hours at 37°C. and chilling overnight at 4°C., the individual allantoic fluids were tested for hemagglutination on a spot plate. Positive allantoic fluids from dilutions infecting 25 per cent or less of the eggs were identified by H.I. tests with ferret antisera. To detect inhomogeneity in the mouse lung suspensions of the viruses, homologous ferret antiserum diluted 1:5, 1:10, and 1:20 was mixed in equal volume with mouse lung virus suspensions diluted 1:50. After 30 minutes at room temperature, 0.2 ml. of each serum virus mixture was inoculated intraallantoically into each of fifteen eggs which were incubated and tested as described above.

RESULTS

The system used to designate the derived antigenic variants of influenza A PR8 virus is presented in Table I. The serological characteristics of the first

four variants, As, Ba, Cb, and Dc have been described (1, 2). Continuation of the series led to the production of three additional variants, Fd, Gf, and Hg. The fifth variant, Fd, was readily distinguishable from its predecessor Dc after 19 passages in mice immunized with Dc. The sixth variant, Gf, was difficult to produce and was maintained in vaccinated mice only by resorting to frequent passages in the lungs of normal mice. After 33 passages there appeared to be only a slight difference between Gf and its predecessor Fd. Therefore, the passages were halted with no assurance that a new variant actually had been obtained. Since it appeared likely that the Gf variant represented the end of the possible antigenic variations in this series by the methods employed, its passage in the lungs of mice given homologous vaccine was undertaken to confirm this expectation. However, without great difficulty

TABLE I

Derivation of Antigenic Variants of Influenza A Virus (PR8-S)

Virus inoculated intranasally*	Virus used for immunization of mice	No. of passages in immunized mice	Variant isolated
PR8-S	PR8-S	22	As
As	As	25	Ba
Ba	Ba	17	Cb
Cb	Cb	26	Dc
Dc	Dc	19	Fd
Fd	Fd	33	Gf
Gf	Gf	33	Hg

* The letter E has been omitted because of its use to denote egg passage.

another antigenic variant, Hg, emerged, and it appeared to be distinct from Gf and Fd variants.

After as many as 20 passages in lungs of normal mice, no change in serological characteristics of the seven antigenic variants could be demonstrated. Clones of each of the variants isolated by the limiting dilution procedure appeared identical with the virus in the first egg passage used as routine for serological tests. No inhomogeneity could be detected by inoculation of eggs with virus mixed with homologous antiserum. However, it is recognized that this technique is not sufficiently sensitive to rule out the presence of a very small proportion of another strain.

Serological Characteristics of the Variant Viruses.—The results of cross-H.I. tests are presented in Table II. The antibody titers of PR8-S antiserum with homologous virus and the seven variant viruses show a progressive decrease which reaches a titer ratio of <1 with Fd, Gf, and Hg variants. On the other hand, the antibody spectrum of the first four variant viruses show little reduction in the proportion of antibody reacting with PR8-S virus compared to homologous antibody, *e.g.* titer ratios decline from 100 with As antiserum, to 50 with Dc anti-

serum against PR8-S virus. Also, the antisera of As, Ba, and Dc cross-reacted with the virus preceding them in the sequence, but only slightly with viruses succeeding them.

Antiserum of the third variant, Cb, did not exhibit this phenomenon, but showed high titers with the fifth and the sixth variants, Fd and Gf, respectively. Examination of the antibody spectrum of Fd antiserum reveals less cross-reaction with PR8-S and the first two variants (As, Ba) than had been found in antisera to the four preceding variants. However, Fd antiserum reacted in high titer with Cb virus as did the Fd virus with Cb antiserum. It appeared, therefore, that these two variants might be similar. Fd antiserum also cross-reacted with Gf virus, and indeed the titer ratios obtained with Gf antiserum and all test viruses closely resembled those obtained with Fd antiserum. On the basis of H.I. tests seen in Table II it might be concluded that no change from the Fd to the Gf variant occurred during passage in mice immunized with homologous vaccine. In contrast to the close relationship between Gf and Fd, the Hg variant derived from Gf could be distinguished easily from the preceding variants, since it reacted only slightly with all antisera as shown by titer ratios ranging from <1 with PR8 antiserum to 19 with Gf antiserum. Moreover, this variant produced antibody with a rather broad spectrum, *e.g.* titer ratios of Hg antiserum with PR8-S and Cb were 67 and 75, respectively, and 25 to 37 with the other variants.

TABLE II

Cross-Hemagglutinin-Inhibition Tests with the Parent (PR8-S) Influenza Virus and the Seven Variant Strains

Test viruses	PR8-S	As	Ba	Cb	Dc	Fd	Gf	Hg
				Ferret antisera*				
PR8-S	**1536**	768	768	384	384	96	128	256
As22	256	**768**	768	192	256	96	64	192
Ba25	48	192	**1024**	256	384	96	128	192
Cb17	32	24	384	**768**	96	384	384	384
Dc26	48	128	768	192	**768**	96	96	192
Fd19	<8	<8	128	512	32	**768**	512	128
Gf33	<8	8	128	384	96	768	**512**	192
Hg33	8	16	128	128	128	64	96	**512**
				Titer ratios‡				
PR8-S	**100**	100	75	50	50	12	25	50
As22	17	**100**	75	25	33	12	12	37
Ba25	3	25	**100**	33	50	12	25	37
Cb17	2	3	37	**100**	12	50	75	75
Dc26	3	17	75	25	**100**	12	19	37
Fd19	<1	<1	12	67	4	**100**	100	25
Gf33	<1	1	12	50	12	100	**100**	37
Hg33	<1	2	12	17	17	8	19	**100**

* Reciprocal of initial dilution of serum partially (±) inhibiting 4 HA units of virus.

‡ $\frac{\text{Heterologous titer}}{\text{Homologous titer}} \times 100$.

The results of cross-neutralization tests with PR8-S and the seven variant viruses are pre-

sented in Table III. In general these tests confirmed the results obtained by the H.I. procedure. There was a decline in reaction between PR8-S antibody and the variant viruses, but the antisera of the variant strains reacted with PR8-S virus in varying degrees. Only Fd and Gf antisera showed significantly lower titers with PR8-S than with the respective homologous viruses. Certain cross-reactions among the seven variants were more prominent in the neutralization test than in the H.I. test. For example, antibody titers of Ba antiserum were lower

TABLE III

Cross-Neutralization Tests with the Parent (PR8-S) Influenza Virus and the Seven Variant Strains

Test viruses	PR8-S	As	Ba	Cb	Dc	Fd	Gf	Hg
				Ferret antisera*				
PR8-S	**2900**	280	1580	256	316	40	32	81
As22	91	**316**	720	160	280	25	32	40
Ba25	20	20	**1780**	200	182	40	20	20
Cb17	12	<8	50	**645**	22	200	64	45
Dc26	22	40	430	182	**400**	11	25	25
Fd19	6	5	50	363	16	**280**	100	40
Gf33	4	6	25	256	20	182	**128**	25
Hg33	8	8	81	128	50	50	32	**91**
				Titer ratios‡				
PR8-S	**100**	88	89	40	79	14	25	89
As22	3	**100**	40	25	70	9	25	43
Ba25	0.6	6	**100**	31	45	14	15	22
Cb17	0.4	<2	3	**100**	5	71	50	49
Dc26	0.7	12	24	28	**100**	3	19	27
Fd19	0.2	2	3	56	4	**100**	78	43
Gf33	0.1	2	1	40	5	65	**100**	27
Hg33	0.3	2	4	20	12	18	25	**100**

* Reciprocal of initial serum dilution preventing infection of half of the eggs when mixed with 1000 EID_{50} of virus.

‡ $\frac{\text{Heterologous titer}}{\text{Homologous titer}} \times 100.$

with Cb virus than with Dc virus, and similarly titers of Dc antiserum were lower with Cb than with Ba. This indication of cross-relationship between Ba and Dc was confirmed by antibody absorption tests to be discussed below. The possible cross-relationship between Cb and Fd variants already shown by cross-H.I. tests was also present in cross-neutralization tests. On the other hand, although Fd and Gf gave similar titer ratios with PR8-S antisera and antisera of the other variants, certain differences appeared in the antibody spectrum of Gf compared with Fd. The titers of Gf antiserum with As and Dc variants were higher than those of Fd antiserum with these variants, and the reciprocal cross-reactions between Fd and Gf did not have a titer ratio of 100 as they did in the cross-H.I. test. Neutralization tests with Hg and its antiserum showed a broad reactivity just as had been shown by cross-H.I. tests.

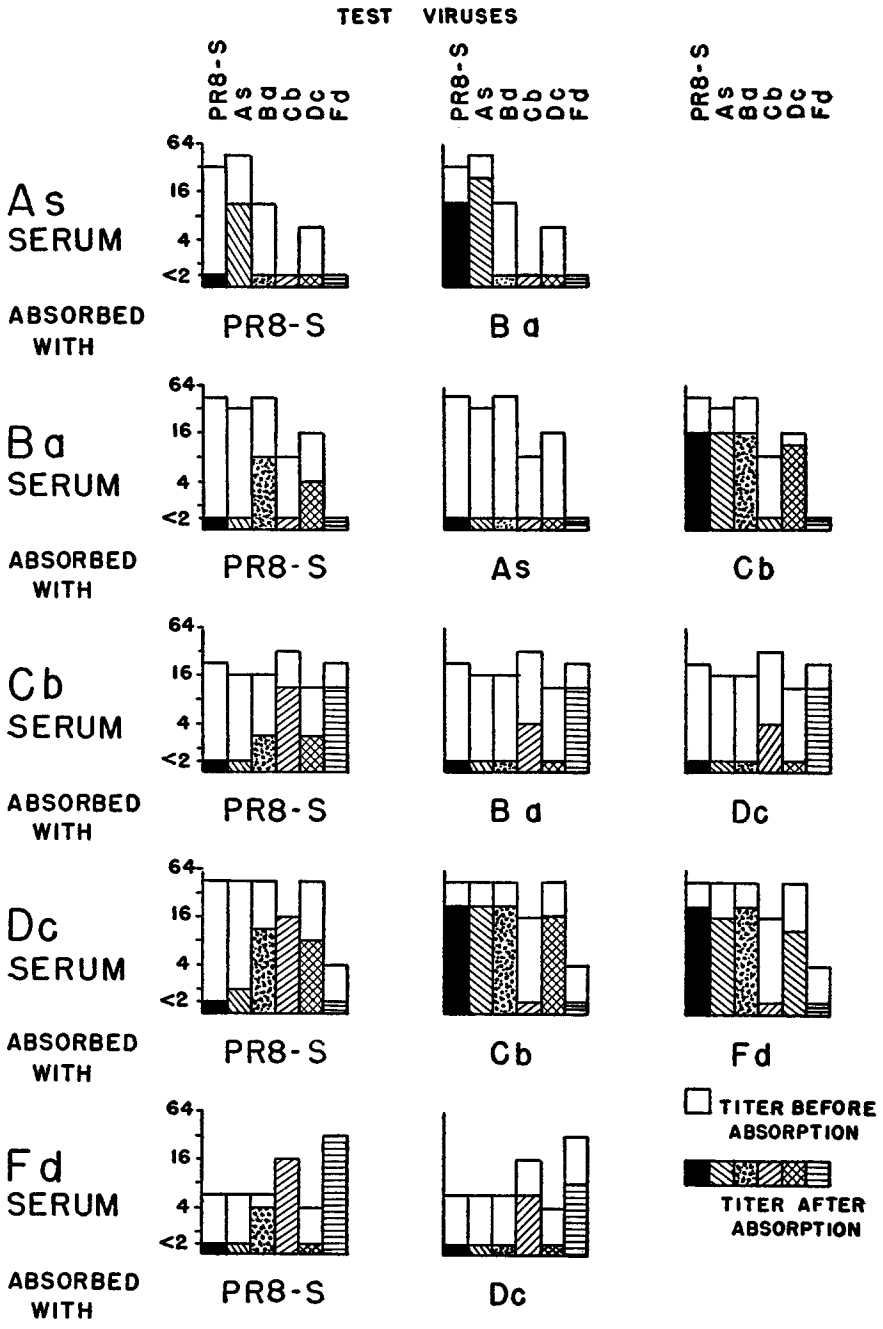

FIG. 1. Antibody absorption tests with the first five variant virus antisera, each absorbed with PR8-S, the preceding and the succeeding variants.

Of particular interest is the increase in titer ratio to PR8-S virus shown by Hg antiserum compared with the ratios of the two preceding variants.

To clarify the extent of antigenic similarities among the variant viruses, antibody absorption tests were carried out.

Absorption of Antibody.—In previous tests (2) absorption of antisera of PR8-S, As, Ba, Cb, and Dc with PR8-S virus removed all antibody from PR8-S antiserum, and all antibody reacting with PR8-S in the four variant antisera, leaving antibody for the homologous viruses.

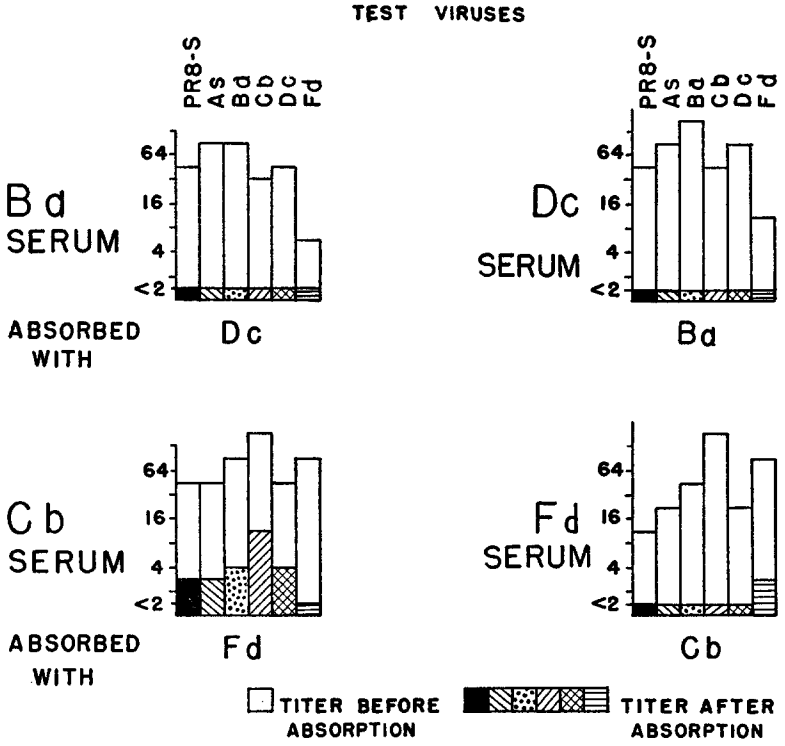

FIG. 2. Cross-absorption tests with Ba and Dc variant viruses and their antisera; and with Cb and Fd viruses and their antisera.

By extending the testing to include five variant viruses including the homologous virus, and PR8-S for each antiserum before and after absorption with PR8-S, homologous virus, and the preceding and succeeding variant viruses, some of the antigenic components in these viruses could be described. It should be pointed out that the designation of a certain component as "new" is not meant in its literal sense. Since all antisera were diluted to have the same H.I. titer, about 64, before absorption, minor antibodies might have been diluted out. Therefore, as used in this discussion, a new antigenic component refers to one that has not been prominent in previous strains in this study.

The bar graphs in Fig. 1 present the results of absorption of antisera of the first five variants. In all absorption tests, homologous virus removed all antibody and is not shown in the figure. In the left column of Fig. 1 is shown the remaining antibody of the five variant viruses in each

serum after absorption with PR8-S virus. Thus, As antiserum contained antibody which reacted only with As virus. Ba antiserum showed titers with both Ba and Dc viruses. Cb antiserum reacted with Ba, Cb, Dc, and Fd virus but highest with Cb and Fd. Dc antiserum showed about the same titer to Ba, Cb, and Dc along with a low titer to As and none to Fd. The titers of Fd antiserum with Cb and Fd viruses remained the same as before absorption with PR8-S, while the titer to Ba was somewhat lower, and all antibody to As and Dc variants

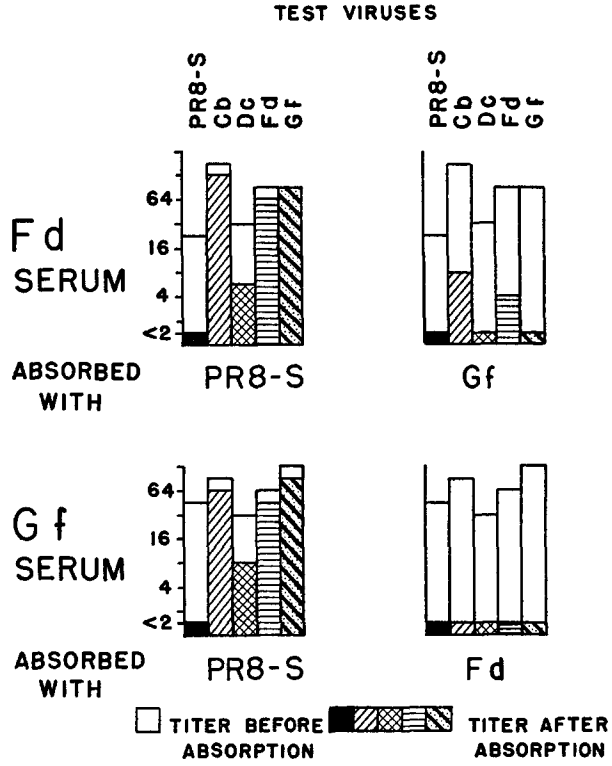

FIG. 3. Antibody absorption tests with Fd and Gf antisera absorbed with PR8-S, Gf and Fd viruses.

was removed. These results suggest that Ba and Dc share a major antigenic component, and similarly Cb and Fd have a common major antigen.

Cross-absorption between As and Ba variants showed that Ba virus failed to remove all antibody in the As antiserum, leaving titers to PR8-S and As, while As virus removed all antibody from Ba antiserum. This could be interpreted as loss of an antigen by Ba virus without the addition of a new component. On the other hand, cross-absorptions between Ba and Cb variants pointed out the differences between these viruses. Cb virus removed from Ba antiserum only the antibody to itself and the absorption of Cb antiserum with Ba virus gave just the opposite reaction, since Ba virus removed titers to PR8-S, As, Ba, and Dc, and left titers to Cb and Fd.

The cross-absorption of Cb and Dc variants gave results very similar to those just described between Cb and Ba. Cb antiserum absorbed with Dc variant had antibody to Cb and Fd

viruses remaining, and the reverse absorption of Dc antiserum with Cb virus left titers to PR8-S, As, Ba, and Dc.

Absorption of Dc antiserum with the fifth variant Fd, left the same antibody spectrum as absorption with the third variant Cb, while absorption of Fd antiserum left titers to Fd and Cb, similar to absorption of Cb antiserum with Ba or Dc viruses. The cross-relationship between alternate variants of this line suggested by cross-H.I. and neutralization tests is confirmed by antibody absorption.

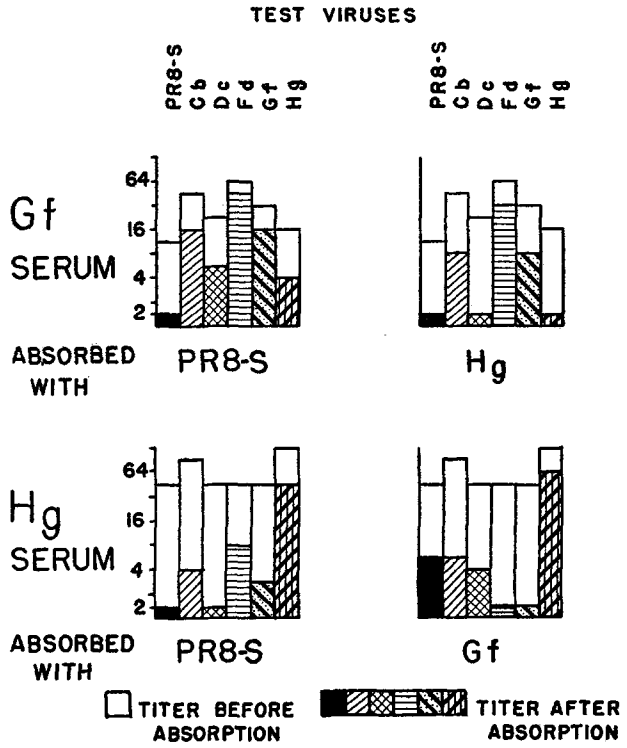

FIG. 4. Antibody absorption tests with Gf and Hg antisera absorbed with PR8-S, Gf and Hg viruses.

Because of the similarity between Ba and Dc on the one hand and Cb and Fd on the other, these pairs of variants were checked by cross-absorption tests, with the results given in Fig. 2. Homologous virus removed all antibody in all tests, and is not shown. Since cross-absorption with Ba and Dc removed all antibody from both antisera, it can be concluded that these variants are very closely related, if not identical. However, absorption of Cb antiserum with Fd virus did not remove all antibody, but left titers to PR8-S, and all variants except Fd thereby indicating that Fd variant is deficient in some of the antigenic components present in Cb. On the other hand, absorption of Fd antiserum with Cb left only a low titer to Fd virus, indicating that most of the components present in Fd variant are shared by Cb.

Absorption of PR8-S antiserum with As variant has been shown to lower the titer of PR8-S antiserum (2). However, absorption of PR8-S antiserum with the other six variants in this

series did not alter the antibody titer significantly. This is in agreement with the results obtained in H.I. and neutralization tests.

On the basis of the results of these absorption tests, the following antigenic components can be listed, named for the viruses in which they are most prominent: (*a*) PR8-S, (*b*) Ba-Dc, (*c*) Cb-Fd.

Cross-H.I. and neutralization tests indicated that Fd and the succeeding variant Gf were closely related. By antibody absorption test, however, it was possible to show a slight difference between them (Fig. 3). Whereas absorption of both antisera with PR8-S left the same antibody spectrum, reacting highest with Cb, Fd, and Gf and lower with Dc, cross-absorption of these antisera with the Fd and Gf variants did not remove all antibody from both sera. Absorption of Fd antiserum with Gf virus left titers to Cb and Fd variants while absorption of Gf antiserum with Fd virus removed all antibody. Thus, Gf virus apparently lacks components present in Fd but contains no new components that are not shared by Fd virus.

TABLE IV

H.I. Titer Ratios of Antisera of Epidemic Type A Influenza Virus Strains Tested with PR8-S, As, Ba, Cb, and Dc Variant Viruses

Test viruses	Titer ratios* of ferret antisera‡ (yr. isolated)										
	Swine 1931	PR8 1934	Mel 1935	Gat 1937	Tal 1937	Alas 1937	Bloom 1937	Gid 1940	Dyk 1941	Hems 1943	Weiss 1943
PR8-S	1.5	**100**	25	12	25	25	25	6	17	25	16
As22	<0.5	17	6	6	9	25	50	2	12	12	12
Ba25	<0.5	3	<3	<2	12	<3	<0.5	1	2	1	2
Cb17	<0.5	2	<3	<2	<1	<3	<0.5	1	4	1	<2
Dc26	<0.5	3	<3	<2	6	<3	<0.5	<1	<1	<1	<2

* $\frac{\text{Heterologus titer}}{\text{Homologous titer}} \times 100.$

‡ Antisera prepared from the following group A or A′ strains of influenza virus showed no H.I. when tested with the above PR8-S and the four variants: WS '33, BH '35, Hickcox '40 DSP '43, Cam '46, FM1 '47, Sweden '50, Boch '51, Wright '53, and Malaya '54.

The seventh variant, Hg, has been shown by serological tests to be distinct from previous variants, although its broad antibody spectrum indicated that it shared antigens present in previous viruses. In Fig. 4, this is brought out by comparing the absorption of Gf and Hg antisera by PR8-S. In Gf antiserum, antibody reacting in varying amounts with Cb, Dc, Fd, Gf, and Hg viruses remained after absorption with PR8-S. In Hg antiserum, the highest titer remaining was to Hg, with lower titers to Fd, Cb, and Gf in that order. Absorption of Gf antiserum by Hg virus removed all the antibody to PR8-S, Dc, and itself. Absorption of Hg antiserum by Gf left low titers to PR8-S, Cb, Dc viruses, and a high titer to Hg virus. From these results it appears that Hg contains considerable amounts of PR8-S antigen as well as a new antigen.

Serological Comparison of Variant Strains with Human Influenza A and A′ Strains.—To explore the possibility that the variant strains of PR8-S virus developed in the laboratory might antigenically resemble some of the other type A and A′ strains, cross-H.I. tests were carried out with all variants, PR8-S and swine, 14 type A, and 11 type A′ influenza strains.[1] Since these tests were performed on different days, only titer ratios will be given. Only the

[1] We are indebted to Dr. Keith E. Jensen, CDC Virus and Rickettsia Section, Montgomery, Alabama, for these strains and their antisera.

first four variants reacted with some of nine type A antisera; all other variants were negative. Titer ratios of antisera of the nine type A influenza strains and four variant viruses are given in Table IV.

TABLE V

H.I. Titer Ratios with Variant Antisera and Type A and A' Influenza Viruses

Test viruses		Titer ratios* of antisera							
Name	Date isolated	PR8-S	As	Ba	Cb	Dc	Fd	Gf	Hg
Swine	1931	<1	<1	<3	<2	<6	<3	<4	<4
Type A									
WS	1933	<1	3	5	25	17	6	33	12
PR8-S	1934	100	100	75	50	50	12	25	67
Mel	1935	25	25	3	25	8	5	16	25
BH	1935	1	<1	3	4	3	12	16	6
Gatenby	1937	3	6	<3	2	<4	5	16	<4
Talmey	1937	12	6	19	4	9	<3	<4	<4
Alaska	1937	25	37	6	4	<6	8	16	<4
Bloom	1937	67	33	6	25	25	33	12	100
Gideon	1940	17	12	2	6	8	4	<2	<12
Hickcox	1940	<1	<1	<3	<2	<6	<4	<2	<12
Dyken	1941	50	25	6	25	25	12	6	100
Hemsbury	1943	25	12	3	2	3	2	<2	25
DSP	1943	<1	<1	<1	<1	<2	<4	<2	<12
Weiss	1943	25	12	2	12	12	4	3	50
Type A'									
Cam	1946	<1	<2	<2	18	<2	<2	<6	<2
FM1	1947	<1	<2	<2	<4	<2	<2	<6	<2
Sweden	1950	<1	<2	<2	<4	<2	<2	<6	<2
Boch	1951	<1	<2	<2	4	<2	<2	<6	<2
Wright	1953	<1	<2	<2	18	<2	<2	<6	<2
Malaya	1954	<1	<2	<2	<4	<2	<2	<6	<2
Alb/1	1955	<1	<2	<2	<1	<2	<2	<3	<4
AA/4	1956	<1	<2	<2	6	<2	<2	<3	<4
Ned/36	1956	<1	<2	<2	<1	<2	<2	<3	<4
Denver/1	1957	<1	<2	<2	<1	<2	<2	<3	<4
Gl/10	1957	<1	<2	<2	<1	<2	<2	<3	<4

* $\frac{\text{Heterologous titer}}{\text{Homologous titer}} \times 100.$

The cross-reactions shown in Table IV probably can be attributed to the PR8-S component in these variants. On the other hand, as seen in Table V, antisera of the variant viruses showed H.I. reactions when type A and A' were employed as antigens. It can be noted in Table V that WS strain, which was not inhibited by PR8-S antisera, gave titer ratios above 20 with Cb and Gf antisera. Among the other type A strains, cross-reactions equal to the cross reaction obtained with PR8-S antisera were also seen with some of the variants antisera. These reactions, however, did not seem to follow any pattern expected of the relative amounts of PR8-S anti-

body in variant antisera. For example, Ba antiserum which gave a titer ratio of 75 with PR8-S virus hardly inhibited Bloom virus at all, while Fd antiserum, with a titer ratio of 12 with PR8-S virus showed a titer ratio of 33 with Bloom virus. However, in the case of Hg antiserum, the high titer ratios obtained with Bloom and Dyken viruses probably reflect the large amount of PR8-S antibody in this variant antiserum.

As seen in Table V, only Cb variant antiserum showed inhibition with any of the A′ strains. Low cross-reactions with this antiserum were noted only with Cam, Wright, and AA/1/55 viruses. Because this variant appeared unique in this respect, Cb antiserum was tested in neutralization tests with Cam and Wright viruses in eggs. However, a titer of less than 16 was obtained. On rechecking the H.I. tests with another lot of Cb ferret antiserum, titer ratios of 12 were obtained with Cam and Wright viruses. No non-specific inhibitors could be detected in any of the RDE-treated antisera by using heated Lee virus. The cross-reaction between Cb antiserum and Cam and Wright viruses probably indicates at most a very minor antigenic relationship.

DISCUSSION

Among the variants of this series, derived in sequence from PR8-S virus, there was a progressive loss of serological reaction between the variant viruses and PR8-S antiserum. Nevertheless the antisera of the variants contained antibody reacting to varying degrees with PR8-S virus. One possible explanation of this apparent paradox could be that the PR8-S antibody produced by the variants represents a common antigen which occupies a minor position in the antigenic complex of PR8-S virus, and that the major antigen of PR8-S virus which provokes the highest proportion of antibody present in PR8-S antiserum is not shared by the variants. This would account for the failure of the variants to react with PR8-S antiserum.

The variants described in this series also contained new antigenic components. In this context "new" does not mean arising *de novo* in the variant viruses, since no techniques exist at present for delineating all antigenic components present in influenza viruses. What are designated here as new antigens could well have been present in quantities too small to detect in the parent PR8-S virus. By absorption of antiserum of the variant viruses with preceding and succeeding viruses in the sequence, it was possible to show a pattern of relationship between alternate viruses. The present study would seem to leave little doubt that antigenic variation in influenza A virus PR8 can be brought about by passage in mice immunized to PR8 virus, but whether the variants follow a predetermined pattern or occur at random cannot be ascertained without repetition of some of the passages. Such experiments are now in progress.

The close relationship between the second variant Ba, and the fourth variant, Dc, could indicate a carry-over of small proportions of Ba virus through the passages leading to Cb variant, and then re-emergence of Ba variant when passages in mice immunized with Cb variant were used for the next series of passages. However, all attempts to detect the presence of Ba variant in mouse lung suspensions containing Cb variant have failed. This consisted of inocu-

lation of eggs with a mixture of Cb virus and large amounts of Cb antiserum, and although either these eggs yielded no virus, or the virus isolated was identical with Cb, it is possible that the technique is not sufficiently sensitive to detect small proportions of Ba virus. Another observation which does not support the hypothesis of carry-over of Ba virus is the number of passages required in immunized mice before Dc variant became established. By H.I. tests, no significant deviation from Cb variant could be detected before the 19th passage. If Ba variant had been present, it seems likely that less than 19 passages in an environment unfavorable for growth of Cb variant would have sufficed to re-establish Ba.

Although the fifth and sixth variants, Fd and Gf, of this line produced significantly lower antibody which reacted with PR8-S virus, the seventh variant, Hg, showed a significant increase in the production of PR8-S antibody. This reappearance of PR8-S component in Hg was not unexpected in view of the lower PR8-S antibody titers of the mice used for this passage. Although attributing the higher PR8-S antibody production by Hg to the lowering of PR8-S antibody in the mice used for its passage may not be justified, it raised the question as to what course antigenic variation would follow in mice given polyvalent vaccine containing PR8-S virus as a constant factor. The production of variants of PR8-S virus under this condition will be described in the following report (10).

The results of cross-protection tests with this series of variants and the original PR8-S virus along with comparative antigenicity tests is the subject of a subsequent report (11). These studies further demonstrate the marked antigenic deviation of the variant viruses when compared with the parent PR8-S strain.

None of the variant viruses appeared identical with any of 25 type A and A′ viruses tested by cross-H.I. reactions. Most of the cross-reactions were obtained with antisera of the variants and the type A and A′ viruses. Without the use of absorbed sera it is difficult to interpret these results. Presumably the variant viruses do not share any major antigens present in any of the type A and A′ strains employed in these tests.

Since Archetti and Horsfall (12) carried out their passages in eggs in the presence of antiserum to a related strain rather than in mice actively immunized with homologous virus, it is difficult to compare the properties of the antigenic variants reported by them with those described here. Recently Edney (13) reported the isolation of three antigenic variants of Cam virus by passage with homologous antiserum in embryonated eggs. The three variants were serologically distinguishable, and two of them were pathogenic for mice by the intranasal route although the original Cam strain was not. The study reported by Magill (14) who also carried out experiments in actively immunized mice is more comparable to the present one, but use of a different

technique for H.I. tests makes comparison difficult. Also, the methods for evaluating variation are quite different, since the variants described by Magill were tested with antisera of several human strains rather than by cross-reactions with antisera of the variant viruses and their parent strains.

SUMMARY

Seven variant strains of influenza A PR8-S virus, each derived from the previous one by serial passage in the lungs of mice immunized with the homologous agent have been produced. With the H.I. and neutralization procedures these variants showed a progressive serological deviation from the parent PR8-S virus. The seven variants provoked antibodies in varying titers to the preceding variants and the parent virus but not in relation to their position in the series. Thus, the seventh variant provoked significantly more antibody to the PR8-S virus than did the fifth variant. A possible explanation for this is presented. The first four variant viruses showed progressively less ability to react with antisera of the preceding variants and the PR8-S virus, and the three most recently derived variants showed essentially no ability to react with PR8-S and first variant antisera. The variant viruses remained antigenically stable through numerous lung passages in normal mice. Cross absorption tests revealed common antigenic components among the variant viruses and also individual characteristics which classify them as being different from one another. The implications of these findings in relation to studies by others have been discussed.

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