ANTIGENIC VARIANTS OF INFLUENZA A VIRUS (PR8 STRAIN)

IV. Serological Characteristics of a Second Line of Variants Developed in Mice Given Polyvalent Vaccine*

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In previous reports a series of seven antigenic variants of type A influenza virus PR8-S strain has been described (1–3). Each new strain was derived by passage in lungs of mice given homologous vaccine. Although the variants in this series showed decreasing serological reactions with PR8 antiserum, all antisera produced by the variants contained varying amounts of antibody which reacted with PR8-S virus. Only two of the seven variants, the fifth and sixth, elicited relatively low PR8-S antibody titers. However, the seventh variant virus derived from the sixth provoked significantly more PR8 antibody (3).

In an attempt to decrease further the serological relationship of the PR8-S virus to its variant offspring, a polyvalent vaccine containing PR8-S virus as a constant component, as well as the variant strain was employed for mouse immunization. This was done so that a high antibody titer (immune environment) to the PR8-S virus, which might serve to suppress selectively the development of variants with PR8-S characteristics, would be present continually. With this procedure a second series of four antigenic variants has been produced. The comparative serological characteristics of the seven variants of the first series and the four variants of the second series employing cross-hemagglutinin-inhibition, neutralization, and antibody absorption tests will be presented. The variants of the second series were also compared with several influenza virus A and A′ strains.

Materials and Methods

In the development of this second series of influenza virus PR8-S variants mice immunized with monovalent (PR8-S virus) and polyvalent (variant virus plus PR8-S) vaccines

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and mice recovered from active PR8-S virus infections were employed. The reasons for use of these procedures will be given in the results section.

The monovalent PR8-S vaccine was prepared in the manner described in detail in the previous paper (3). The polyvalent vaccines were prepared from dialyzed first passage infected allantoic fluid. One part PR8-S fluid was added to nine parts of the variant virus

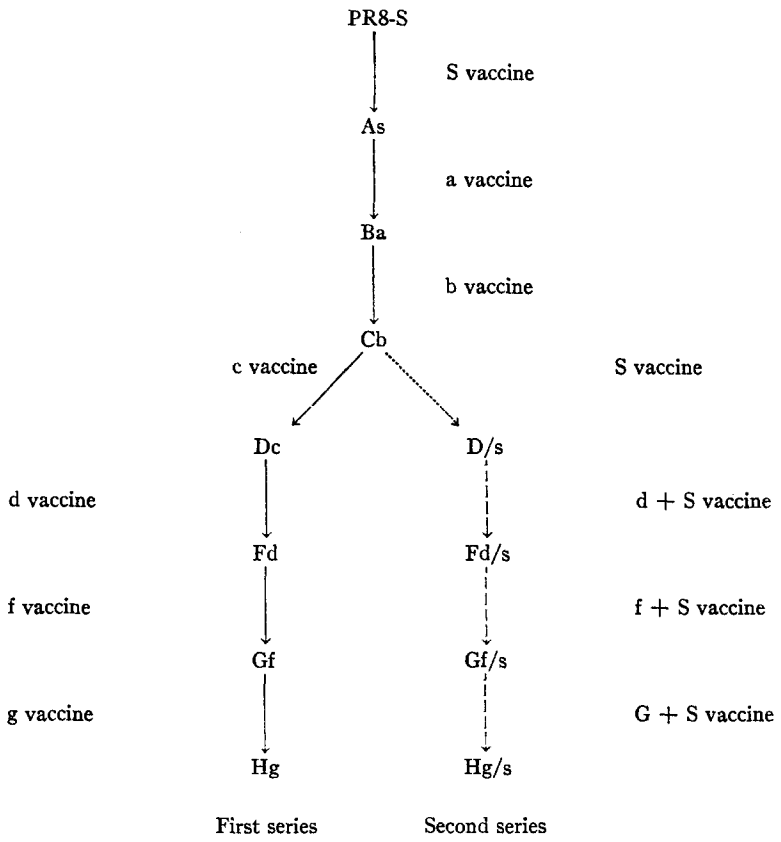

FIG. 1. Nomenclature, relationship, and derivation of the first and second series of influenza PR8-S virus variants.

fluid. Each virus suspension had approximately the same HA units per ml. After mixing, the vaccine was inactivated by the addition of formalin (1:4000 dilution) followed by incubation at 37°C. for 2 hours. Because of the progressive and marked decline in antigenicity of each new emerging variant, it was necessary to concentrate the variant virus component in the vaccines in order to provoke suitable antibody titers for comparative tests. This was done by centrifugation of dialyzed infected allantoic fluid in a Spinco model L centrifuge at 59,000 *g* for 40 minutes. The pellets then were resuspended in one-tenth the original volume of buffered saline. Nine parts of the concentrated variant virus suspension were mixed with one part of unconcentrated PR8-S fluid after which the vaccine was inactivated with formalin as described above.

The technique of passage, criteria for recognition of a new variant, the hemagglutinin inhibition (H.I.), *in ovo* neutralization, and antibody absorption tests are similar to those outlined in detail in the previous report (3).

RESULTS

The relationship of the first and second series of influenza PR8-S variants is shown in Fig. 1. The second series was started by passage of the third variant (Cb) of the first series in the lungs of mice immunized with PR8-S vaccine by intraperitoneal inoculation of three 0.5 ml. doses at weekly intervals. After 32 passages in the lungs of mice with high PR8-S antibody titer, the last passage still elicited considerable PR8-S antibody. Therefore, thirteen additional lung passages were made in recovered mice which previously had been infected with the PR8-S virus by the aerosol technique (4). Such mice when used for passage had H.I. and neutralizing antibody titers ranging respectively from 240 to 960 and 200 to 400. After a total of 45 lung passages no significant antigenic changes had occurred. The passages were then terminated and the variant was designated D/s (Fig. 1, Table I).

TABLE I

Derivation of Second Series of Variants of Influenza PR8-S Virus in Mice Immunized with Polyvalent Vaccines

Virus inoculated intranasally	Viruses used for immunization of mice	No. of passages in immunized mice	Variant isolated
Cb*	PR8-S	45	D/s
D/s	D/s and PR8-S	20	Fd/s
Fd/s	Fd/s and PR8-S	25	Gf/s
Gf/s	Gf/s and PR8-S	33	Hg/s

* Third variant of the first PR8-S series (1–3).

The next three variants, Fd/s, Gf/s, and Hg/s, were derived from lung passage in mice given polyvalent vaccine. The symbols and number of passages employed in the serological procedures are given in Table I. As the variant components in the vaccines became progressively poorer antibody producers (5) the mice through which the passages were made had to be given from one to three 0.5 ml. intraperitoneal inoculations of vaccine at weekly intervals to produce the desired antibody level. The antibody titer in such mice varied from 80 to 240.

Serological Characteristics of the Second Series.—The results of the cross-H.I. and neutralization tests with those of the first and second series of variants are shown respectively in Tables II and III. As in the previous report (3) titer ratios were also employed to make the serological results with the different virus variants more comparable. It can be seen that the first variants D/s of the second series showed essentially the same cross-reactions as did its predecessor (Cb). The following three variants, however, Fd/s, Gf/s, and Hg/s,

TABLE II

Cross-Hemagglutinin-Inhibition Tests with Influenza PR8-S Virus and Its Variants of the First and Second Series

Test viruses	Ferret antisera*											
	First series								Second series			
	PR8-S	As	Ba	Cb	Dc	Fd	Gf	Hg	D/s	Fd/s	Gf/s	Hg/s
First series												
PR8-S	**1536**	768	768	384	256	96	128	256	256	32	16	48
As	256	**768**	768	192	384	96	64	128	384	48	48	16
Ba	48	192	**1024**	256	384	96	128	128	384	32	48	16
Cb	32	24	512	**768**	96	512	384	256	512	96	48	48
Dc	48	128	768	192	**384**	96	96	128	256	24	48	32
Fd	8	<8	128	384	32	**768**	512	128	192	32	48	<8
Gf	<8	8	128	384	32	384	**512**	128	256	32	24	24
Hg	8	16	192	128	96	64	128	**384**	128	32	24	32
Second series												
Cb	32	24	512	**768**	96	512	384	256	512	96	48	48
D/s	16	24	512	512	64	512	384	128	**512**	48	24	24
Fd/s	<8	<8	64	96	24	192	96	128	128	**192**	48	96
Gf/s	<8	<8	32	32	24	24	24	12	64	96	**192**	96
Hg/s	<8	<8	16	8	8	<8	16	48	32	48	64	**128**
	Titer ratios‡											
First series												
PR8-S	**100**	100	75	50	66	12	25	66	50	16	8	37
As	17	**100**	75	25	100	12	12	33	75	25	25	12
Ba	3	25	**100**	33	100	12	25	33	75	16	25	12
Cb	2	3	50	**100**	25	66	75	66	100	50	25	37
Dc	3	16	75	25	**100**	12	18	33	50	12	25	25
Fd	<1	<1	12	50	8	**100**	100	33	38	16	25	<6
Gf	<1	1	12	50	8	50	**100**	33	50	16	12	18
Hg	<1	2	18	16	25	8	25	**100**	25	16	12	25
Second series												
Cb	2	3	50	**100**	25	66	75	66	100	50	25	37
D/s	1	3	50	66	16	66	75	33	**100**	25	12	18
Fd/s	<1	<1	6	12	6	25	18	33	25	**100**	25	75
Gf/s	<1	<1	3	4	6	3	4	3	12	50	**100**	75
Hg/s	<1	<1	1	1	2	<1	3	12	6	25	33	**100**

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

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

TABLE III

Cross-Neutralization Tests with Influenza PR8-S Virus and Its Variants of the First and Second Series

Test viruses	Ferret antisera*											
	First series								Second series			
	PR8-S	As	Ba	Cb	Dc	Fd	Gf	Hg	D/s	Fd/s	Gf/s	Hg/s
First series												
PR8-S	**2560**	360	720	320	360	40	32	81	50	8	5	11
As	91	**400**	720	160	280	25	32	40	32	11	<8	11
Ba	20	20	**840**	200	182	40	20	20	22	16	<8	13
Cb	12	<8	50	**645**	22	200	64	45	182	20	10	<8
Dc	22	40	430	182	**400**	11	25	25	50	11	<8	11
Fd	6	5	50	363	16	**280**	100	40	100	20	<8	11
Gf	4	6	25	256	20	182	**95**	25	50	22	<8	<8
Hg	8	8	81	128	50	50	32	**91**	64	40	<16	<16
Second series												
Cb	12	<8	50	645	22	200	64	45	182	20	10	<8
D/s	4	5	256	316	64	230	50	<16	**182**	16	<8	<8
Fd/s	<4	<8	25	89	22	81	32	22	20	**182**	20	22
Gf/s	6	<8	22	64	22	20	22	12	25	64	**85**	50
Hg/s	11	<8	25	76	25	22	<32	22	32	91	81	**130**
Titer ratios‡												
First series												
PR8-S	**100**	90	85	50	90	14	33	89	27	4	6	8
As	3	**100**	85	25	70	9	33	44	17	6	<9	8
Ba	<1	5	**100**	31	45	14	21	22	12	8	<9	10
Cb	<1	<2	6	**100**	5	71	67	49	100	11	12	<6
Dc	<1	10	51	28	**100**	4	26	27	27	4	<9	8
Fd	<1	1	6	56	4	**100**	100	44	55	11	<9	8
Gf	<1	1	3	40	5	65	**100**	27	27	12	<9	<6
Hg	<1	2	9	20	12	18	33	**100**	35	22	<18	<12
Second series												
Cb	<1	<2	6	100	5	71	67	49	100	11	12	<6
D/s	<1	1	30	49	16	82	52	<17	**100**	8	<9	<6
Fd/s	<1	<2	3	14	5	29	33	24	11	**100**	23	17
Gf/s	<1	<2	3	10	5	7	23	13	13	35	**100**	38
Hg/s	<1	<2	3	11	6	8	<33	24	17	50	95	**100**

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

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

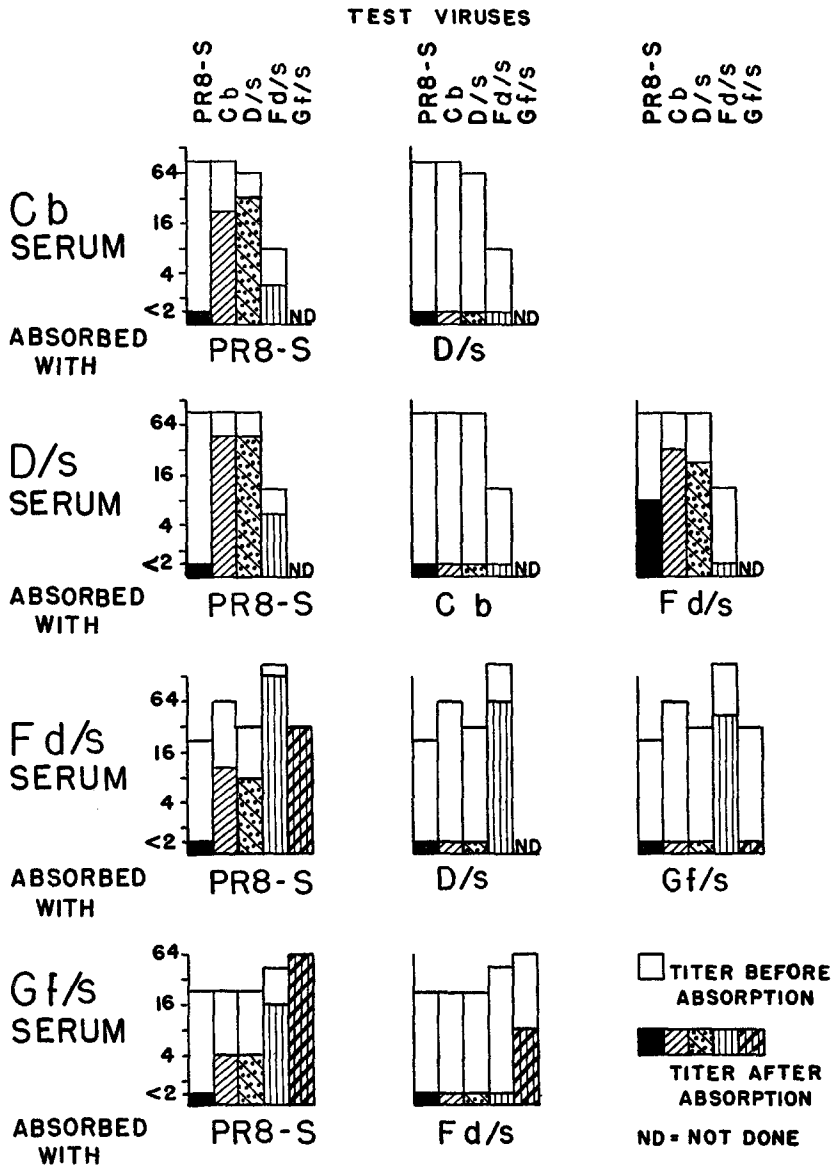

FIG. 2. Cross-absorption tests with Cb and its three variant antisera each absorbed with PR8-S and the preceding and succeeding variants.

showed considerable deviation from the PR8-S virus, the variants of the first series, and the D/s virus. They all failed to react with PR8-S and As antisera and produced only small amounts of antibody which reacted with the PR8-S, the variants of the first series and the D/s variant of the second series. Although the results of the H.I. and neutralization tests suggest that the last three variants of the second series are quite similar, cross-absorption

tests of these antisera with the PR8-S virus and preceding and succeeding variant viruses revealed individual antigenic characteristics.

Antibody Absorption Tests.—The results of the cross-absorption tests with the PR8-S virus and the viruses and antisera of the second series, are shown in Figs. 2 and 3. All homologous absorptions removed all antibody and are omitted from the figures. It can be seen in Fig. 2 that absorption of Cb, D/s, Fd/s, and Gf/s antisera with PR8-S removed all its antibody but left varying amounts which reacted with Cb and the respective Bar-S

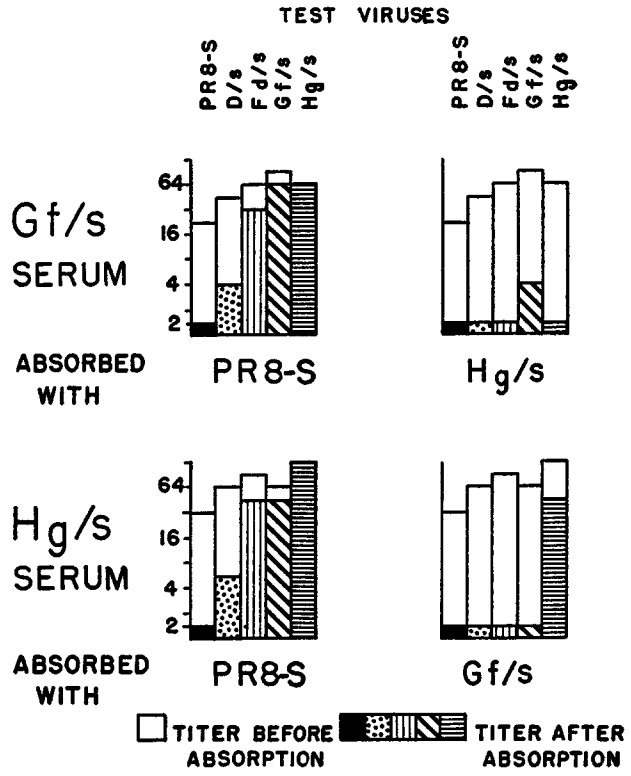

FIG. 3. Cross-absorption of Gf/s and Hg/s antisera with respective antigens and PR8-S virus.

variants. Cb and D/s antisera showed complete cross-absorption by the respective heterologous virus which confirms their close antigenic relationship suggested by the results with cross-H.I. and neutralization tests (Tables II and III). The antibodies in Fd/s antiserum after absorption with PR8-S virus showed a shift in proportions, that is, less titer was left to Cb and D/s viruses, a high titer to homologous virus remained, and the titer to Gf/s virus was unchanged. This trend continued in the results obtained with Gf/s antiserum absorbed with PR8-S virus, but in this case the titer with Fd/s virus was lower than the homologous titer.

Cross-absorption tests between D/s and Fd/s indicated that Fd/s virus had lost components present in D/s since absorption of D/s antiserum with Fd/s virus did not remove all antibody but left components which reacted with PR8-S, Cb, and D/s. However, Fd/s

virus acquired a new antigen because absorption of Fd/s antiserum with D/s virus left essentially all the Fd/s antibody. After absorption of Fd/s antiserum with Gf/s a high titer to Fd/s virus also remained. Absorption of Gf/s antiserum with Fd/s virus, however, removed all except a low titer to Gf/s virus. If these results are examined along with the titers left in these two (Fd/s and Gf/s) antisera after absorption with PR8-S virus, it appears

TABLE IV

H. I. Antibody Titer Ratios of Antisera of Variants of the Second Series Tested with Swine and Type A and A' Influenza Viruses

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

likely that Fd/s contains the antigen which becomes dominant in Gf/s. Thus after PR8-S absorption of Fd/s antiserum, the Fd/s titer is higher than the Gf/s titer, and the reverse is true for Gf/s antiserum (Fig. 2).

The results after absorption of Gf/s and Hg/s antisera with the respective heterologous antigens and PR8-S virus are shown in Fig. 3. It can be seen that similar antibody patterns remained after absorption of these antisera with the PR8-S virus. The absorption of Gf/s antiserum with Hg/s virus left only a low antibody titer to Gf/s antigen; however, after absorption of Hg/s antiserum with Gf/s antigen a high titer to the homologous (Hg/s) virus remained.

The results of the absorption tests indicate that variants Fd/s, Gf/s, and Hg/s of the second series are characterized by a decrease in antigenic components present in the parent Cb and the D/s viruses and the appearance of new components which are shared in varying degrees themselves. In additional experiments not given in table or graph form it was shown that these new components in the absorbed Fd/s, Gf/s, and Hg/s antisera did not react with the Cb and Dc variants of the first series of PR8 variants. Likewise, none of the variants of the second series, when used to absorb PR8-S antiserum, significantly reduced the antibody titer. This confirmed the lack of cross-reaction in the H.I. and neutralization tests between PR8-S antiserum and these viruses.

Serological Comparison of Variants of the Second Series with Other Influenza Type A and A′ Strains.—In cross-H.I. tests none of the variant viruses reacted with any of the antisera of swine, fourteen strains of type A and eleven strains of A′ influenza virus. However, when the antisera of these variants were tested with the variant viruses certain reactions were observed. The results are expressed in antibody titer ratios and presented in Table IV. Antisera of Fd/s and Gf/s variants showed low H.I. antibody titers with the swine virus. This is notable only because none of the variants of the first series (3) or the rest of the variants in the second series showed any reaction with this antigen.

In Table IV it can be seen that generally there was less reaction between the type A strains and the variants succeeding D/s than had been obtained with seven variants of the first series (3). The lower homologous titers of Fd/s, Gf/s, and Hg/s compared to those of the other variants may be responsible for this. It is interesting to note that D/s, which was shown by cross-absorption tests to be very closely related to Cb variant of the original line, did not show a titer ratio with Cam, Wright, or AA/4 as had Cb antiserum (3).

Among the A′ strains, only FM1 reacted with Fd/s, Gf/s, and Hg/s antisera. The titer ratios were low, 12 to 19. No reactions, however, were observed in cross-neutralization tests with these antisera and FM1 virus. This is similar to the reaction between Cb antiserum of the first series and Cam and Wright viruses (3).

DISCUSSION

Experiments reported in this and the previous paper (3) have demonstrated that influenza virus type A, PR8 strain, will alter antigenically when grown in the presence of homologous antibody in mice. This has provided a laboratory model for study of antigenic variation of this virus, which has been well documented in the epidemiology of the disease in man (5–7). In two series of passages in immunized mice; one, in which each variant was derived from the preceding one, and the second, in which each variant was derived from the preceding one, but in the presence of a continuous antibody titer to the original virus PR8, the antigenic composition of the variants differed markedly. Not only did the variants in these two series contain different "new" antigens, but also two variants in the second series had less PR8 component than any of the variants in the first series. This lends support to the hypothesis that the maintenance of antibody levels to PR8 virus in mice will lead to reduction of the PR8 component in the emerging variants. However, while recognizing the importance of the immune state of the host, the precise mechanism in the selection of a new major antigenic component in the emerging variant cannot be assessed at this time. Repetition of some of the passages is neces-

sary to determine whether or not this selection is a random or a regulated one. Such studies are now in progress.

As with the first series of PR8-S variants (3) cross-H.I. tests with several type A and A′ strains of influenza virus and the variants of the second series failed to reveal any close relationships. The only cross-reactions were obtained with antisera of the variants and some of the type A and A′ viruses. Lack of cross-reactions with antisera of the type A and A′ viruses presumably indicates that none of the variants share with them major common antigens.

Further evidence that the serological deviations noted in the two series of PR8-S variants represent a major change in antigenicity is revealed in the cross-protection tests. The results of these tests along with those which measure virulence, antigenicity, and immunogenicity will be presented in the following paper (8).

SUMMARY

A second series of four variants of PR8-S virus has been produced by passage of the variants in the lungs of mice immunized with the PR8-S virus as well as the homologous strain. The PR8-S virus was added as a constant component of the vaccine so that a high antibody titer to it would suppress selectively the development of variants with PR8-S characteristics. Comparative H.I. and *in ovo* neutralization tests with the first and second series of variants revealed that the three variants, Fd/s, Gf/s, and Hg/s, of the second series, failed to react with PR8-S antisera and produced significantly smaller amounts of antibody which reacted with PR8-S, the variants of the first series, and the first variant (D/s) of the second series. Although these three variants reacted quite similarly in the H.I. and neutralization tests cross-absorption of these sera with the PR8-S virus and themselves revealed individual antigenic characteristics. While these studies emphasize the importance of the immune state of the host the precise mechanism in the selection of a new antigenic component in the emerging variant must yet be determined.

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