ANTIGENIC VARIANTS OF INFLUENZA A VIRUS (PR8 STRAIN)

II. SEROLOGICAL AND IMMUNOLOGICAL CHARACTERISTICS OF VARIANTS DERIVED FROM VARIANTS*,‡

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In an attempt to simulate conditions in the human population between epidemics of influenza, the influenza A virus (PR8 strain) has been serially passed in mice partially immunized with this virus. The antigenic variant which emerged in the course of these passages has been described (1). This variant was capable of multiplying in the lungs of vaccinated mice which were fully resistant to challenge with the parent PR8 strain. It was shown by cross-serological tests that the variant contained a new dominant antigen, but that it also retained antigens related to the parent PR8 strain. Because there was no further alteration in the antigenic composition of the variant after the 17th passage in PR8-immunized mice, passages were carried in mice immunized to the variant. The present report describes the production and characterization of a series of three variants, each derived in succession from the previous one, and compares them with the parent PR8 strain and the first variant strain (1).

Materials and Methods

Several of the methods employed are similar to those previously described in detail (1). These will be briefly reviewed in the present report. The additional techniques used include the complement-fixation test and antibody absorption.

The PR8 influenza A strain was initially employed as the parent virus (1). The stock suspensions of PR8-infected mouse lung were prepared from mice infected by the air-borne route in a closed chamber and sacrificed 48 hours later (2). 10 per cent suspensions were frozen in aliquots and stored at −40°C. Broth used for lung suspensions and for all dilutions contained 1,000 units/ml. of penicillin and 1 mg./ml. of dihydrostreptomycin. Viral antigens

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for hemagglutination (H.A.) tests, hemagglutinin-inhibition (H.I.) tests, neutralization tests, antibody absorption tests, and vaccine were prepared in eggs by inoculation of diluted mouse lung suspension intraallantoically. Only first egg passage material was used (1). Virus titrations in eggs were carried out by inoculating 0.1 ml. of falling 10-fold dilutions into four 10 to 11 day old chick embryos for each dilution and testing individual allantoic fluids for chicken red cell hemagglutination after 48 hours' incubation at 37°C. and overnight chilling (3). The EID_{50} was calculated by the method of Reed and Muench (4).

Inoculation of mice for serial passage was by the intranasal route using light ether anesthesia. Mice used for serial passage were vaccinated with one to three intraperitoneal doses of formol-inactivated virus at weekly intervals. From 10 to 14 days after the last dose of vaccine the mice were bled from the tail vein and the sera tested individually for H.I. antibody titers by the modified Salk pattern test (5). In the initial passages of the variants in homologously immunized animals it was necessary to employ mice of low antibody titers. As the number of passages increased, mice with gradually increasing antibody titers were selected until virus growth could be maintained in the presence of high antibody levels. With each passage the immunized mice were inoculated intranasally under light ether anesthesia with a 0.05 ml. of a 10 per cent mouse lung suspension of the respective strains. 48 hours after inoculation the animals were sacrificed. Ten per cent mouse lung virus suspension was then prepared and titrated in eggs before passage into the next group of vaccinated mice.

Antisera for all strains of virus were prepared by infecting ferrets in the cloud chamber and bleeding them 14 to 21 days after infection (1). All sera for H.I. tests were first treated with RDE to remove non-specific inhibitors of hemagglutinin inhibition (6).

Complement-Fixation Tests.—The complement-fixation test described by Lennette *et al.* was employed (7). The viral antigens (600 S fraction) were prepared according to the method of Wiener, Henle, and Henle (8). Fixation was carried out overnight at 4°C. and the titers are expressed as the initial serum dilution showing 50 per cent fixation.

Antibody Absorption Tests.—A modification of the technique described by Walker and Horsfall (9) was used. Freshly harvested allantoic fluid virus was concentrated by adsorption and elution. Three times washed chicken red blood cells were added to chilled allantoic fluid virus in 2 per cent final concentration and the mixture was held at 2°C. for 90 minutes. Following removal of the supernate the virus was eluted at 37°C. into buffered saline, $\frac{1}{20}$ of the original volume, for a period of 2 to 3 hours. The eluate had an H.A. titer of 8,000 to 16,000 per ml. 2 ml. aliquots of the eluate were centrifuged at 39,000 *g* for 40 minutes (Spinco model L preparative centrifuge, rotor No. 40), the supernate was discarded and the virus pellet was resuspended in 2 ml. of ferret antiserum which had been diluted with equal volumes of RDE and nutrient broth to 64 H.I. units per 0.2 ml. The serum virus mixtures were held overnight at 4°C. followed by removal of the virus by centrifugation at 39,000 *g* for 40 minutes. Three successive cycles of absorption were carried out with each serum. Traces of residual viral hemagglutinins and excess RDE were inactivated by heating the supernate at 65°C. for 30 minutes. Antibody absorption was considered satisfactory (*a*) when all homologous antibody was removed in one absorption cycle, and (*b*) when the heterologous titer showed no further changes after two successive absorptions. All sera were titrated by the hemagglutinin-inhibition test and the titers are expressed as the reciprocal of the initial serum dilution causing complete inhibition of hemagglutination of four agglutinating doses of virus.

RESULTS

Nomenclature of Variant Strains.—Upon initiation of experiments to develop variants from the first variant, it became necessary to devise a scheme for designating the new strains

in order to keep the relationship of one to the other clear. This is shown in Table I. The PR8 parent strain is referred to as stock (S), and the first variant, referred to as PR8-T in the previous report (1), is renamed As, or variant A produced by serial passage in PR8-S-vaccinated mice. The second variant, derived from the first, is called Ba, the third Cb, and the fourth Dc. In each case the small letter indicates the strain used to vaccinate the mice. The numbers following these letters give the number of the serial passage in vaccinated mice at which maximum antigenic change occurred (Table I).

The appearance of the influenza virus variants in the course of serial passages in vaccinated mice was a gradual one. In early passages, the virus multiplied only in the lungs of mice with relatively low H.I. titers (1:40 to 1:80). Selection of mice with the same or with higher H.I. titers for the next passage was made on the basis of the egg infectivity titer of the lung suspension from the last passage. Variation in antibody levels among mice receiving the same vaccine made necessary the prior determination of individual titers (1).

TABLE I

Summary of Derivation of Antigenic Variants of Influenza PR8-S Virus

Virus inoculated intranasally	Virus used for immunization of mice for passage	No. of passages*	Variant isolated
PR8-S	PR8-S	14	As‡
As	As	22	Ba
Ba	Ba	15	Cb
Cb	Cb	14	Dc

* Indicates passage number at which maximal antigenic deviation occurred.
‡ Referred to in previous paper (1) as PR8-T.

Antigenic Stability and Homogeneity of Variant Strains of PR8-S Virus.—When further alteration in antigenic composition of the new strain as compared with its parent failed to occur during serial passage, it was passed in normal mice by the intranasal route for 18 to 24 passages. The final normal mouse passage was, in each case, found to be identical antigenically with the last passage in vaccinated mice. In addition, one variant, Cb, was passed in eggs at 10^{-2} dilution serially for twelve passages without any change in antigenic composition.

To determine the extent of antigenic homogeneity of the variants and the parent PR8-S strain, each strain was tested by the limiting dilution technique. Groups of twelve eggs were inoculated intraallantoically with 0.5 log increment dilutions near the EID_{50} of a particular mouse-lung virus suspension. The positive fluids from individual eggs in the group in which 25 per cent or less of the total were infected were again passed at limiting dilutions in eggs.

No serological differences were detectable among the individual fluids tested by H.I. or *in ovo* neutralization tests.

Serological Characteristics of the Variants.—The results of cross-H.I. tests (Table II), cross-complement-fixation tests (Table III), and cross-neutralization tests (Table IV), show the progressively decreasing titers of each successive variant with PR8-S antiserum. This appeared to reach the point of greatest deviation with Cb. Variant Dc had slightly, but consistently, higher titers with PR8-S antiserum in all three tests. A similar pattern of serological

TABLE II

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

Test viruses	Convalescent ferret antisera				
	PR8-S	As22	Ba25	Cb17	Dc26
PR8-S	**2560***	640	160	320	320
As22	320	**1280**	160	160	320
Ba25	40	80	**320**	320	320
Cb17	16	16	32	**640**	320
Dc26	32	40	80	160	**320**
Lee	<10	<10	<10	<10	<10

* Reciprocal of initial dilution.

TABLE III

Cross-Complement Fixation Tests with Parent (PR8-S) Virus and Antigenic Variants

Test viruses	Convalescent ferret antisera				
	PR8-S	As22	Ba25	Cb17	Dc26
PR8-S	**512**	128	128	128	128
As22	64	**128**	128	128	128
Ba25	16	32	**128**	128	128
Cb17	10	16	16	**256**	128
Dc26	16	32	32	64	**256**
Lee	<8	<8	<8	<8	<8

TABLE IV

Cross-Neutralization Tests in Eggs with PR8-S Virus and Antigenic Variants

Test viruses	Convalescent ferret antisera				
	PR8-S	As 22	Ba25	Cb17	Dc26
PR8-S	**2560**	453	57	57	113
As22	80	**1019**	113	64	120
Ba25	28	50	**226**	80	120
Cb17	7	14	12	**510**	80
Dc26	17	57	113	40	**226**

relationship is noted when the variants are compared to one another. Antisera of all of the variants crossed with PR8-S virus to almost the same titer as the homologous virus in the H.I. and complement fixation tests, while in the neutralization test there was less crossing with PR8-S. This is in agreement with previous reports that cross-neutralization tests tend to show greater immunological differences than cross-H.I. tests (10, 11).

A more precise definition of antigenic structure of the variants was obtained by antibody absorption tests. The results, presented in Fig. 1, show that

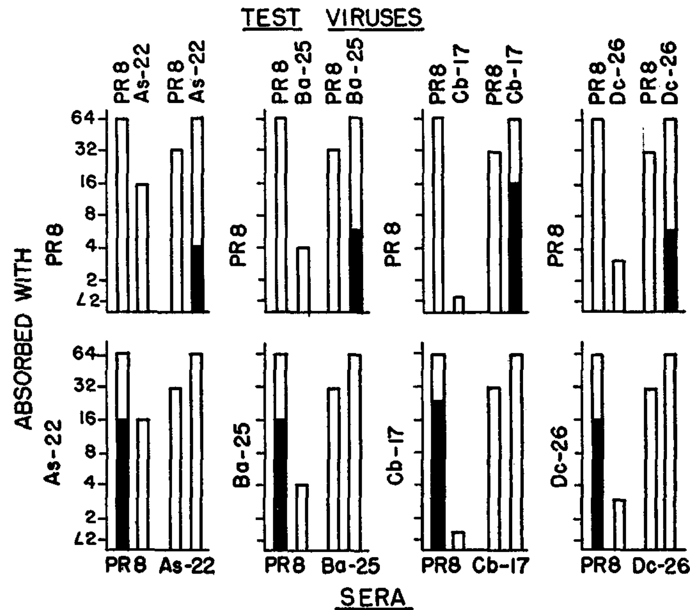

FIG. 1. H.I. titers of ferret antisera before and after absorption with homologous virus or PR8-S virus. (Black columns are titers after absorption.)

absorption of PR8-S antiserum with PR8-S virus removed all PR8-S antibody and, therefore, eliminated the titer of this serum with each of the variant viruses. Absorption of As, Ba, Cb, and Dc antisera with PR8-S removed the PR8-S antibody and reduced the titers to the homologous viruses by varying degrees. It is apparent that Cb antiserum contained less PR8-S antibody than did the antisera of the other variants. However, when the PR8-S antisera and the antisera to each variant were absorbed with homologous virus, as shown in the lower half of Fig. 1, all variants reduced the titer of PR8-S antiserum to about the same extent when tested with PR8-S virus, although the titer to each variant was eliminated. The same absorbing dose of each variant completely removed the antibody in its own antiserum, and the crossing antibody to PR8-S virus.

These results indicated that the variants shared antigens with PR8-S but in addition contained specific components. In Table V which gives the relative amount of antigen shared by each variant with PR8-S, it is again obvious that Cb contains less PR8-S antigen than strain Dc which was derived from Cb.

Immunogenic Properties of the Variant Viruses.—The observation that the variant strains continued to produce considerable amounts of antibody reacting with PR8-S in spite of their decreasing reactivity with PR8-S antiserum might have been a reflection of differences in antigenicity between PR8-S and the new specific components. Results of the serological tests indicated that the variants became progressively poorer antigens, as shown by the lower homologous titers. Furthermore, this was also noted during the development of the strains, when intraperitoneal vaccination of mice with the variants produced lower antibody titers. If the antigenicity of PR8-S is greater than

TABLE V

Homologous H.I. Titers of Ferret Antisera before and after Absorption with PR8-S Virus

Sera	Unabsorbed	Absorbed with PR8-S	PR8-S antibody absorbed	Specific antibody remaining
			per cent	*per cent*
PR8-S	64*	<2	100.0	0.0
As22	64	4	93.7	6.3
Ba25	64	6	90.6	9.4
Cb17	64	16	75.0	25.0
Dc26	64	6	90.6	9.4

* All sera were diluted to 64 H.I. units before absorption.

that of the dominant antigen of each variant, then the reduction in the amount of PR8-S component in the antigenic composition of the variants would not be reflected by a comparable decrease in the titer of PR8-S antibodies produced.

To test this hypothesis, antigenicity tests with each variant and with PR8-S virus were carried out. Mice weighing 20 to 24 gm. were injected with twofold dilutions in buffered saline of allantoic fluid virus having 1024 H.A. units/ml. Six mice were used per dilution, each receiving 0.5 ml. intraperitoneally. 2 weeks later the animals were exsanguinated from the axillary vessels and the blood from each group pooled. The homologous H.I. antibody titers were then determined and the results are presented in Fig. 2. A marked decrease in the ability of variant strains to provoke antibodies as compared with the parent PR8-S virus is evident. Even in high concentrations the variants failed to stimulate antibodies to as high a titer as PR8-S. Furthermore, PR8-S diluted 1:2560 still elicited antibodies while Cb diluted 1:320 failed to do so.

These results indicated that differences in the immunogenic properties are indeed involved in the serological relationship between the parent virus, PR8-S, and the antigenic variants.

Mouse Protection Tests.—For further evidence of the degree of deviation from PR8-S, protection tests in mice vaccinated with PR8-S and challenged with As22, Cb17, and PR8-S, and in mice passively immunized with PR8-S rabbit antiserum and challenged with Cb17 and PR8-S were carried out.

Active Immunization.—

To compare the protection afforded by immunization with PR8-S vaccine against intranasal challenge with PR8-S, As22, and Cb17, mice were vaccinated intraperitoneally with a single dose of 0.5 ml. of formol-inactivated PR8-S vaccine. The H.I. antibody titers, determined 10 days later by bleeding about half of the mice from the tail vein, ranged from 1:320 to 1:640. Because of the large number of animals involved in the experiment, they were divided into two groups and successively challenged. Groups of ten vaccinated mice

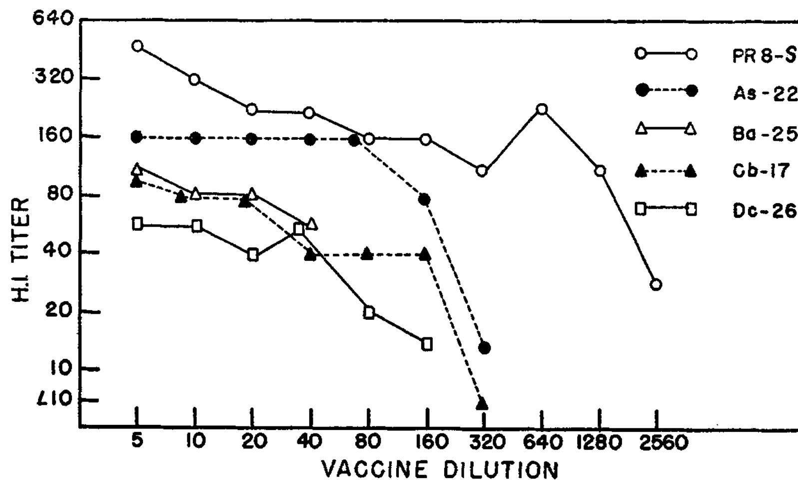

FIG. 2. A comparison of the antigenicity of PR8-S virus with that of the variant strains. The homologous H.I. titers for each virus strain are shown.

and an equal number of controls were challenged intranasally with 10^5, 10^4, and 10^3 LD_{50} of the respective virus. 72 hours later three mice in each group were killed for lung virus titer determinations. The remaining mice were observed for death or survival for 10 days. The results are given in Table VI.

All unvaccinated control mice developed high virus titers in their lungs at 3 days and the remaining mice all died by the 6th day with extensive pulmonary consolidation. Solid homologous protection was indicated by the lack of virus growth and survival of all vaccinated mice challenged with all three doses of PR8-S virus.

The effect of challenge with the variants depended on the amount of virus in the infecting dose. With the highest challenge dose of As and Cb, some of the animals died with extensive pulmonary consolidation, but the significance of the difference in the number of deaths was uncertain in view of the small size

of the sample. No deaths occurred with the two smaller challenge doses. Both variants multiplied in the lungs of immunized mice to similar titers. Thus, PR8-S vaccine failed to protect mice against infection with both variants regardless of challenge dose, and, in some cases, failed to prevent death resulting from infection with the highest dose. However, the fact that Cb reacted with PR8-S antiserum *in vitro* to much less extent than As was not reflected *in vivo* by a higher mortality in PR8-S vaccinated mice challenged with Cb.

Passive Immunization.—Further evidence for cross-protection of Cb-infected mice was obtained by passive immunization with PR8-S antiserum.

Antiserum was prepared by injecting rabbits intravenously with 5 ml. of concentrated PR8-S virus daily for 2 successive days. The animals were bled 15 days after the last injection and the titers of the pooled sera were 640 (H.I.) and 600 (neutralizing) against PR8-S antigen, and 160 (H.I.) and 40 (neutralizing) against Cb antigen. A large group of mice was given two doses, each 0.5 ml., of rabbit antiserum intraperitoneally, one 12 hours and one immediately before infection. These doses were followed by intraperitoneal doses of 0.25 ml. 8, 24, and 48 hours after infection. The passively immunized mice and control mice (given normal rabbit serum in the same way) were each divided into two groups one of which was infected by aerosol (12) with 100 LAD_{50} of PR8-S virus and the other with 100 LAD_{50} of Cb variant.[1] Following challenge in the cloud chamber, ten mice in each group were set aside for observation of death or survival. At intervals three mice from each group were bled for H.I. antibody titer after which the lungs were removed and the virus content titrated in eggs. The lungs of one mouse killed at each interval were fixed and prepared for microscopic study of the pathology. The results of this experiment are shown in Table VII.

TABLE VI

Results of Challenge Test of Mice Immunized with PR8-S Vaccine

Challenge		Vaccinated mice		Unvaccinated mice	
Virus	LD_{50} dose	72 hr. lung virus titer EID_{50}	Deaths at 10 days	72 hr. lung virus titer EID_{50}	Deaths at 10 days
PR8-S	10^5	<1	0/7*	9.0	7/7
“	10^4	<1	0/7	9.0	7/7
“	10^3	<1	0/7	9.0	7/7
As22	10^5	6.0	2/7	8.7	7.7
“	10^4	6.3	0/7	9.2	7/7
“	10^3	5.0	0/7	9.0	7/7
Cb17	10^5	6.3	3/7	9.0	7/7
“	10^4	6.0	0/7	8.7	7/7
“	10^3	5.0	0/7	8.7	7/7

* Numerator = No. of mice which died; denominator = No. of mice inoculated.

The cross-protection noted in actively immunized mice is evident also in this experiment with passively immunized mice. While none of the passively

[1] LAD = lethal air-borne dose.

immunized mice died, those infected with Cb showed considerable growth of virus in the lung. On the other hand, virus was present in the lungs of mice infected with PR8-S only at 48 hours after infection, and then the titer was very low.

TABLE VII

Results of Challenge of PR8-S Passively Immunized Mice with PR8-S and Cb Variant Strains

Time after infection	Control Mice			Passively immunized mice			
	Dead*/Total	Lung‡ titer	H.I. titer	Dead/Total	Lung titer	H.I. titer versus PR8-S	H.I. titer versus Cb
Challenged with 100 LAD_{50} PR8-S							
6 hrs.	0/10	4.2	<10	0/10	<1.0	160	120
24 "	0/10	8.7	<10	0/10	<1.0	160	120
48 "	0/10	9.7	<10	0/10	2.0	240	80
4 days	5/10	9.0	<10	0/10	<1.0	160	80
6 "	10/10		<10	0/10	<1.0	80	60
8 "				0/10		240	60
10 "				0/10		60	30
14 "				0/10		15	20
21 "				0/10		<10	<10
Challenged with 100 LAD_{50} Cb							
6 hrs.	0/10	3.5	<10	0/10	<1.0	160	120
24 "	0/10	8.5	<10	0/10	3.8	160	160
48 "	0/10	8.5	<10	0/10	5.5	240	120
4 days	0/10	8.7	<10	0/10	4.7	120	120
6 "	8/10		<10	0/10	5.0	120	80
8 "	10/10			0/10	4.7	60	40
10 "				0/10	<1.0	60	40
14 "				0/10		30	<10
21 "				0/10		<10	<10

* Cumulative.

‡ EID_{50}.

The gradual disappearance of antibody, without the expected boost in titer which usually follows infection and recovery, has been described in previous experiments (12) and is believed to result from blockage of virus from reaching the sites of antibody formation by the passively circulating antibody.

DISCUSSION

The results reported in this study indicate that successive generations of variants of PR8-S virus exhibit a progressive deviation from the parent virus characterized by decreasing reactivity with PR8-S antiserum. Cross absorption

tests demonstrate that the variants share antigens with the parent PR8-S virus but differ from it by the presence of specific antigenic components which increase in quantity in each successive variant, while the amounts of related antigens show a progressive decrease. Strain Dc26, however, contains more PR8-S component than its parent strain Cb. This would suggest that the development of further variants from variants may demonstrate a closer relationship eventually with PR8-S. These studies are now in progress.

Although the pathogenicity of the variants remains essentially the same as that of the parent virus their ability to stimulate antibodies shows a progressive decrease. This interesting phenomenon is under further study.

The fact that the variants retain the ability to elicit antibody to PR8-S in spite of the progressive decrease of the PR8-S component in their antigenic mosaic is in some respects reminiscent of the P-Q variation described by Van der Veen and Mulder (13). However, the properties of the antigenic variants in this study differ from the P-Q changes for the following reasons:—

(*a*) Fiset and Depoux (14), on the basis of absorption tests, inferred that Q strains contain their major antigens inside the virus particle in a location where it is not readily available for antigen-antibody reactions *in vitro*, but may, nevertheless, cause the formation of antibody when injected into an animal. The results obtained in this study do not indicate the presence of PR8-S antigen in a subsurface position in the virus particle of the variant strains because absorption of variant antiserum by its homologous virus eliminated the titer to both homologous virus and to PR8-S. Had PR8-S antigen been masked, the titer to PR8-S virus would have remained unchanged.

(*b*) According to Isaacs *et al.* (15), P-Q phase variation is a reversible phenomenon. Passage of P virus in fertile eggs in the presence of subneutralizing doses of homologous antiserum results in a change to the Q phase and, conversely, virus reverts to the P phase following few passages in normal mice. In contrast to these findings, the variants described in this study were shown to retain their antigenic character after repeated passages in normal mice, and in eggs (Cb variant).

(*c*) Most Q viruses described by Isaacs are characterized by the production of higher heterologous than homologous antibodies. This was never observed with any induced variants in this investigation.

The results of the antigenicity tests support the hypothesis that some of the PR8 component of the variants is being replaced by different but poorer antigens. Thus, the amount of PR8 antibody produced by the variants continues to be relatively considerable.

The fact that PR8-S vaccine protects mice against fatal infections with large doses (10^4 LD_{50}) of the highly pathogenic variant viruses, although the latter show a progressive decrease in serological reactivity with PR8-S antiserum, points up the necessity for evaluating the significance of antigenic changes with active immunity tests. The results of the challenge experiments indicate

that the variants continue to share antigenic components with the PR8-S strain in sufficient quantities to prevent unrestricted virus growth of the variants in the lungs of PR8-S vaccinated mice and the fatal outcome of the infection.

Since the publication of the first paper (1) of this series, Magill (16) reported the results of his study of influenza A prime virus propagated in immunized mice resulting in the emergence of antigenic variants. However, the differences in techniques employed in the characterization of the variants make a direct comparison of his findings with ours difficult.

The problems encountered in the prophylaxis of influenza by vaccination as a result of strain deviation in the Type A group are well established (17, 18). Recent evidence (19) of major antigenic changes occurring in the B group points up the importance of this phenomenon. Although there are probably many factors influencing the development of antigenic variants of influenza viruses during natural transmission in the human population, the results of this and other studies (10, 16) emphasize the role of the immune state of the host. In view of the limited number of passages carried out in mice in our laboratory, as compared to the large number which occur during a single epidemic in man, it seems possible that the changes shown by these variants represent only a step in the antigenic alteration that can occur in influenza A virus. Therefore, passages of Dc variant in homologously immunized mice, and the succeeding steps in this progression of passage are now in progress.

SUMMARY

Four successive generations of antigenic variants of influenza PR8-S virus, each derived from the previous one by serial passage in the lungs of mice immunized with the homologous agent, were compared with the original parent PR8-S virus with respect to their serological and immunological character. It was demonstrated by means of H.I., complement-fixation and *in ovo*-neutralization tests that the variants exhibited a progressively decreasing reactivity with the parent PR8-S antiserum while retaining the ability to elicit antibody to PR8-S influenza virus and to their respective predecessors. Accompanying these changes was a progressive reduction in antigenicity without any significant changes in pathogenicity for mice. Experimental evidence was presented which indicates that the serological changes observed with the variants are not related to the P-Q phenomenon.

Antibody absorption tests showed that the variants share antigens with PR8-S virus but differ from it by the presence of specific antigenic components; these increase in quantity with each successive variant while the amount of related antigens shows a progressive decrease.

The importance of evaluating the significance of antigenic changes of influenza viruses with active immunity tests was emphasized by the fact that

PR8-S vaccine protected mice against fatal infection with lethal doses of the variant strains although the latter had a progressively decreasing serological reactivity with PR8-S antiserum.

The inheritable character of the new antigenic properties of the variant strains was demonstrated by their persistence in the absence of the selective environment following 18 to 24 serial intranasal passages with large inocula in normal mice and following limiting dilution passage in fertile eggs.

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