STRAIN-SPECIFIC ELEMENTS IN INFLUENZA ANTIGENS

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The viruses of influenza are notable for the extent of antigenic variation which they display, not only with experimental manipulation, but also under natural conditions from epidemic to epidemic and within epidemics. Although all influenza A viruses are immunologically related they are not antigenically identical, as shown by the fact that antibodies against a given strain may be less effective against heterologous than homologous viruses. Reciprocal cross-tests on viruses and their antisera have yielded a wealth of data, showing that both large and small antigenic difference occur even with strains isolated in the same epidemic. In these cross-tests with many strains, there is usually no clear indication of an orderly arrangement of virus subtypes, but rather one gains the impression of an infinite variety of antigenic patterns with exceedingly complex interrelationships. This view is fortified by the somewhat conflicting attempts at compression of influenza A strains into broad groupings (1–3).

Very probably, the surface of a virus is made up of a number of antigenic groups, each of which may undergo variation and it seems unlikely that more definite conclusions regarding these variations can be reached by further tests done with intact viruses and antisera prepared against whole organisms. One obvious way of studying the antigenic composition of influenza viruses is by absorption of antisera with heterologous strains. That this has not already been done extensively is doubtless due in part to technical difficulties and to the large amounts of virus that are necessary.

When a strain difference exists between viruses there are two principal mechanisms by which it may be explained: (1) the same antigens may be present in both agents but in different proportions or (2) each virus may contain antigenic components which are not present in the other. The first attempts to distinguish between quantitative and qualitative antigenic variation were made by Friedewald (4) who used absorbed sera, and his results strongly indicated that there were strain-specific elements in some of the viruses he examined. However, no further experiments have been published since 1944 confirming or expanding his results, either through the use of more extensive absorption procedures or by the examination of a larger array of strains.

In the work reported below, serum absorption was applied on a wide scale to

the examination of influenza viruses which were isolated from man over an 18 year period. The results permit a distinction to be made between quantitative and qualitative antigenic variation and furthermore suggest that this distinction may be of considerable importance in various aspects of the study of influenza. The use of absorbed sera permits the subdivision of the influenza A group into antigenic types and, while the separation of such types is at present very incomplete, the present data offer a clear basis for future studies of obvious interest.

Materials and Methods

Virus Strains.—Ten influenza A strains were selected for intensive study: Swine (Sw), WS, PR8, Melbourne (Mel), Gatenby (Gat), Christie (Chr), Talmey (Tal), Ala-41, NY-43, and FM-1. All except the last were obtained from the Laboratories of the International Health Division of The Rockefeller Foundation in New York City in 1945 and have been used by the author in previous studies. The FM-1 strain was obtained from Dr. J. E. Smadel of the Army Medical School, Washington, D. C. All these strains had been adapted to growth in the allantoic sac and allantoic fluid virus was used for the production of antisera in rabbits, for the absorption of antibodies from immune sera and for the testing of HI (hemagglutination inhibition) titer of immune sera. Many other strains (listed in Table I) were used in HI tests with absorbed sera. These were obtained mainly through the kindness of Dr. T. P. Magill and were part of his collection at the Strain Study Center.

Virus Preparations.—Virus used for serological testing was prepared in the usual way by inoculating a 10^{-5} dilution into the allantoic sac of 11-day old chick embryos and harvesting the allantoic fluid after 48 hours at 36°C. and 16 hours at 4°C. These fluids were dialyzed against buffered saline and stored at 4°C. The same preparations were used for the immunization of rabbits. Virus used for antibody absorption was removed from allantoic fluid by centrifuging at 13,000 R.P.M. for 90 minutes in an angle centrifuge and was resuspended in saline of 1/100 the original volume. This was used without further purification.

Preparation of Antisera.—All antisera were prepared in rabbits. The animals were given one intravenous injection of 10 ml. of dialyzed allantoic fluid virus and were bled from an ear vein 2 weeks later. A second injection of 10 ml. of virus was given intraperitoneally and another bleeding taken after 8 to 14 days. Intraperitoneal inoculation and bleeding were repeated after rest intervals.

Testing of Antisera.—All sera were treated with RDE (receptor destroying enzyme of *Vibrio Cholerae*) before testing. RDE was used in a concentration sufficient to remove any non-specific inhibition in an HI test at a final serum dilution of 1:16 with any of the ten virus strains. All tests for antibody were made with the HI test. Falling twofold dilutions of sera (0.25 ml.) were combined with virus (0.25 ml.) of such strength that the final hemagglutination titer was four units. Fowl red cells (0.5 per cent in saline) were added (0.5 ml.) and the sedimentation pattern read after 45 minutes at room temperature. The sedimentation patterns were read as +, ±, or negative, meaning full, partial, or absence of agglutination. Partial inhibition was taken as the end-point and titers are expressed throughout as the reciprocal of the final serum dilution.

Preparation of RDE.—The Inaba (National Institutes of Health Strain 35A3) or the 4Z (5) strain of *V. cholerae* was grown in peptone broth for 18 hours. $CaCl_2$ was added to the culture and the pH adjusted to 6.0 with HCl according to the method of Stone (6). After heating at 56°C. for 30 minutes the sediment was removed and the supernatant was concentrated 50 to 100 times by pervaporation through cellophane and then dialyzed against saline. The ability of different preparations to remove Mel receptors from red cells was quite variable, but the action against normal serum inhibitor was usually satisfactory.

Absorption of Antisera with Viruses.—The technique used for antibody absorption was essentially similar to that of Walker and Horsfall (7). The sera were diluted 1:3 with saline and RDE was added, 0.1 ml. of RDE concentrate per ml. of undiluted serum. After 2 hours' incubation at 37°C., antibody absorption was carried out by adding the virus in a series of steps. At each stage, the absorbing virus, concentrated 100 times, was added to the serum in a ratio of 1 part virus to 2 parts undiluted serum. After standing overnight at 4°C., the virus was largely removed by centrifugation at 13,000 R.P.M. in an angle head for 90 minutes. Usually several such absorptions were carried out serially after which sodium citrate was added to a final concentration of 2 per cent and the serum heated at 65°C. for 30 minutes. Heating at this temperature nearly always destroyed any residual hemagglutinin left after centrifugation and did not detectably destroy antibody. No antiserum was diluted beyond 1:4 and after this dilution was reached through the addition of virus, further absorptions were carried out by adding the virus in the form of a pellet which was resuspended with the aid of a TenBroeck grinder. It proved necessary to remove the bulk of the absorbing virus before heating, since it was found that virus released absorbed antibody at 65°C.

It happened very often that immune rabbit sera were found to contain hemagglutinins active against chicken cells at dilutions of 1:256 or less. This was especially true of sera from animals that had received long courses of immunization and was presumably the result of inoculation of chick embryo material along with the virus. These hemagglutinins interfered with the specificity of the HI tests at low dilutions. They could be removed with fowl red cells but it was found that they disappeared during the process of antibody absorption, again presumably owing to chick embryo impurities in the absorbing virus.

EXPERIMENTAL

Selection of Strains for Antigenic Comparison

Since one of the principal objectives of this work was to determine whether there are strain-specific antigens among the influenza A group, viruses were chosen for special study which had been shown to differ sharply from each other by the usual methods of cross-testing. In addition, an attempt was made to include in the test strains from most of the major outbreaks since 1933. In order to minimize the difficulties of cross-testing, the number of strains intensively studied was limited to ten and this resulted in some omissions which will have to be corrected in future work.

The strains chosen were as follows: WS (8), PR8 (9), and Mel (10) were the three earliest strains used, dating from epidemics in 1933, 1934, and 1935. They are widely employed for experimental work and in this laboratory show marked differences from one another. Strains Chr, Tal, and Gat were obtained from the English epidemic of 1936–37 and were used by Smith and Andrewes as the representative type strains of that outbreak (2). Ala-41 and NY-43 are the author's strains from American epidemics of 1941 and 1943 and are included because of previous antigenic studies on them (11, 12) which showed that each was fairly representative of the strains then current. Strain FM-1 is the most widely used representative of the kind of virus which was prevalent in 1946 and in subsequent years. The Sw strain was isolated in 1932 by Dr. R. E. Shope and is sometimes designated as Swine-15. Of the foregoing strains only Ala-41 and NY-43 have been shown by previous tests to resemble one another closely. The rest have been considered by most investigators to be widely divergent from each other.

A number of rabbits were immunized with each of the foregoing strains and sera were selected for absorption on the basis of the highest HI titer against the homologous virus. This was usually in the range of 4,000 to 16,000. Extensive preliminary tests were carried out

TABLE I

HI Tests with Influenza A Viruses Done with Absorbed Sera of Seven Virus Types

No.	Strain designation	Year isolated	Place isolated	Isolated by	Method	Strains tested against types						
						Sw	WS	PR8	Mel	Tal	Ala-41	FM-1
1	WS	1933	England	(2)	FME	—	4000	—	—	—	—	—
2	PR8	1934	Puerto Rico	(1)	FME	—	—	2000	—	—	—	—
3	Melbourne	1935	Australia	(1, 10)	FME	—	—	—	2000	—	—	—
4	Philadelphia	1934–35	U. S. A.	(1)	FME	—	—	—	1000	—	—	—
5	Alaska 313	1935	Alaska	(1)	FME	—	—	—	—	—	1500	—
6	Miss.—4, 5	1935–36	NYC	(1)	FME	—	—	—	256	—	—	—
7	Talmey	1937	England	(2)	FME	—	—	—	—	256	—	—
8	Gatenby	1937	England	(2)	FME	—	—	—	2000	—	—	—
9	Christie	1937	England	(2)	FME	—	—	—	2000	—	—	—
10	Oakham	1937	England	(2)	FME	—	—	—	2000	—	—	—
11	Letchworth	1937	New York	(13)	FME	—	—	—	—	—	512	—
12	Patnode	1937	NYC	(1)	FME	—	—	—	—	256	256	—
13	Henry	1936–37	NYC	(1)	FME	—	—	—	—	—	2000	
14	BH	1935	England	(2)	FME	—	—	—	—	—	—	—
15	NY-2	1936	NYC	(13)	FME	—	—	—	—	—	—	—
16	TF	1936–37	NYC	(1)	FME	—	—	—	—	—	—	—
17	Reid-A	1939	Australia	(14)	FME	—	—	—	—	—	64	—
18	Baum	1940–41	NYC	T. Francis, Jr.	FME	—	—	—	—	—	2000	—
19	Coyle	1940–41	NYC	Magill and Sugg	FME	—	—	—	—	—	1000	—
20	Kil-E	1940–41	Miss.	(15)	E	—	—	—	—	—	1000	—
21	Ala-41-E	1940–41	Ala.	(15)	E	—	—	—	—	—	256	—
22	Ala-41-M	1940–41	Aa.	(15)	FME	—	—	—	—	—	128	—
23	Bel-A	1942	Australia	(16)	E	—	—	—	—	—	128	—
24	Hemsbury	1943–44	London	(17)	FME	—	—	—	—	—	512	—
25	Lienhart	1943–44	Calif.	Eaton	E	—	—	—	—	—	256	—
26	Weiss	1943–44	Mich.	(18)	FME	—	—	—	—	—	2000	—
27	L987	1943–44	NYC	Hirst	E	—	—	—	—	—	512	—
28	L999	1943–44	NYC	Hirst	E	—	—	—	—	—	1000	—
29	L1004	1943–44	NYC	Hirst	E	—	—	—	—	—	1000	—

— indicates no inhibition with 4 units of virus and a final serum dilution of 1:16.

A majority of the references indicates papers in which the strain in question was studied and not necessarily a paper referring to the isolation of a strain.

TABLE I—*Concluded*

No.	Strain designation	Year isolated	Place isolated	Isolated by	Method	Strains tested against types						
						Sw	WS	PR8	Mel	Tal	Ala-41	FM-1
30	L1225	1943–44	NYC	Hirst	E	—	—	—	—	—	1000	—
31	CAM	1946	Australia	(16)	E	—	—	—	—	—	—	16
32	IAN	1945–46	Australia	(16)	E	—	—	—	—	—	—	256
33	FM-1	1947	N. J.	(19)	E	—	—	—	—	—	—	256
34	Georgia	1947	Ga.	Friedewald	E	—	—	—	—	—	—	400
35	Ch-F	1947	Wyo.	Smadel	E	—	—	—	—	—	—	400
36	Skopp	1947	Calif.		E	—	—	—	—	—	—	256
37	LF-47	1947	Calif.	Smadel	E	—	—	—	—	—	—	256
38	L2-47	1947	Penn.	Sigel (20)	E	—	—	—	—	—	—	256
39	L3-47	1947	Penn.	(20)	E	—	—	—	—	—	—	128
40	L7-47	1947	Penn.	(20)	E	—	—	—	—	—	—	—
41	L8-47	1947	Penn.	(20)	E	—	—	—	—	—	—	—
42	Jotz	1947–48	NYC	Magill	E	—	—	—	—	—	128	—
43	AZ	1948	NYC	Horsfall	E	—	—	—	—	—	—	—
44	Smith	1947	Cleveland	Feller	E	—	—	—	—	—	—	90
45	Moore	1947–48	Calif.	Lennette	E	—	—	—	—	—	—	1000
46	Ocean Isl.	1947–48	Ocean Isl.	(21)	E	—	—	—	—	—	—	128
47	Wilfong	1948	Calif.	(22)	E	—	—	—	—	—	—	256
48	Devore	1948	New York	Gordon	E	—	—	—	—	—	—	256
49	Augustine	1949–50	Pittsburgh	Salk	E	—	—	—	—	—	—	256
50	Conn.	1949	Conn.	Green	E	—	—	—	—	—	—	64
51	L4–49	1949	Philadelphia	Sigel	E	—	—	—	—	—	—	64
52	A-Nederl.-49	1949	Netherlands	Mulder	E	—	—	—	—	—	—	64
53	Albany-50	1950	New York	Gordon	E	—	—	—	—	—	—	64
54	Warren	1949–50	Wyo.	Gould	E	—	—	—	—	—	—	64
55	Sweden 3/50	1950	Sweden	(23)	E	—	—	—	—	—	—	—
56	Eng. 1/51	1951	England	(23)	E	—	—	—	—	—	—	—
57	Saunders	1951	NYC	Eddy	E	—	—	—	—	—	—	—
58	FM 111/51	1951	N. J.	Hilleman	E	—	—	—	—	—	—	—
59	L590	1951	NYC	Hirst	E	—	—	—	—	—	—	—
60	L615	1951	NYC	Hirst	E	—	—	—	—	—	—	—
61	L629	1951	NYC	Hirst	E	—	—	—	—	—	—	—

with various sera, using a number of viruses for absorption and testing for the removal of homologous and heterologous antibody.

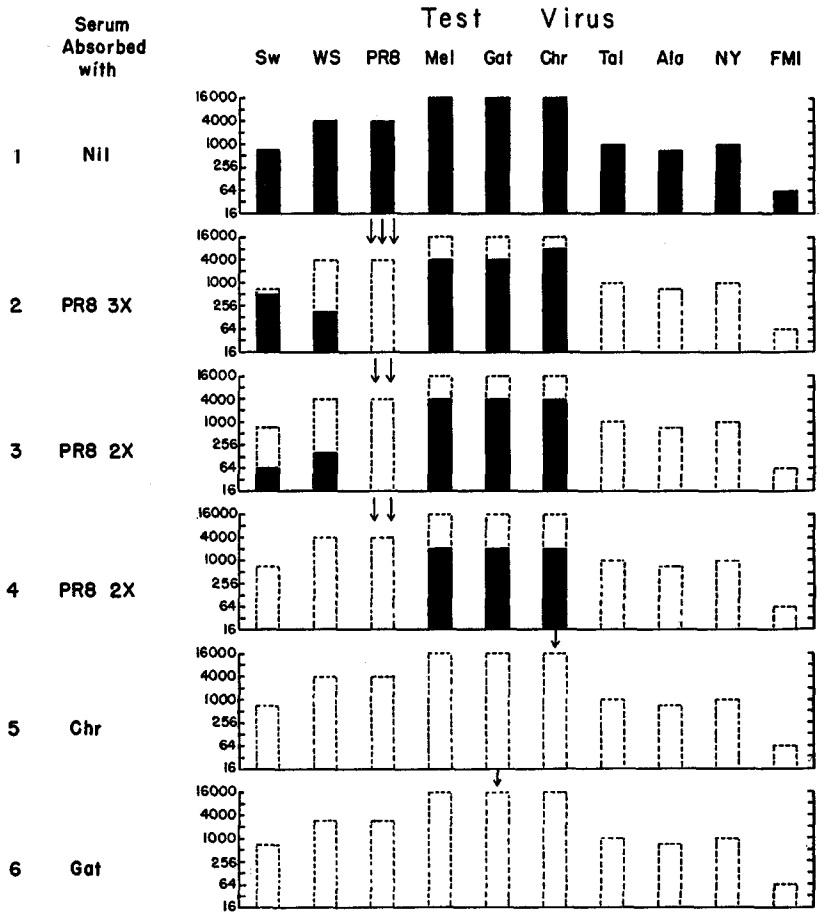

FIG. 1. Absorption of Mel serum with PR8 virus. The solid areas indicate the HI titer against the various viruses at several stages of absorption. The dotted lines indicate the titers of unabsorbed sera. The serum shown in line 4 was absorbed with Chr (line 5) and with Gat (line 6).

Preliminary Absorption Tests with 1936–37 Strains

Results are given below of cross-absorption tests between the English strains Mel, Gat, and Chr. These are given in some detail since they clearly illustrate the relationships found on a larger scale.

The experiment shown in Fig. 1 was carried out with a Mel serum and the 1st line shows the unabsorbed titers against homologous and heterologous strains. The titers against Mel, Gat, and Chr were 16,000, the titer against FM-1 was 64, the other values being intermediate.

The serum was absorbed three times with concentrated PR8 virus and retested for HI titer against the ten standard strains. The results, shown in the second line, indicate that antibody directed against five of the ten viruses was removed below the limit of detectability. However, a high level of antibody remained against strains Mel, Gat, and Chr; reactivity with Sw was only slightly diminished and the titer against WS dropped from 4,000 to 128. Two further absorptions with PR8 virus reduced the titer against Sw and WS still more but in all seven absorptions were necessary to take out these crossing antibodies completely. The serum, as shown in line 4, had a titer of less than 16 against seven strains but a titer of 2,000 against Mel, Gat, and Chr. At this stage, the serum was absorbed separately with heterologous strains

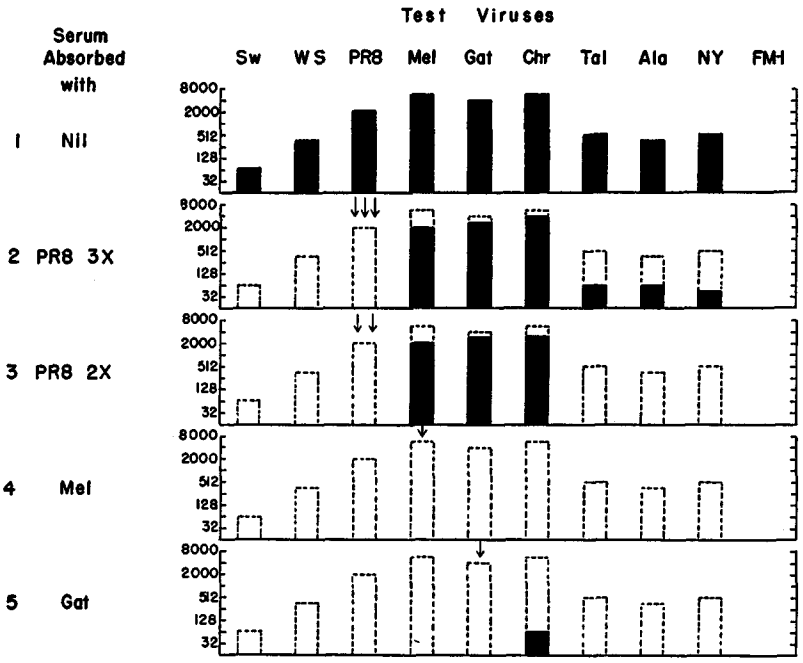

FIG. 2. Absorption of Chr serum with PR8. See Fig. 1.

Gat and Chr (lines 5 and 6). Each removed all of the detectable antibody remaining. Though not shown here the absorption of this Mel serum with Mel virus also removed all antibody.

A similar series of absorptions of serum Chr is shown in Fig. 2. In this case, three absorptions with PR8 left antibody active against strains Ala-41, NY-43, and Tal, as well as higher levels against Mel, Gat, and Chr. Two further absorptions left only antibodies against the latter three viruses, just as occurred in the previous experiment. Here, again, absorption with heterologous strain Mel removed all antibody while a single absorption with Gat left only a trace of Chr antibody. This was easily eliminated in a further absorption. A similar series of tests was carried out with serum Gat. After thorough absorption with PR8 virus antibodies remained only for Mel, Gat, and Chr and these in turn were removed by any of these three viruses.

The results of the foregoing tests were given in some detail because they clearly illustrate two general kinds of strain differences, those which are due to

quantitative differences in the antigenic pattern and those due to sharp qualitative differences in the antigens present. The group Mel, Gat, and Chr is an example of quantitative antigenic difference. Previous studies on these strains (1, 2) have shown that they are different from each other. The fact that absorbtion of antisera for these three viruses with other strains always left antibody in high titer for these viruses and that each of these strains was capable of completely removing antibodies against the others, suggests that Mel, Gat, and Chr belong in the same category or type of influenza A strains. There is no evidence that any one of the three possesses an antigen which the others lack and hence it may be reasonable to assume that the antigenic differences among them are due to quantitative variations in the antigens present. Some evidence in this direction is the fact that the initial absorption of Mel serum with PR8 virus repeatedly left Sw and WS antibody which was very difficult to remove, while a similar absorption of Chr serum left Ala-41, NY-43, and Tal-inhibiting antibodies.

In sharp contrast to the kind of variation displayed within the Mel-Gat-Chr group is the sharp qualitative difference between this group and the seven other viruses tested. Absorption of Mel, Gat, and Chr sera with PR8 invariably failed to remove all the antibodies against these three agents and hence PR8 virus lacked certain antigens present in these strains. Absorption of these sera with still other viruses of these series gave similar results and it therefore seems probable that there are antigens which are peculiar to the Mel-Gat-Chr group. One may think of influenza A viruses as containing two general classes of antigens, the non-specific or group A and the strain- or type-specific antigens. In the illustration given above, it appears that PR8 possessed all of the non-specific antigens present in strains Mel, Gat, and Chr, since it was capable of absorbing all cross-reacting antibody from their sera but it apparently contains some of the non-specific antigens in much reduced amount since it took so much virus to completely remove the cross-reactions, roughly five to ten times the amount necessary to absorb the sera completely with the homologous viruses.

Cross-Relationships among Ten A Strains Isolated between 1932 and 1947

The experiments reported below are similar to those in the previous section but since they cover a wider range of strains the results offer a more comprehensive view of the occurrence of specific antigens from 1932 until the present.

The absorptions in the previous tests were carried out with a single virus strain in order to find out if PR8 actually contained all the antigens necessary for completely removing the crossing antibody. For the practical matter of producing specific sera with the least amount of absorbing virus, most sera were absorbed once or twice with PR8 and once with Mel. This combination usually removed most of the non-specific antibody and that which was left was taken out with the specific crossing strain. This proved to be more efficient than the use of a

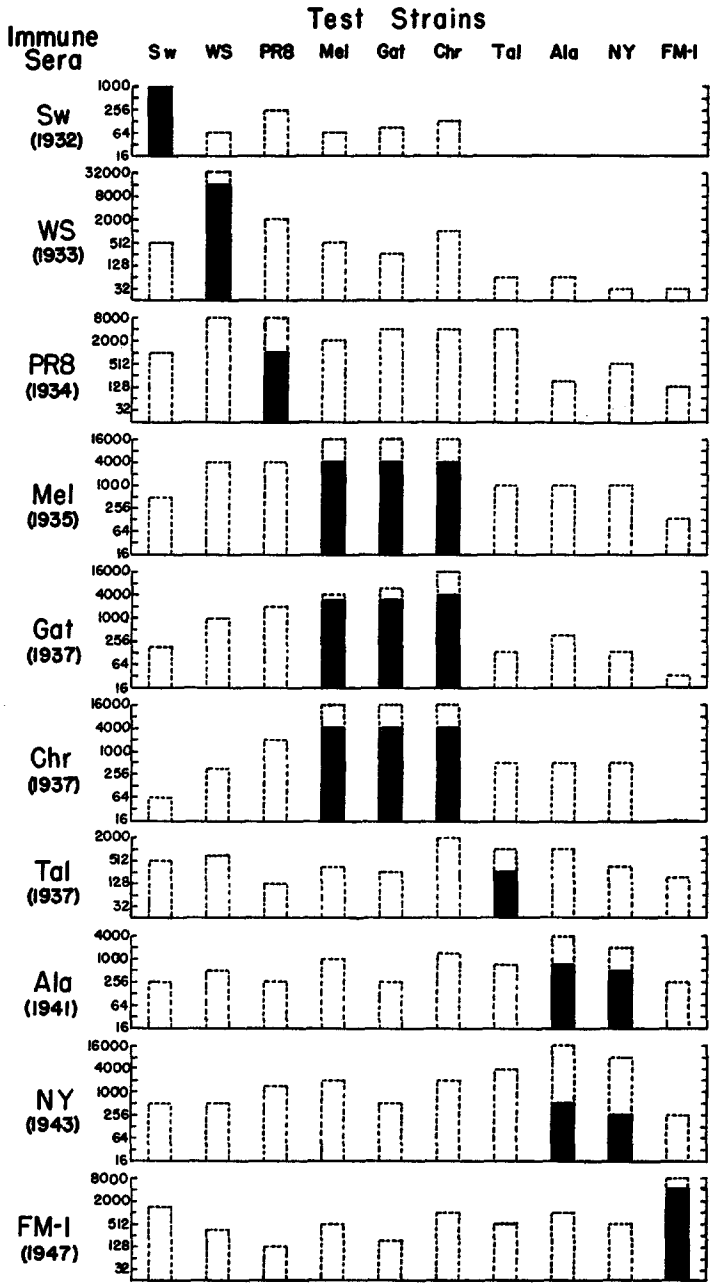

FIG. 3. Complete cross-HI test between ten viruses and their antisera, both absorbed and unabsorbed. Dotted lines indicate serum titer before and solid areas after absorption. The following absorptions were carried out: Serum Sw with PR8 (2) and Mel. Serum WS with PR8, Mel, and Sw. Serum PR8 with Mel and Tal. Serum Mel, Gat, and Chr each with PR8 (5). Serum Tal with PR8, Mel, and NY-43. Serum Ala-41 with PR8, Mel, Chr, and Tal. Serum NY-43 with PR8, Mel, and Tal. Serum FM with PR8 (2), Mel, NY-43, and Sw. Each absorption was carried out with 0.5 vol. concentrated virus per 1 vol. undiluted serum. Numerals in parentheses after name of a virus indicate number of absorptions with that strain.

single absorbing strain. Sera against the ten selected strains were absorbed to the point where non-specific antibody was no longer detectable and a complete cross-HI test involving all the viruses and the unabsorbed and absorbed sera was carried out. The results are shown graphically in Fig. 3.

The crossing antibody was reduced in each case to the point where it was not detectable at a final dilution of 1:16. It is of some interest to see how much homologous antibody was removed to accomplish this result. With sera Sw, WS, and FM-1, the lowering of homologous titer on absorption was small, the specific element accounting for 50 per cent or more of the original homologous titer. This is in line with the relatively high specificity shown by these strains with unabsorbed sera. With most of the other strains, the homologous titer after absorption was 12 to 25 per cent of the titer before absorption, except in the case of NY-43 serum in which it was much less. These percentages varied a good deal, depending on the serum selected for absorption.

As indicated above, Mel, Gat, and Chr formed one group of similar strains. Another though less unexpected group consisted of the 1941 and 1943 strains, which had shown only small antigenic differences by the usual methods. Each virus absorbed completely the antibody from the serum of the other and therefore these two are representatives of the same strain type. Thus, among the ten strains studied there appeared to be seven strain types, of which six came from human epidemics. Sera were obtained against each of these types which after absorption reacted with the homologous strains in a titer usually in excess of 256 and at the same time failed to react with heterologous strains at 1:16.

All the previous tests have been repeated in their entirety at least once with similar, though not quantitatively identical results. The only difficulties in repetition were with Tal. This virus in general is a poor antigen and most antisera were of relatively low titer. Some Tal antisera were exhausted completely of antibody on absorption with PR8 virus, while others, like the one shown, retained low but specific titers. Since Tal virus gave no crossing with other absorbed sera and since the positive results with Tal serum seem more significant than the negative results, it is suggested that Tal is a prototype of a subgroup of the A viruses.

The Typing of Influenza A Strains with Specifically Absorbed Sera

Since the production of absorbed antisera is slow and laborious, the extension of the foregoing system was most readily accomplished by attempting to classify other virus strains with absorbed sera of the seven strain types elucidated above. If other strains reacted specifically with single sera and without cross-reactions, it would greatly strengthen the conception of a true sort of qualitative specificity among influenza A viruses and at the same time gaps or omissions in the present series might be detected.

Absorbed sera were prepared in much the same way as those used in previous

tests. Mel and Ala-41 were used as prototypes of their specific groups and a large array of viruses were tested against the seven type-specific sera at a dilution of 1:16 final. When inhibition occurred, the HI level was titrated. All the absorbed sera used had homologous titers of at least 256 and usually much higher.

The results, given in Table I, were encouraging since only one of 61 strains reacted with more than one absorbed serum, and while many strains were not inhibited by any serum, this may indicate only that some serological types were missing from the series. No strain reacting with Sw serum was found and this was not unexpected since Sw was not of human origin. Likewise, no examples of PR8 and WS types were found and in this connection it should be pointed out that no other strains from the same epidemics were available. There were six strains which were inhibited by Mel serum and this includes examples isolated in England, Australia, and the United States in the years 1935 to 1937. Strain Patnode (No. 12) was the only strain which fell into the Talmey group but it reacted equally well with Ala-41 serum and is the sole example of a strain which reacted with two antisera. This cross-reaction is being further investigated. An example of the Ala-41 type first appeared in an Alaskan strain (No. 5) isolated in 1935 and others were found in the United States in 1936–37 (Nos. 11 and 13) and in Australia in 1939 (No. 17). In numerous examples from American, British, and Australian outbreaks from 1941 through 1944 the Ala-41 type was the only one found, and then it all but disappeared recurring only once much later (1947–48) in what appears to be an exceptional case (No. 42). The FM-1 type strains, as has been previously established, first were found in Australia in 1946, and remained as the only type in numerous epidemics, both here and elsewhere, up until early 1950. Three exceptions (Nos. 40, 41, and 43) will be mentioned below. The influenza outbreak of 1950–51 yielded A viruses which did not react with any absorbed serum and the possibilities of this being a new type are being explored. Three other strains, BH, NY-2, and TF, which were isolated in the years 1935–37 also failed to react with any antiserum, indicating the possibilities of the occurrence of still further types in that period.

Special attention should be called to a few individual strains. Ala-41-E and Ala-41-M (Nos. 21 and 22) are two strains, each adapted to the chick embryo by different methods. The first was isolated in the amniotic sac and adapted to allantoic growth, the other was passed initially in ferrets and then carried through forty mouse passages before being adapted to the egg. Previous HI tests established that the two strains were significantly different from one another in antigenic pattern (15), yet both were inhibited to approximately the same titer by absorbed Ala-41 antiserum and were unaffected by other sera. This illustrates an important point in interpreting data on strain differences since it indicates that mouse passage, though it alters the antigenic pattern, does not necessarily alter the strain type.

Another point of interest concerns the homogeneity of strains within an epidemic. The 1941 epidemic of influenza A in the United States was the first in which numerous strains were isolated that were antigenically homogenous (11) and a similar state of affairs by and large prevailed throughout subsequent outbreaks. There is now little doubt that in most epidemics there has been usually only one antigenic type or occasionally two (23) simultaneously present. There has been, however, a growing number of reports of limited outbreaks in recent years from which older types of A virus (PR8, for example) have been isolated. Isaacs and Andrewes (23) have taken the view that these exceptional isolations are examples of laboratory contamination and cite the fact that all such strains which they have examined were mouse-virulent on initial isolation. The mouse virulence, in their view, was evidence of contamination with a previously mouse-adapted strain and the resemblance to PR8 was due to the wide use of this strain in virus laboratories. It is beyond the scope of the present paper to discuss this problem in its entirety but strains L7-47 and L8-47 (Nos. 40 and 41) are examples of strain heterogeneity in the 1947 outbreak which are not so readily dismissed. These two strains were isolated by Sigel (20) in a school epidemic which was predominantly FM-1 in type (Nos. 38 and 39). He found them (L7 and 8) to be serologically distinct from the FM-1 strains, even when cross-absorption was used. They are not virulent for mice (24) and the present tests confirm his findings since they fail to react not only with FM-1 antisera, but with all the other types as well. Since they show no relationship to a wide variety of types, it seems permissible to conclude that they are not laboratory contaminants and that variations in strains may occur in epidemics, even though this behavior is exceptional.

In Table I it can be noted that there was considerable variation in the titer obtained with various strains against their type serum. This may be an indication that these strains possess the type antigens in varied form or in different amounts, but it seems at least equally likely that the variations may represent an alteration in the "avidity" of the strains for antibody. Hirst (11) first proposed this term to explain the fact that some antisera inhibit heterologous strains to higher titer than the homologous one. The same phenomenon was encountered recently by Van der Veen and Mulder (25) with their P and Q viruses, the latter of which is inhibited in low titer by P and Q sera but can stimulate the formation of antibodies which react in high titer with the P variety. More extensive cross-testing would be necessary to prove that avidity is responsible for the variable titers which were found in the present work.

DISCUSSION

The results of absorption tests with influenza A viruses and their antisera indicate that the antigens of these agents can conveniently be divided into two categories; the group-specific antigens which convey the A character and the

type-specific antigens which are peculiar to certain groups of strains. From ten strains which were studied seven distinct types were segregated on the basis of HI reactions with absorbed sera. Properly absorbed sera gave no cross-reactions among these types and permitted the classification of numerous A strains with very little ambiguity. When large strain differences were found between strains by the usual method with unabsorbed sera, it was generally found that this could be explained on the basis of a difference in type antigens. Where small differences occurred, the strains were often of the same type and it seemed probable that they could be explained on the basis of purely quantitative variations in the antigen content, especially the group antigen. Very limited experiments showed that the group antigens were similar in types PR8 and Mel, although the difficulties of cross-absorption here again suggested quantitative variations in group antigens.

A great deal of the information about strain differences indicates that the situation from 1933 to 1940 is widely at variance with what has prevailed since that time. Prior to 1940, a wide variety of strains were prevalent at one time, even in the same epidemic, while since 1940 the existence of a single variety of strain throughout the world has been the dominant characteristic. Since the earlier work was of necessity done with mouse-adapted strains, the natural but perhaps unwarranted assumption was made (15) that the earlier heterogeneity was largely due to mouse adaptation. If the interpretation, suggested by the tests with Ala-41-E and Ala-41-M, is accepted, that mouse adaptation may induce small but not large changes in antigenic pattern and that it does not alter the virus type, then it follows that at least some of the heterogeneity seen prior to 1940 was in fact due to the concomitant existence of multiple types in the population.

From this angle, it would appear that the first two A types (WS and PR8) disappeared in 1933 and 1934, the length of time in which they were prevalent cannot be determined. Beginning in 1935 at least four and possibly more types occurred at the same time. One of these (Mel) was found between 1935 and 1937. A second, Ala-41, was also found in this period and went on to become the only type existent between 1940 and 1944. A third type (Tal) is so far monotypic and the existence of further types is as yet hypothetical, being based on the non-relationship of a few strains to any of the type representatives. Experience since 1940 suggests the dominance of a single type, Ala-41 until 1944 and FM-1 from 1946 to 1950. The status of the 1950–51 strains is as yet unestablished. If this interpretation is accepted, it is apparent that the experience since 1940 was not necessarily typical of the over-all epidemiological picture of influenza and the time may return when multiple types of virus may be prevalent at one time. Furthermore, the epidemic prevalence of a given strain can be seen to be quite variable in duration, and in these results was as short as 2 years and as long as 9 years.

The occurrence of one type at a time since 1940 has given an excellent opportunity to study the occurrence of variation with a type of virus from epidemic to epidemic. From 1941 to 1943 there was a small antigenic change in the Ala-41 type virus (12) and from 1947 through 1950 Hilleman (22) and Magill (26) have shown small progressive changes in the FM-1 type of virus from year to year. From this rather restricted evidence, it seems that a virus type can change in minor ways from one appearance to the next and that these slow changes may be interrupted by major breaks with disappearance of old and appearance of new types. While there is not much evidence as to the importance of type changes in the problem of influenzal immunity, that which exists suggests that antigenic shifts within a type have relatively little effect, as shown by the fact that immunization of man with FM-1 type virus of 1947 protected individuals against the somewhat different variety of FM-1 infection seen in 1950 (27). On the other hand it was thoroughly established that a vaccine of the Ala-41 type offered no protection whatsoever against an epidemic due to FM-1 type virus (28).

There are, of course, many problems in connection with the foregoing work which need further study. Among these are the completion of the analysis of the available A viruses so that all strains may be included in one system. A similar program should be carried out with B strains. Of greatest importance, however, is the extension of the present methods of study by the use of *in vivo* techniques in the hope of gaining some assessment of the relative importance of group-specific *versus* type-specific antigens in the development of the immune state. Nothing has been done along these lines as yet. Although the importance of a system such as has been proposed is in many respects not yet clear, a grouping of strains based on antigenic composition has proven to be a sound approach to problems in other fields of microbiology. In the study of influenza it may well shed further light on the epidemiological processes at work.

SUMMARY

Rabbit antisera were prepared against ten antigenically different influenza A strains. These sera were absorbed with one or more heterologous strains and in each case all the heterologous or crossing antibody was removed; the antibodies remaining after this treatment were specific for the immunizing strain or group of strains.

On the basis of reactions with absorbed sera, the strains fell into seven groups.

Absorbed specific antisera of these groups were used to test the HI titer against a large number of influenza A viruses. Most of the strains were inhibited by a single serum, a few were inhibited by none of the sera, and only one strain was inhibited by two antisera.

The grouping of strains by this method was less equivocal than classifi-

cations based on previous tests. When more fully developed, this technique promises to be of interest and assistance in the study of influenza, especially from the epidemiological and prophylactic standpoints.

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