SEROLOGICAL RESPONSES TO ASIAN INFLUENZA VIRUSES IN MAN: A COMPARISON OF HAEMAGGLUTINATION-INHIBITION AND COMPLEMENT-FIXATION METHODS

D. HOBSON AND E. PEARSON*

From the University of Sheffield Virus Research Laboratory, Lodge Moor Hospital, Sheffield, 10

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THE serological response to Asian influenza infection demonstrated in man by haemagglutination-inhibition tests appears to be virtually strain-specific in character and limited to the antigen of the Asian (A2) influenza virus (Menon, 1959 ; Zhdanov and Ritova, 1959). Henle, Lief and Fabiyi (1958) showed, however, that complement-fixation techniques with purified strain-specific virus particle (V) antigens (Lief and Henle, 1956*a* and *b*) detected significant antibody responses not only to the homologous infecting virus but to V antigens derived from a variety of strains of influenza virus from the A and A1 families. Further information therefore was sought concerning the range of antibody responses resulting from exposure to Asian influenza virus in Britain by a comparison of haemagglutination-inhibition (HI) tests and complement-fixation tests using virus particle antigens (V-CF tests). The two methods were first compared in tests with antisera from animals and then with sera from two groups of persons. The first group consisted of 25 healthy volunteers (naval recruits aged 20–30) drawn from a larger group inoculated with a monovalent inactivated Asian virus vaccine in the spring of 1959 (Himmelweit, unpublished). In the second group paired sera from 50 cases of influenza occurring between September 1957 and March 1959 were selected on a basis of positive virus isolation or of fourfold or greater rise in antibody to influenza virus soluble antigen (Type A). There was no selection by age in the latter group ; the 50 cases ranged from 8–75 yr.

From each person in the 2 groups paired sera were taken before and after the period of exposure to the virus antigen. In all such pairs, the pattern of antibody response to each of the main families of influenza A viruses was estimated separately, first by HI tests and secondly by V-CF tests.

MATERIALS AND METHODS

Sera

All specimens were kept frozen at − 20°. Convalescent ferret antisera were prepared by intranasal inoculation of infected allantoic fluids (WHO Report, 1953). Guinea-pig "antisoluble" antiserum (anti-S) was produced by intranasal inoculation with live virus of strain WS followed by intraperitoneal injection of "soluble" (S) antigen prepared from PR8 virus (Lief, Fabiyi and Henle, 1958). All human sera were paired specimens ; in the vaccinated group samples were taken one week before and 3 weeks after inoculation ; in the group of clinical cases of influenza, samples were taken when the patient was first seen in the acute phase, and 10–14 days later during convalescence.

* British Council Fellow.

Viruses

The strains of influenza used were Swine, WS and PR8 representative of the family A, FMI as typical of the family A1 and Pakistan I (kindly provided by Dr. F. Himmelweit, the Wright-Fleming Institute of Microbiology, London) as a member of the Asian family. All virus preparations were stored at − 70° until use.

Pools of infected allantoic fluids for HI tests were prepared in eggs by standard methods (WHO Report, 1953). The tests were performed by a modification of the plastic plate technique (WHO Report, 1953) using 0·02 M-phosphate buffered saline at pH 7·0 and a 0·5 per cent suspension of hen red blood cells. Sera for HI tests were treated with commercial cholera filtrate (Philips-Roxane) to remove non-specific inhibitors. Virus suspensions were used at a concentration of 8 haemagglutinating units per test volume.

V antigens were prepared from infected allantoic fluids by 2 cycles of adsorption on to and elution from fowl red blood cells (Lief and Henle, 1956*b*). V-CF tests were performed by a single-line modification of the microdrop method of Fulton and Dumbell (1949) with a fixation period of 1½ hr. at 37°. Sera were inactivated by heat at 56° for 30 min. before testing. Each V antigen was used at an optimum dilution determined by chessboard titrations with known positive ferret antisera.

S antigen was prepared from chorio-allantoic membranes (WHO Report, 1953) using the PR8 virus, and the test procedure was exactly similar to that with V antigens.

In both CF and HI tests antibody titres were expressed in terms of the initial dilution of serum.

RESULTS

Tests with animal antisera

Comparative titrations were performed by HI and V-CF tests with materials prepared from each of the 5 standard virus strains and with S antigen; results are shown in Table I.

TABLE I.—*Comparison of Haemagglutination-inhibition (HI) and Complement-fixation (CF) Titres in Sera from Animals Immunized Against Specific Influenza A Strains.*

Test	Antigen		Antisera: Ferret WS	Ferret PR8	Ferret FMI	Ferret Asian	Guinea-pig anti-S
HI	Allantoic fluids	Swine	0*	0	0	0	0
		WS	*128*†	4	0	0	0
		PR8	4	*128*	0	0	0
		FM1	0	0	*256*	0	0
		Asian	0	0	0	*512*	0
CF	Purified V antigens	Swine	0	0	0	0	0
		WS	*256*	0	0	0	4
		PR8	4	*128*	0	0	4
		FM1	0	0	*512*	0	0
		Asian	0	0	0	*256*	0
	Soluble antigen		128	256	256	128	64

* 0 = no detectable reaction at 1/4 dilution of serum.

† Titres expressed as denominator of dilution. Titres of sera with homologous immunizing virus are in italics.

In each of the 4 ferret antisera, HI antibody was demonstrated only against the homologous immunizing strain with the exception of a very weak reciprocal cross-reaction between WS and PR8. These sera also contained significant and

comparable amounts of CF antibody to the shared soluble (S) antigen. The guinea-pig antiserum contained only anti-S antibody.

The panel of V antigens gave strain-specific reactions with the ferret antisera and titres were closely similar to those obtained in HI tests. There were no heterologous cross-reactions of significance despite the presence of anti-S antibody in all 4 sera ; with the guinea pig anti-S serum, only trace reactions were found confined to the 2 strains used for immunization.

These results suggest that the V antigen preparations were essentially free from detectable S antigen and were capable of demonstrating a strain-specific response to immunization similar to that shown by HI tests. Accordingly, the 2 groups of human sera were similarly investigated with the same batches of reagents.

Tests with sera from vaccinated persons

The pattern of serological response to Asian vaccine is shown in Figs. 1 and 2*a* in which the distribution of the various antibodies in the sera before vaccination is compared with the distribution 3 weeks after immunization.

HI tests.—With the homologous Asian antigen (Fig. 1*a*) a marked rise in antibody was found ; 15 (60 per cent) of the prevaccination sera were negative (titre $<1/4$) and the remainder (40 per cent) showed titres of 1/16 or less, whereas 24 (96 per cent) of the post-vaccination samples were positive and in 18 cases (72 per cent) titres were 1/128 or greater.

All the prevaccination sera contained some antibody to FMI and 13 (52 per cent) were also weakly positive against PR8 antigen ; none were positive with Swine antigen (Fig. 1*a*). In no individual, however, was there a significant (*i.e.* 4-fold or greater) rise in antibody to these strains and the post-vaccination distribution of HI antibodies to all the various viruses was essentially the same as before immunization.

CF tests.—The distribution of antibodies found with V antigens in the same 25 pairs of sera is shown in Fig. 1*b*. With Asian V antigen, a significant rise in antibody was found as with the HI tests. Most prevaccination sera were negative or only weakly positive, but 24 (96 per cent) of the postvaccination samples were positive and 18 (72 per cent) of cases had titres of 1/128 or greater.

With the 4 V antigens from the other strains some antibody was found to one or more antigens in the prevaccination sera (Fig. 1*b*). However, no significant change in the distribution of antibody to any of these earlier strains occurred as a result of immunization.

With S antigen (Fig. 2*a*) only 7 (28 per cent) positive reactions were found in postvaccination specimens and in no case was the titre greater than 1/8.

The overall pattern of results suggests that the only significant response to Asian vaccine was a rise in antibody to the Asian strain and both HI and V-CF tests appeared to show this marked response with equal efficiency (Table II). It may be noted, however, that the lower stationary levels of antibody to viruses recovered before the Asian epidemic were not detected equally well by both methods and both CF positive, HI negative and CF negative, HI positive sera were found.

Tests with sera from cases of influenza

HI tests.—The development of antibody directed against the Asian strain (Fig. 3*a*) was less striking than in the group of vaccinees (Fig. 1*a*) ; 20 cases (40

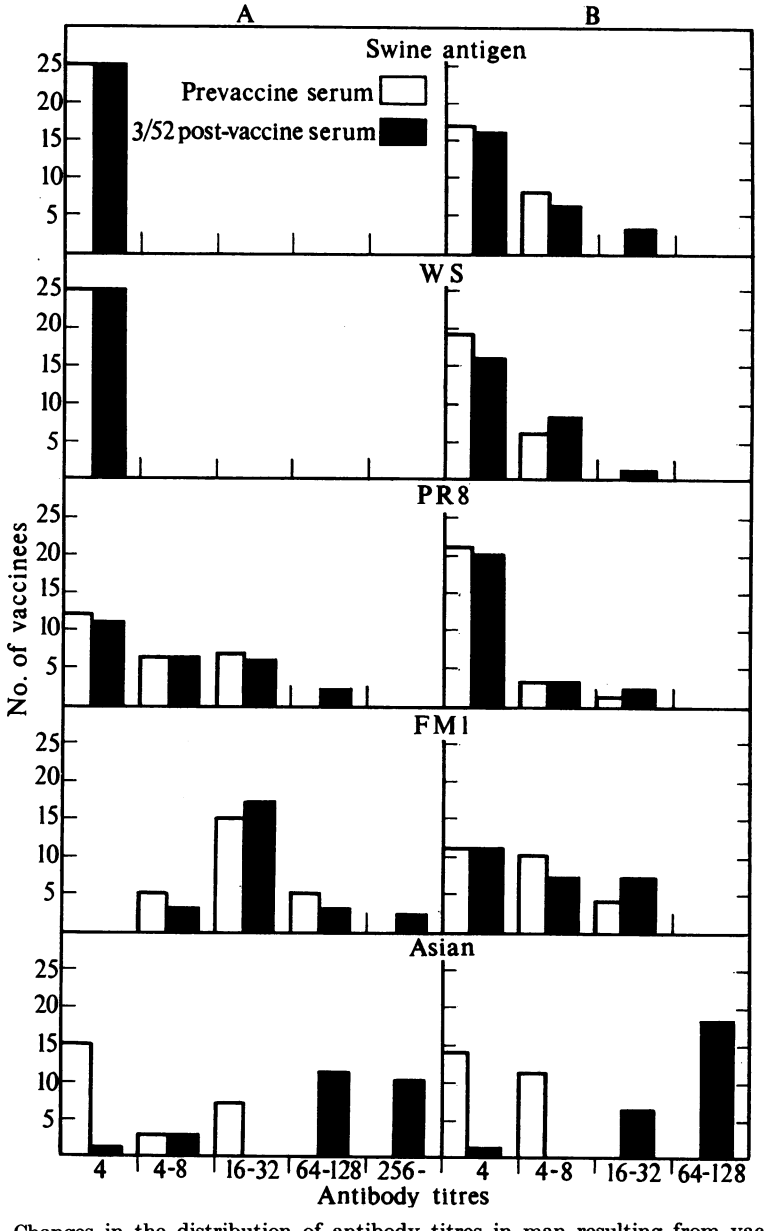

FIG. 1.—Changes in the distribution of antibody titres in man resulting from vaccination with A/Asian strain of influenza virus. Comparative titrations of paired sera from 25 volunteers with haemagglutinating and complement-fixing antigens of 5 influenza A strains.

A = haemagglutination inhibition tests.
B = virus (particle antigen) complement fixation tests.

Antibody titres labelled 4 in fact represent values of <4.

per cent) showed a 4-fold or greater difference in titre between the acute phase and convalescent samples of serum.

HI antibodies to one or more of the other virus strains were found in several cases in both samples (Fig. 3*a*). The distribution of titres to these strains changed

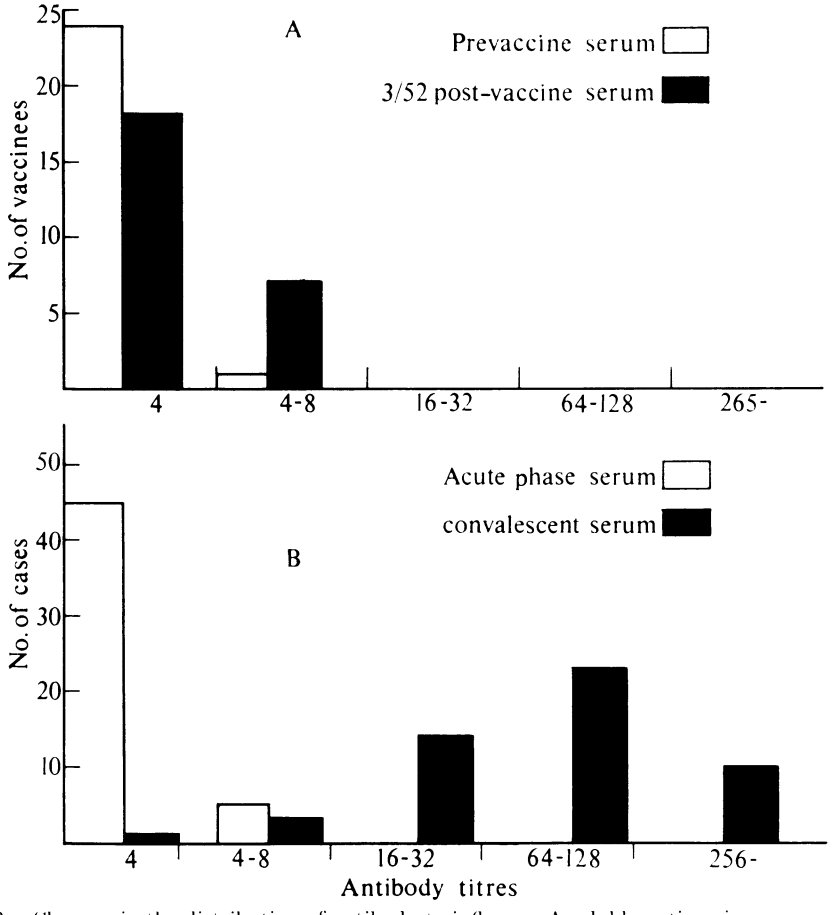

FIG. 2.—Changes in the distribution of antibody to influenza A soluble antigen in man.
A — following vaccination with killed Asian virus.
B — following clinical influenza September, 1957 March, 1959.
Antibody titres labelled 4 in fact represent values of <4.

to only a limited extent as a result of infection : with Swine strain, 8 patients (16 per cent) developed a 4-fold or greater rise in titre between the 2 serum samples. In general however the pattern suggested that the predominant response to Asian virus infection was a rise in strain-specific anti-Asian antibody (Table II).

CF tests.—The distribution of antibodies to V antigens of the 5 virus strains in the same 50 pairs of infective sera is shown in Fig. 3*b*. Of the acute phase specimens, 41 (82 per cent) did not contain detectable antibody to any of the V antigens : in the remaining 9 acute phase sera, only one contained detectable antibody to Asian antigen, but low titres (1/4–1/16) of antibody to one or more of the other virus

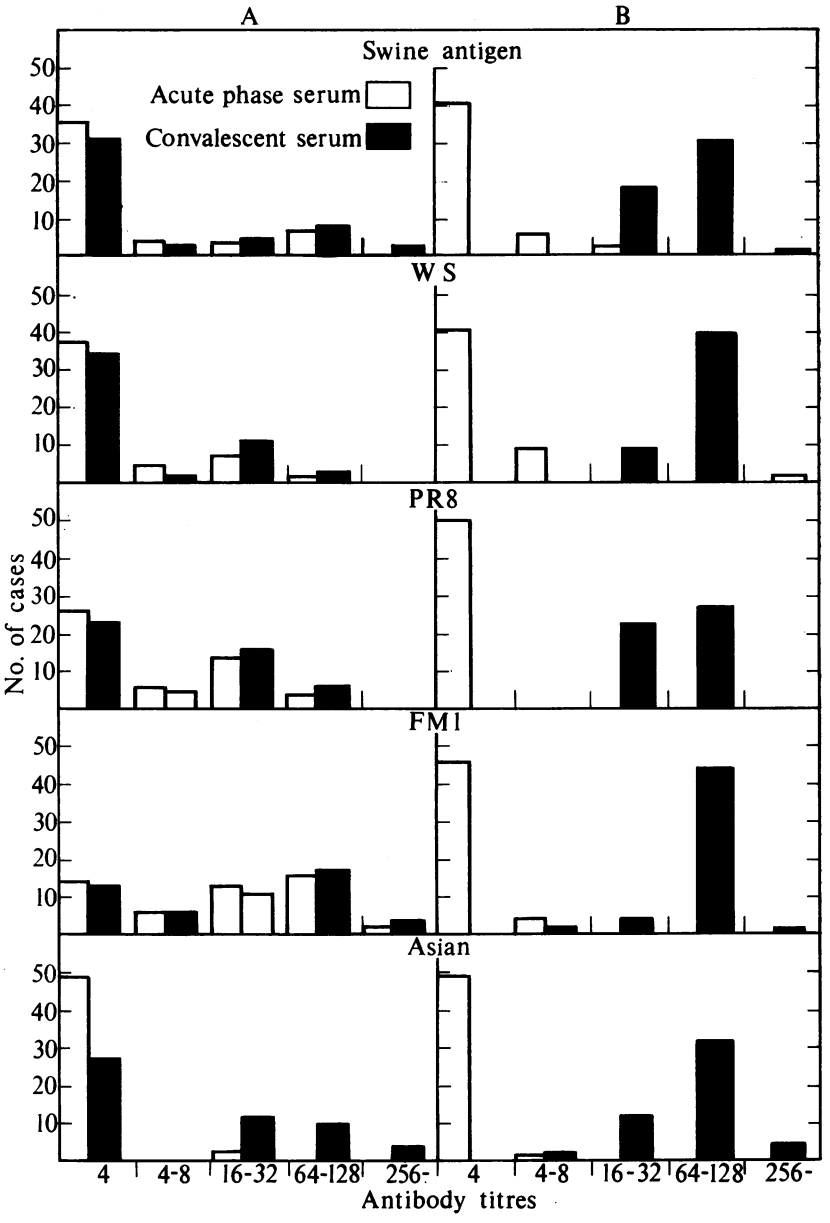

FIG. 3.—Changes in the distribution of antibody titres resulting from influenza infection in 50 cases (September, 1957–March, 1959). Comparative titration of paired sera with haemagglutinating and complement-fixing antigens of 5 influenza A strains.

A = haemagglutination inhibition tests.
B = virus (particle antigen) complement fixation tests.

Antibody titres labelled 4 in fact represent values of <4.

TABLE II.—*The Effect of Exposure to A/Asian Influenza Virus on Antibody Levels to Various Type A Virus Strains in Man.*

Source of human sera	Percentage of serum pairs with > 4-fold rise in antibody titre										
	HI					CF Soluble antigen	CF V antigens				
	Swine	WS	PR8	FM1	Asian		Swine	WS	PR8	FM1	Asian
Vaccination with A/Asian strain (25 cases)	0	0	0	0	92	4	8	0	4	0	92
Clinical influenza infections (50 cases)	16	8	4	10	40	96	100	100	100	100	98

strains were found. As with the group of vaccinated persons these low resting levels of pre-Asian antibodies were not detected with equal efficiency by V-CF and HI tests ; the greatest discrepancy was in the case of the FMI strain where 36 (72 per cent) acute phase sera were found positive by HI tests, but only 4 (8 per cent) by V-CF tests.

In the convalescent sera, the pattern of results obtained with the 5 V antigens differed sharply from V-CF results with post-vaccination sera. All the convalescent sera gave a reaction to high titre with every antigen regardless of the strain from which the antigen had been derived (Fig. 3*b*). Thus in contrast with the pattern found in HI tests with the same serum pairs (Fig. 3*a* and Table II) there was no evidence of any greater response to the homologous Asian strain than to any of the 4 heterologous antigens. With each V reagent the distribution of antibodies changed from an acute-phase pattern of less than 12 per cent positive at 1/4 to a convalescent pattern of 100 per cent positive at 1/32 or more with average titres of 1/64.

In CF tests with S antigen (Fig. 2*b*) there was also a marked change in antibody distribution as a result of infection. Only 5 (10 per cent) of the acute phase sera were positive at a titre of 1/4, whereas 49 (98 per cent) of convalescent specimens were positive with an average titre of 1/64. This finding was to be expected in view of the selection of cases mentioned earlier.

DISCUSSION

The results obtained with haemagglutination-inhibition tests in the present investigation were similar to those described by other workers in cases of Asian influenza (Menon, 1959 ; Zhdanov and Ritova, 1959) or following vaccination with monovalent Asian preparations (Himmelweit, unpublished). They differed in many respects, however, from the serological response to influenza viruses observed before 1957, when it had repeatedly been shown that rises in HI or neutralizing antibody titre following influenza A or A1 infections not only embraced strains other than the homologous infecting strain (Stuart-Harris, Andrewes and Smith, 1938), but might actually be greater for heterologous viruses than for the current epidemic virus (Stuart-Harris and Miller, 1947). With increasing age the reactivity of such sera became increasingly broad, and each successive exposure to a type A or A1 strain by natural infection (Davenport, Hennessy and Francis, 1953 ; Davenport, Stuart-Harris, Hennessy and Francis, 1955 ; Hennessy, Davenport

and Francis, 1955), or by vaccination (Davenport and Hennessy, 1956 ; Jensen, Davenport, Hennessy and Francis, 1956) appeared to stimulate an immunological recall of antibodies resulting from previous infections. Commonly, the highest antibody titres were those against strains first encountered in childhood.

In the present investigation HI antibodies to one or more of the A and A1 virus families were found in sera collected before the exposure of their donors to Asian virus. In the prevaccination samples, all were positive to FMI and 13 of the 25 contained antibody to PR8 ; no antibodies to Swine or WS strains were detected in this group. In the preinfective samples FMI antibodies were less predominant, and almost one-third of all samples contained antibody against PR8, WS and Swine strains, the latter being restricted to those over 35 yr. old. The serological differences between the 2 groups were probably due to differences in the age-structure of the 2 groups and are similar to those found by Davenport *et al.* (1955).

Regardless of age the HI antibody response to Asian virus exposure was remarkably narrow in both groups. The vaccinated individuals all showed a significant rise in HI antibody to the homologous antigen but in no case was there an accompanying rise in antibody to any other strain. In the group of clinical infections, 22 of the 50 cases showed a rise in anti-Asian HI antibody irrespective of the age of the individual concerned. In 8 of these 22 cases, all over 35 yr. old, there was an accompanying rise in pre-existing anti-Swine HI antibody, but in no case did anti-Swine or other heterologous antibody arise *de novo* following infection, and the increase in antibody titre to the homologous Asian antigen was consistently greater than that to Swine antigen.

Thus there is only slight evidence that the recall phenomenon described in earlier epidemics is operative after exposure to Asian viruses. Similarly, direct examination of Asian strains (Menon, 1959 ; Fukumi, 1959) has so far failed to demonstrate in them any of the haemagglutinating antigens known to be shared widely amongst A or A1 strains (Jensen and Francis, 1953) and presumably responsible for the broad spectrum of HI antibody response to these earlier viruses.

The results obtained with the present groups of human sera in V-CF tests differed in several important respects from the HI test results discussed above. In preliminary standardization experiments with animal antisera it was apparent that HI and V-CF tests were each capable of detecting with comparable efficiency a strain-specific response to infection and that the specificity of the V antigens was not obscured by the presence of antibody to the shared S antigen. These results were as would be expected from the basic studies of Henle and his colleagues in this field (Henle and Wiener, 1944, Lief and Henle, 1956*a* and *b* ; Lief, Fabiyi and Henle, 1958 ; Fabiyi, Lief and Henle, 1958 ; Lief, Ostapiak, Fabiyi and Henle, 1958 ; Lief and Henle, 1959).

In the two human groups, however, " resting " levels of antibody to the various earlier (pre-Asian) viruses in the first serum specimens were not detected with equal efficiency by the two methods, and particularly in the case of FMI several sera found positive by the HI test were negative in V-CF tests with antigens derived from the same initial virus seed.

In the vaccinated group, all cases showed a significant rise in CF antibody to the Asian V antigen but in no case was there an accompanying rise or induction of antibody to S antigen or to any of the pre-Asian virus preparations. In pattern, therefore, the actual response of this group to immunization was similar whether judged by HI or by V-CF tests.

In the group of influenza infections, however, the result of Asian virus exposure as judged by V-CF tests was strikingly different from that shown by HI tests in the same set of sera, and contrasted with the pattern of response following vaccination. Few acute-phase sera contained demonstrable antibody to any of the V antigens, whereas in each of the 50 convalescent specimens antibody titres against all these 5 reagents had risen significantly. Antibody titres detected by antigenically-remote strains such as Swine and WS were as high as those shown by the Asian antigen, even in individuals too young ever to have been exposed to infection by such early type A strains. In general, the magnitude of the response to V antigens in these serum pairs seemed comparable to that shown to the shared S antigen; the pattern of response appears to be as non-specific as that obtained by Lennette, Culver and Stevens (1958) with crude egg-fluid antigens. This spectrum of reactivity to V antigens shown in the present investigation could not be attributed to cross-reactions with anti-S antibody in view of the satisfactory behaviour of the same virus reagents with animal sera.

These results suggest that Asian strains of influenza viruses share certain particle complement-fixing antigens in common with earlier A and A1 strains, apart altogether from the shared soluble S antigen. Limited evidence of such relationships have also been demonstrated by direct antigenic analysis with standard specific anti-V sera (Henle *et al.*, 1958), but it is clear that these shared antigenic components are not detected by HI techniques.

In single experimental infections of laboratory animals or in human vaccination with killed virus preparations it would appear that the main immunological response is to major antigens which are effectively strain-specific. This response can be detected equally well by HI or V-CF techniques. Under these artificial conditions of exposure, it would appear that the minor shared particle components are not immunologically active enough to produce detectable antibody.

Under conditions of natural infection in man, however, where antigenic release is prolonged, the antibody response to a given virus strain would be less likely to be restricted to the predominant antigen. Broadly-reactive antibodies directed against antigens shared with other type A viruses would also be induced.

Each subsequent infection with succeeding virus strains would recall previous experience of the same common components. The reactivity of such sera with virus particle suspensions *in vitro* would thus emphasize common factors in V preparations rather than the major antigen characterizing the particular strain, and the V-CF antibody response would become comparable in speed and in degree to the response shown by S antibody, as in the present experiments.

This hypothesis is in keeping with 2 interesting observations of Henle *et al.* (1958). Firstly these authors found that repeated immunization of laboratory animals with a single strain of live virus produces a progressive departure from the apparent strain-specific response to a single inoculation; ultimately, a wide spectrum of anti-V antibodies could be found, often reacting with influenza A strains in which no antigenic sharing could be demonstrated by direct analysis. Secondly, in contrast to their findings with live virus, Lief and her colleagues showed that animals inoculated repeatedly with non-infectious haemagglutinating preparations (probably analogous to vaccine materials) continued to produce only strain-specific antibody responses.

The present experiments show that exposure to Asian viruses yields a pattern of serological response which varies according to the nature of the virus exposure,

and according to the method of *in vitro* examination. As judged by HI tests there is little evidence that Asian viruses can stimulate immunological recall of former A or A1 antibodies, yet recall is clearly suggested by the results of V-CF tests.

It remains to be shown whether the particle antigens which are operative in V-CF tests are necessarily identical to those concerned in haemagglutination-inhibition and whether the anti-V responses demonstrated are correlated in any way with immune resistence to infection.

SUMMARY

Purified particle (V) antigens of A, A1 and Asian influenza viruses gave strain-specific reactions in CF tests with animal antisera.

With human sera from healthy volunteers given monovalent Asian vaccine comparable rises in Asian specific antibody were demonstrated by haemagglutination-inhibition and by complement-fixation with V antigens.

With sera from cases of Asian influenza the antibody response detected by V antigens was not strain specific and was not comparable with HI results in the same group.

The significance of these findings in relation to possible antigenic similarities between Asian and pre-Asian influenza A strains is discussed.

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