CHARACTERIZATION OF INFLUENZA ANTIBODIES BY SERUM ABSORPTION*

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In preceding reports (1, 2) antibody responses to monovalent influenza virus vaccines were characterized for three age groups including children (age 4 to 10), recruits (age 17 to 25) and adults (older than 30). The peculiar patterns noted with sera from each age group indicated that influenza antigens experienced early in life produced a continuing specific effect on antibody formation (3–9). This interpretation has been subjected to test by absorbing sera with virus and measuring the remaining hemagglutination-inhibiting levels with other strains. Using previously described serum absorption technics with virus-coated erythrocytes (10), it has been possible to analyze the sera demonstrating that hemagglutination-inhibiting titers obtained for strains are composites of different antibodies. The data also demonstrated that antibody in some human sera might be highly cross-reactive and inhibitory for distantly related strains, such as Swine-1931 and FM1-1947.

It is now clear that the range of reactivity can be defined by removing that antibody which is reactive with a given strain. In this manner information was obtained concerning the nature of antibody in serum from persons who have had experience with many or few antigenic variants of influenza virus. Immunologic relationships among strains became clearly evident from these results. It was observed that absorption with one Type A strain often removed antibody against all Type A and A-prime strains. The phenomenon has been even more strikingly demonstrated using sera from ferrets that had been successively infected with several antigenically different but related strains of virus. In sera from either experimental animals or man a persistent antibody orientation to the strain of first antigenic experience is demonstrated.

Materials and Methods

Virus.—Strains of influenza virus were chosen from the collections at the Strain Study Center, Commission on Influenza, School of Public Health, Ann Arbor.

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The Type A strains used extensively included a strain of swine influenza virus, Shope/1976 and human strains, PR8 from 1934, and FM1 obtained from the 1947 epidemic.

Serum—Human sera used are those referred to in preceding papers of this series (1, 2). Convalescent ferret sera were obtained by infecting the animals with 2.0 ml. of allantoic fluid virus diluted 1:1000 in broth given intranasally under ether anesthesia. Animals were bled 2 to 3 weeks later, sera separated and stored at 4°C. without preservative.

Hemagglutination-Inhibition Tests.—Antibody titrations were performed using a standard pattern test (11) with 4 hemagglutinating units of virus and 0.5 per cent suspensions of chicken erythrocytes.

Absorption of Antibody from Sera.—Methods used to prepare virus-coated cells for absorption of antibody were similar to those previously reported (10). Pools of infective allantoic fluids in 500 to 1000 ml. volumes were mixed with equal volumes of M/35 potassium periodate. After 1 hour at room temperature, the excess periodate was neutralized with a similar volume of 10 per cent glucose solution. Virus inactivated in this manner firmly adsorbed to erythrocytes. Human red blood cells which had been treated with formalin to increase their stability were mixed with the virus suspensions. After sedimentation and washings, the virus-cell complex was mixed with serum in volumes standardized as to antigen content (10). Antibodies combined with virus antigens and the complete complex were removed by centrifugation. Supernatant serum was then tested for remaining antibody content.

EXPERIMENTAL RESULTS

Absorptive Characteristics of Antibody from Different Age Groups.—Results of preliminary investigations indicated a surprising lack of specificity when human sera were absorbed with strains of virus (10). Strains of Type A influenza virus which appeared antigenically distinct on the basis of results from H.I. tests with animal sera evidently were inhibited by the same antibodies in
human serum. Some of the differences in absorptive characteristics of antibody in the pools of sera from different age groups are evident from data charted in Fig. 1. Sera studied here were obtained prior to vaccination. Titers of antibody observed with the Swine, PR8, and FM1 strains are charted in separate columns for each age group. Antibody levels in sera from persons older than 30 years are indicated by bars marked with diagonal lines; that of recruit age groups are shown as solid black bars. Stippled bars represent antibody of children. Open bars indicate extent of antibody removal after absorptions of sera. The same code will be used in subsequent charts.

Antibody was detected with all three test strains only in sera from the oldest age group. When this serum pool was absorbed with the Swine strain (row 2), all measurable antibody against the three strains was removed, demonstrating that all activities were oriented to react with Swine virus. Absorption of the same serum pool with PR8 (row 3), reduced the levels, but did not completely remove antibody inhibitory for Swine or FM1. In a similar manner, absorption with FM1 (row 4) left antibody for Swine and PR8. Absorptive reactions with sera from the recruit age group, resulted in each case in complete reduction of antibody titers only for the absorbent strain and limited reduction of activities against other strains. Antibody to only FM1 was found in the children's serum, and absorptions with Swine or PR8 strains had little or no effect on the antibody level to FM1.

These results clearly demonstrate that sera from persons in the oldest age group contain a composite of at least three kinds of antibody each of which reacts with and is absorbed by Swine. One reacts only with Swine, another with Swine and PR8, and the third with Swine and FM1. At least two kinds of antibody active against PR8 were found in sera from recruits; one reacts only with PR8 and the other can be absorbed with either FM1 or Swine. Most of the FM1 antibody in children's sera was reactive only with FM1 since absorptions with Swine and PR8 were without significant effect. Similar observations have been made repeatedly with individual sera indicating that this phenomenon cannot be attributed to mixtures found in pools of serum.

Persistent Antibody Orientation in Human Sera.—Serum pools obtained from the three age groups after vaccination with single strains were analyzed with the same absorption methods. Although increased levels of antibody due to the stimulus of vaccination are apparent in each serum pool, adsorption reactions made it evident that antibody response was markedly influenced by preexistent immunologic mechanisms set up during childhood and subsequent antigenic experiences.

Data obtained from hemagglutination-inhibition tests with postvaccination serum pools measured with Swine, PR8, and FM1 after absorptions with each strain are presented in two ways. First, titers demonstrated in sera from each age group after vaccination with a monovalent vaccine are charted in Table I.
The effect of absorptions with homologous and heterologous strains on the antibody patterns found with each serum pool is evident from these data and can be seen to vary remarkably with the age group. It is clear, for example, that antibody in sera from children which neutralizes a strain, Swine virus, is not identical with that antibody in sera from the oldest age group which reacts with Swine. In order to contrast the varied reactive behavior of antibody in each age group after vaccination with a strain, the results are charted in the next three figures so that antibodies measured by H.I. tests are considered with each strain separately.

**Antibodies Titrated with Swine.**--Titers of antibody measured with the Swine strain are charted in Fig. 2. Absorption with the Swine strain (row 2) was included as a positive control and resulted in complete removal of that antibody activity in every case. Several interesting observations were made with these data. First, it was again noted that Swine antibody in serum pools from the

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**TABLE I**

Results of Absorption of Serum from 3 Age Groups after Vaccination

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0, no inhibition by serum at 1:100 dilution.

0, result of absorption with H.I. test strain (homologous system).
oldest age group (third member of each column) is resistant to absorptions by PR8 or FM1, particularly after vaccination with Swine or PR8. Vaccination of this age group with FM1 however, produced a high level of anti-Swine activity which would also react and be largely removed by FM1 and to a lesser extent by PR8. Secondly, a converse situation appeared in sera of children vaccinated with Swine. Here a comparable level of antibody against Swine was completely absorbable with FM1 (column 1, row 4) while PR8 absorptions did not greatly reduce the Swine titers. This suggests that FM1 is more closely related to Swine than is PR8.

These observations appear interpretable in terms of similar mechanisms based on shared antigenic components present in the Swine and FM1 complexes. It is highly probable that persons over 30 years old have had a primary antigenic stimulus with a Swine-like strain of influenza virus. Antigenic materials of the FM1 vaccine which are similar to the Swine strain are emphasized in the antibody response of this group. In contrast, antibody-forming centers of the children were impressed by the similarity of Swine strain antigens to the previously experienced FM1-like strains.

The recruit group (age 17 to 26) probably had a primary orientating experience with strains antigenically similar to PR8. The effect is reflected by the nature of the antibody following vaccination with the Swine strain. Antibody produced in this group measurable with Swine virus was reactive with PR8 as well as with FM1. Antigens shared with Swine are probably not determinant.
in the PR8 and FM1 strains because vaccination of this age group with PR8 or FM1 did not induce Swine antibody.

*Antibodies Titrated with PR8.*—Titers of antibody in the serum samples determined with the PR8 strain are shown in Fig. 3. Vaccination with PR8 (column 2) stimulated high levels of activity against that strain in each age group. Activity in the serum pool from recruits measured with PR8 was not removed except when PR8 was the absorbent strain, as shown by the solid black bars. In contrast, absorption of the children's serum with FM1 greatly reduced the titer against PR8 and points up an essential difference in antibody produced in the two age groups.

![Graph showing antibody titers](image)

**Fig. 3.** Characteristics of postvaccination antibody from 3 age groups measured with PR8 strain.

When Swine was the vaccine strain, the recruit age group produced the highest level of PR8 antibody, a part of which was resistant to absorption with Swine or FM1. It is of interest to note, however, that some activity stimulated by Swine was equally reactive with FM1. Analogous reactions were observed in serum pools from the two older age groups after vaccination with FM1 (column 3). Here again much of the antibody measured with PR8 was also reactive with Swine and FM1. Since vaccination of children with FM1 did not produce activity against PR8 (above) or Swine (Fig. 2), it is clear that the shared antigens are not in a determinant status in each strain. Furthermore, it is evident that cross-reactions between PR8 and FM1 are most readily observed in sera from persons who first have had an antibody-orienting experience with PR8 which is followed by the FM1 antigenic stimulus to produce activities against antigens of both PR8 and FM1.
Antibodies Titrated with FM1.—A composite of the data which also includes antibody measurements with FM1 is presented in Fig. 4. From these data a comparison of adsorption characteristics of antibody from the three age groups may be drawn. Attention is directed to the section on the left where persistence of the lined bars indicates that antibody in serum samples from the oldest age group active against the Swine strain is most often refractory to absorptions with PR8 or FM1. In the middle section, the PR8 antibody in the serum of recruits (black bar) is most specific. On the right are measurements with FM1, and the stippled bar indicates antibody in children is most resistant to absorptions with heterologous strains. These data clearly demonstrate that each age group has a different basic antibody which may be characterized as being refractory to absorptions except when a virus strain with the necessary set of antigens is mixed with the serum. Information concerning the absorptive nature of antibody produced in the three age groups also points out that the antibody of the oldest age group is most complex. This is shown by production of antibody detected with FM1 after vaccination with Swine or PR8 (for right section, first 2 columns) which was highly cross-reactive as shown by complete removal with either Swine or PR8 (rows 2 and 3).
Antibody Activities in Sera from Ferrets after Successive Infections.—Concepts concerning the marked influence the first influenza stimulus may have on antibody produced later in life were derived from results obtained with human sera concerning which the antigenic composition of the first and subsequent virus strains experienced prior to vaccination could only be inferred. Results of experiments with ferrets sequentially infected with known antigenically diverse strains of influenza virus have provided a basis for further understanding of the data obtained with human sera. An example of typical results obtained with sera from a cross-infected ferret is shown in Fig. 5. In this case, the ferret was first infected with the WS strain, given the Weiss strain 7 months after, and again after 6 weeks, infected with the CAM strain. Serum was collected from the animal 2 weeks after each infection and the titers obtained against each strain are charted in the top row. Antibody titers progressively increased until high levels for each strain were found in the last serum sample. Aliquots of serum obtained after the last infection were absorbed and thereafter titrated with each of the three strains. Absorption with strains of the second and third infections (Weiss and CAM) removed homologous antibody in each case and a limited amount of heterologous antibody. When the serum was absorbed with WS strain, however, all antibody was removed, indicating antibody which neutralized Weiss or CAM would also react with WS.

Similar observations have been made with several combinations of Type A strains and with sera from other ferrets after sequential infection by Type B strains. The phenomenon is type-specific though, since absorptions with Type B virus do not remove antibody produced in response to infection with strains of Type A.
The data and the interpretations presented earlier (1–9) concerning persistent levels of antibody in human sera to strains encountered many years earlier seem completely confirmed by the controlled observations from induced infections in ferrets. It is now clear the strains first encountered may so markedly impress antibody-forming mechanisms that subsequent antigenic experiences with related viruses stimulate additional antibody reactive with the earliest strains as well as with later strains; absorption of sera with a strain representative of the initial infections does in fact remove antibody to all strains of the type.

**DISCUSSION**

The striking antibody response of cross-infected ferrets with demonstrations that all type-specific antibodies react with the strain of first infection and the analogous results obtained with human sera describe a phenomenon which may well concern many groups of related antigens in immunology. Although all Type A strains are antigenically similar to some degree with sensitive serological methods, every isolate can be shown to be antigenically distinct (12). When routine laboratory procedures with antisera produced in animals are employed, it is not difficult to observe antigenic differences among strains isolated during two eras, 1933–43 and 1946–55. The strains in the latter group almost never react with antisera produced against the first group of strains. The Swine strain apparently representative of a still earlier time in the history of Type A strains, is not closely related to either group. Nevertheless, each strain has been included in the A type on the basis of results with human sera.

Satisfactory explanations for these phenomena are not yet available, but one may formulate working hypotheses as follows: Virus strains contain several different antigenic materials as surface or subsurface components which are essentially identical in several strains (10). Therefore, cross-reactions with antisera would depend on the site or location of the common antigen. In other instances, certain antigens may both be on the surface, but one is dominant in reaction with antibody although either may direct antibody synthesis. Materials comprising virus particles are probably more or less similar for strains within a broad immunologic type. Antigenic differences defined between any two strains reflect configurational differences observable only with carefully prepared antisera. Since antigenic precision of this degree is not often found with human sera, the immunologic significance of such differences is in doubt. Practically nothing is known concerning what may constitute an antigenic component, prosthetic or determinant group in the several materials of the virus. Thus, a particular grouping of chemical components in a surface material of one virus strain may direct the antibody response while identical structures in another virus matrix may be of secondary serologic importance or occur as a part of a larger prosthetic arrangement. Although these problems
are common for all of immunology, they are particularly embraced in serologic studies with strains of influenza virus.

Contemplation of mechanisms responsible for the antibody activities described in serum from vaccinated persons must include processes involving the dynamics of antigens acting in previously sensitized tissue. Clearly the antibody manufactured in each host is dependent upon previous antigenic impressions and several different activities may be present in any serum sample as described by absorption results. Thus with either human or ferret sera some antibody appeared multivalent and capable of reaction in absorption and H.I. tests with several strains while in other instances the antibody activities inhibited only the strain of first infection. These results suggest the possible formation of antibody at different sites (in various cells within an organ, and in different tissues) in the animal during antigenic experience with a resultant heterogeneity of measurable activities. Perhaps the effect of antigenic stimulation of certain germinal type cells in an antibody-forming organ with antigens similar to those of previous experience calls forth increased manufacture of antibody identical with that formed in the past. Other cells, more synthetically agile, may notice the slight antigenic modifications and prepare antibody capable of reaction with the new antigen, but which retains a configuration that allows reaction with the previously experienced antigens. Whatever the mechanisms may be, it is clear that each antibody response to the various strains is dependent on preexisting immunologic factors and that antigenic similarities are emphasized in human hosts rather than slight antigenic differences which can be demonstrated among influenza viruses.

SUMMARY

Absorptions of sera from groups of persons, both normal and after vaccination, resulted in complete removal of antibody to all strains of influenza virus within a type when a strain of antigenic configuration similar to that presumed to be the strain of first experience was employed. Absorption of these sera with strains encountered after the primary antigenic stimulus, removed antibody to strains recently experienced, but failed to absorb the primary antibody. By these methods, three age groups could be distinguished whose characteristic principal antibody was oriented to react with maximal efficiency either with FM1 or PR8 or Swine/1976 influenza viruses. Persons in these age groups had an initial experience with strains of the respective antigenic characteristics found in the variants of influenza virus mentioned. Basis for understanding these results was obtained with sera from successively infected ferrets, when serum absorptions demonstrated all type-specific antibody was oriented to react only with the strain of first infection. In view of this evidence there can now be little doubt as to the marked persistent influence an antigenic experience with influenza virus has upon the antibody-forming mechanisms of the virgin host.
BIBLIOGRAPHY

2. Hennessy, A. V., and Davenport, F. M., data to be published.