FURTHER OBSERVATIONS ON THE SIGNIFICANCE OF A/EQUINE-2/63 ANTIBODIES IN MAN*

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The finding that the sera of humans 70 or more years of age in 1958 exhibited a unique frequency and concentration of antibody reacting with the 1963 strain of horse influenza has been amply confirmed (1-4). In the discussion of the primary data, it was pointed out that the antibody pattern described probably delineated a period of past prevalence of equine-2/63-like viruses in man extending from about 1880 to 1890. That interpretation was based on the demonstration that episodes in the succession of major antigenic variants of influenza viruses can be reconstructed by ascertaining the age distribution of antibodies to prototype strains currently found in the sera of humans (5, 6). However, it was recognized that the phenomenon observed then with equine-2/63 virus might be alternatively explained as the consequence of accumulated experience with the minor antigens of influenza A viruses, or less likely, might reflect the sporadic cross-species transmission of a respiratory virus from horses to humans (1).

To resolve these questions and if possible to delineate more precisely the period of epidemic prevalence of equine-2/63-like viruses, a series of further studies was carried out. The age distribution of hemagglutination-inhibiting antibodies was ascertained in a larger and more comprehensive set of sera collected in 1964. The interval between the two collections is sufficient to permit an evaluation of whether an antibody pattern changes in concordance with the passage of time (5). Selected sera were examined photometrically for the presence of specific antibody (7). A smaller collection of sera obtained from persons continuously exposed to horses was examined. Finally, the homologous and heterologous antibody response of five age cohorts vaccinated with monovalent vaccines containing either an equine-2/63 or an A/57 isolate was determined.

The findings and the accumulated evidence identify another epoch in the

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annals of antigenic variation among strains of influenza A and sharpen interest in questions concerning the possible relationships between epidemics and epizootics of influenza.

Materials and Methods

Viruses.—The strains utilized were selected from the collection of the Virus Laboratory, Department of Epidemiology, School of Public Health, of the University of Michigan, and are described by currently accepted nomenclature.

Sera.—
(a) Specimens utilized for delineation of the age distribution of horse influenza antibodies in the general population were obtained from patients hospitalized in Michigan during inter-epidemic periods in 1958 and 1964.
(b) Specimens utilized for study of the antibody response to influenza virus vaccines were obtained from patients hospitalized in the fall of 1964. Influenza was not present in this area at that time.
(c) Specimens utilized to ascertain the effects of continued exposure to horses were obtained from a colony of Amish. The samples were drawn between late 1964 and early 1966. Further details concerning each sample are given in the appropriate experimental sections of this report.

Treatment of Sera.—Sera were inactivated by heating at 56°C for 30 min or by use of periodate (8). Periodate treatment was employed for specimens examined photometrically or specimens tested with Asian strains.

Hemagglutination-Inhibition (HI) Titrations.—Determinations were carried out by a standard pattern method with four units of virus and 0.5% erythrocytes (9).

Photometric Titrations.—The procedure developed by Drescher for measuring specifically reacting antibodies was followed exactly (7).

EXPERIMENTAL

Age Distribution of Equine-2/63 Antibodies in Specimens Collected in 1964.—25 sera were obtained from subjects in each 2-yr age class from 55–86 yr of life. The 87–88, 89–90, and 91–97 age groups are represented by only 20, 9, and 8 specimens, respectively, since additional subjects were not available. Fig. 1 shows the birth year of the subjects and the per cent of specimens obtained from each age class that exhibited hemagglutination-inhibiting antibody against the A/equine-2/Milford/2/63 isolate at a level of 1:8 and 1:16 or higher.

The majority of the positive reactions were at the low level of 1:8. The frequency of titers at that value appears to increase gradually and almost progressively from 55–72 yr of age. From 73–90 yr, a higher and quite uniform frequency of titers of 1:8 was maintained. A low frequency and sporadic incidence of titers of 1:16 or higher was found in the age range 55–72. From 73–90 yr, titers of 1:16 or higher were consistently observed, and the frequency of those titers was conspicuously greater than that of subjects 72 yr old or younger. The peak incidence of the higher titers was found in the 77–78 yr old group. The curve of titers 1:16 or higher appears bimodal with a lesser peak at age 87–88. The per cent of positive reactions in the 91–97 yr old group was markedly lower
than that of the 72–90 yr cohort. While the number of specimens available from persons 91 or more years old was small, the decline in the frequency of titers observed in the oldest age group examined is considered significant because it has been a uniform experience that the oldest age class studied exhibits a relative drop in positive reactions to strains of initial infection (1–3, 5, 6).

Once again it was found that the equine-2/63 antibody pattern was distinctly different from that observed in the same set of sera with swine, A, A1, and A2 strains. In addition, none of the sera gave positive reactions with A/equine-1/Prague/1/56. Taken together, the distribution of antibody titers of 1:8 and 1:16 or higher indicate that persons 73–90 yr of age in 1964 have had a richer experience with equine-2/63 antigens than that of younger or older cohorts. By analogy to studies carried out with swine, A, A1, and A2 strains, the findings identify a period of past prevalence of equine-2/63–like viruses which occurred during the childhood of persons born between 1874 and 1891 (2, 5, 6, 10). Alternative explanations for the occurrence of the equine-2/63
Fig. 2. Progression of the age distribution of equine/63 antibodies with time when tested with the A/equine-2/Milford/2/63 strain.
antibody pattern were examined by tests described in the next three sections of this report.

Changes in the Age Distribution of Equine-2/63 Antibodies with the Passage of Time.—If the antibody pattern found with equine-2/63 viruses were the result of broadening in antibody response owing to repeated infections, it would be expected that as time passed and the number of exposures was increased, the pattern would become blurred. In consequence, a progressively younger or older age segment would exhibit equine-2/63 antibodies. Conversely, if the antibody pattern was indelibly imprinted at the initial infections of childhood, it should remain fixed with respect to the age group involved and the pattern should move progressively up the age scale in exact concordance with the passage of time (5). Such synchronous advances have been shown for antibody patterns defined with swine, A, and A_4 strains (2, 5, 6).

To determine whether the same cohort retained their unique antibody characteristics, a comparison was made of the findings in the 1958 collection of sera with those of 1954. For this purpose, it was necessary to regroup the specimens from the 1954 collection by 5 yr age intervals and to advance the age class examined by 6 yr. Fig. 2 shows the frequency of hemagglutination-inhibiting antibodies found in each set of sera when tested with the A/equine-2/Milford/2/63 strain. The titer values selected for analysis are those employed in Fig. 1 and in the initial study (1). Owing to exhaustion of the 1958 samples, it was not possible to test both sets of sera simultaneously. Nevertheless, it can be seen that the general curve of antibody frequency in both sets of sera is similar. Moreover, the age of onset and that of peak frequency of antibody at either level has advanced 6 yr in exact concordance with the time elapsed between collection of the two sets of sera. Similar findings have been reported by Masurel and Mulder (3).

These observations on the constancy with the passage of time in the serologic reactivity of persons exhibiting A/equine-2/Milford/2/63 antibodies lends strong support to the interpretation that the equine antibodies measured are homologous. Clearly, the severe epidemics of Asian influenza encountered in 1950 and 1953 did not distort the characteristic equine-2/63 antibody pattern of the present population.

Photometric Evidence on the Specificity of Equine-2/63 Antibodies Present in the Sera of Humans.—The photometric method developed by Drescher for the quantitative measurement of strain specific antibody distinguishes objectively between the reactions of homologous and heterologous antigen-antibody systems (7). To augment the evidence that the equine-2/63 antibodies found in the sera of humans are homologous, photometric tests were carried out with serum specimens obtained in 1964 from persons 56–95 yr of age. Hemagglutination-inhibiting antibody positive and negative specimens were randomly chosen for testing. The A/equine-2/Milford/2/63 was employed
for analysis using an antibody isotherm generously supplied by Dr. J. Drescher of the Robert Koch Institute, West Berlin, Germany. The findings are recorded in Table I. The results were grouped by the same 5 yr age interval employed for comparison of the frequency of hemagglutination-inhibiting antibodies in the 1964 collection with that of 1958. The age of the donors, the per cent of positive specific reactions found, the range of titers, the ratio of titers greater than 50 antibody concentration units (ACU), and the per cent of these higher values are reproduced. The threshold value of 50 ACU was selected because the majority of the titers, i.e. 61%, fell in the range of less than 50 ACU.

Only three specifically reacting specimens were found in the sample representing the 56–60 yr age group. From 61–75 yr of age a fairly constant frequency of low and high specific antibody titers was found. Note, however, that in the age segment of 76–85 yr, the per cent of positive specimens sharply increased and the high value of the range of titers rose abruptly. The last age class examined showed a relative decline in the frequency and range of specific antibody titers.

These data provide independent support for the interpretations that the equine-2/63 antibody patterns found by hemagglutination-inhibition reflect prior experience with the major antigens of the 1963 strains of horse influenza rather than being the result of a broadened heterologous antibody response. They also emphasize that the specific antigenic experience of persons 76–85 yr of age in 1964 was the richer.

### Table I

| Age      | Fraction positive | Per cent positive | Titer range in ACU | Fraction above 50 ACU | Per cent qualifying |
|----------|-------------------|-------------------|--------------------|-----------------------|---------------------|
| 56-60    | 3/62              | 5                 | 57-68              | 3/62                  | 5                   |
| 61-65    | 6/24              | 25                | 25-77              | 2/24                  | 8                   |
| 66-70    | 11/48             | 23                | 19-81              | 2/48                  | 4                   |
| 71-75    | 15/64             | 23                | 22-118             | 3/64                  | 5                   |
| 76-80    | 15/46             | 33                | 20-508             | 7/46                  | 15                  |
| 81-85    | 11/44             | 25                | 24-307             | 6/44                  | 14                  |
| 86-95    | 6/43              | 14                | 22-81              | 3/43                  | 7                   |

Relation of Equine-2/63 Antibodies to Contact with Horses.—During three prior outbreaks of horse influenza in 1956, 1963, and 1964, transmission of horse strains to humans under natural conditions of exposure was not observed even in stable hands. Nevertheless, experimental infection of volunteers has been reported (11). Therefore, the possibility, however remote, that the equine-
2/63 antibody pattern observed might be the result of cross-species transmission of influenza A from horses to man must be considered.

Two aspects of the present information speak strongly against that possibility. First, the frequency of equine-2/63 antibodies detected by hemagglutination-inhibition in the 73–90 yr old cohort is over 70% (Fig. 1). That finding is not compatible with the thesis of cross-species transmission since such an event is uniformly a sporadic one occurring at a low frequency. Second, model T Ford automobiles were not marketed until 1908, while the decline in frequency and levels of equine-2/63 antibodies occurs in the sera of persons born in 1890–1891. 17 or 18 yr of further exposure of children to horses followed before the horseless carriage began to replace the horse as the common means of transportation.

Nevertheless, direct evidence on the possible relation of exposure to horses and the presence of horse influenza antibodies in humans was sought by examining a set of about 400 sera collected from a population of Amish resident in the state of Illinois. Blood specimens were drawn between 29 December 1964 and 4 January 1966.1 The special significance of the Amish is that by custom they continue to use horses for transportation and farming.

Table II shows, by 5 yr age intervals from 6–80 yr, the number of sera available for testing and the number of positive specimens detected by hemagglutination-inhibition with the A/equine-2/Milford/2/63 strain. None of the specimens from persons 70 or fewer years of age contained equine-2/63 antibodies. In contrast, seven of the nine samples available from persons 71–83 yr

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**TABLE II**

*Distribution of HI Antibodies Against Equine-2/63 Virus in Sera Obtained from an Amish Community*

| Age range | No. of specimens | No. of antibody-positive | Age range | No. of specimens | No. of antibody-positive |
|-----------|------------------|--------------------------|-----------|------------------|--------------------------|
| 6–10      | 14               | 0                        | 46–50     | 16               | 0                        |
| 11–15     | 40               | 0                        | 51–55     | 15               | 0                        |
| 16–20     | 68               | 0                        | 56–60     | 10               | 0                        |
| 21–25     | 49               | 0                        | 61–65     | 10               | 0                        |
| 26–30     | 40               | 0                        | 66–70     | 8                | 0                        |
| 31–35     | 24               | 0                        | 71–75     | 3                | 1                        |
| 36–40     | 20               | 0                        | 76–80     | 4                | 4                        |
| 41–45     | 17               | 0                        | 83        | 2                | 2                        |

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1 These sera were generously furnished by Dr. Charles E. Jackson, Director of Clinical Research of the Caylor-Nickel Research Foundation, Bluffton, Ind.
old were positive. While the number of specimens available from the senior citizens of this sect is small, the antibody pattern observed in the total collection is quite like that found in the larger collections from the general population. The same specimens were tested for antibody against the equine-1/56 prototype. A single specimen obtained from a male aged 41 exhibited hemagglutination-inhibiting antibody at a titer of 1:64. This serum neutralized 63 EID$_{50}$ at a dilution of 1:32 and is the only human serum examined to date in this laboratory which contained antibody against equine-1/56 strains. Only 10 sera were available from horses owned by members of the community. All contained equine-1/56 antibodies, but only two contained equine-2/63 antibodies measured by hemagglutination-inhibition.

These data strongly indicate that continued exposure of a population to horses, rather than to motor vehicles, does not alter the characteristic age distribution of equine-2/63 antibodies found in the population at large, and they speak strongly against cross-species transmission as a likely explanation for the unique antibody pattern found with equine-2/63 strains.

**Antibody Response of Different Age Cohorts to Vaccination with A/Equine-2/Milford/2/63 or A$_1$/AA/1/57 Monovalent Vaccines.**—In previous studies from this laboratory, it was demonstrated that important information about the significance of differences in patterns of age distribution of swine, A, A$_1$, and A$_2$ antibodies found in human sera could be obtained by comparing the antibody response of persons of different ages to monovalent influenza virus vaccines. Moreover, such experiments provide a sensitive method for examining the antigenic relationships of strains isolated in different periods (12, 13). Prompted by these experiences, similar investigations were carried out with monovalent vaccines containing 200 chick cell agglutination (CCA) units of either the Milford strain of A/equine-2/63 virus or the A$_1$/AA/1/57 strain. Groups of 25 persons aged 19–25, 28–34, 47–53, 63–76, or 77–86 were bled before and 2 wk after vaccination with each monovalent preparation. The age ranges chosen represented, in 1964, persons whose childhood infection occurred with A$_3$, A, swine, A$_8$, or equine-2/63-like strains of influenza A. Pre- and postvaccination antibody levels were measured by hemagglutination-inhibition with the vaccine strains and with A/equine-1/Prague/1/56, A$_2$/AA/23/57 A$_1$/PR8/34, and A/swine/31 viruses. The findings for the subjects given the A/equine-2/Milford/2/63 vaccine are summarized in Table III as geometric mean pre- and postvaccination antibody titers and as the proportion of persons exhibiting a fourfold or greater antibody rise. Since only 4% of these subjects showed antibody increase to A/PR8/34 and none to A/swine/31, the details of the findings with those strains are omitted from the table.

In the A$_1$ cohort (19–25 yr), the response to the homologous equine strain was distinct, but of a low order of magnitude. Postvaccination mean titers were unimpressive and less than half of the subjects exhibited a fourfold or
greater increase in antibody levels. In this age group as in all others of the
series, antibody against the Prague strain of equine influenza was not found
in any pre- or postvaccination blood specimen. Obviously that virus is not a
near relative of the influenza A strains examined in this study. Of paramount
interest was the demonstration that by vaccination with an equine-2/63 virus,
antibody increase to A₂ and to A₁ viruses was clearly enhanced. Against A₂/
AA/23/57, the mean titer rose from 51 to 118, and against A₁/AA/1/57 from

TABLE III

| Test strain          | A/equine-2/ Milford 2/63 | A/equine-1/ Prague 1/66 | A₁/AA/23/57 | A₂/AA/1/57 |
|----------------------|--------------------------|-------------------------|-------------|-------------|
| Age                  |                          |                         |             |             |
| 19-25                | <8/<11*                  | <8/<8                   | 51/118      | 10/21       |
| 9/25†                | 0/25                     | 9/25                    |             | 7/25        |
| 28-34                | <8/<8                    | <8/<8                   | 15/28       | <8/<11      |
| 2/25                 | 0/25                     | 5/25                    |             | 4/25        |
| 47-53                | <8/<8                    | <8/<8                   | 12/14       | <8/<9       |
| 3/25                 | 0/25                     | 1/25                    |             | 2/25        |
| 63-76                | <8/44                    | <8/<8                   | 16/21       | <8/<8       |
| 21/25                | 0/25                     | 1/25                    |             | 0/25        |
| 77-86                | <8/50                    | <8/<8                   | 26/30       | <8/8        |
| 13/25                | 0/25                     | 1/25                    |             | 0/25        |

* Geometric mean titer, pre-/postvaccination.
† Proportion showing fourfold or greater rise.

10 to 21. The ratio of antibody increase was essentially the same whether
measured with the equine-2/63, A₂, or A₁ strains. These findings, together
with those acquired through the use of different experimental methods by
Kasel et al. and by Masurel and Mulder, provide conclusive evidence con-
cerning antigenic relationship between equine-2/63 virus and A₂ and A₁ strains
of influenza A and indicate a closer tie to the A₁ family of strains (3, 11).

At ages 28–34 and 47–53, the homologous antibody response to Milford
vaccine was conspicuously less than that observed in younger or older age
groups, and stimulation of A₂ and A₁ antibodies was progressively less pro-
nounced.

At ages 63–76 and 77–86, the response to the homologous Milford strain
was again conspicuously greater than that of the 19–25 yr class. A₁ antibody
increase was not observed in either cohort, and only a single person in each showed a significant A2 antibody rise.

When an A1/AA/1/57 vaccine was given to another set of 25 persons each in the same age groups, no antibody increase to either the 1963 or 1956 strains of equine influenza was observed. Moreover, a group of 44 persons aged 77–84 given the licensed polyvalent influenza virus vaccine of 1964 which contained 600 CCA units per dose also failed to exhibit antibody increase to the two prototype strains of horse influenza.

The results of the vaccination experiments are interpreted as follows: Equine-2/63 virus is definitely, but remotely, related to strains responsible for influenza A in humans. The phenomena observed are best described as a “one-way cross” between the Milford isolate and the A2 and A1 viruses. The homologous response of persons aged 63–76 and 77–86 to equine-2/63 vaccine is compatible with the age distribution of equine 63 antibodies in man and indicates that these persons have had prior experience with strains containing antigens closely related to those of equine-2/63 virus. The modest homologous antibody response of the A1 cohort to Milford vaccine apparently reflects the conditioning effects of previous encounters with Asian strains or with those of influenza A-prime.

DISCUSSION

The evidence marshalled in this and previous studies substantiate and circumscribe a period of past prevalence of equine-2/63-like viruses in man. The unique antibody pattern is reproducible (1–4). With the passage of time, the pattern advances along the age scale yielding a running fix in exact agreement with the number of years that elapsed between the two sights (3, 5). The antibodies react photometrically as specific ones (2, 3, 7). The homologous antibody response to vaccination was more vigorous in the cohorts with prior antigenic experience. Association with horses cannot be correlated with the unique age distribution of equine influenza antibodies.

The present data permit a more precise delineation of the time limits during which equine-2/63-like viruses circulated among mankind. It will be recalled that the age pattern of antibody distribution determined currently in different age groups provides a serologic recapitulation of past infections. With respect to the equine-2/63 antibody pattern, the birth dates of persons who first show the heavier involvement correspond to the years 1890–1891 (Fig. 1). Those dates identify the last years of major prevalence of equine-2/63-like viruses in man. The birth dates of persons who last show the high antibody levels and frequencies correspond to the years 1874–1875. Those dates indicate the earliest years in which equine-2/63-like viruses became the dominant threat to the populace. The bimodal curve seen in Fig. 1 suggests that at least two major visitations of equine-2/63-like viruses occurred in the interval between 1874–1891.
Masurel, on the basis of the finding that equine-2/63 antibodies were present in the sera of persons 60 yr of age in 1958 and 1963, opted for the interpretation that the period of prevalence of equine-like viruses extended from 1895 to 1900 after the pandemic of 1889–1890, which seems related to Asian antigen (3). It is realized that practice of the art of serologic archeology is fraught with many hazards and that the available data are subject to different interpretations (2, 6). It may be that equine-2/63-like viruses were prevalent in the Netherlands at a later time than in the United States, although that occurrence seems doubtful since peak antibody levels were found at the same age in sera collected in both countries. A more plausible explanation for the presence of equine-2/63 antibodies in the sera of persons born after the pandemic of 1889–1890 is that the A~ strain evolved from equine-2/63-like precursors and in subsequent visitations retained, as prominent antigenic characteristics, some of the dominant antigens of the parent strain. It is recognized that the birth dates of persons 73–74 yr of age in 1964 permit the interpretation that the pandemic of 1889–1890 could be attributed to equine-2/63-like viruses as well as to Asian strains. However, the likelihood seems greater that a pandemic would follow the emergence of a new subtype of strains rather than an event occurring at the end of a period of prevalence of a different subtype, since in the latter case the population tends to be saturated with protective antibody. The results of further studies may resolve these questions.

While the antigenic relationships of the horse and human strains of influenza A have been firmly established, the epidemiologic significance of that finding is obscure. The virtual absence of antibodies against strains of horse influenza in the younger cohorts of the population at large and in persons continuously exposed to horses indicates that horses do not constitute an active reservoir of epidemic or pandemic influenza A. The failure to observe cross-species transmission of influenza A virus from horses to man during the epizootics of 1956, 1963, and 1964 is in keeping with this interpretation. Instead, by analogy to findings with swine virus which can be related to the pandemic of 1918–1919, it may be that horses are the repositories of older human strains (14). Alternatively, it might be postulated that the epizootics of 1956 and 1963 resulted from the invasion of horses by strains of human origin. In either case, the further assumption must be made that the mutant or recombinant “variants” simultaneously lost most of the antigens which characterize the influenza A viruses of the opposite species, and the capacity to establish infection in the donor hosts.

Recent evidence indicates an antigenic linkage between strains of influenza A isolated from birds, horses, and man. It has been shown that equine-1/56 and equine-2/63 virus share antigens with the virus of fowl plague and the A/duck/England/62 isolate respectively (15, 16). In a separate study not yet reported in full, it was found that the sera of persons 74 or more yr of age in 1964 exhibit a low frequency of hemagglutination-inhibiting antibodies against
the A/duck/England/62 virus. These antibodies behave as heterologous ones and are considered to represent a serologic crossing with equine-2/63 strains or with an unknown common ancestor (17). It would appear that the pool from which antigenic components may be drawn might encompass at least four species. These findings do not permit a ready interpretation, and it is clear that much additional information is needed before the possible role of extra human reservoirs of antigens or epidemic strains of influenza A can be assessed.

SUMMARY

The antibody pattern of equine-2/63 viruses has been more sharply defined using a large number of human sera collected in 1964. The birth dates of persons exhibiting the richest experience with equine-2/63-like viruses delineate a period of past prevalence in man of equine-2/63-like viruses. The period is believed to have begun in the mid-1870's and to have terminated in 1889-1890 at the time of the first Asian pandemic. The equine-2/63 antibodies found in human sera react specifically in the photometric test of Drescher. The equine-2/63 antibody pattern advances along the age scale in exact concordance with the passage of time. The homologous antibody response of the older subjects to equine-2/63 vaccine is more vigorous, reflecting the conditioning effects of prior exposures to equine-2/63 antigens. A "one-way cross" between equine-2/63 virus and A₂ and A₁ strains has been demonstrated. The antigenic ties between strains of influenza A isolated from humans, swine, horses, and birds is recognized and discussed. It is apparent that horses do not constitute an active reservoir for strains of human involvement. The epidemiologic significance of the antigenic linkages between strains isolated from different species remains obscure.

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