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Studies on the Content of Antibodies for Equine Influenza Viruses in Human Sera

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Previous workers had demonstrated that some of the viruses that caused the influenza epizootics of 1956 and 1963 among horses are antigenically related to certain human influenza viruses. The present authors therefore studied the distribution of antibodies for the equine influenza viruses in human sera. They found that three strains of A/Equi 2 virus (1963 Miami, 1963 Richelieu and 1963 Milford) are antigenically related and that there is a cross-relationship between the mouse line of the equine influenza A/Equi 1 (1956 Praha) antiserum and the 1963 A/Equi 2 strains. Haemagglutination-inhibition (HI) antibodies for A/Equi 2/Richelieu/63 were found in only 2 of 434 sera taken in 1963 from people aged less than 60 years but in 50 of 435 sera from people aged 60 years or more. These results suggest that infection with a virus resembling the A/Equi 2 1963 strain occurred some 7-10 years after the 1889-90 influenza pandemic; this virus was probably a mutant of the influenza A2 virus, which may have been the cause of the 1889-90 pandemic.

In Czechoslovakia in 1956, Sovinová et al. (1958) isolated, from nasal smears and washings obtained during an epizootic among horses, a virus that was shown, by complement fixation, to be related to that which causes influenza type A; it was designated A/Equi 1/Praha/56. By the cross haemagglutination-inhibition (HI) test, the virus was found to be antigenically distinct from the human influenza viruses A and A1 and the swine influenza virus A. No similar infection was observed in people who had been in contact with infected horses and there was no influenza epidemic at that time.

Waddell et al. (1963) isolated in 1963 another equine influenza virus from a widespread epizootic in the USA and Canada; this was designated A/Equi 2/Miami/63. This strain was found to be antigenically distinct from A/Equi 1/Praha/56 virus and the human influenza viruses A, A2 and B. During the same outbreak, Marois et al. (1963) isolated in Canada an equine influenza virus (A/Equi 2/Richelieu/63) which was claimed to be antigenically related to human influenza A2 strains. In the same year Voth & Feldman (1963) reported the finding of some HI antibodies for the equine 1963 influenza virus in the sera of some persons aged 70 years or over. Minuse et al. (1965) found antibodies for the equine influenza virus A/Equi 2/Milford/63 in a proportion of human sera from aged people in Michigan. Schild & Stuart-Harris (1965) obtained similar results with sera from aged persons in Sheffield, using the equine influenza strain A/Equi 2/Miami/63. Neither the American nor the British investigators found antibodies for the equine influenza strain A/Equi 1/Praha/56.

In the present study we have examined the distribution in human sera, by age of the persons from whom the sera were taken, of haemagglutination antibodies for equine influenza viruses; the sera were collected in 1958 and 1963 from people living in the Netherlands. We have also studied the antigenic relationships between the equine influenza viruses and the human and swine influenza viruses, by means of the cross haemagglutination test and by cross reinfection experiments in ferrets.

MATERIALS AND METHODS

Human sera

In the autumn of 1958, 1750 specimens of human sera were obtained from persons of different ages. In the summer of 1963 another 900 specimens were collected. The sera were stored at −18°C.

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Ferret sera

Anaesthetized ferrets were infected by a single intranasal instillation with 1 ml of virus-containing allantoic fluid (0.5 ml per nostril). A blood sample was drawn before the infection and another after 18 days. Each preinfection serum was checked for the presence of antibodies to the representative strains of equine, swine and human influenza virus. Ferrets were reinfected by the different viruses 3-7 months after the first inoculation, when the amount of HI antibody induced by the latter had declined substantially; before reinfection another blood sample was taken.

To prevent contamination, only one virus strain was used on any one day. Each group of infected ferrets was isolated carefully for the duration of the experiment. Sera were obtained by heart puncture and stored at −18°C or freeze-dried in samples of 0.3-1.0 ml. When the human or ferret serum was used in the HI tests, one part of serum was treated with 5 parts of *Vibrio cholerae* filtrate in order to remove non-specific inhibitors.

Mouse neutralization test

Sera were inactivated by heating for 30 minutes at 56°C. Dilutions of the inactivated sera and the virus-containing allantoic fluid (ca 500 HA units) were mixed in equal volumes. The mixture was incubated at 20°C for 1 hour and then 0.05 ml was inoculated intranasally into anaesthetized mice. Positive ferret and negative human antisera were used as controls. In the mouse neutralization test the strain A/Equi 2/Richelieu/63 (passage formula: egg-mouse-egg) was used. Virus with only a few minimum lethal doses per unit volume was used, because only in this way could the low HI titres be confirmed.

Strains of influenza virus

The following equine influenza A strains were used: A/Equi 1/Praha/56 E_{13} and $E_{10}M_{30}E_{4\text{-}16}$; [1] A/Equi 2/Miami/63 E_xE_{11} and $E_xM_{16}E_{6\text{-}20}$; [2] A/Equi 2/Milford/63 E_{13}; A/Equi 2/Richelieu/63 E_{16} and $E_{16}M_{34}E_{3\text{-}12}$.

The human and swine influenza A strains employed were: A-PR8/USA/34 $F_{189}M_{593}E_{55}M_3E_{32}$; [3] A1/Nederland/49 $E_8M_{20}E_{10}M_7E_{17}$; A1/Nederland/56 $E_5M_{23}E_{26}$; A2-305/Japan/57 $E_5F_3M_6E_{46}$; A2-62/Nederland/62 $E_{12}M_{25}E_{25}$; A2-67/Nederland/63 $E_9M_{26}E_8$; A2-68/Nederland/65 $M_{12}E_3$; A/Swine 15/USA/30 $M_{257}E_{27}$.

[1] E = egg, M = mouse; subscript indicates number of passages.

[2] E_x refers to strains received from other institutes, the number of egg passages being unknown.

[3] F = ferret; subscript indicates number of passages.

RESULTS

Antigenic relationships of the influenza viruses

Table 1 shows the results of the cross haemagglutination-inhibition (HI) test with immune ferret sera. It is clear that the strains A/Equi 2/Miami/63, A/Equi 2/Richelieu/63 and A/Equi 2/Milford/63 from the widespread epizootic among horses in 1963 in the USA and Canada are antigenically related. There is also a cross-relationship between the mouse line of the equine influenza A/Equi 1/Praha/56 antiserum and the equine 1963 A/Equi 2 strains. In the HI test the four equine viruses show no relationship with the human influenza viruses A, A1 and A2 or with the swine influenza virus A. These findings are similar to those of Waddell et al. (1963), who used specific immune chicken and guinea-pig sera. Marois et al. (1963) demonstrated a one-way relationship between the guinea-pig antisera of two isolates of A/Equi 2/Richelieu/63 and three influenza A2 isolates obtained in 1957 and 1963. Antibodies against the homologous equine influenza virus and the human influenza A2 virus isolated in 1957 were found in the antisera prepared against the horse virus strains.

Crossed reinfection experiments in ferrets

Table 2 shows the results of experiments with the strain A/Equi 1/Praha/56. Very few ferrets showed an anamnestic recall of titres. These were most clearly present in crossed experiments with the strain A1-39/Nederland/56. No such recall was found when the human pandemic strain A2-305/Japan/57 (Masurel & Mulder, 1962) was used for the first or second infection.

Table 3 gives the results of a further series of crossed reinfection experiments in ferrets, using the strain A/Equi 2/Miami/63. There was no recall in either direction with the swine virus, the human influenza virus A-PR8 and the equine A/Equi 1/Praha/56 strain. This table shows, however, that antibodies to the human influenza virus A1/Nederland/56 and to the A2 viruses both gave an anamnestic response after infection with the equine Miami strain. The crossed experiments with the strains A2-62/Nederland/62 and A/Equi 2/Miami/63 gave the highest anamnestic recall of titres and very low homologous titres against the horse virus. In

TABLE 1

CROSS HAEMAGGLUTINATION-INHIBITION TESTS WITH EQUINE, HUMAN AND SWINE INFLUENZA VIRUSES

Antisera	Haemagglutination-inhibition titre obtained with the strain														
	A/Swine 15/USA/30 FME	A-PR8/USA/34 FME	A1/Nederland/49 EME	A1/Nederland/56 EME	A2-305/Japan/57 EFME	A2-62/Nederland/62 EME	A2-67/Nederland/63 EME	A2-68/Nederland/65 EME	A/Equi 1/Praha/56 E	A/Equi 1/Praha/56 EME	A/Equi 2/Miami/63 E	A/Equi 2/Miami/63 EME	A/Equi 2/Richelieu/63 E	A/Equi 2/Richelieu/63 EME	A/Equi 2/Milford/63 E
A/Swine 15/USA/30 FME	**3 400**								<9	<9	<9	<9	<9	<9	<9
A-PR8/USA/34 FME		**5 400**							<9	<9	<9	<9	<9	<9	<9
A1/Nederland/49 EME			**4 200**						<9	<9	<9	<9	<9	<9	<9
A1/Nederland/56 EME				**1 850**					<9	<9	<9	<9	<9	<9	<9
A2-305/Japan/57 EFME					**6 450**				<9	<9	<9	<9	<9	<9	<9
A2-62/Nederland/62 EME						**15 200**			<9	<9	<9	<9	<9	<9	<9
A2-67/Nederland/63 EME							**4 350**		<9	<9	<9	<9	<9	<9	<9
A2-68/Nederland/65 EME								**9 200**	<9	<9	<9	<9	<9	<9	<9
A/Equi 1/Praha/56 E	<9	<9	<9	<9	<9	<9	<9	<9	**8 700**	11 000	<9	<9	<9	<9	<9
A/Equi 1/Praha/56 EME	<9	<9	<9	<9	<9	<9	<9	<9	1 550	**1 900**	60	20	20	65	500
A/Equi 2/Miami/63 E	<9	<9	<9	<9	<9	<9	<9	<9	<9	<9	**75**	<9	30	85	190
A/Equi 2/Miami/63 EME	<9	<9	<9	<9	<9	<9	<9	<9	<9	<9	<9	**50**	<9	20	35
A/Equi 2/Richelieu/63 E	<9	<9	<9	<9	<9	<9	<9	<9	<9	<9	100	<9	**150**	500	380
A/Equi 2/Richelieu/63 EME	<9	<9	<9	<9	<9	<9	<9	<9	<9	<9	200	85	400	**1 650**	1 550
A/Equi 2/Milford/63 E	<9	<9	<9	<9	<9	<9	<9	<9	<9	<9	50	<9	<9	70	**550**

TABLE 2. CROSSED REINFECTION EXPERIMENTS IN FERRETS WITH STRAINS OF EQUINE INFLUENZA VIRUS A/EQUI 1/PRAHA/56, SWINE INFLUENZA VIRUS A AND HUMAN INFLUENZA VIRUSES A1 AND A2

Strains used		Number of ferrets showing recall (≥4×) of antibody against first virus	Mean geometric titre of ferret antisera showing recall (≥4×) [a]	
Primary infection	Reinfection		A/Equi 1/Praha/56	Other strains
A/Equi 1/Praha/56	A/Swine 15/USA/30	0/11	—	—
A/Swine 15/USA/30	A/Equi 1/Praha/56	1/6	<9/3 250	1 250/6 100
A/Equi 1/Praha/56	A1-39/Nederland/56	4/11	450/5 400	<9/8 200
A1-39/Nederland/56	A/Equi 1/Praha/56	4/10	<9/4 200	300/6 150
A/Equi 1/Praha/56	A1-47/Nederland/56	0/7	—	—
A1-47/Nederland/56	A/Equi 1/Praha/56	2/11	<9/1 050	300/2 100
A/Equi 1/Praha/56	A2-305/Japan/57	0/11	—	—
A2-305/Japan/57	A/Equi 1/Praha/56	0/9	—	—

[a] The numerator is the mean geometric titre of antisera showing recall just before reinfection and the denominator the mean geometric titre 18 days after reinfection.

TABLE 3. CROSSED REINFECTION EXPERIMENTS IN FERRETS WITH STRAINS OF EQUINE INFLUENZA VIRUS A/EQUI 2/MIAMI/63, SWINE INFLUENZA VIRUS A, HUMAN INFLUENZA VIRUSES A, A1 AND A2 AND EQUINE INFLUENZA VIRUS A/EQUI 1/PRAHA/56

Strains used		Number of ferrets showing recall (≥4× of antibody against first virus	Mean geometric titre of ferret antisera showing recall (≥4×) [a]	
Primary infection	Reinfection		A/Equi 2/Miami/63	Other strains
A/Equi 2/Miami/63	A/Swine 15/USA/30	0/8	—	—
A/Swine 15/USA/30	A/Equi 2 Miami/63	0/6	—	—
A/Equi 2/Miami/63	A-PR8/USA/34	0/6	—	—
A-PR8/USA/34	A/Equi 2/Miami/63	0/7	—	—
A/Equi 2/Miami/63	A1/Nederland/49	1/5	20/85	<9/950
A1/Nederland/49	A/Equi 2/Miami/63	0/5	—	—
A/Equi 2/Miami/63	A1/Nederland/56	2/7	12/90	<9/4 900
A1/Nederland/56	A/Equi 2/Miami/63	5/6	<9/550	150/1 200
A/Equi 2/Miami/63	A2-305/Japan/57	1/6	20/140	<9/4 350
A2-305/Japan/57	A/Equi 2/Miami/63	4/6	<9/100	400/2 200
A/Equi 2/Miami/63	A2-62/Nederland/62	5/6	13/325	<9/8 400
A2-62/Nederland/62	A/Equi 2/Miami/63	7/8	<9/80	800/15 000
A/Equi 2/Miami/63	A/Equi 1/Praha/56	0/6	—	—
A/Equi 1/Praha/56	A/Equi 2/Miami/63	0/7	—	—

[a] See footnote to Table 2.

reinfection experiments in a few ferrets, Dowdle et al. (1964) found no measurable anamnestic response between the equine 1963 strain and the human influenza A2/63 virus.

We also used the A/Equi 2/Richelieu/63 strain in reinfection experiments (Table 4). Again no recall was found with the strains of human influenza virus A-PR8 and with A/Equi 1/Praha/56 virus. One ferret reinfected with the swine influenza virus gave a high recall. The main difference between the results of Table 4 and those of Tables 2 and 3 is the absence of any recall with the human influenza A1-1956 strain when the 1963 Richelieu virus is used. The latter showed the most frequent recall with the A2-305/Japan/57 strain. From the crossed reinfection experiments it is concluded that there is a close antigenic relationship between both the equine 1963 viruses (Richelieu and Miami) (Table 4).

Haemagglutination-inhibition antibodies against the equine influenza A strains in human sera

Fig. 1 shows the percentage frequency and height of the HI titres against the A/Equi 2/Richelieu/63 virus of sera obtained in 1963 from people aged 0-84 years. In sera from persons aged 60 years or more there is a gradual increase with age of the percentage of sera containing antibodies for Equi/2 1963 virus. The titres ranged from 1:9 to 1:300; 11.5% of the sera from persons aged 60 years or more and 40% of those from persons aged 70 years or more contained HI antibodies against the strain A/Equi 2/Richelieu/63. Only two sera (from two people aged 40 and 49 years) taken from people less than 60 years old had antibodies against the Richelieu strain. The absence of maternal HI antibodies in the newborn was in conformity with this finding.

TABLE 4

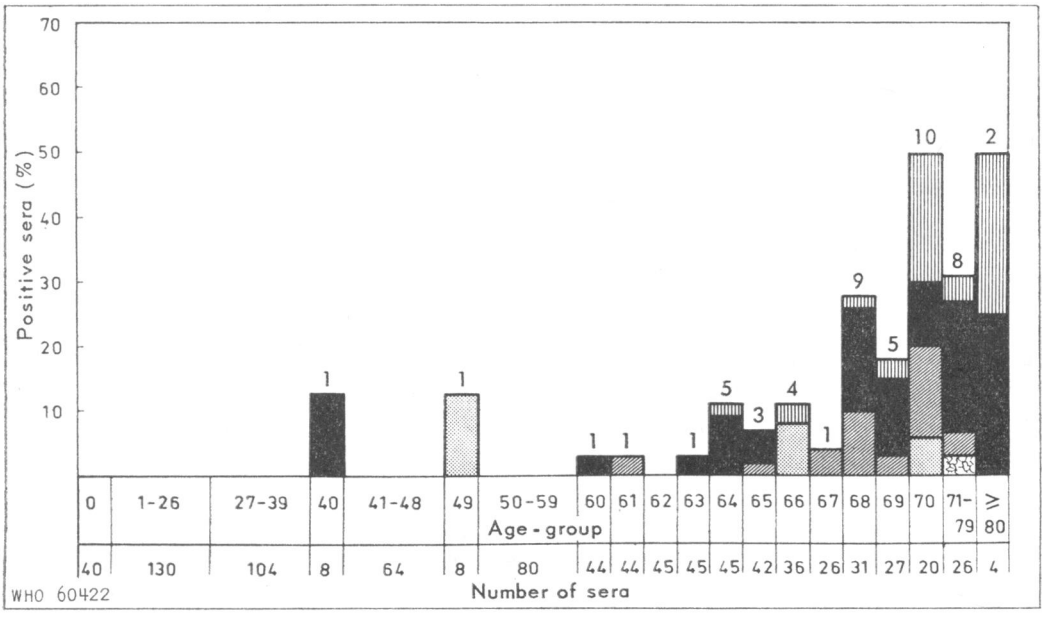
CROSSED REINFECTION EXPERIMENTS IN FERRETS WITH STRAINS OF EQUINE INFLUENZA VIRUS A/EQUI 2/RICHELIEU/63, SWINE INFLUENZA VIRUS A, HUMAN INFLUENZA VIRUSES A, A1 AND A2, AND EQUINE INFLUENZA VIRUSES A/EQUI 1/PRAHA/56 AND A/EQUI 2/MIAMI/63

Strains used		Number of ferrets showing recall (≥4×) of antibody against first virus	Mean geometric titre of ferret antisera showing recall (≥4×) [a]	
Primary infection	Reinfection		A/Equi 2/Richelieu/63	Other strains
A/Equi 2/Richelieu/63	A/Swine 15/USA/30	1/4	230/13 000	<9/8 700
A/Swine 15/USA/30	A/Equi 2/Richelieu/63	0/6	—	—
A/Equi 2/Richelieu/63	A-PR8/USA/34	0/4	—	—
A-PR8/USA/34	A/Equi 2/Richelieu/63	0/4	—	—
A/Equi 2/Richelieu/63	A1/Nederland/56	0/5	—	—
A1/Nederland/56	A/Equi 2/Richelieu/63	0/7	—	—
A/Equi 2/Richelieu/63	A2-305/Japan/57	4/6	250/3 400	<9/1 900
A2-305/Japan/57	A/Equi 2/Richelieu/63	5/7	<9/450	425/6 700
A/Equi 2/Richelieu/63	A2-62/Nederland/62	0/6	—	—
A2-62/Nederland/62	A/Equi 2/Richelieu/63	3/5	<9/650	3 600/20 500
A/Equi 2/Richelieu/63	A/Equi 1/Praha/56	0/4	—	—
A/Equi 1/Praha/56	A/Equi 2/Richelieu/63	0/7	—	—
A/Equi 2/Richelieu/63	A/Equi 2/Miami/63	3/6	225/1 100	20/100
A/Equi 2/Miami/63	A/Equi 2/Richelieu/63	7/7	75/2 950	40/550

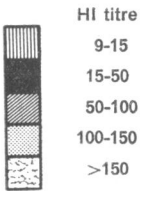
[a] See footnote to Table 2.

FIG. 1

DISTRIBUTION OF HI ANTIBODIES FOR THE EQUINE INFLUENZA VIRUS A/EQUI 2/RICHELIEU/63 IN HUMAN SERA COLLECTED IN 1963



The numbers on the graph indicate positive cases.



The sera from two people aged 65 and 76 years gave an HI antibody titre with the strain A/Equi 2/Miami/63 and both gave a higher titre with the strain A/Equi 2/Richelieu/63, namely, 75 and 290, compared with 20 and 45. HI antibodies for the A/Equi 1/Praha/56 virus were not observed in sera collected in 1963.

Human sera collected in 1958 included more samples from people in the older age-groups. Fig. 2 shows the percentage frequency and height of the HI titres against the strain A/Equi 2/Richelieu/63 in these sera. Antibodies are present in few of the sera of people aged 60-67 years. The highest proportion of sera containing antibodies is found in people aged 69-71 and there is a decline from 72 years onwards. Seven sera containing antibodies against the Richelieu strain show a lower antibody titre with the strain A/Equi 2/Miami/63. No antibody was detected with the virus A/Equi 1/Praha/56 in the sera taken in 1958. Thus the sera from 1958 and 1963 gave similar results.

Fig. 3 shows the percentage frequency of HI antibody for the A/Equi 2/Richelieu/63 virus in two sets of sera obtained in 1958 and 1963, respectively. In this figure the difference of five years between the times of collection of the two sets of sera is taken into account. No correlation was found between the levels of swine, influenza A, A1 or A2 antibodies and those of antibodies for the strain A/Equi 2/Richelieu/63 in individual sera. The presence of equine-virus antibodies thus has no influence on the antibody patterns to the other influenza A viruses.

Mouse neutralization tests with human sera and the mouse-adapted A/Equi 2/Richelieu 63 strain

Mouse neutralization tests were performed to determine whether the inhibition of the equine virus

FIG. 2. DISTRIBUTION OF HI ANTIBODIES FOR THE EQUINE INFLUENZA VIRUS A/EQUI 2/RICHELIEU/63 IN HUMAN SERA COLLECTED IN 1958

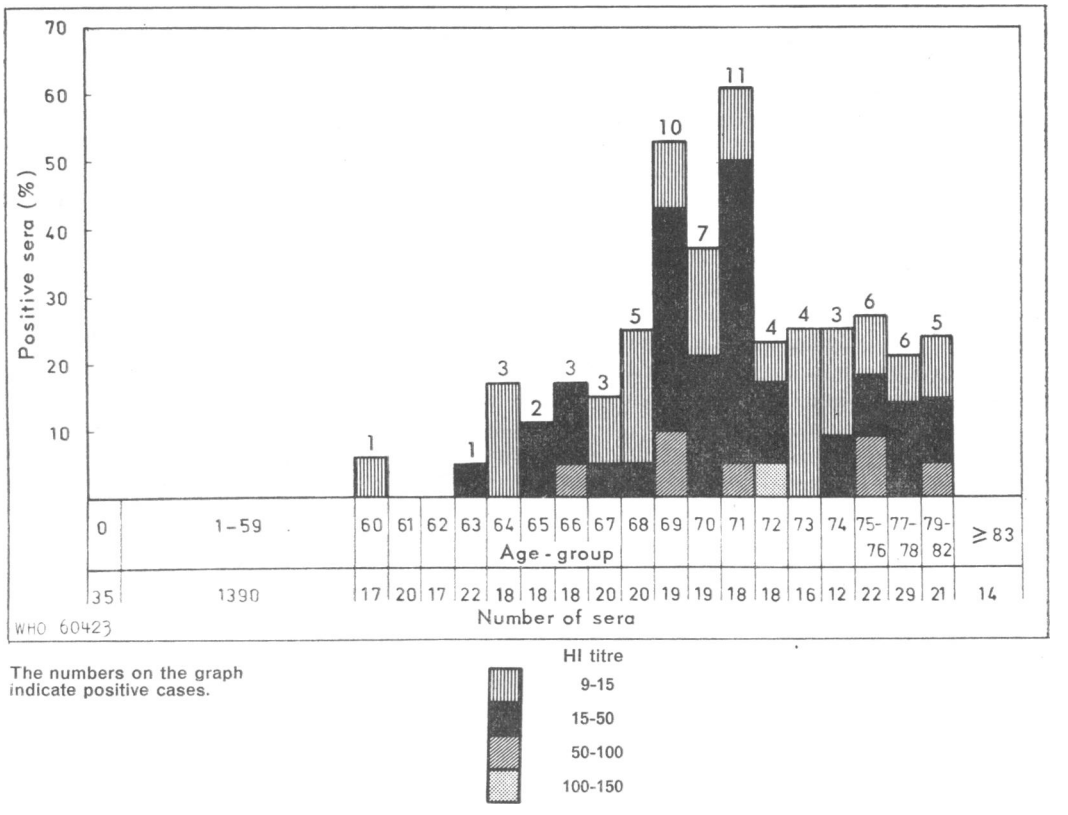
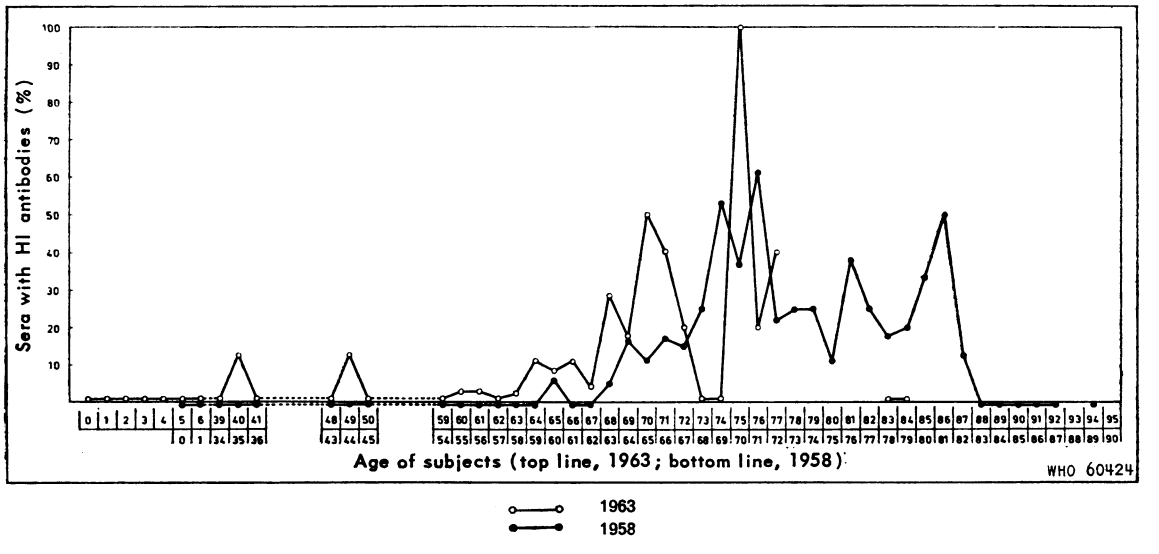


FIG. 3. FREQUENCY DISTRIBUTION OF HI ANTIBODIES FOR EQUINE INFLUENZA VIRUS A/EQUI 2/RICHELIEU/63 IN HUMAN SERA COLLECTED IN 1958 AND 1963



in human serum was in fact due to the presence of antibodies. Sera from subjects in different age-groups with different HI titres were tested by mouse neutralization with the A/Equi 2/Richelieu/63 virus. In all sera, except one with a low HI titre, the presence of antibodies was indicated.

Drescher titrated some of the positive sera with the equine 1963 virus, using the photometric serological method (Drescher, Davenport & Hennessy, 1962; Drescher, Hennessy & Davenport, 1962). They found that the positive sera in our HI test and in the mouse neutralization experiments reacted in a specific manner in the photometric test. This indicates that the antibody had resulted from infection by a strain of virus antigenically related to, or identical with, the equine 1963 virus.

DISCUSSION

In 1957 we demonstrated (Mulder & Masurel, 1958) the presence of antibodies against the influenza A2 virus in the pre-epidemic sera of people aged 70 years or more. By analogy with this study, the present findings, showing the presence of antibodies against the equine 1963 virus in people aged 60 years and more in 1958 or in 1963, suggest that an infection by a virus resembling the equine 1963 strain occurred during 1896-1900, 7-10 years after the 1889-90 influenza pandemic.

Sharing of minor antigens by the equine 1963 virus and the human A2 virus was demonstrated in the reinfection experiments on ferrets. This conclusion was supported by the results of the cross reinfection tests between the human A2 virus strains and the A/Equi 2/Richelieu/63 strain and, to a lesser extent, with the A/Equi 2/Miami/63 strain, which indicated a high anamnestic recall of HI titres. Marois et al. (1963) similarly found an antigenic relationship between the Richelieu strain and the human A2 virus strains. These findings suggest that a virus resembling the equine 1963 virus, which probably caused human infection in the period 1896-1900, was a mutant of the influenza A2 virus, which may have been the cause of the 1889-90 pandemic. It is hardly possible that as many as 40% of persons aged 70 years or more could possess antibodies against the equine 1963 strain simply as a result of sporadic transmission of infection from horses to man. As in the case of the relationship between swine influenza and human influenza, there is now a small link between equine and human influenza. It will be of great interest to continue this study of serological affinity, if only because the serological findings in human sera indicate the temporal relationships of previous human epidemics.

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RÉSUMÉ

Des travaux antérieurs ont montré que certains des virus responsables des importantes épizooties de grippe des chevaux qui se sont produites en Europe en 1956 et aux Etats-Unis et au Canada en 1963 avaient une parenté antigénique avec certains virus de la grippe humaine. Par ailleurs, on avait montré en 1957 que des sérums de personnes âgées de 70 ans et plus possédaient des anticorps contre le virus de la grippe A2 qui peut avoir été la cause de la pandémie de 1889-1890. Les auteurs ont recherché la distribution par âge des anticorps hémagglutinants pour les virus équins de la grippe, dans des sérums humains, au moyen de réactions croisées d'inhibition de l'hémagglutination et de réinfection expérimentale chez le furet. Ils ont prélevé 1750 sérums au cours de l'automne 1958 et 900 au cours de l'été 1963.

Les réactions d'inhibition croisée de l'hémagglutination avec des sérums de furets immunisés ont montré une parenté antigénique entre les souches A/Equi 2/Miami/63, A/Equi 2/Richelieu/63 et A/Equi 2/Milford/63 provenant de l'épizootie de 1963 chez les chevaux ainsi qu'entre la souche A/Equi1/Prague 56 et deux souches équines 1963 A/Equi 2. Les antisérums préparés contre les souches de virus du cheval ont présenté des anticorps contre le virus homologue de la grippe équine et le virus de la grippe humaine A2 isolé en 1957. Des 434 sérums prélevés en 1963 sur des personnes âgées de moins de 60 ans,

deux seulement ont présenté des anticorps inhibant l'hémagglutination pour A/Equi 2/Richelieu/63 alors que 50 en présentaient parmi les 435 sérums provenant de personnes âgées de 60 ans ou plus. Ces résultats suggèrent qu'une infection par un virus ressemblant à la souche A/Equi 2 1963 s'est produite environ 7-9 ans après la pandémie grippale de 1889-1890; le virus a sans doute été un mutant du virus grippal A2.

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