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Experimental Infection in Man and Horses with Influenza A Viruses*

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The recognition of an antigenic relationship between the haemagglutinins of A/Equi-2 and A2/Hong Kong/68 viruses led to experimental studies in man and horses with these virus types.

Human volunteers were inoculated with A/Equi-2/Miami/63 virus and virus shedding ensued in all subjects. The most common clinical response was a febrile illness indistinguishable from naturally occurring human influenza. After administration of A2/Hong Kong/68 virus to 10 ponies there was virus shedding from 9 and a febrile response in 6.

When the human subjects previously inoculated with equine virus were challenged with A2/Hong Kong/68 virus, the frequency of illness and the extent of virus shedding were lower than was observed among control individuals. This immunity was found to be related to the level of heterologous serum antibody to the human virus which developed after equine virus infection. Challenge with A/Equi-2/Miami/63 virus of ponies previously inoculated with A2/Hong Kong/68 virus, in the absence of any measurable levels of heterologous antibody to the human strain, resulted in less shedding of virus among these than occurred in control animals.

An antigenic relationship has been established between the haemagglutinins of A/Equi-2 virus isolated from horses in 1963 and a type A2 virus which caused epidemic illness in human populations during 1968 and 1969 (Coleman et al., 1968; Kasel, Fulk & Couch, 1969). The detection of this relationship suggested that strains from one of the species may have been originally derived from the other species. In order to evaluate this possibility and to determine whether the serological relationship had biological significance, experimental studies in man and horses with A/Equi-2 virus and the new human A2 variant were performed. In man, challenge studies were conducted to assess the protective effect of heterologous serum antibody induced by prior infection with equine influenza virus (Couch et al., 1969).[3] In horses, studies were done to assess the effect of inoculation with the human influenza virus on subsequent challenge with equine virus (Kasel, Fulk & Harvey, 1969).

The purpose of this report is to summarize the results of these studies.

MATERIALS AND METHODS

Subjects

Subjects were adult male volunteers between the ages of 21 and 35 years who lacked detectable serum neutralizing antibody (<1 : 2) to A Equi-2, Miami/63 and had low or no detectable titre to A2/Hong Kong/68 viruses. Medical examinations to ensure good health were performed and written informed consent was obtained prior to viral inoculations.

Horses used in these studies were Chincoteague ponies (*Equus cabullus*) selected from a wild herd on Assateague Island, Virginia. The animals were obtained from a population which was relatively isolated from human beings and had no known previous contact with other horses. The ponies

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lacked measurable serum neutralizing antibody (<1 : 2) to the challenge viruses and were approximately 8 months of age at the time of inoculation.

Inoculum and method of inoculation

The Miami/63 strain of A/Equi-2 virus isolated from a naturally infected horse and a strain of A2/Hong Kong/68 isolated from 2 different persons were used to prepare inoculum pools by previously described procedures (Couch et al., 1969; Cameron et al., 1967; Kasel, Fulk & Harvey, 1969). Embryonated hens' eggs, hamster kidney (HK), African green monkey kidney (AGMK), and human embryonic kidney (HEK) were used to prepare viral inocula. The passage history of the A/Equi-2 virus inoculum pool used in human volunteer experiments was egg_5, HK_2, man_1, HK_4, man_1, HEK_3, and of that used in pony experiments was egg_5, HK_2, $pony_1$, $AGMK_1$, HEK_2, $pony_1$, $AGMK_2$, $pony_1$, HEK_2, $pony_1$, HEK_3, $pony_1$, HEK_3, $pony_1$, HEK_2. Pool 1 and pool 2 of A2/Hong Kong/68 virus were tissue-culture fluid harvests from the sixth and second viral passages in HEK cultures. The human volunteers were given A/Equi-2/Miami/63 or A2/Hong Kong/68 viruses by spray into the nasopharynx and/or by nasal instillation. The animals were given viral inoculum according to the following methods. A2/Hong Kong/68 (pool 1) was sprayed into the nasopharynx by nebulization and also injected into the tracheal lumen by syringe and needle; A2/Hong Kong/68 (pool 2) was sprayed into the nasopharynx and oropharynx by nebulization; and A/Equi-2/Miami/63 was sprayed into the nasal cavities by nebulization.

Clinical and laboratory methods

The procedures employed for clinical evaluation of subjects, virus isolation, and serum neutralizing antibody titres have been described elsewhere (Kasel, Fulk & Harvey, 1969; Couch et al., 1969).

RESULTS

Response of man to equine influenza virus

The illness responses of human volunteers following nasopharyngeal inoculation with $10^{6.75}$ 50% tissue-culture infectious doses ($TCID_{50}$) of A/Equi-2 virus are shown in Table 1. Thirteen of 15 individuals developed illness on the second or third day after inoculation. The most common clinical response was a febrile upper respiratory and systemic illness which lasted 2–4 days; 8 subjects showed this type of illness response. Two volunteers also developed significant symptoms of lower respiratory illness which included paroxysmal episodes of cough, substernal discomfort and tenderness of the trachea. One of these individuals also developed an identical syndrome 10 days after inoculation. Three persons had an afebrile upper respiratory illness only. When symptoms and signs were tabulated and frequencies compared with those described for naturally occurring type A influenza illness (Stuart-Harris, 1965), it was observed that the febrile response was of shorter duration (mean, 1.3 days), nasal discharge and obstruction were slightly more frequent, and there was a slightly less frequent occurrence of cough and sore throat among the subjects inoculated with equine influenza virus. These differences may be attributable to the route of administration of equine virus since it is known that the majority of the virus dose was deposited in the nasopharynx. Nevertheless, the syndrome of sudden onset of febrile systemic illness with rhinitis and cough, the most common clinical response observed in the volunteers, is compatible with clinical descriptions of naturally occurring human influenza.

TABLE 1

ILLNESS RESPONSE OF ADULT MALE VOLUNTEERS FOLLOWING NASAL INOCULATION WITH A/Equi-2/Miami/63 INFLUENZA VIRUS

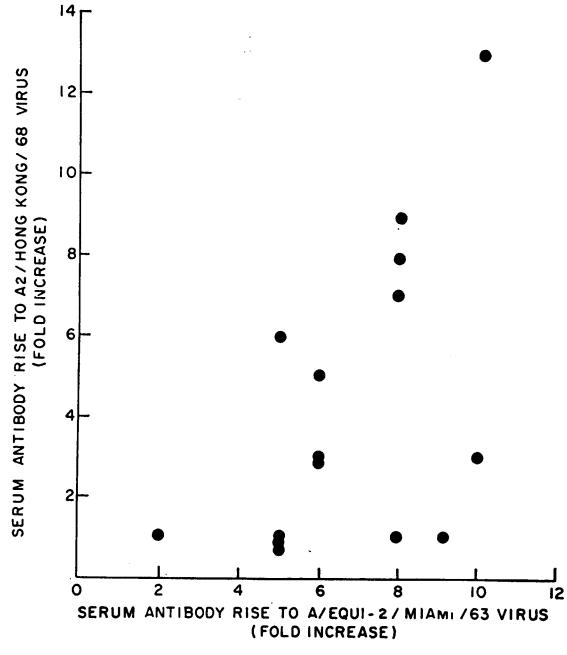
No. of men	Type of illness
8	Febrile upper respiratory and systemic
2	Febrile upper and lower respiratory and systemic
3	Afebrile upper respiratory
2	None

Virus shedding occurred in each of the 15 volunteers. Isolations were obtained beginning on day 1 from 5 men and from all others between days 2 and 4 after inoculation. The duration of virus shedding was limited to the first 6 days except for 4 individuals who yielded a virus-positive specimen on day 10.

Fourteen of the 15 subjects exhibited a 4-fold or greater serum neutralizing antibody response to the infecting virus and, of these, 9 developed heterologous serum neutralizing antibody to A2/Hong Kong/68 influenza virus. All individuals were initially free of measurable antibody to each virus

type except for 4 volunteers who had low titres (1 : 2 or 1 : 4) to the human strain. The relationship between antibody responses to A/Equi-2 and A2/Hong Kong/68 viruses is shown in the accompanying figure. The 28-day post-inoculation serum antibody rises to the equine and human strains ranged from 1 : 2 to $\geqslant$1 : 512 and $<$1 : 2 to 1 : 4096, respectively. A comparison of the magnitude of increases in antibody levels to each of the viruses revealed that persons with a high increase in antibody to A/Equi-2 virus tended also to exhibit a high rise in antibody to the human influenza virus ($r=+0.477$, $P<0.05$, Spearman rank correlation test).

SERUM NEUTRALIZING ANTIBODY TITRES TO A/Equi-2/Miami/63 AND A2/Hong Kong/68 VIRUSES IN HUMAN ADULT VOLUNTEERS 28 DAYS FOLLOWING CHALLENGE WITH A/Equi-2/Miami/63 VIRUS



Response of ponies to human influenza viruses

Responses of the ponies to inoculation with A2/Hong Kong/68 influenza virus are presented in Table 2. For comparative purposes, responses of 5 animals to inoculation with an antigenically dissimilar human-type A2 virus are also included (Kasel, Fulk & Harvey, 1969). Among the 10 ponies inoculated with A2/Hong Kong/68 virus, a febrile response occurred in 6, virus shedding from 9 and a serum neutralizing antibody response in 4. In contrast, among the 5 animals administered the A2/Rockville/65 influenza virus, a human strain known not to possess a virion surface antigen related to A/Equi-2 virus, there were no measurable responses.

Fever was first observed in ponies about 30 hours after virus inoculation and thereafter ranged from 102.5°F to 104.2°F (39.2°C–40.1°C) for 1–3 days.

Six-hour post-inoculation specimens obtained from all ponies were negative for virus. Isolations were first obtained from 5 ponies on day 1, from 3 on day 2 and from 1 on day 3. Three of the animals shed virus for 1 day, 3 for 2 days, 2 for 3 days, and 1 for 5 days.

The serum antibody titres to A2/Hong Kong/68 virus ranged from 1 : 2 to 1 : 8 in the 4 ponies with a serological response. No heterologous antibody responses to A/Equi-2 virus were detected.

Challenge of human volunteers with A2/Hong Kong/68 virus

Four weeks after inoculation with A/Equi-2 virus all 15 of the infected volunteers and 19 serum-anti-

TABLE 2

RESPONSES OF CHINCOTEAGUE PONIES FOLLOWING INOCULATION OF A2/Hong Kong/68 INFLUENZA VIRUS

Virus inoculum	Administered dose ($TCID_{50}$)	No. of animals	No. with febrile illness ($\geqslant$102.5°F) [a]	No. with virus isolations	No. with $\geqslant$2-fold antibody rise
A2/Hong Kong/68 (pool 1)	$10^{6.5}$	5	4	5	3
A2/Hong Kong/68 (pool 2)	$10^{6.2}$	5	2	4	1
A2/Rockville/65	$10^{7.5}$	5	0	0	0

[a] Normal equine temperature is up to 102.5°F (39.2°C).

TABLE 3

OCCURRENCE OF ILLNESS, DISTRIBUTION OF VIRAL ISOLATES AND SERUM ANTIBODY RESPONSES OF ADULT MALE VOLUNTEERS FOLLOWING NASAL INOCULATION OF A2/Hong Kong/68 INFLUENZA VIRUS

Challenge group	No. in group	No. of men with indicated response to virus challenge								
		No. with illness	No. with indicated number of viral isolates							No. with ≥4-fold antibody rise
			0	1	2	3	4	5	6	7	
Prior inoculation with A/Equi-2/Miami/63	15	1	10	2	2	0	0	0	1	0	4
Controls	19	9	8	1	0	1	3	2	3	1	14

body-negative volunteers were simultaneously challenged intranasally with $10^{3.2}$ $TCID_{50}$ of A2/Hong Kong/68 virus (pool 2). The responses to these inoculations are summarized in Table 3. Administration of the human strain to subjects previously inoculated with A/Equi-2 virus was followed by illness in 1, virus shedding in 5 and serum antibody rise in 4. The relative frequency of illness responses among subjects previously given equine virus tended to be less than that seen among the control individuals ($P<0.06$). The difference in the distribution of viral isolates in the equine viral group and that in the control group challenged with the same human strain was clearly significant ($P<0.05$). As shown in Table 4, the resistance to challenge was found to be related to the titre of serum antibody to A2/Hong Kong/68 virus which developed after infection with equine influenza virus. At the time of inoculation with the human strain, 6 volunteers lacked measurable antibody to this strain, 5 exhibited low levels and 4 had high titres. Infection as measured by illness, virus shedding and/or rise in serum antibody titre occurred in 6 subjects. Four of the 6 with no detectable antibody to the challenge virus became infected with this strain, 2 of 5 with intermediate titres became infected, and none of 4 with high titres. The decrease in the occurrence of infection in relation to the titre of serum antibody was statistically significant ($P<0.05$, Yates mean score test).

TABLE 4

OCCURRENCE OF INFECTION AMONG ADULT MALE VOLUNTEERS FOLLOWING NASAL INSTILLATION OF A2/Hong Kong/68 INFLUENZA VIRUS ACCORDING TO PRE-INOCULATION ANTIBODY TITRE

Pre-inoculation serum antibody titre [a]	No. of volunteers	No. infected
<2	6	4
4–32	5	2
256–4 096	4	0

[a] Reciprocal neutralization titre to A2/Hong Kong/68 virus.

Challenge of ponies with A/Equi-2/Miami/63 virus

Seventy-five and 52 days after inoculation of ponies with A2/Hong Kong/68 virus, pools 1 and 2 respectively, these 10 animals and 5 serum-antibody negative controls were given $10^{4.0}$ $TCID_{50}$ of A/Equi-2/Miami/63 virus.

The occurrence of febrile responses and patterns of virus shedding are shown in Table 5. Two ponies that had been previously exposed to pool 2 of A2/Hong Kong/68 virus and one control animal developed a febrile response. Virus shedding of A/Equi-2 virus was less in the previously challenged animals than in the 5 control animals. The difference in the distribution of viral isolates from ponies that had been given pool 1 and from control animals was statistically significant ($P<0.02$). However, the difference was not significant when ponies inoculated with pool 2 and controls were compared ($P>0.10$).

The geometric mean serum neutralizing antibody titres to A/Equi-2 and A2/Hong Kong viruses before and after challenge with equine influenza virus are shown in Table 6. At the time of virus challenge there was no measurable antibody to the equine strain and only low levels to the human strain in the animals that received a prior challenge with A2/Hong Kong/68 virus. Following challenge, each of the 10 animals previously inoculated with A2/Hong Kong/68 virus exhibited a serum antibody rise to both the equine and human strains. All control ponies developed an antibody response to A/Equi-2 virus and of these, 3 showed a rise in titre to A2/Hong Kong/68 virus. The post-challenge geometric mean

TABLE 5

OCCURRENCE OF FEVER AND DISTRIBUTION OF VIRAL ISOLATES OF CHINCOTEAGUE PONIES FOLLOWING NASAL INOCULATION OF A/Equi-2/Miami/63 INFLUENZA VIRUS

Challenge group	No. of animals in group	No. of animals with febrile response (≥102.5°F)	No. of animals with the indicated number of isolates					
			0	1	2	3	4	5
Prior inoculation with A2/Hong Kong/68 (pool 1)	5	0	2	2	1	0	0	0
Prior inoculation with A2/Hong Kong/68 (pool 2)	5	2	3	0	0	1	1	0
Controls	5	1	0	1	0	2	1	1

antibody titres to A2/Hong Kong/68 virus among both groups of ponies previously inoculated with that type were significantly higher than those seen in the control animals ($P<0.01$ for pool 1 and $P<0.02$ for pool 2). The same type of comparison for A/Equi-2 responses showed the mean antibody titre in animals inoculated with only pool 1 to be significantly greater ($P<0.01$). In addition, the mean antibody titre to the equine and human influenza viruses tended to be higher among the ponies that received inoculum pool 1 than among those that received pool 2, and in the case of A/Equi-2 virus, the difference in mean antibody titre was highly significant ($P<0.01$).

TABLE 6

SERUM NEUTRALIZING ANTIBODY RESPONSES OF CHINCOTEAGUE PONIES FOLLOWING NASAL INOCULATION OF A/Equi-2/Miami/63 INFLUENZA VIRUS

Challenge group	No. of animals in group	Geometric mean antibody titre [a, b]			
		A/Equi-2/ Miami 63		A2/Hong Kong/68	
		S_1	S_2	S_1	S_2
Prior inoculation with A2/Hong Kong/68 (pool 1)	5	<2	84.5	1.7	73.5
Prior inoculation with A2/Hong Kong/68 (pool 2)	5	<2	8.0	1.5	24.3
Controls	5	<2	4.6	<2	1.7

[a] S_1 refers to serum specimen collected at the time of challenge with A/Equi-2 virus and S_2 to specimen obtained 25 days after challenge.

[b] Titre expressed as reciprocal of serum dilution.

DISCUSSION

The results reported in the present study indicate that A/Equi-2 influenza virus is capable of infecting man and producing an illness which is indistinguishable from naturally occurring human influenza. The rates of infection and illness in human volunteers given A/Equi-2 virus in the study reported here were higher than those observed in other experiments in man using strains of the same virus type (Alford et al., 1967). Among 33 subjects to whom A/Equi-2 viral inocula had been administered, illness occurred in 4 persons, virus was recovered from 21 and rises in serum antibody were observed in 20. Included in this study group were 6 subjects who had been given the same inoculum as that used in the study described in this report. Following inoculation, there was no illness in any of the individuals, although virus was isolated from 4 and antibody responses occurred in 5. The reasons for these differences in illness response are not known at this time.

The reported results also indicate that A2/Hong Kong/68 influenza virus is capable of infecting horses and producing a febrile illness. Of particular interest are the contrasting results in ponies inoculated with A2/Rockville/65 or A2/Hong Kong/68 viruses. A possible explanation for occurrence of viral infection in the latter ponies and not in those given A2/Rockville/65 virus is the difference in sensitivity to an inhibitor in normal equine serum. It was observed that the A2 strain isolated in 1965 was neutralized *in vitro* by the inhibitor, whereas the 1968 variant was unaffected (Kasel, Fulk & Harvey, 1969).

The known antigenic relationship between A/Equi-2 and A2/Hong Kong/68 viruses was shown to have biological relevance in that human subjects

who were given equine virus and developed serum antibody to A2/Hong Kong/68 virus were protected against a later challenge with the human strain in relation to the magnitude of heterologous antibody that developed. Earlier studies have shown that heterologous serum antibody responses to strains of human type A2 virus occur in horses naturally infected with A/Equi-2 virus (McQueen et al., 1969). This would provide for an additional barrier against transmission of human strains to horses. Moreover, the development of heterologous serum antibody to human type A2 viruses in man or horses exposed to A/Equi-2 virus suggests that surveillance data based on serological surveys alone may not be a reliable indicator of interspecies spread during outbreaks of influenza in either species. This would also be true for epidemics caused by A2/Hong Kong/68 virus.

Immunity against A/Equi-2 virus challenge of 2 groups of ponies previously inoculated with the human strain was limited to a reduction of virus shedding in one group of animals. Thus, little to no protection of ponies was afforded by the prior infection with A2/Hong Kong/68 virus. Since no detectable heterologous serum antibody developed in these animals, we suggest that this is the reason infection ensued following A/Equi-2 virus challenge. In this respect, the ponies were similar to humans who did not develop heterologous antibody in that the occurrence of infection and illness in volunteers without antibody was similar to that which developed in controls.

The presence of A/Equi-2 virus in equine populations for at least 5 years prior to the occurrence of epidemic influenza by A2/Hong Kong/68 virus and the available epidemiological data (Davenport, Hennessey & Minuse, 1967) suggest that horses are not active reservoirs of virus for man. Nevertheless, the appearance in man and horse of type A influenza viruses that are antigenically related suggests that an exchange of viruses between these hosts may on occasion occur in nature. The data presented in this report tend to support that suggestion.

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