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EXPERIMENTAL INFECTION OF HORSES WITH A-EQUI 2/MIAMI/1/63 AND HUMAN A2/HONG KONG/1/68 INFLUENZA VIRUSES I. THE COURSE OF INFECTION AND VIRUS RECOVERY

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Summary. — Eight hundred and twenty five horse sera from animals of different age groups were collected in different regions of Slovakia in 1967. Of these reacted in haemagglutination inhibition (HI) tests: 5.5% with A-equi 1/Praha/1/56 virus, none with A-equi 2/Miami/1/63 virus, 99.9% with the inhibitor-sensitive (IS) variant of human A2/Hong Kong/1/68 virus and 2.3% with the inhibitor-resistant (IR) variant of this virus. After treatment with potassium periodate inhibitors against the IR variant were completely removed from the sera and only 6.4% of sera remained positive against the IS variant.
Four horses infected intranasally with A-equi 2 influenza virus developed clinical disease completely resembling natural infection. Virus from infected horses could be isolated from nasal swabs for at least 5 days after infection (p.i.). Two horses infected intramuscularly did not develop the disease. Very mild disease with non-specific symptoms could be induced in horses infected intranasally with the A2/Hong Kong/1/68 IS human influenza strain. The virus could be recovered from nasal mucosa 5 days p.i. No virus could be isolated from any organs of two horses killed on the 2nd or 4th day p.i.

Introduction

The isolation and identification of the A-equi 2/Miami/1/63 strain of influenza virus (Waddell *et al.*, 1963) and additional strains of horse influenza viruses (Marois *et al.*, 1963; McQueen *et al.*, 1966*a*) from the North American continent stimulated a series of investigations concerning the relationship among influenza group A strains of animal and human origin. It was found that human sera from individuals born before 1900 may contain antibodies against A-equi 2 influenza virus (Voth and Feldman, 1963; Minuse *et al.*, 1965; Schild and Stuart-Harris, 1965; Masurel and Mulder, 1966; Rose, 1966; Tůmová *et al.*, 1968). In addition to specific nucleoprotein (S- or g-) antigen determining the group A influenza viruses, also some common antigens responsible for HI and virus neutralization tests have been established

(Kasel *et al.*, 1965; Lief and Cohen, 1966; Masurel, 1968; Tůmová *et al.*, 1968; Kasel *et al.*, 1969).

Considering the aforementioned facts, as well as previous results concerning experimental pathogenesis of A-equi 1/Praha/1/56 influenza virus in horses (Blaškovič *et al.*, 1966), we attempted to compare experimental infections in horses with A-equi 2/Miami/1/63 and human A2/Hong Kong/1/68 influenza viruses. The immunological response of infected animals measured by HI tests in both infections against homologous and heterologous viral strains was also followed.

A serological survey of horses from Slovakia and testing of their sera with A-equi 1, A-equi 2 and A2/Hong Kong viruses preceded the experimental infection.

Materials and Methods

Viruses. A-equi 1/Praha/1/56 influenza virus was used in its E12 passage. The next egg passage was employed as antigen in HI tests. The haemagglutinin (HA) titre of the allantoic fluid was 128 per ml.

A-equi 2/Miami/1/63 influenza virus was used in its second egg passage in our laboratory; its previous passage history is unknown. Its HA and infectivity titres were 512 per ml and 10^8 EID_{50}/0.1 ml, respectively.

A2/Hong/Kong/1/68 human influenza virus, IS variant, was used in its 5th egg passage; its HA and infectious titres were 1028 per ml and 10^7 EID_{50}/0.1 ml, respectively.

A2/Hong Kong/1/68 human influenza virus, IR variant, was used in its 7th egg passage and had a HA titre of 1024 per ml.

A-duck influenza virus BV/63 isolated by Dr. V. Isachenko, Ivanovsky Institute of Virology, Moscow was used in its 3rd egg passage in our laboratory; its previous passage history is unknown. It had a HA titre of 2048 per ml.

A-equi 1 and 2, A2/Hong Kong/1/68 IS variant and A-duck BV/63 influenza viruses were kindly supplied by Dr. D. Fedová, Czechoslovak Influenza Centre in Prague, and A2/Hong Kong/1/68 IR variant of influenza virus by Dr. E. Farkas, Virus Department, State Institute of Hygiene, Budapest.

Experimental animals and mode of infection. Horses of different age and both sexes were used. Horses Nos 16, 21, 31, 84 (4, 6, 12 and 6 years old, respectively) were infected intranasally (in) into each nostril with 5 ml of allantoic fluid containing A-equi 2/Miami/1/63 influenza virus. Two additional horses, Nos 17 and 90 (11 and 13 years old), were given 10 ml of infectious allantoic fluid with the same virus intramuscularly (im) with adjuvant (arlacel and paraffin oil 1 : 10 mixed with an equal volume of virus).

Six horses, Nos 19, 25, 28, 32, 41 and 840 (9, 17, 10, 9, 21 and 1 year old, respectively), were infected with human A2/Hong Kong/1/68 IS influenza virus. Five ml of infectious allantoic fluid was instilled into each nostril. Allantoic fluid was sprayed in both experiments by a hand sprayer. Two animals of this series (Nos 840 and 41) were killed 48 and 96 hours p.i., respectively.

Horses infected with A-equi 2 influenza virus were boostered with the same virus im on day 50 and on day 90 challenged intravenously (iv) with 10 ml of allantoic fluid containing A2 Hong Kong human influenza virus.

Horses infected with A2/Hong Kong/1/68 influenza virus were challenged iv on day 40 with 10 ml of allantoic fluid containing A-equi 2/Miami/1/63 influenza virus.

Virus recovery from infected horses. Nasal swabs taken daily from nasal mucosa were washed with sterile buffered broth containing 200 units of penicillin and 200 μg of streptomycin per ml. They were left to stay overnight at 4° C for elution. The eluate was centrifuged at 3000 rev/min for 10 minutes and the supernatant inoculated in tenfold dilutions intraallantoically into 10—11-day-old chick embryos, incubated at 35.5° C for 3 days. The presence of virus in the allantoic fluid was tested in haemagglutination tests with 1% rooster red blood cells (rbc).

After the allantoic fluid had been harvested, the chorioallantoic membranes (CAM) were prepared for cryostat sectioning. The sections were fixed for 10 minutes in acetone and stained with labelled hyperimmune gamma-globulin by the direct method as described by Blaškovič *et al.* (1964, 1966).

From two horses, the following organs or tissues were taken at the autopsy: different parts from nasal and conchal mucosa, tracheal mucosa, lung tissue (upper, middle and lower part), peritracheal and mediastinal lymph nodes, spleen and liver. Ten per cent suspensions were prepared in buffered broth and after centrifugation for 10 minutes at 3000 rev/min, the supernatants were inoculated intraallantoically into 10—11-day-old chick embryos. The harvested allantoic fluids were tested for HA with rooster rbc and the CAM prepared for sectioning and staining with fluorescent antibody.

Serology. HI tests were performed by Takátsy's (1955) micromethod. Sera were heated at 56° C for 30 minutes and treated with potassium periodate by the method proposed by the WHO Expert Committee (1959), but a part of the sera was treated with KIO_4 for 1 hour. For comparison, unheated and heated sera, both without periodate treatment, were also employed. Four haemagglutinating units of the respective virus in the form of infectious allantoic fluid were added to twofold dilutions of sera. The serum-virus mixtures were kept at room temperature for 15 minutes. Then 1% of rooster rbc was added and the results were read after 20 minutes.

Results

Serological survey in horses from the territory of Slovakia

Of 840 blood samples collected in different regions of Slovakia in 1967, 825 were suitable for serology. The sera were examined in HI tests with A-equi 1/Praha/1/56, A-equi 2/Miami/1/63, and the IS and IR variants of human A2/Hong Kong/1/68 influenza viruses.

The results of HI tests, summarized in Table 1, suggested a low incidence of influenza infection due to A-equi 1/Praha/1/56 and none with A-equi 2/Miami/1/63 viruses, at least in 1967 or in the preceding years. This enabled us to select non-immune horses for experimental infection with A-equi 2 influenza virus. Our assumption was supported by the finding that 260 unheated horse sera tested in a dilution of 1 : 2 failed to react with A-equi 2 influenza virus.

Table 1. HI tests with horse sera from the territory of Slovakia (1968)

No. of serum samples tested	No. (%) of HI tests positive with virus			
	A-equi 1	A-equi 2	A2/Hong Kong/68 IS	A2/Hong Kong/68 IR
Heated, not treated with KIO_4				
825	45 (5.5%)[1]	0	824 (99.9%)[2]	Not done
735				17 (2.3%)[3]
Heated, treated with KIO_4				
735	Not done	Not done	47 (6.4%)[4]	0

[1] Serum dilutions ranging from 1 : 4—1 : 32.
[2] Serum dilutions ranging from 1 : 4—1 : 512.
[3] Serum dilutions ranging from 1 : 2—1 : 4.
[4] Serum dilutions ranging from 1 : 16—1 : 32.

Practically, all the sera tested inhibited haemagglutination with human A2 Hong Kong IS strain. Treatment with potassium periodate removed antihaemagglutinins which are considered nonspecific inhibitors from 93.6% of the sera. The same treatment had completely removed the low incidence of these inhibitors present in heated sera against A2 Hong Kong IR strain.

This serological screening enabled us to select horses Nos 16, 17, 21, 31, 84 and 90 for experimental infection with A-equi 2 influenza virus and horses Nos 19, 25, 28, 32, 41 and 840 for in infection with human A2 Hong Kong influenza virus.

Experimental infection with A-equi 2/Miami/1/63 influenza virus

Intranasal infection. Four in infected horses (Nos 16, 21, 31 and 84) developed fever on the 2nd day p.i., reaching the highest value of 40.4° C in one 4 years old foal (the youngest in the group). Fever lasted for 4—8 days. On the 2nd or 3rd day p.i., symptoms of inflammatory process of the upper respiratory tract (catarrhal secretion from nostrils, laryngitis) appeared, which was later followed by inflammation of the lower respiratory tract connected with heavy cough.

In general, the clinical symptoms did not differ from those already described (Waddell *et al.*, 1963; Beveridge *et al.*, 1965; Paccaud *et al.*, 1966; Gerber and Löhrer, 1966*a*) and did not substantially contribute to the knowledge of the clinical course of the disease.

Table 2. Recovery of virus from nasal swabs from horses experimentally infected with A-equi 2/Miami/1/63 influenza virus confirmed by positive immunofluorescence

Horse No.	Method of detection*	Days p.i.				
		1	2	3	4	5
16	VI	3.5	4.5	5	5	5
	IF	+	+	+	+	+
21	VI	2.5	5	2		5
	IF	−	+	+		+
31	VI	5	5	5	5	5
	IF	+	+	+	+	+
84	VI	1	5	5	5	5
	IF	−	+	+	+	+

* VI: virus isolation, given in log EID_{50}/0.1 ml values.
IF: immunofluorescence positive (+) or negative (−).

Clinical symptoms included also secondary bacterial infection of two animals which developed perulent rhinitis and pulmonary complications as described by Gerber and Löhrer (1966*b*).

One contact infection of a horse located in the same stable as the four in infected animals was confirmed clinically and by virus isolation. No epizootic of horse influenza occurred either in the environment of this stable or in the country. The contact infection is in good agrement with the repizootology of the disease (Gerber *et al.*, 1966; McQueen *et al.*, 1966*b*; Bryans *et al.*, 1967).

The virus could be easily recovered from nasal swabs from experimentally infected horses during the first 5 days p.i. (the longest interval examined) in titres indicated in Table 2.

The specificity of virus isolation in embryonated eggs was confirmed by staining CAM sections with fluorescent antibody. The cells showed bright specific fluorescence.

The peculiarities of immunological response after the infection will be described in the following paper (Blaškovič *et al.*, 1969).

Intramuscular infection. After im administration of live A-equi 2/Miami/1/63 virus with adjuvants, neither fever nor catarrhal symptoms of respiratory infection occurred. The immunological response will be presented together with that after in infection (see above).

Experimental infection of horses with A2/Hong/Kong/1/68 human influenza virus, IS variant

Five horses (Nos 19, 25, 28, 32 and 41) were in infected with 10 ml of infectious allantoic fluid. Immediately thereafter they were ridden for 15 minutes in a cold, windy weather (March 8, 1969) and then exposed at —8° C for 20 minutes outside the stable. When brought inside, they showed shivering. Horse No. 840 was inoculated similarly, but not stressed.

None but horse No. 840 had elevated body temperature slightly over normal average on the 2nd and 3rd day p.i.

All horses, daily clinically examined, showed the common symptoms: malaise, loss of both appetite and thirst (the latter symptom was not shown by horse No. 840), and mild secretion from nostrils. These symptoms appeared on day 2—3 p.i. and persisted for another 2—4 days. No other clinical symptoms, including cough, were observed.

Table 3. Recovery of virus from nasal swabs from horses experimentally infected with A2/Hong Kong/1/68 human influenza virus confirmed by specific immunofluorescense

Horse No.	Method of detection*	Days p.i.					
		1	2	3	4	5	6
19	HA	—	+	+	+	+	0
	IF	—	+	+	0	0	0
28	HA	0	0	0	0	0	0
	IF	0	0	0	0	0	0
25	HA	+	+	+	+	+	0
	IF	+	+	+	0	0	0
32	HA	+	+	+	+	+	0
	IF	+	0	+	0	0	0
41	HA	+	0	0	0 A		
	IF	+	0	0	0		
840	HA	+	+ A				
	IF	+	0				

* HA: haemagglutination positive (+) or negative (—).
IF: immunofluorescence positive (+) or negative (—) in sections from chick embryo CAM.
A = autopsy.

Horse No. 840 was killed 48 hours p.i. Its nasal mucosa was slightly red and the blood vessels were enlarged. Serous secretion was well detectable. In the trachea, flakes of dense, slightly yellowish mucus were observed. No pathological changes were detected in the bronchi and the lungs. Other organs, including paratracheal lymph nodes, showed no lesions.

Horse No. 41 was killed 4 days p.i. Slight secretion was observed on pink nasal mucosa, which was smooth with enlarged blood vessels. We found no lesions in the trachea, lungs and peritracheal lymph nodes, nor pathological changes in other organs.

Nasal swabs were taken daily and assayed for virus. The results are summarized in Table 3. From CAM of chick embryos inoculated with appropriate tissue or organ suspension and then incubated for 3 days, sections were prepared and stained with specific anti-Hong Kong immunofluorescent conjugate.

The virus was recovered from nasal smears up to the 5th day p.i. from 3 of 4 horses in the first egg passage. Immunofluorescence of CAM sections was positive 3 days p.i., indicating large amounts of virus present in the inoculum.

No virus was recovered from any organ taken at autopsy.

Three out of four horses overcoming the infection developed specific antibodies against the IS and one horse also against the IR variant of A2/Hong Kong/1/68 influenza virus. HI tests revealed the presence of antibodies in serum dilutions of 1 : 16 on the 4th day p.i. (horse No. 25), or on the 7th day p.i. (two horses). Details on antibody response will be presented in the subsequent paper (Blaškovič *et al.*, 1969).

Discussion

The idea to compare in a clinical and virological study on the pathogenicity for horses the A-equi 2/Miami/1/63 and human A2/Hong Kong/1/68 influenza viruses, whose antigenic relationship has been recognized earlier (see above), necessitated a selection of animals non-immune to A-equi 2 influenza virus. Therefore, a serological survey on 825 horses was accomplished with these two viruses, including the IS and IR variants of the Hong Kong influenza strain and with A-equi 1/Praha/1/56 virus.

Surprisingly, no antibodies against A-equi 2 influenza virus were detected in either unheated or heated sera, though in neighbouring countries, Austria and Hungary (F. Bürki and J. Romváry, Budapest, personal communication), an outbreak of A-equi 2 influenza was firmly established in 1968. We were aware of the fact that low antibody titres, or only specific antibody response to an appropriate strain may occur after natural infection with this virus (Rose, 1966). There was no larger influenza epizootic on the territory of Czechoslovakia during the past two years. Since high contagiosity of this infection has been repeatedly proved, this suggested that the absence of antibodies against A-equi 2 virus could be correlated with the absence of an epizootic due to this virus. Successful infection of all horses in the experiment confirmed this assumption.

The low percentage of horses (5.5%) with serum antibodies against A-equi 1/Praha/1/56 virus could be explained by a low incidence of this infection in the past few years.

The IS variant of A2/Hong Kong/1/68 human influenza virus reacted in HI tests with almost all the sera tested. The treatment of sera with potassium periodate significantly lowered the number of samples with positive titres (1:16—1:32), namely from 99.9% to 6.4%. We consider the inhibiting substances remaining in the sera as nonspecific inhibitors in spite of the fact that the sera were treated with KIO_4 for 60 minutes. A more thorough study would be needed to show whether these antihaemagglutinins should be considered as antibodies. The same treatment of sera had completely removed inhibitors to the IR variant occurring naturally in a low percentage.

The egg-passaged A-equi 2 influenza virus proved to be satisfactorily virulent to produce a typical natural infection to the symptomatology of which we have contributed no new data. We regret not to have followed the presence of the virus in nasal mucosa for more than 5 days. According to the titre of the virus recovered from nasal swabs we assume that the period for which the virus might be present is longer than 5 days.

The simultaneous staining of CAM sections with fluorescent antibody has been proved again as very suitable to visualize the virus multiplication and to confirm the specificity of the reproduced virus very soon after harvesting of the allantoic fluid.

We consider the infection of horses with the IS A2/Hong Kong/68 influenza virus as successful because of the presence of the virus in nasal mucosa for 5 days and partially also because of the antibody formation observed. We assume that the virus had multiplied in the cells of nasal mucosa. This is in accordance with our experience concerning inoculation of live influenza virus into man (Styk, 1957). It is difficult to speak about a specific symptomatology of this infection. The symptoms which could be considered as associated with the disease were: serous nasal secretion, malaise, inappetency and loss of thirst. These are rather very common symptoms, not specific for the respiratory infection. It is difficult to resolve whether this type of infection, poor on symptoms and not resembling influenza in horses, is due to species heterogeneity of the virus, or to the history of successive egg passages which might be responsible for a non-characteristic minor disease.

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