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Experimental Equine Influenza in Chincoteague Ponies. (31777)

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In the past decade, influenza in horses has been shown to be caused by 2 immunologically distinct type A influenza viruses. The first type was isolated from horses with respiratory disease in Czechoslovakia in 1956, and is currently classified as A/Equi 1/Prague/56(1). The second was obtained from horses during an epizootic of equine influenza which occurred in the United States in 1963, and is designated as A/Equi 2/Miami/1/63(2). Information relevant to infection and disease in horses due to these viruses has been obtained almost exclusively by the observation of naturally-occurring epizootics. Epidemiologic investigations have provided considerable information about natural equine infection. However, an experimental model has been needed to obtain added information on host-virus relationships and clinical and immunologic responses in equines under controlled conditions. Since previous reports on experimental equine influenza in the natural host indicated failure to produce clinical disease or were lacking detailed information (3,4), experiments were undertaken in Chincoteague ponies using a strain of A/Equi 2 virus for the purpose of developing a satisfactory system for the study of equine influenza in the natural host and to provide materials for studies of equine influenza virus in man. This report describes the results of the pony experiments. The results of human volunteer studies employing materials from the pony experiments will be reported separately(5).

Materials and methods. Subjects. The study was conducted with 24 random-sexed ponies (*Equus caballus*) from a wild herd on Assateague Island, Virginia. The animals were obtained from a population relatively isolated from humans and had no known previous contact with other equines. The approximate age of the ponies employed in the experiments was 1 to 1.5 years. A serologic survey for neutralizing antibody using representative serum specimens collected in recent years from Chincoteague ponies of various ages and those used in this study indicated a complete lack of prior experience with A/Equi 2 virus.

Plan of study. Studies in ponies were conducted in isolation units at the Grayson Laboratory, University of Maryland, College Park. Seven groups of ponies without detectable neutralizing antibody ($<1:2$) were administered A/Equi 2 virus (Table I). The initial inoculum (E-1) was prepared with an isolate derived from a naturally-infected horse and given to 2 ponies. Thereafter, 5 successive groups of ponies were given a viral inoculum prepared with an isolate obtained from a subject in each preceding group. Be-

TABLE I. A/Equi 2 Viral Inocula.

Inoculum*	Virus source	Culture passage history†	Pony No.	Total dose ($TCID_{50}$)‡
E-1	Naturally infected horse	Egg_5, Ham kid_2	1, 2	$10^{5.0}$
E-2	Pony 1	$AGMK_1$, HEK_2	3, 4	$10^{7.0}$
			5–7	$10^{5.2}$
E-3	Pony 3	$AGMK_2$	8, 9	$10^{8.0}$
E-4	Pony 9	HEK_2	10,11	$10^{7.2}$
E-5	Pony 11	HEK_3	12,13	$10^{5.6}$
E-6	Pony 13	HEK_3	14–18	$10^{5.0}$
E-1-TC	Naturally infected horse	HEK_{10}	19–24	$10^{5.7}$

* Letter E refers to equine, numeral indicates virus passage level in equine, and TC indicates additional tissue culture passage of E-1 inoculum pool.

† Egg = embryonated chicken egg. Ham kid = hamster kidney. AGMK = African green monkey kidney. HEK = human embryonic kidney.

‡ 50% tissue culture infectious doses.

cause of low virus titers in nasal specimens from ponies, it was necessary to serially passage isolates in tissue culture to obtain inoculum pools of sufficient titer (Table I). The last group of ponies was inoculated with a virus pool (E-1-TC) that was prepared by passing serially the E-1 pool 10 times in human embryonic kidney tissue culture.

Viral inocula. The equine influenza virus strain used to prepare inocula was A/Equi 2/Miami/1/63.* It was isolated in embryonated chicken eggs from a nasal washing specimen collected from a naturally-infected horse. The isolate was then passed serially 4 times in embryonated chicken eggs before passage in tissue cultures. The virus pools prepared in tissue culture were maintained with Eagle's basal medium to which antibiotics had been added. SV-40 antiserum was added to the maintenance medium when monkey kidney tissue culture was employed. Supernatant fluid from infected cultures was harvested after 3 to 4 days incubation at 36°C and clarified by low speed centrifugation. Bovine serum albumin (Armour) was added to a final concentration of 0.5% and the pools were frozen and stored at —70°C. Each inoculum pool was tested for contaminating micro-organisms as previously described(6). In addition, the test procedure included primary horse embryonic kidney tissue cultures.

Inoculation methods. Each pony received approximately 5 ml of undiluted virus suspension which was delivered into one or both nostrils by means of a small hand atomizer (DeVilbiss No. 127). An additional 5 ml of inoculum was administered intratracheally by a 19 gauge needle. Titrations of the inocula were performed following the administration of virus to ponies. To titrate, 0.2 ml of serial 10-fold dilutions were placed into each of 4 rhesus monkey kidney tissue culture tubes containing 1.0 ml of Eagle's basal medium. After incubation at 36°C, for 5 days, the presence of virus was determined by the hemadsorption technique(7). Endpoints were calculated by the Kärber method(8). Administered doses ranged from 10^5 to 10^8 $TCID_{50}$ (Table I).

Clinical evaluation. The ponies were housed singly or 3 per isolation unit and were kept there for approximately 14 days after administration of virus. Baseline studies were obtained for each pony during a 3 to 9 day pre-inoculation period. Rectal temperatures were taken twice daily prior to and 10 days following inoculation at 8:00 a.m. and at 5:00 p.m. For several days following inoculation 2 additional readings were taken at noon and midnight. Heparinized blood samples were taken daily by jugular venipuncture. Leukocyte counts were

* Obtained from Dr. Michael Sigel, Variety Children's Research Foundation, Miami, Fla.

performed in a Coulter Counter and differential counts determined by microscopic examination of Giemsa-stained blood smears.† Daily examinations were performed by a veterinarian and a report of findings recorded.

Virologic evaluation. Nasal swab specimens from each nostril were taken before inoculation, 6 hours after inoculation, and daily for 10 days thereafter. Swabs were rotated in the nostril and then twirled in a vial containing 6 ml of veal infusion broth with 0.5% bovine serum albumin and antibiotics. Nasal wash specimens were obtained as above from all ponies except the first 2. Specimens were obtained by insertion of a #16 soft rubber catheter into the medial ventral meatus of the nostrils. Ten ml of broth were delivered into each nostril in turn; the uninoculated one was washed first when only one nostril had been inoculated. With the pony's head held downward the effluent broth was collected in a single cardboard container. All specimens, chilled on wet ice, were transported directly to the laboratory. They were clarified by centrifugation at 2000 rpm for 10 minutes at 4°C. Additional antibiotics were added to the material used for egg inoculation.

All specimens were tested in primary tissue culture for presence of virus. Three-tenths ml of each specimen was inoculated into 4 tubes each of rhesus monkey kidney, African green monkey kidney, and human embryonic kidney tissue culture‡ and incubated at 36°C. Eagle's basal medium (1.0 ml) with antibiotics was used for maintenance. The presence of virus was determined by the hemadsorption procedure using 2 tubes of each tissue at 5 and 10 days. Fluid from tubes negative at 10 days was passaged once in the respective tissue culture and examined 6 days later. Several isolates from each horse were identified by the hemadsorption-inhibition neutralization test employing antiserum to a closely related strain (A/Equi 2/Detroit/2/63) prepared in another laboratory.§

Nasal swab specimens obtained from 10 ponies were assayed for the presence of virus in 10-day-old embryonated chicken eggs. One-tenth ml of each specimen was injected into the amnionic sac and 0.2 ml into the chorioallantoic sac of 6 eggs. Combined fluid was harvested after 72 hours incubation at 36°C and the presence of virus determined by hemagglutination of guinea pig red blood cells. Fluid from negative eggs was passaged once.

Serologic evaluation. Antibody titers of serum specimens obtained before and 28 days after inoculation were determined by the hemadsorption-inhibition neutralization test in rhesus monkey kidney tissue cultures (7). Serum specimens were tested against 32-100 $TCID_{50}$ of the inoculum strain. The same procedure was used to determine the presence of antibody to influenza A/Equi 1/Prague/1/56 virus.

Results. Clinical response. Fever of greater than 102.5°F developed in 15 of 24 ponies following inoculation with influenza A/Equi 2 virus. This includes 4 of 6 ponies that had received the inoculum E-1-TC which had undergone multiple tissue culture passages outside of the natural host (Table II). The highest recorded temperature in an animal was 105.2°F. Almost all elevations occurred initially 18 to 24 hours following inoculation and persisted for 1 to 5 days. However, in 1 subject (pony 17) there was a sharp rise and fall in temperature commencing 6 hours following inoculation. The latest onset of fever which was on the third day after inoculation occurred in pony no. 22.

Clinical signs were observed only in those ponies with high fever. These subjects became inactive, experienced anorexia, and in 3, *viz.,* ponies 15, 19 and 21, marked constipation developed that required catharsis. Rapid, deep breathing with rates as high as 80 to 90 per minute and infrequent dry cough were noted. Auscultation revealed coarse pulmonary sounds and occasional

† Performed by Hematology Service, Clinical Center, National Institutes of Health.

‡ Flow Laboratories, Inc., Rockville, Md., and Microbiological Assoc., Bethesda, Md.

§ Communicable Disease Center, Virology Section, Respiratory Virus Unit, Atlanta, Ga.

TABLE II. Responses of Horses to Infection with A/Equi 2 Virus.

Inoculum	Pony No.	Occurrence of fever: Maximum recorded temp (°F)	Occurrence of fever: No. of days of temp ≧102.5	Recovery of virus, No. of days	Neutralization antibody* 28 days after inoc
E-1	1	102.2	0	4	32
	2	102.2	0	0	16
E-2	3	105.2	2	6	8
	4	102.4	0	7	8
	5	102.0	0	6†	8
	6	103.3	3	6	16
	7	101.8	0	7	8
E-3	8	102.2‡	0	6†	32
	9	102.6	1	6†	16
E-4	10	103.0	1	6	4
	11	103.2	2	7	32
E-5	12	102.6	1	7	16
	13	103.9	2	7	32
E-6	14	102.0	0	6	32
	15	104.4	5	7	8
	16	103.2	2	6	16
	17	103.4	1	3	8
	18	103.7	1	1	4
E-1-TC	19	103.3	2	4	4
	20	104.4	3	7	4
	21	104.4	2	5	4
	22	103.6	3	7	16
	23	102.4	0	0	4
	24	102.0	0	1	4

* Reciprocal of serum dilution.
† Virus recovery initially at 6 hr after inoculation.
‡ Six-hr temperature only.

wheezing. Excessive nasal discharge did not occur. Signs of illness disappeared as temperatures returned to normal. No significant changes in leukocyte totals and differentials were evident when the values for all tested animals were compared.

Virus recovery. Virus was isolated from specimens obtained from 22 of 24 exposed ponies. A summary of these results is shown in Table II. Virus was recovered from all exposed ponies except ponies 2 and 23. From 18 subjects, virus was recovered beginning on the first day after inoculation. Virus was recovered on the first day only from 2 ponies (no. 18 and 24). In ponies 1 and 17, demonstrable virus shedding did not commence until the fourth and fifth days after inoculation, and continued for 4 days and 3 days, respectively. Early post-inoculation virus recovery, *i.e.*, from 6-hour specimens, was accomplished in only 3 animals: pony 5 which received 10^5 $TCID_{50}$ of virus and ponies 8 and 9 which received 10^8 $TCID_{50}$.

A marked difference in the number of recoveries of virus from the inoculated and uninoculated nostrils was noted in those ponies in which only one nostril was inoculated. In the 10 ponies evaluated, no. 1-4 and 8-13, a total of 35 swab specimens (excluding 6-hour specimens) from the inoculated nostril were virus-positive. In contrast, only 21 specimens obtained at the same time from the uninoculated nostril were positive.

The frequency of virus isolation was greater by the nasal wash method than by nasal swabs. In 8 ponies, no. 3-4 and 8-13, in which nasal swab and nasal wash specimens were compared on a daily basis, the nasal wash specimens yielded more isolates. Nasal wash specimens were virus-positive in 6 instances in which nasal swab specimens were negative and only once was a swab

specimen positive when a concurrent wash was negative.

A comparison of tissue culture and embryonated egg isolation methods performed on specimens from 10 ponies, indicated that virus recovery rates were approximately the same in those instances in which both assay systems were used. Rhesus and African green monkey kidney tissue cultures were of approximately the same sensitivity; and both were superior to human embryonic kidney tissue culture for isolation of virus.

Serologic response. All ponies developed significant antibody rises to influenza A/Equi 2 inoculum strains. The titer of antibody in post-inoculation serum specimens is shown in Table II. The same serum specimens as well as pre-inoculation serum specimens were found to be without antibody to an influenza A/Equi 1 strain of virus tested (not shown in Table II).

Discussion. In the 24 exposed ponies successful infection was demonstrated by antibody titer rise in all and virus isolation from 22 subjects. Fevers above 102.5°F were charted in 15 ponies during the first and second days post-inoculation and this served as an additional indication of infection. Although 102.0°F is commonly considered the upper limit of the normal equine temperature range, this figure appeared several times in the baseline readings of normal ponies under no observable stress. Therefore, 102.5°F was chosen as the lower limit of any febrile determination.

The disease produced in this experiment was in most instances milder than that occurring in the epizootic from which the original isolation was made. The results may have been due to attenuation of the virus by initial isolation and passage in embryonated eggs as reported in experiments with human influenza A virus(9,10), or due to natural host resistance in the animal system employed. The ponies used were in a resting state at the time of inoculation and thereafter. The natural epizootic described with the virus type employed in this study occurred in race horses at the height of the track season with its accompanying stresses (2). In the current study the incubation period was 1 day as compared to 3 days in horses exposed naturally(11). This may be related to the large amount of the inoculum administered to test subjects.

Sequential passage of the A/Equi 2 virus through 6 pony groups did not appreciably alter the severity of the clinical infection; there was no obvious increase in either the height or length of the febrile response. There was no sequential increase in antibody titers. The clinical response of the 6 ponies of the last group which received an inoculum which had undergone multiple tissue culture passages (10 times in human embryonic kidney tissue culture) was similar to that of ponies that received the pony passage material. This indicates that equine influenza (type A/Equi 2) virus may be maintained in tissue culture for several passages without complete loss of pathogenicity for the natural host. In contrast, it has been repeatedly shown that serial passage in embryonated chicken eggs frequently results in loss of virulence of influenza viruses(9,10).

The lack of neutralizing antibody to A/Equi 1 antibody is of interest. Dowdle *et al*(12) have shown antibody rises to A/Equi 1 virus following naturally acquired virus infection with influenza A/Equi 2 in horses with pre-existing A/Equi 1 antibody. This has been supposed to be antibody formed to a minor antigenic component shared by these types(13). In the current experiment horses which had no detectable neutralizing antibody and almost certainly had no previous exposure to any influenza strain failed to develop A/Equi 1 antibody indicating the response to an initial encounter to the antigens of A/Equi 2 influenza virus.

Summary. A useful model system utilizing equine influenza virus in the natural host is presented. Infection and illness were consistently produced and clinically apparent febrile illness frequently resulted. The implications of the clinical findings and antibody responses were discussed.

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Specific Heterophile Antibody from Infectious Mononucleosis Serum.* (31778)

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The preparation of a specific form of heterophile antibody (HA) would enable better approaches to characterization of this antibody and its site of formation and function. Although this antibody has been found to be present in the macroglobulin fraction (IgM) of infectious mononucleosis serum (1-3), recent evidence indicates that HA constitutes only a small percentage of the increased macroglobulins found in the serum of patients with this condition(4). Standard methods of preparation of macroglobulins, such as chromatography and electrophoresis, would therefore yield macromolecules other than HA.

In order to prepare a specific antibody, advantage was taken of the specific ability of HA to adsorb to beef red cells. This report presents the requirements for obtaining maximal elution of the adsorbed HA, as well as certain immunological and stability characteristics of the purified antibody.

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Materials and methods. Antigens. Beef cell antigen (Balt. Biol. Lab.), freshly obtained beef red cell stroma and beef cell antigen (Hyland Lab., Calif.) were used in preliminary experiments. Beef cell antigen (BBL) gave superior results and was used thereafter.

Test sera. Sera were separated from the blood of patients with infectious mononucleosis and were inactivated at 56°C for 30 minutes prior to testing. Sheep hemagglutination activity and adsorption tests with beef red cell and guinea pig kidney antigens (BBL) were done according to standard procedure(5).

Relationship between amounts of beef cell antigen and the adsorbed and eluted HA. To 5 ml of a 1:2 dilution of serum, previously adsorbed with guinea pig kidney, and to each 2-fold dilution of this serum in pH 7.0 M/15 phosphate buffered saline (PBS) were added varying amounts of beef cell antigen (20 mg to 640 mg). The mixture was incubated for 18 hours at 4°C in a shaker. Each sample was then centri-