A New Murine Hemadsorbing Virus.* (28346)

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In the course of studies on the role of wild animals as reservoirs of human pathogens, an apparently undescribed hemadsorbing virus was recovered from the pooled tissues of 2 white-footed mice *(Peromyscus leucopus)*. The present article describes isolation of the virus and gives information concerning its physical and biological properties and presents serological data suggesting that a number of human beings have experienced infection with the newly isolated organism or an agent antigenically related to it.

Materials and methods. Cell cultures, embryonated eggs and guinea pigs. Cultures of primary rhesus monkey kidney cells and of several lines of continuous cells (rhesus monkey heart and kidney, human embryonic lung, amnion, liver, HEp 2, and HeLa) were used. Both primary and continuous cell cultures were maintained in medium 199. For studies in primary rhesus monkey cells, anti-simian virus 5 (SV_5) rabbit serum was added to the medium to suppress growth of SV_5, a contaminant frequently encountered in primary rhesus kidney cell cultures. Continuous cell lines were maintained in medium 199 without serum. All culture media contained 100 units penicillin and 100 μg streptomycin per ml. Embryonated eggs from Rhode Island Red chickens were purchased from a commercial dealer. Guinea pigs were young adults obtained from the Animal Production Unit, Nat. Inst. of Health.

Specimens for isolation study. Between July, 1960, and December, 1962, 721 white-footed mice *(Peromyscus leucopus)* were trapped in Virginia and shipped alive in individual cages to the Dept. of Rickettsial Diseases, Walter Reed Army Institute of Research. On the day of arrival the animals were bled for serum and then sacrificed to obtain tissues for use in virus and rickettsial isolation studies. Small sections of the brain, liver and spleen (usually from 2 to 5 mice) were ground together in Snyder I diluent(1) containing 100 units of penicillin and 100 μg of streptomycin per ml to make a 20% tissue suspension. The remaining portions of the whole tissues were stored at –60°C for future use, the tissues from each mouse being stored in a separate container. After clarification at 500 rpm for 5 minutes to remove tissue debris, 0.2 ml of the suspension was inoculated into the yolk sac of each of 6 or 8 7-day-old embryonated hens' eggs, and 1 ml was injected intraperitoneally into each of 2 adult male guinea pigs.

Isolation of peromyscus agent. The suspension prepared from the tissues of 2 white-footed mice, trapped in March, 1962, was inoculated into the yolk sac of eight 7-day-old embryonated eggs and into the peritoneal cavity of 2 guinea pigs. At the time the mice were killed both were clinically well and free of gross lesions. One of the inoculated chick embryos was dead on day 8; the remaining ones were alive on day 14 and were discarded. From the dead embryo a transmissible agent was recovered which readily propagated in embryonated eggs when injected by the yolk sac, amniotic or allantoic route. Serial transmission of the agent was also easily accomplished in cultures of monkey heart cells by inoculation of infected embryonated egg materials. Neither of the guinea pigs inoculated with the original

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mouse tissue suspension developed fever or showed any signs of illness over a 28-day-period, when they were discarded.

Serologic procedures. Seed virus. The supernatant fluid obtained on day 7 or 8, from monkey heart cell cultures infected with egg propagated virus after centrifugation at low speed to remove tissue debris, constituted the seed virus. This material, stored in convenient amounts at –60°C, had an infectious titer of $10^{-5.5}$ in tube cultures of monkey heart cells, using 0.2 ml as inoculum.

Hemadsorption and hemadsorption inhibition tests. Presence of hemadsorption was first detected in infected tube cultures of monkey heart cells when the nutrient fluid was replaced on post infection day 3 with 1 ml of 0.4% guinea pig red blood cells in medium 199. Hemadsorption inhibition was accomplished by the procedures described by Vogel and Shelokov(2). Briefly, test serum, diluted 1:5, in 0.3 ml volumes, was mixed with an equal volume of a suspension of virus diluted to contain 48 hemadsorbing units(2). After incubation for one hour at 37°C, 0.2 ml of the mixture containing 16 hemadsorbing units was inoculated into each of 2 culture tubes of monkey heart cells. The cultures were examined for presence or absence of hemadsorption at 3 or 4 days post inoculation. Titers are expressed as the highest serum dilution completely inhibiting the hemadsorbing activity of the test virus. *Complement fixation tests.* Complement-fixing antigen was prepared from infected monkey heart cells grown in medium 199. After 14 days incubation, or 4 to 7 days after cellular destruction had progressed to completion, cells and fluids were harvested, clarified by centrifugation at 2,000 rpm for 15 minutes. and the resultant supernatant fluid was used as antigen. Uninfected monkey heart cultures. treated in the same manner, provided control material. The complement-fixing procedure employed was the standard one used in Army laboratories(3) except that animal sera were inactivated for 30 minutes at 60°C instead of at 56°C. Satisfactory complement fixing antigen was also prepared from the chorioallantoic membranes of embryonated eggs which were harvested on days 4 or 5 after infection by the allantoic route with peromyscus virus of either egg or monkey heart cell origin.

Preparation of antiserum. Antiserum was prepared in young adult guinea pigs by intranasal inoculation of 10^6 $TCID_{50}$ of virus of egg origin. A booster dose of 10^5 $TCID_{50}$ of virus was given by the intraperitoneal route at 21 days, and 7 days later the animals were bled for serum.

Results. Cytopathologic and hemadsorption properties. Inoculation of the peromyscus virus into continuous cultures of rhesus monkey heart cells produced visible changes in 48 hours. The initial change was the appearance of a few to many discrete syncitia depending upon number of infectious particles in the inoculum. With passage of time, the syncitia increased in size and appeared as darkened areas against a background of refractive normal cells. Terminal changes were evident on days 7 to 10 when the infected cells had become detached from the tube wall, and only islands of cells remained attached. Serial passage of the peromyscus virus was also possible in a number of other continuous cell lines of human (embryonic lung, amnion, liver, HEp 2, HeLa) and rhesus monkey (kidney) origin. In all of these lines the cytopathic effect induced by peromyscus virus was similar to that observed in infected monkey heart cells. In primary rhesus kidney cells, however, the cytopathic change was markedly different. In these cells the characteristic changes induced by the peromyscus agent were ballooning and intense vacuolation. These changes were first detectable on post inoculation day 2 or 3 and progressed until day 9 or 10 when cellular degeneration had progressed to completion.

Both continuous and primary cells infected with peromyscus virus showed marked adsorption of guinea pig red blood cells. Hemadsorption patterns observed in primary cell cultures were, however, quite different from that seen in cultures of continuous cells. In infected primary rhesus monkey kidney cell cultures a diffuse pattern of adsorbed erythrocytes was seen which spread more or less

TABLE I. Effect of Temperature on Peromyscus Virus in 199 Cell Culture Medium.

Temp (°C)	Virus titer (log_{10}) at indicated time (hr) 0	½	2	6	24
4	5.5	5.0	5.5	5.5	4.0
22–24	5.5	5.5	5.0	4.0	3.0
37	5.5	5.0	4.5	4.0	2.0
56	5.5	2.5	1.0	1.0	<1.0
60	5 5	<1.0	<1.0	<1.0	<1.0

uniformly over the entire cell sheet, whereas in the continuous lines adsorption appeared to occur only on the syncitia; cells which maintained their morphologic integrity were free of adsorbed red blood cells.

Pathogenicity studies. Peromyscus virus of tissue culture origin was inoculated by the intraperitoneal route (10^5 $TCID_{50}$ contained in 0.03 ml) or intracerebral route ($10^{4.8}$ $TCID_{50}$ in 0.02 ml) into suckling and into weanling animals of the following species: Swiss mice, a laboratory strain of white-footed mice and Syrian hamsters. None of the weanling animals developed signs of overt illness in 3 weeks post inoculation. After intraperitoneal injection, suckling hamsters, and after intracerebral inoculation, suckling hamsters, suckling Swiss mice, and baby white-footed mice developed infections which terminated in death on days 7 to 12. Guinea pigs inoculated either intraperitoneally or intranasally for production of peromyscus virus antiserum failed to show any evidence of illness. However, relatively high titers of hemadsorption-inhibiting and complement fixing antibodies which were attained in the inoculated guinea pigs might be taken as indications that these animals had experienced infection.

Effect of ether and temperature. Approximately $10^{5.0}$ $TCID_{50}$ of peromyscus virus were completely inactivated by 20% ethyl ether after 18 hours in a 4°C refrigerator. However, the agent was relatively resistant to thermal inactivation (Table I).

Serologic reactions of peromyscus virus and hyperimmune animal sera. Attempts to identify the peromyscus virus or to relate it to a number of known viruses were made by hemadsorption inhibition and complement fixation tests. The hemadsorbing property of peromyscus virus was not inhibited by hyperimmune animal sera made against any of the viruses listed in Table II. Furthermore, complement was not fixed by hyperimmune guinea pig serum prepared against peromyscus virus (homologous titer 1:256) in the presence of any of the viral antigens listed in Table III. It thus appears that peromyscus virus is serologically distinct from any of the 10 myxoviruses listed in Tables II and III and also distinct from any of the other viruses with which it was compared.

TABLE II. Hyperimmune Animal Sera Which Failed to Inhibit Hemadsorption in Cell Cultures Infected with Peromyscus Virus.

Influenza A_1	Mumps
" A_2	Simian virus 5
" B	Newcastle disease
	Simian virus 40
Parainfluenza 1 (Sendai)	Polyoma
" 2	Lymphocytic
" 3	choriomeningitis
" 4	

Occurrence of antibody in human and animal sera. In hemadsorption inhibition tests performed with peromyscus virus and paired human sera obtained from patients with clinically diagnosed infectious hepatitis (31 cases) and from serologically proved cases of influenza type A2, 2 cases; type A1, 2 cases; type B, 3 cases; parainfluenza (type 1, 4 cases; types 2, 3 and 4, 1 case each) and adenovirus

TABLE III. Viral Antigens Which Failed to Fix Complement in Presence of Peromyscus Virus Hyperimmune Guinea Pig Serum.*

Influenza A_1	Coxsackie B 1
" A_2	" 3
" B	" 5
Parainfluenza 1 (Sendai)	Reo 3
" 2	Polio 1
" 3	" 2
" 4	" 3
Mumps	Vaccinia
Simian virus 5	Mouse hepatitis (Manaker)
" " 40	K virus (Kilham)
Newcastle disease	Respiratory syncitial (CCA)
Echo 4	Herpes virus hominis
" 9	Adenovirus
" 16	Polyoma
" 20	Psittacosis
Coxsackie A 4	Pneumonia virus of mice
" 8	Lymphocytic choriomeningitis
" 10	
" 24	

* Homologous titer: 1:256.

TABLE IV. Peromyscus Virus Hemadsorption-Inhibiting Antibody in Persons of Different Ages.*

Age group (yr)	Number Tested	Number Positive	Reciprocal of hemadsorption-inhibiting antibody titer
½ – 2	12	0	
3 – 6	14	0	
7 – 9	13	4	20, 10, 10, 10†
10 – 14	16	3	160, 20, 20
15 – 18	7	1	160
>18	12	1	20

* Sera from patients submitted to the Dept. of Virus Diseases (WRAIR) for diagnostic studies. Obtained through courtesy of Dr. E. L. Buescher.

† The 9 patients with peromyscus virus antibody in their sera had the following clinically diagnosed illnesses: aseptic meningitis (3 cases), Rocky Mountain spotted fever (2 cases), mumps, allergic dermititis, erythema multiforme and respiratory illness (1 case each).

infection (types 3, 4 and 7, 1 case each) all failed to show antibody rises to peromyscus virus. It was of interest, however, that sera from 11 (4 hepatitis, 3 influenza, 2 adenovirus, 2 parainfluenza) of the 48 individuals tested had detectable hemadsorption inhibiting antibody against peromyscus virus which ranged in titer from 1:10 to greater than 1:160. In each instance, however, the same level of antibody was present in both early and late serum specimens. Table IV presents the results of hemadsorption inhibition tests to determine the presence of peromyscus virus antibody in different age groups in man. Of the 74 sera tested, 9 contained peromyscus virus antibody. None of the 26 sera from children under 6 years of age contained detectable antibody at 1:10 dilution. However, the sera from 9 of 48 persons who were 7 years or older inhibited the activity of the virus at dilutions ranging from 1:10 to 1:160.

Table V presents the results of hemadsorption inhibition tests performed with peromyscus virus and sera obtained from 11 species of wild animals which were trapped in Virginia in 1960-62 and from 10 strains of laboratory mice obtained from the Animal Production Unit, NIH. All sera were negative for presence of peromyscus virus antibody except 5 of 8 pools of white-footed mouse sera. Each of the 8 pools examined was composed of sera from 4 to 8 animals and represented a total of 45 mice.

Reisolation of peromyscus virus. Several months after the original isolation of the peromyscus virus, tissues of the 2 white-footed mice from which the agent had been recovered were thawed and used to prepare 2 separate suspensions, each containing the tissue of only one mouse. From one of the suspensions, a transmissible agent was recovered which was identified in cross hemadsorption-inhibition and complement fixation tests as an agent indistinguishable from peromyscus virus. No virus was recovered from the other white-footed mouse tissue pool. It may be added here that peromyscus virus was not recovered from any of 207 other tissue pools prepared from materials obtained from 719 white-footed mice examined during this study.

Discussion. It appears that the agent recovered from the pooled tissues of 2 white-footed mice represents a new hemadsorbing virus. The new virus induces cytopathogenic changes in a variety of cultured cells; produces fatal diseases in suckling mice when inoculated intracerebrally and in hamsters

TABLE V. Peromyscus Virus Hemadsorption-inhibiting Antibody in Sera of Wild Animals and in Laboratory Mice.

Species	Tested	Positive	Reciprocal of HAdI titer
Mouse, white-footed (*Peromyscus leucopus*) pool*	8	5	160, 40, 40, 20, 20
Deer	10	0	
Fox, gray	10	0	
" red	10	0	
Marmot	4	0	
Mouse, field (*Microtus*) pool†	7	0	
Mouse, laboratory‡	100	0	
Opossum	10	0	
Rabbit, cottontail	10	0	
Racoon	10	0	
Rat, cotton	3	0	
Squirrel	3	0	

* Each white-footed mouse pool contained sera obtained from 4-8 animals.

† Each field mouse pool contained sera obtained from 2-6 animals.

‡ Serum obtained from 10 mice of each of 10 different strains (GP, CFW, A/LN, Balb/c, C_3H, C57, CAF_1, CDF, DBA, AKR). All strains were obtained from the Animal Production Unit, NIH, Bethesda, Md.

when inoculated either intracerebrally or intraperitoneally; hemadsorbs guinea pig erythrocytes and is inactivated by ether. Although these properties suggest inclusion of the peromyscus agent in the myxovirus group, no antigenic relationship was demonstrated in cross hemadsorption inhibition and complement fixation tests with any of 10 myxoviruses or with any of a number of other viruses with which the new agent was compared.

The occurrence of antibody against peromyscus virus in the sera of white-footed mice and its absence in the sera of any of a number of other wild animals or in the sera of any of 10 strains of laboratory mice indicate that the natural host range of this virus is limited. It is of special interest that antibody directed against peromyscus virus was present in the sera of 20 of 122 human beings examined in this study. This finding suggests that peromyscus virus or a serologically related agent infects man. However, the possible relationship of peromyscus virus to disease in either white-footed mice or man remains to be determined.

Summary. A transmissible agent with biological and physical properties similar to those of viruses in the myxovirus group was recovered from the pooled tissues of 2 white-footed mice (*Peromyscus leucopus*) trapped in Virginia in March, 1962. The recovered agent produced degenerative changes in a variety of primary and continuous cell cultures, induced fatal infections in suckling mice when inoculated intracerebrally and hamsters when inoculated intracerebrally or intraperitoneally. Five of 8 white-footed mouse serum pools contained hemadsorption inhibiting antibody directed against the new agent in titers ranging from 1:20 to 1:160 but sera obtained from laboratory mice and from wild animals other than *Peromyscus* did not. A number of human beings were found to have hemadsorption inhibiting antibody against the new virus in their sera. This observation is taken as evidence that these individuals had experienced infection with the new agent or one closely related to it.

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Isolation of Three Unclassified Enteroviruses from Patients Suffering from Dysentery.* (28347)

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During a study of the gastro-intestinal viral flora of cases of proven or presumed bacillary dysentery, 122 stool specimens from Mexico City, D. F., Mexico, was received through the kindness of Dr. Jorge Olarte, of the Hospital Infantil de Mexico. These specimens were derived from children hospitalized because of diarrheal disease proven or presumed to be bacillary dysentery, and were collected during the acute phase of the disease. Among the numerous viral agents isolated were 3 which, although they appeared to have the characteristics of members of the enterovirus group, bore no demonstrable serological relationship to any of the presently recognized prototypes. This paper describes the more important properties of these 3 agents and discusses the possibility that one or more of them may represent new human enterovirus prototypes.

Materials and methods. The sources of

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