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Isolation and Characterization of a Neurotropic Agent (MHG Virus) From Adult Rats. (28914)

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A transmissible agent has been recovered from laboratory white rats, which by its host range, physical and chemical properties, appears to be distinct from any previously described agent. Preliminary investigations suggest that infection with this agent or an antigenically related agent occurs in human beings.

The isolation was made in the autumn of 1961, from 3 adult white rats of the Sprague-Dawley strain. The rats showed signs indicative of a central nervous system involvement, including circling, incoordination, tremors and torticollis. The animals were not visibly disturbed by sudden noises or when spun by the tail. No gross lesions were observed upon necropsy, and bacteriological examinations of the middle ear canal, cerebrospinal fluid and brain tissues were negative. No significant findings were obtained from parasitological and hematological examinations.

At necropsy, specimens of brain, lung, liver and intestines were collected for biological examination and prepared as 20% tissue suspensions. These suspensions were free of bacterial, leptospiral and pleuropneumonia-like organisms, on cultural examinations.

This colony of rats had been under close supervision for several months and no additions had been made prior to the outbreak of the disease. Information on the isolation of the agent, designated as MHG virus, some of its physical-chemical properties and preliminary serological data are presented here.

Materials and methods. Cell cultures, embryonated eggs and animals. Primary kidney cell cultures of mouse, pig, rat, cow, rabbit, horse, hamster, rhesus monkey and primary cell cultures of whole mouse embryo and whole rat embryo were prepared from trypsinized cell suspensions and were grown in Hanks' balanced salt solution, containing 10% calf serum and 0.5% lactalbumin hydrolysate. Primary cell cultures and other cell lines (L929, human embryonic diploid brain, HeLa and AV-3) were maintained in Eagle's minimum essential medium containing 3% calf serum, 100 units penicillin and 100 μg streptomycin per ml. Cortisone-treated cells were fed with a maintenance medium containing 100 μg hydrocortisone sodium succinate per ml, 24 hours prior to use. This medium was decanted prior to addition of viral inoculum.

The Walter Reed Bagg Swiss strain of mice, the Sprague-Dawley strain of rats, rabbits and hamsters were obtained from the Dept. of Laboratory Animals, Walter Reed Army Inst. of Research. Embryonated eggs were purchased from a commercial source.

Animal tissues were prepared as 20% suspensions in Hanks' balanced salt solution containing 1.5% phenol red base broth, 0.5% lactalbumin hydrolysate (PBLH), 1,000 units of penicillin and 1,000 μg streptomycin. The suspensions were centrifuged at 4,000 rpm for 30 minutes. The supernatant fluid was collected and stored at —70°C until used.

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TABLE I. Example of "Box" Titration of MHG Complement-Fixing Antigen.

Suckling rat brain antigen dilutions (20% suspension)	1:8	1:16	1:32	1:64	1:128	1:256	Anticomplementary control (saline)
1:2	4*	4	4	3	0	0	0
1:4	4	4	4	1	0	0	0
1:8	4	3	±	0	0	0	0
1:16	1	0	0	0	0	0	0

* 4 = 4 plus fixation.

Heat, pH and chemical stability. The method for determining pH stability has been described by Ketler(1). To determine heat stability, vials containing 20% suckling rat brain (SRB) suspensions of virus were held at 50°C for one-half hour, 4°C for 24 hours and 37°C for 2 hours. Tests for ether resistance were according to Andrews and Horstmann(2) and employed absolute diethyl ether. Chloroform resistance was measured by the method of Feldman and Wang(3). Sodium desoxycholate sensitivity was determined according to Sunaga *et al*(4). Infectivity was evaluated in suckling mice and/or suckling rats.

Preparation of antiserum and antigen. Successful production of antisera was accomplished by injecting intraperitoneally (IP) 0.5 ml (rats) and 0.1 ml (mice) of 20% brain suspension weekly for 7 weeks, into survivors of intracranial (IC) challenge. The animals were exsanguinated 7 to 10 days after the last injection. Sera from animals that were test bled after only 5 injections had very low complement-fixing (CF) antibody titers, and were not considered to be satisfactory for our purpose.

High titered MHG seed virus and CF antigen were obtained only when animal tissues were collected from suckling mice and rats showing early symptoms of disease, and when a few litter mates had already died. Satisfactory CF antigen was prepared from suckling rat and suckling mouse brains. Twenty per cent suspensions were made in PBLH, centrifuged at 4,000 rpm and stored at —70°C until used. Control CF antigen was prepared from normal suckling rat brains. The complement-fixing test employed was the standard laboratory procedure(5).

Poliovirus antigens (Types I, II and III) prepared in monkey (rhesus) kidney cell cultures, were obtained from Dr. Francis C. Cadigan, Jr., Virus Dept., Walter Reed Army Inst. of Research. Suckling mouse brain Theiler's GD $\overline{\text{VII}}$ antigen was obtained from Dr. Wallace P. Rowe and Dr. Janet W. Hartley, Nat. Inst. of Health, Bethesda, Md. Suckling rat brain Theiler's GD $\overline{\text{VII}}$ antigen was prepared in our laboratory.

All viruses were verified for identity and purity using their respective antisera. Suckling rat brain and suckling mouse brain antigens were standardized by a "checkerborad" or "block" titration. An example of a block titration of MHG complement-fixation antigen is shown in Table I.

Mouse hyperimmune sera for Theiler's GD $\overline{\text{VII}}$ virus were obtained from Dr. Janet W. Hartley and Dr. Wallace P. Rowe, NIH, Bethesda, Md. Type specific goat hyperimmune sera for poliovirus were obtained from Dr. Paul Gerber, NIH, Bethesda, Md. All other diagnostic sera were obtained from Dr. Francis C. Cadigan, Jr., WRAIR.

Serum neutralization tests were done using 2-fold serum dilutions vs 100 suckling rat IC LD_{50}.

Gradacol filtration. Particle size was determined by using gradacol membranes with an average pore diameter of 60, 75, 110, 160 and 420 mμ. The use and technique of gradacol filtration has been described by Elford (6) and Black(7).

Results. A transmissible agent was readily isolated in suckling mice from brain, lung and intestinal tissues of each of 3 diseased rats tested. However, in suckling rats, the agent was successfully isolated from intestinal material only. No isolations were made from inoculated weanling mice, adult mice, wean-

ling rats, adult rats, suckling guinea pigs or in the rabbit. Pools of virus material were made from brain suspensions of experimentally infected suckling mice and suckling rats, and stored at —70°C for further study.

The MHG agent was found to be infective for one- to two-day-old suckling mice when administered either IC, IP, intranasally or orally. Suckling rats could be infected by the IC route of inoculation only. Suckling hamsters could not be infected by the IC or IP route of inoculation, nor did subcutaneous inoculation of the agent result in tumor formation during the 6 month period the hamsters were observed.

The characteristic signs of disease in suckling rats and suckling mice were paralysis of one or more limbs, disorientation, ruffled fur, arching of the back and emaciation. Depending upon the exposure dose, the incubation period varied from 7 to 9 days. The mortality rate in animals given undiluted virus suspensions was usually 100%. The infectivity titer of 0.01 ml of a 20% tissue suspension ranged from 10^3 to 10^4. Permanent paralysis was common in animals which survived the infection. Paralyzed mice remained sensitive to external stimuli, an indication that sensory nerves were not affected.

On necropsy, hydrocephalus and edema of the brain were frequently seen. Lesions similar to those found in poliomyelitis, *e.g.*, destruction of neurons in the gray matter of the cord or brain stem, were revealed by microscopic examination of tissue sections. Necrosis of neurons, neuronophagia and gliosis were the most constant and striking findings. Perivascular cuffing and meningeal infiltration by cells, although present, were rather minimal. No inclusion bodies could be demonstrated.

The agent has been passed serially 5 times in suckling mice and suckling rats. An increase in virulence has been noted with successive passages. The LD_{50} in suckling mice inoculated IC with 10-fold dilutions of first passage brain suspensions was found to be $10^{2.4}$ per 0.1 ml, whereas the LD_{50} in suckling mice inoculated IC with 10-fold dilutions of fifth passage brain suspension was found to be $10^{4.9}$ per 0.1 ml. The agent can multiply in cell cultures of L929 and mouse kidney cell lines and in an untreated human embryonic diploid brain cell line. Virus replication in tissue culture was verified by IC challenge of suckling rats. Cytopathogenic changes observed in cortisone-treated mouse kidney tissue culture were not seen in the untreated cell sheets although tissue fluids from both cell systems were infective for suckling rats. Viral growth could not be established in cell cultures prepared from kidneys of pig, cow, rabbit, rhesus monkey, rat, hamster, or horse, nor in cultures of mouse embryo, HeLa or AV-3 cell lines. Neither lesions nor death were produced in chick embryos inoculated with the isolate.

The agent measures approximately 17 to 27 mμ in diameter, as determined by gradacol membrane filtration, and is resistant to ether, chloroform and sodium desoxycholate. It is stable at a pH of 3.0 when held for 4 hours at room temperature. There was no appreciable loss in titer when the tissue suspensions were held for 24 hours at 4°C or for 2 hours at 37°C. MHG virus is inactivated after incubation at 50° for 30 minutes.

Antigen prepared from the brain of infected suckling mice, according to the method described by Morris(8) did not agglutinate human type O red blood cells, nor did the suckling mouse brain antigen agglutinate guinea pig erythrocytes, prepared by the method of Olitsky and Yager(9). The virus did not have hemagglutinating (HA) properties when tested with sheep, goose, rat and rooster erythrocytes at a pH range of 6.0 to 7.4.

Attempts to produce MHG hyperimmune sera in rabbits and guinea pigs were unsuccessful. However, by repeated IP challenge, hyperimmune sera were produced in suckling rats and suckling mice, which had survived an initial IC challenge with the virus. The immune sera from rats had neutralization antibody (NA) titers of 1:32 vs 100 rat LD_{50}. Detectible neutralizing antibody titers were not elicited in sera from hyperimmunized mice. Complement-fixing antibody titers of 1:32 and 1:64 were obtained in mouse and rat anti-MHG sera, respectively. Litters of suckling rats borne by hyperimmunized moth-

TABLE II. Serological Comparison Between Theiler's GD VII and MHG Virus by the Complement-Fixation Test.

Antigen	Hyperimmune sera C-F titer: Mouse anti-GD VII	Rat anti-MHG	Mouse anti-MHG	Normal rat§	Normal mouse	AC control saline
GD VII–SMB*	64	4	0‡	0	0	0
GD VII–SRB†	64	4	0	0	0	0
MHG–SRB	8	64	32	4	0	0
MHG–SMB	8	64	64	16	0	0
Normal–SMB	0	0	0	0	0	0
Normal–SRB	0	0	0	0	0	0

* SMB = Suckling mouse brain. † SRB = Suckling rat brain. ‡ 0 = <1:4.
§ Represents findings in each of 4 pools screened.

ers were refractory to IC challenge with MHG virus, suggesting the presence of passive immunity.

Results of serological comparison by complement-fixing tests between Theiler's GD VII virus and MHG are shown in Table II. Pools of normal adult rat sera which had been collected at intervals during the past several years and stored at —30°C, showed a low level CF antibody titer to both SRB and suckling mouse brain (SMB) MHG antigen. Although there was some evidence of cross reactivity between GD VII and MHG, this occurred only at 1/8 to 1/16 homologous titer.

Additional comparative complement-fixing data, utilizing other diagnostic sera, are shown in Table III. The CF titer seen with rabbit ECHO 10 (reovirus) antiserum may possibly indicate an antigenic relationship; however, this pool of serum did not neutralize the MHG virus. The CF antigenic reaction seen with the goat hyperimmune antipoliovirus serum was also found with normal goat serum. Sera from 50 adult human beings were positive for CF antibodies at titers of 1:8 and 1:16, while sera from 9 infants were negative at 1:2. These sera did not react with the antigen prepared from normal suckling rat brains.

High complement-fixing titers were also encountered with sera from adult human beings known to be positive for lymphocytic choriomeningitis and psittacosis. Typical serological findings with the respective agents are shown in Table IV. These reactions parallel findings obtained with sera from adult human beings. A comparison of these results with those obtained with homologous sera (Table II) suggests that the antibody demonstrated in human sera is indicative of previous exposure to the MHG virus or one antigenically related to it. Serological studies are in progress to clarify further the data obtained.

The MHG virus could not be neutralized with antisera of the following agents: Monkey B, Venezuelan Equine Encephalomyelitis, Western Equine Encephalomyelitis, Coxsackie A-1, A-2, A-3, A-9, B-1, B-2, B-3, B-4, B-5 and B-6, 747 hepatoencephalitis virus, Nelson agent reovirus (ECHO 10) and poliovirus

TABLE III. Complement-Fixation Tests with Diagnostic Sera from Various Species and MHG Virus.*

Antiserum	Homologous C-F titer†	MHG titer†
Rat MHG	1:64	1:64
Guinea pig lymphocytic choriomeningitis	1:128	0
Guinea pig parainfluenza I	1:512	0
" " " II	1:128	0
" " " III	1:512	0
" " respiratory syncitial	1:256	0
Monkey measles	1:64	0
Rabbit ECHO 10 (Reovirus)	U	1:16‡
Goat poliovirus Type I	U	1:16‡§
" " " II	U	1:32‡§
" " " III	U	1:32‡§

* MHG antigen—20% SRB dilution 1:2.
† Reciprocal of highest dilution of serum giving a 2 plus or better fixation; 0—No fixation in 1:2 dilution of serum.
‡ These sera failed to show any neutralizing antibodies against MHG virus.
§ Titers of this magnitude were also seen with normal goat sera.
U = Unknown.

TABLE IV. Example of a "Box" Titration of MHG Antigen and Human Diagnostic Sera.*

Antigen dilutions	Lymphocytic choriomeningitis serum dilutions					Psittacosis-LGV serum dilutions				
	1:8	1:16	1:32	1:64	1:128	1:8	1:16	1:32	1:64	1:128
1:2	4†	4	4	3	±	4	4	4	2	±
1:4	2	2	0	0	0	3	2	1	0	0
1:8	0	0	0	0	0	±	±	0	0	0

* Diagnostic sera are pools of adult human sera from convalescent patients.
† 4 = 4 plus fixation.

Types I, II and III (rabbit origin). Serum neutralization tests using hyperimmune rat anti-MHG serum versus poliovirus Types I, II and III, and Theiler's GD VII virus were negative.

Discussion. Although several agents are known to infect the rat, *e.g.*, Kilham's rat virus(10), JHM-H747 virus, MHV(11), Novy rat virus(12) LCM(13), EMC(9), they differ from the MHG virus either in pathogenicity, host range or demonstrable serological reactions. It was not possible to infect adult rats with the isolated agent. However, all rats tested were from the Walter Reed animal colony. Histopathologically, the picture seen with MHG virus resembles that of Theiler's virus(14). However, the MHG virus differed from the Theiler's group (a) by being HA negative, (b) by comparative CF tests (c) by cross-neutralization tests, and (d) by lack of infectivity for weanling mice.

The failure of this agent to produce myositis in infected mice and the lack of CPE production when inoculated onto monkey kidney or HeLa cells as well as the negative serological results of the Coxsackie sera tested serve to exclude this agent as a member of the Coxsackie group. Based on CF results, there is an indication that while the MHG virus may possess some antigens in common with members of Theiler's group, it does not meet all the qualifications for inclusion in this group of viruses as presently classified. It would appear from the characteristics of the virus that the agent belongs in the picornavirus enterovirus subgroup(15).

It is noteworthy that this agent was isolated during a single disease episode involving very few animals of an extensive rat colony. However, significant antibody titers to the agent were found in pools of rat sera obtained from colony rats during the past few years and kept stored at —35°C. These preliminary findings support the viewpoint that this agent may be a common pathogen for rats, but more definitive data in this regard is wanting. Findings of CF cross reactions with adult human sera were particularly interesting. To elucidate these findings, neutralization tests are now in progress. Final classification of this agent and its relationship to disease in man and animal is yet to be determined.

Summary. A transmissible agent (MHG) with biological and physical properties similar to the viruses of the enterovirus group has been isolated from pooled tissues of 3 white rats, Sprague-Dawley strain, obtained from the Walter Reed Army Institute of Research animal colony. The agent produced fatal infections in both suckling rats and suckling mice when inoculated intracranially. A high incidence of complement-fixing antibodies against the new virus was found in adult human sera. The relationship of this virus to disease in man or animal has not been ascertained.

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Prolonged Exposure to Nephritogenic Beta-Hemolytic Streptococcus in Intraperitoneal Diffusion Chambers.* (28915)

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The role of the streptococcus in the pathogenesis of glomerulonephritis has been investigated using diffusion chambers containing beta-hemolytic streptococci, nephritogenic in man(1-4). In these experiments, the chambers remained in the peritoneal cavity for 24 to 72 hours and were then removed. Preliminary experiments in this laboratory with rats and rabbits suggested that the glomerular lesions were enhanced by allowing the chamber to remain *in situ* for periods exceeding the customary 48 hours. A similar observation had previously been noted in mice surviving prolonged streptococcal infection (5). This report deals with results obtained following insertion of diffusion chambers into the peritoneal cavity of rats and rabbits for prolonged periods.

Materials and methods. Diffusion chambers were fashioned from Plexiglass sheets and Millipore paper (H.A. pore size 0.45 μ) as previously described(1). 0.5 ml of a 24-hour broth culture of nephritogenic hemolytic streptococcus type A_{12}, strain No. 35,‡ was delivered into a sterile chamber. The chamber was then inserted free into the peritoneal cavity of 20 rats and 6 rabbits where it remained until the animal was sacrificed.

Twenty-four-hour and 2-hour "fresh" rabbit urine specimens were examined daily for excess proteinuria and hematuria. Blood samples were obtained weekly from the lateral ear vein of the rabbits for estimation of urea nitrogen.

The rats were sacrificed in pairs at 2, 7, 14, 21, 28, 35 and 42 days following insertion of the diffusion chambers. The rabbits were sacrificed in pairs at 21, 28 and 35 days following insertion of the chambers.

At sacrifice the peritoneal surface was cultured immediately on exposure. Heart blood was then collected aseptically, similarly cultured, and the serum separated. The diffusion chamber was recovered and the contents cultured. All cultures were plated on blood agar, incubated at 37°C, and read after 24 hours.

The animals were carefully autopsied and sections obtained from formalin-fixed kidney, heart, lung, liver, spleen, and lymph nodes were stained with hematoxylin and eosin. In addition, appropriate kidney sections were stained by the periodic-acid Schiff technique. Blocks of all rat kidneys were quick frozen and stored at —20°C until used. The sera of rats obtained at sacrifice were tested for rat kidney-localizing activity using the indirect "sandwich" fluorescent technique on both normal and experimental rat kidneys.

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