# Agents Resembling Enteroviruses Isolated in HeLa Cell Cultures from Fecal Specimens.* (24470)

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From an outbreak of respiratory illness occurring at a convalescent home for children in Washington, D. C. during summer of 1956, cytopathogenic agents with some characteristics of enteroviruses(1) were isolated in HeLa cell tissue cultures from fecal specimens. These agents did not produce cytopathic changes in monkey kidney cell cultures and also lacked pathogenicity for suckling mice and other laboratory animals. This report deals with isolation, and biologic characterization of these agents.

*Materials and methods.* Specimens: Pharyngeal, conjunctival, nasal, and anal swabs as well as stools were collected from patients and prepared as previously reported(2). All specimens were kept frozen at –20°C until used. Portions of specimens were inoculated into HeLa and rhesus monkey kidney cell cultures‡ and into 3-day-old Swiss mice. Culture media. Monkey kidney cultures were maintained in medium consisting of 98 parts Medium 199(3) and 2 parts inactivated (56°C for 30 minutes) calf serum. Maintenance medium for HeLa cell cultures consisted of 70 parts Medium 199, 25 parts tryptose phosphate broth, and 5 parts inactivated chicken serum. Maintenance medium for human embryonic fibroblasts (MAF line) was composed of 97 parts Medium 199 and 3 parts inactivated horse serum. All media contained penicillin, streptomycin, and nystatin in final concentration of 250 U, 250 μg, and 125 U/ml respectively. Stock virus pools. One-tenth ml of undiluted fluid harvested from positive tissue cultures was inoculated into tube cultures of HeLa cells. When maximal degeneration occurred the tubes were frozen at –20°C, thawed slowly, and the fluids pooled and centrifuged at 1500 rpm for 20 minutes. The supernatant fluid was decanted and aliquots stored at –50°C in screw-capped vials. Neutralization tests. Two-fold dilutions of serum (inactivated at 56°C for 30 minutes) were incubated for 2 hours at 37°C with equal volume of stock virus diluted to contain 50-100 $TCD_{50}$/inoculum. Two-tenths ml of serum-virus mixture was then inoculated into each of 2 MAF cell cultures. Final readings were made on fifth day after inoculation which was 48 to 72 hours after virus controls developed 2 to 4 plus cytopathic effects (CPE). Complete inhibition of CPE was considered evidence of neutralization. Antiserums to several strains of isolates were prepared in rabbits by intravenous injections. Seven inoculations of 1.5 ml of a stock virus pool known to contain at least $10^5$ $TCD_{50}$/ml were administered within the first 3 weeks. Two booster inoculations were given; the first, at least one month after fifth inoculation and the second, one month later. Serum was collected by cardiac puncture one week after last inoculation.

*Results.* Agents were recovered in HeLa cell cultures inoculated with fecal specimens obtained from 8 of 10 patients. Within 48 hours of specimen inoculation, there was evidence of cellular destruction. CPE and clinical disease were absent respectively in monkey kidney cultures and infant mice. Attempts in these patients to recover virus from specimens other than fecal specimens were consistently negative.

The CPE in HeLa cells could not be distinguished from that produced by poliovirus. One-tenth ml of undiluted stock virus pools produced complete degeneration within 24 hours in HeLa cells, and after inoculation of 0.5 ml there was usually one plus typical CPE within 8 hours. Limiting dilutions usually produced CPE within 72 hours. Titrations of stock virus pools in HeLa cells yielded titers

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‡ Cell cultures obtained from Microbiological Associates, Bethesda, Md.

of $10^{5.5}$ to $10^{7.5}$ $TCD_{50}$ ml. The prototype virus (Pett. strain) was carried through 23 passages in HeLa cells.

MAF cultures were less sensitive for detection of virus, and with some strains showed slow progression of CPE. After several passages in MAF cells, the viruses produced rapidly progressing CPE, and this tissue could then be used for neutralization tests.

To define cell host pattern of virus isolates, 0.2 ml of stock virus pools titering $10^{5.5}$ to $10^{7.5}$ $TCD_{50}$ ml were inoculated into tube cultures of amnion, liver, intestine, conjunctiva, carcinoma (KB strain), heart and fibroblasts of human origin and into heart(4) and kidney cultures of simian origin. For these experiments the medium used for maintenance of HeLa cells was used. CPE was observed in all cell lines with the exception of monkey renal epithelium. Repeated attempts with varied amounts of inocula and progressive passage harvests of positive HeLa cultures consistently failed to provoke CPE in rhesus monkey kidney cultures. A single trial with cynomolgus monkey kidney cultures also failed to induce CPE.

No detectable disease was produced when portions of stock virus pools were inoculated by different routes into one and 3-day-old mice, adult mice, 3-day-old Syrian hamsters, and in adult guinea pigs and rabbits. From the 3-day-old mice, which were inoculated intracerebrally (i.c.) and intraperitoneally (i.p.), 2 mice were killed on fifth day after injection; 10% carcass suspensions were prepared and portions inoculated i.c. and i.p. into other 3-day-old mice. Four serial blind passages were done by this method. Mice were observed for 21 days, and none developed detectable disease. Carcass suspensions at each passage were inoculated into HeLa cells and no virus was recovered. Isolates from 5 patients were tested in this manner.

Undiluted stock virus pools were inoculated amniotically and allantoically respectively into 7- and 10-day-old embryonated hen's eggs. Fluids were harvested individually and tested for presence of hemagglutinins with human Group O and chicken erythrocytes. Fluids from each group were then pooled and passaged to HeLa cells and another set of eggs. Three and 7 serial transfers were made respectively by allantoic and amniotic routes. Of 6 isolates tested by the allantoic method and 4 isolates tested by the amniotic method, no hemagglutination was obtained and no virus was recovered in HeLa cells at any egg passage level. Also no gross lesions were seen in embryos or membranes.

No loss of virus activity was observed after treatment of virus isolates with diethyl ether in a 20% final concentration for 18 hours at 4°C. Infectivity of virus isolates was destroyed at 65°C for 30 minutes. Moreover, a significant loss of titer was noted when isolates were heated at 56°C and 62°C for 30 minutes. The agents tolerated a minimum of at least 15 cycles of freezing and thawing without any apparent loss of titer and remained viable at 4°C for at least 30 days. The agents were recovered from the original fecal specimens after storage for 2 years at -20°C. The virus passed through a Gradocol membrane of apparent pore diameter 55 mmu.

Eight stock virus pools were tested for hemagglutination activity with human type O red cells. Incubation temperatures were varied at 22°C, 37°C, and 4°C and the diluent was adjusted to pH's of 5, 6, 7, and 8. Guinea pig, less-than-3-day-old and adult chicken erythrocytes were also tested at pH 7. Each virus strain was tested more than once, and in no instance was the presence of viral hemagglutinins detected.

Initial attempts to demonstrate neutralization of even 3 $TCD_{50}$ in HeLa cultures by rabbit antiserum and human convalescent serums were unsuccessful, or at most showed only slight delay of CPE. Satisfactory neutralization tests were obtained when the tests were performed in MAF cells with pools of MAF adapted virus. Eight isolates were neutralized by 3 rabbit antiserums prepared from different strains. Also paired serums from 5 patients showed significant antibody rises against the prototype virus.

To determine serologic distinctness of the Pett. virus from known human enteroviruses, rabbit antiserum to the Pett. virus, having homologous neutralizing antibody titer of 1:160, was tested at dilution of 1:10 or 1:20 against 50-100 $TCD_{50}$ or suckling mouse $LD_{50}$

of each of the following: Coxsackie, ECHO, and poliovirus. Neutralization tests with Coxsackie Group A types 1-8, 10, 12, 14, 16, 17, and 19 were done in suckling mice, and tests with Coxsackie Group A types 9, 11, 13, 15, and 18, Coxsackie Group B types 1-5, ECHO types 1-20, and poliovirus types 1-3 were done in MAF or monkey kidney cultures. In no instance was neutralization observed.

*Discussion.* The properties of these agents in regard to size, ether resistance, temperature stability, CPE, recovery from fecal specimens, and isolation during summer would place them in the Enterovirus Group. The failure of these agents to produce CPE in monkey kidney cells and disease in laboratory animals suggested that these agents were different from enteric viruses currently reported. The inability of a prototype rabbit antiserum prepared from one of these isolates to neutralize known members of the enteroviruses added further evidence that they are not antigenically related.

Neutralization of virus isolates by 3 different rabbit antiserums would indicate that these agents are antigenically similar. The presence of specific antibody capable of neutralizing the prototype of this group in convalescent human serums would confirm their original presence in these patients. The spectrum of clinical illness associated with these agents will be described separately.

*Summary.* Agents isolated from fecal specimens in HeLa cell cultures possessed properties similar to enteric viruses. Serologic evidence indicated that these agents were not immunologically related to known members of the Enterovirus Group.

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## Blocking Urinary Electrolyte Effects of Desoxycorticosterone with Progesterone in Rats.* (24471)

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Progesterone causes an increased urinary excretion of Na in Addisonian patients treated with desoxycorticosterone (DOC) possibly by competition between the 2 steroids in the kidney; progesterone was not effective in the absence of DOC treatment(1). The present report on electrolyte effects of progesterone in rats supports these findings and shows, in addition, that DOC-like property of the steroid limits blocking efficiency at large doses.

*Method.* Male Sprague-Dawley rats (150-200 g) were adrenalectomized and maintained with sucrose cubes and tap water overnight. Approximately 24 hours post-operatively, the animals were subcutaneously injected with 2.5 ml 0.85% NaCl solution in shoulder region, and with desoxycorticosterone acetate (DCA) in 0.1 ml Mazola oil. Other animals also received subcutaneous injection of progesterone in 0.5 ml oil. A 4-hour sample of urine was collected in metabolism cages and analyzed for Na and K concentration after dilution to 25 ml with water. Mean values of Na/K ratio were determined after analyses with Beckman flame photometer, using individual or pooled samples of urine. In this method, 12 to 48 μg of DCA cause Na reten-

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