Identification of Reovirus Type 2 in Cell Cultures Inoculated with Hepatitis Sera (33854)

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(Introduced by H. M. Meyer, Jr.)

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In 1961 Hillis reported the detection of serially transmissible cytopathic agents in primary chimpanzee kidney (CK) cells which had been inoculated with sera from two patients in the acute phase of icteric infectious hepatitis (IH) (1). One of the patients, JEC, was at Holloman Air Force Base in New Mexico; the other patient, PEL, was hospitalized at Stead Air Force Base in Nevada and had no known contact with the first patient. Disappearance of the cytopathic effect (CPE) after 4 passages in CK cells postponed further work with these agents until recently when they were found to produce CPE in primary human embryo kidney (HEK) cells (2). The present report describes the identification of these agents as strains of reovirus type 2 and presents data concerning their relationship with human hepatitis.

Experimental methods. Virus stocks were prepared for characterization of the agent from patient JEC after one passage in CK and 5 passages in HEK cells and from patient PEL after one passage in CK and 9 passages in HEK cells. Both agents produced CPE in primary vervet monkey kidney cells, in the FL strain of human amnion cells and in BS-C-1 cells, but not in primary rabbit kidney cells or primary chick embryo fibroblasts. Infected BS-C-1 cells stained by Giemsa's method contained large cytopasmic inclusions. There was surviving infectious virus after heating for 4 hr at 60° and after treatment with chloroform and ether. The agents replicated in the presence of 5-iododeoxyuridine, 50 μg/ml. They passed readily through a 220 mμ Millipore filter but not through a 100 mμ filter. Supernatant fluids from infected cells agglutinated type O Rh negative human erythrocytes but not chick, guinea pig, sheep, or rhesus monkey erythrocytes. The agents were both identified as strains of reovirus type 2 by hemagglutination-inhibition (HI) and neutralization of tissue culture infectivity with type-specific chicken antiserum (supplied by the Biological Reagents Section, National Communicable Disease Center, Atlanta, Georgia). Electron microscopic examination revealed reovirus-like particles in the stock pools of both agents.

At the time of the original detection of CPE in the CK cells inoculated with the sera from JEC and PEL, the same lot of primary CK cells was inoculated with several other materials (Table I). Sera from 2 other hepatitis cases (ARCH and HILL) and from one healthy individual (BER) and stool from a chimpanzee implicated in an outbreak of hepatitis (Y-2-0) did not produce CPE in the CK cells. Passage of the fluids from these cultures to HEK cells did not reveal any evidence of reoviruses.

An agent detected in the fluid of the CK cells inoculated with the chimpanzee stool produced CPE in HEK cells on a roller drum and in stationary WI-38 cell cultures. This agent appeared to be an enterovirus. Its infectivity was (i) filterable through a 50 mμ Millipore filter; (ii) stabilized against thermal inactivation at 50° by 1 *M* $MgCl_2$; (iii) resistant to treatment with ethyl ether and chloroform, and (iv) unaffected by growth in cell cultures maintained with iododeoxyuridine containing medium. Attempts to neutralize the infectivity of this agent with antisera against known human enteroviruses have been unsuccessful, and no neutralizing antibodies have been detected in several lots of human gamma globulin. *Mycoplasma laidlawii* was present in the fluids of PEL

TABLE I. Agents Detected after Inoculation of Chimpanzee Kidney Cells.

Specimen passage	Source	Reovirus type 2	*M. laidlawii*	*M. arginini*	Entero-virus-like agent
JEC (CK-1, HEK-6)[a]	Acute IH serum	+	+	—	—
PEL (CK-1, HEK-10)	Acute IH serum	+	+	+	—
ARCH (CK-1)	Acute (anicteric) IH serum	—	—	—	—
HILL (CK-1)	Acute IH serum	—	—	—	—
BER (CK-1)	Normal serum	—	—	+	—
Y-2-0 (CK-1)	Chimpanzee stool	—	—	—	+

[a] One chimpanzee kidney passage, six human embryo kidney passages.

(CK-1, HEK-10) and of JEC (CK-1, HEK-6); *Mycoplasma arginini*, a newly described species (3), was in the fluids of PEL and BER (CK-1).

Tests for HI antibodies against reovirus types 1, 2, and 3 were done on serial sera from 15 cases of infectious hepatitis (IH), 12 cases of serum hepatitis (SH), and 36 healthy young adult males (5). The lowest serum dilution tested was 1:20 after treatment with kaolin to remove nonspecific inhibitors. In both the hepatitis groups and the control group there were no significant changes in titer and no conversions from seronegative to seropositive during the observation period. The differences noted in incidence of antibodies between patient and control groups were not striking (Table II).

Discussion. It has not been possible to attempt reisolation of the reoviruses from the acute phase sera of JEC and PEL because the sera were exhausted in the original studies. The source of the agents must therefore remain in the realm of speculation. They may have been in the sera of the patients, in the chimpanzee kidney, or contaminants inadvertently introduced in the laboratory.

TABLE II. Hemagglutination Inhibiting (HAI) Antibodies against Reovirus Types 1, 2, and 3 in Patients with Serum Hepatitis (SH) and Infectious Hepatitis (IH) and in Normal Healthy Adult Subjects.

	SH	IH	Control[a]
Reovirus Type 1	1/12[b]	4/15	9/36
2	7/12	7/15	14/36
3	2/12	4/15	4/36

[a] Healthy males, age 18–24.

[b] No. of individuals with HAI titers $\geq$1:20/total cases (2–3 serial sera per patient).

Antibodies against these agents were detected by neutralization and immunofluorescence in serum obtained from JEC during convalescence (1). If the reoviruses were in the patients' sera, they may well have been adventitious agents, as the serologic data do not suggest an association of any of the 3 reovirus types with either IH or SH. Since antibodies against all 3 types of reoviruses are relatively common in American adults (4), only changes from negative to positive or fourfold or greater rises in antibody levels could be considered significant in a serologic evaluation of the role of reoviruses in hepatitis. Viremia was not detected in volunteers infected with reoviruses, and hepatitis has not been a reported feature of naturally occurring reovirus infections in longitudinal surveys or in experimental reovirus infections in volunteers (5–7). The possibility of an association between reoviruses and occasional cases of hepatitis in humans has been raised, however, by McKee (8, 9) and Joske and co-workers (10). The data presented here show that reoviruses are not major causes of either IH or SH.

Reovirus type 1 has been found in uninoculated chimpanzee brain tissue (11) and reoviruses have been recovered from uninoculated cell cultures from monkey kidneys (12, 13). The original experiments by Hillis were all done with cells from the kidneys of a single healthy chimpanzee (1), and the possibility that reovirus type 2 was present in the chimpanzee kidney cells cannot be excluded. Failure to detect reovirus in the CK

cells inoculated with the other materials is the major evidence against this possibility.

The enterovirus-like agent in the cells inoculated with the chimpanzee stool is probably a chimpanzee enterovirus, and the mycoplasmas are felt most likely to be tissue culture contaminants. None of these 3 agents has ever been associated with any human diseases or recovered directly from persons.

Summary. Two cytopathic agents detected in chimpanzee kidney cells which had been inoculated with sera from patients in the acute phase of viral hepatitis have been identified as strains of reovirus type 2. The source of the agents is unclear, but they are not considered to have a major etiologic role in viral hepatitis.

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1. Hillis ,W. D., Proc. Soc. Exptl. Biol. Med. **108**, 813 (1961).

2. Hillis, W. D., Johns Hopkins Med. J. **120**, 176 (1967).

3. Barile, M. F., Del Giudice, R. A., Carski, T. R., Gibbs, C. J., and Morris, J. A., Proc. Soc. Exptl. Biol. Med. **129**, 489 (1968).

4. Tillotson, J. R. and Lerner, A. M., New Engl. J. Med. **276**, 1060 (1967).

5. Rosen, L., Am. J. Hyg. **71**, 242 (1960).

6. Rosen, L., Hovis, J. F., Mastrota, F. M., Bell, J. A., and Huebner, R. J., Am. J. Hyg. **71**, 258 (1960).

7. Rosen, L., Evans, H. E., and Spickard, A., Am. J. Hyg. **77**, 29 (1963).

8. Burlingham, B. T. and McKee, A. P., Bacteriol. Proc. **1965**, 101.

9. McKee, A. P., personal communication.

10. Joske, R. A., Keall, D. D., Leak, P. J., Stanley, N. F., and Walters, M. N. I., Arch. Internal Med. **113**, 811 (1964).

11. Rogers, N. G., Basnight, M., Gibbs, C. J., and Gajdusek, D. C., Nature **216**, 446 (1967).

12. Hull, R. N., Minner, J. R., and Smith, J. W., Am. J. Hyg. **63**, 204 (1965).

13. Malherbe, H. and Harwin, R., Brit. J. Exptl. Pathol. **38**, 539 (1957).

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