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Disease in Macacus Monkeys Inoculated with ECHO Viruses.* (25000)

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Several ECHO viruses have been implicated as causative agents of neurologic disease in man(1). Paralysis has occurred in individual patients infected with some of these viruses(2,3,4,5). The recent reports of Steigman(6), Dalldorf(7) and of Habel and Loomis(8) leave no doubt that paralysis and neuronal damage can be induced in monkeys experimentally infected with Coxsackie viruses representative of both A and B groups. These investigators observed microscopic changes in the central nervous system of inoculated animals which were indistinguishable from those caused by poliovirus. Wenner and Chin(9) in monkeys used for preparation of antiserum against ECHO prototype strains described degenerative changes in neurons after the animals had received several injections of virus and mineral oil adjuvant. Our experiments were undertaken to determine whether 2 types of ECHO virus could produce disease of the central nervous system in monkeys.

Materials and methods. Tissue cultures. Cultures were prepared according to previously described technics(10,11). Cultures of human amnion cells were used in attempts to demonstrate virus in materials derived from inoculated monkeys and in neutralization tests with their serum. The *viruses* employed were ECHO Type 6, strain SHO(2) and ECHO Type 16, strain HAR(2). Clinical manifestations in patients infected with these 2 strains have been described by Kibrick *et al.*(2). The strains were recovered in tissue culture from fecal specimens. The type 6 strain was isolated in human kidney cells and subsequently passed once in this medium and twice in human amnion cells. The fluid from the last of these passages had an infectivity titer of $10^{-6.5}$/0.1 ml in amnion cells and was used as inoculum in the experiments to be described. The type 16 strain was recovered in human kidney cells, passed twice in human embryonic skin and muscle tissue, and twice in human amnion cells. The fluid from the last of these passages had an infectivity titer of $10^{-3.5}$/0.1 ml in cultures of human amnion cells, and was used as inoculum. *Monkeys.* Seven rhesus and 3 cynomolgus monkeys were studied. Before each was injected, specimens of blood for antibody determination, heparinized blood for viral culture, and peripheral blood for white blood cell counts were obtained. Then a #22 gauge hypodermic needle was introduced into the basal cistern, and about 1-2 ml of cerebrospinal fluid was withdrawn for white cell count and for viral culture. Eight of the monkeys were inoculated with 0.5 ml of undiluted tissue culture fluid containing the virus which was introduced into the basal cistern. In half of the animals

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0.1 ml of the fluid was also injected intraspinously into the lumbar enlargement. Two animals serving as controls were inoculated with infected tissue culture fluid that had been heated at 65°C for ½ hour. No active virus was demonstrated in these fluids upon subsequent addition to cultures of human amnion cells. To one control animal 0.5 ml of heated ECHO 16 virus was administered intrathecally and 0.1 ml intraspinously; to the other 0.5 ml of heated ECHO 6 virus was given intrathecally. Specimens of blood and spinal fluid for leucocyte counts and for detection of virus, and samples of feces for detection of virus were obtained every other day during the first week and every third day thereafter until termination of experiment. The animals were examined daily for muscle weakness or paralysis. At intervals varying from 6-21 days after injection, the animals were sacrificed. The brains and spinal cords were immediately removed; portions were preserved in the CO_2 cabinet to test later for presence of virus. The remainder of each tissue was

TABLE I. Results of Tests for Presence of ECHO Virus.

Day after inj.	Source of specimen	No. of animals* Type 6 virus	Type 16 virus	Control
2	Blood	4/4	0/4	0/2
	CSF	"	3/4	"
4	Blood	0/4	0/4	"
	CSF	4/4	1/4	"

* Numerator = No. positive for virus. Denominator = No. tested.

fixed in 10% formalin. *Neutralization tests.* Following centrifugation serum was removed from the clotted blood and stored at −20°C until tested. Undiluted serum was inactivated at 56°C for one half hour, then a series of dilutions, increasing by a factor of 2, was prepared. To each serum dilution was added an equal volume of suspension which contained virus in approximately 100 $TCID_{50}$/0.1 ml. The mixture was incubated at 4°C for one hour, at which time 0.2 ml of the virus-serum mixture was added to each of 3 tubes containing cultures of human amnion cells. Control tubes containing cultures inoculated with a diluted suspension of virus only and uninoculated cultures were included

TABLE II. Neutralizing Antibody Response to Injected Virus.

Virus	Monkey	Before inj. Day	Titer*	After inj. Day	Titer*
ECHO 6	control 1	0	<2	8	<2
	2	"	"	21	64
	3	"	"	"	"
	4	"	"	8	32
	5	"	"	"	16
ECHO 16	control 6	"	"	21	2
	7	"	"	"	32
	8	"	"	"	16
	9	"	"	6	4
	10	"	"	"	"

* Reciprocal of final serum dilution.

in each test. The neutralization titer of a serum was determined as the reciprocal of that dilution which prevented the appearance of cytopathic effect for 14 days.

Results. The results of tests for presence of virus in blood and spinal fluid are shown in Table I. From blood of the 4 animals injected with unheated type 6 virus the agent was recovered on second day but not thereafter. The presence of virus was also detected in the cerebrospinal fluid in specimens obtained on the second and fourth days after inoculation. The agent was not demonstrated in blood of any of the 4 animals injected with active ECHO 16 virus although in all but one instance virus was recovered from the cerebrospinal fluid. Virus was not detectable in *22* blood specimens and *22* cerebrospinal fluid specimens collected between sixth and twenty-first days. No virus was detected in stools of any of the monkeys, nor in specimens of tissue taken at autopsy.

That infection occurred in these animals is also indicated by development of neutralizing antibodies to the injected type of virus. The undiluted serum from all animals obtained at

TABLE III. Muscular Weakness in Relation to Infection with ECHO Virus.

Extremities involved Leg	Arm	No. animals	ECHO virus Type 6	Type 16
—None—		2	1	1
1		1		1
2		1		1
1	+ 1	1	1	
2	+ 1	3	2	1
Total		8	4	4

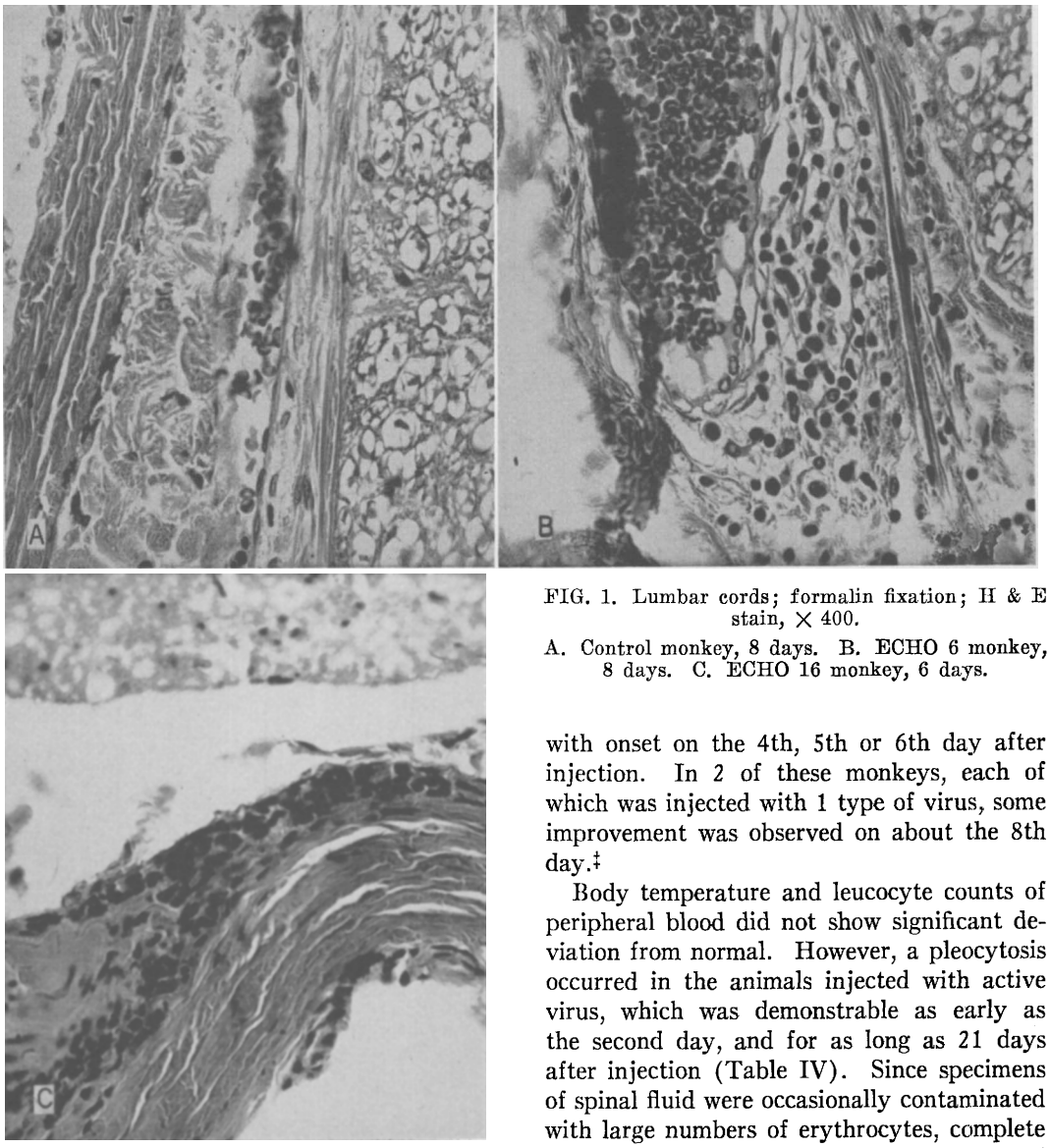

FIG. 1. Lumbar cords; formalin fixation; H & E stain, × 400.

A. Control monkey, 8 days. B. ECHO 6 monkey, 8 days. C. ECHO 16 monkey, 6 days.

time of injection failed to neutralize approximately 100 ID_{50} of the homologous virus. A significant increase in homologous antibody occurred in all the monkeys injected with active type 6 virus (Table II). A significant antibody response also occurred in animals injected with active type 16 virus (Table II).

The incidence and location of muscular weakness are recorded in Table III. Three of the animals injected with active ECHO 6 and 3 of those injected with active ECHO 16 developed definite signs of muscular weakness, with onset on the 4th, 5th or 6th day after injection. In 2 of these monkeys, each of which was injected with 1 type of virus, some improvement was observed on about the 8th day.‡

Body temperature and leucocyte counts of peripheral blood did not show significant deviation from normal. However, a pleocytosis occurred in the animals injected with active virus, which was demonstrable as early as the second day, and for as long as 21 days after injection (Table IV). Since specimens of spinal fluid were occasionally contaminated with large numbers of erythrocytes, complete series of white cell counts on each animal cannot be presented. The cell count of the spinal fluid at its peak varied from 64 to 598 cells/mm^3. Variations of this order of magnitude were recorded in both groups of monkeys inoculated respectively with the 2 types of ECHO virus. In the cerebrospinal fluid of the 2 animals that received heated tissue

‡ We are grateful to Dr. David Grice, formerly of Orthopedic Service, Children's Medical Center, for indispensable aid in evaluating muscle weakness in these animals.

TABLE IV. Leukocytes in CSF of Monkeys Inoculated with Active ECHO Viruses Types 6 and 16.

Cells/mm³ CSF	No. of monkeys								
500-599			1		1				
400-499		2							
300-399		2							
200-299		1		1	2				
100-199		2	2						
21- 99		1	1	1	1	1	1	2	2
0- 20	8					1	3	2	2
	0	2	4	6	7	8	10	15	21
	Day after inoculation								

Maximal WBC count in 2 control monkeys receiving heat-inactivated viruses was 20/mm³.

culture fluid, the highest count observed was 20 cells/mm³.

Microscopic examination of the spinal cords revealed no convincing evidence of neuronal damage. In sections from certain monkeys that received active virus, however, slight round cell infiltration of the meninges was observed in a few areas (Fig. 1).§

Discussion. Mild or moderate muscle weakness has been observed in human beings infected with ECHO types 2(6), 4(4), 6(2), 9(5), and 16(4) virus. It is therefore of interest that in monkeys experimentally inoculated with ECHO 6 and 16 certain muscles were affected. The findings here reported also indicate that, just as in man, these 2 representative ECHO viruses are capable of producing in rhesus and cynomolgus monkeys a mild meningitis characterized by the presence of virus in the spinal fluid and a predominantly mononuclear pleocytosis.

Summary. In monkeys injected intrathecally with ECHO virus 6 and 16, clinical signs of muscle weakness, pleocytosis of cerebrospinal fluid and development of homologous neutralizing antibodies were observed. These reactions were not detected in 2 animals inoculated in comparable manner with suspensions of these viruses which had been heated at 65°C for one half hour.

§ We are indebted to Doctors D. E. Denny-Brown, Joseph M. Foley and John M. Craig for examining sections of CNS tissues.

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Lipemia-Induced Acceleration of Intravascular Clotting.* (25001)

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Reports of changes in coagulability of blood associated with post-prandial lipemia have thus far been based on test tube methods (1,2). Lipemia following ingestion of cream, as well as intravenous infusion of cottonseed oil emulsion were found by one of us to result in significant shortening of whole blood clotting time in siliconized tubes and of plasma clotting time with Russell's Viper Venom(3). The question presented itself as to whether these *in vitro* findings reflected an increased tendency to clot formation *in vivo*. Therefore, a study was undertaken of the effects of

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