Coxsackie A7 Virus and the Russian "Poliovirus Type 4". (23304)

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In February 1956, Drs. M. P. Chumakov and M. K. Voroshilova from the Poliomyelitis Research Institute in Moscow made available to us their "Type 4 poliovirus." Details of the isolation of this virus in 1952 and its characteristics were subsequently published by these authors(1). In summary, 3 strains of a virus had been isolated from the stools of Russian children during the acute phase of a clinical illness characteristic of paralytic poliomyelitis. Strain AB IV, the strain supplied to our laboratory, was isolated by inoculation of monkeys with an admixture of stools from 2 patients with bulbar polio, which subsequently proved to be fatal. The virus caused paralysis and typical histological lesions of poliomyelitis after intramuscular (I.M.) or intracerebral (I.C.) inoculation of monkeys. It also caused paralysis and death in suckling mice and in both suckling and adult cotton rats. This virus was not neutralized by antisera against Types 1, 2, or 3 polioviruses, and showed no cross immunization with these viruses on I.C. challenge. It was reported to be cytopathogenic for human fibroblasts in tissue culture (T.C.) but to have low titer and with a 10 to 12-day incubation period.

The Russian virus was supplied to us in the form of glycerinated brains of the 24th suckling cotton rat passage, and no antiserum was made available at that time. This report details the studies conducted with the AB IV strain of the Russian virus, which have shown it to be identical with Coxsackie A7 virus and confirmed its properties as reported by the Russian investigators. Also reported here are studies on 2 strains of Coxsackie A7 virus isolated in the United States, and evidence is presented to indicate that these strains, like those of Chumakov and Voroshilova, are capable of producing in the central nervous system (CNS) of some monkeys lesions which are indistinguishable from those caused by polioviruses.

Methods. Animal inoculation. Suckling Swiss mice less than 24 hours old were inoculated either intraperitoneally (I.P.) or I.C. with 0.03 and 0.02 ml respectively. Either brain or whole carcass was harvested for passage material. Rhesus monkeys were inoculated with 1.0 ml, either I.C. into the thalamic area or I.M. in the thigh. *Tissue culture.* Freshly prepared monkey kidney roller tube cultures (MKTC) grown and maintained in lactalbumin-calf serum-Hanks' solution medium were inoculated with 0.1 ml of original materials and passages were made with 0.1 ml of undiluted culture supernates. *Neutralization tests.* Routinely 100 infectious doses of virus were mixed with equal volumes of antiserum dilutions, incubated for 1 hour at room temperature and then inoculated into either 2 T.C. tubes or 8 suckling mice. Tubes were examined daily for 10 to 14 days, and mice for 14 days. *Pathology.* The methods used in preparation of the CNS of test monkeys for histological examination, and the procedure for sectioning and examination, were those recommended by the Technical Committee on Poliomyelitis Vaccine(2).

Results with Russian AB IV Strain. The original 24th passage suckling cotton rat brains were washed free of glycerin and emulsified at 10% concentration and used as inoculum for monkeys, suckling mice and tissue culture.

Suckling mice developed paralysis of the extremities after a 4 to 5-day incubation period and usually died in the subsequent 24 hours. The appearance of the mice was typical of Coxsackie A infection. On subsequent passages from these mice it was found that both brain and carcass had a titer of 10^5 by either I.P. or I.C. inoculation. This passage virus caused no symptoms when inoculated I.C. or intraspinally (I.S.) into adult mice.

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One *rhesus monkey* (73542) inoculated I.C. with original cotton rat virus, developed a slight elevation of temperature on the third to seventh days and moderate generalized tremors on the fourth day which persisted for one week. This monkey was bled on the 28th day, then given 5 ml of 20% suckling mouse brain passage 2 virus intramuscularly and bled on day 35, at which time it was killed for pathological examination. A second rhesus monkey (72929) received 1.0 ml of original virus I.M. in the right thigh. On the 3rd day it developed fever; tremors on the 4th day; and definite weakness of both legs on the 5th day. The muscle weakness had not progressed by the next day, so the animal was sacrificed. An emulsion of the lumbar cord of this monkey produced typical disease in suckling mice. An attempt to infect 5 rhesus monkeys by I.M. inoculation with this cord suspension (1 ml of 20% suspension) was clinically negative. No attempt was made to pass it intracerebrally in monkeys. The histological findings in both these monkeys were typical of polio, but in one only the spinal cord was involved.

Tissue cultures, including HeLa, human skin epithelium, human amnion, human fibroblasts and monkey kidney (MK) epithelial cells, were inoculated with the original cotton rat brain virus. There was no apparent cytopathogenic effect (CPE) except in MKTC. Here, after an incubation period of 8 to 10 days, a few scattered individual cells were seen to be round and refractile. Subsequent holding of the cultures did not result in any extension of this effect to the remainder of the cell sheet. The same results were obtained when the emulsified carcasses of the first suckling mouse passage were inoculated into MKTC. Subsequent passage of these materials again gave very few scattered cells showing CPE until the 7th passage. At this time the line established from suckling mouse virus showed widespread CPE after an incubation of 3 days. This T.C. line of AB IV virus has been carried through 33 passages and now titers to 10^6, and with heavy inocula produces CPE within 24 hours. Passage 22, with a titer of 10^5, was shown to produce CPE in HeLa, human amnion, and human fibroblast cells, but not in human skin cultures. MKTC passage 8 was positive on I.C. inoculation into suckling mice, but passage 27 was negative.

Characteristics of the virus were investigated using the suckling mouse passage strain and it was found to be ether resistant, inactivated by 60°C for 10 minutes, filterable through a Seitz EK pad and a Selas 03, and not affected by penicillin, streptomycin or achromycin. It was negative on attempted passage in chicken embryos.

Identification of the Russian AB IV virus was attempted by neutralization, complement fixation and hemagglutination-inhibition tests. Since no specific antiserum had been supplied with the original virus strain, the only antiserum available during the primary investigations was that from the monkey convalescing from I.C. inoculation of the original cotton rat virus after receiving a booster dose of suckling mouse passage virus. This serum neutralized 100 LD_{50} of suckling mouse virus at a 1/25 dilution in mice and 100 $TCID_{50}$ at a 1/25 dilution in MKTC. The serum was tested in neutralization tests in suckling and adult mice and in MKTC against the viruses indicated in Table I. It was negative against Types 1, 2, and 3 polioviruses and all others tested except Coxsackie A7 and A8. This same monkey antiserum was also tested by complement fixation and hemagglutination-inhibition against the viral antigens shown in Table I with negative results.‡

At a later date we obtained a sample of AB IV antiserum from the Russian virologists in the form of immune monkey serum. This was shown to neutralize our suckling mouse virus at a 1/625 dilution and the T.C. virus at 1/25. It also neutralized standard A7 virus in suckling mice.

The AB IV suckling mouse virus was tested for neutralization by the standard antisera listed in Table I with negative results except for Coxsackie A7 serum and normal human gamma globulin produced in the U.S.

Follow-up neutralizations, in view of the positive results with our AB IV antiserum

‡ The hemagglutination-inhibition tests were kindly performed by Dr. Max Theiler of the Rockefeller Foundation Virus Laboratory and Dr. Leon Rosen of the N.I.H.

TABLE I. Immunological Tests Made to Identify Russian "Type 4 Poliovirus."

AB IV virus (SM) *vs* immune sera in SM	AB IV immune monkey serum *vs* viruses in— Adult or suckling mice	MK tissue culture	Complement fixation	Hemagglutination inhibition
Coxsackie A1, 2, 3, 4, 5, 6, 7,* 8, 9, 10	Mouse encephalomyelitis-TO,FA	Herpes	Polio 1, 2	Dengue 1, 2
Coxsackie B1, 2, 3, 4, 5	GD VII	Vaccinia	Coxsackie A10	West Nile
Mengo	Coxsackie A1, 2,	Echo 1, 2, 3, 4,	Adeno 7	Yellow fever
EMC	3, 4, 5, 6, 7,*	5, 6, 7, 8, 9, 10,	Mumps	WEE
LCM	8,* 9, 10	11, 12, 13, 14		EEE
St. Louis encephalitis		Polio 1, 2, 3		Ilheus
Polio 1, 2, 3				St. Louis enceph.
Human gamma globulin A*				
" " " B*				

All negative except those starred. All neutralization tests with undiluted sera against 100 infectious doses of viruses.

against Coxsackie A7 and A8 viruses, showed that both the suckling mouse and T.C. viruses were neutralized by standard A7 immune mouse serum, but not by A8 antiserum. This seemed to establish the fact that the AB IV virus which we had isolated from the original cotton rat material by passage in both suckling mice and MKTC contained Coxsackie A7 virus. In view of the original isolation by the Russian investigators having been made from "paralytic polio" patients and the typical polio lesions produced in monkeys, we felt that further work was necessary to rule out an admixture of Coxsackie A7 with some other virus, especially a poliovirus.

Our monkey antiserum was shown to be negative against polioviruses 1, 2, and 3, and the AB IV virus negative in mice and T.C. against a mixture of antisera against these 3 viruses. The original cotton rat virus supplied by the Russian investigators as well as the monkey, T.C., and suckling mouse passages of it were completely neutralized by standard Coxsackie A7 antiserum. Furthermore, the 4th suckling mouse passage and 26th T.C. passage viruses were carried through 5 ultimate dilution passages and then shown to be neutralized with A7 antiserum. Antisera were produced in adult mice against these 2 ultimate dilution passage viruses and were shown to cross-neutralize each other in the 2 test systems, and were also positive against standard A7 virus in mice. The 5th ultimate dilution suckling mouse passage and the 23rd MKTC passage viruses were sent to Dr. Albert Sabin who kindly tested them for monkey pathogenicity by intraspinal inoculation. Dr. Sabin reports that although no definite clinical paralysis developed in these animals, they showed poliomyelitis-like lesions with a distribution different from poliovirus infection.

Results with U.S. strains of Coxsackie A7. Frozen stool emulsions collected by Dr. Robert Parrott, formerly of N.I.H., from 2 children with aspetic meningitis in 1952 and reported in our studies of nonparalytic poliomyelitis(3) as positive for Coxsackie A7 virus, were available for study. These same stool specimens had been tested by us in MKTC in 1954 with negative results, and Dr. Parrott had isolated A7 virus from them in 1952(4). The stool emulsions were again shown to contain A7 virus by inoculation of suckling mice. Each was also inoculated I.C. into a rhesus monkey. One monkey remained afebrile and showed no symptoms (79287), while the other (79286) had a temperature elevation from the 2nd to the 6th day, during which time there appeared some reluctance to use the left arm, but no definite weakness could be demonstrated. These monkeys were observed for 35 days when they were killed for histological

TABLE II. Results of Neutralization Tests with Sera of Different Age Groups against AB IV Virus (Coxsackie A7).

Ages (yr)	Neutralization test results No. tested	No. positive
1- 5	10	6
5-10	12	7
10-15	7	3

1/4 dilution of sera against 100 LD_{50} virus in suckling mice. Sera from children in Washington, D. C. area.

TABLE III. Degree of Involvement and Distribution of CNS Lesions in Infected Monkeys.*

Monkey No.	Par. and NP polio†	72929	73542	80951	79286	79287	79900 (SMP-1)	79901 (SMP-1)
Inoculated with	Poliovirus	AB IV, IM	AB IV, IC	Stool 6509, IC	Stool 6509, IC	Stool 6617, IC	Stool 6509, IC	Stool 6617, IC
Day after inoc.		6	35	7	35	35	37	37
Clinical signs		Tremors, fever, weak legs	Tremors, fever	Fever, weak arm	Fever, weak arm	None	Fever, ? tremors	Fever, ? weak arm
Regions of CNS								
Spinal grey	++(++)	+++(+)	+	++(+)	++(+)	++(+)	+(+)	+(+)
Cranial nerve nuc.	+(+++)	+++	0	+(+)	++	+	±	±
Retic. formation	+(+++)	++	0	+	+	±	0	0
Midbrain and subthal.	+(+++)	+++	0	+(+)	++	+(+)	+	+
Substantia nigra	+(++)	++	0	++	+++(+)	+	+	+
Hypothalamus	+(+)	++	0	++	+	++	±	0
Dorsal thalamus	+(++)	+	0	+	+	+	±	0
Cerebellar areas:								
Cortex	+	0	0	0	±	0	0	0
Roof nuclei	+(+++)	++	0	++	+++	++	+	+
Dentate nucleus	+(+)	++	0	+	+++	++	+	+
Pontine nuclei	±			+	0	+	0	0
Corpus striatum:								
Caudate	0	0	0	0	+	+	0	0
Putamen	0	0	0	0	++	+	+	0
Amygdala	+	0	0	±	+	+	±	0
Pallidum	+(++)	0	0	±	+	0	0	0
Olfactory centers	±							
Cerebral cortex:								
Frontal	±	±	0	0	+	+	+	0
Pre-central	+(++)	++	0	++	+	++	+	0
Post-central	+	0	0	0	+	++	+	0
Parietal	0	0	0	0	+(+)	+(+)	+	0
Temporal	0	0	0	0	±	+	+	0
Occipital	0	0	0	0	±	+	±	0
Hippocampus	0	0	0	0	0	+	±	0
White matter	±	+	0	±	+(+)	+	+	0
Meninges	+(+)	+	0	+	+	+	0	0

* Symbols: ++(++) = Range of ++ to 4+; 0, no lesions; ±, absent or minimal lesions; +, mild or minimal inflammatory or glial infiltrations; ++, moderate infiltration and minimal neuronal damage; +++, moderate infiltration and moderate neuronal damage; ++++, moderate or severe infiltration and neuronal damage.

† From Table II, collection of D. Bodian, *Am. J. Hyg.*, v64, 104, 1956, Technical Committee on Poliomyelitis Vaccine and Sub-committee on the Monkey Safety Test.

examination of the CNS. Sera obtained before inoculation and at time of sacrifice were checked for antibodies against each of the 3 types of poliovirus with negative results and were shown to have developed neutralizing antibodies against our T.C. strain of AB IV (Coxsackie A7) virus. Typical polio lesions were found in the CNS of these 2 animals, but as indicated in Table III there were also lesions in "non-polio" areas. The first suckling mouse passage of the virus from the same 2 patients' stools were also each inoculated I.C. into a single rhesus monkey (79900 and 79901) with identical clinical and histopathological results except that the latter had lesions completely indistinguishable from those of poliomyelitis. Monkey 80951 was inoculated I.C. with one of the original stool suspensions and became febrile on the 5th day with slight weakness of the left arm, and was sacrificed on the 7th day. The CNS lesions were identical with severe poliomyelitis. An emulsion of brain stem titered 10^2 in suckling mice and the cord to 10^4 and both were neutralized by Coxsackie A7 serum. The cord suspension was negative in MKTC.

Results of serum survey for Coxsackie A7 antibodies. Sera from 29 normal children of different ages in the Washington, D.C. area (ages 1 to 15 years) were made available by Dr. Robert Huebner of N.I.H. These sera at a ¼ dilution were tested against 100 LD_{50} of AB IV (Coxsackie A7) suckling mouse passage virus in mice. The results summarized in Table II show that at each age level a good proportion of the sera possessed specific neutralizing antibodies against Coxsackie A7 virus. It had previously been found that one batch of U.S. produced gamma globulin had a neutralizing titer of 1/5 and another of 1/25 against this virus.

Pathologic picture in CNS of infected monkeys. The histopathological results summarized here are based on the detailed examination of the CNS tissues of 7 monkeys. Two monkeys (72929 and 80951) killed on the 7th postinoculation day showed lesions predominately in the anterior and lateral columns of the spinal cord. Degrees of neuronal degeneration varying from chromatolysis to vacuolization of cytoplasm, nuclear pyknosis and neuronophagia were seen. Additional lesions were focal gliosis of grey matter accompanied by polymorphonuclear leukocytic and lymphocytic perivascular infiltrations. The remaining 5 monkeys (73542, 79286, 79287, 79900 and 79901) were killed between 30-35 days postinoculation, and presented lesions of a sub-acute type. The lesions were comprised of glial foci and lymphocytic perivascular infiltrations in grey matter. There was some loss of neurons in the spinal cord. Inflammatory and glial lesions occurred also in white matter. The intensity and distribution of lesions are shown in Table III.

The qualitative aspects of the pathologic lesions seen in sections of the CNS of these monkeys were not different from those seen in poliovirus infected animals nor from those suffering infection with some other neurotropic viruses. No inclusion bodies were demonstrated.

The distribution of lesions within the various areas of the CNS has been emphasized as the important differentiating criterion in determining whether a particular animal shows histological evidence of poliovirus infection (2). The distribution pattern of lesions in the 7 experimental monkeys is summarized in Table III, and for comparison purposes, that considered typical of poliovirus infection is also included. In summary, the degree of involvement and the distribution of lesions in 3 monkeys were identical to what might be seen in poliovirus infected monkeys. These include monkey 72929 killed in the acute stage of infection with the Russian strain, 79901 convalescent after receiving suckling mouse passage of one of the American strains, and 80951 killed in the acute stage after being infected by inoculation with human stool virus (Fig. 1).

The lesions confined to the spinal cord of monkey 73542 were qualitatively indistinguishable from those of poliomyelitis but not enough areas of the CNS were involved for distribution of lesions to be used as a differentiating characteristic. However, in 3 monkeys (79286, 79287, and 79900) the lesions were distributed in such a manner that permitted differentiation of their infections from those caused by polioviruses. The caudate

nucleus, putamen, or both, of these 3 animals showed lesions (Fig. 2 and 3). Additionally, 2 of the animals had definite though minimal lesions of hippocampal and occipital cortex. These areas are rarely or never involved in poliovirus infection. The approximately

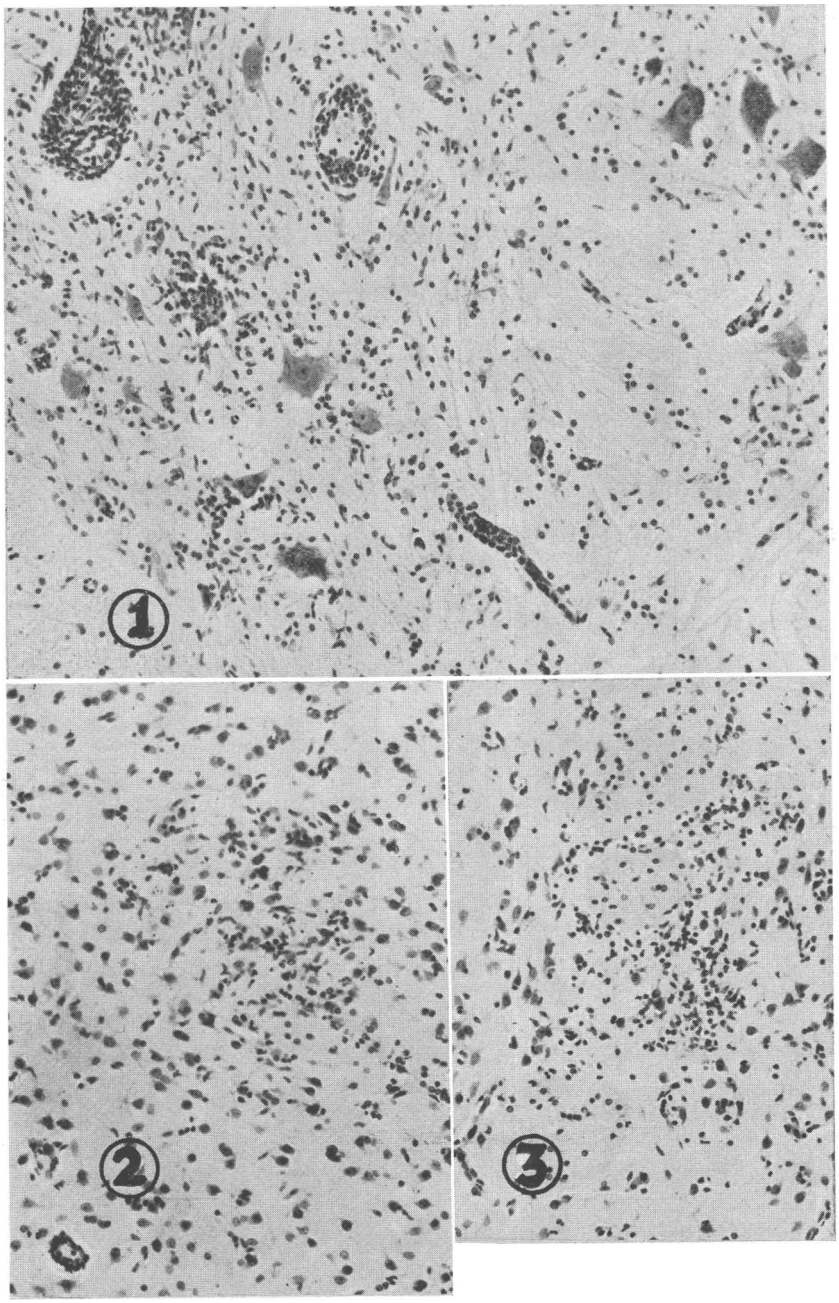

FIG. 1. Lesions caused by Coxsackie A7 in anterior horn of lumbar spinal cord. Perivascular infiltration, focal gliosis, chromatolysis and neuronophagia are illustrated. Monkey No. 80951, Gallocyanine stain, 143 ×.

FIG. 2. A glial focus in caudate nucleus of Monkey No. 79286, Gallo, 143 ×.

FIG. 3. A glial focus in putamen of Monkey No. 79286, Gallo, 143 ×.

equal degree of involvement of the motor (precentral) and of the anterior parietal (postcentral) cortex also was helpful in the differentiation.

Pathological examination of a few infected suckling mice showed typical Coxsackie A type myositis and no involvement of the pancreas or interscapular fat. In 2 suckling mice slight CNS involvement was observed, composed of sparse perivascular cuffing and small glial patches in cerebral cortex.

Discussion. The possibility that viral agents other than the 3 known types of poliovirus may cause a clinical illness in human beings similar to poliomyelitis has by now been substantiated insofar as nonparalytic polio is concerned. A number of agents have been shown to cause an aseptic meningitis clinically indistinguishable from nonparalytic polio(3,5,6,7).

The evidence for the etiological association of Coxsackie B types with the aseptic meningitis or nonparalytic poliomyelitis syndrome is quite well established(6,8,9) whereas for the A group the evidence is only suggestive (8,10). The high frequency with which group A types are isolated from children with minor illnesses or in good health makes their presence in poliomyelitis cases difficult to evaluate unless large numbers of proper controls are tested. In such a controlled experiment (3) we recovered A9 virus 5 times more frequently in the "nonparalytic poliomyelitis" patient group than the expected rate calculated on the basis of overall incidence in this group and 3 control groups of children.

In this same study Coxsackie A7 virus was isolated from the stools of 6 children hospitalized as nonparalytic poliomyelitis patients in Washington, D.C. in 1952. These isolations were made by inoculation of suckling mice by Dr. Robert Parrott, whereas the same stool specimens when tested by us in MKTC were negative. However, from different stool specimens on 2 of these cases we isolated type 2 polio and Echo 6 viruses in T.C. In the case of 4 of these 6 children, the A7 isolation had been made on specimens collected more than 7 days after admission to the hospital ward, during which time other patients with A7 in their stools were present on the same ward. On this basis these 4 whose admission stools had been negative were considered to be hospital infections. The other 2 A7 cases could not be so explained. Johnsson(11) had 6 isolations of A7 virus in suckling mice inoculated with stools from 354 cases of aseptic meningitis, and Svedmyr at the Central Bacteriological Laboratory of Stockholm has isolated 2 strains of A7 in T.C. from children with aseptic meningitis. Stanley and Dorman(12) have described a virus isolated from the cord of a fatal case of paralytic poliomyelitis which is pathogenic for monkeys and suckling mice. The virus was not identified, but did not produce myositis and was not types 1 or 2 poliovirus.

In large-scale surveys of stools of children by suckling mouse inoculation, A7 isolations have been relatively few compared to other A types, yet the presence of specific antibodies in gamma globulin and the high incidence of positive neutralization tests with sera of young children found in the present study indicate that infection in the U.S. with this virus is probably a common experience during childhood.

In contrast to the ever-growing number of viruses capable of producing the nonparalytic poliomyelitis syndrome in humans, there are no known viruses, other than polioviruses, which have definitely been proven capable of causing the paralytic form of the disease in man. Yet as more complete investigations of paralytic poliomyelitis cases are carried out on larger numbers of individuals there will probably be more instances reported where poliovirus cannot be isolated from the stools and paired serum samples show no poliovirus antibody rise. Such paralytic cases have already been found(13,14). Likewise, except for the pathological findings in the Coxsackie A7 infected monkeys reported in this paper which confirm the Russian observations, no other human viruses have been found to cause histological lesions whose nature and distribution in the CNS of some infected monkeys are indistinguishable from those produced by polioviruses(2).

Chumakov *et al.*, have reported the isolation of a so-called "type 4 poliovirus" on the basis that the source was stools of acutely

paralysed children; the virus caused paralysis in monkeys with typical poliomyelitis lesions and antibodies were shown to develop in the blood of convalescent paralytic cases. The virus was shown to be immunologically distinct from the 3 known types of poliovirus. This "type 4 poliovirus" isolated by the Russian workers has now been shown to be a Coxsackie A7 strain in our laboratory and also by Dr. Torsten Johnsson in the State Bacteriological Laboratory at Stockholm. The mistaken identification by the Russian workers is understandable since they did not have specific Coxsackie virus typing sera available at the time.

We have confirmed the results of the Russian workers insofar as demonstrating that their A7 virus is capable of producing polio-like lesions in the monkey CNS and we have further demonstrated this to be true of 2 strains of A7 virus isolated directly from stools of children with aseptic meningitis in the U.S. Actually, on the basis of criteria established by 2 groups of poliomyelitis research workers(15,16), the A7 Coxsackie virus might well be considered a poliovirus. It has been isolated from cases of "nonparalytic" and "paralytic poliomyelitis"; it causes clinical and histological signs in monkeys consistent with poliomyelitis; it has physical properties similar to those of polioviruses; the range of host susceptibility is the same; and pooled human sera contain neutralizing antibodies against it. However, in addition to having these properties in common with polioviruses, it differs immunologically, has only a low level of pathogenicity for T.C., is more pathogenic for suckling mice and not at all for adult mice, and produces a myositis in the sucklings. In other words it is also a typical Coxsackie A virus.

The pathologic picture produced in monkeys by the Coxsackie A7 strains studied, both Russian and American, could not be distinguished from that caused by poliovirus in 4 of the 7 animals examined. The remaining 3 presented lesions with a distribution differentiating their infection from that caused by poliovirus.

The findings of the Russian virologists, confirmed here, add one more facet to the broad spectrum represented by the Coxsackie, poliomyelitis, and certain Echo viruses where the properties of one group blend sometimes rather extensively with those of the others, and all seem capable of causing related and sometimes identical clinical and histological manifestations in experimental animals and man. Certainly our present knowledge of these relationships should emphasize the necessity of search for these other viruses in paralytic cases where the specific etiology of poliovirus cannot be established. It must be remembered, however, that in the case of Coxsackie A7, at least, isolation can be accomplished more easily by inoculation of suckling mice or even monkeys than by currently available T.C. methods. Furthermore, the results reported here make it desirable that more extensive studies than those thus far reported(17) be carried out with Coxsackie viruses in monkeys or chimpanzees to determine if these characteristics may be found in types other than A7.

Conclusions. 1. The Russian Type 4 poliovirus is Coxsackie A7. 2. The Russian virus is capable of causing polio-type lesions in the monkey CNS. 3. American strains of Coxsackie A7 are likewise pathogenic for monkeys and in certain inoculated animals produce histological changes in the CNS which are indistinguishable from those caused by polioviruses. 4. Coxsackie A7 infections are probably prevalent in the U.S.

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A Proposed Potency Test for Smallpox Vaccine. I. Evaluation of Chick Embryo LD_{50} Method. (23305)

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The official potency requirement in force today, at least in the United States, for release of smallpox vaccine is based on a modification of the method of Calmette and Guérin(1,2), suggested by Force and Leake (3). This method has been most valuable over the years, but those engaged in the production and distribution of smallpox vaccine are familiar with the irregularities to which the rabbit test is subject and are aware of the lack of quantitative criteria for interpretation and recording of results. In fact, only relative values of the potency of a vaccine under test can be estimated, since a control vaccine of acceptable potency always has to be titrated in parallel on the same animal. The desirability, therefore, of a test method less susceptible to variation and capable of measuring the absolute titer of smallpox vaccine is quite evident. A titration method for vaccinia virus based on the number of pocks formed on the chorioallantoic membrane of embryonated hen eggs was first described by Burnet(4). Although this technic was found most effective for titration of vaccinia virus preparations of relative purity, it has not generally proved very satisfactory with the comparatively coarse smallpox vaccine suspensions. Furthermore, even with purified vaccinia virus, difficulties have been encountered with this method unless applied under specified optimal conditions(5).

Observations in this laboratory over the years(6) have indicated that embryos inoculated on the chorioallantoic membrane with dilutions of smallpox vaccine eventually die from the infection, and that death is invariably associated with the presence of pocks on that membrane. Moreover, there seems to be a linear relation between log-dilution of vaccine and reciprocal embryo survival time, similar to that reported for the Psittacosis-LGV group of viruses(7,8).

The present communication reports results of repeated titrations of the same smallpox vaccine preparation by the chick embryo LD_{50} method over a period of two years.

Materials and methods. Virus strain. The virus used in this study originated from human vaccination scabs obtained from the New York City Board of Health in 1909. It has since been maintained in this laboratory by irregular, alternating passages through calves, rabbits and humans, and has been used continuously for commercial production of calf lymph vaccine by the standard procedure. *Smallpox vaccine.* All titrations to be reported here were carried out with the same *glycerinated control vaccine virus* prepared in accordance with the minimum requirements set forth by the National Institutes of Health, Bethesda, Md., and meeting the requirement