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Pathogenicity of Coxsackie A-21 Virus for Suckling Mice (32987)

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The prototype strain of Coxsackie A-21 virus (Kuykendall strain), first isolated in 1955, produced a marked myositis and flaccid paralysis in suckling mice (15). Its classification as a Coxsackie group A virus was in part based on these findings. Four strains of Coe virus, recovered in 1958 by Lennette and co-workers from persons with respiratory disease, and subsequently shown to be serologically indistinguishable from Coxsackie A-21 virus, failed to produce paralysis in suckling mice even after multiple blind passages (14). Similar findings were reported by Pereira and Pereira (12) using a Coe virus strain recovered in Great Britain. In 1962 Underwood and co-workers (17) demonstrated that Coe virus could be adapted to growth in suckling and older mice after serial passage. The opportunity to investigate the effect of several newly isolated naturally occurring strains of Coxsackie A-21 virus was provided when numerous strains were recovered during an outbreak of mild acute upper respiratory illness among military personnel at Camp Lejeune, North Carolina in September and October, 1960 (2 and 7). This report describes the quantitative relationships between virus dose and the production of paralysis and myopathy in suckling mice for several naturally occurring and tissue culture adapted strains of Coxsackie A-21 virus.

Materials and Methods. Animals. General purpose Swiss strain mice approximately 24 hours old (either sex) were used. Each litter was housed in a separate cage and provided with water and Purina laboratory chow *ad libitum*.

Tissue culture. Roller tube cultures of HEp-2 and primary human embryonic kidney (HEK) cells were purchased from commercial sources. The maintenance medium for HEK cell cultures was medium 199 with 2% inactivated (30 min at 56°C) chicken serum and 0.002 *M* glutamine, and for HEp-2 cell cultures it was Eagle's Basal Medium with 5% inactivated chicken serum and 0.002 *M* glutamine (11). An antibiotic mixture was added to all media containing at final concentration: aqueous penicillin, 100 units/ml; streptomycin, 100 μgm/ml; mycostatin, 50 units/ml; and tetracycline, 100 μgm/ml. Maintenance medium was changed at 3–4 day intervals, and all cultures were incubated for 18 days at 33°C on a rotating drum revolving at 12 rph.

Virus strains. The strains of Coxsackie A-21 virus used in these studies have been described in a previous communication (11). All strains were recovered from the upper respiratory tract of patients during an epidemic of Coxsackie A-21 virus-associated mild upper respiratory illness at Camp Lejeune, North Carolina (2 and 7). Each strain was

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TABLE I. Multiplication of Serial Passage HEp-2 Coxsackie A-21 Virus in Suckling Mice.

Day post-inoculation	Total quantity of virus recovered per mouse (log_{10})		Paralysis		Histologic evidence of myopathy	
	Expt. 1	Expt. 2	Expt. 1	Expt. 2	Expt. 1	Expt. 2
0	6.3 (Inoculum)	2.6 (Inoculum)	—[a]	—	NT[b]	NT
1	5.4	2.2	—	—	—	—
2	6.5	3.5	—	—	+[c]	—
3	6.6	3.8	—	—	+	—
4	7.3	3.8	—	—	+	—
5	7.9	4.9	+	—	+	—
6	7.9	6.7	+	—	+	—
7	8.1	5.8	+	—	+	—
8	7.9	5.9	+	—	+	—
9	8.2	5.2	+	—	+	—

[a] —, absent; +, present.

[b] NT = not tested.

[c] One animal showed questionable evidence of myopathy; the second animal had a well defined lesion.

reisolated and identified by hemagglutination-inhibition.

Infectivity titrations. Serial tenfold dilutions of virus were made in Hanks' balanced salt solution (HBSS) containing 0.5% gelatin. Each of 2–4 cell culture tubes were inoculated with 0.2 ml/dilution and the cultures were observed at 3–4 day intervals for cytopathic effect (CPE) for 18 days. Each of 8 mice were inoculated with 0.03 ml intracerebrally and 0.06 ml intraperitoneally per dilution. Two mice at each dilution were harvested at appropriate times after inoculation and the surviving animals were examined daily for 14 days. Infectivity titers were calculated by the method of Reed and Muench (13).

Preparation of mouse carcass suspension. Mice were sacrificed by rapid freezing at —60°C and stored at this temperature until processed. Two animals from each dilution were decapitated, skinned, and eviscerated, and the carcasses of both animals were homogenized in a Ten Broeck homogenizer using an appropriate volume of HBSS to give a 20% final suspension (w/v). Cellular debris was removed by centrifugation at 2000 rpm for 10 min. The supernatant was decanted and inoculated into cell cultures almost immediately; the remainder of the supernatant was stored at —20°C.

Pathological examination. All tissues were fixed in 10% buffered formalin (pH 7) or Bouin's solution and processed in an Auto-Technicon. Paraffin imbedded sections were cut at a thickness of 6.5–7.0 μ and stained with hematoxylin and eosin. The following tissues were examined: brain, heart, lungs, liver, spleen, pancreas, kidneys, and the skeletal musculature of the head, truck, one foreleg, and one hind leg.

Results. Multiplication of early tissue culture passage Coxsackie A-21 virus in suckling mice. In this experiment, one group of 18 suckling mice was inoculated with a high ($10^{6.3}$ TCD_{50} per mouse) dose and a second group with a low ($10^{2.6}$ TCD_{50} per mouse) dose of second HEp-2 tissue culture passage Coxsackie A-21 virus (Camp Lejeune strain 5). At 1-day intervals after inoculation 2 animals from each group were sacrificed and the quantity of virus present in the pooled carcasses was determined by titration in rotated HEp-2 cell cultures (Table I). The amount of virus recovered per mouse after inoculation increased until the sixth day and then it remained level (high dose group) or decreased slightly (low dose group). After 5 days postinoculation, paralysis was evident in the mice given the high dose of Coxsackie A-21 virus and in these animals, $10^{7.9}$ TCD_{50} or greater of virus was recovered per mouse.

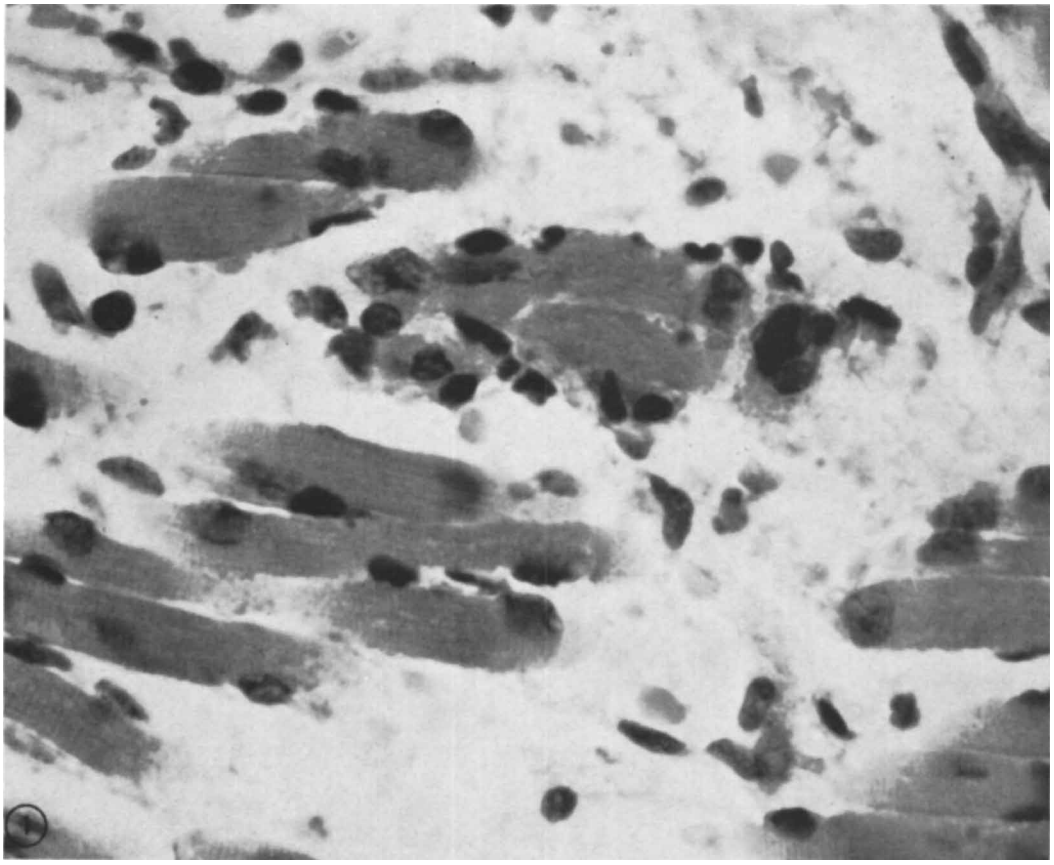

FIG. 1. Section through hind limb skeletal muscle of a Swiss strain newborn mouse 2 days after intraperitoneal inoculation with Coxsackie A-21. Note segmental and focal character of early lesion; hematoxylin and eosin; ×680.

Microscopic evidence of a degenerative myopathy was temporally related to increasing virus yields from the animals given the high multiplicity of virus. The maximum yield of virus in the animals inoculated with the low dose did not exceed $10^{6.7}$ TCD_{50} per mouse and a myopathy or paralysis did not develop in this group. These findings suggest that the production of a degenerative myopathy and flaccid paralysis in suckling mice inoculated with early tissue culture passage Coxsackie A-21 virus is directly proportional to the quantity of virus inoculated and the titer to which the virus multiplied.

Pathologic examination of mice inoculated with early tissue culture passage Coxsackie A-21 virus. Pathological examination of the limb musculature revealed a generalized degeneration demonstrable only in the animals inoculated with the high dose of Coxsackie A-21 virus (Camp Lejeune strain 5). Early changes were first evident on the second day after inoculation and increased in severity thereafter (Fig. 1). No abnormal findings were detected at any time in the skeletal tissues or visceral organs of the group of animals inoculated with the low multiplicity of virus.

Animals inoculated with the high dose of Coxsackie A-21 virus and harvested 2, 3, and 4 days later, when at least $10^{6.5}$ TCD_{50} of virus was recovered per mouse, had microscopic evidence of myopathy, but no paralysis. The general appearance and gait of these animals was normal. The areas of involvement were focal and generally restricted to the lower extremities. The lesion was characterized by proliferation of sarcolemmal and

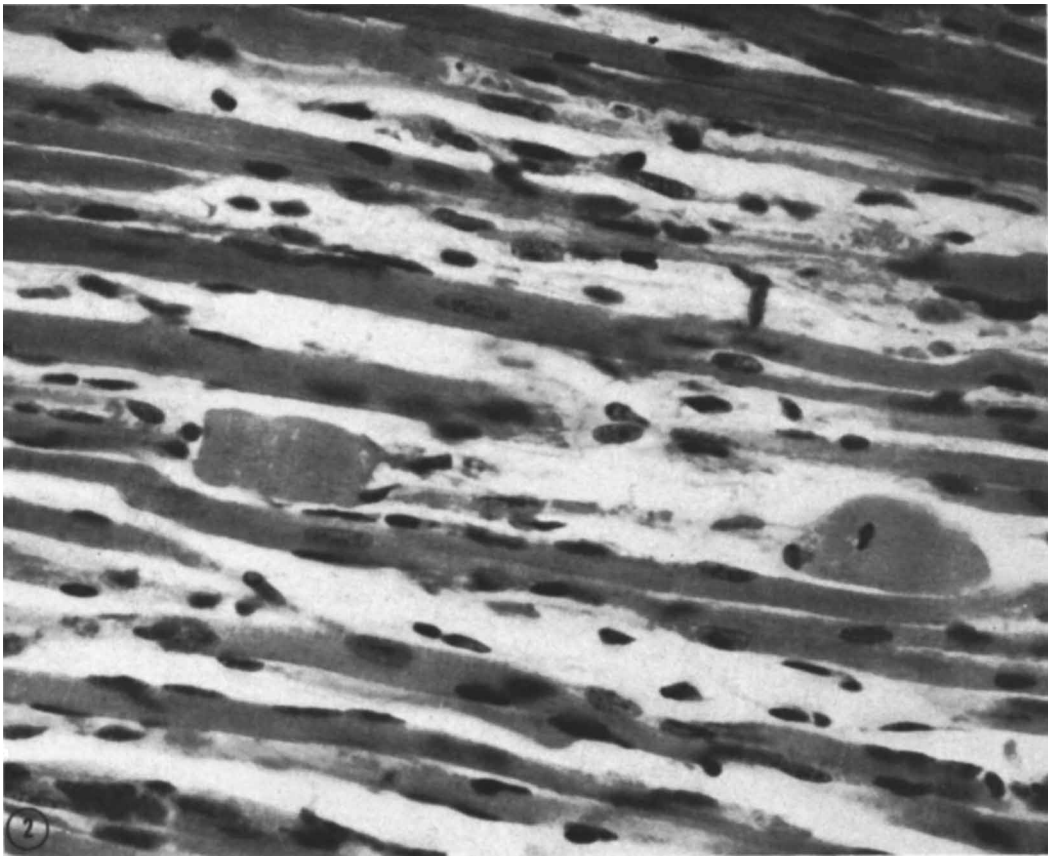

FIG. 2. Section through hind limb skeletal muscle of a Swiss strain newborn mouse 3 days after intraperitoneal inoculation with Coxsackie A-21 virus. Note segmental loss of sarcoplasm; hematoxylin and eosin; ×480.

muscle nuclei, intra and extracellular edema, and focal necrosis of individual muscle fibers. Some fibers assumed an "hour glass" appearance as a result of segmental loss of sarcoplasm and collapse of the sheath between areas of the fiber which were hyalinized and swollen (Fig. 2). The swelling of fragments often resulted in compression and distortion of adjacent, otherwise morphologically normal fibers. Crumbling of the hyalinized, necrotic segments was generally associated with the presence of macrophages, although few inflammatory cells were present in the minimal (early) lesions. Significant degenerative changes in the absence of cellular infiltration was common.

The onset of paralysis, 5 days after inoculation, was associated with a moderately severe, widespread degeneration of the lower limb skeletal musculature. Although a marked disorganization of the normal architecture was noted in many of the muscle groups, in some areas the lesions were limited to focal degenerative changes involving a relatively small percentage of the fibers (Figs. 3 and 4). There was only minimal involvement of the nuchal and intrinsic trunk musculature. Areas of severe involvement were characterized by degenerative changes in most muscle groups, fissuring, fragmentation, and segmental hyalinization of fibers with loss of cross striations and a cellular infiltrate composed of a large number of macrophages, scattered lymphocytes and a few polymorphonuclear leukocytes. Nuclear destruction was generally seen only after advanced cytoplasmic degeneration. Regeneration and restoration, present in the minimal lesions, was well developed in

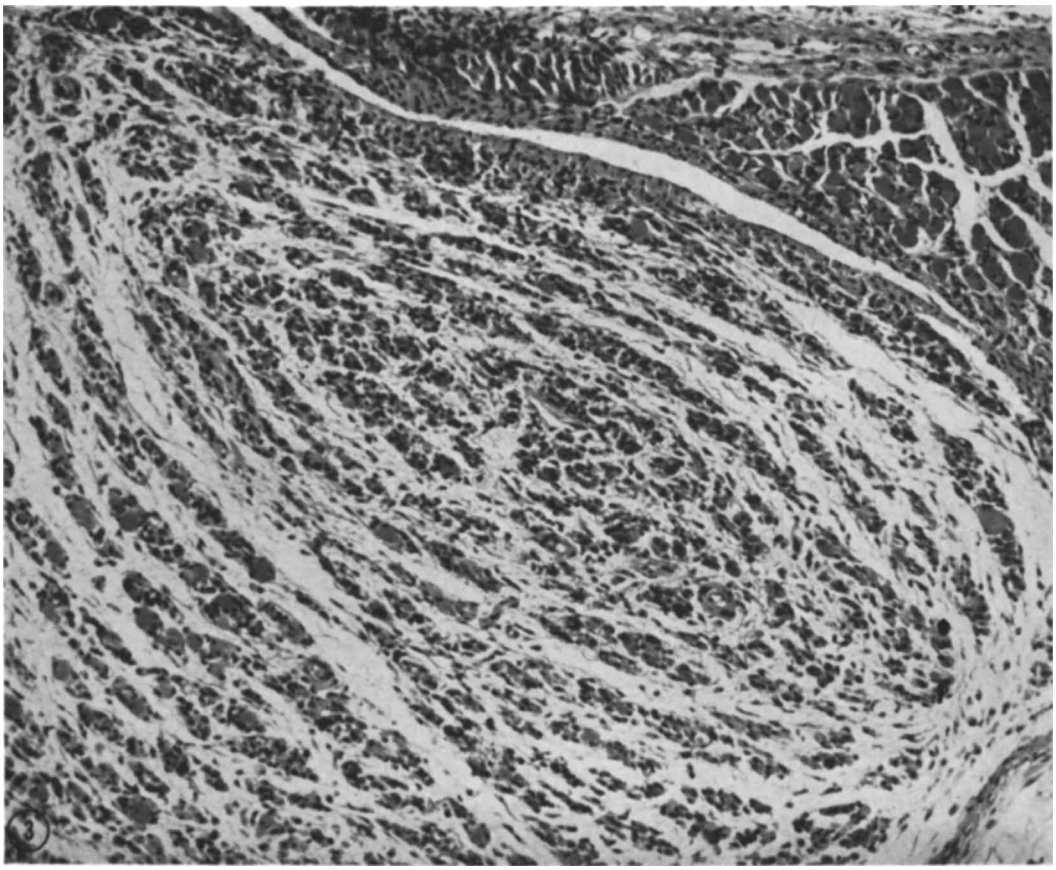

FIG. 3. Section through hind limb skeletal muscle of a Swiss strain newborn mouse 5 days after intraperitoneal inoculation with Coxsackie A-21 virus. Note focal character of lesion; hematoxylin and eosin; ×130.

areas of advanced destruction. It was evidenced by proliferation of sarcolemmal elements and muscle nuclei at the ends of intact fiber segments or within the sheath of severely involved fibers.

In 1 of 2 animals examined 7 days after inoculation a minimal focal pancreatitis was noted. In the area of the lesion there was collapse of glandular elements, degeneration of acinar epithelium, and widespread infiltration with polymorphonuclear leukocytes. The islets were spared and there was no evidence of fat necrosis. No lesion of the pancreas was noted in any other animal.

A nephropathy was present in 3 of 4 animals examined 8 and 9 days after inoculation. The lesion was characterized by the deposition of large amounts of granular eosinophilic material in Bowman's space and the convoluted tubules. In some instances the tubular epithelium was fragmented, coarsely granular, or entirely absent leaving a denuded basement membrane. No inflammatory reaction was associated with the renal lesions.

Examination of the heart, lungs, thymus gland, spleen, and brain failed to reveal any abnormality. The livers of animals with severe myopathy had markedly dilated sinusoids filled with granular eosinophilic material similar to that noted in the renal tubules and in scattered blood vessels throughout the animal. The hepatocellular elements were normal.

Pathogenicity of naturally occurring strains of Coxsackie A-21 virus for suckling mice. Four strains of naturally occurring Coxsackie A-21 virus (Camp Lejeune strains 1–4), selected at random from those recovered during

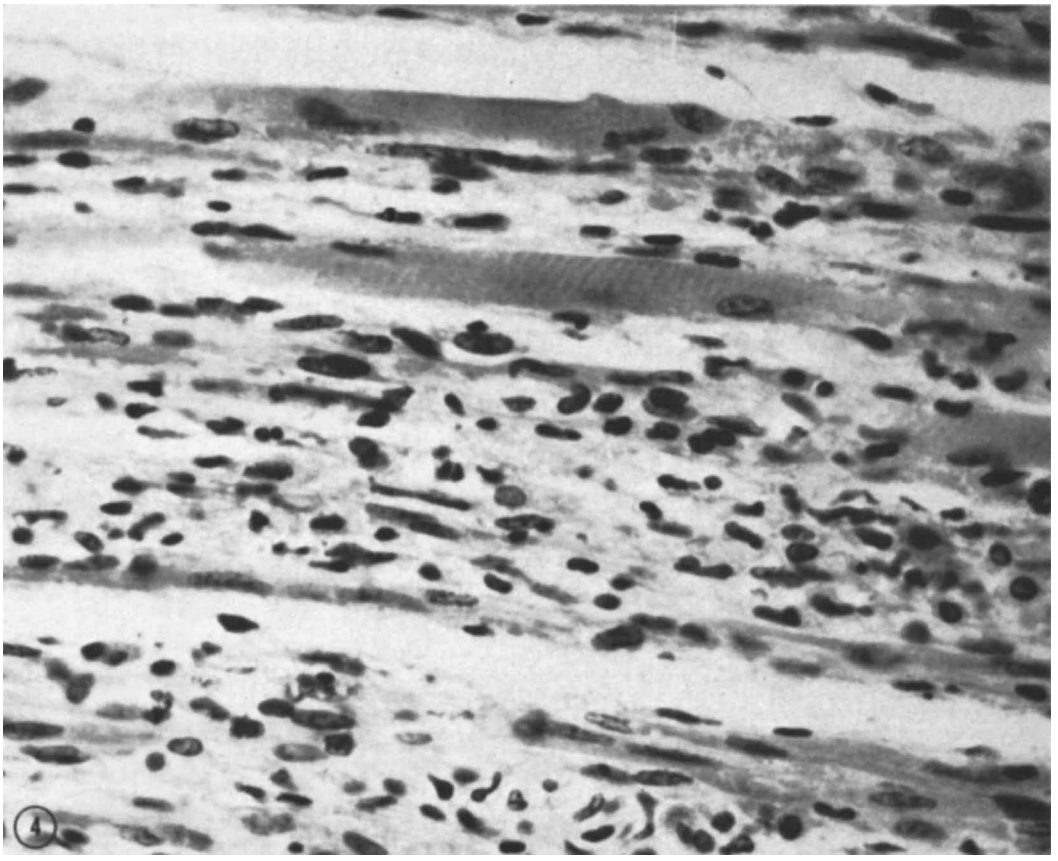

FIG. 4. Section through hind limb skeletal muscle of a Swiss strain newborn mouse 8 days after intraperitoneal inoculation with Coxsackie A-21 virus. Note advanced degeneration of muscle fibers, infiltration with histocytic elements, and proliferation of sarcolemmal nuclei; hematoxylin and eosin; ×520.

the outbreak of upper respiratory illness at Camp Lejeune in 1960, were titrated simultaneously in suckling mice and HEp-2 cell cultures. In this experiment original throat swab fluids were used as the source of naturally occurring virus. Two animals at each dilution were sacrificed 6 days after inoculation (because in the previous experiment it had been shown that maximum virus titers were reached by this day) and the quantity of virus in the pooled carcass suspensions determined by titration in rotated HEp-2 cell cultures. The remainder of the animals in each litter were examined daily for 18 days. Only the 2 strains of Coxsackie A-21 virus (Camp Lejeune strains 1 and 2) which had infectivity titers for rotated HEp-2 cell cultures greater than $10^{2.7}$ TCD_{50} per 0.1 ml multiplied in mice (Table II). In no instance was the quantity of virus recovered per mouse greater than $10^{3.7}$ TCD_{50}, and none of the animals developed histologic evidence of myopathy or paralysis. For the 2 strains (Camp Lejeune strains 1 and 2) of Coxsackie A-21 virus which multiplied in suckling mice, the virus yield was apparently the same regardless of the inoculum size. The other 2 strains of virus did not multiply in mice despite the inoculation of a quantity of virus which successfully initiated infection with Camp Lejeune strains 1 and 2.

The effect of serial tissue culture passage of the mouse pathogenicity of Coxsackie A-21 virus. To ascertain the effect on mouse pathogenicity of Coxsackie A-21 virus after serial tissue culture passage, tenfold dilutions

TABLE II. Multiplication of Naturally Occurring Coxsackie A-21 Virus Strain in Suckling Mice.[a]

Strain	Suckling mouse inoculum: Dilution of throat swab fluid	Suckling mouse inoculum: $Log_{10}TCD_{50}$ per 0.1 ml determined in HEp-2 cultures	Quantity of virus recovered from mice on day 6[b] (log_{10} TCD_{50} per mouse)
1	10^{0}	3.9	3.7
	10^{-1}	2.9	3.4
	10^{-2}	1.9	3.7
	10^{-3}	0.9	<1.0
2	10^{0}	2.7	3.4
	10^{-1}	1.7	3.4
	10^{-2}	0.7	<1.0
3	10^{0}	1.9	<1.0
4	10^{0}	1.9	<1.0

[a] There was no paralysis or histologic evidence of myopathy.
[b] Determined by titration in HEp-2 cultures.

of 4 strains of virus in the third or fourth (early) and tenth (late) passage in HEK were each inoculated into 8 suckling mice. Two mice from each group were harvested 6 days after inoculation and infectivity titers were determined in rotated HEp-2 cell cultures (Table III). Paralysis tended to occur in those mice inoculated with undiluted tissue culture harvests of Coxsackie A-21 virus. In most instances the virus yield from these animals was $10^{6.7}$ TCD_{50} per mouse or greater. When virus multiplication occurred in animals inoculated with tenfold dilutions of virus, the yield was usually less than $10^{6.7}$ TCD_{50} per mouse, and paralysis did not develop. Serial passage in HEK cell cultures failed to alter the mouse virulence of the four strains tested. Similarly, passage of Camp Lejeune strain 1 in HEp-2 cell cultures did not result in the selection of virus virulent for mice (Table IV).

Two strains of Coxsackie A-21 virus (Camp Lejeune strains 1 and 5) were passaged 5 to 6 times in suckling mice and the quantity of virus present in animals harvested 6 days after inoculation was determined. Only 1 of the 2 strains showed a definite increase in mouse pathogenicity (Table IV).

Discussion. Using recent isolates of Coxsackie A-21 virus, the quantitative relationships between inoculum dose, extent of virus multiplication, and the production of paralysis in suckling mice have been investigated. The development of paralysis and a degenerative myopathy in suckling mice was shown to be directly related to the level of virus multiplication after inoculation with tissue culture passaged strains of Coxsackie A-21 virus. Paralysis usually developed in mice from whom $10^{6.7}$ TCD_{50} or greater of virus was recovered. Although virus multiplication occurred in animals inoculated with $10^{6.0}$ TCD_{50} or less per mouse, the quantity of virus recovered was usually less than about $10^{6.7}$ TCD_{50} per mouse and paralysis did not develop. Naturally occurring strains of Coxsackie A-21 virus appeared to vary in their infectivity for mice. Two strains initiated infection when $10^{1.7}$ to $10^{1.9}$ TCD_{50} was inoculated, whereas two other strains did not.

Lennette and co-workers (10), and Pereira and Pereira (12) did not observe paralysis in suckling mice inoculated with several strains of Coxsackie A-21 virus. These differences may be due in part to the use in our experiments of a high titered inoculum of virus to infect suckling mice. Lennette and co-workers (10) and Pereira and Pereira (12) did not report whether or not virus infection occurred without signs of paralysis. Eggers and Sabin (4) also demonstrated that high multiplicities of ECHO-9 virus produced paralysis in suck-

TABLE III. Quantity of Coxsackie A-21 Virus Required to Paralyze Suckling Mice: Effect of Passage in Tissue Culture.

Strain	Passage level	Suckling mouse inoculum: Dilution of tissue culture fluid	Suckling mouse inoculum: $Log_{10} TCD_{50}$ per 0.1 ml determined in HEp-2 cultures	Quantity of virus recovered from mice on day 6 (log_{10} TCD_{50} per mouse)	No. of animals paralyzed/no. inoculated
1	HEK_4	10^0	6.2	8.2	7/8
		10^{-1}	5.2	6.7	1/8
		10^{-2}	4.2	6.7	0/8
		10^{-3}	3.2	6.2	0/8
		10^{-4}	2.2	5.2	0/8
		10^{-5}	1.2	0.7	0/8
		10^{-6}	0.2	<0.7	0/8
	HEK_{10}	10^0	6.7	7.2	8/8
		10^{-1}	5.7	6.7	0/8
		10^{-2}	4.7	6.2	0/8
		10^{-3}	3.7	4.7	0/8
		10^{-4}	2.7	4.2	0/8
		10^{-5}	1.7	<0.7	0/8
		10^{-6}	0.7	<0.7	0/8
2	HEK_3	10^0	6.4	6.2	0/8
		10^{-1}	5.4	5.7	0/8
		10^{-2}	4.4	5.7	0/8
		10^{-3}	3.4	3.7	0/8
		10^{-4}	2.4	1.7	0/8
		10^{-5}	1.4	<0.7	0/8
		10^{-6}	0.4	<0.7	0/8
	HEK_{10}	10^0	6.4	7.7	8/8
		10^{-1}	5.4	6.2	1/8
		10^{-2}	4.4	5.2	0/8
		10^{-3}	3.4	5.2	0/8
		10^{-4}	2.4	2.7	0/8
		10^{-5}	1.4	<0.7	0/8
		10^{-6}	0.4	<0.7	0/8
3	HEK_3	10^0	6.7	6.7	4/8
		10^{-1}	5.7	6.7	0/8
		10^{-2}	4.7	6.7	0/8
		10^{-3}	3.7	5.2	0/8
		10^{-4}	2.7	4.2	0/8
		10^{-5}	1.7	4.2	0/8
		10^{-6}	0.7	2.7	0/8
		10^{-7}		<0.7	0/8
	HEK_{10}	10^0	6.4	7.2	8/8
		10^{-1}	5.4	7.2	3/7
		10^{-2}	4.4	5.2	0/8
		10^{-3}	3.4	5.7	0/8
		10^{-4}	2.4	4.7	0/8
		10^{-5}	1.4	<0.7	0/8
		10^{-6}	0.4	<0.7	0/8

TABLE III (continued)

Strain	Passage level	Suckling mouse inoculum: Dilution of tissue culture fluid	Suckling mouse inoculum: $Log_{10}TCD_{50}$ per 0.1 ml determined in HEp-2 cultures	Quantity of virus recovered from mice on day 6 (log_{10} TCD_{50} per mouse)	No. of animals paralyzed/no. inoculated
5	HEK_1	10^0	6.4	6.7	8/8
		10^{-1}	5.4	6.2	0/8
		10^{-2}	4.4	5.2	0/8
		10^{-3}	3.4	4.2	0/8
		10^{-4}	2.4	4.2	0/8
		10^{-5}	1.4	<0.7	0/8
		10^{-6}	0.4	<0.7	0/8
	HEK_{10}	10^0	6.4	7.2	8/8
		10^{-1}	5.4	6.2	0/8
		10^{-2}	4.4	5.2	0/8
		10^{-3}	3.4	4.2	0/8
		10^{-4}	2.4	4.2	0/8
		10^{-5}	1.4	<0.7	0/8
		10^{-6}	0.4	<0.7	0/8

ling mice, whereas low multiplicities infected the animals but failed to cause paralysis. In their experiments the titer of virus recovered from paralyzed mice was generally equal to or greater than 10^8 TCD_{50} per mouse.

Multiple passages of several strains of Coxsackie A-21 virus in HEK cell cultures did not alter the pathogenicity of the virus for suckling mice. Eggers and Sabin (4) demonstrated that serial passage of ECHO-9 virus in primary cynomologous monkey kidney cell culture did not apparently result in selection of virus particles with enhanced mouse virulence because the development of paralysis was also directly proportional to the inoculum dose. Lehmann-Grube and Syverton (9) demonstrated that serial passage of several Coxsackie group A and B viruses in primary human amnion cell cultures resulted in the loss of pathogenicity for suckling mice. These investigators postulated that passage in human amnion cells selected particles which were genetically different.

TABLE IV. Quantity of Coxsackie A-21 Virus Required to Paralyze Suckling Mice: Effect of Passage in Tissue Culture and Mice.

Strain	Passage level	Log_{10} per ml: TCD_{50} for rotated HEp-2 cultures	Log_{10} per ml: PD_{50} for mice
1	HEp_1[a]	5.2	1.5
	HEp_{10}	8.2	1.8
	HK_3[b]	8.2	2.3
	HK_1SM_5[c]	7.2	2.4
5	HK_3	7.7	2.4
	HK_{10}	8.2	1.5
	HEp_2	7.7	2.5
	HEp_2SM_6	7.2	5.2

[a] HEp-2 tissue culture passage.

[b] Human embryonic kidney culture passage.

[c] Human embryonic kidney culture passage and suckling mouse passage.

It has been suggested that paralysis in suckling mice might not be a valid parameter for the classification of Coxsackie group A viruses. Along this line, other investigators have noted the failure of some naturally occurring strains of Coxsackie A-21 virus to produce paralysis in suckling mice (10, 12, 17). In our experiments, virus multiplication was detected in mice inoculated with 2 of 4 naturally occurring strains of Coxsackie A-21 virus, although paralysis failed to develop. In those instances in which suspected new strains of Coxsackie group A viruses fail to cause paralysis in suckling mice, the demonstration of virus multiplication in mice might provide additional taxonomic evidence.

The histopathology of the lesions associ-

ated with Coxsackie type A virus infection in young mice has been described by several investigators (1, 3, 5, 6, 8). The pathologic changes are generally restricted to the skeletal musculature of the lower extremities and the lesion is a myopathy which may be grouped under the category of Zenker's hyaline or waxy degeneration. The distribution of the lesion may be a function of a relative predilection of Coxsackie A-21 virus for the limb musculature or it may be related to the amount of virus present at any given time. Only following proliferation of the virus in the limb musculature was there apparently sufficient virus to cause pathologic lesions in other sites.

In animals with severe and widespread muscle degeneration, large quantities of circulating, esosinophilic granular material were noted in the blood vessels of most organs. Deposits of a similar staining material could be detected in Bowman's space and renal tubules. The most likely source of this material was the sarcoplasm of the degenerating skeletal muscle. The renal lesion was similar to that associated with a crush injury (myoglobinuric nephrosis). Gadeke (5) also noted lesions of this type in suckling mice following Coxsackie group A infection. The finding of a focal pancreatitis in one animal with severe myositis is unexplained. Although lesions of the pancreas have been described in mice infected with Coxsackie group B viruses such lesions have not been noted in infection with group A strains (6, 8).

Summary. The production of a myopathy and paralysis in mice inoculated with tissue culture passaged Coxsackie A-21 virus was directly related to the inoculum dose and extent of virus multiplication. The virus yield from paralyzed mice was usually about $10^{6.7}$ TCD_{50} or greater per mouse. Four strains of naturally occurring virus did not produce paralysis of suckling mice but virus multiplication occurred in mice with 2 of the 4 strains. Serial passage of Coxsackie A-21 virus in HEK cell cultures did not alter its virulence for suckling mice. The histopathologic changes associated with Coxsackie A-21 virus infection in mice were characterized by a waxy degeneration of the skeletal musculature which was generally confined to the lower extremities.

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