AGE AND SUSCEPTIBILITY OF MICE TO COXSACKIE A VIRUSES*

BY A. MARTIN LERNER,‡ M.D., HOWARD S. LEVIN, M.D., AND MAXWELL FINLAND, M.D.

(From the Thorndike Memorial Laboratory, Second and Fourth (Harvard) Medical Services and Mallory Institute of Pathology, Boston City Hospital and the Department of Medicine, Harvard Medical School, Boston)

PLATE 60

(Received for publication, November 8, 1961)

The Coxsackie viruses were first characterized by their ability to produce illness and death regularly in suckling mice and their failure to affect older mice (1). Infection in the newborn mouse is lethal only if initiated during the first 10 days of life with Coxsackie A viruses, or during the first 48 hours after birth with Coxsackie B viruses (2). Human adults, on the other hand, are frequently infected with Coxsackie viruses although the resulting illnesses are usually less severe than in infants and children (3–5). The increasing numbers of reports of the great variety of manifestations of Coxsackie virus infections in humans would seem to indicate that they have much in common with those described in suckling mice (5). However, whereas infections with Coxsackie B viruses in adult mice have been described, very little has been reported about the effects of Coxsackie A viruses in the adult mouse. The present report deals primarily with host-virus relationships in mice of various ages infected with Coxsackie A virus, type 9. These investigations were stimulated by recent studies of an outbreak of infections with this virus affecting both adults and children (3).

Materials and Methods

Mice.—Swiss mice of the Webster[1] or Hauschka and Marand Institute of Cancer Research[2] strains and ranging in age from birth to 8 months were used.

Viruses.—Three strains of Coxsackie A virus were employed:

Type 9, Strain 13 was isolated in monkey (rhesus) kidney tissue culture (MKTC) in this laboratory from the lung of a 16 month old girl who died of interstitial pneumonia (3); it was used in primary and first MKTC passage.

Type 9, Strain NIH-1 was originally received in this laboratory by Dr. Christo-

* Aided by a grant (E-1695) from the National Institutes of Health.

‡ Research Fellow of the Medical Foundation, Inc.

[1] Obtained from New Animal Farm, Harvard Medical School.

[2] Supplied by Charles River Breeding Laboratories, Inc., Boston.

pher M. Martin from the Laboratory of Infectious Diseases, National Institutes of Health in March 1956 and was used after 3 additional passages in MKTC. *Type 4, High Point strain* (6) was obtained from Dr. Leon Rosen of the same laboratory as a 20 per cent mouse leg suspension and was used in this laboratory as a similar suspension after one additional passage. Strains 13 and NIH-1 were cytopathogenic in MKTC but the High Point strain was not; all 3 strains produced flaccid paralysis and death on intraperitoneal (i.p.) inoculation in suckling mice.

Neutralization Tests.—Conventional MKTC tube tests, as in previous studies (3), or the immunoinactivation modification (7) of this test (IA) which was found to enhance the titers 2- to 4-fold, were used in the assays of antibody to the Coxsackie A9 viruses. Protection tests (8) in suckling mice were used in the assays of antibody to the Coxsackie A4 virus.

Tissue Culture and Titration of Viruses.—Reisolations and titrations of Coxsackie A type 9 strains were done in MKTC[3] using Eagle's basal medium (9) to which 5 per cent horse serum and antibiotics (250 units of penicillin, 250 μg of streptomycin and 100 units of nystatin per ml.)[4] were added. Hanks' balanced salt solution (BSS) containing antibiotics were used for suspensions and dilutions. Cultures were examined for cytopathic effects (cpe) every other day for 14 days and the medium was replenished at 5 day intervals. Isolated viruses were identified by neutralization procedures (10).

Tissues were minced, ground with alundum, and 20 per cent suspensions were made in BSS. Tissue minces from animals in which viremia had been demonstrated were first washed 5 to 7 times in 10 ml amounts of BSS before grinding; the fluid of the last wash was then assayed along with the final tissue suspension and the difference in amount of virus found in these assays was taken to indicate the titer of virus in the tissue free of virus from blood.

All tissues, suspensions, and fluids for assay were kept at $-20°C$ until used. For the viral titrations of Coxsackie A9, 0.1 ml amounts of 10-fold dilutions of the thawed materials were each inoculated into 3 MKTC tubes and the infectious titer estimated by the 50 per cent endpoint (11). Similar materials from mice infected with the Coxsackie A4 strain were inoculated in 0.05 ml amounts i.p. in mice less than 1 day old, using 2 litters of 8 to 10 mice for each titration; the mice were observed daily for 3 weeks.

Experimental Infections.—Groups of 30 to 60 mice of the same age, and often, in the case of older animals, also of the same sex (mostly female) were used for each experiment. Infection was produced by i.p. inoculation of 0.05 ml of suspension containing approximately 10^5 TCD_{50}[5] or LD_{50} for suckling mice.

[3] Obtained from Microbiological Associates (MBA), Bethesda.

[4] Antibiotics in these concentrations were used in all tissue culture media and solutions, and twice these concentrations were used in suspension media for storage of specimens.

[5] TCD_{50} is the amount of virus that produces cpe in 50 per cent of inoculated tubes of MKTC.

The mice were observed for signs of illness daily for 21 days. At regular intervals beginning on the 4th day, groups of 6 mice were sacrificed. They were lightly anesthetized with ether and exsanguinated by partially skinning, then cutting deeply into the axilla, and collecting the blood that accumulated between the skin and thoracic cage. Serum thus obtained was used for neutralization tests. Two of the mice were skinned and then fixed *in toto* in 10 per cent formalin for histologic studies; the others were autopsied and pools of the hind limbs, hearts, blood[6] and, in some instances, feces (rectal swabs)[7] were collected in separate screw-capped vials and stored at −20°C. Randomly selected specimens of limbs, heart, or blood were used to reisolate and identify the virus. There was no evidence of contamination in the laboratory with these or other viruses during the course of these studies, appropriate positive and negative controls being included in each experiment.

Pathology.—The formalin-fixed tissues from one or both of the mice of each age group and each time interval after inoculation were examined microscopically. Tissues of uninoculated mice obtained soon after birth, and at 10, 20, 40, and 50 days and 8 months old were also studied in the same manner. In the suckling mice, transverse sections were prepared at the level of the tongue, shoulder girdle, heart, upper and lower abdomen, and pelvic girdle, and additional sections were made of brain, neck, and fore and hind limbs (12). The older mice were dissected and in addition to transverse sections cut at the level of the tongue and heart, sections were made from fore and hind limbs, liver, kidney, pancreas, spleen and intestine. Sections of stomach were obtained from animals that were inoculated with Coxsackie A9, strain 13, at 20 days, 40 days, and 8 months of age. All sections were embedded in paraffin and stained with hematoxylin and eosin.

RESULTS

Infections of Mice at Various Ages with Coxsackie A9 virus, Strain 13.—As shown in Table I, suckling mice uniformly succumbed to the i.p. inoculations, whereas none of the older mice showed evidence of illness. The virus, however, was uniformly isolated from hind limbs of inoculated mice of all ages, and from the hearts of the newborns as well as of those 40 days or 8 months old at the time of inoculation. In these experiments, assays of the tissues for viral content indicated that the hind limbs of younger mice (those inoculated 20 days or less after birth) had 100 to 1000 times as much virus as those of the older animals.

Microscopic sections, which will be described in greater detail below, showed

[6] Residual cardiac blood was collected and defibrinated in 10 volumes of fluid consisting of BSS, 1 per cent skimmed milk and antibiotics.

[7] These were each placed in 1 ml of the same fluid as that used for blood.

diffuse myositis in the suckling mice and only focal lesions of striated muscle in the older ones. No lesions were seen in the sections of the 40-day-old mice that were examined although Coxsackie A9 virus was isolated from hind limbs and heart in these animals. In the 8 month old mice, replication of virus in the heart was associated with the demonstration of acute myocarditis with subsequent healing.

Tests for neutralizing antibody, done by immunoinactivation with serum of surviving animals of each age 21 days after inoculation, showed a titer of 1:80.

TABLE I

*Infections in Mice of Different Ages with Coxsackie A Virus, Type 9, Strain 13**

Age of mice‡	Virus isolation§		Abnormal tissue		Antibody‖ in serum 21 days after inoculation	No. died / No. inoculated	Pathological findings
	Hind limb	Heart	Striated muscle	Heart			
A. Sucklings							
<24 hrs.	+	+	+	0	—	21/21	Acute myositis
10 days	+	—	+	0	—	21/21	" "
B. Others							
20 days	+	—	+	0	80(20)	0/21	Myositis with regeneration
40 "	+	+	0	0	80	0/21	None
8 months	+	+	+	+	80	0/21	Focal myositis and myocarditis
C. Uninoculated							
All ages¶	0	0	0	0	<10	0/21	None

* Isolated from lung of a fatal case of pneumonia and used as first passage in MKTC; each mouse received about 10^5 TCD_{50} in 0.05 ml.

‡ At time of inoculation.

§ +, positive, 0, negative; —, not attempted.

‖ Reciprocal of immunoinactivation titer (neutralizing titer in tubes shown in parentheses).

¶ Includes mice of each of the same ages used for infection.

Uninoculated mice of the same ages showed no evidence of illness, yielded no virus, showed no microscopic lesions, and their serum failed to neutralize the virus.

This experiment indicated that mice of all ages were susceptible to Strain 13 of Coxsackie A9 virus, but that subclinical infection and recovery with development of antibody occurred in those infected at 20 days of age or later. It also showed that the virus replicated in the heart of 8 month old mice and produced myocarditis in them. The effect of this myocardial involvement on longevity in these mice was not studied. In the 40-day-old animals, the virus apparently multiplied in the heart (10^2 TCD_{50} per ml. of the 20 per cent suspension after 48 hours) but foci of myocarditis were not seen.

Infections with Strain 13 in Suckling Mice: Relation of Age and Antibody to Susceptibility.—The results of the i.p. inoculations in suckling mice at various ages are summarized in Table II. Groups of 3 litters were used for each of 4 age levels and the mice were observed for 7 days, noting the numbers that died

TABLE II

*Relation of Age to Incubation Period and Antibody Production in Suckling Mice Infected with Coxsackie A Virus, Type 9**

Age of mice at time of inoculation	Litter No.	No. of mice surviving 24 hrs. after inoculation‡	Days after inoculation					
			2	3	4	5	6	7
days								
2	1	7	2§	5				
	2	9	3	6				
	3	8	3	1	3	1		
5	1	7	3	0	4p	4		
	2	8	0	0	0	3, 5p	5	
	3	9	0	0	0	5, 4p	4	
10	1	9	0	0	9p	9		
	2	8	0	0	8p	8		
	3	7	0	0	2, 3p	3	0	0‖
14	1	10	0	0	0	0	0	0
	2	10	0	0	0	0	0	0
	3	10	0	0	0	0	0	0
Antibody titer¶.....			—	—	<10	—	—	160

* Strain 13 (see Table I, footnote *).

‡ Deaths occurring in first 24 hours after inoculation were considered nonspecific.

§ No. of mice that died; the number surviving but manifesting paralysis are indicated by "p."

‖ 2 mice survived without symptoms in litter 3 of the 10-day-old group, as did all that were not sacrificed on days 4 and 6 among those inoculated when 14 days old.

¶ Reciprocal of immunoinactivation titer in 14 day old mice sacrificed for this purpose; —, not done.

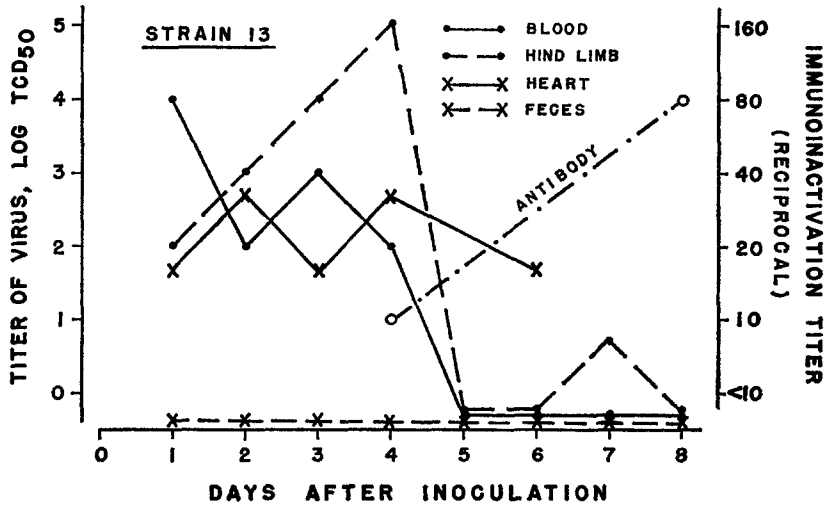
or showed evidence of flaccid paralysis each day.[8] Serum was collected for neutralization tests from groups of 14-day-old mice 4 and 7 days after the i.p. injections of the virus.

The 2-day-old mice all died and the average survival time after injection was 3 days. Animals inoculated on the 5th or 10th day after birth showed evidence of paralysis or died on or before the 5th day after infection and 2 in the

[8] The usual order of appearance of muscular paralysis in mice infected with Coxsackie A virus is: pelvic, abdominal, vertebral, intercostal and finally scapular (12).

10-day-old group survived. None of the mice inoculated at 14 days of age died or showed any evidence of illness. Thus, the disease was most virulent in the youngest mice. Antibodies were not detected in the serum by IA tests on the 4th day but were present in a titer of 1:160 on the 7th day. The fatal infections, therefore, developed in the susceptible mice before antibody could be demonstrated.

Infections with Strain 13 in 31-Day-Old Mice.—Text-fig. 1 shows the findings in 31-day-old mice that were inoculated i.p. with this strain of Coxsackie A9 virus. Viral assays were done on blood, skeletal muscle, heart, and feces of



TEXT-FIG. 1. Coxsackie A-9 virus infection in 31-day-old mice.

groups of animals sacrificed each day for 8 days. Neutralizing antibody was determined by IA in other mice that were sacrificed 4 and 8 days after the inoculations. None of these mice showed evidence of illness. A high titer of Coxsackie A9 virus (10^4 TCD_{50} per ml) was found in the blood 1 day after inoculation and the viremia was maintained through the 4th day but could no longer be demonstrated on the 5th day. The amount of virus in hind limb preparations increased steadily for 4 days and reached a titer of 10^5 TCD_{50} per gm. From the 5th day on, virus could no longer be demonstrated in the limb except in a barely detectable titer on the 7th day. Virus was demonstrated in the heart from the first through the 6th day, but the titers during the first 4 days were lower than in the limb. In contrast to the latter, the heart still had a moderate amount of virus 6 days after inoculation and 2 days after antibody had already been demonstrated in the serum. Virus was not detected in feces obtained by rectal swab at any time. By the 8th day, virus could not be demon-

strated in any of the materials tested. Neutralizing antibody was found (in a titer of 1:10) in pooled serum obtained at the end of the 4th day and increased to a titer of 1:80 in the serum obtained 8 days after infection. The abrupt fall in titer of virus in the blood and limbs was thus associated with the appearance and rise in titer of neutralizing antibody.

Coxsackie A9 Infection in 7-Month-Old Mice.—An experiment of similar

TABLE III

*Infection of 7-Month-Old Mice with Coxsackie A Virus, Type 9, Strain 13**

Days after inoculation	TCD_{50} of virus		
	Heart	Hind limb	Blood
2	10^3	0	0
3	10^4	5	0
4	10	5	0
6	$10^{2.5}$	5	0
7	10	5	0
8	0	0	0

* Each mouse received 10^5 TCD_{50} i.p.

TABLE IV

Summary of Observations in Adult Mice Inoculated Intraperitoneally with NIH-1 (Type 9) and High Point (Type 4) Strains of Coxsackie A Virus

	NIH-1	High Point
Age of mice, *days*	33	40
Pathological findings	Acute myositis	None
Virus isolation (from hind limb)	+	0
Antibody in serum (21 days after inoculation)	1:20*	<1:10‡
Manifest illness	None	None

* Immunoinactivation titer.

‡ Protection titer in suckling mice.

design but limited in scope to assays for viral content of blood, hind limbs, and heart was carried out with Strain 13 in 7-month-old mice. The results are shown in Table III. By the method used, viremia was not detected at any time, and only small amounts could be demonstrated in the hind limbs of these animals between the 3rd and 7th days. By contrast, the hearts of these older animals yielded moderate to high titers of virus. The apparently marked susceptibility of the myocardium and relatively low susceptibility of striated muscle in the older animals is of particular interest in relation to the pathological findings.

Susceptibility of Adult Mice to Other Coxsackie A Viruses.—In order to

determine whether other Coxsackie A viruses behave in adult mice like strain 13, an experiment was carried out with two other strains,—the NIH-1 strain of type 9, and the High Point strain of type 4,—using groups of mice 33 days old and 40 days old, respectively. The results are summarized in Table IV.

None of these animals showed any signs of illness. The mice inoculated with the NIH-1 strain of Coxsackie A9 virus behaved like those of similar age infected with Strain 13; their hind limbs yielded infectious virus and showed acute myositis, and neutralizing antibody was demonstrated in their serum. In the mice infected with the High Point strain of Coxsackie A4, on the other hand, the virus could not be recovered from the hind limbs, lesions could not be demonstrated in striated muscle of these limbs, and protective antibody was not detected in the serum 3 weeks after inoculation of the virus. The insusceptibility of these adult mice to this herpangina strain thus may involve some other mechanism.

Pathological Findings

Although all major organs including brain were examined histologically, the abnormal findings in mice of all ages infected with Coxsackie A9 viruses were limited to skeletal muscles except for the myocarditis noted in the 8-month-old mice. The lesions of skeletal muscle varied histologically with the age of the animal and the duration of the infection. The findings will therefore be described for each group since lesions have rarely been observed in non-suckling animals infected with Coxsackie A viruses.

Newborn Mice (Strain 13).—Suckling mice inoculated with strain 13 on the 1st day of life (Day 0) showed signs of weakness, instability, and decreased motility by day 2 or 3. By the next day, the animals showed hyperextension of hind limbs, increased weakness, and shallow breathing. Most animals died by the end of day 4. Early microscopic changes were present on day 3. At this time scattered groups of muscle fibers showed eosinophilic smudging, swelling, and focal necrosis with varying degrees of infiltration by mononuclear and polymorphonuclear inflammatory cells. Variation in muscle group involvement was considerable with the most severe involvement evidenced in the shoulder girdle (Fig. 1). By day 4 more muscle groups were involved and showed increased necrosis of fibers, characterized by loss of cross-striation, fragmentation, loss of sarcoplasm and markedly increased inflammatory infiltration.

10-Day-Old Mice (Strain 13).—Animals inoculated at this age produced lesions identical with those described above. Focal and diffuse lesions were present on day 4, and by day 5 muscle involvement was more uniformly diffuse. In areas of necrosis, spindle-shaped basophilic cells were prominent. All animals were dead by the end of day 5 (Fig. 2).

Twenty-Day-Old Mice (Strain 13).—In contrast to the previous groups, animals inoculated at 20 days of age showed no objective signs of illness, but

histologically there was an evolving myositis. Animals were first examined histologically on day 3. Focal areas of involved muscle showed moderate interstitial edema, eosinophilic smudging of muscle fibers, and loss of sarcoplasm. Within some necrotic fibers were polymorphonuclear leucocytes and histiocytes. These cells were also present in the interstitium. Basophilic spindle-shaped sarcolemmic cells were numerous in some areas (Fig. 4). On day 6, areas of myositis were more numerous and necrosis more severe. Segments of some muscle fibers contained larger numbers of inflammatory cells which, in some cases, distended the sarcolemmic sheaths. Segments of other fibers in affected areas appeared as empty tubes lined with basophilic sarcolemmic nuclei (Fig. 3).

In most of the muscles examined, only a few fibers were affected, but in the hind limb large and diffuse areas of myositis were seen. The predominant inflammatory cells varied from area to area but consisted chiefly of polymorphonuclear leucocytes or histiocytes. On day 8 only a single focus of involved muscle was identified. In this area there was loss of muscle fibers, fibrosis, and a moderately dense infiltration by histiocytes and lymphocytes. On day 11 there were foci of basophilic muscle fibers containing vesicular nuclei, some of the fibers arranged in rows. Between some fibers were occasional lymphocytes and histiocytes (Fig. 5). The sections available from the mouse sacrificed on day 13 showed no abnormal lesions. On day 16 individual fibers were noted to contain histiocytes, occasional polymorphonuclear leucocytes and lymphocytes. In addition, fusiform swellings containing granular or clumped basophilic substance were found, but their exact nature and their location in relation to muscle fibers could not be determined. Around some of these nodules there appeared to be some basophilic, pyknotic nuclei. No histologic alterations were noted on day 20.

40-Day-Old Mice (Strain 13).—No muscle or visceral lesions were demonstrable histologically.

8-Month-Old Mice (strain 13).—These mice, in contrast to those of other groups showed both skeletal and myocardial lesions although they manifested no signs of illness.

Lesions of Skeletal Muscle.—On day 3 two small foci in paraspinal musculature showed fragmentation and shrinkage of muscle fibers in the presence of basophilic spindle cells and interstitial infiltration by mononuclear and occasional polymorphonuclear inflammatory cells. No muscle lesions were noted in the materials obtained on day 6. On Day 9, however, there was involvement of a large area of hind limb muscle and adjacent fat. The muscle fibers were necrotic with infiltration of both fibers and interstitium by polymorphonuclear leucocytes. Sarcolemmic nuclei were spindled and basophilic (Fig. 6). No skeletal muscle involvement was demonstrated in animals examined on days 13, 16, or 21.

Myocardial Lesions.—The myocardium of the 8 month old mice examined on

day 3 appeared normal. On day 6, however, the hearts showed scattered foci of myocardial involvement. In these areas there was rupture and fragmentation of myocardial fibers, slight hemorrhage, and infiltration by polymorphonuclear leucocytes, lymphocytes, histiocytes, plasma cells, and occasional large mononuclear cells (Fig. 7). On day 9 small foci of myocardial fibers were necrotic. These fibers showed loss of sarcoplasm and infiltration by inflammatory cells, chiefly histiocytes. No cardiac lesions were demonstrated in the animals examined on day 13. Of two animals examined on day 16 one showed no myocardial lesions, whereas the other showed small areas of patchy interstitial myocardial fibrosis. In these areas there were scattered lymphocytes, occasional plasma cells and histiocytes (Fig. 8).

33-Day-Old Mice Infected with NIH-1 Strain of Coxsackie A9.—Animals were examined on days 3, 6, 9, 13, and 17, but only a single focus of myositis was demonstrated; the animal examined on day 9 showed involvement of a single diaphragmatic muscle fiber over a segment of which there was loss of sarcoplasm, spindling of sarcolemmic nuclei, and distention of the fiber by infiltrating lymphocytes and polymorphonuclear leucocytes. Adjacent muscle fibers were unremarkable.

Infection of 40-Day-Old Mice with High Point Strain of Coxsackie A4.—No muscle or visceral lesions were demonstrable.

DISCUSSION

The discovery of the Coxsackie viruses was made possible by the marked susceptibility of suckling mice to these agents (13). The greater susceptibility of young animals has also been noted with other viruses (14–20). Various factors have been considered as contributing to this effect of age. One is the influence of age on antibody formation. Freund (21), in 1930, showed that the formation of agglutinins against typhoid bacilli, hemolysins against sheep cells, and precipitins against horse serum and egg white was strikingly less intense in rabbits under 20 days old than in adult rabbits. Baumgartner (22) suggested that similar differences might explain the greater susceptibility of the young to certain infectious diseases. Overman and Kilham (19) also related the changes in resistance of hamsters against mumps virus with age to the maturation of the antibody forming mechanism.

Certain poorly understood, non-specific or localized cellular barriers have been implicated in resistance to infection and perhaps also in the increasing resistance with age on primary exposure to infectious agents (17). Thus, the physiologic state of the host cells or some factors in their environment may influence the multiplication of certain bacteriophages in several B/4 mutants of *Escherichia coli* (23), the multiplication of mature animal viruses in tissue culture cells (24), or the appearance of cellular degeneration once virus multiplication has occurred (25). Holland and McLaren (26) showed that the

susceptibility of HeLa and other cells depends on the ability of such cells (or their insoluble debris) to adsorb, receive and eclipse poliovirus. This function was postulated to reside in organized cytoplasmic lipoprotein structures not possessed by insusceptible cells. Kunin and Jordan (27) extended this hypothesis to poliovirus infection *in vivo* and Kunin (28) showed that the relative abundance of these specific viral receptors for Coxsackie B viruses in mice decreased as the mice matured.

Choppin and Philipson (29, 30) suggested that the reaction between some enteroviruses and erythrocytes or tissue culture cells involves sulfhydryl group interchanges between the reactants, and that these linkages might be the biochemical basis of the initial interaction between specific cell receptors and the viruses. On the other hand, Hsiung and Melnick (31) found that several enteroviruses were adsorbed equally well on *rhesus* and *patas* kidney cells regardless of the degree of susceptibility of these cells to the viruses; the ability to synthesize the adsorbed virus seemed most important in their experiments. The role of interferon, presumably by inhibiting viral synthesis, and its possible role in recovery from viral infections (32), requires further study, but the decreased production and effectiveness of interferons in young tissues (33) is consistent with their greater susceptibility.

In the present study, replication of Coxsackie A9 virus occurred in mice ranging in age from 1 day to 8 months. In the 31-day-old animals, the example shown in Text-fig 1, the virus was adsorbed and replicated to a high titer in the hind limbs and antibody was demonstrated on the 4th day after the i.p. inoculation. In younger (14-day-old) animals antibodies appeared later (Table II). The incubation period, as judged from the time of death or of obvious paralysis, appeared to increase with age but was usually less than 5 days in the suckling mice infected when 10 days old or earlier. In the 40-day-old animals the virus multiplied in the cardiac muscle without producing demonstrable lesions.

By contrast, the observations in the 7- and 8-month-old mice revealed only slight viral multiplication in the hind limb preparations, but large amounts of virus and definite inflammatory changes in the myocardium. The decreased susceptibility of skeletal muscle and increased susceptibility of myocardial tissue to this virus in these animals could have been related either to changes in specific receptor sites at the surface of the cells, or to differences in the ability of these tissues to synthesize the adsorbed Coxsackie A9 virus. The present data do not resolve this problem, nor do they indicate whether this effect is peculiar to this strain.

Experimental infections of adult mice with other Coxsackie viruses have been studied previously. Strains of Coxsackie B viruses produced lesions in mice limited to the pancreas (34). However, exposure of such animals to 4°C (35) or to ionizing radiation (36, 37), or the administration of cortisone (38) converted this localized and only rarely lethal infection to a severe and usually

fatal generalized disease involving the heart and liver in addition to the pancreas. These effects may have been the indirect result of inhibition of antibody synthesis (39) but this has not been definitely established. Dalldorf (40), using a strain of Coxsackie A virus, type 14, that was adapted to adult mice, produced in them extensive poliomyelitis as well as minute foci of muscle degeneration, the latter not unlike those seen here. In humans, myocarditis and pericarditis have been reported in infants and young adults infected with Coxsackie B viruses (5), but have not been observed in infections with Coxsackie A viruses.

Involvement of the entire musculature and the selective susceptibility of suckling mice have thus far been associated primarily with Coxsackie A infections. Howes (2), from calculations of LD_{50} and ID_{50}, concluded that this susceptibility is apparently lost soon after the age of 3 weeks. The lesions of skeletal muscle found here in animals inoculated with strain 13 of Coxsackie A9 at birth, and at 10 and 20 days of age were identical with those described by others (41, 42) in experimental infections with other Coxsackie A viruses. For animals of the first two age groups the diffuse myositis was uniformly fatal; in them the lesions of skeletal muscle were patchy, often segmental and characterized by hyalinization, progressive necrosis, infiltration by inflammatory cells and proliferation of basophilic sarcolemmic cells. In the 20-day-old animals the lesions were similar in the early stages, but in them, recovery was accompanied by a variable evolution of the microscopic picture of the myositis in the later stages. The muscles of the older animals showed various stages of healing, whereas in the younger animals, at the time of death, the skeletal muscles were necrotic and showed diffuse inflammation, relatively scant phagocytosis and only early evidence of regeneration.

Inasmuch as only single animals were usually examined at most intervals, it is not possible to elaborate the details of the degenerative and regenerative sequence within involved fibers. However, in the 20-day-old mice, there was a strong suggestion that the lesions evolved at different rates, some resolving as others were in earlier stages of regeneration. The presence of interstitial inflammatory cells on the 8th and 11th days, regenerative activity on the latter day and a yet unresolved area of inflammation on the 16th day suggests both sequential and "crop-like" involvement.

In mice inoculated at 40 days there was viral multiplication, but inflammatory changes were not seen and neutralizing antibody was demonstrated early. In the 33-day-old mice inoculated with the NIH-1 strain of Coxsackie A9, only a single focus of myositis was found and a low titer of neutralizing antibody was demonstrated in the serum by immunoinactivation. The Coxsackie A4 virus inoculated in 40-day-old mice produced no lesions and no significant titer of protective antibody.

Of particular interest was the finding of myocarditis and myositis in the 8-month-old mice infected with strain 13 of Coxsackie A9. In younger animals, myocarditis was not seen in histologic sections although multiplication of this

virus in the heart was demonstrated. Myocardial lesions have been described in mice infected with Coxsackie B viruses (41–43) and with encephalomyocarditis (EMC) virus (44). The Coxsackie B viruses produced focal necrosis with fragmentation and hyaline degeneration of myocardial fibers, whereas EMC virus produced a more varied picture, the lesions being either focal or diffuse, beginning with mild perivascular monocytic infiltration and progressing to swelling, extensive necrosis, fibrosis, and calcification (44). The cellular infiltrations were predominantly with lymphocytes and histiocytes (45). EMC virus produced lesions of skeletal muscle only if inoculated directly into the muscle, and Coxsackie B virus produced inflammation of both myocardial and skeletal muscle only in baby mice. Thus both of these viruses differed from Coxsackie A9 which in the present study was shown to produce myocarditis and myositis after i.p. inoculation.

A number of workers have reported spontaneous occurrence of cardiac lesions in mice (46–48). These have been variously described as involving epicardium, myocardium, and cardiac valves. Some of the myocardial lesions consisted of interstitial infiltration by mononuclear cells, whereas others have shown degeneration and necrosis of myocardial fibers with infiltration by inflammatory cells, thus resembling the lesion described here. Such lesions were not found among the control animals in the present study. Moreover, the isolation of virus from myocardium and hind limb, the demonstration of myocarditis and myositis, and the development of neutralizing antibody in the serum of the 8-month-old mice infected with Coxsackie A9 virus leave little doubt as to the relation of these lesions to the infection.

In some respects, the myocardial tissue of the young mouse in which small amounts of virus are present without producing lesions, may be said to resemble an *in vitro* carrier culture (49). The low yield of virus from the heart of mice infected at 40 days of age or earlier may be a reflection of a smaller proportion of susceptible myocardial cells as compared with 8-month-old mice. A low proportion of susceptible cells is an essential feature of the *in vitro* carrier culture (50). The brevity of the infection, however, precludes further comparison.

In the case of Coxsackie A9 strains a change occurred with age, in the ability of skeletal and cardiac muscle either to adsorb virus, which involves specific receptor sites and possibly sulfhydryl group interchanges, or to replicate complete virus. No evidence of either viral multiplication or homologous antibody synthesis was obtained after comparable exposure of adult mice to the High Point strain of Coxsackie A4. In the latter system either lack of adsorption or inability to synthesize the virus could have accounted for the insusceptibility of adult mice and hence also the absence of antibody production.

SUMMARY AND CONCLUSIONS

Mice varying in age from 1 day to 8 months were inoculated intraperitoneally with Coxsackie A virus, type 9 and studies were made of the quantity of virus

in striated muscle and myocardium, the presence of neutralizing antibody in the serum, and the pathological changes in the tissues.

The hind limbs of young (1-to 20-day-old) mice yielded high titers of virus and showed diffuse myositis, whereas only low yields of virus and focal myositis were obtained in older mice. In the 20-day-old mice the skeletal lesions were not accompanied by manifest symptoms and histologically showed evidence of regeneration progressing from the 3rd to the 11th day after inoculation. Older mice showed no symptoms and only focal myositis and low yields of virus were found in their hind limbs.

Coxsackie A9 virus replicated to relatively low titers in the hearts of young (1- to 40-day-old) mice without producing any demonstrable lesions whereas frank myocarditis with high yields of virus were demonstrated in mice infected at 8 months of age.

The data suggest that at least for the 2 strains used, the adult mouse should be considered susceptible to subclinical infection with Coxsackie A9 virus. Neither subclinical infection, nor antibody formation was demonstrable in young adult mice inoculated with a strain of Coxsackie A4 virus.

The authors are indebted to Miss Priscilla Bills for technical assistance and to Dr. Harold S. Ginsberg for helpful advice. They are also grateful to Dr. Derek Denny-Brown and Dr. Flaviu Romanu for their help in reviewing the microscopic sections.

BIBLIOGRAPHY

1. Dalldorf, G., The Coxsackie group of viruses, *Science*, 1949, **110,** 594.
2. Howes, D. W., Comparison of age-susceptibility relationships in mice, *Australian J. Exp. Biol. and Med.*, 1954, **32,** 253.
3. Lerner, A. M., Klein, J. O., Levin, H. S., and Finland, M., Infections due to Coxsackie virus group A, type 9 in Boston, 1959. With special reference to exanthems and pneumonia, *New England J. Med.*, 1960, **263,** 1265.
4. Lerner, A. M., Klein, J. O., and Finland, M., Laboratory outbreak of infections with Coxsackie virus, group A, type 9, *New England J. Medicine*, 1960, **263,** 1302.
5. Lerner, A. M., and Finland, M., Coxsackie viral infections, *A.M.A. Arch. Int. Med.*, 1961, **108,** 329.
6. Huebner, R. J., Beeman, E. A., Cole, R. M., Beigelman, P. M., and Bell, J. A., The importance of Coxsackie viruses in human epidemics, particularly herpangina and epidemic pleurodynia, *New England J. Med.*, 1952, **247,** 249 and 285.
7. Gard, S., Immuno-inactivation of poliovirus, *Arch. Virusforsch.*, 1957, **7,** 499.
8. Lennette, E. H., General principles underlying laboratory diagnosis of virus and rickettsial infections, *in* Diagnostic Procedures for Virus and Rickettsial Diseases, *New York*, American Public Health Association, 2nd edition, 1956, 1–51.
9. Eagle, H., Nutritional needs of mammalian cells in tissue culture, *Science*, 1955, **122,** 501.

10. Committee on Enteroviruses, National Foundation for Infantile Paralysis, Enteroviruses, *Am. J. Pub. Health*, 1957, **47,** 1556.
11. Reed, L. J., and Muench, H., Simple method of estimating fifty per cent endpoints, *Am. J. Hyg.*, 1938, **27,** 493.
12. Camain, R., and Bres, P., Contribution a l'étude du virus de Coxsackie: évolution des lésions chez le souriceau, *Ann. Inst. Pasteur*, 1959, **96,** 376.
13. Dalldorf, G., and Sickles, G. M., An unidentified, filtrable agent isolated from the feces of children with paralysis, *Science*, 1948, **108,** 61.
14 Sigel, M. M., Influence of age on susceptibility to virus infections with particular reference to laboratory animals, *Ann. Rev. Microbiol.*, 1952, **6,** 247.
15. Morgan, I. M., Influence of age on susceptibility and on immune response of mice to eastern equine encephalomyelitis virus, *J. Exp. Med.*, 1941, **74,** 115.
16. Theiler, M., Studies on the action of yellow fever virus in mice, *Ann. Trop. Med. and Parasitol.*, 1930, **24,** 249.
17. Sabin, A. B., and Olitsky, R. K., Influence of host factors on neuroinvasiveness of vesicular stomatitis virus. III. Effect of age and pathway of infection on the character and localization of lesions in the central nervous system, *J. Exp. Med.*, 1938, **67,** 201.
18. Casals, J., Influence of age factors on susceptibility of mice to rabies virus, *J. Exp. Med.*, 1940, **72,** 445.
19. Overman, J. R., and Kilham, L., The inter-relation of age, immune response and susceptibility to mumps virus in hamsters, J. Immunol., 1953, **71,** 352.
20. Sigurdsson, B., The influence of age of host and temperature of incubation on infection of the chick embryo with vesicular stomatitis virus, *J. Exp. Med.*, 1943, **78,** 17.
21. Freund, J., Influence of age upon antibody formation, *J. Immunol.*, 1930, **18,** 315.
22. Baumgartner, L., The relationship of age to immunological reactions, *Yale J. Biol. and Med.*, 1934, **6,** 403.
23. Luria, S. E., and Human, M. L., A nonhereditary host-induced variation of bacterial viruses, *J. Bact.*, 1952, **64,** 557.
24. Mogabgab, W. J., and Holmes, B., 2060 and JH viruses in secondary monkey kidney cultures, *J. Infect. Dis.*, 1961, **108,** 59.
25. Ginsberg, H. S., The significance of the viral carrier state in tissue culture systems, *Progr. Med. Virol.*, 1958, **1,** 36.
26. Holland, J. J., and McLaren, L. C., The mammalian cell-virus relationship. II. Adsorption, reception, and eclipse of poliovirus by HeLa cells, *J. Exp. Med.*, 1959, **109,** 487.
27. Kunin, C. M., and Jordan, W. S., Jr., *In vitro* adsorption of poliovirus by noncultured tissues. Effect of species, age and malignancy, *Am. J. Hyg.*, 1961, **73,** 245.
28. Kunin, C. M., Virus-tissue union and the pathogenesis of enterovirus infections, *J. Immunol.*, 1962, in press.
29. Choppin, P. W., and Philipson, L., The inactivation of enterovirus infectivity by the sulfhydryl reagent p-chloromercuribenzoate, *J. Exp. Med.*, 1961, **113,** 713.

30. Philipson, L., and Choppin, P. W., On the role of virus sulfhydryl groups in the attachment of enteroviruses to erythrocytes, *J. Exp. Med.*, 1960, **112,** 455.
31. Hsiung, G. D., and Melnick, J. L., Adsorption, multiplication and cytopathogenicity of enteroviruses (poliomyelitis, Coxsackie, and ECHO groups) in susceptible and resistant monkey kidney cells, *J. Immunol.*, 1958, **80,** 45.
32. Isaacs, A., and Hitchcock, G., Role of interferon in recovery from virus infections, *Lancet*, 1960, **2,** 69.
33. Isaacs, A., and Baron, S., Antiviral action of interferon in embryonic cells, *Lancet*, 1960, **2,** 946.
34. Pappenheimer, A. K., Kunz, L. J., and Richardson, S., Passage of Coxsackie virus (Connecticut-5 strain) in adult mice with production of pancreatic disease, *J. Exp. Med.*, 1951, **94,** 45.
35. Boring, W. D., ZuRhein, G. M., and Walker, D. L., Factors influencing host-virus interactions. II. Alteration of Coxsackie virus infection in adult mice by cold, *Proc. Soc. Exper. Biol. and Med.*, 1956, **93,** 273.
36. Cheever, F. S., Multiplication of Coxsackie virus in adult mice exposed to roentgen radiation, *J. Immunol.*, 1953, **71,** 431.
37. Cajal, N., Mateescu, S., and Capelovici, Y., Susceptibility of adult mice to the pathogenic action of Coxsackie virus following ionizing irradiation (X and β), *Acta Virologica*, 1959, **3,** Supplement, 107.
38. Kilbourne, E. D., and Horsfall, F. L., Lethal infections with Coxsackie virus of adult mice given cortisone, *Proc. Soc. Exp. Biol. and Med.*, 1951, **77,** 135.
39. Germuth, F. G., Oyama, J., and Ottinger, B., The mechanism of action of 17-hydroxy-11-dehydrocorticosterone (Compound E) and of the adrenocorticotropic hormone in experimental hypersensitivity in rabbits, *J. Exp. Med.*, 1951, **94,** 139.
40. Dalldorf, G., Neuropathogenicity of group A Coxsackie viruses, *J. Exp. Med.*, 1957, **106,** 69.
41. Gifford, R., and Dalldorf, G., The morbid anatomy of experimental Coxsackie virus infection, *Am. J. Path.*, 1951, **71,** 1047.
42. Melnick, J. L., and Godman, G. C., Pathogenesis of Coxsackie virus infection: multiplication of virus and evolution of muscle lesion in mice, *J. Exp. Med.*, 1951, **93,** 247.
43. Pappenheimer, A. M., Daniels, J. B., Cheever, F. S., and Weller, T. H., Lesions caused in suckling mice by certain viruses isolated from cases of so-called nonparalytic poliomyelitis and pleurodynia, *J. Exp. Med.*, 1950, **92,** 169.
44. Schmidt, E. C. H., Virus myocarditis: pathologic and experimental studies, *Am. J. Path.*, 1948, **24,** 97.
45. Warren, J., Infections of minor importance: encephalomyocarditis in Rivers, T. M., and Horsfall, F. C., Jr. (Editors) Viral and Rickettsial Infections of Man, Philadelphia, J. B. Lippincott Company, 3rd edition, 1959, pp. 903–907.
46. Lenke, S. E., and Loewe, L., Cardiac lesions resembling Aschoff bodies in mice, *Am. J. Path.*, 1941, **17,** 857.
47. Angevine, D. M., and Furth, J., A fatal disease of middle-aged mice characterized by myocarditis associated with hemorrhage in the pleural cavity, *Am. J. Path.*, 1943, **19,** 187.

48. Gray, F. G., Spontaneous cardiac lesions in mice, their bearing on attempts to produce experimental carditis, *Am. J. Path.*, 1949, **25,** 1215.
49. Henle, G., Deinhardt, F., Bergs, V. V., and Henle, W., Studies on persistent infections of tissue cultures. I. General aspects of the system, *J. Exp. Med.*, 1958, **108,** 537.
50. Takemoto, K. K., and Habel, K., Virus-cell relationship in a carrier culture of HeLa cells and Coxsackie A9 virus, *Virology*, 1959, **7,** 28.

EXPLANATION OF PLATE 60

All of the sections are from mice inoculated intraperitoneally with Coxsackie A virus, type 9, strain 13, and were stained with hematoxylin and eosin.

FIG. 1. Cross-section of newborn mouse 3 days after inoculation. Muscle fibers vary markedly in size and morphology,—some are small and shrunken and occasional ones are large, eosinophilic, and waxy. Within the interstitium there are a few mononuclear and polymorphonuclear inflammatory cells. × 400.

FIG. 2. Longitudinal section of skeletal muscle of 10 day old mouse on day 5 showing severe myositis. × 100.

FIG. 3. Cross-section of skeletal muscle of 20-day-old mouse, 6 days after inoculation. Muscle fibers are markedly necrotic. Sarcolemmic tubes are infiltrated chiefly with mononuclear phagocytes, the cellular infiltrate extending to the edematous interstitium. × 200.

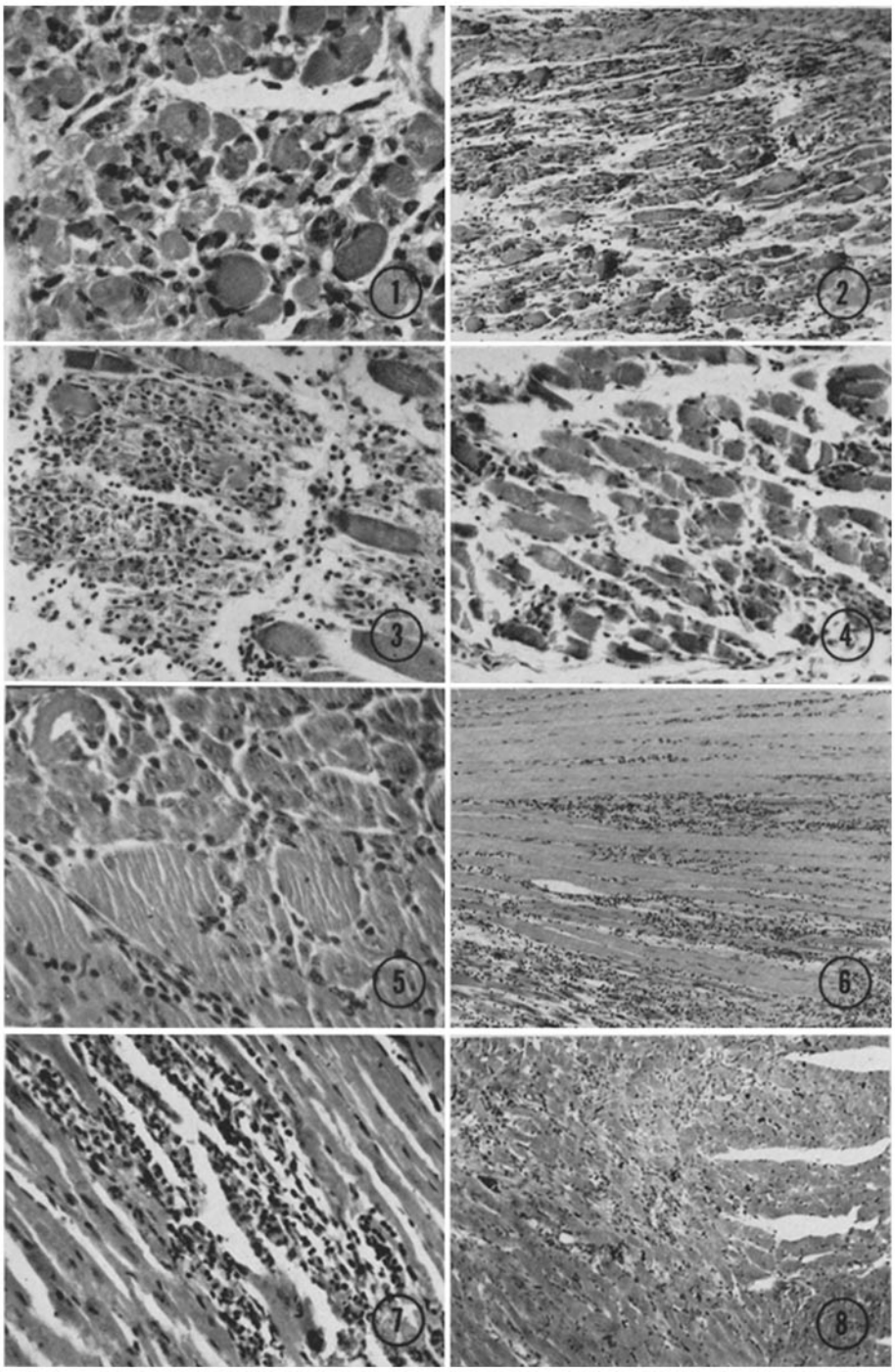
FIG. 4. Cross-section of skeletal muscle of 20-day-old mouse 3 days after inoculation. The muscle fibers vary in size and are eosinophilic. There is interstitial edema and moderate infiltration with mononuclear cells. × 200.

FIG. 5. Skeletal muscle of 20-day-old mouse 11 days after inoculation. There appears to be proliferation of small basophilic muscle fibers, some of which contain centrally located vesicular nuclei. Mononuclear inflammatory cells are scattered among the fibers. × 400.

FIG. 6. Longitudinal section of skeletal muscle of 8-month-old mouse 9 days after inoculation. There is moderately severe myositis and considerable interstitial infiltration with inflammatory cells. × 100.

FIG. 7. Myocardium of 8-month-old mouse, 6 days after inoculation, showing necrosis of myocardial fibers and infiltration by mononuclear and degenerating polymorphonuclear cells. × 200.

FIG. 8. Cardiac muscle of 8-month-old mouse 16 days after inoculation showing patchy areas of fibrosis and mononuclear inflammatory cells. × 100.



(Lerner *et al.*: Age and susceptibility to Coxsackie A viruses)