Differing Susceptibility of Adolescent and Adult Mice to Non-lethal Infection with Coxsackievirus B_3[1] (35596)

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Clinical observations (1–6) suggest that the severity of disease associated with virus infections increases with increasing age, if the generally greater susceptibility of the neonatal period and the contribution of previously acquired specific immunity to adult resistance be excluded. Although much has been published on the influence of host age on the outcome of experimental viral infection, most studies (i) have contrasted the experience in infant and adult animals; (ii) have employed large challenge doses of virus (often by grossly abnormal routes); and (iii) have relied upon lethal (LD_{50}) end points for measurement of effects.

In the present study, we compare the course of infection with coxsackievirus B_3 in young growing but sexually mature (adolescent) mice with that of adult animals. Severity of this nonlethal infection has been measured by quantitation of tissue concentrations of virus and scoring of lesions in target organs. This system has proved valuable in extensive studies in this laboratory (7, 8) of the effects of malnutrition on experimental virus infection.

Methods. Mice. MF-1 pathogen-free male mice (supplied by Manor Farms, Staatsburg, New York) were used in all experiments reported herein. Ages varied between 4 weeks and 8 months as indicated under results of specific experiments. From 5 to 10 mice were included in each experimental group. Animals designated "adult" or "older" were more than 2 months old while "adolescent" or "younger" mice were less than 2 months of age.

Viruses. Coxsackievirus B_3 (Nancy strain) was employed throughout. Seeds were prepared in HEp-2 cells grown in milk dilution bottles in BME enriched with 10% fetal calf serum (Microbiological Associates, Bethesda, Md.). Infectivity titers of the seed virus were determined by inoculation of serial 10-fold dilutions in tube cultures of HEp-2 cells and expressed as TCD_{50}.

Mice were infected by intraperitoneal (ip) inoculation with either 1000 or 5000 TCD_{50} of the virus, in a volume of 0.5 ml of phosphate buffered saline (PBS), as indicated under results of specific experiments.

Newcastle disease virus (NDV) (Hickman strain) was propagated in 11-day-old embryonated hens eggs, and infectivity is expressed in plaque-forming units (pfu) determined in clone 1-5C-4 of the Wong-Kilbourne variant of the aneuploid Chang human conjunctival cell, as described previously (9). Interferon was induced by intravenous injection of NDV in 0.2 ml of PBS.

Vesicular stomatitis virus (VSV) (Indiana strain) was prepared and assayed in clone L-929 cells.

Viral titrations. Organs from infected mice were obtained aseptically under Nembutal anesthesia at different intervals following infection. Ten percent by wet weight organ extracts were prepared by homogenizing individual organs or organ pools suspended in maintenance medium in grinding tubes, and removing tissue debris by centrifugation (9000 rpm in Lourdes centrifuge Model AX

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with the 9RA head). Supernates were removed, quick-frozen in a dry ice–ethanol mixture, and stored at —90° in a mechanical freezer (Revco) prior to viral titration. Titrations were carried out in replicate in HEp-2 cells (2 tubes/dilution) and expressed in terms of $\log_{10}$ TCD_{50}/0.1 g of tissue.

Lesion scores in organ sections. As indicated under Results, in some experiments, portions of the infected organs were removed, fixed in Bouin's solution, sectioned, and stained with hematoxylin and eosin; they were then used for histologic evaluation, employing the grading system summarized in legend to Fig. 2. Scores for a given organ from each experimental group were tabulated, "cumulative lesion score," and divided by the "maximum possible score." The latter value was derived by multiplying the maximal score obtainable, *i.e.*, 4+, by the number of animals in the group. The result of these calculations multiplied by 100 was referred to as the "lesion index." This scoring system was derived from one described by Horsfall (10).

Interferon determinations. Interferon titrations were carried out by the 50% plaque reduction method employing VSV as the indicator virus in clone L-929 cells as previously reported (9). Interferon determinations were carried out on sera obtained from blood removed by cardiac puncture and pooled for each experimental group. No effort was made to inactivate or remove coxsackieviruses from sera of infected mice prior to interferon titration, since, in preliminary experiments, it was determined that even high multiplicities of the virus did not replicate in L-929 cells, neither did they interfere with VSV plaque formation in these cells. Because, in preliminary experiments, only minimal amounts of interferon were found in tissue extracts, no extensive analyses for difference in interferon levels in these samples were carried out.

Results. Viral multiplication in adult and adolescent mice. In preliminary experiments, groups of mice 2 and 4 months of age were infected by intraperitoneal inoculation of 5×10^3 TCD_{50} of coxsackievirus B_3. At 2, 5, and 9 days following injection of virus, viscera known to be sites of multiplication of the virus (7) were removed for titration of infective virus. Results of this experiment, summarized in Table I, demonstrate consistently higher titers of virus in the hearts and livers of adult mice, with detection of virus in the brains only of older animals.

TABLE I. Comparative Titers of Coxsackie B_3 Virus in Organs of Adult[a] and Adolescent[b] Mice.

Age of mice	Organ	Viral titers[c] after viral inoculation Days: 2	5	9
Adolescent	Heart	2.8	2.9	1.9[d]
Adult	Heart	3.4	4.6	2.6
Adolescent	Brain	<2.0	<2.0	<2.0
Adult	Brain	<2.0	2.3	<2.0
Adolescent	Liver	3.0	<2.0	<2.0
Adult	Liver	3.9	2.0	<2.0
Adolescent	Pancreas	7.4	4.7	<2.0
Adult	Pancreas	6.7	4.2	<2.0

[a] Adult = 4 months old.

[b] Adolescent = 2 months old.

[c] $\log_{10}$ TCD_{50}/0.1 g of tissue; geometric mean titers, 5 animals/group.

[d] 2/5 hearts negative for virus at 10^{-1} dilution.

In another experiment of similar design, 10^3 TCD_{50} of coxsackievirus B_3 were inoculated into groups of 2- and 5-months-old mice

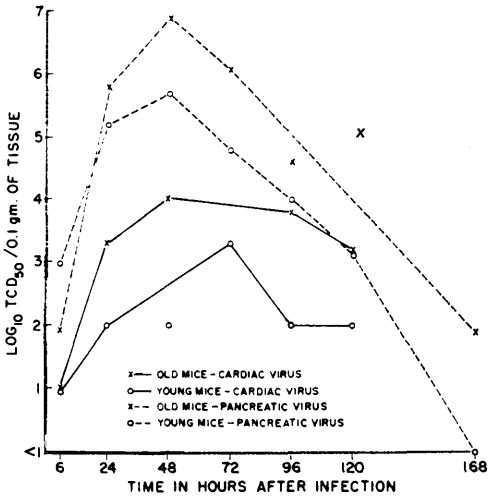

FIG. 1. Comparison of cardiac and pancreatic viral titers after infection of young (2 months old) and adult (5 months old) MF-1 mice with 5×10^3 TCD_{50} of coxsackievirus B3 (curves fitted by eye in 2 instances).

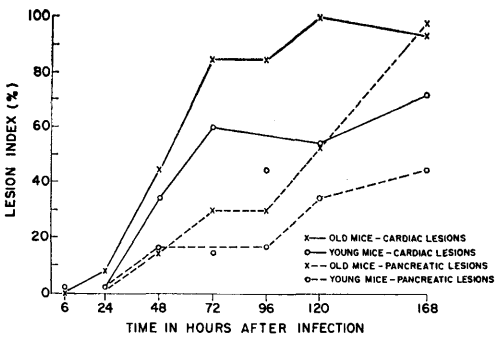

FIG 2. Comparison of lesion severity in adolescent and adult mice following infection with coxsackievirus B3 (data from same experiment as that yielding viral replication curves illustrated in Fig. 1): scoring system for grading histologic lesions in organs of mice infected with coxsackievirus B_3 (6–9 sections of each organ were examined): heart: ± = necrosis, inflammation or fibrosis involving <25% of organ; 1+ = necrosis, inflammation, fibrosis involving 25% of organ; 2+ = necrosis, inflammation, fibrosis involving 50% of organ; 3+ = necrosis, inflammation, fibrosis involving 75% of organ; 4+ = most of the organ involved; pancreas: + = questionable involvement; 1+ = scattered foci of necrosis and connective tissue replacement; 2+ = 50% of organ replaced by connective tissue; 3+ = 75% of organ replaced by connective tissue; and 4+ = total replacement by connective tissue.

and concentrations of cardiac and pancreatic virus were studied at more frequent intervals, as illustrated in Fig. 1. Persistently increased concentrations of virus were present in both the heart and pancreases of older animals in this experiment.

Neutralizing antibody response in older and mice. Histologic assessment of pancreatic and cardiac lesions in animals from the same experiment described above (for which curves of viral replication are presented in Fig. 1) establishes a general concordance of lesion severity with levels of infective virus, with a 3-day interval between peak titers and peak lesion severity (Fig. 2).

Neutralizing antibody response in older and younger mice. To determine whether differences in antibody response might account for the greater susceptibility to coxsackie infection seen in older animals, neutralizing antibody titers were compared for older and younger mice. The results are presented in Fig. 3. Note that the viral titer in the hearts of older animals again exceeded in this experiment by at least 10-fold the viral titer in the hearts of younger animals at 5 and 9 days following infection. The differences in the geometric mean serum neutralization titers between these two groups were not significant; however, when differences in antibody levels were observed (at 12 and 16 days) titers were higher in the older animals. These data suggest that differences in antibody synthesis probably do not explain the greater vulnerability of older mice to the effects of coxsackievirus infection.

The relation of age to serum interferon levels. Concentrations of serum interferon were determined in mice of different age groups. In the first series of experiments, Newcastle disease virus (NDV) which has been previously shown to elicit high levels of serum interferon in mice when injected intravenously (11), and which does not replicate completely in this host system (12, 13), was chosen as the inducer of interferon. Groups of mice (consisting of 6 mice/group) varying in age from 2 to 32 weeks were inoculated intravenously with NDV, 7.7–8.3 $\log_{10}$ pfu/mouse (dose was adjusted per g of body wt). Bloods were collected 6 hr following inoculation and serum assayed for interferon. It was found that the highest interferon response (16,386 units/2 ml of serum) occurred in mice 2 months of age, *i.e.*, adolescent mice (Table II). Mice at 18 and 32 weeks of age ("old mice") had serum interferon levels 8 and 4 times less than the

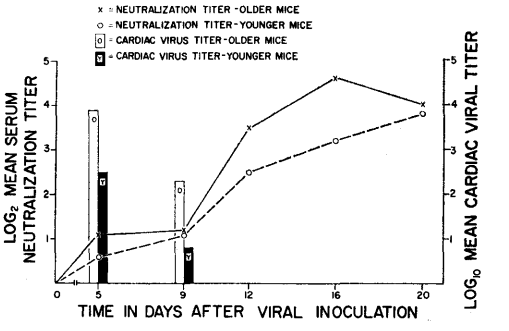

FIG. 3. Geometric mean neutralizing antibody titers of MF-1 mice infected with 5×10^8 TCD_{50} of coxsackievirus B_3. Older mice are 5 months of age; younger mice are 2 months old. Geometric mean cardiac virus titers are shown by the bars.

TABLE II. Comparison of NDV Induced Serum Interferon Levels in Adult and Adolescent Mice.

Age of mice (months)	Expt. no.:	Interferon titer (units/2 ml of serum) 1563	1597	1612
2		9600	6400	16,386
4		2000	1500	3000

8-week-old mice. Two- and 4-week-old mice also had interferon levels somewhat lower than 8-week-old mice, but in each instance, these titers were still higher than those in the old animals.

The difference in interferon titers between the young and the old animals was substantiated in several other experiments when comparisons were limited to the age groups that have been utilized for the majority of coxsackie B_3 experiments, namely the 2- and 4-month-old animals. Serum interferon titers of the young animals were consistently about 4-fold higher than those in the sera of the old animals.

In a second set of experiments, interferon responses in coxsackievirus-infected mice were determined and compared to the viral titers in the 2- and 8-month-old animals. Mice were inoculated intraperitoneally with 10^3 TCD_{50} of coxsackievirus B_3. Groups (composed of 5 mice) were sacrificed at days 2 and 4 following infection; serum pools and organ extracts from these mice and from controls (day 0) were prepared as described above. Figure 4 presents the serum interferon levels in relation to the cardiac virus titers in young and old animals. The usual difference in the cardiac viral titers between the older and the younger mice was again observed at 2 and 4 days following infection. Serum interferon levels were considerably lower than those elicited by NDV. Moreover it was found that both at 2 and 4 days following infection, the interferon levels were consistently higher in the sera of old mice, and thus bore a positive (but not proportional) relationship to the degree of viral multiplication (rather than an inverse one) in a manner similar to that observed for the antibody response (Fig. 3).

Discussion. The limited data presented herein permit the conclusion that *in vivo* replication of coxsackievirus B_3 in certain tissues of adult mice infected by the intraperitoneal route exceeds that observed in adolescent animals under similar conditions, and that more extensive lesions occur in adult animals in association with the increased viral replication. These findings are in accord with the clinical observations in man (1–6) which led to the present study. However, as noted in the introduction to this paper, the emphasis of most studies of experimental viral infection in animals has been upon the greater resistance of older animals.

Our studies are not alone in demonstrating the vulnerability of older mice to coxsackievirus infection. Higher titers of coxsackievirus B_3 were reported by Howes (14) in 8–9-week-old compared with 6–7-week-old mice, and Grodum and Dempster noted the "moderate susceptibility" of 6-month-old mice to myocarditis (15). Also pertinent are the studies of Lerner *et al.* (16), which have demonstrated that coxsackievirus A-9 induces myocardial lesions only in older mice (*i.e.*, more than 40 days of age). When 1-year-old mice were compared with younger animals, a progressive increase in myocardial virus concentration was noted with age, and a coincident reciprocal decrease in skeletal muscle virus (17). McLaren and Sanders (18) noted greater susceptibility to infection with the Connecticut 5 strain of coxsackievirus B_1

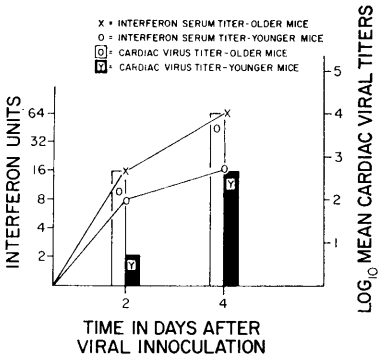

FIG. 4. Interferon levels (units/2 ml) in sera of MF-1 mice infected with 10^3 TCD_{50} of coxsackievirus B_3. Older mice are 8 months of age; younger mice are 2 months old. Cardiac virus titers (pooled organs) are shown by the bars.

and a shorter mean survival time in 20-day-old than in 15-day-old mice.

Our model does seem to have a parallel in human disease; the maximal susceptibility of the 1–2-day-old mouse corresponds to the frequently observed susceptibility of the human neonate to viral disease; the refractory period of the second week of life (19) parallels the relative resistance of the young child; while the returning susceptibility of the weanling period of the mouse (20) coincides with the development of sexual function (21) and is equivalent to adolescence in man. This entire period is one of rapid growth, and analysis of growth patterns of individual organs might be instructive in gaining understanding of changing susceptibility.

Our data on interferon formation in response to coxsackievirus infection suggest that adult mice produce *relatively* less interferon in relation to the amount of virus in their tissues than do younger animals. Furthermore, the absolute amount of interferon yielded by adult animals in response to intravenous (nonreplicating) NDV was less than that found in adolescent mice—a finding independently remarked by De Maeyer and De Maeyer-Guignard during the course of our studies (22).

We report our studies on interferon with no implication that these data supply an adequate explanation of the greater vulnerability of adult mice infected with coxsackievirus B_3. It is unlikely that the matter of age and susceptibility has interferon (or indeed any other factor) as a single determinant. It is notable, however, that in the present system no physiologic "barriers" (23) or failure of viral access to tissues (14) are involved and that virus multiplication occurs in the same organs of young and old mice. Resistance and susceptibility in the present system is thus an expression of relative tissue concentrations of virus and the reaction of the host to its presence. Future research must define the nature of the intrinsic susceptibility of the target tissues.

Summary. Nonlethal infection of adult (4–5 month old) MF-1 mice with coxsackievirus B_3 results in greater amounts of infective virus in their hearts and pancreases and more extensive lesions in these organs than are observed in adolescent (2 month old) animals. While significant differences in neutralizing antibody response to coxsackievirus infection were not demonstrated, adult animals produced relatively less interferon in response to infection and absolutely less in response to administration of Newcastle disease virus than did the younger animals.

Present evidence is inconclusive that differences in interferon formation account for the age-related difference in viral susceptibility observed in this model system.

The findings of the present study are in accord with clinical and epidemiologic evidence that the severity of viral infection increases with advancing age, if the contributions to resistance of the neonatal state or previously acquired specific immunity be excluded from consideration.

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