PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE **151**, 333-338 (1976)

Interferon and Neutralizing Antibody in Sera of Exercised Mice with Coxsackievirus B-3 Myocarditis (39204)

MILAGROS P. REYES AND A. MARTIN LERNER[1]

Hutzel Hospital Medical Unit, Department of Medicine, 432 E. Hancock, Detroit, Michigan 48201, and Wayne State University School of Medicine, Detroit, Michigan

Virulence of murine coxsackievirus B-3 infections is increased by a number of factors, including a decrease in ambient temperature to 4° (1), ionizing radiation (2), undernutrition (3, 4) or administrations of corticosteroids (5), cyclophosphamide (6), or reserpine (7). We have reported that when weanling mice are infected with coxsackievirus B-3 and forced to swim in a preheated pool (33°), myocardial virus replication multiplies 530 times and hearts dilate, hypertrophy, and become grossly necrotic. Mortality increases from 5.5 to 50% (8).

Thirty years ago Levinson, Milser, and Lewin showed that if during the incubation period of experimental poliomyelitis monkeys are forced to swim until exhausted, they develop a higher incidence and more severe paralysis than controls (9). In 1950, Horstman correlated the amount of physical activity and severity of resulting illness in 411 patients from three epidemics of poliomyelitis. When about the time of onset of major illness, physical activity is performed, it is associated with significant increases in incidence and severity of paralysis. Moreover, a higher percentage of nonparalytic than paralytic patients have a history of bed rest or minimal activity during early stages of major illness (10).

These observations relate exercise to a worsening of several viral infections. In preliminary observations, we report interferon, neutralizing antibody, and adrenal corticoids in severely exercised young mice with coxsackievirus B-3 myocarditis.

Materials and methods. Mice. Pregnant albino Swiss ICR mice were obtained at term. After delivery, each mother with its brood was housed in a separate cage. Nurslings were weaned at 3 weeks and, thereafter, were fed standard Rockland rat chow.

Viruses. Coxsackievirus B-3 (Nancy) was used (8). Coxsackievirus was passed in tube cultures of rhesus kidney and a pool of stock virus prepared. Five-tenths milliliter samples of coxsackievirus B-3 were placed in screw-capped vials and stored at −70° until use. Each vial contained 3.75 × 10^5 plaque-forming units (PFU) per milliliter. Similarly, vesicular stomatitis virus (VSV) stocks were prepared in tissue cultures of mouse embryo fibroblasts (MEF). When cytopathic effects were 3-4+, cells were scraped and fluids centrifuged at 1000*g* for 10 min at 4°. Supernates were divided into samples of 1 ml and stored (−70°). The VSV stock contained 1.6 × 10^6 PFU/ml.

Experimental infections. At 14 days of age, three groups of 180 baby mice were formed. Until weaning at 21 days mothers were kept with their young. Mice in Group I were inoculated ip with 0.5 ml of a 10^{-1} dilution of coxsackievirus stock. From the 14th day of life, mice in Group I were forced to swim in a plastic pool (33°) for increasing intervals, beginning for 5 min once a day. When nurslings could swim for 30 min uninterruptedly, they then were exercised for 30 min in the morning and afternoon. At exhaustion, when drowning, mice were rescued with a net, and this swimming period was shortened. Sucklings in Group II were inoculated with coxsackievirus B-3 as above; they were not exercised. Animals in Group III were neither inoculated nor exercised.

After inoculations and unless they died earlier, 20 mice from each group under ether anesthesia were sacrificed by exsanguination at 8, 24, 48 and 72 hr. This procedure was repeated on 6, 9, 13, 20, and 40 days. In order to obtain sufficient volumes, pools of sera and heparinized plasma from

---

[1] Address reprint requests to A. Martin Lerner, Department of Medicine, Hutzel Hospital, 432 E. Hancock, Detroit, Michigan 48201.

Copyright © 1976 by the Society for Experimental Biology and Medicine
All rights reserved.

at least five mice were collected. Hearts, kidneys, brains, and fore and hind limbs were placed in screw-capped vials and kept at −20° until assay. Sera and 20% suspensions of hearts in Eagle's medium (EM) were titered for coxsackievirus B-3 using an agar overlay method in 30-ml screw-capped plastic rhesus-kidney-tissue culture flasks as described (8). Sera and tissues were assayed for interferon. Samples of plasma from each group were taken at 4 PM daily for the first 9 days after inoculations and tested for content of corticosterone.

Tissue cultures of mouse embryo fibroblasts. Primary MEF tissue cultures were prepared from decapitated and eviscerated 14- to 18-day-old mouse embryos. Carcasses were minced, trypsinized, and grown in 30-ml plastic flasks at a concentration of 25 × 10^4/ml in EM with 5% fetal calf serum containing 100 μg of penicillin G and 50 μg of streptomycin/ml.

Assay of interferon (11). Sera and 20% suspensions of tissues were diluted in EM. Primary mouse embryo fibroblasts were washed once with EM and inoculated with 2-ml suspensions of tissues in 1 ml of sera, using four plaque bottles per serial twofold dilution. Flasks were incubated (37°, 24 hr), washed with EM, and inoculated with 50 PFU of VSV/0.4 ml.

Cultures were again washed with another 2 ml of warm EM (37°) and finally overlaid with 2 ml of twice-concentrated EM with 10% fetal calf serum (37°) and 2 ml of distilled water with 1.5% Difco nutrient agar and 0.4% sodium bicarbonate (42°). Forty-eight hours later, we added 0.01% neutral red to each culture, and after a further 3 hr at 23°, plaques were counted. In preliminary experiments similar to Rytel (12), we showed that the concomitant presence of coxsackievirus B-3 in sera or suspensions of tissues does not interfere with the formation of PFU by VSV. Therefore, we did not inactivate coxsackieviruses from specimens. The titer of interferon was read as the highest dilution of the sample producing a 50% reduction in VSV PFU compared to appropriate controls (virus-EM and virus-noninfected suspensions of tissues). Titers were not compared to standard preparations of interferon.

The interfering substance was identified as interferon. It was stable on dialysis (24 hr) versus glycine buffer at pH 2; not sedimentable on ultracentrifugation at 105,000*g* for 2 hr (4°) in the presence of 0.06% gelatin; sensitive to destruction by trypsin (0.05 mg/ml) (37°, 1 hr) and species specific. The interfering substance did not inhibit formation of VSV PFU in similar cultures of chick embryo fibroblasts.

Neutralizing antibodies. Conventional neutralizing antibodies (N). Sera from experimental groups were inactivated in a water bath (56°, 1 hr), and twofold dilutions in EM were made. Three-tenths milliliter of appropriate dilutions of sera, 0.3 ml of stock coxsackievirus B-3 containing 25 PFU/0.1 ml, and 0.1 ml of a 1:4 dilution of guinea pig serum (GPS) which had been inactivated (56°, 1 hr) were incubated in stoppered-test tubes at 23° for 1 hr. A control of 0.3 ml of virus, 0.3 ml of EM, and 0.1 ml of heat-activated GPS was included with each test. Thereafter, 0.25 ml of each mixture was inoculated into two plaque bottles of rhesus-kidney-tissue culture. After a further incubation (37°, 1 hr) agar overlays were added, and, subsequently, plaques were counted. The titer of N was read as the highest dilution of serum completely inhibiting plaques of coxsackievirus B-3.

If we had used a 50% reduction in PFU as the endpoint, it may have provided a more sensitive measure of these antibodies. However, this was not possible because of the large size of our coxsackievirus plaques. Decreasing the inoculum size also increases the sensitivity of tests for neutralizing antibodies. We used an inoculum of 25 PFU.

Complement-requiring neutralizing antibodies (CRN). In infections with herpes simplex virus type 1, IgM or early IgG neutralizing antibody has been found, which requires complement. After primary immunization, complement-requiring neutralizing antibody often appears before conventional neutralizing antibodies (13). Therefore, we attempted to measure CRN to coxsackievirus B-3. CRN were measured as described for N except that fresh guinea pig serum containing approximately 2.5 hemolytic units of complement was not inactivated.

Immunoinactivating neutralizing antibod-

ies (IA). Gard used the term immunoinactivation to designate another technique for increasing sensitivity of tests for conventional neutralizing antibody (14, 15). Here, virus-antibody incubations are prolonged to allow neutralization to reach completion. Three-tenths milliliter of virus and 0.3 ml of dilutions of serum were incubated at 23° for 18 hr. Controls contained coxsackievirus and EM. Thereafter, 0.25 ml of the reacting mixtures were inoculated into two rhesus-kidney plastic flasks and incubated (37°, 1 hr). Finally, agar overlays were added, plaques counted, and antibody titers recorded.

Assays of corticosterone. Kilbourne, Wilson, and Perrier induced gross myocardial lesions in mice by giving cortisone to adult mice infected with coxsackievirus B-3 (5). Therefore, corticosteroids in mice of the three groups were determined. The major corticosteroid in mice is corticosterone. As in man, diurnal variations in concentrations occur, but murine peaks are in late afternoon (16). A modified Porter–Silber method was used to determine plasma corticosterone (17). In a preliminary experiment, concentrations of corticosterone in pooled specimens of heparinized plasma from five normal weanling Swiss ICR mice were 67 μg % at 8 AM and 108 μg % at 4 PM.

Results. Virological findings. Coxsackievirus was isolated from sera of infected and exercised mice (Group I) and the infected but not conditioned nurslings (Group II) as early as 8 hours and 1, 2, 3, and 6 days after inoculations. At 8 hr after inoculations there were 10^2 PFU in pooled sera from both infected groups. Viremias reached peaks at 24–48 hr, but were 10 × greater in mice of Group I (10^6 PFU/ml versus 10^5 PFU/ml). Titers of coxsackievirus B-3 declined somewhat in sera from both infected groups on Days 3–6, but total amounts of coxsackievirus B-3 were on the average 75 × greater in weanlings which were swimming. Virus was no longer present in sera on the ninth day of infection (Figs. 1, 2).

Coxsackievirus B-3 was not present in hearts of any experimental mice at 8 hr, but was found at 24 hr (10^3 PFU/g, Group I versus 10^2 PFU/g, Group II). Virus persisted in high titers in both groups through Day 9, but from Days 4–9 quantities of virus in hearts of exercised nurslings were considerably greater. On Day 6 myocardial virus of exercised mice reached a peak of 10^7 PFU/g, and was 1000-fold greater than that in the infected, but not swum mice (Fig. 2). By the 13th day, virus was no longer present in any hearts. No virus was isolated from sera or hearts of mice in Group III. In general, these data confirm those of our earlier report, but add details of viral kinetics during the initial 72 hr of infection (8).

Interferon. At 8 hr there was no interferon in sera of any group, nor was this activity found in sera of mice of Group I at 24 hr after infection. However, pooled sera of nonexercised baby mice had an interferon (IF) titer of 1/10 u/ml (Fig. 1). By 48 hr, titers were 1/40 (Group II), but were 1/80 (Group I). Therefore, exercised mice had no circulating IF at 24 hr after infection when nonexercised mice did.

By 72 hr and later, IF was no longer present in sera of any mice which were infected but not conditioned. However, high titers in sera of the swum mice continued on Days 3 (1/320 u/ml) and 6 (1/320 u/ml), but IF was absent on Day 9.

Until specimens were diluted fourfold, IF was not found in sera of Group I from Days 3 and 6. Other samples did not require this dilution. It is possible that in these samples an "anti-interferon" was produced (18). Unfortunately, volumes of the available specimens prevented us from characterizing the interfering substance further.

Interferon was not found in hearts of mice from either Group I or II, at 8 and 24 hr. However, by 48 hr titers were $^1/_{16}$ and $^1/_{32}$ u/ml in Groups I and II, respectively. Among infected but not exercised mice, IF in hearts then declined sharply. At 72 hr, 6 days, and 9 days, titers were $^1/_4$, $^1/_2$, and $<^1/_2$ u/ml. In conditioned mice, IF in the heart declined more slowly, and titers were $^1/_{32}$, $^1/_4$, and $<^1/_2$ u/ml at these times. No sample of serum or heart from Group III, or hind or fore limb, brain or kidney of any of the groups had detectable IF.

Neutralizing antibodies. Conventional neutralizing antibodies were not found in sera of any group at 3, 6, 9, 13, 20, and 40

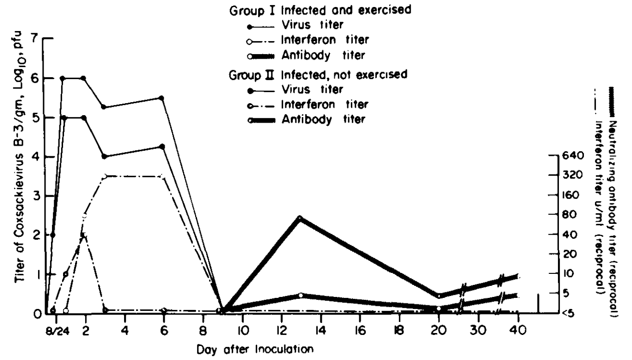

FIG. 1. Coxsackievirus B-3, interferon, and neutralizing antibody in sera of swimming (I) and resting (II) weanling mice.

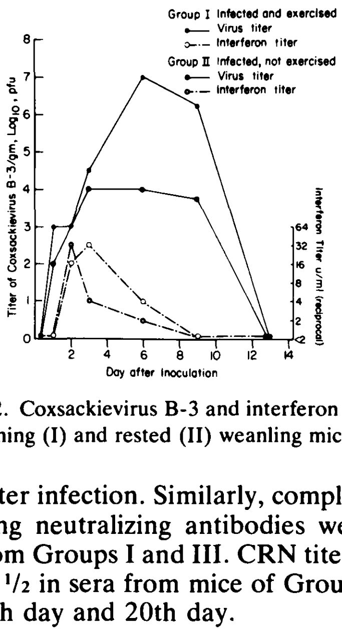

FIG. 2. Coxsackievirus B-3 and interferon in hearts of swimming (I) and rested (II) weanling mice.

days after infection. Similarly, complement-requiring neutralizing antibodies were absent from Groups I and III. CRN titers were 1/4 and 1/2 in sera from mice of Group II on the 13th day and 20th day.

Using the immunoactivation method, titers from Group I mice were <1/4, <1/4, <1/4, 1/4, <1/4, and 1/4 at 3, 6, 9, 13, 20, and 40 days. IA titers were higher in infected, but not exercised mice appearing on the 13th day (1/64) and being 1/4 and 1/8 on the 20th and 40th days. On the 13th day, IA titers were 16 × greater in the nonswum than in the swum mice (Fig. 1).

When compared to previous experiments, usually with older mice, in this and other laboratories, the appearance of neutralizing antibodies was delayed in both Groups I and II (19, 20). While we have no definite explanation for this delay, antibodies may have been diminished by storage (−20°), or by freezing and thawings of specimens to measure coxsackievirus or IF.

Values of corticosterone in sera (Fig. 3). No differences among the three groups of mice were evident.

Discussion. Rager-Zisman and Allison have shown that after infections with coxsackievirus B-3 neutralizing antibodies and macrophages collaborate to limit the spread of virus (20). Antibodies neutralize coxsackievirus B-3 by attachment to viral protein 4 (VP 4) of the capsid (21). Macrophages have receptors for IgM, IgG, and complement on their surfaces, and virus combines with antibody as potentially infective complexes. Macrophages immobilize and inactivate the complexes.

In our studies, viremia ceased on Day 9, and several days later coxsackievirus B-3 was no longer present in the heart. We found circulating neutralizing antibody on the 13th day of infection, but a more sensitive technique, such as radioimmunoassay, may have detected antibody earlier.

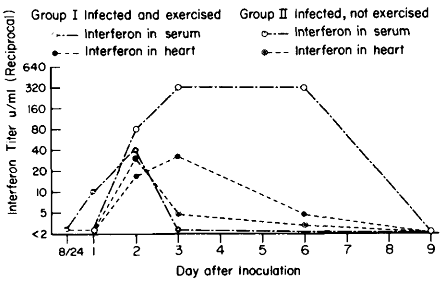

FIG. 3. Interferon in sera and hearts of swimming (I) and rested (II) weanling mice infected with coxsackievirus B-3.

Interferon titers generally parallel virus and once infection is established, particularly with enteroviruses or togaviruses, circulating antibody and macrophages are considered to play a major role in recovery (6, 20). In this regard, immunosuppression of coxsackievirus B-3 infected young adult CBA mice with cyclophosphamide leads to lethal infections which can be passively prevented after infection by administration of serum antibody (6). Immuno-suppressed animals show transient production of IgM, but no IgG neutralizing antibodies.

On the other hand, Baron, Buckler, Friedman, and McCloskey (22) and Norris and Loh (23) demonstrate the protective effects of circulating interferon in the absence of antibody during murine infections with encephalomyocarditis, vesicular stomatitis, Germiston (Bunyamwera group, arbovirus), vaccinia, and coxsackievirus B-3. With coxsackievirus B-3 a single ip inoculation of poly I:C either 12–48 hr before, or 24 hr after inoculation of virus results in a significant protection from myocarditis. When this double-stranded synthetic polynucleotide is given 48 hr after coxsackievirus B-3, no protection occurs. Poly I:C stimulates phagocytosis, formation of antibodies, and cell-mediated immune responses of which interferon is one of the major effector molecules (24). Certainly, the prophylactic use of poly I:C precludes a protective role for neutralizing antibody.

Likewise, DeMaeyer and DeMaeyer (25), Smart and Kilbourne (26), and Rytel (12) emphasize the role of circulating interferon *early* in several viral infections. Later, interferon is less able to inhibit multiplication of virus, and titers of IF and virus increase together. For instance, during initial replicative cycles in influenza-infected chorioallantoic membranes, hydrocortisone inhibits release of preformed interferon and its synthesis. For as long as 50 hr after infection within the chorioallantoic membrane and in allantoic fluid, IF is depressed. Stevens and Merigan also emphasize the apparent importance in the elimination of intravesicular varicella-zoster virus of a delay in the appearance of interferon within vesicular fluid. Delay in local IF is associated with disseminated disease (27). It is possible that there may be, at least with some virus infections, sequentially critical roles for first interferon (during early replicative viral cycles), and later for specific neutralizing antibody.

Chronic exercise produces a depletion of catecholamines. The onset of this depletion relates to intensity of the exercise, but the final level (about 30% of normal) is approached after 2 weeks of intensive exercise, and is achieved after 6 weeks of a less intensive program (28). Endogenous levels of cAMP play a role in controlling to what extent an activated immuno-competent cell is "turned on," or is "turned off" (24). Levels of cAMP are modified by the activity of intracellular adenyl cyclase. Among the stimulators of adenyl cyclase are catecholamines (norepinephrine, epinephrine, and isoproterenol (24).

The present experiments confirm that after ip inoculation with the Nancy strain of coxsackievirus B-3, swimming nursling abino Swiss ICR mice results in a remarkable increase in virus multiplication in the hearts. When compared with infected, but not swum controls an increase in magnitude of viremia also results. Concentrations of corticosterone at 4 PM daily are similar in infected and exercised (Group I), infected, not exercised (Group II), and uninfected, not exercised (Group III) mice.

Sera of the mice which are infected and forced to swim had no circulating interferon 24 hr after infection, while sera from similarly infected (not swum) mice did. Later IF in serum and hearts of mice from Group I are consistently higher than in other mice of Group II. Neutralizing antibodies were demonstrable on the 13th day after infection and were 16 × greater in the nonexercised mice.

Summary. Weanling ICR albino Swiss mice were inoculated ip with 1.9×10^4 PFU of coxsackievirus B-3 (Nancy) and subsequently forced to swim vigorously daily in a preheated pool (33°). Viremias and virus in hearts of exercised mice were respectively 75 × 1000 × greater than in infected, but not exercised mice. At 24 hr after inoculation, pooled serum from mice that had been swum had no circulating interferon, while infected but not swum mice had interferon activity at a dilution of 1:10. At 72 hr after

infection, circulating interferon disappeared from infected (not swum) mice, but continued to be present in high titers through the sixth day in sucklings forced to swim. Interferon was first detected in the hearts of both groups at 48 hr. Quantities in both infected groups were generally similar.

Neutralizing antibodies were found in these baby mice on the 13th day of infection and were 16 × greater in nurslings that were not exercised. Measures of corticosterone taken at 4 PM daily were similar in infected, infected-swum, and uninfected mice.

The work presented in this paper was aided by PHS Grant from the National Institute of Allergy and Infectious Diseases (No. AI 00261-11); a grant from the American Heart Assn. (No. 73-773); and a grant from Skillman Foundation for general support of research in infectious diseases.

We wish to thank Elizabeth Jane Bailey, Vera Tripp, Susie Harvey, and Babette Jackson for their assistance in these studies.

---

1. Boring, W. D., ZuRhein, G. M., and Walker, D. L., Proc. Soc. Exp. Biol. Med. **93,** 273 (1956).
2. Cheever, F. S., J. Immunol. **71,** 431 (1953).
3. Woodruff, J. F., and Kilbourne, E. D., J. Infec. Dis. **121,** 137 (1970).
4. Woodruff, J. F., J. Infec. Dis. **121,** 164 (1970).
5. Kilbourne, E. D., Wilson, C. B., and Perrier, D., J. Clin. Invest. **35,** 362 (1956).
6. Rager-Zisman, B., and Allison, A. C., J. Gen. Virol. **19,** 339 (1973).
7. Grodums, E. I., Canad. J. Microb. **18,** 577 (1972).
8. Gatmaitan, B. G., Chason, J. L., and Lerner, A. M., J. Exp. Med. **131,** 1121 (1970).
9. Levinson, S. O., Milzer, A., and Lewin, P., Amer. J. Hyg. **42,** 204 (1945).
10. Horstman, D. M., J. Amer. Med. Assoc. **142,** 236 (1950).
11. Riley, B. P., Toy, S. T., and Gifford, G. E., Proc. Soc. Exp. Biol. Med. **122,** 1142 (1966).
12. Rytel, M. W., J. Infec. Dis. **120,** 379 (1969).
13. Lerner, A. M., Shippey, M. J., and Crane, L. R., J. Infec. Dis. **129,** 623 (1974).
14. Gard, S., Arch. Ges. Virusforsch. **7,** 449 (1957).
15. Lerner, A. M., Remington, J. S., and Finland, M., J. Clin. Invest. **41,** 805 (1962).
16. Halberg, F., Peterson, R. E., and Silber, R. H., Endocrinology, **64,** 222 (1959).
17. Porter, C. C., and Silber, R. H., J. Biol. Chem. **185,** 201 (1950).
18. Galliot, B., Moreau, M. C., Renard, N., and Chany, C., Proc. Soc. Exp. Biol. Med. **142,** 266 (1973).
19. Lerner, A. M., Levin, H. S., and Finland, M., J. Exp. Med. **115,** 745 (1962).
20. Rager-Zisman, B., and Allison, A. C., J. Gen. Virol. **19,** 329 (1973).
21. Philipson, L., Beatrice, S. T., and Crowell, R. L., Virology, **54,** 69 (1973).
22. Baron, S., Buckler, C. E., Friedman, R. M., and McCloskey, R. B., J. Immunol. **96,** 17 (1966).
23. Norris, D., Loh, P., Proc. Soc. Exp. Biol. Med. **142,** 133 (1973).
24. Braun, W. *in* "Cyclic AMP, Cell Growth and the Immune Response" (W. Braun, L. M. Lichtenstein, and C. W. Parker, eds.), p. 4. Springer-Verlag, New York, Heidelberg, Berlin (1974).
25. DeMaeyer, E., and DeMaeyer, J., Nature (London) **197,** 724 (1963).
26. Smart, K. M., and Kilbourne, E. D., J. Exp. Med. **123,** 299 (1966).
27. Stevens, D., and Merigan, T., J. Clin. Invest. **51,** 1170 (1972).
28. DeSchryver, C., and Mertens-Strythagen, J., Pfluegers Arch. **336,** 345 (1972).

Received September 10, 1975. P.S.E.B.M. 1976, Vol. 151.