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Modification of Severe Coxsackievirus B_3 Infection in Marasmic Mice by Transfer of Immune Lymphoid Cells

(viral-host defense/immunocompetence/pathological lesions/virus titration)

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ABSTRACT **Coxsackievirus causes severe disease in adult mice subjected to sustained post-weaning undernutrition (marasmus). Virus-infected marasmic mice have an increased incidence of mortality, severe lesions, and elevated and persistent viral titers in target organs. Transfer of immune lymphoid cells 30 min or 24 hr after viral challenge significantly reduced the incidence of death and lesions. The protective capacity of immune cells was further manifested by reduced titers of virus in the target organs. Since lymphoid tissues are severely atrophic in marasmic mice, these results indicate that this deficiency contributed significantly to the impaired ability of these hosts to recover from viral disease. These observations support the idea that the acquisition of lymphocyte-mediated defense mechanisms is essential for normal recovery from certain primary viral infections.**

Coxsackievirus B_3 infection, which is tolerated in normal adult mice (1), produces servere disease in mice subjected to sustained post-weaning undernutrition (marasmus) (2). Marasmic mice show persistence of infective virus in the heart, spleen, liver, and pancreas, and an increased occurrence of massive hepatic necrosis, gross cardiac lesions, and death. These animals can be significantly protected from the severe sequellae of viral infection by the institution of an optimal diet at the time of viral challenge. Complete protection is achieved if the animals are fed an optimal diet for about 1 month before viral infection (2).

Marasmic animals have marked atrophy of lymphoid tissues and severe lymphocytopenia (3). Moreover, the infiltrates of lymphocytes and macrophages, which normally appear in the heart 7 days after Coxsackievirus infection, are markedly reduced or absent. Although the spleens of marasmic animals contain confluent collections of macrophages, spleen virus-titers are nonetheless abnormally high. Also the protection afforded by the introduction of an optimal diet at the time of infection is associated with a normal inflammatory infiltrate in the target organs on day 7 and is correlated with a substantial increase in the concentration of circulating lymphocytes (2, 3).

These observations suggest that depletion of lymphocytes might be a crucial factor in the failure of marasmic mice to generate effective antiviral recovery mechanisms (3). This proposition has been investigated by transferring to these mice suspensions of lymphoid cells, free of phagocytic cells, obtained from the spleens of normal mice recovering from Coxsackievirus B_3 infection. The data demonstrate that adoptive transfer of immune lymphoid cells increased the resistance to viral infection.

Abbreviation: MEM, minimum essential medium.

MATERIALS AND METHODS

Animals

Specific pathogen free, male, outbred, albino mice were obtained from Purina Laboratory Animals, Vincentown, N.J. The degree of histoincompatibility among these animals is unknown. Mice were housed individually with free access to water and fed the diets described below.

Recipient marasmic mice

Mice were received in the laboratory at 24 days of age and were fed 1.5 g/day of D&G Research Animal Laboratory Diet (2) for Rats and Mice (Price-Wilhoite Co., Frederick, Md.). This regimen provides about 25% of the optimal diet required by these animals for normal growth. Mice maintained on this diet for 30–40 days become marasmic, with an average body weight of 16 g.

Donor mice

Mice used as donors of normal or immune lymphoid cells were 7–8 weeks of age and weighed about 35 g. They were fed a regular mouse diet *ad lib.*

Virus propagation

Two tissue culture cell lines were used in these experiments. H.Ep. no. 2 cells, obtained from Flow Laboratories, Inc., Rockville, Md., were serially passaged in this laboratory and used to grow and titrate Coxsackievirus B_3, and to detect neutralizing antibodies to Coxsackievirus B_3 (3). The NCTC (clone 929) strain of L cells (CCL-1) used for interferon titrations, was obtained from Dr. Jan Vilcek. The methods used to grow both cell lines have been described (2 and 3).

Two viruses were used in these experiments. Coxsackievirus B_3 (Nancy strain) was used to infect mice and as challenge virus in titrations of neutralizing antibody. Each seed had a titer of $10^{6.5\pm0.5}$ tissue culture $dose_{50}$ (TCD_{50}) of Coxsackievirus B_3/ml. Vesicular stomatitis virus (VSV, Indiana strain) was used as indicator virus in the interferon titrations. The stock VSV seed used (obtained from Dr. Jan Vilcek) was grown in a primary chick-embryo-cell monolayer and had a titer of 1.5×10^8 plaque-forming units (PFU)/0.4 ml on the NCTC clone 929 strain of L cells. Interferon was titrated as described previously (4).

The procedures used to prepare whole-organ suspensions and to determine whole-organ virus titers were described (2).

Preparation of immune and normal lymphoid cells

Mice used as donors of immune lymphoid cells were immunized by an intraperitoneal (i.p.) injection of Coxsackievirus B_3 (10^6 TCD_{50}) in 0.5 ml of minimum essential medium (MEM). 7 days later, infected mice were killed by cervical dislocation, and their spleens were removed aseptically and teased apart in cold MEM containing 2% heat-inactivated fetal-calf serum. This suspension was passed through sterile wire-mesh and then filtered through a column (1 by 5 cm) of acid-washed glass wool to remove phagocytic cells. The cells recovered in the effluent were sedimented by centrifugation (1000 rpm for 5 min) at 4°C, washed twice with cold MEM-serum, counted, and resuspended at a concentration of 7–9 × 10^7 cells/0.4 ml of MEM. Examination of wet preparations and Wright-stained smears showed that essentially all cells were erythrocytes and lymphocytes. At least 90% of the cells excluded trypan blue.

Supernatants obtained from two separate suspensions of immune lymphoid cells were examined for interferon and for Coxsackievirus B_3 neutralizing antibody. At the lowest dilutions tested (antibody 1/32 and interferon 1/64), no virus-specific neutralizing antibody or interferon was detected.

Heat-killed immune lymphoid cells were prepared by incubation of cell suspensions in a 56°C water bath for 20 min; 95% of the incubated cells were stained with trypan blue.

Normal lymphoid cells were collected from spleens of uninfected mice as described above.

Experimental design

All marasmic mice were inoculated i.p. with 10^6 TCD_{50} of Coxsackievirus B_3 in 0.5 ml of MEM. 30 min or 24 hr later, groups of these mice were transfused intranvenously (i.v.) with 0.4 ml of medium, 7–9 × 10^7 immune lymphoid cells, 7–9 × 10^7 normal lymphoid cells, or 7–9 × 10^7 heat-killed immune lymphoid cells, and the percent cumulative mortality was calculated. All mice that died 3 days or more after viral challenge were autopsied.

In another experiment, mice challenged with Coxsackievirus B_3 were transfused 24 hr later with 8 × 10^7 immune lymphoid cells or with 0.4 ml of medium. On the third, sixth, and eighth days after viral challenge, the heart, spleen, liver, and pancreas were removed aseptically for the determination of whole-organ virus titers.

Histology

Mice were injected, within 24 hr after viral infection, with the medium alone or with immune lymphoid cells. 5–8 days after infection, liver sections were fixed in 10% formalin solution, processed, and stained with hematoxylin and eosin. Massive hepatic necrosis in these sections has been described (3).

Statistical analysis

Variance analysis was used to analyze differences in organ virus titers. Chi-square testing was used to evaluate significant differences in the incidence of mortality, massive hepatic necrosis, and gross cardiac lesions in mice injected with Coxsackievirus B_3 only (controls), and those also injected with immune lymphoid cells.

RESULTS

Percent cumulative mortality

In two separate experiments, marasmic mice were injected with Coxasackievirus B_3 ($10^{6.0}$ TCD_{50}, i.p.), and 30 min later were transfused, i.v., with medium alone, or with 7–9 × 10^7 immune lymphoid cells. Between the third and twelfth day after infection, 63% of the medium-injected mice died, as compared to only 20% of mice receiving immune lymphoid cells (Fig. 1, $P < 0.01$). In two other experiments, groups of mice were transfused 24 hr after virus infection with medium alone, or with immune lymphoid cells. By day 12, 47.6% of the medium-treated mice and 15.7% of the mice injected with immune lymphoid cells were dead ($0.05 > P > 0.02$). Thus, transfer of immune lymphoid cells significantly protected these animals against the lethal effects of Coxsackievirus B_3 infection (see Fig. 1).

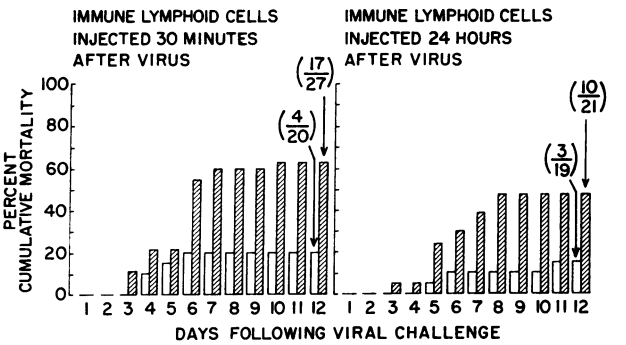

FIG. 1. Percent cumulative mortality of virus-infected marasmic mice that received immune lymphoid cells (*open bars*) or medium alone (*hatched bars*).

Transfer of 7–9 × 10^7 normal lymphoid cells or the same number of heat-killed immune lymphoid cells within the first 24 hr after inoculation of the virus did not significantly reduce the incidence of mortality. Thus, 69% (9/13) of the recipients of normal lymphoid cells and 59% (10/17) of the recipients of heat-killed immune lymphoid cells were dead by the twelfth day after virus inoculation.

Whole-organ virus titers

Virus titers per heart, spleen, liver, and pancreas were simultaneously determined in two groups of infected mice. 24 hr after viral inoculation, 16 mice were injected i.v. with medium alone, and 12 mice were injected each with 8 × 10^7 immune lymphoid cells. Organs were assayed for virus on the third, sixth, and eighth day after infection. The results are depicted in Fig. 2. At each interval, the average virus titer in the spleens, hearts, and livers of the recipients of immune lymphoid cells was less than that in control (medium-treated, virus-inoculated) mice. On day 8, the differences in average virus titers in hearts, spleens, and pancreases of the two groups were statistically significant (P0.05–0.02). In addition, on day 8, essentially no virus was detected in the livers of 5/5 recipients of immune lymphoid cells, although 4/7 livers of control mice contained virus, and three of these had virus titers ranging from $10^{5.6}$ to $10^{6.9}$ TCD_{50}.

Massive hepatic necrosis

Massive hepatic necrosis is an index of the enhanced severity of Coxsackieviral disease in marasmic mice (2). Transfer of immune lymphoid cells within the first 24 hr after viral inoculation significantly decreased the incidence of these lesions by the eighth day of infection (see Table 1). The present study also showed that nearly all mice (19/20) that died by the

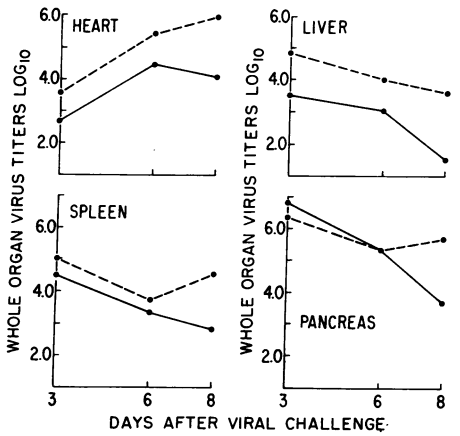

FIG. 2. Average viral titers in the heart, spleen, liver, and pancreas of infected animals that received immune lymphoid cells (——) or medium alone (- - -). Numbers of mice examined at 3, 6, and 8 days in the immune cell-treated group was 2, 5, and 5, and 2, 7, and 7 in the medium-treated group. Differences between average heart, spleen, and pancreas virus titers in the two groups were statistically significant on day 8 ($P \leq 0.05$).

eighth day of infection, and three sick animals killed at this time, had massive hepatic necrosis. Titration of virus in the livers of 28 mice, 5–8 days after infection, revealed that this lesion was invariably associated with virus titers of $10^{4.3}$ TCD_{50} or higher.

Gross cardiac lesions

Gross cardiac lesions, consisting of myocardial necrosis and calcium deposition, are found in Coxsackievirus-infected marasmic mice. Immune lymphoid cells did not prevent the development of these lesions (see Table 1). This is consistent with the observation that gross cardiac lesions can be induced in animals by various nonspecific stress stimuli, such as surgery or challenge with Newcastle disease virus (2).

DISCUSSION

The present study shows that the spleens of mice who have recovered from Coxsackievirus B_3 infection contained a population of lymphoid cells capable of transferring to recipient animals protection against this viral disease. Transfer of immune lymphoid cells to virus-infected recipients significantly reduced the incidence of death and massive hepatic necrosis. The protective capacity of immune lymphoid cells was further manifested by the reduced titers of virus recovered in the target organs. Protection was not transferred by lymphoid cells from unimmunized mice, which suggests that donors specifically produced protective cells in response to Coxsackievirus infection.

Recipients in these experiments were all adult marasmic mice. These animals are unable to establish effective antiviral recovery mechanisms. For example, in normal mice, Coxsackievirus B_3 replicates to maximal titers in the heart, spleen, and pancreas by the third day of infection; by the seventh or eighth day, virus is not detected in these organs. This sequence of events indicates that recovery mechanisms in the normal host become operative after the third day of infection and are fully effective by the eighth day. In contrast, Coxsackievirus causes death and severe lesions in a substantial proportion of marasmic mice. These animals, moreover, show persistence of high virus titers in the target organs after the first week of infection, at a time when normal mice have become fully resistant to this virus (2). The demonstration that immune lymphoid cells conferred an increased capacity to resist viral replication indicates that defective lymphocyte-mediated recovery mechanisms contributed significantly to the increased severity of viral disease in marasmic mice. The concept that the recipients were to some degree immunologically incompetent is also consistent with the finding of extreme atrophy of lymphoid tissues in these animals (3).

Also, the recipients were begun on the dietary deprivation just after weaning. The enhanced severity of viral disease in these animals correlates with the clinically recognized synergistic association of post-weaning malnutrition with severe viral disease in children (5). It is possible that in man, malnutrition induces changes in host defense mechanisms similar to those described here. For example, tuberculin skin reactivity was depressed after bacillus Calmette-Guerin (BCG) vaccination of malnourished children (6, 7).

The mechanisms by which immune lymphoid cells transfer protection are unknown. While neither interferon nor neutralizing antibody was detected in supernatants obtained from suspensions of immune cells, these cells may, after transfer, lead to the synthesis of interferon or neutralizing antibody. In preliminary experiments, however, it was found that the incidence of death of marasmic mice 12 days after virus in-

TABLE 1. *Incidence of mortality, massive hepatic necrosis, and gross cardiac lesions in Coxsackievirus-infected marasmic mice injected with immune lymphoid cells*.*

	Mortality		Massive hepatic necrosis		Gross cardiac lesions	
	No. dead / Total	Percent	No. afflicted / Total	Percent	No. afflicted / Total	Percent
Mice injected† with virus and MEM	17/35	48.57	18/35	51.42	15/35	42.85
Mice injected with virus,‡ and immune lymphoid cells	3/21	14.28	4/21	19.04	8/21	38.09
P	$.02 > P > .01$		$.05 > P > .02$		$.95 > P > .90$	

* Data are from two separate experiments terminated at the end of the eighth day after viral challenge.

† Mice received MEM, i.v., within 24 hr after challenge with Coxsackievirus B_3.

‡ Mice received 7–9 $\times$ 10^7 immune lymphoid cells, i.v., within 24 hr after Coxsackievirus B_3 challenge.

fection was not reduced by transfer of exogenous interferon (Woodruff, J. J. and J. F. Woodruff, unpublished observations).

It cannot be determined from the present studies whether humoral neutralizing antibody mediated the protection induced by the donor cells. Previous studies showed that marasmic mice maintained on 25% of the optimal diet have a severely depressed serum neutralizing antibody response 7–8 days after Coxsackievirus infection (3). However, similar mice that were fed an optimal diet at the time of infection, have significantly less virus in their target organs on day 7, even though the serum neutralizing antibody response remains significantly reduced. Also, animals maintained on 50% of the optimal diet, have, after Coxsackievirus infection, "normal" titers of serum-neutralizing antibody, but significantly elevated titers of virus in the target organs on day 8. These observations suggest that factors other than, or in addition to, serum-neutralizing antibody are required for recovery from Coxsackievirus infection. The consistent association of lymphocytes with macrophages at the site of viral-induced lesions (2, 3) suggests that if neutralizing antibody contributes to recovery from this primary viral infection, part of its major effect may be achieved by interaction with these inflammatory cells at the site of virus replication.

These observations on the antiviral effect of immune lymphoid cells are in accord with the study by Frenkel that demonstrated decreased mortality after rhinopneumonitis virus infection in recipients of immune cells (8). Several reports have documented the increased mortality of virus-infected animals previously treated with antithymocyte or antilymphocyte sera (9–11). Recently, it has been shown that antithymocyte-treated recipients infected with mouse pox virus have normal organ virus titers 4 days after infection, but elevated titers by days 6-7 (12). This effect of antithymocyte serum has led to the suggestion that thymus-dependent lymphocytes are essential for recovery from certain viral infections.

There is thus evidence that the acquisition of lymphocyte-mediated defense mechanisms is essential for normal recovery from certain primary viral infections. Since activated macrophages can inhibit viral replication (13), interaction between immunologically competent lymphocytes and macrophages may provide a decisive mechanism for the elimination of virus. This system may be similar to that described for recovery from infection with facultative intracellular parasites (14).

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