Laboratory Investigation

Monoclonal Antibody Therapy for Prevention of Acute Coxsackievirus B3 Myocarditis in Mice

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The efficacy of monoclonal antibodies against T cell subsets in the therapy of experimental myocarditis caused by coxsackievirus B3 (CB3) was investigated. Two-week-old male C3H/He mice were inoculated with CB3 virus. Treatment was begun in the viremic stage (starting on the day of inoculation) in experiment 1 and in the later aviremic stage (starting on day 10) in experiment 2. Rat anti-mouse monoclonal antibodies, Lyt 1 (helper/inducer T) at 1 µg/mouse (group 2 in experiment 1; group 6 in experiment 2), Lyt 2 (suppressor/cytotoxic T) at 1 µg/mouse (group 3 in experiment 1; group 7 in experiment 2), and Lyt 1 at 1 µg plus Lyt 2 at 1 µg/mouse (group 4 in experiment 1; group 8 in experiment 2), were administered subcutaneously daily for 2 weeks. The treatment groups were compared with infected controls (group 1 in experiment 1; group 5 in experiment 2). In experiment 1, the survival rate in group 4 was higher (p<0.01) than in group 1. In experiment 2, mice treated with Lyt 1 plus Lyt 2 (group 8) survived significantly longer (p<0.05) than did controls (group 5). In experiment 1, myocardial virus titers on days 5 and 6 did not show any significant differences among the four groups. Serum-neutralizing antibody titers between group 1 and group 4 in experiment 1 or between group 5 and group 8 in experiment 2 did not differ significantly. Histologic examination showed extensive myocardial necrosis and cellular infiltration in untreated groups: there was less infiltration in group 4 and in group 8 and less severe necrosis in group 8. The in vivo efficacy of the administered monoclonal antibodies was shown by immunostaining methods in uninfected mice receiving the same treatment as in experiment 1. Thus, monoclonal antibody therapy for total T cells, but not for each T cell subset, partially suppressed acute myocarditis in mice, independent of myocardial CB3 virus replication. (Circulation 1989;79:1300–1308)

With the use of endomyocardial biopsies, myocarditis has become recognized more widely.1–3 Virus infections initiate the disease although the suspected antigen is rarely isolated from patients with myocarditis.4–7

Current therapy for viral myocarditis is symptomatic and supportive because our understanding of the pathogenesis of myocarditis in humans is limited. For example, the efficacy of immunosuppressive therapy for viral myocarditis has been controversial, based upon both clinical8,9 and experimental studies.10–13

Recently, monoclonal antibody therapy has been explored in some diseases, focusing upon the etiologic factors.14–16

Coxsackievirus B has been established as the predominant cause of viral myocarditis in humans.5–7 The role of the immune system in acute coxsackievirus myocarditis and its sequelae remains uncertain.1–7 Previous work of our laboratory showed the significance of T cells in the development and pathogenesis of murine viral myocarditis.17,18

In this study, we investigated the effects of monoclonal antibodies against T cells in murine coxsackievirus B3 myocarditis.

Methods

Virus and Cell

Coxsackievirus B3 (CB3) (Nancy strain) (American Type Culture Collection, Rockville, Maryland) was used; the virus stock was prepared in cultures of VERO (kidney of African green monkey) cells in Eagle’s minimum essential medium (EMEM). Virus suspensions were centrifuged after the cytopathic

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effect had developed. Virus stock had a titer of more than $10^6$ plaque forming units (pfu) per 0.1 ml determined by plaque assay. Virus fluid was stored at $-70^\circ$ C until use.

Viral titers were determined by plaque formation on VERO cell monolayers. Cells were suspended at a concentration of $1 \times 10^6$ in EMEM with 3% fetal calf serum (FCS) plus 100 $\mu$g/ml of penicillin and streptomycin in six-well plastic plates and were allowed to grow for 2 days at $37^\circ$ C in 5% CO$_2$.

Volumes (0.1 ml) of decimal dilutions of virus suspended in EMEM were absorbed to VERO cell monolayers for 60 minutes at $37^\circ$ C in 5% CO$_2$. After adsorption, the cells were overlaid with 3 ml medium containing 3% FCS and 1% methyl cellulose. After 2 days of incubation at $37^\circ$ C in a humidified atmosphere containing 5% CO$_2$, cells were fixed with acetic acid and methanol (at a ratio of 1:3) and stained with crystal violet (1%); plaques were counted with an inverted microscope.

**Monoclonal Antibodies**

Rat anti-mouse monoclonal antibodies, Lyt 1 (53-7.3) and Lyt 2 (53-6.7), were obtained as culture supernatants of the respective hybridoma cells originally supplied by the Cell Distribution Center, Salk Institute, San Diego, California. The efficacy of these antibodies for immune staining has been reported previously.

Before injection into mice, 0.1% NaN$_3$, a preservative, was removed by dialysis.

**Virus Inoculation and Treatment of Mice**

Two-week-old male C$_3$H/He mice (Jackson Laboratory, Bar Harbor, Maine) were inoculated intra-peritoneally with 0.1 ml CB3 virus diluted in EMEM to a concentration of $3 \times 10^6$ pfu/ml. Mother mice were supplied for the maintenance of 2-week-old mice. Rat anti-mouse monoclonal antibodies were administered subcutaneously daily, beginning immediately (experiment 1, see Table 1) or on the 10th day (experiment 2, see Table 2) after virus inoculation for 2 weeks, at a dose of Lyt 1 at 1 $\mu$g/mouse/day (group 2, n=15, in experiment 1; group 6, n=10, in experiment 2), Lyt 2 at 1 $\mu$g/mouse/day (group 3, n=15, in experiment 1; group 7, n=10, in experiment 2), and Lyt 1 at 1 $\mu$g plus Lyt 2 at 1 $\mu$g/mouse/day (group 4, n=15, in experiment 1; group 8, n=10, in experiment 2). Control mice (group 1, n=20, in experiment 1; group 5, n=20, in experiment 2) were injected with normal rat immunoglobulin at a dose of 1 $\mu$g/mouse/day. Mice were observed daily, and when found dead, complete necropsies were performed. Surviving mice were killed on day 14 or 24, in experiment 1 or 2, respectively. Organs were processed for histologic study. In experiment 1, to match for stage, three mice in each group were killed on day 6 for the determination of myocardial virus titers. Hearts were sectioned into two equal cross sections in the short axis, and half of the heart was processed for histologic study; the other half was processed for virologic study.

In parallel with these experiments, uninfected 2-week-old male C$_3$H/He mice received the same treatment as in experiment 1 [rat immunoglobulin (n=7), Lyt 1 (n=3), Lyt 2 (n=3), and Lyt 1 plus Lyt 2 (n=7) received groups], and they served as antibody-treated, uninfected controls. They were killed on days 10–14. The peripheral blood and spleen were processed for immunofluorescence study, and the thymus and spleen were frozen and processed for immunoperoxidase staining. Other organs were preserved for histologic study.

Earlier studies had shown that the dosage of monoclonal antibodies used in this experiment is appropriate.

**Immunostaining**

These studies were performed to determine the in vivo effects of monoclonal antibodies upon the peripheral blood and lymphoid organs of uninfected controls. Immunofluorescence methods were similar to those described previously. In brief, the spleen was minced gently with a sterile stainless mesh. After mincing, the cell suspension was rapidly pipetted with a sterile Pasteur pipette into 20–25 ml Hanks’ balanced salt solution (HBSS), filtered through nylon mesh to eliminate debris, and centrifuged at 1,500 rpm for 5 minutes. The cells were washed twice with HBSS. Peripheral blood (0.8–1.1 ml) was collected in heparin and washed twice with HBSS. The lymphocyte fractions of these samples were obtained by Ficoll gradient centrifugation; the suspensions were layered carefully on 4 ml Ficoll and centrifuged at 2,000 rpm for 15 minutes. Cell viability was determined by the trypan blue dye exclusion test. The cells were finally suspended at a concentration of $1 \times 10^6$ cells/ml in RPMI-1640 media with 2.5% fetal calf serum and 0.1% NaN$_3$. T cell subsets were stained using monoclonal rat anti-Lyt 1, rat anti-Lyt 2, and rat anti-Lyt 1 plus rat anti-Lyt 2 as the first layer of antibodies and fluorescein isothiocyanate-labeled rabbit anti-mouse immunoglobulin as the second. Fluorescein isothiocyanate-labeled anti-mouse immunoglobulin was diluted 10-fold, and rat monoclonal antibodies were diluted 10-fold. The cells were put into V-bottomed tubes and centrifuged at 1,500g for 3 minutes. After centrifugation, the cell pellet was suspended in 100 $\mu$l RPMI-1640 media with 2.5% fetal calf serum and 0.1% NaN$_3$ on the first layer of antibodies. After incubation for 30 minutes at 4°C, the cells were washed three times and suspended in 1.0 ml RPMI-1640 media with 2.5% fetal calf serum and 0.1% NaN$_3$. The percentage of positive fluorescent cells in each specimen was determined by the examination of at least 100 cells under a fluorescence microscope. The percentages of T-cell subsets were obtained by subtracting the percentage of positive cells stained with the second antibody from those obtained after staining with each monoclonal
TABLE 1. Percentages of Positive Cells in Each Lymphocyte Subset in the Peripheral Blood and Spleen Treated With Monoclonal Antibodies

<table>
<thead>
<tr>
<th>Group (Regimen)</th>
<th>Peripheral blood (%)</th>
<th>Spleen (%)</th>
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<tbody>
<tr>
<td></td>
<td>Lyt 1+Lyt 2</td>
<td>Lyt 1</td>
</tr>
<tr>
<td>Group 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Control*)</td>
<td>39.3±3.8</td>
<td>31.0±7.0</td>
</tr>
<tr>
<td>Group 4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Lyt 1 at 1 μg/day plus Lyt 2 at 1 μg/day)</td>
<td>11.7±6.3†</td>
<td>14.6±7.1‡</td>
</tr>
</tbody>
</table>

Values are mean±SD.
TLC, total lymphocyte counts per mouse.
* Rat immunoglobulin G=1 μg/day.
† p<0.001, §p<0.05, ‡p<0.01 vs. group 1.

rat antibody and fluorescein isothiocyanate-labeled rabbit anti-mouse immunoglobulin.

For immunopathologic study, six micrometer sections were cut from the frozen blocks on a cryostat at −20°C, placed on glass slides, air-dried for 1 hour, and fixed in 95% cold methanol. Cell surface markers were shown in situ by 3,3'-diaminobenzidine tetra-hydrochloride immunoperoxidase staining, using the same series of monoclonal rat anti-mouse alloantigen antibodies as in experiments 1 and 2 as the first layer of antibodies, and peroxidase-conjugated rabbit anti-rat immunoglobulin (Dako, Santa Barbara, California) as the second. Antibodies were diluted 10 times. Incubation of samples with antibodies was performed for 1 hour in a moist chamber. Sections were counterstained with Mayer's hematoxylin (Fisher, Medford, Massachusetts) or methyl green (Sigma Chemical, St. Louis, Missouri), dehydrated, and mounted in Permount (Fisher) under a glass coverslip. Control sections were treated with phosphate-buffered saline for the first layer of antibodies.

Histopathology

Organs were fixed in 10% formalin solution, embedded in paraffin, and stained with hematoxylineosin stain in serial slides. For myocardial lesions, cellular infiltration, myocardial cell necrosis, and calcification were scored blindly by us on a scale of 1+ to 5+ in terms of severity. The mean value was cited. The scores were 1+ (mild): one or two small foci; 2+ (slight): several small foci; 3+ (moderate): multiple small foci or several large foci; 4+ (severe): multiple large foci; 5+ (extremely severe): diffuse inflammation, necrosis, or calcification over the entire heart.

Sections of thymus and spleen were inspected for cellularity and its depletion, those of lungs and liver for congestion, and kidney and pancreas for virus-induced lesions.

Virus Titer of Hearts

For infectivity assays, hearts were removed aseptically, and aliquots were weighed and homogenized in 2 ml EMEM. After centrifugation at 1,500g for 15 minutes at 4°C, supernatants (0.1 ml) were inoculated into VERO cell monolayers, and plaque assays were performed.

Serum Neutralizing Antibody Titers

Neutralizing antibody titers were determined in groups 1 and 4 in experiment 1, and in groups 5 and 8 in experiment 2, at the end of each experiment.

Blood was obtained under sterile conditions from the retro-orbital plexus, and the serum was inactivated at 56°C for 30 minutes. Each sample was titrated serially by determining the fourfold dilution in 5.0 ml EMEM supplemented with 3% FCS that protected VERO cell monolayers against a challenge of 100 pfu CB3 virus. Tubes were observed daily throughout a period of 7 days for the appearance of characteristic cytopathic effects.

Statistics

Differences between the control and treated survival curves were evaluated by the Kaplan-Meier method.¹⁹ The differences of the percentages of lymphocyte subsets were examined by unpaired t tests. Virus titers of the heart and histologic scores were examined by one-way analysis of variance with multiple sample comparisons.²⁰

Results

Immunostaining

The results of the immunofluorescence study are listed in Table 1. In both the peripheral blood and the spleen in animals treated with Lyt 1 plus Lyt 2 antibodies, the percentages of all the subsets were decreased compared with mice treated with rat immunoglobulin. In immunopathologic studies, uninfected mice treated with rat immunoglobulin (n=3) showed a positive reaction with a majority of thymocytes or cells of the T cell zone (paracortical area and periarterial sheath) in the spleen in the Lyt 1, Lyt 2, and Lyt 1 plus Lyt 2 stains. In contrast, uninfected mice treated with Lyt 1 plus Lyt 2 antibodies (n=3) showed a positive reaction with a minority of thymocytes or of the T cell zone in the spleen in the same staining series. Uninfected mice treated with Lyt 1 (n=3) or Lyt 2 (n=3) antibody tended to show an intermediate positive reaction of thymocytes or of the T cell zone in the spleen in the same series. Thus, the in vivo efficacy of the monoclonal antibody used was shown.
**Experiment 1**

**Mortality.** Group 4 mice survived longer than controls \((p<0.01\) vs. group 1) (Figure 1). Survival rate on day 14 in each group was as follows: group 1 was 17.6\% (three of 17), group 2 was 25.0\% (three of 12), group 3 was 25.0\% (three of 12), and group 4 was 75.0\% (nine of 12).

**Histology.** The incidence of myocarditis was 100\% in each group (Figure 2, Table 2). Cellular infiltration was significantly less severe \((p<0.05\) in group 4 compared with controls; there were no statistically significant differences between group 1 and groups 2 or 3. The degree of myocardial necrosis and calcification was not different among the four groups.

**Myocardial virus titers.** Myocardial virus titers did not differ among the 4 groups (Table 2).

**Neutralizing antibody titers.** There were no significant differences between group 1 and group 4 (Table 2).

**Experiment 2**

**Mortality.** Group 8 mice survived significantly longer than controls \((p<0.05\) vs. group 5) (Figure 3). Survival rate on day 24 in each group was as follows: group 5 was 55.0\% (11 of 20), group 6 was 60.0\% (six of 10), group 7 was 80.0\% (eight of 10), and group 8 was 90.0\% (nine of 10).

**Histology.** The incidence of myocarditis was 100\% in each group (Figure 4, Table 3). Both cellular infiltration and myocardial necrosis were significantly less severe \((p<0.05\) in group 8 compared with group 5; however, there were no significant differences between group 5 and groups 6 or 7. Calcification did not differ significantly among the four groups.

**Myocardial virus titers.** Myocardial virus was not detected in dead mice on days 10–14 in any of the four groups.

**Neutralizing antibody titers.** Neutralizing antibody titers were not different between groups 5 and 8.

**Other Organ Involvement in Experiments 1 and 2**

Overall, pancreatic lesions likely of viral origin were noted in three or four mice in each group, and these lesions may have contributed to the observed clinical course. No viral lesions were noted in the lung, liver, kidney, thymus, and spleen.

**Uninfected Controls**

None of the uninfected mice in the four groups (groups receiving rat immunoglobulin, Lyt 1, Lyt 2, and Lyt 1 plus Lyt 2) showed any histologic abnormalities in the heart, lung, liver, kidney, and pancreas.

**Discussion**

This study shows the beneficial effects of monoclonal antibody therapy against total T cells, but not for each T cell subset, consisting of partial protection against the development of CB3 virus myocarditis in the acute stage of illness, independent of myocardial virus replication.

The murine model of CB3 virus myocarditis shows a progression from viral infection to myocardial dysfunction: a biphasic disease process results when mice are infected with CB3 virus. During the acute phase, viral replication in the myocardium results in myocardial necrosis and inflammation. After the virus has been eliminated from the myocardium, a chronic inflammatory reaction may lead to progressive myocyte damage and hypertrophy, ventricular dilatation, and even heart failure. The chronic phase has been postulated to result from cell-mediated immune responses to a neoantigen that developed during the acute phase of the illness.1–7

To clarify the immune mechanisms in myocarditis in CB3 virus murine models, we already showed the different kinetics of lymphocyte subsets between the peripheral blood and the heart, their serial changes in the course of the disease, and the significance of myocardial Thy 1.2+ (pan T) and Lyt 1+ (helper/inducer T) cells in the development of myocarditis.17 Similar results were also shown in encephalomyocarditis virus-induced murine myo-
FIGURE 2. Sections of myocardium in experiment 1. Fourteen days after virus inoculation, marked inflammatory cell infiltration and extensive myocardial necrosis are seen in a mouse of group 1 (Panel A). On the other hand, infiltration and necrosis are less severe in the heart of a mouse in group 4 (Panel B). Hematoxylin-eosin stain. Original magnification, ×380.
TABLE 2. Results of Experiment 1

<table>
<thead>
<tr>
<th>Group (Regimen)</th>
<th>Cardiac histology</th>
<th>Myocardial virus titers (pfu/mg tissue)</th>
<th>Neutralizing antibody titers (log 2)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>I</td>
<td>N</td>
<td>C</td>
</tr>
<tr>
<td>Group 1 (Control*)</td>
<td>3.2±1.0</td>
<td>3.4±0.9</td>
<td>3.2±0.8</td>
</tr>
<tr>
<td>(n=20)</td>
<td></td>
<td>(n=20)</td>
<td>(n=20)</td>
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<tr>
<td>Group 2 (Lyt 1 at 1 µg/day)</td>
<td>2.9±0.5</td>
<td>2.9±1.0</td>
<td>2.9±1.0</td>
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<tr>
<td>(n=15)</td>
<td></td>
<td>(n=15)</td>
<td>(n=15)</td>
</tr>
<tr>
<td>Group 3 (Lyt 2 at 1 µg/day)</td>
<td>3.1±1.1</td>
<td>3.0±1.1</td>
<td>2.7±1.0</td>
</tr>
<tr>
<td>(n=15)</td>
<td></td>
<td>(n=15)</td>
<td>(n=15)</td>
</tr>
<tr>
<td>Group 4 (Lyt 1 at 1 µg/day plus Lyt 2 at 1 µg/day)</td>
<td>2.3±0.9†</td>
<td>2.5±0.9</td>
<td>3.0±0.7</td>
</tr>
<tr>
<td>(n=15)</td>
<td></td>
<td>(n=15)</td>
<td>(n=15)</td>
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</tbody>
</table>

Values are mean±SD.
Each group included three hearts of mice killed on day 6.
I, infiltration; N, necrosis; C, calcification; pfu, plaque forming units.
*Rat immunoglobulin G at 1 µg/day.
†p<0.05 vs. group 1.

Carditis models. These data led us to the present study of the effects of monoclonal antibodies upon murine CB3 virus myocarditis.

In experiment 1 (viremic stage) of this study, significant improvement of survival and reduction in cellular infiltration were shown in group 4, but not in groups 2 and 3, compared with controls. The lack of change in myocardial necrosis or calcification in group 4 may be related to the longer survival rate; indeed, nine mice survived until day 14. Murine myocardial calcification is accompanied by cell necrosis and increases with time. In experiment 2 (aviremic stage), significant improvement of survival and a decrease in cellular infiltration and necrosis were seen in group 8.

With regard to the assigned functions of each murine lymphocyte subset, the Lyt 1+ and Lyt 2+ subset (Lyt 1+, 23+ subset) is thought to be the immature T cells and precursor of other T cell subsets; it has been shown to be an alloreactive and syngeneic cytotoxic lymphocyte subset in addition to the Lyt 1− and Lyt 2− subset (Lyt 1−, 23− subset). The Lyt 1+ and Lyt 2− subset (Lyt 1+, 23− subset) is not only a helper/inducer in antibody and cytotoxic lymphocyte inductions but also an effector of delayed-type hypersensitivity in which many lymphokines and monokines are involved.

To consider the role of T cell subsets, as mentioned above, the depletion of Lyt 1 or Lyt 2 subset alone may not suffice for therapy because the immature and precursor cells, that is, Lyt 1+, 23+ subset, can develop into other subset populations (i.e., Lyt 1 or Lyt 2 subset). Thus, it is reasonable that administration of both Lyt 1 and Lyt 2 antibodies together yielded the clear effectiveness in the present study.

The most commonly identified agents in cases of human myocarditis are enteroviruses, which contain a simple RNA genome surrounded by a capsid composed of four distinctive polypeptides with cubic symmetry. Coxsackievirus B3 (CB3) is the predominant cause of viral myocarditis in humans. The critical role of neutralizing antibody in the protection from virus entrance into the host has already been shown. However, previous reports have shown that serum neutralizing antibody production in experimental CB3 virus infection in mice is not affected by the absence of T cells, that is, it may be controlled only by B cells. Indeed, the neutralizing antibody titers found in the present study support this. Thus, monoclonal antibody therapy...
FIGURE 4. Sections of myocardium 24 days after virus inoculation. Extensive myocardial necrosis is seen in a mouse from group 5 (Panel A). On the other hand, necrosis is less severe in the heart of a mouse in group 8 (Panel B). Hematoxylin-eosin stain. Original magnification, ×380.
against T cells does not suppress neutralizing antibody formation in the host. Future work should address the question whether the administration of monoclonal antibodies affects the population of myocardial infiltrating lymphocytes in each group. Therapy for clinical viral myocarditis has remained symptomatic and supportive, and specific treatment has not been available. The use of immunosuppressive agents,\textsuperscript{8-13} including cyclosporines, is still controversial, and clinicians have to recognize the side effects of these agents. Antiviral agents, such as ribavirin\textsuperscript{23,24} or interferon,\textsuperscript{25,26} can be used only as long as the virus is present in the host; in clinical settings, the likelihood is small. Thus, monoclonal antibody therapy against pan T cells may be useful for the treatment of patients with myocarditis, irrespective of the presence of the virus, even though its effect may be only partial. Further investigations in different strains of mice infected with this and other strains of coxsackievirus are indicated, with ultimate aim to explore this therapeutic approach in early cases of identified coxsackievirus myocarditis in humans.

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KEY WORDS: myocarditis • coxsackievirus B3 • murine model • monoclonal antibody therapy • immune system
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