Anti-CD2 Monoclonal Antibodies Prevent the Induction of Experimental Autoimmune Myocarditis

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SUMMARY
We investigated the effect of a monoclonal antibody against CD2 molecules (OX34) in preventing the induction of experimental autoimmune myocarditis (EAM) induced by immunizing Lewis rats with cardiac myosin. Administration of OX34 before immunization, on Days -6, -4, -2 and 0, completely prevented EAM. On the other hand, treatment with OX34 just before the appearance of myocardial lesions, on Days 9, 11, 13 and 15, had only a partial effect in preventing the disease. Flow cytometric analysis of lymph node cells showed that CD3+ T cells were immediately depleted with the administration of OX34 but had largely recovered on Day 21. Lymph node cells in OX34-treated rats had no proliferative responses to cardiac myosin-rod, but the proliferation was restored when recombinant IL-2 was added. Ultimate production of the anti-myosin antibody was not inhibited by the treatment with OX34. These results suggest that the prevention of EAM by administering the anti-CD2 monoclonal antibody OX34 resulted from T cell depletion during the induction phase, and might in addition result from T cell anergy of Th1, but not Th2 cells. (Jpn Heart J 2000; 41: 507-517)

Key words: Autoimmune myocarditis, Antibody therapy, Anergy, Cytokine, Myosin, Cardiomyopathy

An autoimmune mechanism has been found to be involved in some types of human myocarditis or dilated cardiomyopathy.1,2) Experimental autoimmune myocarditis (EAM) is a T-cell mediated autoimmune disease model produced by immunization of rats or mice with cardiac myosin.3,4) T cell recognition of an antigen requires a trimolecular complex consisting of a major histocompatibility complex (MHC) molecule associated with a peptide and the T cell receptor (TCR).5) In addition, optimal activation of T cells by ligation of this TCR com-
plex requires co-stimulatory signals, which are provided by the adhesive interaction between receptor molecules on T cells and their counterreceptors on antigen-presenting cells. Several immunotherapies in T-cell mediated autoimmune diseases are based on blocking these two types of signal transductions. We have already reported that immunotherapies using a monoclonal antibody (mAb) against TCRαβ or a combination of mAbs against leukocyte function-associated molecule-1 (LFA-1) and intercellular adhesion molecule-1 (ICAM-1) prevent the induction of rat EAM.

CD2 has been reported to function as both an adhesion and signal molecule in T cell recognition. Treatment with anti-CD2 mAb has been shown to induce T cell unresponsiveness in vivo. Preoperative and postoperative administration of anti-CD2 mAb induced long-term survival of cardiac allograft in the rat. However, there have been no reports concerning immunotherapy with administration of anti-CD2 mAb preventing the induction of autoimmune disease except for spontaneous and adoptive transfer of diabetes in the BB/Wor rat.

This study examined whether treatment with anti-CD2 mAb OX34 in the induction or afferent phase can prevent EAM in Lewis rats. Furthermore, we also demonstrated that the alteration of cellular and humoral autoimmune responses, which might be induced by inhibition of some helper T (Th) functions, are associated with prevention of the disease.

**METHODS**

**Animals:** Inbred 6-week-old Lewis rats were purchased from Charles River Japan Inc. (Kanagawa, Japan) and maintained at the Facilit for Comparative Medicine & Animal Experimentation, Niigata University School of Medicine.

**EAM induction:** Cardiac myosin was prepared from the ventricular muscle of porcine hearts according to the methods of Murakami, et al. The antigen was dissolved in a solution of 0.6 M KCl at a concentration of 10.0 mg/ml. Rats were immunized once in their footpad with 1.0 mg cardiac myosin in an equal volume of Freund's adjuvant containing 10.0 mg/ml Mycobacterium tuberculosis H37Ra (Difco Laboratories, Detroit, MI, USA).

**Preparation of mAb:** Hybridoma cells that produce mouse mAb against CD2 molecule (OX34) were obtained from the European Collection of Animal Cell Center (Salisbury, UK) and were grown as ascites in BALB/c mice. The antibody was isolated from the ascites by ammonium sulfate precipitation. The content was quantified by optical density and fluorescence...
cence-activated cell-sorter analysis.

The mAbs, FITC-conjugated W3/25 (anti-CD4)\(^{16}\) and FITC-conjugated OX8 (anti-CD8)\(^{17}\) were purchased from Serotec (Blankthorn, Bicester, Bucks, UK). FITC-conjugated G4.18 (anti-CD3)\(^{18}\) was purchased from Pharmingen (San Diego, CA, USA).

**Treatment protocols:** One milliliter of OX34 in phosphate-buffered saline (PBS) at a concentration of 1.0 mg/ml was administered intraperitoneally once daily according to the 2 protocols described below. Control groups were injected with 1 ml of PBS in the same manner. In the first protocol, rats received anti-CD2 mAb or PBS before immunization with cardiac myosin, on Days -6, -4, -2, and 0. In the second protocol, treatment was started shortly before the appearance of myocardial inflammation, on Days 9, 11, 13, and 15.

**Histopathology:** Rats were sacrificed under ether anesthesia on Day 21. The heart weight was determined by measuring the cardiac ventricle, excluding the atrium and other tissues. Macroscopic findings were graded according to the following system: 0 (normal appearance), 1 (a discolored focal area), 2 (multiple discolored areas), or 3 (diffuse discolored area). The ventricles were transversely sliced and fixed in 10% formalin, embedded in paraffin and stained with hematoxylin-eosin. Microscopic findings were scored as previously described.\(^{19}\)

**Flow cytometry analysis:** Lymph node cell suspensions were prepared by passage through a 200-gauge stainless steel mesh. Peripheral blood (1 ml) was diluted in PBS, and lymphocytes were recovered by Ficoll-Hypaque (Pharmacia, Uppsala, Sweden). For detecting the expression of surface phenotypes of the lymphocytes, the cells were incubated with FITC-conjugated mAbs (CD3, CD4, and CD8) or PE-conjugated anti-mouse IgG (Vector Laboratories, Burlingame, CA, USA). Fresh viable stained cells (1 × 10\(^4\)) were analyzed by a FACScan (Becton-Dickinson, Mountain View, CA, USA).

**Proliferation assay:** Viable lymph node cells prepared as previous stated were cultured in triplicate in 0.2 ml of RPMI 1640 (Gibco BRL, Grand Island, NY, USA) containing 10% fetal bovine serum at 2 × 10\(^4\) cells / well in 96 well U-bottomed microtiter plates (Coster Co., Cambridge, MA, USA) with or without 25 µg/ml of the rod fraction of porcine cardiac myosin, which included major pathogenic epitopes and was prepared as previous reported.\(^{19}\) We used the rod portion as a stimulating antigen because whole myosin induced milder proliferation of lymphocytes in EAM rats than the rod.\(^{19}\) In addition, the cell suspensions were cultured in the presence or absence of 10 U/ml of recombinant human interleukin-
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2 (rhIL-2, Shionogi & Co., Ltd., Osaka, Japan). Incorporation of 0.5 mCi of methyl-3H thymidine was determined after 72 h of incubation in 5% CO₂ and air at a temperature of 37°C. After further incubation for 18 h, the cells were harvested and radioactivity assessed by the liquid-scintillation counting method.

**Anti-myosin Ab measurement:** Anti-myosin antibodies in sera from rats were measured using an enzyme-linked immunosorbent assay. Individual wells in polystyrene microtiter plates (Nunc, Roskilde, Denmark) were coated with 2 µg of porcine cardiac myosin in 0.1 ml of PBS. After overnight incubation at 4°C, the plates were washed three times with PBS containing 0.5% Tween-20. Twenty-fold diluted sera was added to each well and incubated overnight at 4°C. After five washes, 0.1 ml of HRP-labeled rabbit anti-rat IgG (Dako Japan, Kyoto, Japan) diluted × 10,000 was added and incubated for 40 min at 37°C. Thereafter, the plates were washed five times and then incubated at room temperature with 0.1 ml of o-phenylenediamine dihydrochloride solution at a concentration of 0.4 mg/ml. After 5 min, the optical density value at 492 nm was measured in an autoreader (Flow Laboratories Inc., McLean, VA, USA).

**Statistical analysis:** Data are presented as mean ± SD. Differences in inducibility of myocarditis were assessed by Fisher's exact probability test. The two sample t-test was used to analyze the activity of myocarditis and proliferative responses of lymph node cells.

**RESULTS**

**Effect of in vivo treatment of anti-CD2 mAb on EAM development:** Administration of OX34 before immunization, on Days -6, -4, -2 and 0, completely prevented the induction of EAM (Table). Microscopic examination demonstrated that there were no infiltrations of inflammatory cells in the myocardium (Figure 1A). All control rats treated with only PBS showed

<table>
<thead>
<tr>
<th>Day</th>
<th>Treatment</th>
<th>Myocarditis (diseased / total)</th>
<th>Macroscopic scores</th>
<th>Microscopic scores</th>
</tr>
</thead>
<tbody>
<tr>
<td>-6, -4, -2, 0</td>
<td>OX34 1 mg / day</td>
<td>0 / 7</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>PBS</td>
<td>5 / 5*</td>
<td>2.40 ± 0.55**</td>
<td>1.48 ± 0.26**</td>
</tr>
<tr>
<td>9, 11, 13, 15</td>
<td>OX34 1 mg / day</td>
<td>4 / 5</td>
<td>1.90 ± 1.52</td>
<td>1.14 ± 0.81</td>
</tr>
<tr>
<td></td>
<td>PBS</td>
<td>5 / 5</td>
<td>2.40 ± 0.58</td>
<td>1.22 ± 0.21</td>
</tr>
</tbody>
</table>

*p < 0.05, **p < 0.01 vs OX34 treated group.
severe myocarditis, which showed that the lesions were composed of the infiltrations of various inflammatory cells including multinucleated giant cells and degenerated myocardial tissue (Figure 1B). However, the treatment with OX34 started shortly before the appearance of myocarditis on Days 9, 11, 13 and 15, had little effect in preventing inflammation. Three of five treated rats elicited myocarditis as severe as the control. It was

Figure 1. Histological examination of myocardial samples obtained from rats treated with OX34 (A) or with PBS (B) on Days -6, -4, -2 and 0. (A) no myocardial lesion, (B) severe myocarditis with multinucleated giant cells was observed (hematoxylin-eosin staining, original magnification x 33).
also ineffective when rats were administered five doses of OX34 (data not shown).

**Phenotypic analyses of lymphocytes from lymph nodes:** Preliminary examination had demonstrated that intraperitoneal injection of 1 mg of OX34 markedly decreased CD3⁺ T cells in naive Lewis rats 1 day after treatment. The percentages of CD3⁺ T cells of the lymph node and peripheral blood in the treated or untreated rat were 15.8% vs 78.4% and 10.8% vs 68.8%, respectively. We also investigated the surface phenotypes of lymphocytes from lymph nodes at sacrifice to examine the recovery of depleted lymphocytes (Figure 2). In rats treated with OX34 before immu-

![Figure 2](image-url)
nization, PE-conjugated mouse IgG was not detected, which demonstrated that OX34 administered *in vivo* had not attached to the cell surface by Day 21. The decrease in the percentage of T lymphocytes which expressed CD3 was only slight compared with the control. On the other hand, CD4⁺ cells were still partially depleted but CD8⁺ cells had already recovered to normal in OX34-treated rats.

**Proliferative responses of lymph node cells to cardiac myosin-rod:** Lymph node cells had marked proliferative responses to the rod fraction of cardiac myosin in the control, as previously demonstrated in myosin-immunized rats.¹⁹ In the OX34 treated group, however, lymph node cells showed no significant proliferation to these antigens. But the capacity to induce a proliferative response was restored when rhIL-2 was further added to the cultures (Figure 3).

**Humoral response to cardiac myosin:** The anti-myosin antibody level was elevated from Days 4-8 in the control. In OX34-treated rats, an increase in the antibody titer was delayed for about 4 days compared to the control, however, ultimate production of anti-myosin antibody was not inhibited on Day 21 (Figure 4).

![Figure 3. Proliferative responses of lymph node cells to 25 µg/ml of porcine cardiac myosin-rod. Lymph node cells were obtained from rats treated on Days -6, -4, -2 and 0 with PBS (control) or with OX34 (OX34-Tx). The cells were cultured with or without 10 U/ml of human recombinant IL-2.](image)
DISCUSSION

Most organ specific autoimmune disease models induced by immunization with self-antigens are predominantly mediated by T lymphocytes.20) Immunotherapy directed against T cells, therefore, can be efficacious in the treatment of these diseases. In this study, we demonstrated that anti-CD2 mAb OX34 completely prevented the induction of EAM. As previously reported,12,21) administration of OX34 in vivo resulted in immediate depletion of T cells, especially CD4+ cells, rather than sequestration or trafficking of CD2 molecules. Treatment with OX34 before immunization of cardiac myosin could prevent the expansion of autoreactive CD4+ T cells in the induction phase. However, the ability of depleting pathogenic T cells was not complete, and autoimmune disease might be induced by residual autoreactive cells. In the BB/Wor rat model, nondepleting anti-CD2 mAbs could prevent spontaneous development of T cell-mediated autoimmune diabetes.13) Therefore, we propose an additional mechanism for preventing diseases: clonal anergy of pathogenic T cells.

As shown in Figure 3, T cell proliferative responses to cardiac myosin-rod were diminished in lymph node cells from treated rats. These were investigated on Day 21, when the number of T cells in the lymph nodes had recovered to approximately the same levels as the controls. In the

Figure 4. Kinetics of serum anti-myosin antibody titers. Serum samples from rats treated on Days -6, -4, -2 and 0 with PBS (control) or with OX34 (OX34-Tx) were used for measurement by ELISA.
presence of recombinant IL-2, the antigen-specific proliferative responses
of lymph node cells from the treated rats increased to amounts comparable
to controls, suggesting that OX34 prevented myosin-specific immunogenic
cells from secreting sufficient IL-2 to induce proliferation. Insufficient sig-
nal transduction from either TCR or costimulatory molecules has been
shown to induce T cell anergy. 22) Anergic T cells have a reduced ability
to produce IL-2 in response to antigen stimulation 22) and the addition of
exogenous IL-2 restores antigen responsiveness. 23) Our data here may indi-
cate that the administration of OX34 induced anergy in lymph node T cells
by blocking costimulatory signal transduction through CD2 molecules.

Murine CD4+ T cells are comprised of two subsets: Th1 cells and Th2
cells. It is believed that similar T cell subsets also exist in humans 24) and
rats. 25) Th1 cells, which produce IL-2, IFN-γ and TNF-α are responsible
for cell-mediated immune reactions. 26) In autoimmune diseases 27) including
EAM, 28) these products of Th1 cells have been implicated in disease pro-
gression. On the other hand, Th2 cells have been found to have protective
effects in autoimmune diseases 29) through the production of IL-4, IL-5, IL-
6 and IL-10, and are also involved in antibody production. 26) The finding
that these were no differences in ultimate production of anti-myosin Ab
between these two groups could not imply Th2 cell unresponsiveness. Our
in vitro experiments, therefore, indicated that OX34 administration might
induce T cell anergy of Th1 but not Th2 cells. It has been reported that
Th1, but not Th2 clones can be rendered anergic in vitro by signaling
through the TCR complex in the absence of a costimulatory signal deliv-
ered by an accessory cell. 30, 31) This phenomenon can resolve the discrep-
ancy between cellular and humoral immunity observed in OX34-treated
rats. Krieger, et al. 32) demonstrated upregulation of both Th1 and Th2
cytokine profiles in surviving allografted heart tissue, but not in the
periphery, in OX34 treated rats. They speculated that negative regulatory
cells observed in surviving grafts might locally suppress the graft rejec-
tion. However, it is difficult to compare the potential mechanism of OX34-
induced tolerance in EAM with that in allograft rejection because no cell
infiltrations were observed locally in heart tissue in OX34 treated EAM.
Several mechanisms have been reported: that anti-CD2 mAbs induce sup-
pressor T cells, 33) abrogate killer T cell function, 34) or impair NK cell func-
tion. 35) Further studies are required to assess the contribution of these
various factors to disease suppression.

It has been reported that immunotherapies using mAb T cell mole-
cules such as CD4 are also efficacious in human autoimmune diseases, for
instance, rheumatoid arthritis and multiple sclerosis. 36) In human myocardi-
tis and DCM, there is a controversy concerning the effects of immuno-suppressive therapies. However, it is possible to make immunotherapies effective in some cases of myocarditis and DCM with myocardial infiltration of inflammatory cells, as previously shown in treatment with OKT3 mAb. Therefore, immunotherapy with an antibody against CD2 may be applicable to treatment in some cases of human myocarditis and DCM.

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