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A study on OX39, a murine anti-rat interleukin 2 receptor antibody

A report on receptor binding and effects on allograft survival

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Abstract. OX39, a murine IgG1 monoclonal antibody (MoAb) that recognizes the 55 kDa alpha chain of the rat interleukin 2 receptor (R-IL2), was studied in vitro for its ability to interfere with IL2 binding and IL2-induced proliferation on rat concanavalin A (ConA) blasts and in vivo in a model of rat heart allografts. In vitro studies indicated that OX39 MoAb interacts with a single class of sites on the alpha chain of the rat R-IL2 with a high affinity (K_D = 0.8 nm) and competes with IL2 binding on this chain (K_I = 0.53 nm). In contrast, OX39 MoAb was found to be 10-20 times less efficient in competing with IL2 binding to the high-affinity R-IL2 (K_I ~10 nm). It is proposed that the epitope recognized by OX39 on the alpha chain (low-affinity R-IL2) is modified on (or buried in) the high-affinity R-IL2 configuration. Accordingly, OX39 was found to be a weak inhibitor in vitro on IL2-induced proliferation and in vivo on allograft rejection. Allograft survival was unaffected by doses of OX39 of 20 and 50 μg/rat for 9 days; only a borderline effect was noted when doses as high as 250 μg/rat were used. A significant, but restricted, effect of OX39 could be further detected when combined with low doses of cyclosporine A (1.5 mg/kg), which were ineffective by themselves. Together, our data suggest that in order to be efficient in vivo, anti-R-IL2 MoAbs must bind with high affinity to epitopes involved in the high-affinity IL2 binding site.

Key words: Anti-rat R-IL2 - IL2 binding - Proliferation - Allograft survival.

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Activated T lymphocytes display a receptor for IL2 (R-IL2) upon antigenic stimulation [4, 19, 20]. The level of R-IL2 expression is also regulated by monokines [10, 13] and lymphokines such as IL2 itself [1, 7, 12, 16, 26]. R-IL2/IL2 interaction is the major stimulus for the growth of activated lymphocytes. Two classes of R-IL2 have been characterized. The more numerous are low-affinity receptors (K_D ~30 nm). Binding of IL2 to low-affinity receptors does not trigger mitosis. Approximately 2%-5% of R-IL2 can bind IL2 with high affinity (K_D ~10 pm). This high-affinity receptor has been shown to require the association of an alpha chain (55 kDa) of low affinity for IL2 when expressed alone and of a newly characterized beta chain (75 kDa) component which, when complexed to the alpha chain, gives rise to high-affinity receptors [21, 25, 28, 29]. This binding is followed by an internalization of the IL2/R-IL2 complex and a triggering of the growth signal [17]. In addition, it has recently been shown that a truncated soluble R-IL2 (S-R-IL2) form is produced by activated T cells [8, 18, 22]. This S-R-IL2 binds IL2 with low affinity only [8] and is of unknown biological relevance.

Monoclonal antibodies that are directed against R-IL2 epitopes involved in the high-affinity site of IL2 have been produced in various species and have been shown to prevent the rejection process in rodents [9], monkeys [24], and, more recently, in man [2, 27]. In this paper, we report on the effect of a murine antibody directed against the alpha chain of the rat R-IL2. This MoAb, with high affinity for its epitope on the alpha chain, was nevertheless unable to prevent organ rejection and exhibited a low inhibitory effect on both IL2 high-affinity binding and IL2-driven lymphoblast growth. These results suggest that in order to be efficient in vivo, anti-R-

IL2 MoAbs must interact efficiently with an IL2 high-affinity binding site.

Materials and methods

Monoclonals

Ascites of mice injected with OX39 hybridoma producing an IgG1 MoAb directed against rat R-IL2 were kindly provided by Dr. Williams of the Medical Research Council (Oxford, UK) [15]. The MoAb was purified on Affigel-Protein A column and kept at −80 °C until use. An "irrelevant" IgG1 MoAb (anti-*Escherichia coli*), kindly supplied by M. Hirn (Immunotech, Marseille, France), served as the control.

Animals

Lewis rats and (Lew × BN) F1 hybrids (~250 g) were purchased from the animal center of the Centre National de la Recherche Scientifique (Orléans, France) or obtained from P. Druet (Hôpital Broussais, Paris, France).

Cellular suspension

Splenocytes were harvested from Lewis rats by perfusion of the spleen with 50 ml RPMI 1640 (Gibco, Glasgow, UK). Mononuclear cells were obtained by centrifugation over a 65% Percoll Gradient (Pharmacia, Uppsala, Sweden). Concanavalin A (ConA)-activated T lymphocytes were obtained by stimulating mononuclear splenocytes with 5 μg/ml ConA (IBF, Villeneuve La Garenne, France) for 4 days in a culture medium composed of RPMI 1640 supplemented with 10% fetal calf serum (FCS) (Flow Laboratories, Puteaux, France) and $5 \times 10^{-5} M$ 2-mercaptoethanol (Prolabo, Paris, France).

Inhibition of IL2-driven proliferation

ConA blasts were washed twice in RPMI and seeded at a concentration of 25,000 cells/well in 160 μl of culture medium in 96 round bottom titer plates (Nunc, Roskilde, Denmark). The OX39 MoAb and irrelevant IgG1 MoAb were tested at concentrations ranging from 1500 to 0.7 nm in the presence of different concentrations of recombinant IL2 (r-IL2). After 48 h of incubation (37 °C, 5% CO_2), cultures were pulsed with 0.5 μCi/well tritiated thymidine (3H-TdR) (TRK 61 Amersham AS: 22 Ci/mmol) for 4 h, harvested, and counted for radioactivity.

Binding of radioiodinated OX39 and IL2

ConA blasts were washed twice for 2 h at 37 °C in RPMI containing 10% FCS and resuspended in phosphate buffered saline containing 0.1% bovine serum albumin (BSA) (Sigma Chemical, St. Louis, Mo.). They were seeded (2×10^5 cells/well) in 96 round bottom titer plates with increasing concentrations of ^{125}I-labeled OX39 MoAb. OX39 was labeled with iodine-125 (Centre Energie Atomique, Saclay, France) by the Iodogen procedure at a specific radioactivity of about 1500 cpm/fmol. The final volume was 50 μl/well. After incubation for 40 min at 37 °C under agitation, the cells were collected; cell-bound and unbound fractions were separated on a layer of dibutylphtalate-paraffin oil and counted as described [7]. The equilibrium dissociation constant (K_D) of OX39 for its binding site and its cellular density (B_M) were calculated by Scatchard analysis after substraction of nonspecific binding. Binding of ^{125}I-labeled IL2 (New England Nuclear Company, Boston, Mass.) was carried out in a similar way and analyzed; however, the cellular density was 2.10^6 cells/well. ^{125}I-IL2 was used either undiluted in the picomolar range (specific radioactivity around 800 cpm/fmol and 15-min incubation at 37 °C) or in the nanomolar range after isotopic dilution with unlabeled recombinant IL2 (specific radioactivity around 40 cpm/fmol and 30-min incubation at 37 °C).

Inhibition of labeled OX39 and IL2 binding

Inhibition of labeled OX39 binding was carried out at a low saturation of cell receptors using a concentration of labeled OX39 (0.36 nm) below its K_D. The competitors (unlabeled OX39 or IL2) were added at the time of labeled OX39 addition to cells, and cell-bound and unbound fractions were determined after being incubated for 40 min at 37 °C. The true inhibition constant (K_I) characterizing the competitor was calculated according to the equation

$$K_I = I_{0.5} \times F_o \times (B_M - B_o)/[F_o \times B_M + B_o \times (B_M - B_o/2)]$$

where $I_{0.5}$ is the concentration of the competitor, giving 50% inhibition of bound, labeled OX39; B_M is the concentration of total receptors; and B_o and F_o represent the concentrations of bound and unbound ^{125}I-OX39 in the absence of a competitor.

Conversely, inhibition of labeled IL2 binding was carried out either in the presence of a low concentration of ^{125}I-IL2 (100 pm) to label the high-affinity receptors or in the presence of a high concentration of ^{125}I-IL2 (14 nm) to label the low-affinity receptors. Unlabeled OX39 was added as a competitor at increasing concentrations. Incubation, processing of samples, and K_I derivation were carried out as described for the inhibition of ^{125}I-OX39 binding.

Heart transplantation

Heterotopic heart grafts (Lew/BN heart into Lew) were performed according to Ono and Lindsey [14]. MoAbs were given IV for the first 3 days following surgery and IP thereafter until day 9. Graft survival was monitored daily by heart palpation through the abdominal wall. Three experimental groups were formed according to the dose of OX39 injected per day to recipients (10, 50, and 250 μg). An additional group of 6 animals received 250 μg per day of the irrelevant control IgG1. Finally, another group of recipients was treated with varying doses of cyclosporin A (CyA) (1.5, 3, and 10 mg/kg for 15 days), alone or in combination with 50 or 250 μg OX39 per day for 9 days or with 250 μg of the irrelevant control IgG1 per day for 9 days.

Results

Interaction between OX39 and IL2 binding on membrane rat IL2-receptors

As shown in Fig. 1 A, the ^{125}I-labeled OX39 MoAb bound to a single class of receptor sites on 4-day-activated rat ConA blasts. Scatchard analysis (Fig. 1 B) gave a dissociation constant (K_D) of 0.8 nm and a maximal capacity of binding (B_M) of 70,000 sites/

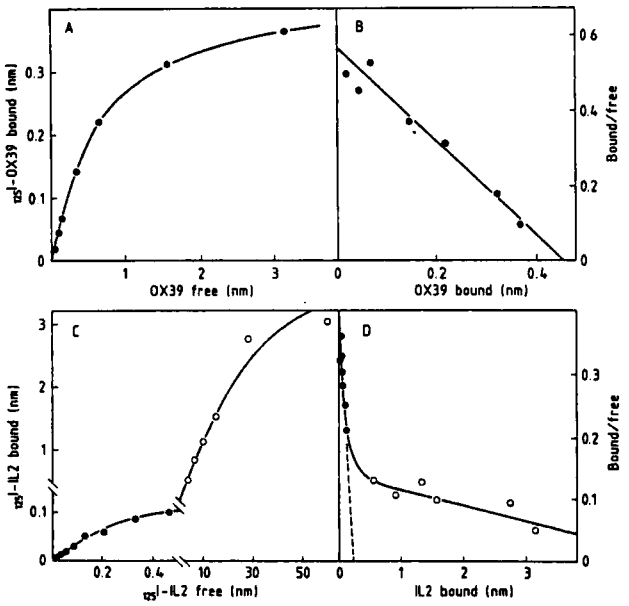

Fig. 1 A-D. Binding of ^{125}I-labeled OX39 MoAb and ^{125}I-labeled IL2 to rat ConA blasts. A Direct binding curve of ^{125}I-labeled OX39 after subtraction of nonspecific binding evaluated in the presence of 660 nm of unlabeled OX39 (less than 10% of total binding). B Scatchard plot analysis of A. C Specific binding of ^{125}I-labeled IL2 used either in the picomolar (-●-) or the nanomolar (-O-) range. Nonspecific binding was determined in the presence of 133 nm (picomolar range) or 1.3 μM unlabeled IL2 (nanomolar range). D Scatchard plot analysis of C

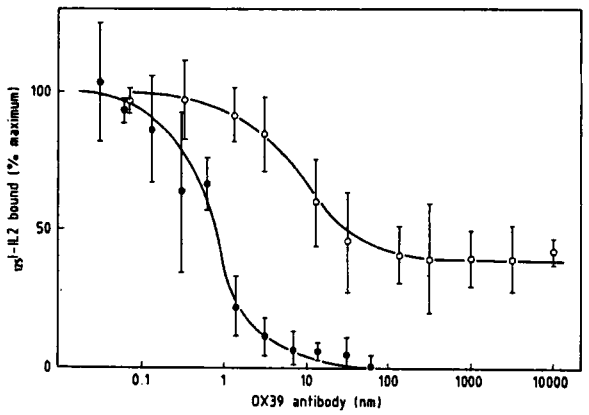

Fig. 2. Inhibition of ^{125}I-labeled IL2 binding by unlabeled OX39. ^{125}I-labeled IL2 was used at a concentration of 100 pm (-O-) or 14 nm (-●-)

cell. In contrast (Fig. 1C, D), ^{125}I-labeled human recombinant IL2 bound to at least two distinct classes of receptors on the same cells: a high-affinity component ($K_D = 640$ pm, $B_M = 3800$ sites/cell) and a low-affinity component ($K_D = 39$ nm, $B_M = 92000$ sites/cell).

OX39 MoAb was tested for its capacity to interfere with IL2 binding on high and low components (Fig. 2). Binding of ^{125}I-IL2 at a high concentration

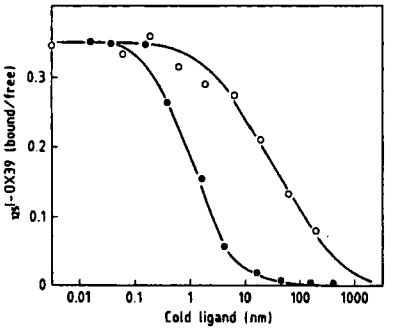

Fig. 3. Inhibition of ^{125}I-labeled OX39 binding by unlabeled OX39 (-●-) and IL2 (-O-). Nonspecific binding determined in the presence of 1.3 nm unlabeled OX39 (2% of total binding) was subtracted

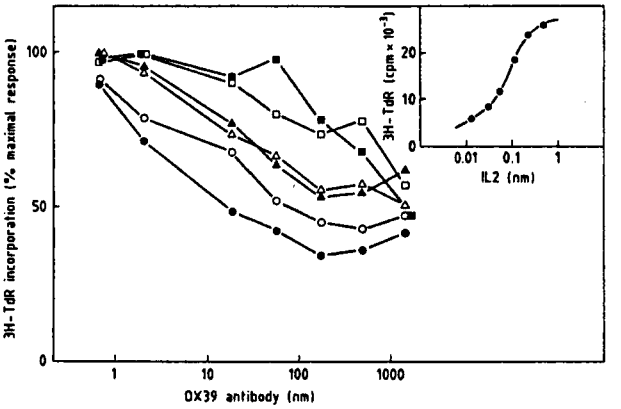

Fig. 4. Inhibition by OX39 MoAb of IL2-induced proliferation of rat ConA blasts. *Main panel:* dose-response curve of OX39 on (3H)thymidine incorporation induced by increasing amounts of IL2: 16 pm (-●-), 62 pm (-O-), 125 pm (-▲-), 250 pm (-△-), 500 pm (-□-), 2 nm (-■-). *Inset:* dose-response curve of IL2 on (3H)thymidine incorporation in the absence of OX39

(14 nm), which allowed for the almost selective labeling of low-affinity IL2 receptors, was completely reversed when increasing concentrations of OX39 were included in the binding test. The inhibition constant derived from this curve had a value of 0.53 nm, a value compatible with the K_D of OX39 (0.8 nm). In contrast, when ^{125}I-IL2 was used at a low concentration (100 pm), which allowed for the selective labeling of high-affinity receptors, OX39 only partially (60%) inhibited this binding with a markedly increased inhibition constant ($K_I = 8.4$ nm).

Conversely, unlabeled r-IL2 was tested for its ability to interfere with labeled OX39 binding (Fig. 3). Inhibition by IL2 was complete with an inhibition constant of 26 nm, a value compatible with the dissociation constant of IL2 for the low-affinity receptors ($K_D = 39$ nm). Unlabeled OX39 also completely displaced ^{125}I-labeled OX39 binding with a $K_I = 0.96$ nm, a value compatible with its K_D.

Effect of OX39 on IL2-induced proliferation

As shown in the inset of Fig. 4, IL2 induced proliferation of rat ConA blasts in a dose-dependent and saturable manner. Fifty percent of maximal proliferation was achieved with IL2 concentrations around 200 pm, a value compatible with the K_D of interaction of IL2 with its high-affinity receptors.

OX39 was analyzed for its ability to interfere with this proliferation at various concentrations of IL2 along its dose-response curve. The results (Fig. 4), expressed in percentages of the maximal responses observed in the absence of OX39, indicate that OX39 inhibited IL2-driven proliferation dose-dependently, but that the maximal extent of inhibition was limited to about 60%, regardless of the concentration of IL2 used in the assay. In the presence of low concentrations of IL2 (16-66 pm), the doses of OX39 necessary to induce half of the maximal inhibition approach 10 nm. As the concentration of IL2 is increased, the doses of OX39 required to produce a similar inhibition are gradually increased to reach 300-500 nm in the presence of 2 nm IL2.

Effect of OX39 on the survival of allogenic heart transplantation

Table 1 indicates that no significant prolongation of graft survival could be obtained when concentrations ranging from 20 μg to 50 μg/rat for 9 days were used. Animals (~250 g) injected with a very high dose (250 μg/rat for 9 days) do, however, show a borderline, yet significant ($P<0.05$), increase in graft survival when compared with recipients injected with the same dose of irrelevant IgG1. Irrelevant IgG1 MoAb did not affect graft survival in the same allograft combination. Table 1 also shows that when CyA was administered for 10 days after grafting at a very low dose (1.5 mg/kg, graft survival was not modified. This dose was nevertheless able to significantly increase allograft survival when OX39 was administered (≥ 50 μg/rat for 9 days); however, this effect remained of low magnitude. A low dose of CyA, combined with an irrelevant IgG1 (250 μg), did not affect graft survival.

Table 1. Effect of OX39 on the survival time of $(Lew/BN)_{F1}$ heart allografts grafted into Lew recipients. * $P<0.05$ (Wilcoxon test) compared to group 1 and <0.01 compared to group 5, ** $P<0.05$ (Wilcoxon test) compared to group 4, *** $P<0.01$ (Wilcoxon test) compared to group 3

Treatment	Graft survival in days	$\bar{x} \pm SD$
1. None	7, 9, 8, 9, 11, 12, 12	9.6±2
2. OX39 20 μg[a]	6, 7, 7, 8, 8	7.2±0.8
3. OX39 50 μg[a]	6, 7, 7, 7, 7	6.9±0.4
4. OX39 250 μg[a]	10, 11, 11, 14, 12, 15	12.2±1.9*
5. Anti-*E. coli* (250 μg) (IgG1) control MoAb[a]	7, 8, 8, 8, 8	7.8±0.4
6. CyA (1.5 mg/kg × 10 days)	7, 8, 10, 10, 11, 11	9.5±1.64
7. CyA (3 mg/kg × 10 days)	12, 12, 12, 12, 13, 13	12.3±0.52
8. CyA (10 mg/kg × 10 days)	26, 33, 39, 39, 41	35.6±6.2
9. CyA[a] (1.5 mg/kg × 10 days and OX39 250 μg)	13, 14, 14, 14, 15, 15	14.2±0.75**
10. CyA[a] (1.5 mg/kg × 10 days and OX39 50 μg)	11, 11, 11, 11, 12	11.2± 0.45***
11. CyA[a] (1.5 mg/kg and ir.-IgG1 250 μg)	9, 9, 9, 11, 11	9.8±1.1

[a] MoAb is given daily at the indicated doses for 9 days

Discussion

OX39 is a murine IgG1 which has been shown to immunoprecipitate the alpha (55 kDa) chain of rat IL2 receptors [15]. In this study, we have demonstrated that OX39 binds to a single class of sites on activated ConA blasts with a high affinity: ^{125}I-labeled OX39 binding occurs with a dissociation constant of 0.8 nm, and unlabeled OX39 competes with this binding with an inhibition constant of 0.96 nm.

OX39 binding is related to OX39 interaction with the low-affinity configuration since IL2 competes for its binding with an inhibition constant of 26 nm in the range of the K_D of IL2 for low-affinity receptors (39 nm). Conversely, OX39 inhibits the binding of IL2 to IL2 receptors. However, the inhibitory potency of OX39 is markedly different when IL2 is used to label the high-affinity configuration and when it is used to label the low-affinity configuration. While OX39 competes with IL2 binding to the low-affinity form with a $K_I = 0.53$ nm that is compatible with its K_D for the 55 Kd Tac chain, it only partially (60%) inhibits IL2 binding to the high-affinity form and its K_I is markedly enhanced (8.4 nm).

These results can be interpreted on the basis of recent data concerning the molecular structure of IL2 receptors as demonstrated in the human [21, 25, 28, 29] and murine [23] systems. In these models, the IL2 receptors are comprised of at least two independent glycoproteic entities, the well-known alpha (55 kDa) chain (Tac antigen) and a novel beta (75 kDa) chain. Both chains alone are able to bind IL2, and their presence together in the membrane gives rise to the high-affinity IL2 receptor configuration. If this model holds for the rat system, our data suggest the following:

1. OX39 recognizes the alpha chain with a high affinity ($K_D = 0.8$ nm) and competes with IL2 for binding on this chain. OX39, therefore, probably interacts with an epitope close to the IL2 binding site on the alpha chain.

2. OX39 does not interact with the beta chain as indicated by immunoprecipitation studies [15].

3. When the IL2 receptor is in the high-affinity alpha/beta configuration, the epitope for OX39 on the alpha chain is more or less buried or has an altered conformation so that the affinity of OX39 for this epitope is reduced by a factor of 10-15.

4. High concentrations of OX39 (100-1000 nm) displace IL2 binding from the alpha/beta conformation receptor and, in doing so, may promote the dissociation of the alpha and beta chains. The residual 40% binding that is not displaceable by OX39 is probably due to residual IL2 binding to the beta chain.

Proliferation studies support this proposed mechanism. IL2-induced proliferation is driven at concentrations corresponding to the saturation of high-affinity IL2 receptors. OX39, by interfering with IL2 binding to these receptors, inhibits this proliferation. Furthermore, there are close relationships between the pattern of inhibition of proliferation and that of inhibition of IL2 binding. In both cases, the maximal inhibition induced by OX39 is about 60%. This would support the observation that the beta chain alone is able to induce a proliferative signal [30]. Another possibility would be that another lymphokine receptor pathway is involved in the residual proliferation. The concentration of OX39 required to inhibit proliferation (at low IL2 concentrations) is in the range of 10-20 nm, a value corresponding to its apparent affinity for the alpha chain when the receptor is in the alpha/beta (high-affinity) configuration. When the IL2 concentration used to promote proliferation is increased, concentrations of OX39 necessary to inhibit proliferation are increased, due to the fact that IL2 and OX39 compete for binding to the alpha chain.

Monoclonal antibodies against R-IL2 have been shown to prevent allograft rejection in various animal [9, 24] and human [2, 27] models. So far, this effect has been related to the ability of MoAb to interfere with the binding of IL2 on its high-affinity receptor, although detailed molecular interaction has seldom been observed. Thus, the only borderline effect of OX39 in the prolongation of allograft survival - even when used at very high doses (250 μg/rat per day) - was expected from the molecular interaction. The same antibody, however, has recently been shown to protect animals from autoimmune encephalitis (AIE) [6]. Although the antibody in these experiments was administered on the basis of ascites dilutions and, as a result, no dose-effect relationships could be established, it is suggested that the delayed hypersensitivity reactions involved in AIE and the immune machinery leading to graft rejection may have different sensitivities to anti-R-IL2 reagents. Interestingly, the same low dose of CyA that was unable to prolong allograft survival by itself did potentiate OX39. This phenomenon, which has previously been found with an anti-R-IL2 capable of interacting with the IL2/R-IL2 interaction and of preventing autoimmune diabetes in rats [5], remained restricted to doses of OX39 that were $\geqq 50$ μg/rat for 9 days. The low synergistic effect which could be predicted from the low OX39-R-IL2 functional interaction observed in vitro is most likely related to the inhibition of IL2 production [11] and R-IL2 expression [3], both of which are likely to facilitate the weak effect of OX39 on lymphocyte growth.

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