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SYNERGISTIC ACTION OF MONOCLONAL ANTIBODIES DIRECTED AT p55 AND p75 CHAINS OF THE HUMAN IL-2-RECEPTOR[1]

MARIE AUDRAIN, FRANÇOISE BOEFFARD, JEAN-PAUL SOULILLOU, AND YANNICK JACQUES[2]

From the Institut National de la Santé et de la Recherche Médicale (INSERM U211), "Unité de Recherche sur les effecteurs lymphocytaires T" 1, rue Gaston Veil, 44035 Nantes Cedex, France

TU27, a mouse IgG1 mAb directed at the p75 chain of the human IL-2R, was analyzed for its ability to interact with IL-2 binding on isolated p75 chains (YT-2C2 cells) and high affinity p55/p75 receptors (human alloreactive T cell clone 4AS), to inhibit IL-2-induced proliferation (4AS cells) and to cooperate with an anti-p55 chain mAb (33B3.1) for inhibiting IL-2 binding and proliferation. TU27 and IL-2 bound to the isolated p75 chain expressed by YT-2C2 cells with respective dissociation constants (K_d) of 1.3 and 1 nM. They cross-inhibited each other for binding with inhibition constants (K_i) in agreement with their respective K_d values. The nature of the interaction was, however, not purely competitive and suggested nonidentical epitopes for the two ligands on the p75 chain. On 4AS cells, IL-2 bound with high affinity (K_d = 50 pM) and TU27 with an affinity similar to that found on YT-2C2 cells. The binding of TU27 and IL-2 were also mutually exclusive on 4AS cells. However, the mechanism of interaction of TU27 with IL-2 was complex since the inhibitory potency of the antibody depended on temperature, antibody preincubation and time of assay. Data obtained at 4°C in the presence of suboptimal, tracer-like concentrations of IL-2 indicated that the intrinsic affinity of TU27 for the high affinity configuration was 15-fold lower than for the isolated p75 chain and argued in favor of the affinity-conversion model (as opposed to the preformed complex model) in which p55 and p75 are dissociated in the absence of IL-2. At 37°C, TU27 inhibited IL-2 binding only on short time assays (6 min). Longer time (30 min) of IL-2 binding resulted in an almost complete disappearance of the effect of TU27, suggesting that internalization of the high affinity p55/p75/IL2 complex enables the cells to escape from the inhibitory effect of TU27. In the presence of the 33B3.1 mAb, the interaction of TU27 with IL-2 ressembled the one observed on YT-2C2 cells, suggesting that 33B3.1 is able to inhibit the IL-2-induced association of p55 and p75. Both antibody were found to synergize on 4AS cells, as a result of a cooperative mechanism in which 33B3.1 blocks the formation of the high affinity complex hence allowing TU27 to bind with higher affinity, and TU27 blocks IL-2 binding to the p75 chain. Proliferation studies corroborated the binding experiments. IL-2-induced proliferation of 4AS cells (37°C) was not affected by TU27, with or without antibody preincubation. When 33B3.1 was present, a clear inhibitory effect of TU27 was then observed. Conversely, TU27 markedly facilitated the effect of 33B3.1 (40-fold shift in the dose-response curve). Finally, our data indicate that the p75 chain, like the p55 component, is up-regulated upon Ag or mitogen stimulation. Its level of induction (3–4-fold) is, however, much lower than for the p55 chain (30–100-fold).

Among the cytokines which are involved in the regulation of the immune system, IL-2 (1, 2) plays a central role, and lot of effort has been made to elucidate its structure (3–6), receptors (7–11), and mode of action (12–14). At least two membrane-anchored glycoproteins are involved in IL-2 binding. A 55-kDa component (Tac Ag) (15, 16) which by itself binds IL-2 with low affinity (K_d = 10–8 M) (17) and a 75-kDa chain which displays intermediate affinity for IL-2 (K_d = 9–10 M) (18–21). Both chains combine to form the high affinity IL-2R) (K_d = 10–11 M) (11, 18–21). There is increasing evidence that the p75 chain is responsible for signal transduction. The cDNA encoding the p55 chain (9) and more recently the p75 chain (11) have been identified. Unlike the p55 component, the p75 chains has a large cytoplasmic domain which may contain a functional domain for signal transduction. Furthermore, cells bearing only the p75 chain, in contrast to those expressing only the p55 chain, have been shown to respond to IL-2 (22–24) and to internalize IL-2 (25).

Several mAb directed at the p55 chain in humans or animals have been obtained. Some of them have been used in the treatment of diseases associated with lymphocyte activation such as experimental autoimmune diseases (26, 27), T cell leukemia (28), and to a larger scale in organ transplantation (29). We have demonstrated for the first time in a pilot study (30, 31) followed by a randomized study vs antilymphocyte globulins (32) the efficacy of an anti-p55 mAb (33B3.1) in the prevention of early cellular rejection kidney allograft recipients. The data accumulated to date in animals and humans designate IL-2R as a major functional target in the modulation of alloreactivity and highlight the specificity of this strategy. In this context, antibodies directed to the p75 chains may offer alternative immunosuppressive tools, either alone or in combination with anti-p55 antibodies. We have tested this possibility in vitro by using a

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[2] Address correspondance and reprints requests to: Yannick Jacques, INSERM U211, 1 rue Gaston Veil, 44035 Nantes Cedex, France.

recently described anti-p75 mAb (TU27) (33, 34). Its characteristics of interaction with the different forms of IL-2R as well as its functional effects are described. A possible synergistic effect between this antibody and 33B3.1 is also analyzed.

MATERIALS AND METHODS

Cells. The YT.2C2 clone is derived from a human leukemic NK-like cell line (20) and was cultured in RPMI 1640 (GIBCO, Glasgow, Scotland) supplemented with 8% FCS (SEBAK, Paris, France) at 37°C in a 5% CO_2 humidified atmosphere. The 4AS is one of a series of clones established by limiting dilution from a rejected kidney allograft (35, 36). It was cultured in RPMI 1640 supplemented with 10% human serum (CTS Nantes, France), and 1 nM rIL-2 (Roussel-Uclaf) and was weekly stimulated with irradiated EBV-transformed B cells prepared from splenocytes of the kidney donor. This cell line (referred to as DAB cell line) was cultured in RPMI 1640 supplemented with 10% FCS. Con A-activated PBL were prepared by culturing PBL in RPMI 1640 supplemented with 5% human serum and 1 μg/ml of Con A (Sigma Chemical Co., St. Louis, MO) for 5 days.

Antibodies and IL-2. TU27 is a mouse IgG1 directed against the p75 chain of the human IL-2R (33). It was purified from ascitic fluid (kindly given to us by Dr. K. Sugamura, Tohoku University, Japan) by affinity chromatography on a protein A column (Bio-Rad, Richmond, CA), dialyzed against PBS, and aliquots were stored at −20°C. 33B3.1 is a rat IgG2a directed against the p55 chain of the human IL-2R (37, 38). IL-2 (highly purified recombinant molecule produced in *Escherichia coli*) was a kind gift of Roussel-Uclaf Laboratories (Romainville, France).

Radiolabeling of IL-2 and mAb. The radiolabeled ^{125}I-IL-2 used in binding assays was from New England Nuclear (Boston, MA). Its specific radioactivity was in the range of 300 to 1000 cpm/fmol. TU27 and 33B3.1 were radiolabeled with ^{125}I by using the Iodogen method (39). Their specific radioactivities were between 1500 and 2500 cpm/fmol.

Binding assays. YT.2C2 cells and Con A-activated PBL were centrifuged, washed three times, and resuspended in PBS containing 0.5% BSA. 4AS cells were similarly prepared except that they were washed from IL-2 by preincubation for 2 h at 37°C in an IL-2-free medium before being processed. Aliquots of 25 μl per well of the cell suspension (between 0.5 and 2.106 cells per well, depending on the experiment) were seeded in 96-multiwell plates (NUNC, Roskilde, Denmark), with increasing concentrations of the radiolabeled ligand in a final volume of 50 μl per well. After a 45-min (antibodies) or 30-min (IL-2) incubation at 37°C under agitation, cells from each well were layered on top of a layer of dibutylphtalate-paraffin oil, centrifuged, and cell bound and unbound radioactive fractions separated and counted as described elsewhere (40). When indicated, binding experiments were conducted at 4°C. Nonspecific binding was determined in the presence of a 50- to 100-fold excess of unlabeled ligand. Determination of maximal binding capacities for the ligands and of their respective dissociation constants (K_d) was determined by Scatchard analysis.

Competitions. Cells were prepared and seeded as above. The radiolabeled ligand was added at a fixed concentration (as indicated) together with increasing amounts of the unlabeled competitors. When indicated, the cells were preincubated 1 h at +4°C with the competitors before adding the radiolabeled ligand. After incubation at 4°C for 1 h (IL-2 or antibody) or at 37°C for 5 to 30 min (IL-2) or 1 h (antibody), cell bound and unbound fractions were determined as described above.

Proliferation assays. 4AS cells were seeded in 96-multiwell plates (NUNC, Roskilde, Denmark) at a density of 10,000 cells per well in 200 μl of RPMI 1640 plus 10% human serum. They were added with Ag (20,000 irradiated DAB cells per well) and various concentrations of rIL-2 as indicated in *Results*. When tested for their inhibitory effects, 33B3.1 and TU27 mAb were either preincubated with 4AS cells for 1 h at 4°C or added at the time of DAB and IL-2 additions. After 72 h of incubation at 37°C in the incubator, the cells were pulsed for 16 h with [^{3}H]thymidine (0.25 μCi per well) (Amersham, Les Ulis, France) harvested (PHD harvester, Watertown, MA), and counted for thymidine incorporation.

RESULTS

Interaction between TU27 and IL-2 on the YT.2C2 lymphoma cells. The YT.2C2 clone derived from the human NK-like YT lymphoma cell line expressed the p75 chain of the IL-2R without expressing the p55 component (Tac Ag) (20). Indeed, whereas the anti-p55 mAb 33B3.1 did not bind to the YT.2C2 cells (not shown) the anti-p75 TU27 bound to a single class of binding sites on YT.2C2 cells with a dissociation constant K_d = 1.3 nM and a maximal binding capacity of 26,000 sites per cell (Fig. 1*A*). rIL-2 inhibited 125I-TU27 binding to the same nonspecific level as the one obtained with excess unlabeled TU27 (Fig. 1*B*). The effect of IL-2 was caracterized by an inhibition constant K_i = 1 nM. Conversely, radiolabeled IL-2 bound to a single class of sites on YT.2C2 cells (16,540 sites per cell) with a dissociation constant K_d = 1.31 nM (Fig. 1*C*) and this binding was completely inhibited by increasing doses of TU27 (Fig. 1*D*) with an inhibition constant K_i = 1 nM, a value very close to the dissociation constant of the antibody. Experiments in Figure 1 were conducted at 37°C. Similar results were obtained at 4°C. In addition, the inhibitory effect of TU27 on IL-2 binding was observed with a similar inhibition constant whether or not TU27 was preincubated with YT-2C2 cells for 1 h at 4°C before the addition of radiolabeled IL-2 (not shown). The interaction between TU27 and IL-2 on the p75 chain of the IL-2R was further analyzed at different concentrations of the ligands and at 4°C. As shown in Figure 2, the nature of the interaction was not of a pure competitive type. Indeed (Fig. 2*A*), when IL-2 was added at concentrations of 1 and 10 nM, the number of TU27 binding sites per cell dropped from 27,700 in the absence of IL-2 to 25,800 and 13,800, respectively. Conversely, concentrations of 2 and 10 nM of TU27 induced a dose-dependant decrease of the number of IL-2 binding sites (from 18,125 to 14,155 and 9,585, respectively).

Binding of TU27, IL-2, and 33B3.1 on activated nor-

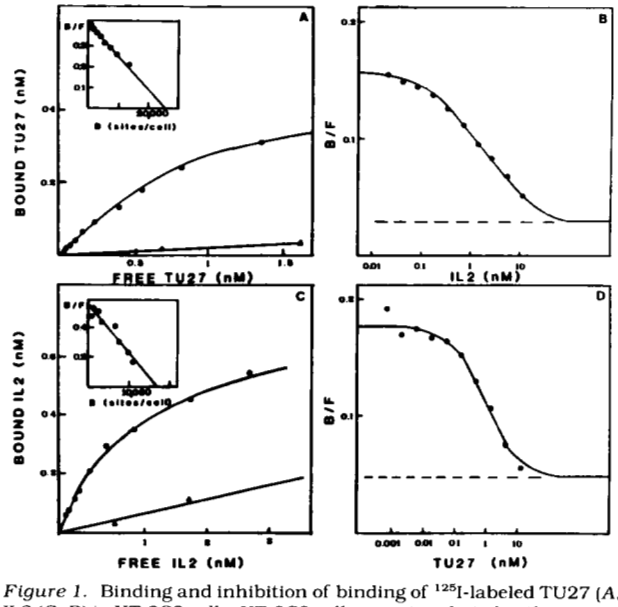

Figure 1. Binding and inhibition of binding of ^{125}I-labeled TU27 (*A*, *B*) or IL2 (*C*, *D*) to YT.2C2 cells. YT.2C2 cells were incubated with increasing concentrations of ^{125}I-labeled TU27 (*A*) for 45 min at 37°C, or with increasing concentrations of ^{125}I-labeled IL-2 (*C*) for 30 min at 37°C. Bound and free ligands were separated as described in *Materials and Methods*. Nonspecific bindings (▲) were measured with a 100-fold excess of the respective unlabeled ligands. Scatchard analysis of the specific binding components are shown in the *insets*. For inhibition, cells were preincubated for 1 h at +4°C with increasing concentrations of unlabeled IL-2 (*B*) or TU27 (*D*) before adding, respectively, 1 nM ^{125}I-labeled TU27 for 45 min at 37°C or 0.5 nM ^{125}I-labeled IL-2 for 30 min at 37°C. Bound and free fractions were separated as described. *Dashed lines* represent nonspecific bindings obtained with 100-fold excess of unlabeled TU27 (*B*) or IL-2 (*D*), respectively.

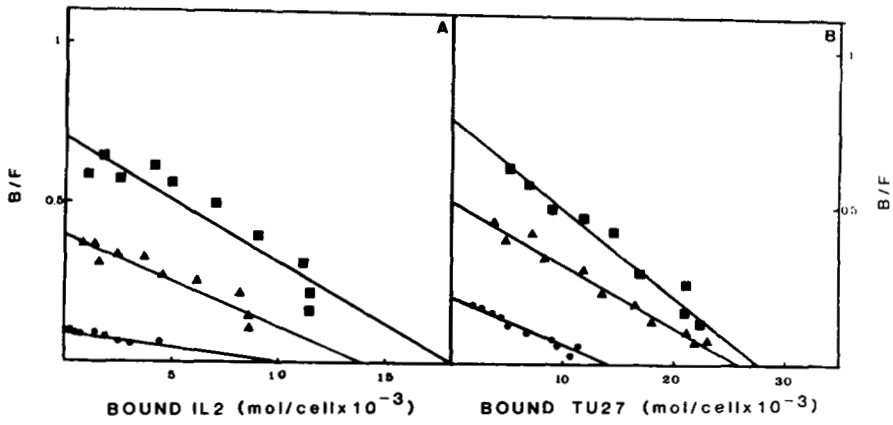

Figure 2. Competition between TU27 and IL-2 for binding to YT.2C2 cells. (*A*) YT.2C2 cells were incubated with increasing concentrations of ^{125}I-labeled IL-2 for 30 min at +4°C, in the absence (■) or the presence of 2 nM (▲) or 10 nM TU27 (●). (*B*) YT.2C2 cells were incubated with increasing concentrations of ^{125}I-labeled TU27 for 45 min at +4°C in the absence (■) or the presence of 1 nM (▲) or 10 nM (●) IL-2. Bound and free fractions were separated as described and nonspecific binding was substracted from total binding before Scatchard analysis.

mal T cells. Binding studies were conducted on two cell types: Con A-activated PBL and the 4AS human T cell clone collected 2 days after stimulation with the DAB cell line. The p55 and p75 chains of the IL-2R were targeted with the 33B3.1 mAb and TU27 mAb, respectively, whereas the p55/p75 high affinity receptors were measured with labeled IL-2 at low (pM range) concentrations. Table I summarizes the results obtained. The dissociation constant of 33B3.1 on the p55 chain expressed by activated T cells was in the range of 0.3 nM, a result in agreement with previous experiments by using this antibody (38, 40, 41). The dissociation constant of TU27 on these activated T cells was also comparable to the value measured on YT.2C2 cells. High affinity IL-2 binding was characterized by a dissociation constant of about 50 pM. As already reported on activated T cells, the number of 33B3.1-binding sites was far higher than the number of high affinity IL-2R on Con A-blasts (55-fold higher) and the 4AS T cell clone (85-fold higher). TU27-binding sites were slightly more numerous than high affinity IL-2R on Con A-blasts (1.5-fold) and 4AS cells (2.0-fold). A similar finding was found on YT.2C2 cells (1, 57) (Fig. 1).

Interaction of TU27 and 33B3.1 with IL-2 high affinity binding. The effect of TU27 and 33B3.1 on IL-2 binding were first analyzed at 37°C and by adding the competitors at the same time as labeled IL-2 (Fig. 3). In the range of concentrations studied (up to 3 nM), IL-2 binding was characterized by a predominant high affinity component (K_d = 60 pM, 2600 sites/cell) and the beginning of a lower affinity component. At a concentration (26 nM) which induced a complete inhibition of IL-2 binding on YT.2C2 cells, TU27 only slightly inhibited IL-2 binding on the 4AS cells, and the K_d of the high affinity IL-2-binding component was only slightly affected (K_d = 75 pM) (Fig. 3*B*). 33B3.1, which was inactive on the YT.2C2 cells, had a much larger inhibitory effect on IL-2 binding. As revealed by Scatchard analysis (Fig. 3*B*), 33B3.1 induced a complete disappearance of the high affinity component. IL-2 binding in the presence of 33B3.1 was characterized by an intermediate affinity (K_d = 1.36 nM) comparable to the binding of IL-2 with the p75 chain of YT.2C2 cells. The number of these intermediate affinity IL-2-binding sites (2500 sites/cell) was similar to the number of high affinity IL-2-binding sites measured in the absence of 33B3.1, suggesting that the effect of 33B3.1 is to convert the high affinity structures into these intermediate affinity components. Furthermore, this intermediate affinity IL-2-binding component observed in the presence of 33B3.1 was completely inhibited by TU27 (Fig. 3) with a half-maximal effect observed in the 1 nM range (not shown), suggesting that this component is related to isolated p75 chains.

TABLE I

High-affinity IL2, TU27, and 33B3.1 binding sites on activated PBL and 4AS cells[a]

		IL2	TU27	33B3.1
PBL	K_d (pM)	50	1,300	350
	B_{max} (sites/cell)	530	800	29,300
4AS	K_d (pM)	56	2,300	290
	B_{max} (sites/cell)	1,060	2,116	90,500

[a] PBL were cultured for 5 days with 1 μg/ml Con A, and 4AS were stimulated for 2 days with the DAB cell line and 1 nM IL-2. After washing, cells were incubated with increasing concentrations of IL-2 for 30 min at 37°C, or mAb TU27 or 33B3.1 for 45 min at 37°C. The respective dissociation constants (K_d) and maximal binding capacities (B_{max}) were determined by Scatchard analysis.

We next examined the reverse situation, i.e., the effect of IL-2 on TU27 binding (Fig. 4). rIL-2 did completely inhibit TU27 binding to the 4AS cells and this effect was observed at low concentrations of IL-2 (inhibition constant K_i = 20 pM) corresponding to its high affinity binding. 33B3.1, by itself, had no effect on TU27 binding and conversely (not shown). When 33B3.1 was present, IL-2 still inhibited TU27 binding, but at about 50 times higher concentrations. In that case, the inhibition constant was K_i = 890 pM, a value in agreement with the interaction of IL-2 with intermediate affinity sites, a pattern which was very similar to the one observed on YT.2C2 cells (Fig. 1*D*).

The absence of a significant inhibitory effect of TU27 on IL-2 high affinity binding was in contrast to previous experiments with this antibody (33). In an attempt to resolve this discrepancy, the effects of temperature, antibody preincubation, and kinetics were analyzed. The results are summarized in Figure 5. Radiolabeled IL-2 was used at a low, tracer-like concentration (20 pM) 2.5-fold below its dissociation constant for the high affinity receptors. At 4°C (Fig. 5*A*) and when TU27 was added together with labeled IL-2 for 1 h, i.e., under equilibrium conditions, TU27 did inhibit IL-2 binding. However, the half-maximal effect was observed at 20 nM, a concentration 15- to 20-fold higher than its dissociation constant on YT-2C2 or 4AS cells, or than its inhibition constant of IL-2 binding to YT-2C2 cells under comparable experimental conditions (concentrations of labeled IL-2 lower than its dissociation constant for the p75 chain) (Fig. 1*D*). When TU27 was preincubated for 1 h at 4°C before the addition of IL-2, its inhibitory effect was markedly poten-

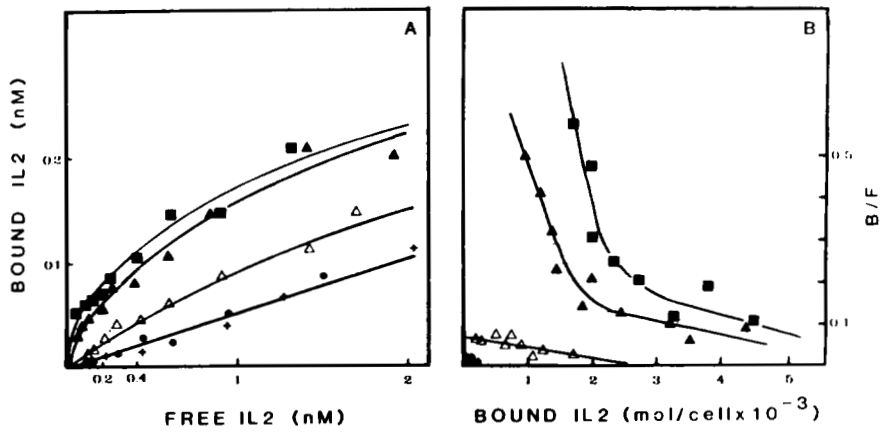

Figure 3. Effect of TU27 and 33B3.1 on IL-2 high affinity binding to 4AS cells. 4AS cells were incubated for 30 min at 37°C with increasing concentrations of ^{125}I-labeled IL-2 alone (■) or in the presence of TU27 (40 nM) (▲), 33B3.1 (20 nM) (△) or both in combination (●). Nonspecific binding measured in the presence of a 100-fold excess of unlabeled IL-2 is also indicated (+). (*A*) Binding curves. (*B*) Scatchard analysis after substraction of nonspecific binding.

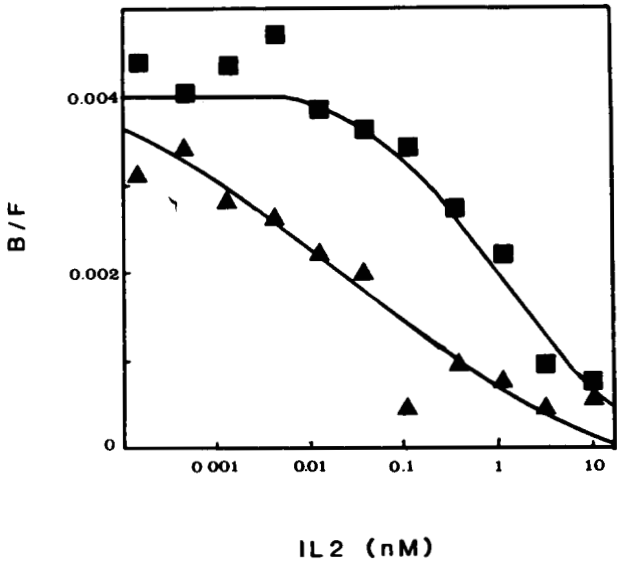

Figure 4. Inhibition by IL-2 of ^{125}I-labeled TU27 binding to 4AS cells. 4AS cells were incubated for 45 min at 37°C with 2 nM ^{125}I-labeled TU27 and with increasing concentrations of IL-2, in the absence (▲) or the presence (■) of 20 nM 33B3.1. Bound and free fractions were separated as described.

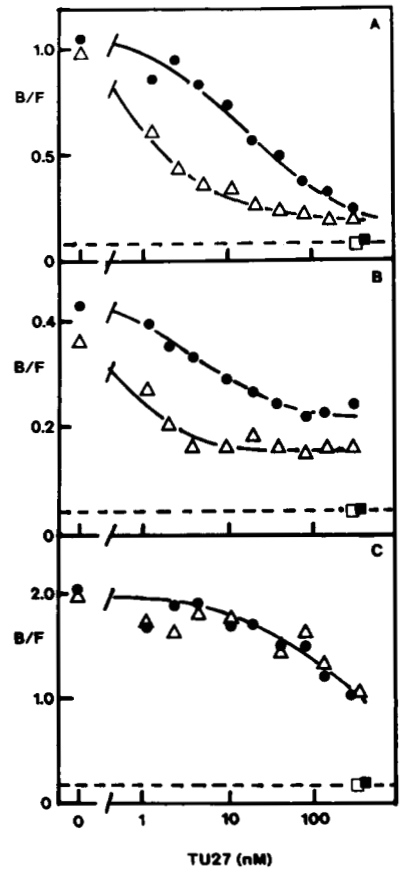

Figure 5. Effect of antibody preincubation, temperature and time of assay on TU27 inhibition of IL-2 high affinity binding. Labeled IL-2 was used at a fixed concentration (20 pM). Increasing concentrations of TU27 were added to 4AS cells at the same time as labeled IL-2 (●) or preincubated 1 h at 4°C with 4AS cells before the addition of labeled IL-2 (△). The IL-2 binding assay was performed at 4°C for 1 h (*A*), at 37°C for 6 min (*B*) or at 37°C for 30 min (*C*). Bound and free fractions were separated as described in *Material and Methods*.

tiated. Half-maximal inhibition was then observed at a concentration (1.5 nM) compatible with the dissociation constant of the antibody. At 37°C, the results also differed whether the binding assay was performed on a short time (6 min) (Fig. 5*B*) or on a longer time (30 min) (Fig. 5*C*) and whether TU27 was preincubated or not. On a short time without preincubation, the inhibitory effect of TU27 was only partial and observed at high concentrations. Preincubation of TU27 enhanced its inhibitory potency to nanomolar concentrations. At a longer time, there was an almost complete disappearance of the inhibitory effect of TU27, 50% of inhibition being reached at the highest dose tested (320 nM). Preincubation of 4AS cells with TU27 did not change this result. In contrast to TU27, 33B3.1 inhibited IL-2 high affinity binding at 4°C and 37°C and whether or not the antibody was preincubated (not shown).

Kinetics of IL-2R cell surface expression after antigenic stimulation. Freshly isolated human PBL had undetectable IL-2 high affinity binding sites. In contrast, they expressed low but detectable levels of p55 chains and p75 chains (as revealed by the 33B3.1 ans TU27 mAb, respectively) (Fig. 6*A*). Stimulation with Con A was followed by a gradual increase in the density of 33B3.1 epitopes peaking at day 3 to 4 and declining thereafter. There was a concommitant increase in the density of TU27-binding sites and high affinity IL-2R. The extent of stimulation was much higher in the case of 33B3.1 (30-fold) than of TU27 (3.6-fold).

The alloreactive human T cell clone 4AS collected 10 days after antigenic stimulation had a low but significant level of high affinity receptors (320 sites/cell) as well as p55 and p75 chains (Fig. 6*B*). As found upon Con A stimulation of PBL, antigenic activation by irradiated DAB cells in the presence of IL-2 resulted in a general

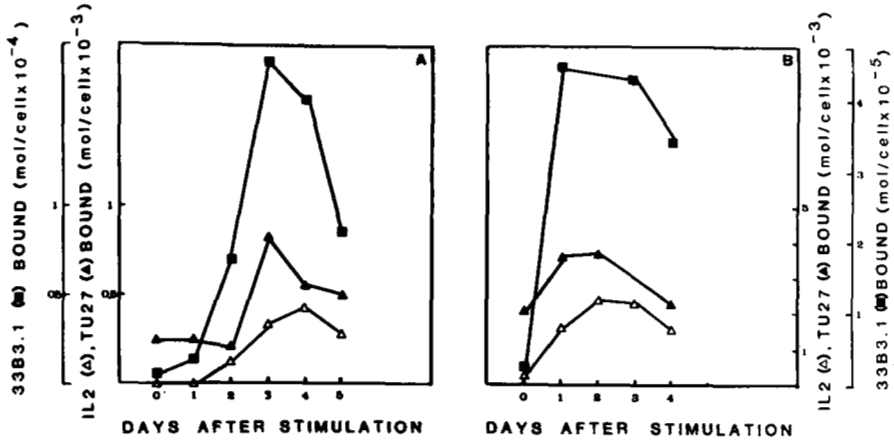

Figure 6. Kinetics of TU27, 33B3.1 and high affinity IL-2R expression after mitogen or Ag stimulation. PBL were stimulated at time zero with 1 μg/ml Con A and 4AS cells with 1 nM IL-2 and irradiated DAB cells. Maximal capacities of high affinity IL-2 (△), TU27 (▲) or 33B3.1 (■) binding sites were measured every day by Scatchard analysis as described in Table I.

increase of 33B3.1 binding sites (p55 chain), TU27-binding sites (p75 chain) and high affinity IL-2R. The levels of antibodies and IL-2-binding sites were higher on the 4AS clone than on Con A-blasts, but the extents of activation were of the same order of magnitude. Additionally, the peak of antibodies binding sites following antigenic stimulation of the 4AS clone was earlier (days 1 to 2) than the corresponding response following Con A stimulation of PBL (days 3 to 4).

Effect of TU27 and 33B3.1 on IL-2 dependent proliferation of the 4AS cells. When stimulated with alloantigen (DAB cell line), the 4AS cells proliferated in a dose-dependent response to IL-2 (Fig. 7). Half-maximal proliferation was observed at 200 pM IL-2, a value in agreement with its binding to the high affinity receptors. Addition of excess 33B3.1 (660 nM) in the culture medium resulted in a complete inhibition of proliferation. In contrast, TU27 had no significative effect on IL-2-dependant proliferation of the 4AS T cell clone even at low, suboptimal concentrations of IL-2 and whether the antibody was added at the time of IL-2 addition or was preincubated for 1 h with the cells before IL-2 addition. Experiments performed on other T cell clones derived from independant cloning experiments gave similar results (not shown).

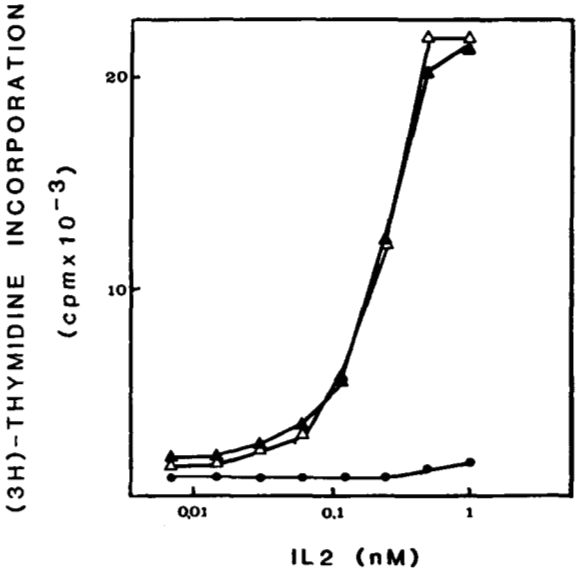

Figure 7. Effect of TU27 and 33B3.1 on IL-2-dependant cell proliferation. 4AS cells were seeded in 96-well plates with irradiated DAB cells and increasing concentrations of IL-2, in the absence (▲), or in the presence of 70 nM TU27 (△) or 660 nM 33B3.1 (●). After 72 h of incubation at 37°C, [^{3}H]thymidine incorporation was measured as described in *Materials and Methods*.

Antibodies dose response curves showed that 33B3.1 inhibited IL-2-driven proliferation of the 4AS cells (Fig. 8*A*) with a half-maximal effect observed at 7.5 nM, a value which agreed with previously reported data (38, 40). However, when TU27 was present at a fixed concentration of 70 nM, the dose-response curve of 33B3.1 was very markedly shifted toward lower concentrations. In that case, half-maximal inhibition of proliferation was achieved with only 0.2 nM of 33B3.1, i.e., about 40 times less antibody. Converse experiments (Fig. 8*B*) showed that whereas TU27 alone, over a wide concentration range (50 pM to 320 nM) and with or without preincubation, had no effect on IL-2-induced proliferation, it did show an inhibitory effect when tested in the presence of 33B3.1. 33B3.1 at 25 nM induced about 70% inhibition of proliferation and the remaining 30% was completely abrogated by concentrations of TU27 (half-maximal effect observed at 1 nM), very compatible with its binding to the p75 chain.

DISCUSSION

YT.2C2 cells express only the p75 chain of the human IL-2R (20). Indeed, YT.2C2 cells bound IL-2 with an intermediate affinity (K_d = 1.31 nM) but not the anti-p55 antibody 33B3.1. TU27 bound to YT.2C2 cells with a dissociation constant of 1.3 nM and the number of TU27-binding sites, although slightly higher, was comparable to the number of IL-2-binding sites. Competition experiments showed that TU27 and IL-2 cross-inhibited each other for binding to the YT.2C2 IL-2R. IL-2 inhibited TU27 binding with an inhibition constant (K_i = 1 nM) corresponding to its affinity for the p75 component. Conversely, TU27 inhibited IL-2 binding with a K_i = 1 nM in agreement with its binding properties. Similar values were measured whether the competitor was preincubated or not before adding the labeled ligand. The nature of this interaction appeared to be slightly different from a pure competitive mechanism, because IL-2 induced a reduction of the number of TU27-binding sites and conversely. Internalization of the p75 molecule seems not to be responsible for this behavior because it was observed at 4°C. Although the mechanisms underlying this type of interaction remain to be clarified, they suggest that the binding site of TU27 on the isolated p75 chain is not identical to the IL-2 binding site and that cross-inhibition between the two ligands occurs via conformational

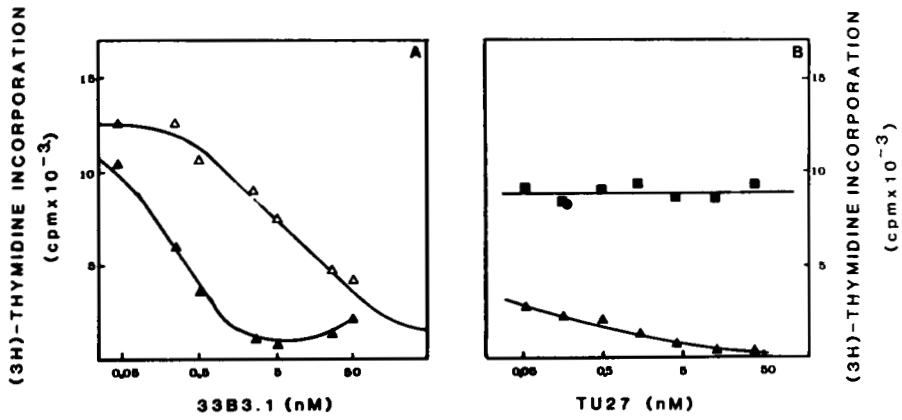

Figure 8. Synergy between TU27 and 33B3.1 for inhibition of IL-2-dependent cell proliferation. Cells were seeded in 96-well plates with irradiated DAB cells, 250 pM IL-2 and (*A*) increasing concentrations of 33B3.1 in the absence (△) or in the presence (▲) of 70 nM TU27 or (*B*) increasing concentrations of TU27 in the absence (■) or in the presence (▲) of 25 nM 33B3.1. After a 72-h incubation at 37°C, incorporation of [^{3}H]thymidine was measured.

changes in the receptor structure.

We next investigated the interaction of TU27 with the IL-2R expressed by alloantigen-activated human T cell clones. As expected, these cells expressed a high amount of p55 chains as detected by the 33B3.1 mAb (38, 40) and 100 times less high affinity IL-2R (K_d = 50 pM). TU27 bound to 4AS cells with an affinity (K_d = 2.3 nM) similar to the one found on YT-2C2 cells, suggesting that the epitope recognized by the antibody was equally accessible on both types of cells, at least in the absence of IL-2. The number of TU27-binding sites was slightly higher (1.5- to 2-fold) than the high affinity IL-2-binding sites, a finding which could suggest the existence of isolated p75 chains not participating in the formation of high affinity structures. However, as already mentioned, a similar ratio of TU27 vs IL-2 binding was found on YT.2C2 cells which do not express p55 chains. This difference of stoichiometry seems therefore to be directly related to the isolated p75 chain. There could be two TU27 binding sites for one IL-2 binding site on the p75. Alternatively, the stoichiometry of IL-2 binding sites could be underestimated because of a slightly overestimated value of the specific radioactivity of the commercial source of radiolabeled IL-2 we are using. In any case, the fact that a similar stoichiometry between TU27- and IL-2-binding sites was found on YT.2C2 (p75 chains) and activated 4AS cells support the notion that the majority of p75 chains in 4AS cells can participate in the high affinity configuration. Similar numbers of TU27- and high affinity IL-2-binding sites have also been demonstrated on a series of human HTLV-I-infected cell lines (33).

IL-2 completely inhibited TU27 binding on 4AS cells and this effect was achieved at low concentrations (20 pM) corresponding to the high affinity IL-2 binding. This result further strengthens the idea that most of the p75 chains on activated 4AS cells can participate to the formation of the high affinity receptors. Competition experiments between TU27 and labeled IL-2 turned out to be more complex than expected because different results were obtained whether the antibody was preincubated or not, whether the assay was conducted at 4°C or 37°C, and, in that latter case, whether the assay was carried out on a short or longer time period. At 4°C, when both reactants were added together, TU27 competed with IL-2 high affinity binding. However, the inhibition constant describing this effect (K_i = 20 nM) was about 15-fold higher than the dissociation constant of the antibody on 4AS cells. This difference could be linked to a saturation effect by IL-2. This explanation is unlikely, because labeled IL-2 was used at a concentration (20 pM) 2.5-fold below its dissociation constant (K_d = 50 pM). Furthermore, on YT-2C2 cells under comparable conditions (4°C, ligands added together, concentration of IL-2 twofold below its K_d for the p75 chain) such a difference was not seen: TU27 inhibition of IL-2 binding was characterized by a K_i (2.5 nM) similar to the K_d of the antibody for the isolated p75 chain. A second explanation for the difference observed between K_i and K_d of TU27 on 4AS cells would be that the inhibition binding assay of Figure 5*A* did not reach steady state. Indeed IL-2, even at low concentrations, has an association rate which is fast whereas, on the other hand, a very slow dissociation rate constant (22, 42). One could therefore argue that, if the association rate of TU27 is slow enough compared with that of IL-2, the receptors would be preferentially occupied by IL-2 and TU27 would associate very slowly, because its rate of association would be mainly determined by the dissociation rate of IL-2. In other words, steady state binding of TU27 and IL-2 would not be reached during the 1-h binding assay, which would explain the low inhibitory potency of TU27. In order to assess the merit of this explanation, kinetic binding experiments at concentrations of IL-2 and TU27 corresponding to the 50% inhibition point in Figure 5*A* have been carried out, namely, association of labeled IL-2 (20 pM) in the presence of 20 nM TU27 and conversely, association of labeled TU27 (20 nM) in the presence of 20 pM IL-2. In both cases, steady state binding levels were reached after 20 min of binding with similar association rates (0.88 and 0.34 min^{-1} for IL-2 and TU27, respectively) (data not shown). Therefore, the results of Figure 5*A* cannot be attributed to differences in binding kinetics between TU27 and IL-2 and the absence of steady state conditions.

As a matter of fact, under the conditions used in Figure 5*A* (ligands added simultaneously, tracer-like concentration of labeled IL-2, steady state conditions, absence of internalization), the inhibition constant K_i is a measure of the affinity of TU27 for the p75 chain engaged in the p75:IL2:p55 high affinity structure. In addition to showing that TU27 and IL-2 are also mutually exclusive on this high affinity structure, a result which agrees with the original work describing the TU27 mAb (33), our data therefore show that the intrinsic affinity of TU27 for the p75 chain is reduced when this chain is engaged in the high affinity complex. There are two molecular shemes which have been proposed for the formation of the high

affinity complex: the preformed binary complex model which assumes the preexistence of a p55/p75 complex which binds IL-2 with high affinity and the affinity conversion model in which IL-2 first binds to p55 and the resulting complex then associates with p75. Although some investigators, with the use of bifunctionnal cross-linking agents, have been able to materialize p55:p75 complexes in the absence of IL-2 (43, 44), others have provided steady state (45) or kinetic binding data (46, 47) that are not compatible with the preexistence of a stable p55:p75 complex. In this controversial debate, our data showing that 1) the affinity of TU27 for 4AS cells in the absence of IL-2 is similar to its affinity for isolated p75 chains and 2) the intrinsic affinity of TU27 for the high affinity conformation is 15-fold lower, indicate that the p75 chains on 4AS cells are not engaged in the high affinity structure in the the absence of IL-2 and therefore argue in favor of the affinity-conversion model. When TU27 was preincubated for 1 h at 4°C before adding labeled IL-2 (Fig. 5*A*), the inhibition dose-response curve was shifted toward lower concentrations compatible with the dissociation constant of TU27. Such a shift, which was not observed on YT-2C2 cells, is also predictable by the affinity conversion model, in contrast to the preformed complex model. Indeed, upon preincubation, TU27 saturates p75 chains dose-dependently according to its K_d. Due to the low dissociation rate constant of TU27 at 4°C (not shown), the subsequent binding of IL-2 at low doses does not modify this equilibrium and is proportionnal to the fraction of p75 chains not occupied by TU27. The resulting inhibition curve therefore reflects the binding of TU27 to p75 during its preincubation and is characterized by a K_i close to the K_d of the antibody. In the preformed complex model, the inhibition curve at low doses of IL-2 would reflect the affinity of TU27 for the preformed p55/p75 complex and, as found on the isolated p75 chain, there would not be any significant difference with of without preincubation of the antibody.

The results obtained at 37°C on a short time of IL-2 binding assay were similar to the ones obtained at 4°C, i.e., a low inhibitory constant for TU27 when both ligands were added together and a shift toward lower concentrations when TU27 is preincubated. At a longer time of assay, there was an almost complete disappearance of the inhibitory effect of TU27, whether the antibody was preincubated or not. These results suggest that internalization of the IL-2/IL-2R complexes at 37°C enables the cells to escape from the inhibitory effect of TU27. Preliminary kinetic experiments (unpublished) analyzing the fractions of cell-associated and internalized IL-2 at 37°C indeed indicate that, whereas the acid-removable IL-2 fraction is inhibited by TU27, the kinetic of IL-2 internalization is much less affected.

In contrast to TU27, the anti-p55 mAb 33B3.1 inhibited IL-2 high affinity binding at 4°C and 37°C. This is in agreement with earlier observations (38) which suggested that 33B3.1, in addition to its ability to interfere with IL-2 binding to the p55 component, could also act by preventing the association of p55 and p75. When 33B3.1 was present in the assay, TU27 binding to 4AS cells was not affected. IL-2 still inhibited TU27 binding but at concentrations (K_i = 890 pM) 50-fold higher than when 33B3.1 was absent, and in agreement with its interaction to the p75 intermediate affinity receptor. TU27, under conditions (37°C, 30 min) in which it was poorly active when tested alone, was then able to inhibit IL-2 binding to 4AS cells at concentrations (nanomolar range) compatible with its affinity for the p75 chain. These results therefore demonstrate that, in the presence of 33B3.1, TU27 and IL-2 interact in a way similar to that observed on YT.2C2 cells. In view of the affinity-conversion model proposed above, this reinforces the idea that 33B3.1, by inhibiting IL-2 binding to the p55 chain, prevents the association of the p55 and p75 subunits.

The analysis of the effect of TU27 on IL-2-induced proliferation on 4AS cells completely corroborated the binding studies. Indeed, as expected from the competition studies at 37°C, TU27 did not affect the proliferation of 4AS cells and preincubation of the antibody did not change this result. This lack of effect of TU27 was observed in a range of IL-2 concentrations covering the saturation of the high affinity receptors. These data are in some contrast with original experiments with the use of TU27 (33). In these studies, the authors showed that TU27 could inhibit IL-2-induced proliferation at low, suboptimal concentrations of IL-2. Although we have no clear explanation for this discrepancy, it could be linked to differences in the respective cellular material used. When the 33B3.1 antibody was present and again in agreement with the binding studies, a clear inhibitory effect of TU27 could be evidenced which occurred at nanomolar concentrations. Conversely, the inhibitory effect of 33B3.1 on IL-2-induced proliferation was greatly facilitated in the presence of TU27, 50% inhibition of proliferation being obtained at concentrations of 33B3.1 40 times lower than in the absence of TU27. According to the affinity-conversion model, this synergistic effect can be explained by a concerted action in which 33B3.1 inhibits IL-2 binding to the p55 chain and the formation of p55/p75 complexes therefore allowing TU27 to bind with higher affinity and inhibit IL-2 binding to the p75 chain.

Other mAb directed at p75 components of the human IL-2R have been described. mAb Mikβ1, Mikβ2 (48), as well as mAb 2-RB (49), like TU27, completely inhibited IL-2 binding to the isolated p75 chain. They also ressembled TU27 in that they were shown to inhibit IL-2 high affinity binding without being able to affect IL-2 induced proliferation, and exerted an inhibitory effect only when anti-Tac was simultaneously present. mAb Mikβ3 was described as interacting with an epitope not engaged in IL-2 binding to the p75 component (48). The YTA1 and YTA2 mAb, which recognize 75-kDa components on T cells, exert mitogenic activities and accelerate internalization of the IL-2-IL-2R complex (49). More recent data, however, indicate that the YTA1 Ag is a unique surface molecule different from the IL-2R p75 chain, although functionally associated with this latter (50). As found on the p55 chain (51–53), there seems therefore to exist multiple epitopes on the p75 chain which, upon targeting with their respective antibodies, differently affect IL-2 binding and/or IL-2 signal transduction.

Based on its high in vitro activity, mAb 33B3.1 has been used in the prophylaxis of kidney graft rejection in human and proved to be as efficient as antilymphocyte globulins (32). Most of the literature concerning the use of anti-p55 mAb in organ transplantation in animals or humans indicate that only mAb able to inhibit functional

IL-2/IL-2-R interaction are efficient (29, 53). However, some anti-p55 mAb not belonging to this class and ineffective by themselves in vivo were shown to synergize with low doses of the former antibodies (54). Our results therefore raise the attractive possibility of combining anti-p75 antibodies and anti-p55 antibodies to enhance the effectiveness of the functional immunosuppression in vivo.

Freshly isolated human PBL expressed low amounts of TU27- and 33B3.1-binding sites, but undetectable high affinity IL-2R. This result indicates either that p55 and p75 chains are expressed by separate cell subpopulations in PBL or that p55 and p75 coexpression by all or part of these cells does not lead to the formation of high affinity structures. In favor of the first hypothesis are reports indicating a preferential association of the p75 chain by the CD16-positive LGL/NK population (34). Upon Con A activation, there was a large induction in the number of p55 chains (30-fold increase). The number of TU27 binding sites also increased (3.6-fold) together with the appearance of high affinity structures. The kinetics associated to the expression of these three IL-2R structures (p75, p55, and IL-2 high affinity complex) were similar with a peak of expression on days 3 and 4 and a subsequent decline. The 4AS T cell clone is a population of cells already primed in vivo against an allogenic kidney graft and subjected to repetitive restimulation in vitro with the specific antigen for more than 2 yr (36). Similar increases of 33B3.1, TU27, and high affinity IL-2-binding sites were also obtained after antigenic stimulation of these cells, except that the peak of expression was observed earlier (days 1 to 2) on these already primed cells. These results demonstrate for the first time that the p75 chain, like the p55 component, is up-regulated upon Ag or mitogen activation of T cells. They corroborate the data recently reported which showed such a transient increase (2.5-fold) of the p75-mRNA after stimulation of PBL with PHA (11).

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