ANTIBODIES TO INTERLEUKIN 2

Effects on Immune Responses In Vitro and In Vivo

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A large body of experimental data indicates that T cell growth factor, interleukin 2 (IL-2),[1] participates in a number of in vitro assays for immune responses. For example, IL-2 has been shown to maintain the in vitro proliferation of antigen-specific, cytolytic, and helper T cell lines (1, 2); to contribute to the formation of cytotoxic T lymphocytes in cultures stimulated with alloantigen (3); to enhance thymocyte mitogenesis, and to mediate the development of erythrocyte-specific antibody-producing cells in T cell–deficient cultures (4–6). Based on these activities in vitro it seems likely that IL-2 will also play a role in cell-mediated immunity in vivo.

Tests for the in vivo relevance of IL-2 would be greatly facilitated by the development of specific reagents that could detect the molecule independent of its biological activity, or block its action, or both. Specific antibodies to IL-2 might be expected to have such properties; in addition, they could be valuable specific immunoadsorbents in IL-2 purification and characterization, and in studies of IL-2 biosynthesis. With this in mind, we have raised antibody in rabbits using as antigen a highly purified fraction of mouse IL-2 whose isolation from spleen cell cultures we have recently described (7). The characterization of these anti-IL-2 antibodies, and some of their effects in vitro and in vivo, are reported here.

Materials and Methods

Male BALB/c, C57bl/6 and athymic (nude) BALB/c mice were obtained from Charles River Breeding Laboratories, Inc., Wilmington, MA. [^{3}H]Thymidine (sp act 17.5 Ci/mM), [^{35}S]methionine (sp act 1,000 Ci/mM) and ^{51}Cr (sp act 331 mCi/mg) were purchased from Schwarz/Mann Div., Becton Dickinson and Co., Orangeburg, NY; Dulbecco's minimal essential medium (MEM) and fetal bovine serum from Grand Island Biological Co., Grand Island, NY; acrylamide, bis-acrylamide, $N,N',N'N'$-tetramethylethylene-diamine, and ammonium persulfate from Eastman Kodak Co., Rochester, NY; three times crystallized concanavalin A (Con A) from Miles Laboratories, Inc., Elkhart, IN. Protein A-Sepharose and activated CNBr-Sepharose were obtained from Pharmacia Fine Chemi-

[1] *Abbreviations used in this paper:* BSA, bovine serum albumin; Con A, concanavalin A; CTL, cytotoxic T lymphocyte; FCS, fetal calf serum; IL-1, interleukin 1, IL-2, interleukin 2, IL-3, interleukin 3; MEM, Dulbecco's minimal essential medium; MLR, mixed leukocyte reaction; PBS, phosphate-buffered saline; PFC, plaque-forming cell; PHA, phytohemagglutinin; PMA, α-phorbol 12-myristate, 13-acetate; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SRBC, sheep erythrocytes.

cals, Piscataway, NJ; trasylol, bovine serum albumin (BSA), α-phorbol 12-myristate 13-acetate (PMA) from Sigma Chemical Co. (St. Louis, MO); oligo (dT) cellulose type 77 from PL Biochemicals; tissue culture plasticware from Falcon Labware Div. of Becton, Dickinson and Co., Oxnard, CA. Alu Gel S (2% suspension) from Accurate Chemical and Scientific Corp., Westbury, NY. All reagents were of the highest grade commercially available.

Antibody Production. Antibodies to IL-2 were raised in rabbits. Purified IL-2 fraction VI (7) (1–10 μg) was mixed 1:1 with Alu Gel and injected intradermally on the back of the animal in 30 different sites. Identical booster injections were administered every 4 wk. Antibody responses were detectable 2–3 months after the first injection.

Mixed Leukocytic Reaction (MLR). The effect of anti-IL-2 on antigen-induced T cell proliferation was tested in primary MLR using replicate 100 μl cultures (MEM, 5% heat-inactivated fetal calf serum (FCS), 5 × 10^{-5} M 2-mercaptoethanol, and antibiotics) of 1 × 10^6 cells/ml C57Bl/6 responder spleen cells and 1.5 × 10^6 cells/ml BALB/c irradiated (1,000 rad) splenic stimulator cells. Replicate cultures were supplemented with varying concentrations of either anti-IL-2 Ig or preimmune Ig. After 5 d of culture (37°C in a humidified atmosphere of 10% CO_2, 7% O_2, and 83% nitrogen), cells were exposed to a 4-h pulse of 1.7 μCi [^{3}H] TdR. The cultures were then collected onto glass fiber strips using a Brandell cell harvester and [^{3}H] TdR incorporation was determined by liquid scintillation counting in liquifluor toluene. For CTL response viable effector cells were tested for their ability to lyse ^{51}Cr-labeled allogeneic P815 target cells.

Preparation of Conditioned Medium Rich in IL-2. Conditioned medium from Con A–activated spleen cells were prepared as described elsewhere (7). Conditioned medium from blast cells was prepared by plating 2 × 10^6 3-d Con A–stimulated spleen cells in 35-mm cultures in 1 ml of MEM medium containing 5 × 10^{-5} M 2-mercaptoethanol, and 5 μg/ml Con A. After 2 h the Con A–induced cell monolayers were washed to remove Con A and the medium replenished with 2 ml of MEM plus 5 × 10^{-5} M 2-mercaptoethanol. Cell-free supernatant media were harvested at the desired time, centrifuged to remove cell debris, and stored at −20°C.

Assay for T Helper Activity. Antibody synthesis was measured as described by Mishell and Dutton (8). Spleen cells from BALB/c, BALB/c nu/nu, and C57bl/6 mice were resuspended in MEM supplemented with 5% FCS to a density of 5 × 10^6 cells/ml and distributed into 35-mm plates; 50 μl of 0.5% sheep erythrocytes (SRBC) were added as antigen to each culture. Immune or preimmune Ig or IL-2 were added at the desired concentration on day 0 or day 2. The culture was incubated with gentle rocking for 5 d and the cells were harvested and assayed for the antibody production in the Jerne plaque assay as modified by Mishell and Dutton (8). The results are expressed as the number of lytic plaques formed by 10^6 cells.

Assay for IL-2. IL-2 activity in conditioned media was assayed on 3 d Con A–stimulated spleen cells as described previously (7). 1 U was defined as the amount of IL-2 required to double the [^{3}H]-TdR incorporation observed in untreated cultures. Con A supernatants (containing ~10 U/ml of IL-2) were incubated for 2 h with or without serial dilutions of anti-IL-2 or preimmune serum and then mixed with T cell blasts (4 × 10^5 cells/ml). 20 h after culture initiation cells were exposed to a pulse of [^{3}H]-TdR for 4 h.

IL-1 Assay. IL-1 was assayed for its ability to stimulate the proliferative response of C3H/HJ thymocytes to phytohemagglutinin (PHA). Several dilutions of IL-1-containing supernatants produced by the J7774 macrophage cell line stimulated with lipopolysaccharide, were dispersed to individual microculture wells in 100 μl and then 100 μl of thymocytes suspension (1.5 × 10^7/ml) was added to each well. After 72 h cells were pulsed for 4 h with [^{3}H]-TdR.

IL-3 Assay. A FDC/D_1 cell line, described by Ihle (9) was used as the responding cell in a proliferative assay. 2 × 10^4 cells were cultured in microwells with several dilutions of supernatants from the WEHI-3 cell line and the response was measured after 20 h by [^{3}H] TdR incorporation (4 h).

Interferon Assay. Con A supernatants were titrated for their inhibition of the cytopathic effect of vesicular stomatitis virus on mouse fibroblasts line L929/B. Confluent monolayers

were seeded in microtest wells with threefold dilutions of supernatant or fractions to be assayed. After 20 h of incubation an infecting dose of vesicular stomatitis virus was added. The plates were examined under the microscope to determine the last dilution of supernatant that inhibits the lysis of one-half the cells.

B Cell Stimulating Factor Assay. Assay for B cell helper factors was done as described by Inaba et al. (10) using 3×10^6 purified B cells as responder and SRBC as antigen. The development of anti-sheep plaque-forming cells (PFC) requires helper factors and antigen and is antigen specific (10).

Cytotoxic Differentiation Factor. Assay for factors necessary for the development of cytolytic T cells was performed according to Granelli-Piperno and Steinman (manuscript in preparation). 5×10^5 thymocytes from Swiss mice (4 wk old) were suspended in MEM medium supplemented with 10% heat-inactivated FCS, 5×10^{-5} M β-mercaptoethanol, and 1 μg/ml Con A, and cultured in microwells with several dilutions of conditioned media. Polyclonal cytolytic T lymphocyte (CTL) response was measured after 72 h using ^{51}Cr-labeled P815 as targets in the presence of 10 μg/ml PHA. Addition to the cell cultures of purified or recombinant IL2 (Biogen), γ-IFN (Genentech), or IL-1 only did not support CTL response in this assay.

Polyacrylamide Gel Electrophoresis (SDS-PAGE). SDS-PAGE was performed as described by Laemmli (11). Conditioned medium was concentrated 10-fold over an Amicon PM-10 membrane. Samples were then mixed with an equal volume of double-strength sample buffer and electrophoresed in a 12.5% polyacrylamide slab gel. One lane of the gel was cut into 1-mm slices, eluted in phosphate-buffered saline (PBS) for 18 h at 4°C, and assayed for IL-2 activity. Protein bands were visualized by silver staining techniques (12).

Anti-IL-2 Affinity Chromatography. Serum from immunized rabbits was diluted 1:1 with PBS and loaded onto a protein A–Sepharose column. The column was washed with PBS containing 0.4 M NaCl and the Ig fraction was eluted with 0.1 M glycine HCl, pH 2.3 and immediately neutralized with 1 M Tris. Immunoglobulin G (7.5 mg) was extensively dialyzed against coupling buffer (0.05 M MES, pH 6.5), coupled to 1.5 ml of swollen activated CNBr Sepharose, and the mixture shaken for 12–15 h at 4°C. Residual activated groups were quenched by incubating the resin for 2 h in 1 M Tris/HCl pH 9. Unmodified conditioned medium containing IL-2 activity was passed through the column at neutral pH, the resin was washed with 70 ml of PBS containing 0.4 M NaCl, 1% Triton X-100, 0.2% SDS, 1 mM EDTA followed by 50 ml of PBS, and IL-2 was then eluted with 0.1 M glycine HCl, pH 2.3.

Immune Precipitation of Biosynthetically Labeled IL-2. 2×10^7 spleen cells were incubated for 2 h with Con A (5 μg/ml) in serum-free medium, and the supernatant removed; methionine-deficient Dulbecco's medium containing 1/20th of the usual level of methionine and 50 μCi/ml [^{35}S]methionine was added and incubation continued for a further 20 h. Immunoprecipitation was then performed as follows: culture medium was made 1% in SDS and boiled for 5 min, after which Triton X-100 was added to a final concentration of 5%. The samples were immunoprecipitated for 16 h at 4°C, protein A–Sepharose was added, the mixture incubated for 30 min at room temperature and then centrifuged.The pellets were washed three times with NETTS buffer (0.5 M NaCl, 0.2% SDS, 1% Triton X-100, 1 mM EDTA, 50 mM Tris HCl, pH 7.4) and once with 0.5 M NaCl, 0.1% SDS, 50 mM Tris HCl, pH 7.4. Proteins were eluted by boiling the pellets in electrophoresis sample buffer and applied to 12.5% SDS-PAGE.

Popliteal Lymph Node Assay. The popliteal lymph node response to antigen stimulation was measured according to Lafferty et al. (13). 30 μl of PBS containing 3×10^6 C57BL/6J (H-2^b) spleen cells were injected into the left hind footpad of BALB/c (H-2^d) recipients. As control, the right hind footpads were injected with 30 μl of PBS alone. The animals were then either given daily intravenous injections of anti-IL-2 IgG (0.4 mg/d) or an equivalent dose of preimmune IgG, or were left uninjected. After 2, 3, and 4 d the draining popliteal lymph nodes were removed and assayed for cytotoxic activity against EL-4 (H-2^b) targets. Results are expressed as log 10 cytotoxic units (C-U) per node according to Lafferty et al. (13).

Isolation of Poly A$^+$ RNA. mRNA was extracted from cultured cells by the method of

Lizardi and Engelberg (14). For the preparation of RNA from EL-4 culture cells (5 × 10^5 cells/ml) were stimulated for 5 h with PMA (10 ng/ml) and Con A (5 μg/ml), collected by centrifugation, washed twice with PBS, and lysed with buffer A (25 mM Tris/HCl, pH 7.4, 0.1 M NaCl, 7.5 mM EDTA, 25 mg/ml polyvinyl sulfate, 2.4% SDS 350 μg/ml proteinase K; 3 ml for 1 × 10^8 cells) at 37°C for 15 min; then 2/3 volume of 3.5 M NaClO_4 was added and the viscosity of the solution was reduced by passing it twice through a 21-gauge needle, after which nucleic acids were precipitated with 4 vol of EPR (NaClO_4 saturated ethanol/water, 4:1) kept at 4°C for 60 min, and the precipitate collected by centrifugation at 6,000 rpm. The pellet was dissolved in buffer B (25 mM Tris/HCl, pH 7.4, 1 mM EDTA, 0.2% SDS; 8 ml for 1 × 10^8 cells), NaCl, was adjusted to 0.2 M, 0.6 vol of isopropanol was added, and the solution was stored at −20°C overnight. The precipitate was collected by centrifugation, the pellet resuspended in H_2O, 0.2 M NaCl added, and nucleic acids reprecipitated with 2.5 volumes of ethanol and stored at −20°C overnight. The precipitate was collected as above and the pellet dissolved in H_2O to a concentration of 20 OD_{260} U/ml. Lithium dodecyl sulfate was added to 0.07%, LiCl was added to 0.2 M, and the solution was left at 4°C for 4 h. The precipitated RNA was collected by centrifugation and reprecipitated twice with ethanol and salt. For the preparation of control RNA, cells were lysed in buffer A immediately before the addition of PMA and Con A and processed in the same way.

The preparation of poly A^+ RNA was accomplished by batchwise fractionation using oligo (dT) cellulose. RNA was dissolved in H_2O to 100 OD_{260} U/ml, heated at 60°C for 2 min, cooled quickly, an equal volume of 2 × HSB-buffer (HSB; 10 mM Tris/HCl, pH 7.4, 0.12 M NaCl, 1 mM EDTA, 0.20% SDS) and prewashed oligo (dT) cellulose (1 mg cellulose for 1 OD_{260} units) was added. After the solution was shaken for 1 h at room temperature, the resin was washed 6 times with HSB, twice with HSB minus SDS, then poly A^+ RNA was eluted with H_2O three times. For each wash the resin was collected by centrifugation at 3,000 rpm for 3 min. The eluate was precipitated with 3 vol of ethanol after adjusting 0.3 M NaCl, and the sedimented RNA dissolved in H_2O and stored at −80°C. From 5 × 10^8 cells 150 μg of poly A^+ RNA was obtained.

Microinjection of Oocytes.   40 ng of poly A^+ RNA were injected in *Xenopus laevis* oocytes. Groups of 20 injected oocytes were incubated in microwells containing 200 μl of Barth's incubation medium (15) supplemented with BSA (0.5 mg/ml) and Trasylol (50 U/ml). After 20 h the medium was collected and centrifuged for 5 min at 12,000 g in an Eppendorf microcentrifuge, and aliquots of supernatants were assayed for IL-2 activity.

Results

To characterize the anti-IL-2 antibodies, the properties of either unfractionated antisera or purified immunoglobulins were evaluated in a series of biochemical and biological tests.

Inhibition of IL-2-dependent Lymphoblast Proliferation.   Antibodies in rabbit sera were initially detected by an IL-2 neutralization test in which the antibody blocked the proliferation of T lymphoblasts mediated by the conditioned medium from Con A–stimulated spleen cell cultures. The action of one pool of antisera is shown in Fig. 1, where it can be seen that immune serum completely suppressed the growth-promoting action of conditioned medium, while the same amount of preimmune rabbit serum was inert. Identical results (not shown) were obtained with the respective immunopurified immunoglobulin at final concentration of 5–10 μg/ml.

The specificity of this inhibition for IL-2 was established by the failure of the antisera to block the action of other lymphokines, including γ-interferon, IL-3 (Table I), and B cell stimulating factor (10).

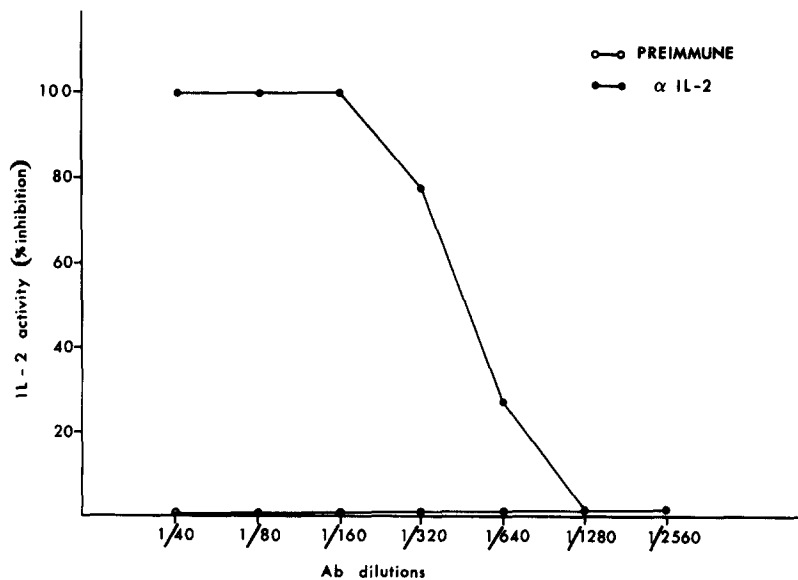*Immunoprecipitation of Biosynthetically Labeled IL-2.*   The IL-2 neutralizing

FIGURE 1.   Inhibition of IL-2 activity in conditioned medium from Con A–stimulated spleen cells. Aliquots of conditioned medium were incubated for 2 h with serial dilution of preimmune (O) or anti-IL-2 antiserum (●) and then mixed with T cell blasts; after 20 h of incubation the cultures were exposed to a 4-h pulse of [^{3}H]-TdR. Results are expressed as percentage of IL-2 activity inhibited by antibodies.

TABLE I

Specificity of α-IL-2 Neutralization Assay

Lympho-kine	Source of Factor	Preimmune IgG added			α-IL-2 IgG added		
		50	25	12.5	50	25	12.5
		μg/ml			*μg/ml*		
IL-2*	Con A sup	127,000	125,000	129,000	1,450	1,630	1,890
IL-3‡	WEHI-3 sup	97,000	107,100	103,000	115,950	98,800	111,400
IFN§	Con A sup	300	300	300	300	300	300

Aliquots of conditioned media were preincubated with antibody for 2 h at 37°C and then assayed for lymphokine activity.
*‡ Results are expressed as [^{3}H]-TdR cpm incorporated.
§ Results are expressed as interferon units.

action of antisera prompted us to test whether they could be used to precipitate biosynthetically radiolabeled lymphokine. The supernatant of mouse spleen cells cultures stimulated by addition of Con A and incubated for 20 h with [^{35}S]-methionine were immunoprecipitated with purified anti-IL-2 IgG and the washed precipitate was analyzed by SDS-PAGE. The autoradiogram in Fig. 2 shows that the immune IgG precipitate (but not that from preimmune IgG) contained a radioactive protein whose mobility yielded an apparent M_r of 23,000, which corresponds exactly with the migration of active IL-2 under the same conditions (7). Taken together with the preceding results, this finding indicates that the antibodies both neutralize IL-2 activity and precipitate the lymphokine.

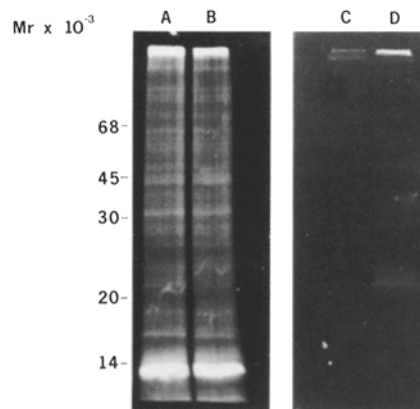*Neutralization of Oocyte-translated IL-2.*   As a basis both for exploring the

FIGURE 2. Immunoprecipitation of biosynthetically labeled IL-2. Autoradiogram of labeled proteins remaining in supernatant after IL-2 precipitation with preimmune Ig (lane A), or anti-IL-2 Ig (lane *B*); immunoprecipitate obtained with preimmune IL-2 Ig (lane *C*) or anti-IL-2 Ig (lane *D*).

TABLE II

Inhibition by Anti-IL-2 of IL-2 Activity Produced by Microinjected Xenopus Oocytes

Source of mRNA	[³H]-TdR cpm	
	−α-IL-2	+α-IL-2
None	1,020 ± 15	NT
EL-4	1,170 ± 12	1,050 ± 9
EL-4 stimulated	15,310 ± 114	1,370 ± 7

Conditioned medium from injected Xenopus oocytes (see Materials and Methods) was incubated for 2 h with or without anti-IL-2 IgG (5 μg/ml) and then mixed with T cell blasts. 20 h after cultures initiation cells were pulsed for 4 h with [³H]-TdR.

feasibility of cloning mouse IL-2 gene, and for characterizing the nature of IL-2 activity synthesized in heterologous translation systems, we assayed the effect of anti-IL-2 on incubation medium from cultured *Xenopus laevis* oocytes that had been injected with mRNA preparations extracted from an IL-2–producing T cell line (EL-4). In this experiment poly A⁺ RNA was extracted from EL-4 cultures 5 h after IL-2 production was stimulated by addition of PMA. Following purification and isolation of mRNA (see Materials and Methods) Xenopus oocytes were microinjected with 40 ng of RNA from either PMA-stimulated or unstimulated cultures. After 24 h of incubation the oocyte incubation medium was assayed for IL-2 activity (Table II). IL-2 activity was detectable only in the medium conditioned by oocytes that had received poly A⁺ RNA from PMA-treated cultures, and the IL-2 was completely neutralized by the anti-IL-2 Ig. This result demonstrates the faithful translation of IL-2 mRNA in oocytes and the immunological cross-reaction of the product with lymphokine purified from spleen cell culture medium.

Antibodies Neutralize the Activity of Two Forms of IL-2. The major species of IL-2 present in spleen cell conditioned medium, which after purification, served

as immunizing antigen for raising the antisera used in this study, migrates in SDS-PAGE under nonreducing conditions with apparent M_r 23,000 (7).

While exploring the responses of other mouse lymphoid cell populations, we observed that lymphoblasts upon stimulation with Con A released an IL-2 whose rate of migration in SDS-PAGE corresponded to M_r 20,000. This component was clearly separated from the 23-kdalton protein both during initial and subsequent electrophoresis. Although the significance of the lower molecular weight form has not been defined, we found that its mitogenic action on lymphoblasts is just as sensitive to inhibition by antibody as is that of the slower moving species. This suggests that the two are closely related, the electrophoretic difference possibly being due to differences in glycosylation or limited proteolysis. Molecular heterogeneity of IL-2 observed here is not unique because human IL-2 (16) and other cytokines, such as GM-CSF (17), have been shown to exhibit different molecular characteristics dependent on the experimental conditions used.

Immunoaffinity Purification of IL-2.   The anti-IL-2 immunoglobulins could be used for construction of affinity columns that effectively retained IL-2 activity. When IL-2 fractions, concentrated and slightly purified by chromatography on hydroxylapatite columns (7), were passed through such an affinity column the IL-2 activity was retained and could be recovered in good yield (20–50%) in the usual dissociating buffer (0.1 M glycine HCl, pH 2.3) (Table III, Fig. 3). The 23-kdalton component in these eluates, though highly purified, was contaminated

TABLE III

Purification of Mouse IL-2 Using Mouse Anti-IL-2 Sepharose

Fraction	Volume	Total protein	Total units	Sp act	Yield
	ml	*mg*	*U*	*U/mg*	*%*
Con A supernatant	800	24	550	23	100
Hydroxylapatite	30	6.3	386	61	60
Anti-IL-2 Sepharose	9	0.00135	120	88,888	21.8

For determination of protein recovered in each fraction, BSA was used as a standard in the Bio-Rad assay.
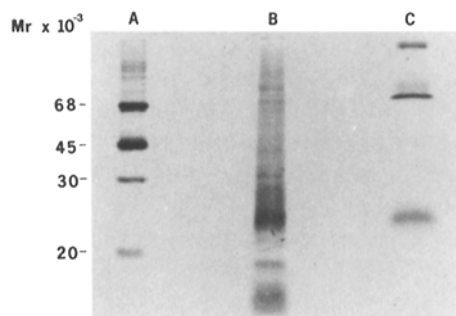

FIGURE 3.   SDS-PAGE of hydroxylapatite and anti-IL-2 Sepharose purified IL-2. The protein bands were visualized by silver staining. (*a*) Standard protein: bovine serum albumin (M_r 68,000), ovalbumin (M_r 43,000), carbonic anhydrase (M_r 30,000) and soybean trypsin inhibitor (M_r 20,000). (*b*) Hydroxylapatite purified IL-2. (*c*) Anti-IL-2 purified IL-2.

with two other major proteins that gave bands corresponding to M_r of 60–65,000 and 90–100,000. However, mitogenicity for lymphoblasts was associated entirely with the 23-kdalton species. We note that IL-2 activity was unstable after immunoaffinity purification, with 50% loss of activity occurring within 15 d, at either acid or neutral pH, and at either 4°C or −20°C. If these fractions were further purified by SDS-PAGE, the resultant preparation were stable, without detectable loss of activity, for at least 10 months at 4°C. Human IL-2 could not be adsorbed onto anti-mouse IL-2 column, consistent with the known antigenic differences between the two species (18).

Interaction of Antibodies with IL-2 and Not with Other Lymphokines. Anti-IL-2 antibodies do not neutralize or absorb other soluble mediators; Table IV shows the specificity of the anti-IL-2 Ig Sepharose column. Various cell culture supernatants were fractionated on anti-IL-2 Ig Sepharose and unbound and eluted fractions assayed for the specific lymphokines. The results clearly show that the anti-IL-2 Sepharose column absorbed and purified only IL-2. All the other lymphokines tested—IL-1, IL-3, γ-IFN, B cell stimulating factor, and cytotoxic differentiation factor—were found in the unbound fractions.

Biological Effects of Anti-IL-2 Antibodies

Inhibition of T Cell Mitogenesis by Anti-IL-2. Given the capacity of anti-IL-2 antibody to inhibit IL-2-dependent T lymphoblast growth, it was of interest to determine its effect on more complex multicellular responses. As seen in Fig. 4, the addition of anti-IL-2 Ig to MLR strongly suppressed T cell multiplication as assessed by [³H]-TdR incorporation, whereas preimmune Ig was without effect. The same pattern was observed in cultures exposed to mitogenic stimulation by Con A: once again, spleen cell proliferation was markedly inhibited by anti-IL-2 Ig (50% reduction of [³H]-TdR labeling at 50 μg/ml of antibody protein) but not by preimmune Ig. The generation of alloreactive CTL on MLR was also affected by anti-IL-2 antibody (Fig. 5).

Effect of Anti-IL-2 on T Cell Helper Activity. Previous work (7) has shown that

TABLE IV
Specificity of α-IL-2 Immunoabsorption

Lymphokine	Source of factor	Fraction tested:		
		Start	Unbound	Eluted
IL-2*	Con A sup	2.7	0	1.6
IL-3‡	WEHI-3 sup	0.70	0.65	0
IFN§	Con A sup	300	300	0
IL-1∥	J774 + LPS sup	7,354	7,127	0
B cell stimulating factor¶	Syn MLR sup	4,920	3,750	3
Cytotoxic factor**	Con A sup	48	32	1

Supernatants were applied to a α-IL-2 IgG Sepharose column. Aliquots of the fractions were tested for lymphokines activity.
*‡§ Results are expressed respectively as U/ml of IL-2, IL-3, and IFN activity.
∥ Results are expressed as [³H]-TdR incorporated during a 4-h pulse by C3H/J thymocytes.
¶ Results are expressed as PFC for individual culture.
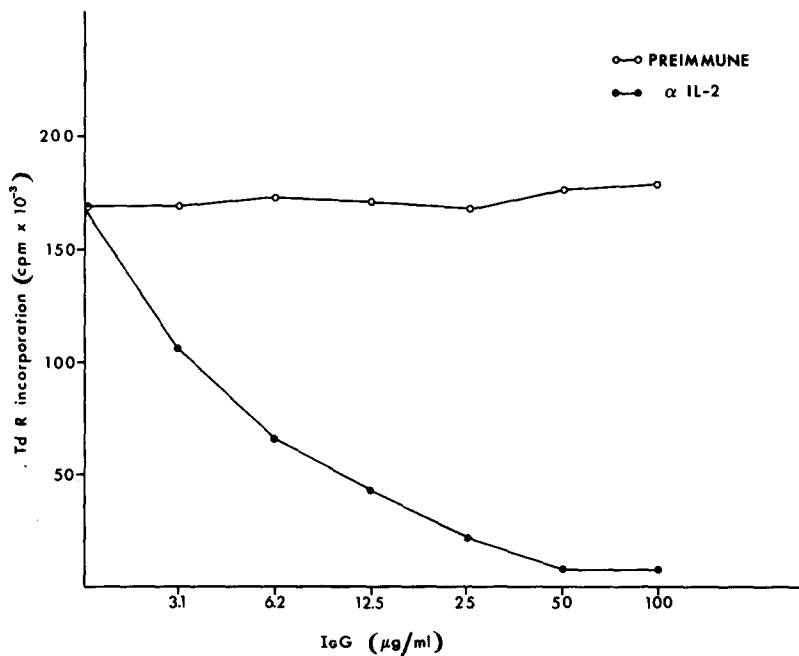** Results are expressed as % of ⁵¹Cr released in a 4-h lectin-dependent cytotoxic assay.

FIGURE 4. Effect of anti-IL-2 on proliferation of mixed leukocyte reaction. 1 × 10⁶ spleen cells/ml from C57Bl/6 mice were mixed with 1.5 × 10⁶ irradiated BALB/c spleen cells/ml (1,000 rad), and 100 μl of the resulting cell suspension were plated in microtest wells. Six replicate MLC were conducted containing varying concentrations of either anti-IL-2 (●) or preimmune IgG (○). After 4 d, cultures were pulsed for 4 h with [³H]-TdR and the incorporation into DNA was determined.

purified IL-2 could promote antibody production in cultures of nude mouse spleen cells. The availability of specific antibody enabled us to test whether IL-2 was actually produced and required under ordinary conditions of assay for antibody synthesis in culture. The addition of antibody significantly reduced the plaque-forming titer of spleen cultures exposed to SRBC (Fig. 6). The inhibitory action of antibody was equally pronounced (70–85% reduction in plaque number) when the antibodies were added together with SRBC, at time 0 or after two days of culture (Fig. 6). When antibody was added to the cultures for the first 2 d and then removed, there was subsequent full recovery of plaque-forming activity. As expected, the effect of antibody could also be reversed by addition of excess IL-2, and preimmune Ig was inert.

Effect of Anti-IL-2 In Vivo. In view of the preceding results, that anti-IL-2 specifically affect IL-2, and not other cytokines, and could block the MLR, it seems desirable to explore the consequences of injected antibodies in situ, particularly since this might produce evidence about a possible role for IL-2 in modulating T cell functions in vivo. We used an immunization protocol known to generate alloreactive CTL in vivo (13), in the course of which some of the mice received daily injections of anti-IL-2 Ig, while control mice were injected either with saline or with preimmune Ig. Control determinations were performed to assess the level of circulating anti-IL-2 and stable titers of circulating activity
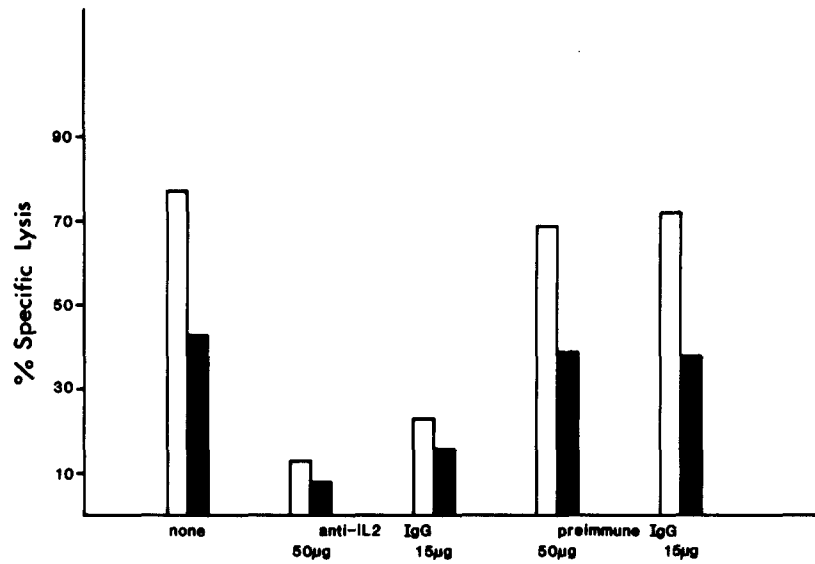
FIGURE 5. Effect of anti-IL-2 on mixed leukocyte reaction:generation of CTL. 1×10^6 spleen cells/ml from C57Bl/6 mice were mixed with 1.5×10^6 irradiated BALB/c spleen cells/ ml (1,000 rad) and 1 ml of cell suspension was plated in 16mm macro wells and anti-IL-2 or preimmune IgG added at the indicated concentration. After 5 d viable effector cells were counted and tested for lytic reactivity against ^{51}Cr-labeled P815 tumor target cells. White bar, E/T 1:50; black bar, E/T 1:15.
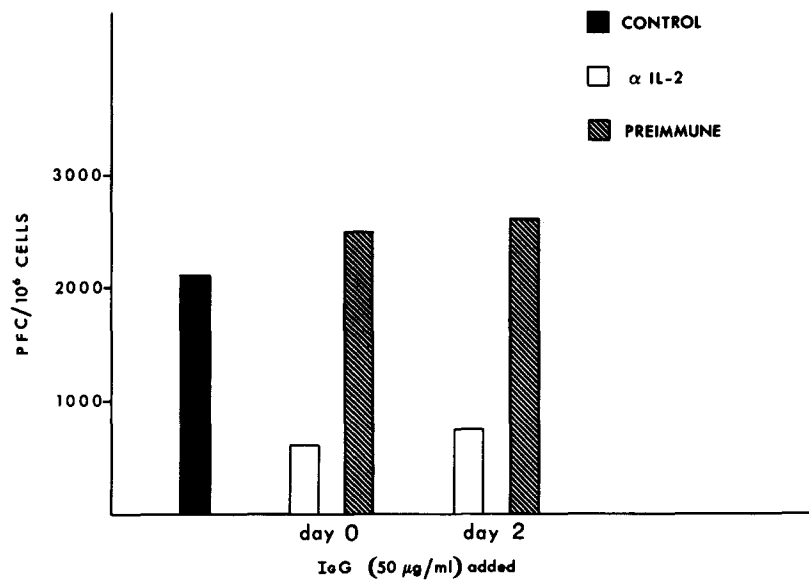

FIGURE 6. Effect of anti-IL-2 on T helper activity. IgG were added to the cultures on day 0 or day 2. Helper activity was measured with PFC assay on day 5.
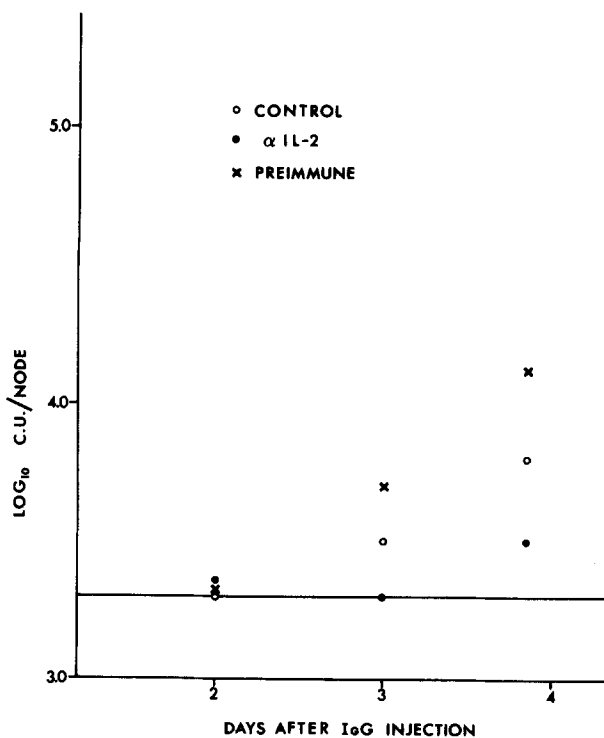
FIGURE 7. Ability of IL-2 antibody to inhibit cytotoxic T cell formation in vivo. BALB/c mice were inoculated in the right hind foot pad with 3 × 10⁶ C57Bl/6 spleen cells. Three groups of 18 mice each were either left uninjected (O), or were given daily intravenous injections of anti-IL-2 Ig (0.4 mg/ml) (●) or preimmune Ig (0.4 mg/ml) (X). At the indicated times lymph node cells from the three groups of animals (6 each) were tested for lytic activity against ⁵¹Cr-labeled EL-4 target in a 4-h cytotoxicity assay. Lymph nodes draining control footpads which were not inoculated with antigen gave no detectable cytotoxic response (<10³·³ C.U/node). Experiments were repeated 5 times with no significant difference in the results.

were observed throughout the course of the experiments. As seen in Fig. 7, lymph node cells from mice injected with anti-IL-2 Ig showed a significant reduction of specific CTL responses to allogeneic cells compared with lymphoid cells from mice that were injected with saline or with preimmune antibody. The cytolytic activity mediated by the lymph node cells was specific for the immunized allogeneic cell type, as shown by the fact that inappropriate target cells were not lysed. These results strongly implicate IL-2 function during specific cytotoxic T cell responses in situ.

Discussion

Our results show that the anti-IL-2 antibodies that we have raised in rabbits are potentially useful reagents both for isolating mouse IL-2 and for probing the functions of this molecule in immune phenomena in vivo and in vitro.

The antibodies were raised in response to injection of highly purified antigen; they are highly specific and may well even be monospecific, but we have not rigorously established this. The elution of three bands of protein from the

immunoaffinity column might cast some doubt on the monospecificity of the Ig, but nonspecific absorption of protein to immunoaffinity columns is a well-known phenomenon; furthermore, the presence of IL-2 sequences in the apparent contaminants have not yet been excluded, and additional experiments are required to characterize antibody specificity in greater detail. In spite of this uncertainty, it is clear that the antibodies effectively remove all detectable IL-2 activity from solution and precipitate a protein whose migration on SDS-PAGE is indistinguishable from that of authentic IL-2; further, whatever the limitations of their specificity, the antibodies do not interact with the other lymphokines such as IL-1, IL-3, γ-IFN, or B cell stimulating factor(s) or cytotoxic differentiation factor(s).

The anti-IL-2 antisera and the Ig purified from them inhibited a variety of immune responses. These included antigen- and lectin-stimulated T cell mitogenesis, IL-2-dependent lymphoblast proliferation, cytotoxic lymphocyte generation in culture, and T cell helper function in an assay for antibody production in culture. In addition, the antibodies reduced the development of alloreactive cytotoxic cells in vivo; this finding is, so far as we are aware, the first direct evidence that *endogenous* IL-2 may be important for cytotoxic T cell development in vivo. Since this and other responses occur only in mixtures of cells and relatively complex media, the availability of anti-IL-2 should facilitate the further dissection of the cellular and hormonal pathways that mediate these reactions both in vivo and in vitro. The antibodies are therefore likely to be useful as a mechanistically specific immunosuppressive agent in experimental contexts involving a wide range of immune phenomena. They should also be of value in other contexts where tests for IL-2 are needed; these include studies of biosynthesis of IL-2, and the molecular cloning of its cDNA.

Summary

Antibodies to highly purified mouse interleukin 2 (IL-2) were raised in rabbits; a 1:500 dilution of antiserum completely blocked the in vitro mitogenic effect of 10^{-9} M IL-2. The antisera functioned effectively to immunoprecipitate biosynthetically labeled IL-2 and the purified immunoglobulins were useful in the construction of affinity columns for the adsorption and one-step immunopurification of IL-2. The antibodies were apparently specific for IL-2 among the lymphokines, they did not block the biological effects of IL-1, IL-3, γ-IFN, B cell stimulating factor(s), and cytotoxic T cell differentiation factor(s).

When anti-IL-2 was added to the in vitro reactions, it blocked mixed leukocyte reactions (MLR) and associated lymphocyte proliferation, the in vitro generation of cytotoxic T cells, and antibody formation as assessed by erythrocyte-specific plaque-forming cells (PFC).

When injected into mice, anti-IL-2 antibodies also reduced the formation of cytotoxic lymphocytes in response to allogeneic cells, suggesting that endogenous IL-2 participates in such reactions in vivo.

Taken together, the results indicate that these IL-2 antibodies will be useful adjuncts in the analysis of immune response both in vivo and in vitro.

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