

# Antibodies of predetermined specificity against chemically synthesized peptides of human interleukin 2

(synthetic peptides)

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**ABSTRACT** This communication describes the preparation of antibodies to human interleukin 2 (IL-2), using as immunogens synthetic peptides derived from the predicted amino acid sequence of IL-2. Rabbits and mice were immunized with protein carrier conjugates of eight chemically synthesized IL-2-derived peptides, each consisting of 13–15 amino acids. The immune antisera were screened in a solid-phase ELISA for reactivity to a native human IL-2. Antibodies to four of the eight peptides were found by a variety of biological and immunochemical criteria to react against human IL-2. Furthermore, an affinity-purified antibody to one of the IL-2 peptides (peptide 84) specifically stained the cytoplasm of phytohemagglutinin-stimulated human peripheral blood lymphocytes or T-leukemic cells (Jurkat). Antibodies to synthetic IL-2 peptides should serve as useful probes for studying this lymphokine and for developing quantitative assays for measuring its levels in biological fluids and its association with disease.

Interleukin 2 (IL-2) or T-cell growth factor (TCGF), a T-cell-derived glycoprotein shown originally to support the long-term growth of activated T cells (1, 2), is believed to provide a universal signal for the proliferation of mature T cells (3) through its binding to specific cell-surface receptors (4). Furthermore, IL-2 was shown to induce antigen-specific cytotoxic T lymphocytes (5–7), natural killer cells (8), or lymphokine-activated killer cells (9), all of which are implicated as effector cells in surveillance against malignancy, to reconstitute functional T-cell responses in athymic nude mice (10) and to assist in the immune T-cell- and chemotherapy-induced elimination of established murine syngeneic leukemias (11). These activities have suggested the potential use of IL-2 as an immunotherapeutic agent.

Additional clinical interest in IL-2 arose in view of the reported associations between abnormalities in IL-2 production and/or response and several diseases. Such defects were identified in mice (12, 13) and humans (14, 15) with systemic lupus erythematosus, in aged individuals (16, 17), in children with primary immunodeficiencies (18), in bone marrow transplant recipients undergoing a graft vs. host reaction (19), and in parasite-infected mice (20). In view of its role in immune function and its possible disease association, a great deal of work on IL-2 has recently been done, resulting, among others, in the establishment of B-cell hybridomas producing monoclonal antibodies to human IL-2 (21–23) and determination of its primary sequence via molecular cloning techniques (24).

Recent studies have shown that synthetic peptides can be used to induce antibodies specific for predetermined primary amino acid sequences in intact proteins (reviewed in refs. 25 and 26). Such antibodies can be raised even against peptides that are not immunogenic in the context of the native protein

molecule. Anti-peptide antibodies are, therefore, powerful tools for analyzing the fine structure of complex proteins. On the basis of this information, we have prepared antibodies against several synthetic peptides derived from the deduced amino acid sequence of a human IL-2 molecule (24). In this communication, we describe the preparation and properties of several such antibodies, and we present several lines of evidence that the anti-peptide antibodies bind to, and recognize, human IL-2.

## MATERIALS AND METHODS

**Preparation and Bioassay of Human IL-2.** IL-2 was prepared by stimulating human tonsillar lymphocytes ( $2 \times 10^6$ /ml) for 42 hr with 1% (vol/vol) phytohemagglutinin (PHA; GIBCO), phorbol myristate acetate at 50 ng/ml (P-L Biochemicals), and irradiated (5000 rad; 1 rad =  $1 \times 10^{-2}$  gray) human lymphoblastoid cells ( $0.5 \times 10^6$ /ml) (Raji). IL-2 was concentrated and purified by methods essentially similar to those described by Robb *et al.* (4). Samples were titrated for IL-2 activity as described (27) using the IL-2-dependent line, CTLL-2, as a source of indicator cells.

**Synthesis and Carrier Conjugation of Peptides.** Peptides were derived from the published sequence of a human IL-2 gene (24) and synthesized by the Merrifield solid-phase method (28, 29). The positions of the different peptides along the published IL-2 sequence are given in Table 1. A cysteine residue was added to the amino or carboxyl terminus of some of the peptides to allow coupling to a protein carrier. The composition of all peptides was confirmed by amino acid analysis. Synthetic peptides were coupled to keyhole limpet hemocyanin or tetanus toxoid by two methods. In the first, the carrier was activated with *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester and subsequently coupled to the peptide through its cysteine residue (30). In the second method, the peptide was coupled to the carrier through free amino groups, using a 0.04% glutaraldehyde solution.

**Immunization and Purification of Antibodies.** Rabbits were injected intramuscularly with 300–400  $\mu$ g of conjugate in complete Freund's adjuvant, incomplete adjuvant, or in alum (5 mg/ml) on days 0, 14, or 21, respectively. Mice were immunized in a similar way using 1/10th of the above dose per injection. Animals were bled 7 and 14 days after the last injection and, in some cases, were given further booster injections (in alum) and bled as necessary. Anti-peptide antibodies were purified on peptide affinity columns. Peptides were coupled to Affi-Gel 10 (Bio-Rad) according to the instructions of the manufacturer. Antisera were passed through the columns, and bound antibodies were eluted with a glycine/HCl buffer, pH 2.5, dialyzed, and concentrated.

**ELISA.** Antisera or affinity-purified antibodies were tested in an ELISA in round-bottom Immulon 2 microtiter plates

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Abbreviations: IL-2, interleukin 2; TCGF, T-cell growth factor; PBL, peripheral blood lymphocytes; PHA, phytohemagglutinin.

(Dynatech, Alexandria, VA) coated previously with solutions of peptides (500 ng/ml), partially purified TCGF (50–100 units/ml), or 1% bovine serum albumin. The plates were incubated (1 hr, 37°C) with serial dilutions of antisera in 1% bovine serum albumin buffer, washed, and treated for 1 hr at 37°C with a 1:2000 dilution of affinity-purified, horseradish peroxidase-conjugated goat anti-mouse IgG and IgM or goat anti-rabbit IgG antibodies (Tago, Burlingame, CA). The washed plates then reacted with a solution of *o*-phenylenediamine (0.4 mg/ml)/0.006% hydrogen peroxide (both from Sigma) in a (pH 5.0) citrate-phosphate buffer. Plates were analyzed after 15–30 min at 490 nm in a model MR600 Microplate Reader (Dynatech).

**Immunoblot Analyses.** Protein constituents in crude IL-2-containing supernatants (concentrated 100×) were separated by NaDodSO<sub>4</sub>/polyacrylamide gel electrophoresis as described (31), and then electrophoretically blotted onto nitrocellulose paper (32). The blots were blocked, treated with affinity purified anti-p84 antibody (5 μg/ml), washed, and labeled with <sup>125</sup>I-labeled staphylococcus protein A at a concentration of 1 × 10<sup>6</sup> cpm per gel lane using a modification of a standard procedure (33). They were then washed extensively, dried, and subjected to autoradiography. A parallel gel was cut into 2-mm slices and the slices were eluted by mincing and incubating them in 5% fetal calf serum-supplemented culture medium (300 μl) for 16 hr at 37°C. The medium was incubated at 4°C for 4 hr to precipitate the NaDodSO<sub>4</sub> from the gels. The precipitate was removed by centrifugation and the supernatants were tested for IL-2 activity as described above.

**Immunofluorescence.** Human peripheral blood lymphocytes (PBL), separated from heparinized blood by Ficoll-Hypaque centrifugation, were cultured at 2 × 10<sup>6</sup>/ml in RPMI 1640 medium plus 5% fetal calf serum in the absence or presence of 0.5% PHA. The cells were harvested 2 days later, washed two times, and pelleted on glass slides using a cytocentrifuge. The slides were fixed for 5 min with 95% cold ethanol, washed, and incubated for 30 min at room temperature with anti-IL-2 peptide at 10–50 μg/ml (or control) antibody in phosphate-buffered saline/10% fetal calf serum. After the slides were washed with water, they reacted with a 1:50 dilution of fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (Cappel Laboratories, Cochranville, PA) for 30 min at 4°C, washed, and observed in a Zeiss microscope under phase and fluorescence microscopy.

## RESULTS

**ELISA Reactivity of Anti-Peptide Antisera.** All peptides, except peptide 83, elicited high-titered anti-peptide antibodies. Moreover, four of the eight peptides elicited antibodies that reacted with a tonsil-derived, 100-fold-purified IL-2 preparation (Table 1). Anti-IL-2 titers were lower than the respective anti-peptide titers. No crossreactivity among the different IL-2-derived peptides was observed (not shown). Binding was specific since a given anti-peptide antibody bound to plates coated with the immunizing peptide or IL-2, but not to an irrelevant peptide. Moreover, preimmune serum or hyperimmune antiserum to unrelated peptides did not bind to IL-2 plates to any significant degree (not shown). The same four peptides that elicited IL-2-reactive antibodies in rabbits also induced the formation of anti-IL-2 antibodies in mice (data not shown).

Fig. 1 shows that the reactivity of anti-p84 antiserum against solid phase p84 or IL-2 was specifically inhibited by the homologous peptide in solution (50% inhibition at 10–100 ng of peptide per ml) but not by another peptide, p81. This result indicates that the restricted population of anti-peptide antibodies is also the one reacting with the IL-2.

The antisera that showed reactivity against partially puri-

Table 1. Representative antibody titers of rabbit anti-IL-2 peptide antisera

Peptide	Residues <sup>†</sup>	Antibody titer*	
		Anti-peptide	Anti-IL-2
10	65–78	4 × 10 <sup>4</sup>	<10
11	94–108-C	2.5 × 10 <sup>4</sup>	<10
12	111–125	1.2 × 10 <sup>5</sup>	1 × 10 <sup>2</sup>
13	C-126–138	3 × 10 <sup>5</sup>	<30
81	18–32-C	1 × 10 <sup>6</sup>	1 × 10 <sup>2</sup>
82	139–153	2 × 10 <sup>5</sup>	5 × 10 <sup>3</sup>
83	51–64-C	Neg	Neg
84	79–92	1.2 × 10 <sup>5</sup>	5 × 10 <sup>2</sup>

Neg, negative.

\*Reciprocal dilution producing 50% of maximal OD (490 nm).

<sup>†</sup>Based on the published IL-2 sequence (24). C indicates a cysteine residue added for coupling purposes.

fied tonsil-derived IL-2 were purified on peptide-coupled affinity columns. Anti-peptide and anti-IL-2 coeluted from the column after addition of a glycine/HCl buffer, pH 2.5 (not shown). The affinity-purified antibodies were tested in an ELISA against Jurkat-derived IL-2 purified by gel chromatography and isoelectric focusing and considered to be >90% pure (4), as well as against recombinant human IL-2 (rIL-2), a gift from Biogen. Antibodies against peptides 82 and 84 and, to a lesser extent, those against peptides 12 and 81 reacted to these IL-2 preparations (not shown).

**Correlation of IL-2 Biological Activity with ELISA Immunoreactivity.** Four liters of IL-2-containing crude tonsil supernatants were fractionated on an Aca-44 column and individual fractions were tested for biological activity in a TCGF bioassay and for immunoreactivity with anti-peptide antibodies in a solid-phase ELISA. Fig. 2 shows results of a representative experiment with an affinity-purified rabbit anti-p84 antibody. An excellent correlation between biological activity and immunoreactivity was found and the peaks of the two activity profiles were similar. In contrast, Aca-44-derived fractions of a mock IL-2 preparation did not react to any significant extent with the anti-peptide antibody. Moreover, an excellent correlation between biological activity and immunoreactivity profiles was found after two additional purification steps—namely, Affi-gel blue and DEAE-Sephacel chromatographies (not shown). These results strongly suggest that the antigen recognized by the anti-peptide

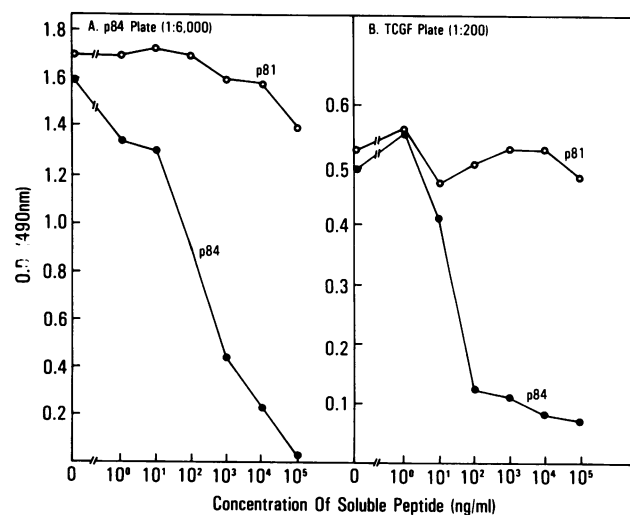


Fig. 1. Inhibition of rabbit anti-p84 antiserum-mediated ELISA by soluble peptides. Rabbit anti-p84 antiserum was incubated in p84- or TCGF-coated plates in the absence or presence of increasing concentrations of soluble p81 or p84.

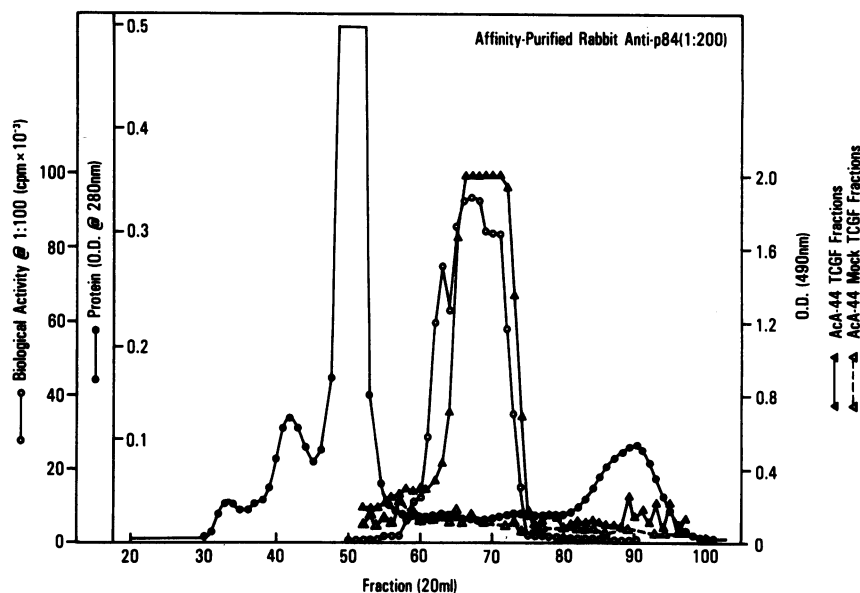


FIG. 2. Biological activity and ELISA reactivity profiles of ACA-44-fractionated tonsil-derived TCGF. Samples of 20-ml fractions were used undiluted to coat microtiter plates in the ELISA or were tested at a dilution of  $1:10^2$  for biological activity. Fractions of a mock TCGF preparation that lacked biological activity were tested in parallel in the ELISA. Affinity-purified rabbit anti-p84 (1:200) was used in the ELISA.

antibodies in the partially purified IL-2 preparations is IL-2 itself.

**Immunoblot Analysis.** Crude concentrated tonsil-derived supernatants subjected to immunoblot analysis revealed two closely positioned protein bands, in the molecular weight range of 15,000 to 18,000, which reacted with the anti-p84 antibody (Fig. 3). Preimmune serum or an irrelevant antibody did not react in a similar way (not shown). Furthermore, biologically active TCGF was eluted in a sharp peak from an almost identical position in the gel (Fig. 4). These results show that (i) anti-p84 reacts with a protein(s) migrating in a position corresponding to the size of human IL-2, and (ii) this protein and the biological activity of IL-2 comigrate on the gel.

**Immunofluorescence of PHA-Stimulated Cells.** PHA-stimulated (or control) human PBL or leukemic Jurkat cells were fixed, treated with an affinity-purified rabbit anti-p84 anti-

body, and then treated with a fluorescein isothiocyanate-conjugated goat anti-rabbit IgG. Cytoplasmic staining of PHA-stimulated cells was observed (Fig. 4). Specificity of this reaction was indicated by our findings that (i) the staining was abolished by preincubation of the anti-p84 antibodies with soluble p84 (20  $\mu\text{g}/\text{ml}$ ), but not with p81 (not shown); (ii) unstimulated PBL were not stained; and similarly (iii) PHA-stimulated PBL, treated with an anti-p72 (hepatitis B) antibody, were not stained under the same conditions (not shown). Details of this procedure and its application to the enumeration of IL-2-containing cells will be described in a separate communication (unpublished results).

## DISCUSSION

The data presented here show that antibodies reactive with native human IL-2 can be readily raised in rabbits and mice immunized with synthetic peptides derived from the predicted amino acid sequence of a human IL-2 molecule. This sequence was deduced from a recently cloned cDNA coding for human IL-2 (24) and confirmed, at least partially, by direct protein sequence analysis (22, 23).

Several lines of evidence clearly indicate that the anti-peptide antibodies recognize native human IL-2. (i) Anti-IL-2 peptide antiserum, but not the preimmune serum or a hyperimmune antiserum against an irrelevant peptide, bind to tonsil-derived IL-2 (as well as to the immunizing peptide), but they do not bind to an unrelated peptide or to a mock IL-2 preparation; furthermore, the binding to IL-2 is specifically inhibited by the soluble immunizing peptide. (ii) An excellent correlation between the profiles of biological activity and immunoreactivity is observed when crude IL-2-containing supernatants are fractionated by several independent steps based on different separation principles (i.e., ACA-44, Affigel blue, or DEAE-Sephacel chromatographies). (iii) Antipeptide and anti-IL-2 activity coelute from a peptide immunoaffinity column. (iv) Affinity-purified anti-peptide antibodies bind to electrofocused Jurkat-derived IL-2 or to recombinant IL-2. (v) IL-2 biological activity and an immunoreactive protein (identified by blot analysis) migrate in an almost identical position when a crude tonsil-derived, IL-2-containing supernatant is separated by NaDodSO<sub>4</sub>/PAGE. (vi) An affinity-purified antibody against one of the human IL-2 peptides gave a specific staining reaction on mitogen-

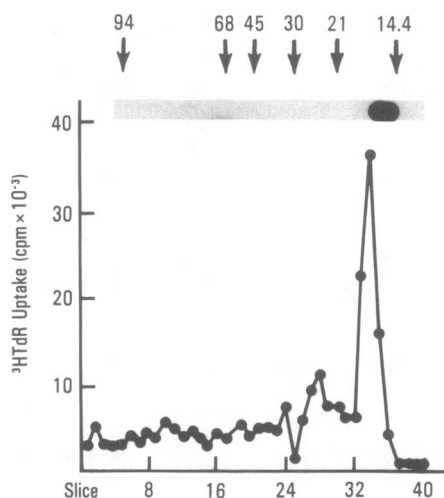


FIG. 3. Immunoblot analysis of crude tonsil-derived IL-2-containing supernatants using an affinity-purified anti-p84 antibody. Electrophoretically transferred proteins from a supernatant (concentrated  $100\times$ ) reacted with affinity-purified rabbit anti-p84 antibody (5  $\mu\text{g}/\text{ml}$ ). Eluates of 2-mm slices of a parallel gel were tested for IL-2 activity at a dilution of 1:100. Numbers above arrows are  $M_r \times 10^{-3}$ .

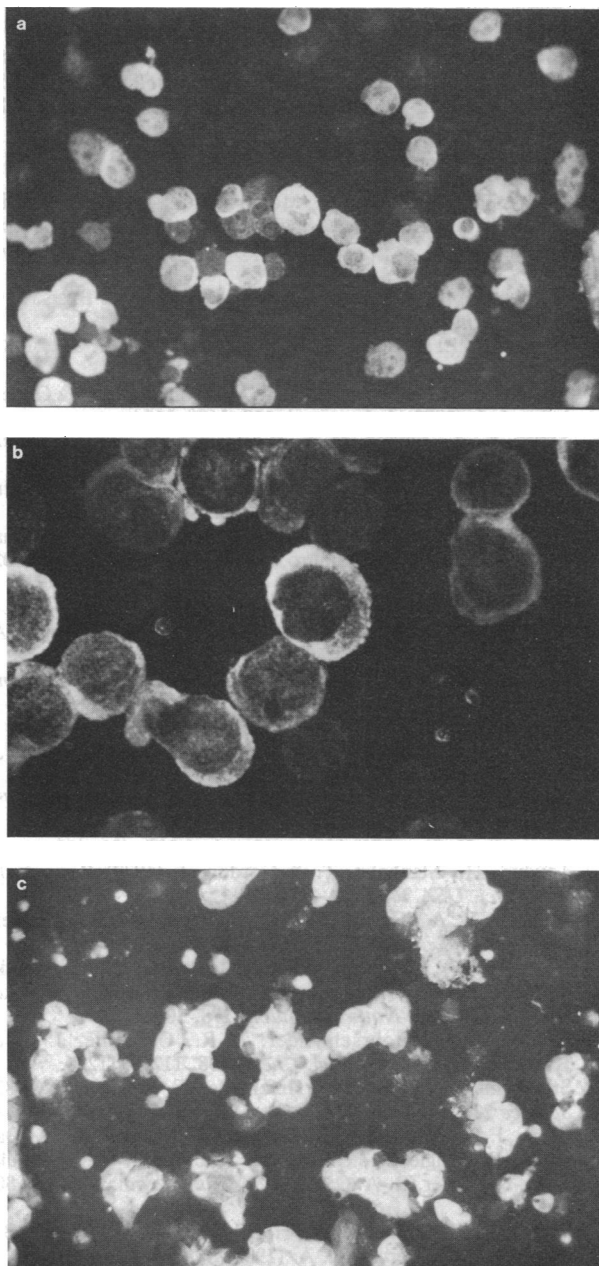


FIG. 4. Immunofluorescent staining of PHA-stimulated human cells with affinity-purified anti-p84 antibody. (a) PBL, 48 hr stimulation. ( $\times 280$ .) (b) Same as a. ( $\times 600$ , with a subsequent  $\times 1.4$  enlargement of the picture.) Positive and negative cells are present. Note the cytoplasmic perinuclear staining. (c) Jurkat cells, 16 hr stimulation. ( $\times 280$ .) The majority of cells are positive and clumped together.

activated human peripheral blood lymphocytes or T-leukemic cells. Taken together, these independent findings show that the anti-peptide antibodies described herein recognize native human IL-2 molecules.

Interestingly, the affinity-purified antibodies to peptides 12, 81, 82, or 84 failed so far to neutralize the biological activity of human IL-2 or to immunoprecipitate it from an IL-2-containing supernatant (unpublished observations). This could be due to one of the following: (i) An amino acid sequence required for biological activity could be missing (completely or partially) from the four peptides that elicited anti-IL-2 antibodies. The need for such a critical sequence to elicit antibodies that protect against foot and mouth disease (34) or inhibit the tyrosine-specific kinase activity of the

pp60src transforming protein (35) has been shown. (ii) The failure of the antibodies to inhibit IL-2 activity, or to react with it in solution, may represent an affinity problem. It has been shown that, even in the case of antibodies raised against native IL-2, a large excess of antibody is required to neutralize IL-2 activity (22). This is most probably due to the fact that the affinity of interaction between IL-2 and its cellular receptor (4) is greater by several orders of magnitude from the affinity of interaction between IL-2 and its respective antibody (22). The preparation of antibodies to other IL-2 peptides may clarify this issue.

The establishment of monoclonal hybridomas producing antibodies to human Jurkat-derived IL-2 was recently reported (22, 23). One of the critical requirements to ensure success was the use of sufficiently large quantities of purified IL-2 for immunization, thus requiring the preparation and processing of relatively large volumes of culture supernatants (22). The three monoclonal antibodies characterized in one of these studies (22) appeared to react with distinct epitopes on the IL-2 molecules, but these epitopes have not been identified. In a second study (23), the monoclonal antibody appeared to react with a sugar moiety on the IL-2 molecule. This antibody reacted with Jurkat- but not with tonsil-derived IL-2.

In contrast, our approach in this study has been to prepare antibodies against predetermined primary amino acid sequences of the human IL-2 molecule. The decision to use synthetic peptides was based on the recent demonstration in many laboratories that, using synthetic peptides appropriately coupled to protein carriers, it is possible to elicit antibodies to practically any region of a protein molecule, including those that are not immunogenic in the context of the native molecule (reviewed in refs. 25 and 26). Such antibodies offer two important advantages. First, the use of an easily prepared pure peptide bypasses the need to prepare sufficiently pure native antigen in the large quantities needed for immunization. Second, because the amino acid sequence of synthetic peptides is known in advance, they elicit antibodies of predetermined specificity.

Moreover, because antibodies can be raised against synthetic primary amino acid sequences that are antigenically "silent" in the context of the native protein (25, 26), the array of immunogenic epitopes and, hence, the spectrum of antibodies to the IL-2 molecule should be significantly expanded by the use of synthetic peptides. The most obvious example of the value of IL-2-reactive anti-peptide antibodies would be their use to identify those peptide moieties in the IL-2 molecule that mediate biological function and/or binding to the cell-surface receptors. A relevant example is a recently described antibody against a synthetic peptide deduced from the nucleotide sequence of a cloned *src* gene (35). Using this antibody, it was possible to identify a pentadecapeptide that is necessary for the tyrosine-specific kinase activity of the transforming protein pp60src (35).

Our findings that anti-p84 antibodies can stain in a specific manner the cytoplasm of PHA-stimulated human PBL or Jurkat cells strongly suggest that these antibodies may serve as sensitive and reliable tools to enumerate IL-2-producing cells in relation to disease. This finding is similar to that reported in a recent study using a monoclonal antibody made against native rat IL-2 (36).

Changes in the primary amino acid sequence of IL-2, resulting from mutations, etc., and leading to a loss or modification of biological activity, could theoretically be associated with certain diseases. If such IL-2 variants exist, anti-peptide antibodies may allow us to identify them. The high discriminatory power of anti-peptide antibodies was clearly illustrated by the ability of such antibodies to discriminate between subtypes of the hepatitis B virus surface antigen (37) idiotypic determinants in the hypervariable region of

two dextran-binding myeloma proteins (38), or between alloantigenic determinants differing in a single amino acid (39).

The ability of IL-2 to correct some immunodeficient states (10), to assist in tumor rejection *in vivo* (11), and to enhance or stimulate the induction of various types of killer lymphocytes *in vitro* (5–9) aroused great interest in this lymphokine and suggested that IL-2 may provide an immunotherapeutic tool. Moreover, association of IL-2 abnormalities with various diseases (12–20) emphasized the need for sensitive and reliable quantitative immunoassays for human IL-2 in a clinical setting. The relative ease in preparing anti-IL-2 antibodies by using synthetic IL-2-derived peptides is encouraging and strongly suggests that such antibodies may be useful in various studies on IL-2, including the establishment of more accurate IL-2 assays and the enumeration of IL-2-producing cells in health and disease.

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