ety scales, and the Global Assessment Scale (*5*). Outpatients were diagnosed according to DSM-III, after a Schedule for Affective Disorders and Schizophrenia (SADS) interview (*5*). Both patient groups included only major depressive disorders of the unipolar type (MDD). In healthy volunteers 16 years or older, the PAA excretion averaged 141.1 ± 10.2 mg per 24 hours, with 70 percent of the subjects excreting between 70 and 175 mg per 24 hours (Table 1). Low values for PAA excretion were found at admission in patients (N = 31) with unipolar depression: average = 68.7 ± 7.0 mg per 24 hours. Fifty-five percent of these depressed subjects excreted less than 70 mg per 24 hours, whereas only 15 percent of the controls excreted as little. There were no cases of high PAA excretion in this group (15 percent of the controls excreted more than 175 mg per 24 hours). The PAA excretion was equally low in untreated unipolar depressed inpatients and in patients who had received antidepressant medication prior to admission without clinical improvement. Ongoing longitudinal studies during treatment (*6*) showed that effective antidepressant treatment increases PAA excretion. Outpatients with less severe forms of MDD who still meet DSM-III criteria as shown by scores in SADS interviews excreted PAA in a range intermediate between that of more severely depressed inpatients and that of controls.

These results suggest that measurement of PAA urinary excretion may be a valuable diagnostic test in affective disorders, particularly because PAA is excreted in very high amounts (milligram range) in comparison to other amine metabolites. Since PAA is the major metabolite of PEA, the observed differences between control and depressed subjects supports the PEA theory of affective behavior. However, there may be other sources for the PAA found in urine, such as dietary and bacterial transformation of phenylalanine by the gut flora. Several lines of evidence support the view that urinary PAA reflects mainly PEA metabolism in the body rather than in the gastrointestinal tract. Stein *et al.* (*7*) reported no difference between the amounts of PAA excreted by normal individuals while fasting and those on a normal diet; they concluded that diet has no effect on PAA excretion. Seakins (*8*) reported that an oral phenylalanine load did not increase PAA excretion. It has also been observed that after the ingestion of neomycin, which kills gut flora, the PAA urine excretion is not altered (*9*). Moreover, PAA excretion has been reported low in some chronic cases of schizophrenia (*10*), and high PAA concentrations in plasma were observed in aggressive psychopaths (*11*).

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4. Urine was collected over a 24-hour period, the volume was measured, and 250-ml portions were kept at 4°C until the day of analysis. For each urine sample, 2 ml was acidified with 2 ml of 6*N* HCl. Phenylpropionic acid (300 μg) was added to each sample as an internal standard. All samples were heated at 100°C for 4 hours to hydrolyze conjugated PAA (PAA-glutamine). The samples were then cooled in an ice bath and extracted with 1 ml of ethyl acetate. Then 25 μl of the organic layer were derivatized with 25 μl of *bis*-trimethylsilyl trifluoro-acetamide. Between 1 and 5 μl of this mixture was injected into a Packard gas chromatograph with flame-ionization detector. The column (180 cm long) was packed with 3 percent OV-17. The oven temperature was constant at 175°C. Retention times under these conditions were 60 seconds for the PAA derivative and 90 seconds for the internal standard derivative.
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Serological Visualization of Interleukin 2

Abstract. *Interleukin 2, a lymphokine that acts as a second signal of cellular immune response by way of its action as a T-cell growth factor, was morphologically identified by immunoperoxidase staining. With the use of a monoclonal antibody to interleukin 2 and several complex-forming antisera, the lymphokine was readily distinguished in cytocentrifuge preparations of peripheral blood leukocytes stimulated with a T-cell mitogen. When preparations of cloned interleukin 2 producer and responder cells were stained by the same procedures, discrete patterns of both responder and producer cell phenotypes were revealed. Interleukin 2 producer T cells exhibited a characteristic intense, ringlike cytoplasmic staining, whereas the responder cells (as exemplified by interleukin 2–dependent cell lines) exhibited a less intensive, spotlike membrane staining. In addition, intense membrane localization of interleukin 2, reminiscent of potential capping phenomena, could be observed in stained preparations of cloned responder cells.*

The lymphokine interleukin 2 (IL-2), also referred to as T-cell growth factor, is an obligatory soluble factor that promotes mitogen- and antigen-induced T-cell proliferation (*1*). Interleukin 2 acts to promote the differentiation of cytolytic effector cells both in vitro and in vivo (*2*). Investigators at our laboratory recently described the generation of B-cell hybridomas whose antibody products neutralized IL-2 in several discrete experiments, including inhibition of T-cell mitogenesis and generation of alloreactive cytolytic T cells (*3*). On the basis of recent studies documenting the existence of IL-2 surface receptors on lymphokine responsive cell lines (*4, 5*), we questioned whether a monoclonal immunoglobulin G (IgG) antibody to IL-2 could also be used for the morphological demonstration of IL-2. By means of such a monoclonal antibody and an immunoperoxidase staining procedure we were able to visualize IL-2 deposits both in the cytoplasm and on the surface of producer and responder subpopulations of human T cells. In this report we summarize our findings and describe the differential staining patterns associated with IL-2 producer and responder cell phenotypes.

The monoclonal IgG antibody to IL-2, of the γ_2b subclass, was produced and purified as described (*6*). One hybridoma clone (4E12B2F7) produced particularly high titers of the antibody in the supernatant and ascites; these were therefore used as the starting materials for the

purification of the antibody by Protein A Sepharose affinity chromatography. The antibody, designated anti-IL2, produced by the 4E12B2F7 cell line inhibited both the proliferation of IL-2–dependent T cells and the generation of alloreactive cytotoxic T-lymphocyte concentrations as low as 10 μg/ml.

Cytocentrifuge preparations of several different cell types were used in the immunoperoxidase staining experiments. Murine IL-2–dependent CTLL-2 cells which have been cultured in the presence of IL-2 for some 5 years, were used as a representative culture of cloned IL-2 responder cells. The CTLL-2 cells were first washed extensively with several volumes of warm, conditioned medium containing human IL-2 to promote receptor saturation with active lymphokine. H33HJ-JA1 cells, a leukemia cell line cloned from Jurkat-FHCRC cells, were used as an example of human IL-2 producer T cells. These cells produced large quantities of human IL-2 after stimulation with 1 percent phytohemagglutinin (PHA) and phorbol myristate acetate (PMA, 10 ng/ml). The cells were harvested for cytocentrifuge slide preparation 8 hours after the induction of IL-2 biosynthesis and secretion by PHA and PMA. Because normal peripheral blood leukocytes stimulated by PHA are known to include both IL-2 producer and responder cell populations, cytocentrifuge samples of appropriately stimulated peripheral blood leukocytes were prepared at various time points after mitogen stimulation. Lipopolysaccharide-activated human leukocytes, as well as cultures of ARH-77 human myeloma cells, served as negative control populations.

After the cells were fixed, a circle was etched in the slide immediately surrounding the cytocentrifuge smear to ensure that the surface tension of staining solutions would promote maximum contact of the antibody reagents with the cell populations. In the staining procedure, the slides were first exposed (for 30 minutes at room temperature) to a solution of anti-IL2 (0.1 to 10 μg/ml). They were then washed extensively and reacted with peroxidase-coupled, rabbit antiserum to mouse IgG (Dako Scientific), washed again, and exposed to sheep antiserum to rabbit IgG (Dako Scientific). The next reagent in the staining procedure was a complex of horseradish peroxidase and antibody to peroxidase (Dako Scientific). To guard against nonspecific binding, all the antibody solutions contained 25 percent human AB serum. Further controls included identical slides in which monoclonal antibody anti-IL2 was either omitted or substituted by an equivalent concentration (total protein) of diluted control ascites fluid (Bethesda Research Laboratories) or an irrelevant monoclonal antibody (against murine leukemia virus gp70) of the same isotype (subclass γ_2b). After a final washing the slides were developed with a solution of phosphate-buffered saline (*p*H 6.8) containing diaminobenzidine tetrahydrochloride (Sigma) and hydrogen peroxide. The slides were then counterstained with methyl green to facilitate the visualization of the nuclei and cytoplasm.

This procedure enabled us to demonstrate various types of cellular deposits of IL-2 in both producer and responder cells. Approximately one-third of the Jurkat-FHCRC cells stimulated with PHA and PMA showed strong cytoplasmic staining (Fig. 1). The nucleus of the positive cells appeared surrounded by a broad rim of intensely stained cytoplasm. Previous studies have shown that IL-2 activity usually occurs in the supernatants of Jurkat-FHCRC cells 6 to 8 hours after induction (*7*). Immunoperoxidase staining of cells exposed to mitogen for longer periods revealed a progressive increase in the number of positively stained cells. The most intense staining appeared in cells exhibiting somewhat frayed or disrupted membranes. Both Jurkat-FHCRC and LBRM33 murine IL-2 producer cells have been found to die

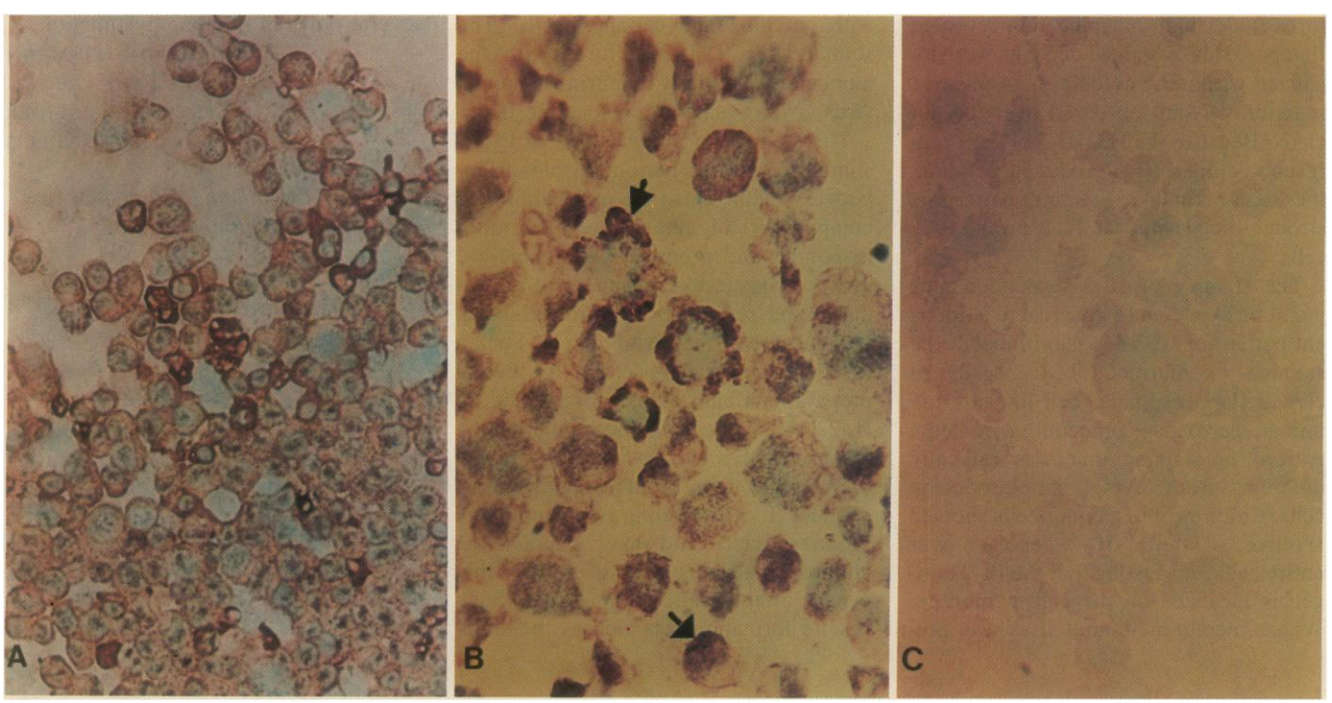

Fig. 1. (A) Cloned Jurkat-FHCRC cells stained by anti-IL2 and immunoperoxidase 8 hours after stimulation with PHA and PMA. Approximately one-third of the cells show strong cytoplasmic staining. The broad rim of IL-2 deposits surround the centrally located nuclei. The stained IL-2 producer cells range in size from large blastic forms to medium-sized and small cells showing intense staining. These intensely stained cells may be those that eventually die, releasing large concentrations of IL-2 into the milieu (×400). (B) Cultures of CTLL-2 cells stained with anti-IL2 and immunoperoxidase. The cells show surface staining and capping phenomena (arrows). Note the spotlike, positive reaction scattered over the nucleus and cytoplasm of almost all the cells (×400). (C) Cultures of CTLL-2 cells stained with a monoclonal antibody to murine leukemia virus gp70. No detectable staining pattern is visible (×400).

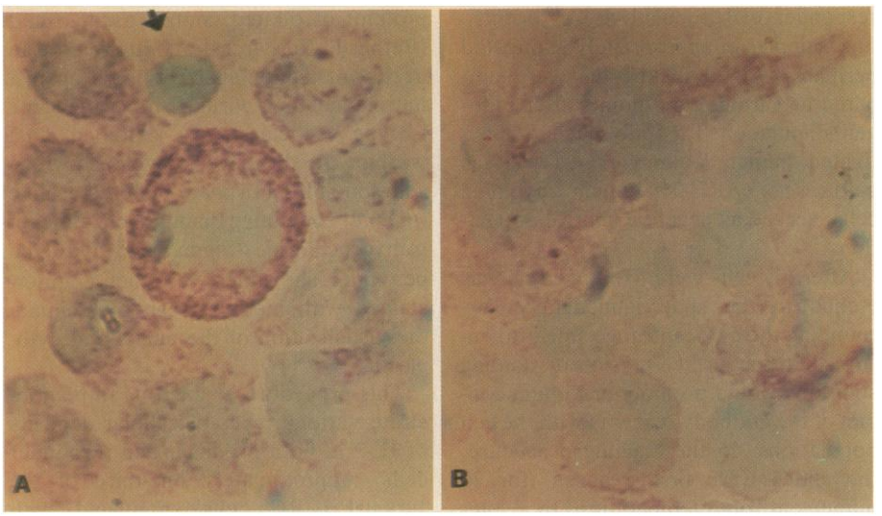

Fig. 2. (A) Human peripheral blood leukocytes stained with anti-IL2 and immunoperoxidase. The cells had been stimulated with PHA and cultured for 5 days. An IL-2 producer cell is shown surrounded by IL-2 responders and an IL-2–negative small lymphocyte (arrow) in a typical rosette formation. Note the central location of the nucleus of the IL-2 producer T cell and the complete absence of any positive reaction within the nuclear field. This strongly suggests a cytoplasmic localization of IL-2 in these cells. In contrast, responder cells show a positive, spotlike reaction in a scattered fashion, indicative of membrane localization (×1000). (B) Human myeloma ARH-77 cells stained with anti-IL2 and immunoperoxidase. No IL-2 staining is visible, indicating the absence of IL-2 in the cytoplasm and nucleus (×1000).

after induction of IL-2 biosynthesis and secretion (*7, 8*). Similar high-density ringlike IL-2 staining of lymphocytes was observed in cultures of PHA-stimulated peripheral blood leukocytes (Fig. 2).

Examination of the H33HJ-JA1 cultures under high magnification revealed that peroxidase-stained deposits were localized within the cytoplasm. A positive reaction was not observed over the nucleus. Because the H33HJ-JA1 cells represent a cloned IL-2–producing cell line, the dense ringlike staining pattern is strongly suggestive of IL-2 producer T cells.

The staining of IL-2 responder cells might be expected to give rise to a different pattern of IL-2 deposits. Indeed, the staining of murine CTLL-2 cells (a cloned IL-2 responder cell line) by the immunoperoxidase procedure revealed a pattern of staining that was radically different from lymphokine-producing cells. Cultures of responder cells showed spotlike, scattered IL-2 deposits over entire cells, suggesting that the IL-2 antigen is localized on the cell membrane. Where membrane-bound IL-2 was present in high concentrations as in many CTLL-2 cells, a potential capping and patching pattern could be visualized. Thus there was a distinct difference between the staining patterns of producer (dense, ringlike cytoplasmic staining) and responder (diffuse, total cell deposits with patching and capping) cells. The staining of CTLL-2 cultures with the monoclonal antibody to murine leukemia virus gp70 revealed no detectable IL-2.

The staining of preparations of PHA-stimulated human peripheral blood leukocytes with anti-IL2 and additional complex-forming antisera revealed both responder and producer cells. Figure 2 shows a rosette-like arrangement of producer and responder cells including an IL-2–negative small lymphocyte. Such a rosette array was observed in several preparations and almost always had an IL-2 producer cell in the center of the rosette. Such a cluster suggests a model of interaction of proliferating IL-2 responder T cells feeding off lymphokine producer cells and may explain the clumping and clustering of T cells that occurs after mitogen stimulation. Such rosette formations, as well as the staining indicative of IL-2 producer cells, were not found in 7- to 12-day-old cultures of mitogen-stimulated peripheral blood leukocytes. This again suggests that IL-2 producer cells do not survive for long periods after being induced to produce the lymphokine, a hypothesis that is supported by the finding that cloned IL-2 producer tumor cells die soon after ligand stimulation (*7, 8*). In further support of this hypothesis, we observed that the percentage of producer cells stained increased after mitogen stimulation, peaked some 96 hours after lectin sensitization, and then decreased until the producer phenotype was no longer evident 12 days after lectin stimulation. Also, as shown in Fig. 2, identical staining of the B-cell myeloma (ARH-77) resulted in no visualization of IL-2 determinants on the surface or within the cytoplasm of the cells. Similar lack of peroxidase-mediated color development was apparent in all slides (even of cloned producer and responder cells) when the monoclonal antibody anti-IL2 was either eliminated or substituted with an identical concentration (0.1 to 10 μg) of a control ascites preparation.

These results suggest that intracellular staining of IL-2 may eventually become useful in the diagnosis of certain diseases in humans. It should now be possible to quantify IL-2 producer and responder cells in various cell populations and tissues taken from patients suffering from several types of immune dysfunction.

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8 March 1983