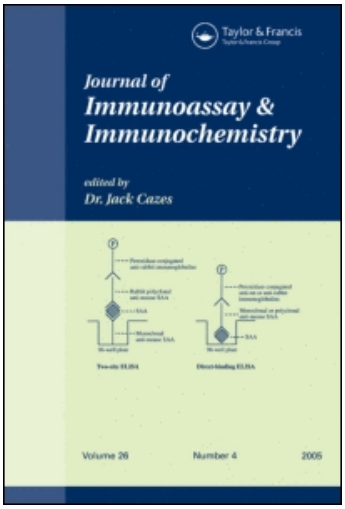

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A Simple Radioimmunoassay of Human Interleukin-2

T. P. Lee[a]; B. K. Mookerjee[a]; J. L. Pauly[b]
[a] Department of Medicine, Veterans Administration Medical Center and State University of New York at Buffalo, Buffalo, NY [b] Department of Molecular Immunology, Roswell Park Memorial Institute, Buffalo, NY

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A SIMPLE RADIOIMMUNOASSAY OF HUMAN INTERLEUKIN-2

T. P. Lee*, B. K. Mookerjee* and J. L. Pauly+

* Department of Medicine, Veterans Administration Medical Center and State University of New York at Buffalo, Buffalo, NY

+Department of Molecular Immunology, Roswell Park Memorial Institute, Buffalo, NY

ABSTRACT

We have developed a liquid phase radioimmunoassay of human interleukin-2 which uses inexpensive commercially available reagents exclusively. The assay is simple, reproducible and specific in detecting different batches of human interleukin-2 of natural as well as recombinant origin, but not detecting recombinant murine interleukin-2. The assay is sensitive to a concentration of approximately 0.05 ng/ml and can be used in measurement of IL-2 in serum containing culture media. (KEY WORDS: Radioimmunoassay, human IL-2).

INTRODUCTION

Interleukin-2 (IL-2) is a glycoprotein that serves as a growth factor for activated T cells (1,2). There is intense interest in this hormone and in measurement of its concentration in culture media and in biological fluids. Abnormalities in the

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Dr. Basab K. Mookerjee
VA Medical Center
3495 Bailey Avenue
Buffalo, NY 14215

ability of T cells to produce and secrete IL-2 have been detected in several pathologic states including acquired immunodeficiency state (AIDS), malignancies and auto-immune disorders (3,4,5). Incubation of human lymphocytes with IL-2 in vitro results in the generation of lymphokine activated killer cells (LAK cells). Recently, it has been shown that administration of LAK cells along with IL-2 to human patients having certain types of advanced metastatic malignancies leads to impressive tumor regression (6).

The most commonly used bioassay depends on the ability of test material (culture medium) to support the growth of IL-2 dependent cell lines (7). This assay is subject to various types of interference from substances present in cultures as experimental variables thereby creating difficulties in assay standardization. Measurement of IL-2 in human serum or other biological fluids is frequently confounded by the presence of IL-2 inhibitory substances often present in such fluids (5). Therefore, there is need for a reliable, simple and highly sensitive assay based on the immuno-chemical specificity of the structure of IL-2. Several assays have been described, which depend on the ability of IL-2 in unknown samples to compete for binding of radioisotope labelled IL-2 either to cells having IL-2 receptors or to monoclonal antibodies to IL-2 in ELISA type assays (5,8). Many of these assays are dependent on the availability of specialized reagents, e.g. monoclonal antibodies, special cell lines some of which are expensive, other not so readily available, or require complex maintenance technologies or expensive kits.

We describe here an radioimmunoassay developed by use of commercially available polyclonal antibody to IL-2 and iodinated IL-2. The assay can be set up in any laboratory, is specific, sensitive and highly reproducible, and can be performed so that results are obtainable in 18-24 hrs.

MATERIALS AND METHODS

Materials:

Recombinant interleukin-2 was obtained from Cetus Corporation, Emoryville, CA. Recombinant murine IL-2 was obtained from GENZME Corp., Boston, MA. Affinity purified natural human IL-2 obtained from human cell line JURKAT was kindly donated by Dr. Richard J. Robb, DuPont Corporation, Glenolden, PA.

Iodinated interleukin-2 ([^{125}I-IL-2], specific activity 38.7 uCi/ug) and tritiated thymidine (TdR-[^{3}H], 7.6 Ci/mmol) were obtained from DuPont-NEN, Boston, MA. Goat antibody to rabbit IgG and bovine serum albumin (BSA) were obtained from Sigma, St. Louis, MO. Standard commercial sources were used for general supplies, media, etc.

Polyclonal rabbit antibody (purified IgG) to human IL-2 was obtained from Collaborative Research Inc., Bedford, MA (Lot #851367), supplied at concentration of 1 mg IgG in 370 ul of phosphate buffered saline (PBS) per vial. According to the manufacturer, 43 ng of this IgG fully neutralized 1 half maximal unit of human IL-2 of either recombinant or natural origin by bioassay

using TdR-[^{3}H] incorporation of the CTLL cell line. The antibody did not interact at all with recombinant human interleukin-1 (IL-1) in the bioassay of IL-1 activity using mouse thymocytes. Furthermore, the antibody reacted equally well with recombinant or natural human IL-2 but not at all with recombinant human IL-1 in the ELISA assay (Mr. Andrew Lahey, Collaborative Research, Inc., personal communication).

Radioimmunoassay

Assays were performed in 5 ml (12 x 75 mm) glass tubes in a total assay volume of 1.0 ml. For the construction of standard curves, assay mixtures contained 0.1 ml of a solution of rabbit antibody (IgG) against human IL-2 (commercial stock solution diluted 1:1000 in PBS, pH 7.4 containing 0.02% sodium azide and 0.05% Tween 20), 0.1 ml of [^{125}I]-IL-2 (3 x 10^{-9} Ci/tube, 0.1 ng), 0.1 ml of unlabelled IL-2 at designated concentration diluted in PBS and 0.2 ml of PBS. To this was added 0.5 ml of medium 199 and 10% human blood group AB serum to make the volume up to 1 ml. For assay of supernatant fluids of lymphocyte cultures, 0.5 ml of each supernatant (medium 199 + 10% human blood group AB serum) was added to 0.5 ml of an assay mixture that contained 0.1 ml of rabbit antibody (IgG) to human IL-2, 0.1 ml of [^{125}I]-IL-2 (3 x 10^{-9} Ci/tube) and 0.3 ml of PBS. Assay mixtures were incubated overnight at 4°C and 0.1 ml of a solution of purified rabbit IgG (1 mg/ml) was added per tube as carrier. Rabbit IgG was then

precipitated with goat antibody to rabbit IgG (0.1 ml of commercial stock, titer 1:16 per tube), by 4 hr incubation at 4°C and the mixture centrifuged at 10,000 x g for 30 min to separate unbound IL-2 from precipitated bound IL-2. The pellet was counted by a gamma counter and the percentage of radioactivity bound determined. Under these conditions, in absence of added unlabelled IL-2, approximately 30-40% of the [^{125}I]-IL-2 added was found in the precipitate fraction. The non-specific binding, measured in the presence of 10,000-fold excess unlabelled IL-2 accounted for less than 4% of the radioactivity added.

All pipets, tips and tubes used for the RIA were first coated with BSA by incubation for 1 hr at 37°C with a solution containing 20 mg/ml BSA in a coating buffer (50 mM sodium bicarbonate, 0.02% sodium azide, pH 9.6) following which they were twice rinsed with double distilled water.

Lymphocyte Culture

Human peripheral blood lymphocytes were obtained from normal healthy volunteers by Ficoll Hypaque density gradients as described earlier (9,10). After 3 washes, they were set up in tube cultures (Falcon plastic tubes) in medium 199 and 10% of autologous serum. Each tube contained 2 x 10^6 lymphocytes in 4 ml of medium (11). Phytohemagglutinin (PHA, 5 ug/ml) and sodium azide (designated concentrations) were also added. Samples were cultured in a humidified tissue culture incubator at 37°C. At the

end of 24, 48, 72 and 96 hours, duplicate cultures were terminated by centrifugation and collection of supernatant fluids. The cells were harvested at the end of 48 and 96 hours, washed (x 3) and set up in cultures in microtiter plates (10^5 cells/well in 0.2 ml of medium 199 and 10% autologous serum). The microcultures were immediately pulsed with TdR-[^{3}H] (0.1 uCi/well) and cultured overnight at 37°C. The cultures were now terminated, harvested by an automatic device (Otto Hiller, Inc., Madison, WI) and the mean thymidine uptake was determined by scintillation counting as described (9,10).

RESULTS

Neutralization of Mitogenic Effect of IL-2 by Antibody used in the RIA

The results of an experiment designed to determine the specificity of the rabbit antibody to human IL-2 are depicted in Table 1. We and others have previously shown that human IL-2 is mitogenic to a subset of T cells in normal human peripheral blood lymphocytes (HPBL) (11-15) and that the mitogenic effect of IL-2 is completely blocked by a monoclonal antibody directed against the IL-2 receptor in human T cells (11). We show here that the polyclonal rabbit antibody (IgG) to human IL-2 used to develop the radioimmunoassay is specific in being able to completely abrogate the mitogenic effect of human recombinant IL-2. About 50% inhibition (K_I) of IL-2 induced (IL-2 conc. 50 u/ml) proliferative responses occurred at a concentration of around 25 ng/ml.

TABLE 1

Neutralization of Effect of IL-2 by Rabbit IgG antibody to Human IL-2[a]

IgG antibody concentration	Lymphocyte proliferative responses in TdR-[^{3}H] uptake (mean c.p.m. ± SEM)	
	Control	IL-2
None	330 ± 63	2779 ± 414
25 x 10^3 ng/ml	244 ± 2	266 ± 38
2.5 x 10^3 ng/ml	329 ± 75	262 ± 28
250 ng/ml	258 ± 29	172 ± 19
25 ng/ml	153 ± 12	1392 ± 212

a) Human peripheral blood lymphocytes set up in micro cultures containing 10^5 cells/well in 0.2 ml of medium 199 and 10% autologous serum, with or without IL-2 (50 u/ml) and designated concentration of IgG antibody. Cultures were pulsed on day 6 with TdR-[^{3}H] and the mean thymidine uptake of triplicate cultures determined on day 7.

Specificity of the Assay and Standard Curve of the RIA

Figure 1 depicts the standard curve obtained from a series of 3 experiments. In these 3 separate experiments, affinity purified natural human IL-2 obtained from DuPont Corporation was used in concentrations indicated in the standard curve. When recombinant human IL-2 obtained from Cetus Corporation was used, this preparation yielded a standard curve of virtually identical shape (data not presented). The manufacturer indicated this preparation had the specific activity of 3 x 10^6 units/mg protein by bioassay. The specificity of this RIA was further established by using murine recombinant IL-2 as an unknown sample. The RIA did not

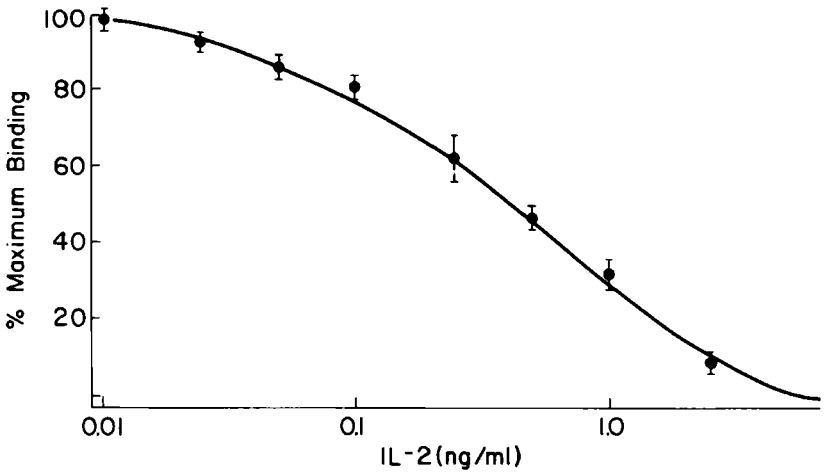

FIGURE 1. Standard curve of the radioimmunoassay. Small circles with standard error bars represent the standard curve established from affinity purified natural human IL-2. Each point represents mean of 3 experiments (± SEM). In each experiment, mean of duplicate tubes was used for each concentration of IL-2. Vertical axis depicts binding of iodinated IL-2 to rabbit antibody in the presence of test samples, expressed as maximum binding in absence of added unlabelled IL-2. Horizontal axis shows concentration of unlabelled IL-2 used in ng/ml.

detect recombinant murine IL-2 at concentrations up to 1000 ng/ml (data not shown). Human blood group AB serum up to a concentration of 50% did not affect the RIA, nor did phytohemagglutinin (5 ug/ml) (data not presented).

Validation of Assay by Measurement of IL-2 Production by PHA Stimulated Lymphocytes

Human peripheral blood lymphocytes from apparently healthy laboratory personnel were set up in culture in Falcon plastic tubes (2×10^6 lymphocytes in 4 ml of medium 199 and 10% autolo-

gous serum). Cultures were started with or without PHA (5 ug/ml), and PHA plus sodium azide in 2 concentrations (0.01, and 0.001%). At designated times following the initiation of these cultures (days 1, 2, 3 and 4), control and PHA-stimulated cultures were terminated, the supernatant fluids separated by centrifugation and assayed for presence of IL-2, as depicted in Figure 2. TdR-[^{3}H] uptake of these cells were also determined at days 2 and 4. It can be seen that detectable amounts of IL-2 were present in the PHA stimulated cultures at the end of 24 and 48 hours. The amount of IL-2 in supernatant fluids of stimulated cultures declined and were no longer detectable by 72 hours or later. IL-2 activity was not detectable at any time in the supernatant fluids of the corresponding unstimulated control cultures. The IL-2 levels in supernatant fluids of PHA stimulated human lymphocyte cultures performed in the presence of sodium azide yielded results very similar to those reported by Feldman et al. (16). Thus, 0.001% azide significantly augmented the concentration of IL-2 detectable at the end of 24 hours and this level was maintained in the supernatant fluids at the end of 48, 72 and 96 hours. TdR-[^{3}H] uptake of PHA stimulated lymphocytes was significantly inhibited by azide concentrations of 0.01%, thymidine incorporation at 48 and 96 hours was totally abrogated. However, the amount of IL-2 detectable in the supernatant was higher than that without azide and that with 0.001% azide, the concentration being highest in the supernatant at 96 hours.

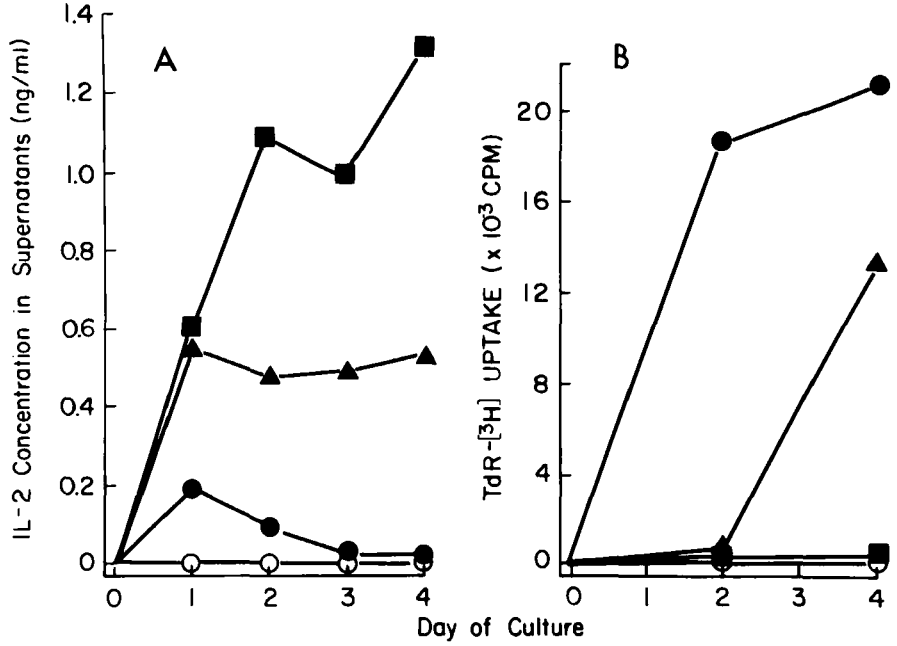

FIGURE 2. Kinetics of IL-2 production and cell proliferation. The graph on the left hand side (A) depicts IL-2 concentration measurable in supernatant fluids of human lymphocyte cultures, while the graph on the right hand side (B) depicts thymidine uptake by lymphocytes after designated periods of culture (plotted on horizontal axis for both graphs). (A) Each point represents mean IL-2 concentration of supernatants in ng/ml, plotted on vertical axis. (B) Each point represents mean thymidine uptake of triplicate cultures, vertical axis depicting thymidine uptake in c.p.m. (x 10^{-3}). For both graphs (A and B) open and closed circles represent unstimulated and PHA stimulated lymphocytes, respectively, both cultured in absence of sodium azide, while triangles and squares represent that of PHA stimulated lymphocytes cultured in the presence of 0.001 and 0.01% sodium azide, respectively.

DISCUSSION

Several methods for measurement of IL-2 in biological fluids or culture media have been described, e.g. biological assay dependent on survival and growth of IL-2 dependent cell lines (7), competitive binding of isotopically labelled IL-2 to its receptor(s) (5), sandwich radioimmunoassay using a monoclonal antibody that can detect IL-2 bound to a receptor on hematopoietic cell line (8), etc. IL-2 has been used in clinical trials in AIDS, and is gaining increasing acceptance in therapy of malignant disease (3,6). Therefore, the need for relatively simple methods that utilize readily available reagents for purposes of correlating levels of IL-2 in serum or in biological fluids with clinical parameters in different diseases which may be expected to increase. We describe here a radioimmunoassay which uses inexpensive commercially available reagents exclusively, and is highly sensitive, reproducible and simple to perform. It can also measure IL-2 in serum containing culture media, not being affected by serum or additives such as PHA.

The standard curve illustrated in Figure 1 suggests that the maximum sensitivity of the assay is the detection of IL-2 at concentrations of about 0.05 ng/ml. The bioassays described originally by Gillis et al. can accurately quantitate IL-2 down to a concentration of 0.2 ng/ml. The sandwich ELISA described by Gehman and Robb is much simpler than the bioassay, but is not as sensitive, being able to measure IL-2 concentrations over the

range of 10 ng/ml to 10^5 ng/ml (5). The commercially available ELISA kit marketed by GENZYME (Intertest) can measure IL-2 to 0.05 ng/ml. Thus, the RIA technique described in this manuscript is at least as sensitive as any available at this time. As with other immunochemical assays, the described assay is easy to standardize and is not affected by inhibitory or stimulatory effects of either contaminating or biologically produced substances simultaneously present in the sample. In addition, the assay can be performed more quickly compared to the 3-6 days needed to complete the bioassay and offers much greater range for the same number of sample dilutions. The described assay is specific in detecting human IL-2 of both natural and recombinant origin, but not detecting murine recombinant IL-2.

The results presented confirm and extend the report of Feldman et al., that sodium azide at concentrations of 0.01 and 0.001% significantly increases the amount of IL-2 detectable in supernatants of PHA stimulated lymphocytes (16). These workers attributed this either to increased production due to deregulation, inhibition of action of regulatory cells or factors, or to under utilization of IL-2 in the presence of azide.

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