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# THE DEVELOPMENT OF A MICRO ASSAY FOR NATURAL KILLER AND LYMPHOKINE-ACTIVATED KILLER ACTIVITY AND ITS USE IN MONITORING THE PURIFICATION OF AN INTERLEUKIN-2 INHIBITOR

By

Loraine H. Anderson

A THESIS

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May, 1989

Dedicated to

my father,

WENDELL S. ANDERSON

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# CHAPTER 1

## INTRODUCTION AND LITERATURE REVIEW

The treatment of cancer is a constantly changing field and has recently expanded to include immunotherapy. One of the recent advances in cancer immunotherapy involves the infusion of lymphokine-activated killer (LAK) cells. The LAK cells are derived from the patient's own lymphocytes which are incubated with interleukin-2 (IL-2) for a few days. The IL-2 augments the cytotoxicity of a subpopulation of these lymphocytes, increasing their ability to kill tumor cells. As an adjunct to the LAK cells, interleukin-2 (IL-2) is often administered intravenously to maintain the LAK cells' enhanced cytotoxicity. Unfortunately, the high doses of IL-2 required for this LAK maintenance are often toxic to the patient. It has been postulated that an IL-2 inhibitor is present in the circulation and plays a role in localizing the effects of IL-2 to the site of production. This inhibitor may neutralize IL-2 in circulation, therefore high doses of the lymphokine would be required to overcome this neutralization effect. If the activity of such an inhibitor could be controlled, lower doses of IL-2 would be as effective in maintaining the increased cytotoxicity of the LAK cells as the high doses currently used and thus decrease the toxicity to the patients.

The purpose of this research was to partially purify the IL-2 inhibitor from normal mouse serum. Since the IL-2 inhibitor has been postulated to have multiple mechanisms, two different assay systems were used to monitor the purification of the inhibitor; the inhibition of proliferation of IL-2 dependent cells, and the inhibition of IL-2 enhanced natural killer (NK) cell cytotoxicity. A micro assay to measure the inhibition of IL-2 enhanced NK cytotoxicity was developed to reduce media requirements and to reduce manipulation of the cells. To confirm the validity of such an assay, it was compared to the prior art technology, the bulk assay.

The partially purified IL-2 inhibitor was used to induce antibody formation. The polyclonal antiserum was tested for its neutralizing capability and will be used to further purify and characterize the inhibitor.

Overview of the Immune Response

The immune response is a complex chain of events involving the interaction of several cells and the soluble factors they produce. In review, the antigen presenting cell (APC) phagocytizes and processes the antigen, which is then presented on the surface of the APC, in conjunction with major histocompatibility complex (MHC). Two signals are required to activate the T helper cell. Binding of the antigen/MHC complex by the T cell receptor on the T helper (CD4+) cell is one signal, and interleukin 1, produced by the APC, acts as

the second signal. The activated T helper cell then begins production of high affinity IL-2 receptors and also secretes IL-2 (1). This IL-2 can function in a variety of ways:

1) Stimulation of activated T helper cells or T cytotoxic cells, causing proliferation (1-4)
2) Enhancement of natural killer (NK) and lymphokine-activated killer (LAK) cells, increasing their cytotoxic ability (5, 6)
3) Induction of B Cell Growth Factor-1 (interleukin-4) formation by T cells, which in turn stimulates both the proliferation and differentiation of B cells and the production of antigen-specific antibody (4, 7, 8)
4) Induction of interferon-gamma release by T cells (9)

Interleukin-2 and Lymphokine-Activated Killer Cells

IL-2 has recently been used in cancer therapy. This therapy consists of removing the patient's lymphocytes, incubating these cells with IL-2 for 2-3 days, and reinfusing these lymphokine-activated killer (LAK) cells. Large doses of IL-2 are administered intravenously with the cells and are continued for several days after the cell infusion to maintain *in vivo* lymphokine activation of the LAK cells (10-15).

This therapy developed from the observations by Henney, et al. (5) in 1981 who reported the augmentation of NK activity by IL-2.

They found that culturing murine splenocytes in an IL-2 containing media for 24 hours resulted in an increased NK cytolysis against NK sensitive target cells, but not against NK resistant targets. The cytolytic activity peaked after 24-30 hours and had declined by 96 hours. Later the same year, Lotze and colleagues (16) reported that human peripheral blood lymphocytes cultured in media containing IL-2 could lyse fresh autologous tumor target cells, which freshly isolated NK cells were not able to lyse. Grimm and coworkers (17) further characterized this IL-2 induced antitumor cytotoxicity phenomenon and introduced the term "Lymphokine-Activated Killer Cells" (LAK cells). They demonstrated that IL-2 was responsible for the ability of these cells to lyse fresh tumor targets.

The major features of LAK cells reported by Grimm were:

1) The LAK cells were able to lyse NK-resistant, fresh, solid tumor cells.
2) The LAK cells were generated in 2-3 day cultures in media containing IL-2.
3) Interferon was not the primary stimulus for LAK formation.
4) Neither the LAK precursor nor effector cell was adherent to plastic or nylon wool.
5) The LAK precursor cell was present in an NK void population.
6) LAK development was radiation sensitive.
7) The LAK cells expressed a T cell phenotype (OKT-3).

The data suggested that LAK cells are not classic NK cells, monocytes, or B cells; however, they did not rule out the possibility that the activities described for LAK cells could be accessory functions of any of these cells (17).

These in vitro observations led investigators to attempt in vivo enhancement of NK activity by IL-2. In mice, IL-2 induced proliferation and enhanced the function of cultured T cells in vivo (18). However, when IL-2 was administered intravenously in humans, no enhancement of T cell function was demonstrated (19).

The lack of success with in vivo activation led investigators to infuse in vitro activated (LAK) cells, which retained their enhanced cytolytic activity. LAK cells have been used in combination with IL-2 as therapy for cancer patients since 1983 (10, 12-15, 20, 21). Approximately one-fifth (34 of 172) of the patients treated with combined IL-2 and LAK therapy had partial or complete reduction of their tumor burden (14, 15, 21). This therapy was most successful in treatment of non-Hodgkin lymphoma (21), renal cell carcinoma (15, 21), melanoma (21), and colo-rectal carcinoma (21). These results are especially encouraging because these patients had failed to respond to other, more conventional, therapies. Unfortunately, the administration of IL-2 was toxic when given in the high doses required for maintenance of LAK activity. Side effects of IL-2 administration included fever, chills, headaches, nausea, vomiting, malaise, weight gain, diarrhea, rash, arthralgia, myalgia, fluid retention, anemia,

hypotension, dyspnea, and hyperbilirubinemia (21-23). There was no apparent toxicity in mice (24). The high doses of IL-2 used in IL-2/LAK therapy may be required to overcome IL-2 inhibitor(s) in the plasma.

## Interleukin-2 Inhibitor

IL-2 inhibitors have been postulated to be mechanisms for regulating IL-2 activity and have been described by several investigators over the past several years (25-28). Hardt et al. (29) and Klassen et al. (25) were among the first to report an IL-2 specific inhibitor present in normal mouse serum (NMS). NMS was shown to inhibit IL-2 dependent processes, including the generation of cytotoxic T cells (29), the proliferation of IL-2 dependent T cells (25, 27), and the IL-2 enhancement of NK activity (25). NMS had no inhibitory effect on the proliferation of IL-2 independent, malignant T lymphoid cells, demonstrating that the effect of NMS on IL-2 dependent cells was not due to a cytotoxic factor (27).

The effects of the IL-2 inhibitor in serum were also reported to be dose dependent, i.e. the greater the concentration of the inhibitor, the greater the inhibition (25-27, 29-31). The effects of the inhibitor could be overcome *in vitro* by increasing the concentration of IL-2 (25, 26, 29, 30, 32).

What Is IL-2 Inhibitor ?

In an effort to elucidate the origin of the IL-2 inhibitor in the serum, the investigators examined the effects of serum from nude mice. Nude mice lack thymus glands, so the effects of nude mouse serum would indicate any contribution to IL-2 inhibition by thymus dependent cells. Serum from nude mice failed to inhibit the generation of cytotoxic T lymphocytes (CTL) (29) or the proliferation of IL-2 dependent T cells (25, 26). However, the nude mouse serum did inhibit the IL-2 enhancement of NK activity (25). These findings suggested that the means of regulating the IL-2 enhancement of NK function is produced by cells other than thymus dependent cells. These data also suggested the production of the inhibitor of IL-2 dependent proliferation and CTL generation is regulated by thymus dependent cells. This hypothesis was further supported by the findings of Hardt et al. (29) who demonstrated that the inhibitor of CTL generation disappeared within three days after total body irradiation (950 rad). Normal inhibitor levels were restored within three days by transplanting allogeneic Lyt-2,3+ T cells into the irradiated mice; however, the inhibitor activity fell to undetectable levels by days 7-12. Transplantation of syngeneic cells showed no restoration of inhibitor. Hardt et al. (29) postulated that the T cell implant underwent a graft versus host reaction, and the activated Lyt-2,3+ T cells produced the inhibitor. Since the Lyt-2,3+ T cell population included suppressor cells, they considered this to be indirect evidence that the suppressor cells were producing the IL-2

inhibitor. In further support of this theory, Hardt et al. (29) observed reduced inhibitor activity in serum following treatment with cyclophosphamide, which selectively paralyzed the suppressor T cells. They proposed the relative concentrations of IL-2 and IL-2 inhibitor in vivo would determine if IL-2 would bind to the IL-2 receptor and activate the cytotoxic T cell precursor. Honda et al. (32) provided further information concerning the role of T cells in inhibitor production when they reported the presence of a specific IL-2 inhibitor in the supernatant of spleen cell culture stimulated with concanavalin A (Con A). They demonstrated an inhibitor in supernatants taken from either L3T4+ or Ly-2+ T cells, but not in supernatants from B cells.

Maca (27) and Lelchuk and Playfair (26) theorized that a soluble IL-2 receptor may inhibit the action of IL-2. Maca proposed his theory when he observed an increase in the IL-2 inhibitory activity of serum after adsorbing the serum with activated T cells which were expressing IL-2 receptors (27). Lelchuk and Playfair were unable to adsorb the inhibitor with cells expressing IL-2 receptors, and therefore postulated that the inhibitor binds to IL-2 and is a "serum analogue of the IL-2 receptor" (26). The soluble IL-2 receptor has been shown to be released from activated lymphoid cells after exposure to various lectins or specific antigens (33-36) or from alloreactive lymphocytes (37). It is possible that Maca's adsorption process caused the receptors to be released into the serum, increasing the inhibitory activity of the serum. His theory was further supported by

the observations that soluble IL-2 receptors have been found in low levels in the serum of healthy individuals, and in much higher levels in the serum of patients with malignant diseases (36). The inhibitor levels in serum have been reported to increase following antigen exposure (26, 29, 32), which would correlate with increased soluble receptor expression in diseased individuals. The best evidence that the soluble IL-2 receptor acts as an IL-2 inhibitor was reported by Symons et al. (38). Rheumatoid arthritis (RA) patients were found to have more than twice the serum IL-2 inhibitory activity as healthy, age-matched controls. These investigators then determined the soluble IL-2 receptor levels and the IL-2 inhibitory activity in RA synovial fluid, and found a positive correlation. To determine if the IL-2 inhibitor and the IL-2 receptor were actually the same proteins, gel filtration was used to size fractionate the synovial fluid proteins. The soluble IL-2 receptor eluted as a single peak at approximately 100 kilodaltons (kDa), as did the IL-2 inhibitor. These results were surprising because the soluble IL-2 receptor is known to have a molecular weight of approximately 45 kDa, not 100 kDa. The investigators postulated that the receptor either polymerized or bound to other synovial fluid components, forming a larger particle. To determine the validity of this hypothesis, they combined the supernatants of Con A activated peripheral mononuclear cells, which contained the receptors, with synovial fluid and repeated the fractionation. The receptor eluted in fractions containing proteins with molecular weights from 50-200 kDa, confirming the earlier data. When the Con A supernatant alone was fractionated, the receptor eluted

in fractions containing proteins with molecular weights of less than 50 kDa fraction. These findings indicate that the soluble IL-2 receptor in synovial fluid was complexed, either to itself or to other components of the synovial fluid.

Recently, Nagler et al. (39) demonstrated yet another IL-2 inhibitor, IL-4. IL-4 inhibited the IL-2 activation of NK cells. This report was the first to show down-regulation of IL-2 activation of NK cells by a lymphokine and suggested that IL-4 contributes to the inhibitory activity of normal serum, or that the IL-2 inhibitor may actually be IL-4. Future investigators should consider IL-4 when identifying serum IL-2 inhibitors.

The Effect of Antigen Stimulation on IL-2 Inhibitor Levels

Hardt et al. (29) hypothesized that nonimmunized mice have IL-2 inhibitor present in their serum due to constant, low level antigen stimulation of the cells producing the IL-2 inhibitor. They reached this conclusion after they showed there was no inhibitory activity in the serum of newborn mice; however, the serum inhibitory activity was present at adult levels by the age of only seven days. Lelchuk and Playfair (26) provided supporting evidence for the role of low level antigenic stimulation in IL-2 inhibitor production when they demonstrated that mice in a specific pathogen free (SPF) environment had no IL-2 inhibitor activity. However, three weeks after removal from the SPF conditions, these mice had inhibitor levels equivalent to

those caged in a normal environment. In addition, malaria-infected mice had increased IL-2 inhibitor levels compared with non-infected mice (26), indicating that mice which received higher levels of antigenic exposure responded with higher inhibitor levels.

Gautum et al. (30) found that the IL-2 inhibitor, as measured by proliferation of an IL-2 dependent cell line (CT-6), was neutralized following injection of minor histocompatibility locus (Mls)-disparate splenocytes. Since these investigators also measured increased IL-2 levels in the lymph nodes of these animals, they hypothesized that the additional IL-2 neutralized the IL-2 inhibitor. Several investigators (25, 26, 29, 30, 32) have shown IL-2 can overcome the inhibitor *in vitro*, which would support the hypothesis that increased IL-2 in plasma could override IL-2 inhibition *in vivo*.

## IL-2 Inhibitor and Autoimmune Disease

Systemic lupus erythematosus (SLE) and rheumatoid arthritis are autoimmune diseases associated with disordered immunoregulation. Investigators have measured IL-2 inhibitor levels in humans with these autoimmune diseases to determine if there was any correlation between the inhibitor levels and disease activity. Levels of IL-2 inhibitor were almost twice as high in RA patients as compared to healthy controls. In patients with SLE, the opposite was found, with levels of IL-2 inhibitor at approximately one-fourth of those found in healthy controls (40). The reported data suggested decreased IL-2

inhibitor levels in patients with SLE resulted in a systemic IL-2 overactivity, which could account for the widespread nature of this disease. In contrast, the increased levels of IL-2 inhibitor in the serum of patients with RA may account for the localized nature of this systemic disease (40).

## Biochemical Characterization

Few investigators have attempted the difficult process of biochemically characterizing the inhibitor. The molecular weights reported for the mouse serum inhibitor varied from 10 to 80 kDa, as shown in Table 1. This variation may indicate multiple IL-2 inhibitors or multiple forms of a single inhibitor. Using gel filtration, Honda et al. (32) isolated two different molecular weight fractions that demonstrated IL-2 inhibitory activity; however, they also isolated a single, low molecular weight fraction using reverse phase high pressure liquid chromatography (RP-HPLC) followed by gel filtration (Table 1, column 3). A possible explanation for these results was that the low pH used in the RP-HPLC may have disassociated a complex containing the lower molecular weight IL-2 inhibitor. The IL-2 inhibitor from human serum was reported to have a molecular weight between 70 and 220 kDa (40). The IL-2 inhibitor in human synovial fluid has a similar molecular weight, 100 kDa (41) or 150 to 180 kDa (38). The difference in molecular weights may be due to polymerization of the inhibitor or its binding to other components in the synovial fluid (38).

The data reported for other tests used to biochemically characterize the IL-2 inhibitor are also controversial. These differences may again be due to multiple IL-2 inhibitors or to the complexing of the IL-2 inhibitor to itself or to other plasma components. The isoelectric point of the IL-2 inhibitor in mouse serum was reported to be 6.2 (42). Another team of investigators determined there was inhibitory activity in human synovial fluid at three different isoelectric points, 6.8, 5.4, and 4.8 (43). The mouse serum inhibitor eluted from a cation exchange column just prior to albumin (5.2 mmhos) (42). Reports of heat stability of the IL-2 inhibitor are also varied, ranging from stable at 56$^{o}$C for 30 minutes (32) to partially labile (35% decrease in activity) under the same conditions (40, 42). The stability of the inhibitor in acid is more consistent. Male et al. (42) reported stability at pH 3.0, while Honda et al. (32) reported stability down to a pH of 2.1.

Table 1. Biochemical Characteristics of the IL-2 Inhibitor in Mouse Serum.

| | Reference[a] | | |
|---|---|---|---|
| | Hardt et al. | Male et al. | Honda et al. |
| Molecular Weight (kDA) | 50 | 25 | 10-12 |
| | | 50 | 60-80[b] |
| SAS Precipitation[c] | 50% sup | 50% ppt | 40-70% ppt |
| Stable at 56°C | NT[d] | no | yes |
| Labile pH | NT | < 3 | < 2.1 |

[a] Complete reference citations for Hardt et al. (29), Male et al. (42) and Honda et al. (32) are listed in the Reference List.

[b] Two peaks of IL-2 inhibitory activity were seen when gel filtration was used; however, when reverse phase HPLC was used, IL-2 inhibitory activity was contained only in the smaller molecular weight peak.

[c] saturated ammonium sulfate precipitation, sup refers to supernatant and ppt refers to precipitate

[d] not tested

# CHAPTER 2

# MATERIALS AND METHODS

## Animals

All animals were maintained on water and laboratory chow ad libitum.

### Mice

The mice used were female, age 2 to 4 months, and were obtained from the National Cancer Institute (Bethesda, MD) via an interagency agreement (GA V0101 (134A)P-77014) with the Veteran's Administration. The source of splenocytes for comparison of the two NK assay methods was C57Bl/6 (C57) strain mice. This strain was readily available at this time and known to have NK activity. Serum and splenocytes to test interstrain variations were obtained from mice of strains Balb/c, C3H/HeN (C3H), and C57Bl/6 x DBA/2J F1 (BDF1). The source of splenocytes for the inhibitor studies was strain BDF1 mice and C3H mice were the source of serum. This combination gave the greatest inhibitory activity of those tested.

Rats

The Sprague-Dawley rats used for antibody production were obtained from SASCO (Omaha, NE). At the time of initial immunization, the female rats weighed approximately 250 grams.

Reagents

Chemicals obtained from Mallinckrodt, Inc. (Paris, KY) were glacial acetic acid, ammonium sulfate, calcium chloride ($CaCl_2$-$2H_2O$), calcium lactate, 95% ethanol, ether, ethylenediaminetetraacetic acid (EDTA), glycine, concentrated hydrochloric acid (HCl), magnesium chloride ($MgCl_2$-$6H_2O$), potassium chloride (KCl), potassium phosphate monobasic ($KH_2PO_4$), sodium bicarbonate ($NaHCO_3$), sodium barbital, sodium chloride (NaCl), sodium hydroxide (NaOH), sodium phosphate dibasic ($Na_2HPO_4$), and sodium phosphate monobasic ($NaH_2PO_4$-$H_2O$). Sodium azide was obtained from Fisher Scientific Co. (Fair Lawn, NJ).

Reagents obtained from Hazelton Biologicals, Inc. (Lenexa, KS) were essential amino acids (100X), Eagle's Minimal Essential Media (MEM) with Hank's Salts, Hank's balanced salt solution (HBSS) (10X), Hank's balanced salt solution powder, 1 M HEPES buffer, MEM vitamins (100X), nonessential amino acids (100X), RPMI 1640, 7.5% sodium bicarbonate ($NaHCO_3$), sodium glutamine (100X), sodium pyruvate (100X), and fetal bovine serum.

Phosphate buffered saline (PBS) was prepared by dissolving 0.276 gram (g) $NaH_2PO_4$, 1.133 g $Na_2HPO_4$, and 8.783 g NaCl in distilled, deionized water (d/d water) to give one liter, followed by adjusting the pH to 7.0-7.2 using concentrated HCl.

Tris-HCl buffer was prepared by dissolving the disodium salt of tris(hydroxymethyl)aminomethane (Sigma Chemical Company, St. Louis, MO) in d/d water to give the required molarity (i.e. 6 grams per liter for 50 mM Tris-HCl). The pH of the solution was then adjusted to pH 8.0 using concentrated HCl.

Glycine-HCl was prepared by adjusting 0.1 M glycine to pH 2.3 using concentrated HCl.

Saturated ammonium sulfate (SAS) was prepared by heating ammonium sulfate in d/d water to 80°C. Ammonium sulfate was added just until crystals remained in the suspension. The hot suspension was filtered through a Whatman #1 filter with negative pressure and allowed to cool to room temperature. The pH of the cooled suspension was adjusted to 6.5. The solution was stored on the crystals that formed as the solution cooled.

Three IL-2 preparations of differing purity were used in this study. Rat T Cell Polyclone (RTP) (Collaborative Biologicals Inc., Cambridge, MA) was used as an IL-2 source for long term growth of

IL-2 dependent cells. T Cell Growth Factor (TCGF) (Cellular Products, Inc, Buffalo, NY) was the IL-2 source for proliferation assays. Recombinant IL-2 (rIL-2) was provided by the Cetus Corporation (Emeryville, CA) and was used in all IL-2 boosted NK studies. When reconstituted, the stock solution contained 20,000 units (U) rIL-2 per milliliter (ml).

Fetal bovine serum (FBS) from Hazelton Biologicals Inc. was used in all proliferation assays, as it provided optimal proliferation. Hyclone FBS (HyClone Laboratories, Logan, UT) was used for studies comparing the assay systems. FBS (Biofluids, Inc, Rockville, MD and Gibco Laboratories, Grand Island, NY) was used in early IL-2 boosted NK assays. Newborn bovine serum (NBS) (HyClone Laboratories) was used in later IL-2 boosted NK assays, to reduce cost. Testing of fetal and newborn bovine serum was done and minimal differences were seen. All serum was heat-inactivated by incubation at 56°C for 40 minutes prior to use.

Media

Hanks balanced salt solution (HBSS) was prepared as directed from powder and used to wash cells.

A modified Eagle-Hanks media called EHAA (44) was prepared from stock solutions for use in the proliferation assays. The nucleic acid

precursors were made by combining 0.5 gram of each nucleic acid; adenine, guanosine, cytosine, and uridine (Gibco Laboratories) in 500 ml d/d water. This solution was filter sterilized through a 0.2 micron (um) filter (Costar Corp., Cambridge, MA) using negative pressure and stored at -20°C. The EHAA Stock Additives were prepared by combining 80 ml essential amino acids (100X), 80 ml nonessential amino acids (100X), 50 ml sodium pyruvate (100X), 20 ml MEM Vitamins (100X), 40 ml HBSS (10X), 40 ml sodium glutamine (100X), 50 ml nucleic acid precursors (prepared above), 3 ml 1 M HEPES, and 2 ml gentamicin (50 mg/ml) (Whittaker M.A. Bioproducts, Walkersville, MD). The solution was filter sterilized through a 0.2 um filter (Costar Corp.) using negative pressure and stored in 18.25 ml aliquots at -20°C. The 1X EHAA media was prepared for use by combining one tube of thawed EHAA Stock Additives, 1 ml of $5 \times 10^{-3}$ M 2-mercaptoethanol (Eastman Chemical Company, Rochester, NY), 81.75 ml Eagle's Minimal Essential Media with Hank's Salts, and 1.33 ml 7.5% $NaHCO_3$. The pH was adjusted to approximately 7.2 (salmon pink color) using 1 M NaOH. FBS (Hazelton Biologicals, Inc.) was added at a final concentration of 5%. The media was then filter sterilized for use.

The tissue culture media, TC-10, was used for culturing cells, and for the IL-2 boosted NK assays. TC-10 was prepared by supplementing RPMI 1640 with 10% bovine serum, 0.1 mg/ml gentamicin, and 15 mM HEPES buffer. For long term culture, 2% L-glutamine was added.

Preparation of Dialysis Bags

Dialysis bags (3500 m.w.) (Spectrum Medical Industries, Inc., Los Angeles, CA) were prepared by boiling the bags in 2% sodium bicarbonate (w/v), 1 mM EDTA for ten minutes. The bags were rinsed with d/d water and boiled another ten minutes in 1 mM EDTA. The dialysis bags were allowed to cool to room temperature and were stored at 4°C in 1 mM EDTA. Each bag was rinsed with d/d water prior to use (45).

Serum Collection

Blood was obtained from ether-anesthetized mice by retro-orbital puncture. It was allowed to clot at room temperature, and then stored at 4°C for several hours. The blood was centrifuged at 2200 x g for ten minutes. The serum was removed and stored at -20°C until needed. After the serum was thawed, it was filter sterilized with a 0.22 um filter (Millipore Corp., Bedford, MA). Unpublished data indicated that room temperature storage for two days caused an increase in the inhibitory activity. Therefore, to obtain activated serum, the serum was allowed to stand at room temperature for two days.

High Pressure Liquid Chromatography (HPLC)

HPLC (BioRad Laboratories, Richmond, CA) was performed using the Bio Gel TSK DEAE-5 PW ion exchange column (BioRad Laboratories). The

buffers used were 25 mM Tris-HCl, pH 8.0 and 25 mM Tris-HCl with 0.5 M NaCl, pH 8.0. The linear salt gradient began at fraction 5 and increased to fraction 50, where a concentration of 0.25 M NaCl was reached. This concentration of NaCl was held constant for 15 fractions. The gradient resumed at fraction 65 and the salt concentration was 0.5 M NaCl at fraction 75. This concentration was held for the remainder of collection. Eighty fractions of forty drops (1.3 ml) were collected and, due to low protein concentration, they were combined into 26 pools of five consecutive fractions each. The protein in each pool was concentrated in a 50% saturated ammonium sulfate precipitate.

Isoelectric Focusing

Isoelectric focusing of serum protein in a liquid phase was done using the Rotofor (BioRad Laboratories). A slight modification of the protocol supplied by the manufacturer was used. Briefly, the serum was dialyzed against d/d water overnight to remove salts. The suspension was centrifuged at 2200 x g for 10 minutes to remove the precipitate. The supernatant was diluted with d/d water to 35 ml. Ampholytes (Pharmalyte, pH 3-10, Sigma Chemical Company) were added to a final concentration of 1%. This solution was injected into the focusing chamber and, after the voltage had stabilized, was focused at 12 watts constant power for two hours (a total of approximately three

and one-half hours). The samples were harvested and the pH of each fraction was determined.

Protein Determination

Protein concentrations were determined according to Groves (46). Briefly, samples were diluted in PBS and the optical density (OD) of each sample was measured at wavelengths of 224 and 233 nanometers, using PBS as a reference blank. The change in the OD of each sample was plotted on a standard curve to determine the concentration of protein. The standard curve was prepared by plotting the change in OD of each standard made from bovine serum albumin (Sigma Chemical Company) at 10, 20, 40, 80, and 100 micrograms/milliliter (ug/ml) against the concentration of that standard.

Ammonium Sulfate Precipitation

Saturated ammonium sulfate (SAS) was added to the protein solution at the specified concentration and the suspension was refrigerated overnight. The suspensions were centrifuged at 13,000 x g for 10 minutes. The precipitate was resuspended in a minimal volume (500 microliters) of d/d water and dialyzed against 5 mM Tris-HCl, pH 8.0 overnight at room temperature. The SAS supernatant was similarly dialyzed.

Precipitate Preparation

Water precipitate (water ppt) was the precipitate formed when serum was dialyzed against d/d water overnight. The supernatant was called water supernatant.

To make the pH precipitate (pH ppt), the pH of the water supernatant was lowered to 4.0, using 0.1 M HCl. This suspension was allowed to stand at room temperature for 30 minutes, then centrifuged at 2200 x g for 10 minutes. The supernatant was carefully removed and the precipitate was set aside. The pH of the supernatant was raised to 4.5, using 0.1 M NaOH, and this suspension was allowed to stand at room temperature for 10 minutes. This process was repeated at half pH unit increments until a pH of 6.0 was reached. The five precipitates (pH 4.0, 4.5, 5.0, 5.5, and 6.0) were each washed with 0.5 ml d/d water, and pooled in 0.1 M NaOH. The final pH was 10.5 - 11.0. The suspension was allowed to stand at room temperature approximately 30 minutes, or until the precipitate was solubilized. The pH was then lowered to 6.7 - 7.0 using 0.1 M HCl.

To make the SAS/pH precipitate, a 40% ammonium sulfate precipitate of the pH precipitate was prepared and resuspended in a minimal volume (usually 500 microliters) of d/d water. The suspension was dialyzed against 5 mM Tris-HCl, pH 8.0 overnight. To assure there was no particulate matter injected onto the HPLC column, the 40% SAS

precipitate was centrifuged at 13,000 x g for 10 minutes. The very small precipitate, referred to as the post dialysis precipitate (PDP), was reserved. The supernatant was injected into the DEAE HPLC column. Fractions were collected and pooled as described in the HPLC section. The protein in each pool was concentrated using 50% SAS.

Immunoglobulin Production

A Sprague Dawley female rat was immunized intraperitoneally (IP) with 100 ug PDP in 200 microliters (ul) of 5 mM Tris-HCl, with 100 ul Freund's Complete Adjuvant (Difco Laboratories, Detroit, MI). Boosting with 20-25 ug PDP in 500 ul 5 mM Tris-HCl with 100 ul Freund's Incomplete Adjuvant (FICA) (Difco Laboratories) was done IP at three and nine weeks. At 15 weeks, the antigen used for boosting was isolated by immunoaffinity purification, using the polyclonal antibody obtained after the second boost. The antigen (20 ug) was suspended in 500 ul 5 mM Tris-HCl with 25 ul FICA. This mixture was divided for injection at two sites, subcutaneously at the nape of the neck and IP. Rat serum was obtained as described for mouse serum.

A second rat was immunized with protein from a DEAE pool; however, it died secondary to anesthesia before antiserum could be obtained.

Partial Purification of Immunoglobulins

The rat serum was diluted 1:3 with PBS. SAS was then added to give a final concentration of 50%. The suspension was incubated at room temperature for 30-45 minutes and then centrifuged at 13,000 x g for 10 minutes. The precipitate was resolubilized in d/d water equivalent to the volume of the PBS-diluted serum. SAS was added to give 40% concentration. After a second room temperature incubation and centrifugation, the precipitate was resuspended in d/d water (volume equivalent to 1/2 original serum volume) and dialyzed overnight against 50 mM Tris-HCl containing 0.1 M NaCl at final pH of 8.0.

A control immunoglobulin preparation was made from rat antiserum to proteins unrelated to the IL-2 preparations.

Immunoaffinity Purification

BioRad Affi-Gel Hz Immunoaffinity kit (BioRad Laboratories) was used to prepare immunoaffinity columns. Immunoglobulin was coupled to an agarose support through the carbohydrate moieties in the Fc portion of the molecule.

The following protocol was supplied with the immunoaffinity kit. The polyclonal preparation described on the previous page was desalted using a sizing column (Econo-Pac 10DG, supplied with kit). The

protein peak was combined with 97 mM sodium periodate and rotated in the dark for one hour to oxidize the sugars to aldehydes. The periodate was removed using the sizing column. The oxidized, desalted immunoglobulin was coupled to hydrazide gel overnight at room temperature. A small aliquot of the oxidized, desalted immunoglobulin was reserved for quantitation of immunoglobulin G (IgG). After the coupling reaction was complete, the column was made by pouring the gel slurry into a 3 ml syringe barrel plugged with nylon wool. The eluate was collected. The column was washed with 5 mM Tris-HCl with 0.5 M NaCl, pH 8.0. This wash was combined with the previous eluate and the total volume measured. Quantitation of IgG before and after coupling was done by radial immunodiffusion (RID), and used to determine the efficiency of coupling. The column was stored at 4°C in 5 mM Tris-HCl, pH 8.0 with 0.02% azide (w/v) added.

Prior to use, the column was washed sequentially with 5 mM Tris-HCl, pH 8.0; 5 mM Tris-HCl with 0.5 M NaCl, pH 8.0; 0.1 M glycine-HCl, pH 2.3; and 5 mM Tris-HCl, pH 8.0. The antigen sample, in 5 mM Tris-HCl, pH 8.0, was then applied to the column. The column was washed with 5 mM Tris-HCl with 0.5 M NaCl, pH 8.0 followed by 5 mM Tris-HCl, pH 8.0 to remove any unbound material. The bound antigen was eluted with 0.1 M glycine-HCl, pH 2.3, followed by 5 mM Tris-HCl, pH 8.0. The column was regenerated in 5 mM Tris-HCl, pH 8.0.

Radial Immunodiffusion (RID) Assay

A modification of the RID assay of Mancini, et al. (47) was used. The most significant modification was the use of a template rather than punched wells for antigen loading. Other differences were the agarose formulation, the use of a humidity chamber to prevent evaporation during diffusion rather than maintaining the gel under paraffin oil, and the measurement of precipitin ring diameter rather than calculation of ring area by weight. In detail, the RID plates were prepared from 0.75% agarose (L'Industrie Biologique, Francaise, France) in 16 mM barbitol buffer containing 2 mM calcium lactate and 0.1% (w/v) sodium azide, pH 8.5. Goat anti-rat IgG (Miles-Yeda Ltd., Israel) was added to melted agarose at 56$^{o}$C at a concentration of 0.25%. The agarose was poured between two glass plates separated by 0.75 mm thick spacer on the sides and bottom. After the agarose had solidified, the top plate was removed, and the gel surface was dried at room temperature for 5 minutes. A template was placed over the agarose, and 5 ul of sample was added to each well. The gel was incubated overnight at room temperature in a humidity chamber. The template was removed and the gel was dried. The drying process consisted of two steps. Firstly, filter paper was placed over the gel and the entire sandwich of paper towel-gel-glass plate was inverted onto approximately one inch of dry paper towels and weighted with a five pound weight, for one hour at room temperature. After this initial drying, the gel was soaked in PBS for approximately 15 minutes to smooth the gel surface. The final drying of the gel was done by

blowing hot air across the gel surface until it was dry. The gel was stained with 0.05% Coomassie Brilliant Blue R-250, (EM Laboratories, Elmsford, NY) and then destained in a 95% ethanol, d/d water, and glacial acetic acid (45:45:10) mixture. The ring sizes were measured, and the concentration of each sample was determined from the standard curve. The standard curve was made by plotting the concentration of each standard against the diameter of its ring. The standards used were rat IgG (Pel-Freeze, Rogers, AR) at 100, 80, 60, 40, 20, and 10 ug/ml.

Cell Counts

Non-radiolabeled cells were counted using the Coulter Zf cell counter (Coulter Diagnostics, Hialeah, FL). The following settings were used: Amplification = 1/2, Low Threshold = 15, Upper Threshold = 100, and Aperture Current = 1. Each sample was diluted in Hematall Isotonic Diluent (Fisher Scientific, Pittsburgh, PA) and lysed with Hematall LA-Hgb Reagent (Fisher Scientific) before counting. Cell counts from microtiter wells were done by adding the lysing agent directly to each well. The entire contents of each well were then diluted in isotonic diluent. When necessary, the cells were further diluted to give 2000 to 10,000 cells/ml. (Lysis directly in the well allowed the counting of adherent cells.)

Radiolabeled cells were counted on the hemocytometer, after dilution in 0.05% erythrosin B (Fisher Scientific Co., Fair Lawn, NJ),

in PBS. Erythrosin stains the non-viable cells, while viable cells remain unstained.

Splenocytes

Mice were sacrificed by cervical dislocation and the spleens were removed and placed in HBSS kept cold on ice. The spleens were mashed through a metal screen (30 mesh) using a syringe plunger and the screen was rinsed with cold HBSS. The spleen fragments were repeatedly expelled through a 20 gauge needle to produce a single cell suspension. The cells were transferred to a 50 ml conical polypropylene centrifuge tube (Sarstedt, Inc., St. Louis, MO) and cold HBSS was used to bring the volume to 50 ml. The cell suspension was centrifuged in a refrigerated ($5^{o}C$) centrifuge for 10 minutes at 248 x g. The supernatant was discarded and the pellet resuspended in 50 ml cold HBSS. After a second centrifugation, the pellet was resuspended in cold TC-10 (approximately 3 ml for each spleen). The cells in this suspension were then counted and diluted to $1.5 \times 10^7$, $3 \times 10^7$, or $6 \times 10^7$ cells/ml, as required. The cells were held on ice, to prevent loss of cell viability when delays were encountered.

Cell Culture

CTLL-2 cells (American Type Culture Collection, Rockville, MD) are cytotoxic T cells derived from a C57Bl/6 mouse and are dependent on IL-2 for growth (48). These cells were maintained in TC-10

supplemented with 5% RTP, 2 uM sodium pyruvate, and $10^{-5}$ M 2-mercapto-ethanol.

YAC-1 cells (American Type Culture Collection) are derived from a murine lymphoma induced by inoculation of Maloney leukemia virus into a newborn A/Sn Mouse (48). These cells were maintained in TC-10.

CTLL-2 Proliferation Inhibition Assay

The proliferation inhibition assay described by Lelchuk and Playfair (26) was modified by the use of commercially prepared IL-2 and a higher concentration of CTLL-2 cells. In detail, 50 ul aliquots of the inhibitor-enriched sample were plated in triplicate in the first row of a 96 well, flat bottom, microtiter plate (Corning Glass Works, Corning, NY), and were two-fold, serially diluted in EHAA media to a total of four rows. The flat bottom wells were used to give maximum cell separation and thus avoid contact inhibition. Fifty microliters of a 1:200 dilution of TCGF were added to each well. This was sufficient IL-2 to cause half-maximal stimulation of [$^3$H]-thymidine incorporation into cells. CTLL-2 cells were washed twice in RPMI 1640 with 0.1 mg/ml gentamicin to remove residual IL-2, and were resuspended at 5 x $10^5$ cells/ml. A 100 ul aliquot of these cells was added to each well. After incubation for 18-20 hours at 37$^{o}$C with 5% $CO_2$, the plates were pulsed with 10 ul [$^3$H]-thymidine (Research Product International Corp., Mount Prospect, IN) at 0.1 microCurie/ml. The plates were further incubated four hours and then harvested onto

glass fiber paper (Whittaker M.A. Bioproducts) using the PHD Cell Harvester (Cambridge Technology, Inc., Watertown, MA). Each sample was placed in scintillation vials (Sarstedt, Inc.) with 2 ml ScintiVerse E (Fisher Scientific) and the radioactivity was determined by the LS1800 liquid scintillation counter (Beckman Instruments, Inc, Irvine, CA).

Target Cells

Target cells were labelled with $^{51}$Chromium ($^{51}$Cr) using a modification of the method described by Kay, et al (49). A 15 ml overnight culture of cells was centrifuged in a 15 ml conical, polypropylene centrifuge tube (Sarstedt, Inc.) at 200 x g for 10 minutes. After removal of the supernatant, 70-80 ul $^{51}$Cr (1 milliCurie/ml) (New England Nuclear, Boston, MA) and 200 ul heat-inactivated FBS were added to the cell pellet. This suspension was incubated 90 minutes in a 37$^{o}$C water bath with gentle mixing every 10-15 minutes. The cells were resuspended in 5 ml TC-10 and incubated 20-30 minutes in a 37$^{o}$ water bath. The cell suspension was centrifuged for 10 minutes at 200 x g. The pellet was resuspended in 5 ml fresh TC-10 and incubated in the water bath again for 20-30 minutes. After another 10 minute centrifugation, the pellet of cells was resuspended in 10 ml TC-10 and counted on the hemocytometer. Viability was determined using 0.05% erythrosin B, and was greater than 90% in all assays. Cells were diluted to 5 x $10^4$/ml before use in the assay.

Bulk IL-2 Boosted NK Assay

To evaluate the effect of various incubation concentrations, splenocytes were seeded into separate 25 $cm^2$ flasks (Corning Glass Works, Corning, NY) at final concentrations ranging from 1.5 x $10^6$/ml to 3.0 x $10^7$/ml in TC-10 with FBS. Recombinant IL-2 was added to each flask at a final concentration of 1000 U/ml. The cultures were incubated 20-24 hours at 37°C in 5% $CO_2$. After incubation and without decanting the media, the bottom of each flask was scraped with a teflon cell scraper (Costar Corp.) and the contents of each flask were transferred to a 15 ml conical polypropylene tube. An aliquot of each suspension was removed for determination of cell counts before washing. The cells were washed twice and another aliquot was removed from each suspension for cell count. After the cells were counted, the washed cells were then resuspended at final concentrations equivalent to the original incubation concentrations used for the IL-2 boosting. These suspensions were seeded in quadruplicate to the first row of a round bottom, 96 well microtiter plate (Corning Glass Works) and two-fold serially diluted, for a total of six rows. The round bottom wells were used to promote maximum effector cell to target cell contact to ensure maximum lytic activity. Radiolabeled target cells were added to give 5000 cells/well. After incubation at 37°C in 5% $CO_2$ for four hours, 200 ul of each supernatant were removed and transferred to vials (Sarstedt, Inc.). These vials were placed on the LKB1274 gamma counter (LKB-Wallach RiaGamma 1274, Wallach Oy,

Finland) to determine counts per minute (cpm) due to $^{51}Cr$ released by lysed cells.

Bulk LAK Assay

The procedure for the bulk IL-2 boosted NK assay was modified as follows: IL-2 was used at a final concentration of 4000 U/ml, flask caps were taped to reduce evaporation, flasks were incubated 5 days in 5% $CO_2$ at 37°C, and TC-10 with 4000 u/ml IL-2 was added at 3 days to double the original volume.

Micro IL-2 Boosted NK Assay

This procedure differed from the bulk assay in that fresh splenocytes were seeded directly into the microtiter wells (no flasks were used) and incubated with IL-2. The cytotoxicity assay was done in the same wells with no transfer of the IL-2 boosted cells. In detail, fresh splenocytes were seeded in quadruplicate to the first row of a 96 well, round bottom, microtiter plate at a final concentration of $3.0 \times 10^7$ cells/ml. The round bottom wells were used to promote maximum effector cell to target cell contact to ensure maximum lytic activity. Two-fold serial dilutions were done to provide various incubation concentrations down to $9.4 \times 10^5$ cells/ml. Recombinant IL-2 was added at a final concentration of 1000 units/ml. The plates were incubated 20-24 hours at 37° C in

5% $CO_2$. Radiolabeled target cells were then added to give 5000 cells/well. The plates were again incubated at 37° C for four hours. A 200 ul aliquot of the supernatant was transferred from each well to a counting vial. These vials were placed on the LKB1274 gamma counter to determine counts per minute (cpm) due to $^{51}Cr$ released by lysed cells.

Micro LAK Assay

The procedure for the micro IL-2 boosted NK assay was modified as follows: IL-2 was used at a final concentration of 4000 U/ml, plates were taped to reduce evaporation, plates were incubated 5 days in 5% $CO_2$ at 37°C, and after 3 days incubation, 100 ul of each supernatant was removed and replaced with 100 ul of warm TC-10.

Assay of Selected Cell Populations

To determine the independent contribution of adherent cells, non-adherent cells, and soluble factors to the total NK cytotoxicity, a micro assay was set up as described on page 33, with some modifications. After the overnight incubation with IL-2, the supernatant and nonadherent cells in each well were gently mixed and moved to an empty well. Fresh TC-10 was added back to the adherent cells in the original wells. The plates were centrifuged approximately 10 minutes at 200 x g to sediment the nonadherent cells. A 150 ul aliquot of the supernatant was removed from each well and placed in an empty

well. Variations of this procedure were used to provide adherent cells only, nonadherent cells only, supernatant only, adherent plus nonadherent cells, and total (no manipulation). Radiolabeled target cells were added to each well and the assay was completed as described in the Micro IL-2 Boosted NK Assay section.

NK Inhibition Assay

The inhibitor-enriched sample to be tested (40 ul) was placed in each of four wells of a 96-well, round-bottom microtiter plate and 60 ul TC-10 were added. The contents of each well were mixed, and two-fold serial dilutions were made. Recombinant IL-2 was added to each well to give 1000 U/ml final concentration. Splenocytes were added to each well at a final concentration of $7.5 \times 10^5$ cells/ml. (The final volume per well was 200 ul.) The plates were then incubated 20-24 hours at $37^{\circ}$ C with 5% $CO_2$. Radiolabeled YAC-1 target cells were added, and the assay was completed as described in the Micro IL-2 Boosted NK Assay section.

Assay for IL-2 Inhibitor-Neutralizing Antibody Activity

The assay for inhibitor-neutralizing antibodies (the antibodies blocking the effect of the IL-2 inhibitor) was done in a manner similar to the inhibitor assay. Two-fold serial dilutions of the

SAS/pH precipitate (inhibitor) were made in the microtiter wells, beginning with a concentration previously found to cause 90-95% inhibition. Semi-purified antibody was added at various concentrations and allowed to incubate 45-60 minutes at room temperature. IL-2 and splenocytes were then added and the assay was completed as shown for the NK inhibition assay (page 35). The semi-purified antibody was diluted so the concentration during incubation with SAS/pH precipitate was equivalent to serum diluted 1:25, 1:250, 1:2500, and 1:25000.

Calculations

**Inhibition of CTLL-2 proliferation** was calculated as follows:

$$\% \text{ Inhibition} = 100 \times \left(1 - \left[\frac{\text{Exp cpm} - \text{Cells Only cpm}}{(\text{Cells} + \text{IL-2 cpm}) - \text{Cells only cpm}}\right]\right)$$

Exp cpm = cpm due to [$^3$H]-thymidine incorporation in experimental wells, containing cells, IL-2 and inhibitor sample.

Cells Only cpm = cpm due to [$^3$H]-thymidine incorporation when no IL-2 was added to the wells.

Cells + IL-2 cpm = cpm due to [$^3$H]-thymidine incorporation when IL-2 was added to the wells.

**NK or LAK Cytotoxicity** was calculated as follows:

$$\% \text{ Cytotoxicity} = 100 \times \left[ \frac{\text{Exp release - spont release}}{\text{max release - spont release}} \right]$$

Exp release = cpm due to $^{51}Cr$ released when labeled target cells were lysed by effector cells.

Spont release = cpm due to $^{51}Cr$ released when labeled target cells were incubated in media alone.

Max release = cpm due to $^{51}Cr$ released when detergent was added to the labeled target cells to lyse the cells.

To facilitate the interpretation of cytotoxicity, lytic units were used to reduce multiple points of cytotoxicity to a single number. Lytic $Unit_{20}$ ($LU_{20}$) is defined as the number of cells required to cause 20% target cell lysis and is expressed as $LU/10^6$ cells (50).

**Inhibition of IL-2 Boosted NK Activity** was calculated with the following formula:

$$\% \text{ Inhibition} = 100 \times \left(1 - \left[ \frac{\text{cytotoxicity with Inhibitor}}{\text{cytotoxicity with IL-2}} \right]\right)$$

Cytotox with IL-2 = cytotoxicity of splenocytes incubated in IL-2 alone.

Cytotox with Inh = cytotoxicity of splenocytes incubated with IL-2 plus inhibitor-enriched sample.

**Neutralizing Units** (NU) were calculated as the inverse of the dilution of inhibitor-enriched sample required for 50% inhibition, using the following formula:

$$NU/mg = \frac{200}{HP - \left[ (HC - 50) * \frac{HP - LP}{HC - LC} \right]}$$

HC = Percent inhibition just higher than 50% inhibition

LC = Percent inhibition just lower than 50% inhibition

HP = Micrograms of protein in the well giving HC

LC = Micrograms of protein in the well giving LC

Statistical Analysis

Data from within individual assays was examined using Student's t-test and analysis of variance (ANOVA).

# CHAPTER 3

# RESULTS

## Assay Comparison

Before utilizing the micro assay to determine IL-2 boosted NK and LAK activity, it was necessary to assure that it was an acceptable substitute for the bulk assay, the current state of the art method. We compared the two assay systems based on the cell survival after incubation with IL-2, the cell survival after the cells were prepared for assay, and the cytotoxicity of the cells assayed. The splenocytes incubated in IL-2 overnight were considered to have IL-2 boosted NK activity, while the splenocytes incubated with IL-2 for five days had LAK activity.

### Cellular Viability in the Bulk Assay

To determine the effect of the incubation concentration, spleen cells were incubated at various concentrations in five milliliters of culture media. Firstly, after overnight incubation with IL-2 in bulk culture, 54-63% of the splenocytes originally seeded into the flask were recovered depending in the incubation concentration (Table 2).

Table 2. The Effect of The Bulk Assay on Cell Viability After Overnight Or Five-Day Incubation With IL-2.

| Inc Con[a] | Overnight | | Five Day | |
|---|---|---|---|---|
| | Before Wash[b] | After Wash[c] | Before Wash[b] | After Wash[c] |
| | [mean % (SE)[d]] | | [mean % (SE)] | |
| $3.0 \times 10^7$ | 55 (2) | 39 (2) | 48 ( 8) | 24 (8) |
| $1.5 \times 10^7$ | 54 (3) | 40 (3) | 54 ( 9) | 28 (9) |
| $7.5 \times 10^6$ | 59 (1) | 34 (3) | 48 ( 8) | 24 (4) |
| $3.0 \times 10^6$ | 63 (8) | 32 (5) | 46 (12) | 18 (3) |
| $1.5 \times 10^6$ | 63 (7) | 27 (2) | 43 (13) | 12 (3) |

There was a significant difference ($p < .05$) between the number of cells recovered before washing and after washing, at all incubation concentrations and for both overnight and five-day incubations.

[a] Incubation concentration expressed as cells/ml.

[b] The splenocytes were counted after overnight incubation, but before they were washed.

[c] The splenocytes were counted after overnight incubation and after they were washed twice with warm media.

[d] Mean % refers to the percentage of seeded cells that were viable after incubation. The mean and the standard error (SE) of the mean from three separate experiments are given.

As expected, the percentage of splenocytes recovered was even lower (43 - 54%) when the splenocytes were cultured for five days. There was no significant effect of incubation concentration on the cell survival. The cultured splenocytes were washed twice and counted again. At this point in the procedure, 27-40% of the originally seeded cells were recovered after overnight incubation, and 12-28% of the cells incubated for five days (Table 2). There was a significant difference ($p < .05$) between the number of cells recovered before washing and after washing, at all incubation concentrations, and for both overnight and five day incubations. This decrease was postulated to be due to a combination of the centrifugation and the removal and replacement of the media required in the washing process. To observe the effect of centrifugation alone on cell survival, cultured splenocytes were "washed" without changing the media. Twenty-seven per cent of the originally seeded cells survived (data not shown), suggesting that centrifugation was the primary cause of cell loss during the washing process.

## Cellular Viability in the Micro Assay

After overnight incubation with IL-2 in the micro assay, 46-56% of the splenocytes seeded to the wells survived (Table 3), similar to that observed in the bulk assay. However, in contrast to the bulk assay, the number of cells recovered before washing and after washing in the micro assay were approximately equal. When the splenocytes were incubated with IL-2 for five days, 39-53% of the cells

Table 3. The Effect of The Micro Assay on Cell Viability After Overnight Or Five-Day Incubation With IL-2.

| Inc Con[a] | Overnight | | Five Day | |
|---|---|---|---|---|
| | Before Wash[b] | After Wash[c] | Before Wash[b] | After Wash[c] |
| | [mean % (SE)[d]] | | [mean % (SE)] | |
| $1.5 \times 10^7$ | 46 (3) | 45 (2) | 46 ( 5) | 42 ( 8) |
| $7.5 \times 10^6$ | 48 (8) | 50 (5) | 48 ( 8) | 44 ( 6) |
| $3.8 \times 10^6$ | 55 (2) | 52 (3) | 53 (12) | 46 (11) |
| $1.9 \times 10^6$ | 54 (4) | 56 (2) | 48 (13) | 44 (12) |
| $9.4 \times 10^5$ | 56 (5) | 61 (2) | 39 ( 9) | 38 ( 9) |

There was no significant difference ($p > 0.5$) between the Before Wash counts and the After Wash counts.

a Incubation concentration, expressed as cells/ml

b The splenocytes were counted after overnight incubation, but before they were washed.

c The splenocytes were counted after overnight incubation and after they were washed twice with warm media.

d Mean % refers to the percentage of seeded cells that were viable after incubation. The mean and the standard error (SE) of the mean from three separate experiments are given.

survived (Table 3). When these LAK cells were washed, this number dropped slightly, but not significantly, to 38-46%.

## Comparison of Cell Recovery in the Bulk and Micro Assays

When the cell survival in the two assays was compared after either overnight or five day incubation, the percentage of cells recovered was approximately the same in both assays (Tables 2 and 3). The difference in cell recovery between the two assays was seen after the cells were washed, indicating the wash process was the critical phase. After washing, the percentage of cells recovered in the bulk system was significantly less ($p < 0.05$) than the percentage recovered in the micro assay, regardless of the incubation concentration or time of incubation.

## Lytic Activity

After incubation in IL-2, the cytotoxicity of the cultured cells was determined in a chromium release assay and the data was used to calculate the lytic activity. The lytic unit (LU) was used to facilitate the interpretation of the cytotoxicity data in the expression of lytic activity. LU represented the number of cells required to lyse a specified percentage of the target cells, in this case 20% ($LU_{20}$), and was expressed as $LU/10^6$ cells so the value increased as the cytolytic activity increased (50).

After overnight incubation with IL-2, the lytic activity of the cells measured in the micro assay (4.4 $LU_{20}/10^6$ cells) was nearly double that of the cells cultured in the bulk system (2.3 $LU_{20}/10^6$ cells). The data from the individual experiments are shown in Table 4. In the five day assay, the results were similar (Table 5), although the difference between the two assays was amplified. The lytic activity of the cells in the micro assay (14.4 $LU_{20}/10^6$ cells) was approximately four times that of the cells in the bulk assay (3.6 $LU_{20}/10^6$ cells).

Since NK activity is primarily found in the adherent population (51, 52), the selective loss of the adherent cells could account for the difference in lytic activity between the bulk and micro assays. To confirm this, we examined the cytolytic activity of adherent cells and nonadherent cells. After overnight incubation, the adherent cells had three to four times more lytic activity than the nonadherent cells (Table 6). After five days (Table 7), this difference had dropped to less than twofold, but it was significant ($p < 0.05$). The supernatant alone did not have cytotoxic activity. These data suggested that the selective loss of the adherent cells may be responsible for the difference in lytic activity between the two assay methods. It was unclear why there was such wide variation in the absolute values from different days; however, during portions of this study, mouse hepatitis virus (MHV) and sendai virus infected the mouse colony. The relative difference between the lytic activity of the adherent and nonadherent cells was not affected.

Table 4. The Effect of the Assay System on the Lytic Activity of Splenocytes Cultured With IL-2 Overnight.

| Assay Number | Bulk[a] (SD[b]) | Micro[c] (SD) | p |
|---|---|---|---|
| 1 | 3.0 (0.3) | 5.2 (0.8) | |
| 2 | 1.4 (0.4) | 2.6 (0.4) | |
| 3 | 2.6 (0.3) | 5.5 (0.7) | |
| Mean | 2.3 (0.8) | 4.4 (1.6) | .05 |

[a] The lytic unit$_{20}$/$10^6$ splenocytes as measured in the bulk assay.

[b] Standard Deviation

[c] The lytic unit$_{20}$/$10^6$ splenocytes as measured in the micro assay.

Table 5. The Effect of the Assay System on the Lytic Activity of Splenocytes Cultured With IL-2 For Five Days.

| Assay Number | Bulk[a] (SD[b]) | Micro[c] (SD) | p |
|---|---|---|---|
| 1 | 6.1 (1.1) | 16.0 (5.2) | |
| 2 | 1.0 (0.2) | 12.9 (3.2) | |
| Mean | 3.6 (3.6) | 14.5 (2.2) | .06 |

[a] The lytic unit$_{20}$/$10^6$ splenocytes as measured in the bulk assay.

[b] Standard Deviation

[c] The lytic unit$_{20}$/$10^6$ splenocytes as measured in the micro assay.

Table 6. The Lytic Activity of Adherent and Nonadherent Populations of Splenocytes As Measured in The Overnight Micro IL-2 Boosted NK Assay.

| Assay Number | Adherent[a] (SD[b]) | Nonadherent[c] (SD) |
|---|---|---|
| 1 | 42.9 (12.3) | 9.9 (1.4) |
| 2 | 11.8 ( 2.6) | 3.8 (0.6) |

There was no statistically significant difference ($p = .35$) when the lytic activity of the adherent and nonadherent populations was compared, using paired analysis by Student's t-test.

[a] Adherent cells were those cells remaining in the wells after the contents were mixed and transferred other wells. Units are lytic $unit_{20}/10^6$ splenocytes.

[b] Standard Deviation

[c] Nonadherent cells were those cells that were in suspension after the contents of each well were mixed. These cells were transferred to other wells. Units are lytic $unit_{20}/10^6$ splenocytes.

Table 7. Lytic Activity of Adherent and Nonadherent Populations of Splenocytes in The Micro LAK Assay.

| Assay Number | Adherent[a] (SD[b]) | Nonadherent[c] (SD) |
|---|---|---|
| 1 | 41.9 (13.4) | 26.2 ( 3.8) |
| 2 | 60.0 (13.5) | 41.7 (18.8) |

There was a statistically significant difference ($p < .05$) in the lytic activity of the adherent and nonadherent populations.

[a] Adherent cells were those cells remaining in the wells after the contents were mixed and transferred other wells. Lytic activity is expressed in lytic $unit_{20}/10^6$ cells.

[b] Standard Deviation

[c] Nonadherent cells were those cells that were in suspension after the contents of each well were mixed. These cells were transferred to other wells. Lytic activity is expressed in lytic $unit_{20}/10^6$ cells.

## The Effect of the Cell To Surface Area Ratio

Adherent cells were shown to have much greater lytic activity than the nonadherent cells, and adherence required the cells to have contact with the bottom of the culture vessel. Therefore, it was important to determine the cell to surface area ratio (C:SA) and evaluate its effect on the lytic ability of the cells. The C:SA may have affected the lytic activity in two ways, surface contact by the splenocytes needed for adherence, and the effector cell to target cell contact required for lysis. To assure that the difference in the lytic activity in the bulk and micro assays was not due to a difference in the C:SA, we calculated the surface area of the flat bottom of a flask and a flat bottom well. In a round bottom well, the cells adhered to the slanted surfaces in addition to the bottom of the well, covering almost half the area; therefore, one-half the surface area of the bottom half-sphere was used in the determination of C:SA for the round bottom wells. As shown in Figure 1, there is no significant difference in the cytotoxicity of the cells regardless of the type of culture vessel. Even if the surface area of the round bottom well covered by the cells were one-third to one-fourth of the total surface area, there would still be no significant effect due to the bottom surface area of the culture vessel.

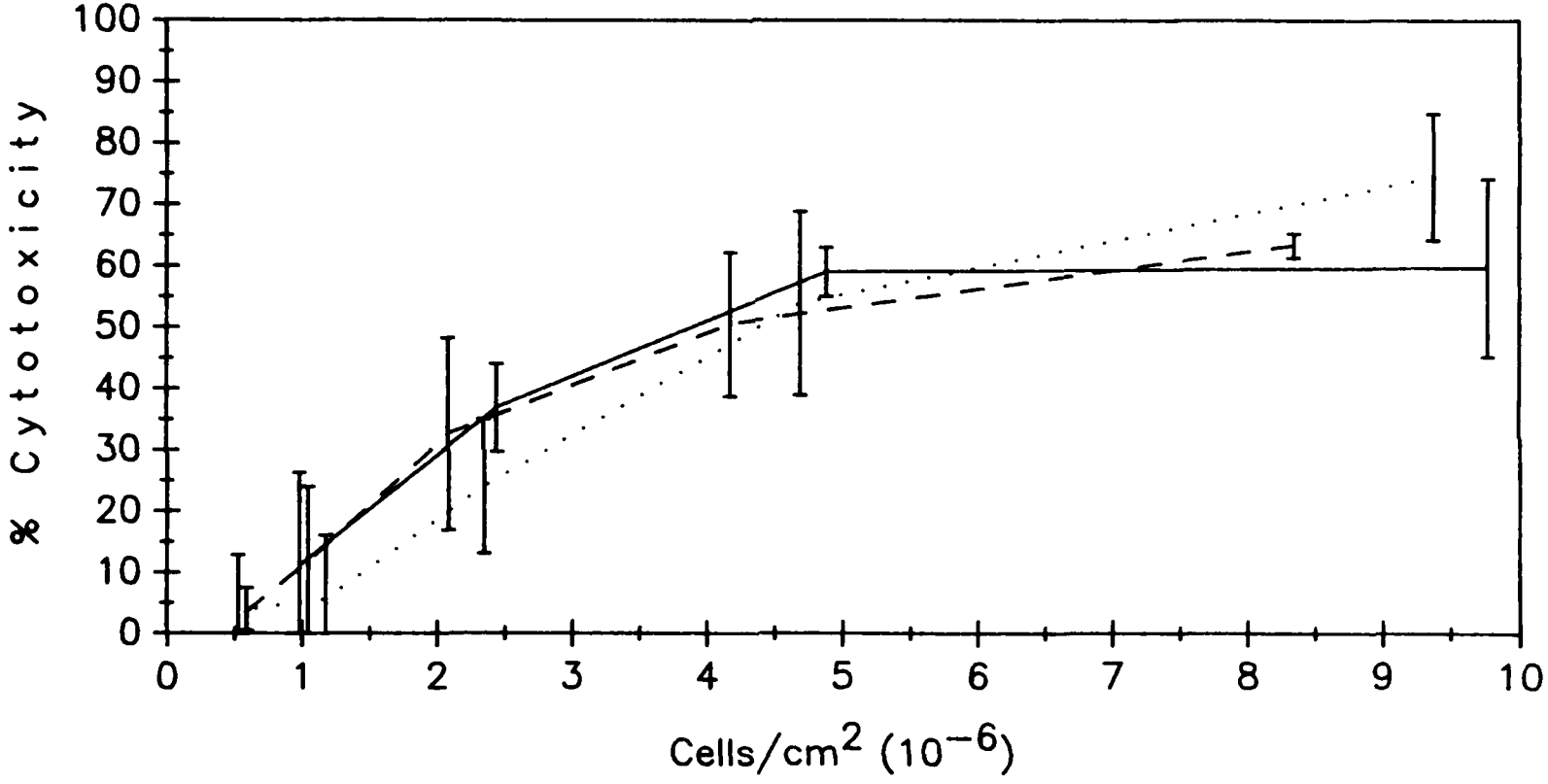


Figure 1. The Effect of Surface Area on The Cytotoxicity of Splenocytes Incubated With IL-2 Overnight. The splenocytes were incubated in either flasks ( —— ), or in microtiter plates containing either flat bottom wells ( – - ), or round bottom wells ( · · · ). The error bars indicate the standard error of the mean.

## The IL-2 Inhibitor

### The Activation of IL-2 Inhibitor in Serum

Results obtained in our laboratory have shown that room temperature storage of normal mouse serum (NMS) for two days activated the IL-2 inhibitor, i.e. the inhibitory activity was increased when compared to fresh serum, or to serum stored frozen. The only reference to this phenomenon in published reports was when Male, et al. (42) reported "more activity was retained if serum was not frozen". To verify this activation phenomenon, the IL-2 inhibitory activity of activated serum was compared to the inhibitory activity of serum stored at -20°C and to the inhibitory activity of fresh serum. These data (Table 8) demonstrated that serum stored at room temperature had a significantly ($p < 0.01$) higher level of inhibitory activity than fresh or frozen serum. The frozen serum had significantly ($p < 0.01$) higher inhibitory activity than did fresh serum, a result apparently due to slow activation that occurred even at low temperatures. The mechanism of this activation is not known. Based on these results, activated serum was used for all studies.

### Assay and Diluent Selection

Ion exchange chromatography (DEAE) was used to fractionate activated mouse serum. All eighty fractions were assayed for inhibitory activity in the CTLL-2 proliferation inhibition assay.

Table 8. Comparison of Activated, Fresh, and Frozen Serum in a CTLL-2 Inhibition Assay.

| Serum [a] | % inhibition[b] (SD[c]) | |
|---|---|---|
| Fresh (n=3) | 48.5 (2.1) | $p < 0.01$ |
| Frozen (n=2) | 66.7 (3.1) | $p < 0.01$ |
| Activated (n=3) | 85.0 (0.0) | |

[a] All serum was from BDF1 mice and at a final concentration of 25% (v/v) in the assay. Fresh serum was used within one hour of bleed, frozen serum was stored at -20°C for several weeks, and activated serum was stored at room temperature for two days.

[b] The percentage of inhibition of proliferation caused by mouse serum when compared to cells incubated with IL-2 in the absence of mouse serum (0% inhibition), and to cells incubated with no IL-2 (100% inhibition).

[c] Standard Deviation

Each of the fractions caused proliferation of the CTLL-2 cells greater than the IL-2 alone, indicating some stimulatory action rather than the expected inhibition. One common feature of the fractions was that all contained Tris-HCl which was used as the buffer for DEAE chromatography. Therefore, Tris-HCl and other potential diluents were assayed to determine the effect of each in this assay. Bovine serum albumin (BSA) was included as a protein replacement to determine if the protein concentration in the final suspension was critical to cellular responses. At a final concentration of 0.6 mM, Tris-HCl caused greater than 80% inhibition of the cellular proliferation, while saline had a stimulatory effect (-20% inhibition). The effects of PBS varied from -24% to 25% inhibition of proliferation. EHAA was used as a baseline control since it was the culture media used for this assay. As expected, it had little effect (7% inhibition) on the proliferative response. The addition of BSA had no modulating effect on the inhibition caused by Tris-HCl. These findings indicated this assay system would not be suitable to follow the purification of the IL-2 inhibitor, since most of the inhibitor-enriched samples assayed would be in a buffer. Therefore, these diluents were then evaluated in the micro IL-2 boosted NK assay. Tris-HCl (5 mM) caused the least effect and resulted in baseline levels of inhibition of cytotoxicity (Table 9). PBS and d/d water affected this assay slightly, -0.2% and 4% inhibition respectively. Therefore, the micro IL-2 boosted NK assay was used for determination of IL-2 inhibitory activity and 5 mM Tris-HCl was used as the sample diluent.

Table 9. The Effect of Various Diluents in the Micro IL-2 Boosted NK Assay.

| Diluent | % Inhibition at 10% diluent (v/v) | | |
|---|---|---|---|
| | Mean | SD[a] | n[b] |
| 5 mM Tris-HCl | 0 | 12 | 13 |
| 5 mM Tris-HCl with 0.5 M NaCl | 17 | 34 | 3 |
| PBS | 0 | 17 | 5 |
| d/d Water | 4 | 14 | 6 |

[a] Standard Deviation

[b] Number of replicates. The high number of Tris-HCl replicates was a result of the inclusion of this diluent as a control during later assays.

When normal mouse serum was added to the IL-2 boosted NK assay at a final concentration of 10% (v/v), the mean inhibition of cytotoxicity was 88%. This confirmed the observations of Klassen and his colleagues (25) that NMS was capable of inhibiting IL-2 boosted NK activity. To determine if the observed inhibition of cytotoxicity was due to serum toxicity, viable cell counts were determined after splenocytes were incubated overnight with IL-2, without IL-2, or with IL-2 plus 10% activated mouse serum. Of those cells incubated with IL-2 and serum overnight, 54% survived; compared to 57% of those incubated with IL-2 alone, or 54% of the cells incubated without IL-2. There was no significant difference in the cell viability, which showed that the inhibition was not due to serum cytotoxicity.

## Selection of Splenocyte and Serum Sources

Having selected the assay system and sample diluent, the next step was to select the strains of mice to use for the source of the inhibitor and the NK cells. The preliminary experiments had been done using BDF1 serum as the source of the IL-2 inhibitor and BDF1 splenocytes as the source of NK cells. The mouse strains that were readily available were C3H, BDF1, and Balb/c. IL-2 boosted NK inhibitor assays were set up using splenocytes from each of the three strains. Activated serum from each strain was tested in each of the three assays. As shown in Table 10, BDF1 splenocytes were more sensitive to the inhibitory effects of serum than C3H or Balb/c splenocytes and therefore, were the splenocytes of choice for future

Table 10. The Effect of Different Mouse Strains Used As Sources of Serum and Splenocytes In the Inhibition of IL-2 Boosted NK Cytotoxicity.

| Serum (10% v/v) | Splenocyte Source | | |
|---|---|---|---|
| | BALB/c | BDF1 | C3H |
| BALB/c | 18 | 38 | 15 |
| BDF1 | 4 | 84 | -1 |
| C3H | 49 | 64 | 32 |

assays. Although, C3H serum did not have as much inhibitory activity as BDF1 serum, the difference was not appreciable. C3H serum was selected for use because there were insufficient numbers of BDF1 mice to use as a source of both splenocytes and serum. The combination of C3H serum and BDF1 splenocytes was used throughout the rest of the study.

### The Use of a Negative Control

To assure the IL-2 inhibitory activity was not an artifact of the purification procedure, a control serum without IL-2 inhibitor was needed. Fetal bovine serum (FBS) was our first choice and non-activated FBS showed no inhibitory activity. However, when FBS was activated for two days at room temperature, its ability to inhibit the IL-2 enhancement of NK activity rose to 90% inhibition. When the activated serum was further purified to the pH precipitate, the inhibitory activity was 77%. These data indicate the FBS contained the IL-2 inhibitor, and could not be used as a negative control. As a substitute for any serum, protein solutions containing either IgG, bovine serum albumin, or both were tested, but no protein could be isolated by the purification procedures used for the IL-2 inhibitor, e.g. the formation of the pH precipitate. As a result, a negative control was not used to monitor the purification of IL-2 inhibitor.

The Purification Process

Initial attempts to purify the IL-2 inhibitor were by preparative isoelectric focusing in a liquid phase with the BioRad Rotofor. The majority of the inhibitory activity was in the precipitates that formed during the focusing process in the pH range of 4.2 to 5.7. There was little or no activity in the supernatants. Since the IL-2 inhibitory activity was associated with the precipitate that formed at pH 4.0 to 6.0, we investigated using dialyzed serum and pH adjustment as a rapid purification method. The serum was first dialyzed against d/d water and clarified by centrifugation. Serial pH precipitates ranging from 4.0 to 6.0 were formed by adjusting the pH of the supernatant HCl and NaOH. The precipitates were pooled, solubilized in 0.1 M NaOH sodium hydroxide, and analyzed for inhibitor activity. These pooled precipitates had approximately ten times the specific activity present in serum (3.8 NU/mg protein compared to 0.4 NU/mg).

Since the precipitate represented the majority of inhibitor activity, the pH precipitate was used as the source of inhibitor for further purification by DEAE. When the material was dialyzed against DEAE starting buffer for equilibration small precipitate formed. After removal of the precipitate, the supernatant was injected onto the DEAE HPLC column. A representative graph of protein profile and inhibitory activity of this fractionated material is shown in Figure 2. A shallow NaCl gradient was used so a sharp peak of IL-2

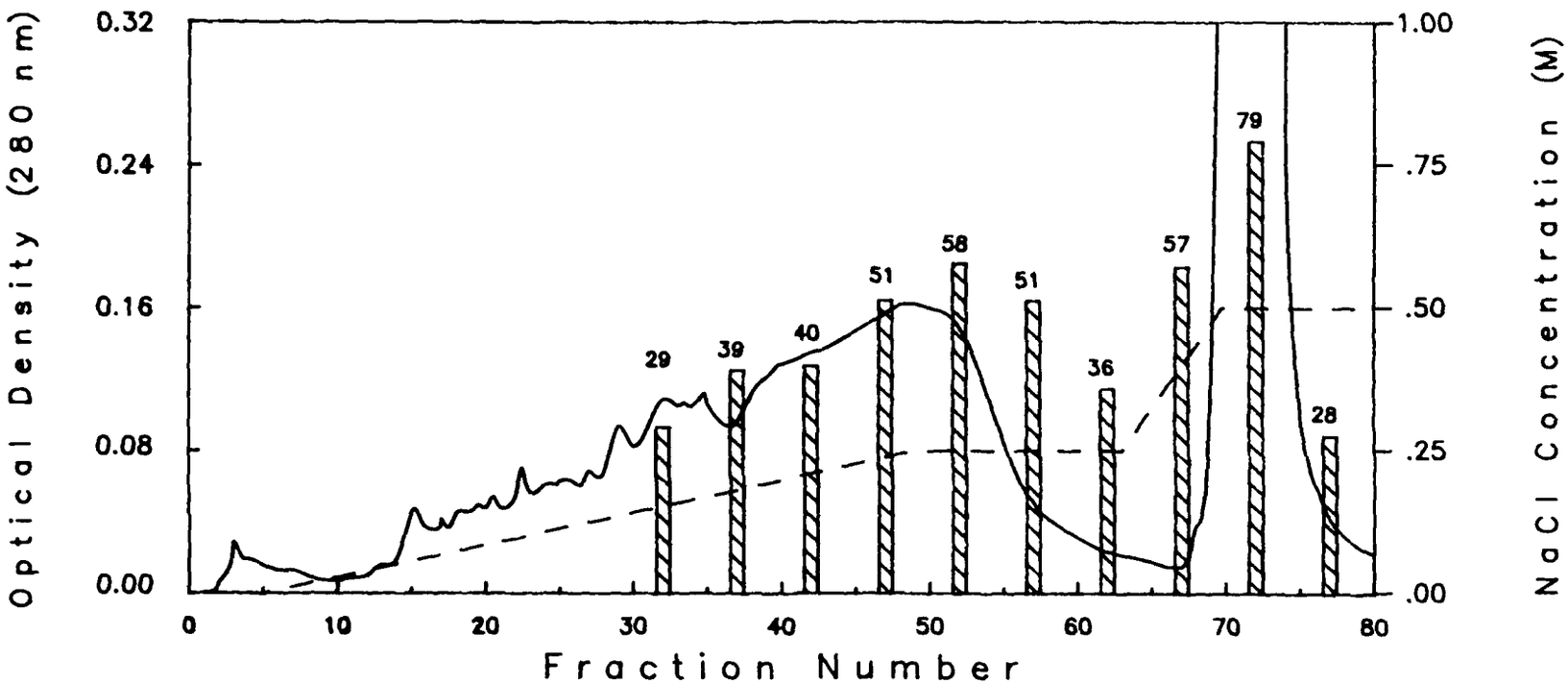


Figure 2. Partial Purification of IL-2 Inhibitor by DEAE Chromatography. The graph depicts the protein profile (——) and the NaCl gradient (– –) from DEAE HPLC purification of an IL-2 inhibitor-enriched sample. The bars indicate the inhibitory activity of five-fraction pools as measured by inhibition of IL-2 boosted NK function. The activity is expressed as percent decrease of NK cytotoxicity and is represented by the numbers above the bars. (100% inhibition would be equivalent to splenocytes cultured without IL-2.)

inhibitory activity would form. As can be seen in Figure 2, this did not occur, instead there was a very broad peak of inhibitory activity, spread over 30 fractions, centered at 0.25 M NaCl. When the NaCl concentration was increased to 0.5 M NaCl to elute all proteins, a second peak eluted, which also contained inhibitory activity. It was not clear from the preliminary data whether or not this second peak was real. It was not present on the initial run, but was present on the second run. This difference may have been due to the amount of protein loaded on the column (5.5 mg versus 10 mg), but the location of the peak indicated that the proteins in this peak had different binding characteristics than the proteins in the broad peak. These results suggested that DEAE chromatography was not the ideal way to purify the inhibitor.

As can be seen in Table 11, the specific activity of each IL-2 inhibitor-enriched sample increased during the purification process. There was an approximately 200-fold (0.4 NU/mg to 77 NU/mg) increase in specific activity as the IL-2 inhibitor was purified. However, as expected this increased specific activity was at the expense of yield, the final yield was only 16%. The five-fraction pools from DEAE chromatography each yielded approximately 5%, which further suggests that DEAE chromatography was not a good method for use in IL-2 inhibitor purification.

Table 11. Specific Activity of IL-2 Inhibitor at Different Steps Of Its Purification.

| | NU/mg[a] | n[b] | % Yield[c] |
|---|---|---|---|
| Activated Serum | 0.4 | 11 | |
| pH ppt[d] | 3.8 | 3 | 24 |
| SAS/pH ppt[e] | 8.1 | 6 | 171 |
| PDP[f] | 76.9 | 2 | 16 |

[a] Neutralizing units per milligram of protein.

[b] The number of replicates. The large number of serum replicates resulted from including serum in every assay, regardless of the purpose of the assay. Similarly, the SAS/pH ppt was included in all assays of antibody activity.

[c] Percentage of neutralizing units present in the original serum that were present in the inhibitor-enriched sample.

[d] The pH precipitate. See page 23 in Materials and Methods.

[e] The 40% saturated ammonium sulfate precipitate of the pH ppt, dialyzed against 5 mM Tris-HCl overnight.

[f] The small precipitate formed when the solubilized SAS/pH ppt was dialyzed against 5 mM Tris-HCl.

## Antibody Production and Testing

One of the important objectives of this study was the development of a purified form of the IL-2 inhibitor with high specific activity, for use as an immunogen to develop an antibody probe. Of all the inhibitor-enriched samples, the one that fit the criteria was the PDP, the precipitate that formed when the SAS/pH ppt was dialyzed against 5 mM Tris-HCl. PDP had the highest specific activity (77 NU/mg), with the least protein, and therefore was used as the immunogen. The antiserum that was produced from immunization with PDP was partially purified using saturated ammonium sulfate precipitation. The semi-purified antisera was tested for its ability to neutralize the IL-2 inhibitor in the SAS/pH precipitate and was bound to an immunoaffinity gel in an attempt to purify the inhibitor. The semi-purified antisera was diluted and added to the inhibitor-enriched sample (SAS/pH ppt) in an IL-2 boosted NK inhibition assay. The SAS/pH ppt used in this assay had 10.7 NU/mg. At a 1:25 dilution, the semi-purified antisera from the first bleed reduced the inhibitor activity by two-fold (10.7 NU/mg to 4.6 NU/mg). The semi-purified control antisera from a rat immunized with proteins unrelated to the IL-2 inhibitor showed little blocking ability (10.7 NU/mg to 9.0 NU/mg).

The rat was boosted two more times and the semi-purified antisera obtained after the last boost showed a decrease in blocking activity, indicating the neutralizing antibodies were no longer present. This

antisera were used to immunoaffinity purify the inhibitor from both serum and the SAS/pH ppt. The eluted fractions had inhibitory activity, and there was a decrease in the amount of inhibitor in the nonbound fractions, when compared to the starting material. This indicated the anti-inhibitor antibodies were present in the antisera, even though they were not neutralizing antibodies. The nonbound fraction (5.5 mg/ml) inhibited 61% of the IL-2 boosted cytotoxicity, after one passage of activated mouse serum over the hydrazide gel. After a second passage over the gel, the nonbound fraction's activity dropped to 37% inhibition at approximately the same protein concentration. The bound fraction from the two passages of serum (600 ug/ml) inhibited 49% of the IL-2 boosted cytotoxicity. In contrast to serum, when the SAS/pH ppt was passed through the affinity column, the eluted fraction inhibited only 17% of the IL-2 boosted cytotoxicity, at 500 ug/ml, while the nonbound fraction inhibited 75% at 2.7 mg/ml. A second passage of the nonbound fraction did not change either the inhibitory activity or the protein concentration. These values were given as percentage inhibition rather than neutralizing units because the inhibition was generally less than 50%, so neutralizing units could not be calculated.

# Chapter 4

# Discussion

## Assay Comparison

The purpose of this portion of the study was to develop a micro assay for IL-2 boosted NK activity. We needed a reliable and rapid assay for use in the determination of the IL-2 inhibitory activity of numerous samples. After developing this new procedure, we compared it to the more traditional bulk assay, to confirm the validity of the new assay. In the bulk assay, a large number of cells were incubated in large culture vessels (flasks). After incubation, the cells were transferred to microtiter wells for a cytotoxicity assay. This transfer involved the scraping of the adherent cells from the bottom of the flask, followed by the washing of the entire cell population, which included both adherent and nonadherent cells. In the micro assay, a small number of cells were incubated in small culture vessels (microtiter wells) and washed. The cytotoxicity assay was done in the same wells, without any cell transfer.

## Cell Survival

Our initial observations concerned the cell survival after incubation with IL-2. In either assay, approximately 50-60% of the cells originally seeded to the culture vessel survived. This reduction in cell numbers was due to cell death which normally occurs when these cells are cultured. However, after the cells were washed, the number of cells surviving this procedure in the bulk assay decreased by another 50%, while there was little change in the cell numbers in the micro assay. The reason for this difference in survival was thought to be cell damage caused when the cells were scraped off the bottom of the flask. This harsh procedure was only done in the bulk assay, as there was no cell transfer required in the micro assay. The fact that the number of cells recovered in the two assays were significantly different only after washing suggested that the scraping and cell transfer did not directly lyse the cells, but made them more fragile and thus more susceptible to the effect of centrifugation.

The adherent cells were less than 10% of the cell population in the micro assay (data not shown). Therefore, if this relationship were also true in the bulk assay, the reduction in cell survival after washing was not entirely due to the loss of the adherent cells. There were simply not enough adherent cells to account for the entire reduction in cell survival. This indicated that another mechanism of cell destruction occurred in the bulk assay. A possible explanation

of this reduced cell survival was inadequate centrifugation, i.e. the centrifugation speed was too low, the time of centrifugation too short, or a combination of the two. Since the cells had to travel farther when washed in a conical tube than in the microtiter well, inadequate centrifugation speed and/or time would leave cells in the supernatant during washing of cells in the bulk assay, but not during washing of cells in the micro assay.

## Lytic Activity

The cells that remained after washing were used as effector cells in a cytotoxicity assay. Those cells cultured in the micro assay had two to four times more lytic activity than did those cells cultured in bulk. The reason for this may have been that the cells cultured in bulk had a lower percentage of adherent cells present, due to the damage which was thought to occur when the cells were removed from the flask. Since adherent cells had been reported to have more NK function than nonadherent cells (51, 52), the individual contribution of each of these subpopulations was determined. Our data showed that, after overnight incubation, the adherent cells had at least three times more lytic activity than the nonadherent cells. Therefore, the decreased contribution of adherent cells to cytolysis in the bulk assay may explain the difference in lytic activity observed in the two assays.

Since our data showed there was more contribution to cytolysis by adherent cells in the micro assay, we wanted to ascertain if the increased contribution by adherent cells was due to an increased availability of surface area for attachment. We hypothesized that the difference in lytic activity in the two assays was due to a difference in the cell to surface area ratio of the different culture vessels. If more surface area were available for each cell, the cell's opportunity to adhere to the bottom surface would increase, and result in an increased percentage of adherent cells in the cell population. Since our data and others' (51, 52) had shown that adherent cells have more lytic activity than nonadherent cells, the increase in the percentage of adherent cells would increase the lytic activity of the cell population. This hypothesis proved to be incorrect, as there was no significant difference in the cytotoxicity of cells due to the type of culture vessel used. (Figure 1).

From our data, it was apparent that the cell populations in the two assays were different. This difference implied that the two assays measured the cytotoxicity of two different cell populations; the total population in the micro assay, and the adherent-depleted population in the bulk assay. The depletion of adherent cells within the cell population results in reduced lytic activity in any experiment performed using the bulk assay. This difference in the cell populations measured by the two assays is important when attempting to compare results obtained using the two assays. The results obtained using the micro assay include the effects of the

adherent cells, which would be missing from the results obtained using the bulk assay. Such a difference may or may not be important, depending on the cell population being tested. In experiments involving biological response modifiers, which are often cell-type specific, the loss of any cell type could significantly affect the results. The micro assay more reliably reflects the in vivo environment because no selective destruction occurs. If the NK activity of nonadherent cells, or the effects of a specific treatment on this population of cells were of interest, the bulk assay would be the better assay.

## Advantages of the Micro Assay

The micro assay has numerous advantages over the bulk assay. The most significant advantage is that the micro assay reflects the in vivo situation more accurately than does the bulk assay. The reason for this is that the micro assay measures the cytotoxicity of the cells initially seeded, without selection for or against a specific subpopulation of cells, whereas the bulk assay actually selects against the adherent cells, a result of the cell transfer required in this assay. The micro assay also reduces the cost of an assay by reducing the technician time and the volume of media, reagents, and disposable supplies required. In addition, the effects of various biological response modifiers or other soluble factors can be easily determined. In the micro assay, it is easy to alter the constituents of the media within an assay by adding different components to

different wells. Due to the decreased volume of media, the amount of these factors required is also decreased

The IL-2 Inhibitor

Regulation of the immune response is highly complex. The existence of multiple IL-2 inhibitors or multiple mechanisms of IL-2 down regulation has been postulated by several investigators (25, 26, 28). This is logical since IL-2 has such a key role in the immune response that the regulation of its production and degradation would also regulate the immune response. It also follows that the mechanism(s) of regulating IL-2 concentrations would include a down-regulatory mechanism in addition to the well-known up-regulatory signals, to prevent overactivity of IL-2. Since IL-2 has a variety of actions in the immune system, it may be that different control mechanisms exist for its different functions. Such a system of control would allow for the down-regulation of B cell responses without effecting the T cell responses, or vice versa. In a similar manner, the NK cells could be activated without activation of T cells or B cells. The IL-2 inhibitors could have any of a variety of methods of controlling IL-2 function:

1) Competition between the IL-2 and the IL-2 inhibitor, which may be an IL-2 analogue.
2) Steric blockage of the IL-2 receptor

3) Blockage of the IL-2 active site, preventing its binding to the IL-2 receptor. The inhibitor may mimic IL-2 receptor structure.

4) Effect on the events occurring after the IL-2 binds to the membrane-bound IL-2 receptor, such as signal transduction or the action of a second messenger.

## Possible Source of IL-2 Inhibitor

Hardt et al. (29) postulated that the IL-2 inhibitor was produced by T suppressor cells. They first showed that nude mouse serum lacks IL-2 inhibitory activity, and correlated this with the lack of thymus dependent cells (T cells) in the nude mouse. They then demonstrated that the IL-2 inhibitor was produced by an Lyt 2,3+ cell population, which contained suppressor cells. As further evidence of the role of suppressor cells in the production of IL-2 inhibitor, Hardt et al. (29) also demonstrated that the production of IL-2 inhibitor was sensitive to cyclophosphamide, which destroys suppressor cell activity. Klassen et al. (25) confirmed the lack of IL-2 inhibitory activity in nude mouse serum when measured in an IL-2 dependent proliferation assay, but reported inhibition of IL-2 enhanced NK activity by the same nude mouse serum. These results were thought to indicate different mechanisms of IL-2 inhibitor action (25); however, they may indicate the different sensitivities of the two assays. In fact, our observations suggest the two assays do have different sensitivities to the IL-2 inhibitor, with the IL-2 boosted

NK assay more sensitive to the effects of the IL-2 inhibitor. Ten percent activated serum (v/v) caused 88% inhibition of IL-2 boosted NK cytotoxicity (page 54), but it required 25% serum to produce 85% inhibition of IL-2 dependent proliferation (Table 8). The nude mouse serum may contain the IL-2 inhibitor at lower levels than in normal mouse serum. The nude mouse does not completely lack T cells as thought by Hardt, et al.; there are actually low levels of these cells present in the nude mouse (reviewed in 53), which may provide low levels of IL-2 inhibitor.

## Activation of IL-2 Inhibitor

The activation of the IL-2 inhibitor in serum stored at room temperature for a few days is an interesting phenomenon and has never been reported in the literature. Although the exact mechanism of this activation is unknown, possibilities include:

1) A circulatory form of IL-2 inhibitor that has low activity, but is converted to the more active form by enzymes or other factors present in the serum. This process may be analogous to an *in vivo* activation which would provide greater action at sites requiring IL-2 down-regulation.
2) Some of the IL-2 inhibitor is bound to IL-2 in the circulation, preventing the action of IL-2. These complexes are degraded *in vitro*, perhaps due to the lability of IL-2 at room temperature. More free IL-2 inhibitor is thus available to block the IL-2 function within the assay.

The serum was sterile prior to activation, ruling out the contribution of bacterial enzymes in the activation process.

This activation phenomenon contributed to the difficulty encountered in locating a suitable IL-2 inhibitor-negative control. Activated fetal bovine serum contained IL-2 inhibitory activity. The presence of an IL-2 inhibitor in bovine serum was not surprising, as Viet and Michael reported finding immunosuppressive activity in sera from several mammalian sources such as mice, rats, gerbils, guinea pigs, rabbits and monkeys (54). Normal human serum (38, 40) and bovine serum (55) also contained IL-2 inhibitory activity. The low levels of IL-2 inhibitor in fetal bovine serum correlated with the low levels in fetal and newborn mouse serum as reported by Hardt et al (29). These results suggested the IL-2 inhibitor is conserved throughout mammalian evolution, and lacks species specificity, as does the IL-2 it inhibits.

## IL-2 Inhibitor Purification

The activation phenomenon continued during the purification of the IL-2 inhibitor. The yield of inhibitor increased dramatically when the SAS/pH ppt was formed (171% of the original serum). It was not surprising that the activation phenomenon continued, since the activation of the serum occurred at room temperature and the purification process included overnight dialysis at room temperature.

However, the yield declined significantly (one hundred-fold) as the inhibitor was further purified and specific activity increased (ten-fold).

When DEAE chromatography was used in an attempt to further purify the SAS/pH ppt, the IL-2 inhibitor eluted over a broad range of NaCl concentrations, ranging from 0.15 M to 0.35 M, and the majority eluted at 0.25 M. The location of this peak of IL-2 inhibitory activity contradicted the data reported by Male et al. (43). They reported the elution of the IL-2 inhibitor just prior to albumin, whereas the inhibitor discussed here eluted after albumin. The reason for the difference in the elution profiles was unknown, but further indicated that DEAE chromatography was not a suitable method for use in the purification of IL-2 inhibitor. These results suggested the existence of multiple IL-2 inhibitors, which may consist of polymers of IL-2 inhibitor, complexes containing IL-2 inhibitor and other compounds, or totally unrelated proteins. The presence of IL-2 inhibitor complexes in synovial fluid was demonstrated by Symons et al. (38), so the presence of similar complexes in the serum would not be unusual.

## Antibody Production and Use

In order to further characterize IL-2 inhibitor, IL-2 inhibitor antiserum was produced to immunoaffinity purify the inhibitor. The antisera obtained from the rat immunized with the IL-2 inhibitor had neutralizing activity after the initial boost, which decreased with

subsequent boosts. The antigen for the third boost was immunoaffinity purified inhibitor (using antibodies obtained after the second boost), This change in the antigen used for boosting may have caused the proliferation of a different population of B cells which did not produce neutralizing antibodies. When the semi-purified antibodies, which lacked neutralizing activity, were bound to hydrazide gel, and incubated with the sample containing IL-2 inhibitor, IL-2 inhibitor was removed from serum and from the SAS/pH ppt. The IL-2 inhibitor was more effectively removed from the serum than from the SAS/pH ppt. After the serum was passed through the gel twice, more than half of its IL-2 inhibitory activity had been removed. The serum components that were eluted from the immunoaffinity column inhibited 49% of IL-2 boosted cytotoxicity, indicating the antiserum contained IL-2 inhibitor-specific antibodies which were not neutralizing antibodies. However, when the inhibitor-enriched sample (SAS/pH ppt) was passed over the column, the eluted components inhibited only 17% of the IL-2 boosted cytotoxicity. This was surprising, since the SAS/pH ppt had a much higher specific activity. The difference in the inhibitory activity of the eluted material was not due to a difference in protein concentration, as might be thought, since the protein concentration of the two was almost the same (600 ug/ml and 500 ug/ml). A possible cause of the difference in the inhibitory activity of the two eluted fractions may have been the inactivation of the inhibitor from the SAS/pH ppt that bound to the affinity column by exposure to the low pH (2.3) of the glycine-HCl. In contrast, the inhibitor present in serum may have been protected by other protein components in the serum, such

as albumin. This explanation does not explain why the nonbound components of the SAS/pH ppt caused 75% inhibition, while the nonbound components of the serum caused 37% inhibition. If the inhibitor were damaged after it was removed from the sample, why wasn't there a greater loss in the inhibitor activity of the SAS/pH ppt? One explanation which would address this question is that the IL-2 inhibitor aggregated with itself or other proteins, during the purification. This aggregation may have caused a conformational change in the inhibitor, such that the antibody binding affinity was altered, but not the inhibitor's function. Thus the inhibitor would remain in the nonbound fraction of the SAS/pH ppt, but would be in the bound fraction of the serum, causing a decrease in the inhibitory activity of the serum's nonbound fraction.

## Summary

The purpose of this study was to partially purify IL-2 inhibitor and produce antisera to the IL-2 inhibitor, using the IL-2 inhibitor-enriched sample as the immunogen. In order to monitor the purification, we developed the micro assay for IL-2 boosted NK activity. This new assay method was compared to the traditional bulk assay, to confirm the validity of the micro assay. The evaluation of comparability between the two assays was based on the number of cells that survived incubation and the lytic activity of those cells. Our results indicated that the micro assay was a better assay to measure the lytic activity of the total cell population than the bulk assay.

This conclusion was based on the fact that the bulk assay selected against the adherent cell population, which we demonstrated had higher lytic activity than the nonadherent cell population, whereas the micro assay did not select for or against any specific cell population. The micro assay was then used to monitor the purification of IL-2 inhibitor.

Prior to purification, preliminary experiments were done to determine the optimal source of the IL-2 inhibitor. The results indicated that the IL-2 inhibitor in serum was activated at room temperature, and that the levels of IL-2 inhibitor varied depending on the mouse strain. Therefore, the C3H strain was selected as the source of serum and the serum was activated prior to purification. The IL-2 inhibitor was partially purified using various precipitation techniques, such as saturated ammonium sulfate precipitation, and pH-dependent precipitation. The IL-2 inhibitor-enriched sample was fractionated by DEAE chromatography; however, when the fractions were assayed for IL-2 inhibitory activity, this activity was not well isolated. Therefore, DEAE chromatography was not thought to be a feasible method for IL-2 inhibitor purification.

One of the goals of this work was the development of IL-2 inhibitor antisera. Therefore, a rat was immunized with the precipitate that formed when the SAS/pH ppt was dialyzed against 5 mM Tris-HCl. Semi-purified antisera neutralized approximately one-half the IL-2 inhibitory activity in the SAS/pH ppt and was also used to

affinity purify the IL-2 inhibitor. The affinity purified material was used as an antigen for boosting and the new antisera lacked the neutralizing activity. This was thought to be due to the difference in the antigen used for boosting, which may have caused the proliferation of a separate subpopulation of B cells. Although there was no neutralizing activity, the antisera was used to prepare an immunoaffinity column. The serum components that were eluted from this column effectively inhibited IL-2 boosted NK cytotoxicity, indicating that this antisera did contain antibodies to IL-2 inhibitor. Therefore, the goals of this study were met; the IL-2 inhibitor was partially purified and an IL-2 inhibitor antisera was produced.

## Recommendations for Future Experiments

As research of the IL-2 inhibitor continues, further purification of the IL-2 inhibitor is needed. A purified inhibitor would make it possible to determine the following:

1) The molecular weight, isoelectric point, and other biochemical characteristics of the IL-2 inhibitor. Comparison of its physicochemical properties to those of known proteins will determine if the IL-2 inhibitory action is a secondary function of a known protein, or if the IL-2 inhibitor is a previously unidentified protein.

2) The mechanism of activation. Activation may be due to the effects of other serum components, or internal enzymatic activity may cause self-activation.

3) The effect of inhibitor on the binding of IL-2 to the IL-2 receptor. Such information will show whether or not the inhibitor blocks binding of IL-2 to its receptor, and thus aid in the determination of the mechanism of the IL-2 inhibition.

4) The effect of IL-2 inhibitor in vivo. Infusion of the IL-2 inhibitor into autoimmune mice, raising serum concentrations, may alleviate the SLE-like symptoms in these mice. If effective, such treatment could then be used in human SLE patients.

Although the IL-2 inhibitor is thought to have a significant role in the immune process, there has been little done to characterize it, or to determine its action. Just as IL-2 has been well studied, the IL-2 inhibitor must be investigated in further detail. This further investigation is made imperative by the effect of the IL-2 inhibitor on IL-2 function, and the delicate balance that must exist between the two. The mechanism of the activation phenomenon, the mode of inhibition, and the source of the inhibitor are all unknown and must be determined for full understanding of IL-2 inhibitor. Further IL-2 inhibitor research will provide medically significant data and impact on millions of lives in the future treatment of cancer and autoimmune diseases.

# Appendix

## Abbreviations

| | |
|---|---|
| APC | antigen presenting cell |
| BDF1 | B6D2F1 - a mouse strain |
| BSA | bovine serum albumin |
| $cm^2$ | square centimeter |
| cpm | counts per minute |
| C:SA | cell to surface area ratio |
| Con A | concanavalin A, a mitogen |
| CTL | cytotoxic T lymphocyte |
| CTLL-2 | a cell line dependent on IL-2 for survival |
| C3H | C3H/HeN - a mouse strain |
| C57 | C57Bl/6 - a mouse strain |
| d/d water | distilled and deionized water |
| DEAE | diethylaminoethane |
| EDTA | ethylenediaminetetraacetic acid |
| EHAA | a tissue culture media used in the CTLL-2 proliferation assay |
| FBS | fetal bovine serum |
| FICA | Freund's incomplete adjuvant |
| g | gram |

| | |
|---|---|
| HBSS | Hank's Balanced Salt Solution |
| HPLC | high pressure liquid chromatography |
| IgG | immunoglobulin with gamma heavy chains |
| IL-2 | interleukin-2 |
| Inc Con | incubation concentration |
| kDa | kilodalton |
| LAK | lymphokine-activated killer |
| LU | lytic unit |
| M | molar |
| mg | milligram |
| MHC | major histocompatibility complex |
| ml | milliliter |
| mls | minor histocompatibility locus |
| mM | millimolar |
| m.w. | molecular weight |
| NBS | newborn bovine serum |
| NK | natural killer |
| NMS | normal mouse serum |
| OD | optical density |
| PBS | phosphate buffered saline |
| PDP | the post dialysis precipitate, formed when the pH ppt was dialyzed against 5 mM Tris-HCl overnight |
| pH ppt | the pool of precipitates formed when the pH of the water supernatant was lowered to 4.0, and then raised in increments to 4.5, 5.0, 5.5, and 6.0. The pool was solubilized in NaOH. |

| | |
|---|---|
| ppt | precipitate |
| RA | rheumatoid arthritis |
| RID | radial immunodiffusion |
| rIL-2 | recombinant IL-2 |
| RTP | rat T cell polyclone, a type of IL-2 |
| SAS | saturated ammonium sulfate |
| SAS/pH ppt | the 50% SAS precipitate of the pH ppt |
| SD | standard deviation |
| SE | standard error of the mean |
| SLE | systemic lupus erythematosus |
| SPF | specific pathogen free |
| sup | supernatant |
| TCGF | A cell growth factor, another term for IL-2, used here as manufacturer's name for a specific preparation of IL-2 |
| TC-10 | tissue culture media with 10% serum, used to culture cells, and as media for NK assays |
| Tris-HCl | a buffer containing tris(hydroxymethyl)aminomethane and HCl |
| Water ppt | The precipitate formed when serum was dialyzed against water overnight |
| U/ml | units in each milliliter |

| | |
|---|---|
| um | micron |
| ug | microgram |
| ul | microliter |
| x g | times the gravitational force of the earth |
| YAC-1 | a tumor cell line used as NK or LAK target cells |

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