Toxoplasma inhibitory factor and interleukin 2 : assessment of their effects on toxoplasma gondii replication within a continuous macrophage-like cell line
by Anita Susan Hagemo

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Veterinary Science
Montana State University

Abstract:
A rapid, reproducible, and accurate microassay was developed to study the effects of soluble lymphocyte products on Toxoplasma replication with in parasitized cells. The assay system is based upon radioactive uracil incorporation into nucleic acids of intracellular T. gondii and employs a homogeneous macrophage-like cell line, RAW 264. Supernatant fluids prepared from antigen- and Iect in-stimulated murine spleen cells effectively inhibited Toxoplasma replication within RAW 264 cells and induced proliferation of the interleukin 2 (IL2)-responsive T-cell line, CTLL2C175. These results indicated that both Toxoplasma inhibitory factor (TIF) and IL2 were present in unfractionated supernatant fluids. Removal of IL2 activity from antigen- and lectin-prepared supernatant fluids after adsorption by CTLL 2C17 5 cells had little effect on inhibiting Toxoplasma replication within RAW 264 cells. Supernatant fluids prepared from concanavalin A-stimulated FS6-14.13 cells, an IL2-producing T-cell hybridoma, induced proliferation of CTLL 2C17 5 cells, but failed to inhibit T. gondii replication within RAW 264 cells. Fractionation of lectin-prepared supernatant fluids by gel-filtration using Sephadex G-100 column chromatography showed that TIF activity eluted in a broad peak in the region of 39,000 to 58,000 daltons, whereas IL2 activity eluted in the molecular size range of 28,000 to 30,000 daltons. Therefore, both TIF and IL2 were found to be present in the same antigen- and lectin-prepared supernatant fluids and experimental results suggest that these soluble lymphocyte products are two distinct and unique molecules.

TOXOPLASMA INHIBITORY FACTOR AND INTERLEUKIN 2: ASSESSMENT OF THEIR EFFECTS ON *TOXOPLASMA GONDII* REPLICATION WITHIN A CONTINUOUS MACROPHAGE-LIKE CELL LINE

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12/2/82
Date

Paul C Baker
Chairperson, Graduate Committee

Approved for the Major Department

12/2/82
Date

David M Young
Head, Major Department

Approved for the College of Graduate Studies

12-6-82
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Michael S. Malone
Graduate Dean

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ABSTRACT

A rapid, reproducible, and accurate microassay was developed to study the effects of soluble lymphocyte products on Toxoplasma replication within parasitized cells. The assay system is based upon radioactive uracil incorporation into nucleic acids of intracellular T. gondii and employs a homogeneous macrophage-like cell line, RAW 264. Supernatant fluids prepared from antigen- and lectin-stimulated murine spleen cells effectively inhibited Toxoplasma replication within RAW 264 cells and induced proliferation of the interleukin 2 (IL2)-responsive T-cell line, CTLL2C115. These results indicated that both Toxoplasma inhibitory factor (TIF) and IL2 were present in unfractionated supernatant fluids. Removal of IL2 activity from antigen- and lectin-prepared supernatant fluids after adsorption by CTLL2C115 cells had little effect on inhibiting Toxoplasma replication within RAW 264 cells. Supernatant fluids prepared from concanavalin A-stimulated FS6-14.13 cells, an IL2-producing T-cell hybridoma, induced proliferation of CTLL2C115 cells, but failed to inhibit T. gondii replication within RAW 264 cells. Fractionation of lectin-prepared supernatant fluids by gel-filtration using Sephadex G-100 column chromatography showed that TIF activity eluted in a broad peak in the region of 39,000 to 58,000 daltons, whereas IL2 activity eluted in the molecular size range of 28,000 to 30,000 daltons. Therefore, both TIF and IL2 were found to be present in the same antigen- and lectin-prepared supernatant fluids and experimental results suggest that these soluble lymphocyte products are two distinct and unique molecules.

CHAPTER 1

INTRODUCTION

Toxoplasma gondii is a coccidian protozoan capable of infecting a wide range of vertebrate cells (2,30,31). Although the mechanism of entry of this obligate intracellular parasite into host cells was demonstrated to occur by phagocytosis (52,80), the predominant means of cellular entry was recently established to occur by active invasion (80). Intracellular tachyzoites reside in cytoplasmic vacuoles and replicate asexually with a generation time of five to ten hours (52), leading eventually to cell rupture and liberation of parasites, which then infect adjacent cells. Even though the cells are equipped with extensive biochemical machinery including Golgi apparatus, ribosomes, and endoplasmic reticulum (40), extracellular replication of *T. gondii* was never reported.

Although macrophages are highly phagocytic scavenger cells that kill and degrade many microbes, *T. gondii* was reported to survive and multiply within these mammalian cells (53). After phagocytosis of this intracellular parasite by macrophages, it was demonstrated that living *T. gondii* alter the phagosomal membrane such that fusion with lysosomes does not occur (52). In contrast, dead

Toxoplasma or Toxoplasma coated with antibody are readily engulfed and degranulated by lysosomal constituents after phagocytosis by macrophages (52).

The mechanism(s) of resistance to T. gondii is not completely understood. Humoral immune reactions are of diagnostic importance in infections with Toxoplasma, but the antibody titer does not necessarily signify immune status against the agent (27). It was shown that antibody inactivates or lyses Toxoplasma in the presence of a complement-like "accessory factor" obtained from Toxoplasma antibody-free human serum, which is the basis of the Sabin-Feldman dye-test (93). Antibody is effective, however, only against free or extracellular organisms, whereas the intracellular organisms are protected. Since most of the life cycle takes place intracellularly, humoral immunity does not play a major role in protection.

Several investigators (12,28,48,54,87) reported that cell-mediated immunity is important in resistance to infection with this obligate intracellular pathogen. Lymphoid cells adoptively transferred from Toxoplasma-immune donors confer specific immunity to Toxoplasma infection in hamsters (28). In addition, activated macrophages are important effectors of immune protection to infection with T. gondii (74,87). Activated macrophages from mice chronically

infected with I. gondii had an enhanced capacity for killing the parasite when compared with macrophages from normal mice (87).

Shirahata and coworkers (101) observed that nonimmune peritoneal mouse macrophages cultivated in vitro with supernatant fluids prepared from lymphocytes of Toxoplasma-immune mice stimulated with Toxoplasma lysate antigen significantly inhibit the intracellular replication of I. gondii. In contrast, a marked proliferation of organisms was noted in the same cells incubated with supernatant fluids obtained from immune lymphocytes cultured without antigen. It was later shown that this antitoxoplasma activity observed is due to a soluble lymphocyte product, called Toxoplasma growth inhibitory factor (Toxo-GIF) (102) or Toxoplasma inhibitory factor (TIF) (55), present in antigen-prepared supernatant fluids. This lymphokine, with a reported molecular weight of 38,000 to 55,000 daltons (79, 103), can also be generated by stimulating lymphocytes of nonimmune animals with the T-cell lectin, concanavalin A (Con A) (102).

In addition to sharing roughly similar molecular weights, the production of TIF occurs under conditions similar to those required for production of another T-cell derived product previously known as T-cell growth factor (TCGF) (35), and currently referred

to as interleukin 2 (IL2) (1). For example, TIF was reported to be elaborated by *in vitro* reactivation of lymphocytes from dye-test negative, streptokinase-streptodornase (SK-SD) positive individuals with SK-SD, or from lymphocytes of dye-test positive individuals with *Toxoplasma* lysate antigen (5). Similarly, production of IL2 was demonstrated to result from *in vitro* reactivation of lymphocytes from ovalbumin-immune animals with ovalbumin (56). Moreover, both TIF and IL2 were reported to be generated by stimulating murine spleen cells with mitogenic doses of Con A (35,102).

Thus, in the present study it was hypothesized that TIF, a lymphokine which inhibits the intracellular replication of *T. gondii* within parasitized phagocytic cells (101), might be the same as IL2, a lymphokine which was previously reported to only activate other T-cells (35). To determine whether TIF and IL2 were actually one and the same molecule, a rapid, reproducible, and accurate microassay using a homogeneous macrophage-like cell line, RAW 264, was developed to study the effects of these soluble lymphocyte products on *Toxoplasma* replication within parasitized cells. This microassay, which was based upon the observation by Pfefferkorn and Pfefferkorn (85) and Schwartzman and Pfefferkorn (96) that radioactive uracil is not significantly incorporated into nucleic acids of host cells, but is incorporated into nucleic acids of

actively replicating tachyzoites, has proven to be a useful method for determining the relationship among TIF and IL2. Experimental results indicated that (1) both TIF and IL2 were present in the same antigen- and lectin-prepared supernatant fluids; and (2) these soluble lymphocyte products are in fact two distinctly separate molecules.

CHAPTER 2

LITERATURE REVIEW

Toxoplasma gondii, an obligate intracellular coccidian protozoan, is a common cause of infection and disease in many vertebrates including man (2,31). Serological studies indicate that about 40% of the United States population has had toxoplasmosis by age 50 (31). Although toxoplasmosis is commonly asymptomatic, it can produce serious illness in congenitally infected children (18,25, 69) and in immunosuppressed patients (14,32,81). In veterinary medicine, it is a significant cause of abortions in sheep (10) and is therefore of economic importance.

The life cycle of *T. gondii* is similar to most coccidia because it has an enteroepithelial cycle in a specific host, resulting in the formation of oocysts (29). Only domestic cats and certain other members of the family *Felidae*, the definitive hosts for *T. gondii*, were reported to produce *Toxoplasma* oocysts (70).

Unlike most other coccidia, *T. gondii* has an extra-intestinal or tissue cycle. These stages, which also occur in cats, appear to constitute the entire life cycle in nonfelines (31). In acute visceral infections, rapidly

dividing Toxoplasma (tachyzoites) multiply in any cell of the intermediate host (nonfelines) and in nonintestinal epithelial cells of the definitive host (21). Cysts containing slowly multiplying organisms (bradyzoites) are characteristic of chronic infections and occur in the brain, heart, skeletal muscle, and other tissues (31).

The three known infective stages of T. gondii are bradyzoites (in cysts), tachyzoites (in pseudocysts), and sporozoites (in oocysts) (21). It was reported that the three modes of Toxoplasma transmission, carnivorism, fecal contamination, and transplacental or congenital infection, are linked to the life cycle (29). These modes of transmission involve the different stages as follows: ingestion of bradyzoites, tachyzoites, or both; contamination with feline feces containing sporozoites of sporulated oocysts; or transplacental infection of the fetus with tachyzoites after ingestion of encysted bradyzoites or sporulated oocysts by the mother (21).

T. gondii is capable of infecting a wide range of vertebrate cells (30). Intracellular tachyzoites reside in cytoplasmic vacuoles and replicate asexually with a generation time of five to ten hours (52), leading eventually to cell rupture and liberation of parasites, which then infect adjacent cells. Even though the cells are equipped with extensive biochemical machinery including

mitochondria, Golgi apparatus, ribosomes, and endoplasmic reticulum (40), extracellular replication of I. gondii was never reported.

Although the mechanism of entry of I. gondii into host cells was demonstrated to occur by phagocytosis (52, 80), the predominant means of cellular entry was recently established to occur by active invasion (80). When examined ultrastructurally, it was clear that different events occur during these two entry processes (80). Nichols and O'Connor (80) reported that microfilament aggregates are present beneath attached organisms during phagocytosis, whereas such aggregates are absent from phagocytic cells during invasion. The absence of subplasmalemmal filament aggregates and disruption of host cell membrane during invasion indicated that cellular penetration is due to effects exerted by the parasite (80). Although lacking definitive evidence, membrane disruption seen during invasion is speculated to be due to the release of lytic enzymes from specialized organelles in I. gondii called rhoptries (82).

Another feature distinguishing phagocytic from invasive entry is the presence of two distinct types of parasite-containing vacuoles in Toxoplasma infected cells. During phagocytosis of I. gondii by host cells, the parasites are immediately enclosed in typical phagocytic vacuoles (80). In contrast, as parasites disrupt the host

cell plasma membrane during invasion, they are not immediately enclosed in intact vacuoles. Instead, large parasitophorous vacuoles quickly form around the parasites which invade (80). Vacuoles formed after invasion have tubules protruding into their cavities, whereas phagocytic vacuoles have less free space around the parasites and absence of tubules (80). Since during invasion there is no evidence of an invaginated host cell plasmalemma external to the unit membrane of the parasite (80), the formation of a classical phagocytic vacuole does not occur during this entry process.

Although macrophages are highly phagocytic scavenger cells that kill and degrade many microbes, I. gondii was reported to survive and multiply within these mammalian cells (53). After phagocytosis of this intracellular parasite by macrophages, it was demonstrated that living I. gondii alter the phagosomal membrane such that fusion with lysosomes does not occur (52). In contrast, dead Toxoplasma or Toxoplasma coated with antibody are readily engulfed and degranulated by lysosomal constituents after phagocytosis by macrophages (52).

The mechanism(s) by which living I. gondii can survive within phagocytic cells remains largely unknown. Since lysosomal constituents are not delivered to phagocytic vacuoles harboring living organisms, I. gondii is speculated to secrete some substance that alters the vacuolar

membrane and prevents lysosomal fusion (53). Jones and Hirsch (53) suggested that the fate of intracellular Toxoplasma may reflect cellular factors in which there are variations in the capacity of macrophages to destroy this pathogen. However, when highly activated macrophages containing large numbers of lysosomal granules were compared with unstimulated macrophages containing fewer granules, no differences were observed in the capacity of these cells to kill ingested organisms (53).

Jones and Hirsch (53) observed that early after phagocytosis and throughout the course of parasite multiplication in the cell, phagocytic vacuoles harboring living organisms are enveloped by endoplasmic reticulum and mitochondria. The mechanism accounting for this phenomenon is unknown, but these investigators suggested that attraction for these cytoplasmic organelles might reflect an alteration in the vacuolar membrane.

Another morphologic feature indicating an alteration in the vacuolar membrane by I. gondii is the presence of microvillus protrusions of the membrane into the vacuole forming arrays of small vermiform structures (100). Since these microvillus protrusions markedly increase the surface area of the vacuolar membrane, Jones and Hirsch (53) suggested that they may serve a role in transferring to the phagocytic vacuole some cytoplasmic factors required for parasite survival or multiplication.

Several different mechanisms might be responsible for parasite survival in phagocytic cells, but morphologic evidence indicates that vacuolar membrane alteration by I. gondii may be of importance in relation to the absence of lysosomal fusion (53). Such membrane alteration may also play a role in protecting the host cell or in nourishing the parasite.

The mechanism(s) of resistance to I. gondii is not completely understood. Humoral immune reactions are of diagnostic importance in infections with I. gondii, but the antibody titer does not necessarily signify immune status against the agent (27). It was reported that antibody inactivates or lyses Toxoplasma in the presence of a complement-like "accessory factor" obtained from Toxoplasma antibody-free human serum, which is the basis of the Sabin-Feldman dye-test (93). Antibody is effective only against free or extracellular organisms, whereas the intracellular parasites are protected.

Studies on potential immune mechanisms associated with resistance to I. gondii showed that at least two types of humoral antibodies, complement-fixing and cytoplasm-modifying, are elicited by animals inoculated with live parasites (114). Subsequent immunity to reinfection was reported to develop in these animals (114). However, only one type of humoral antibody, the cytoplasm-modifying antibody of Sabin and Feldman (93), was found to be elicited by animals

inoculated with heat-killed Toxoplasma antigen, and no immunity to reinfection develops (58).

Cutchins and Warren (15) reported that a cytoplasm-modifying antibody titer and protection against reinfection are demonstrated in guinea pigs inoculated with a killed vaccine. They also found that inoculation of these animals with a killed vaccine, to which an adjuvant had been added, results in the production of a higher cytoplasm-modifying antibody titer and active protection against reinfection, as well as a substantial complement-fixing antibody titer.

According to the report by Foster and McCulloch (27), neither vaccination nor recovery from infection completely prevents the persistence of T. gondii in various organs of guinea pigs. These investigators showed that even in the presence of very high antibody levels, it is still possible to isolate living Toxoplasma from the tissues of these animals. It was therefore suggested that immunity to toxoplasmosis in guinea pigs and probably man involves a restriction or repression of growth of the parasites, rather than complete destruction (27).

Others later showed that immunization of mice with a few cysts of a low virulent strain of T. gondii elicits both a humoral and cellular immunity which are manifested in a resistance to intraperitoneal challenge with a virulent strain of the parasite (54,98). Antibody titers, detectable

by the Sabin-Feldman dye-test, are then evident throughout the life of the animal.

Cell-mediated immunity is important in resistance to infection caused by intracellular pathogens (12,28). Lymphoid cells adoptively transferred from Toxoplasma-immune donors confer specific immunity to Toxoplasma infection in hamsters (28). In addition, activated macrophages are important effectors of immune protection to infection with I. gondii (74,87). Activated macrophages from mice chronically infected with I. gondii had an enhanced capacity for killing this parasite when compared with macrophages from normal mice (87). The mechanism whereby these cells acquire their enhanced ability to inhibit intracellular replication or kill this pathogen is still unclear.

A number of investigators (4,12,47,48,54,59,98,101) reported that macrophages cocultivated with sensitized lymphocytes and mitogen or antigen, to which the lymphocytes were previously sensitized, acquire the ability to kill or inhibit replication of the obligate intracellular parasite, I. gondii. Jones and coworkers (54) demonstrated that peritoneal macrophages obtained from Toxoplasma infected animals are unable to kill or inhibit the intracellular replication of I. gondii. Reexposure of these cells in vitro to lymphocytes of Toxoplasma-immune mice and specific antigen (54) or supernatant fluids obtained from immune lymphocytes stimulated with Toxoplasma

lysate antigen (12) restores the ability of immune macrophages to inhibit the organism. Exposure of normal macrophages to the same lymphocytes and antigen or supernatant fluids does not result in macrophage activation since no antitoxoplasma activity is observed in these cells (12,54). Thus, for induction of immunity, these investigators reported that macrophages must be in a state of "stimulate" before they are exposed to such antigen-stimulated immune lymphocytes or their products.

Shirahata et al. (101) showed that when glycogen-induced peritoneal macrophages from nonimmune mice are cultured *in vitro* with supernatant fluids prepared from lymphocytes of *Toxoplasma*-hyperimmunized mice stimulated with *Toxoplasma* lysate antigen, significant inhibition of intracellular replication of *T. gondii* results. In contrast, a marked proliferation of organisms was noted in the same cells incubated with supernatant fluids obtained from immune lymphocytes cultured without antigen (101). These investigators reported that stimulation of immune lymphocytes with specific antigen is necessary for the production of biologically active products capable of conferring cultured nonimmune macrophages the ability to kill *T. gondii* or inhibit its intracellular replication (101).

According to the report by Sethi and coworkers (97), about 80% of the macrophage population obtained from thioglycollate-stimulated nonimmune mice could kill and digest

intracellular *T. gondii* when cocultivated with *Toxoplasma*-immune lymphocytes and *Toxoplasma* lysate antigen. They also noted that only 20% of the macrophage population obtained from unstimulated nonimmune mice were able to destroy intracellular organisms, although the cells were cultured in the presence of the same lymphocytes and antigen.

In human cases, Borges and Johnson (12) reported that supernatant fluids obtained from antigen-stimulated lymphocytes of *Toxoplasma*-immune subjects induce macrophages from immune and nonimmune subjects to inhibit intracellular replication of *Toxoplasma*. They also showed that these macrophage populations cocultivated with *Toxoplasma*-immune lymphocytes and specific antigen inhibit parasite replication as effectively as antigen-stimulated immune lymphocyte supernatant fluids.

Anderson et al. (5) also demonstrated that human macrophages incubated with supernatant fluids prepared from antigen-stimulated lymphocytes of individuals who were positive in the Sabin-Feldman dye-test acquire the ability to inhibit intracellular *T. gondii* replication. Supernatant fluids obtained from nonsensitized lymphocytes of Sabin-Feldman dye-test negative subjects and *Toxoplasma* lysate antigen are ineffective. However, incubation of macrophages with supernatant fluids prepared from lymphocytes of dye-test negative, streptokinase-streptodornase (SK-SD) positive individuals when cultured in the presence

of SK-SD or concanavalin A (Con A) were found to induce a significant, but lesser activation (5).

According to the report by McLeod and Remington (66), a variety of *in vivo* and *in vitro* conditions initiates or modulates stimulation of macrophages by different mechanisms, leading to specific or nonspecific effects. These investigators showed that immunization of mice with *Corynebacterium parvum*, a stimulus unrelated to *Besnoitia jellisoni* and *T. gondii*, results in the ability of the host's macrophages to nonspecifically kill both protozoa. Nonspecific microbicidal processes of macrophages were also observed in cells stimulated with a variety of agents and substances including infection with intracellular microorganisms (9,26,34,49,61,115), endotoxin (3), RNA (6), and soluble lymphocyte products elaborated from antigen- or mitogen-stimulated lymphocytes (4).

Biologically active moieties, other than soluble antibody, present in the supernatant fluid of antigen- or lectin-stimulated lymphocytes, are termed "lymphokines" (88). These mediators of cellular immunity are soluble products of lymphocytes and are responsible for the multiple effects of a cellular immune reaction. Regarding lymphokines in relation to *Toxoplasma* infection, Shirahata and coworkers (101) reported that in response to antigenic stimulation, spleen cells from *Toxoplasma*-immune mice elaborate a soluble factor which is capable of inhibiting

the intracellular replication of T. gondii within immune and nonimmune peritoneal mouse macrophages. This soluble factor, termed Toxoplasma growth inhibitory factor (Toxo-GIF) (102) or simply Toxoplasma inhibitory factor (TIF) (55), can also be generated by stimulating spleen cells from nonimmune animals with the T-cell lectin, Con A (102).

In order to determine the molecular characteristics of TIF, supernatant fluids prepared from lectin- and antigen-stimulated murine spleen cells were fractionated by gel-filtration using Sephadex G-100 column chromatography and assayed for TIF activity (79,103). Factor activity was reported to elute from Sephadex G-100 in the molecular size range of 38,000 to 55,000 daltons (79, 103). When factor activity in these column fractions was further examined after purification by isoelectric focusing (IEF, pH 4.0 - 7.0 gradient), TIF activity was found to fractionate with an isoelectric point of pH 4.9 to 5.9 (79,103).

Chinchilla and Frenkel (13) reported that in vitro reactivation of lymphocytes from Toxplasma-immune hamsters with Toxplasma lysate antigen results in the production of a soluble factor which is capable of inhibiting the intracellular replication of T. gondii within immune and nonimmune peritoneal hamster macrophages. In contrast to murine TIF, this soluble factor, with a reported

molecular weight of 4,000 to 5,000 daltons (13), is also effective against Toxoplasma infection in hamster kidney cell and fibroblast cultures.

Antitoxoplasma activity in macrophages exposed to lymphocytes of Toxoplasma-immune mice and specific antigen or supernatant fluids obtained from these antigen-stimulated lymphocytes was previously assessed by direct microscopic observation of parasite multiplication or inhibition of multiplication (98,101,102). Since visual evaluation of Giemsa-stained preparations was both time-consuming and subject to potential observer bias, McLeod and Remington (65,67) developed a new method for evaluating the capacity of phagocytic cells to kill or inhibit multiplication of intracellular T. gondii.

This new method was based on the observation by Pfefferkorn and Pfefferkorn (85) and Schwartzman and Pfefferkorn (96) that radioactive uracil is not significantly incorporated into nucleic acids of host cells, but is incorporated into nucleic acids of actively replicating Toxoplasma tachyzoites. These investigators reported that cells infected with T. gondii incorporate much more uracil into their nucleic acids than uninfected cells and that nearly all of the label is associated with the parasites. They ascribed the observed differences in uracil incorporation in uninfected and infected cells to differing levels of uridine phosphorylase, an enzyme that converts

uracil into uridine, which is then incorporated into nucleic acids. Since intracellular Toxoplasma have substantially more uridine phosphorylase than host cells, they incorporate more uracil into their nucleic acids.

McLeod and Remington (65,67) subsequently reported that when phagocytic cells are cultured with TIF-containing supernatant fluids and are infected with T. gondii, incorporation of radioactive uracil by parasites is significantly less than identical cultures incubated with control supernatant fluids. They also found that uptake of radiolabel correlates with Toxplasma multiplication or inhibition of multiplication in Giemsa-stained preparations, and that extracellular organisms do not incorporate sufficient amounts of uracil to affect interpretation of the assay. This technique made it possible to more accurately assess antitoxoplasma activity in phagocytic cells activated in vitro by TIF-containing supernatant fluids.

Since lymphokines are produced in minute quantities, and are therefore difficult to purify, analyses of unpurified crude supernatant fluids, which could theoretically contain two or more active moieties, has led to confusion in ascertaining the relationship among soluble mediators. For example, supernatant fluids prepared from antigen-stimulated splenocytes of Toxplasma-immune mice were shown to contain a variety of biologically active substances including TIF (55), immune interferon (IF) (104), and

macrophage migration inhibitory factor (MIF) (79,103), which were distinguished primarily by their functional and biochemical characteristics.

In addition to sharing roughly similar molecular weights, the production of TIF occurs under conditions similar to those required for production of another T-cell derived product previously known as T-cell growth factor (TCGF) (35), and currently referred to as interleukin 2 (IL2) (1). For example, TIF was reported to be elaborated by *in vitro* reactivation of lymphocytes from dye-test negative, streptokinase-streptodornase (SK-SD) positive individuals with SK-SD, or from lymphocytes of dye-test positive individuals with *Toxoplasma* lysate antigen (5). Similarly, production of IL2 was demonstrated to result from *in vitro* reactivation of lymphocytes from ovalbumin-immune animals with ovalbumin (56). Moreover, both TIF and IL2 were reported to be generated by stimulating murine spleen cells with Con A (35,75). It appears that TIF, a lymphokine which inhibits the intracellular replication of *T. gondii* within phagocytic cells (101), may be similar to IL2, a lymphokine which was previously reported to only activate other T-cells (35).

Supernatant fluids prepared from Con A-stimulated murine splenocytes were shown to contain a variety of biologically active substances including T-cell growth factor (TCGF) (35), costimulator (84), T-cell replacing factor

(TRF) (94), and killer helper factor (KHF) (37). All four lymphokines were reported to act as communication signals between leukocytes (1).

Gillis and Smith (35) reported that exogenously supplied TCGF is responsible for the continuous proliferation of antigen-specific cytotoxic murine T-cells. Paetkau et al. (84) demonstrated that costimulator induces mitogenesis in primary thymocyte cultures, whereas Con A in itself is unable to stimulate these cells to proliferate. Schimpl and Wecker (94) described a soluble factor which is capable of completely replacing T-cells in the immune response of B-cells to T-cell dependent antigens. This lymphokine, termed TRF, was shown to restore the in vitro antibody response of murine B-cells to sheep red blood cells (SRBC), a T-dependent antigen in T-cell depleted or in nude mouse splenocyte cultures (94). Finally, Gillis et al. (37) reported that KHF is capable of influencing maturation, proliferation, or both of prothymocytes present in athymic nude mice. They showed that dual stimulation of nude mouse spleen cells with mitomycin-inactivated, allogeneic tumor cells and KHF induces the proliferative expansion of allo-reactive, Thy-1 antigen-positive, cytotoxic lymphocytes.

Although TCGF, costimulator, TRF, and KHF displayed distinctive functions, several investigators (38,72,112, 113) collaborated on the purification of these four

lymphokines in an attempt to establish their biochemical relationships. Supernatant fluid prepared from Con A-stimulated murine splenocytes was successively fractionated by gel-filtration using Sephadex G-100 column chromatography and isoelectric focusing (IEF, pH 4.0 - 7.0 gradient) and assayed for TCGF, costimulator, TRF, and KHF activity (24,99,113). All four activities were reported to co-purify to a single molecular weight class of 30,000 daltons and separate by charge into two components with isoelectric points of pH 4.3 and 4.9 (113). Although TRF was found to display an additional activity which fractionates in the pH range of 3.0 to 4.0 (113), all four lymphokines are considered to be the same molecule. Thus, based on the decision by participants at the Second International Lymphokine Workshop (Ermatingen, Switzerland, May 27-31, 1979), TCGF, costimulator, TRF, and KHF are collectively referred to as interleukin 2 (1).

In order to study the kinetics of IL2 production and utilization, Gillis et al. (36) developed a rapid microassay for the quantitative determination of IL2 activity in supernatant fluids prepared from lectin-stimulated mononuclear cells. Their microassay is based upon tritiated thymidine incorporation into nucleic acids of proliferating murine tumor-specific cytotoxic T lymphocytes (CTLL 1 and CTLL 2) (35) cultured in the presence of IL2-containing supernatant fluid. The relative amount of IL2 present in active