PARTIAL CHARACTERIZATION

OF

SHEEP T CELL GROWTH FACTOR

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by

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Harold Frank Latta, III. PARTIAL CHARACTERIZATION OF SHEEP T CELL GROWTH FACTOR. (Under the direction of Dr. Leonard S. English, School of Medicine) Department of Biology, July, 1983.

T cell growth factor (TCGF) is a lymphokine which plays an important role in the regulation of the immune response and has been partially characterized in man, mouse and rat. The unique property of TCGF is its ability to support the growth of activated T cells. In this work, the following characteristics of sheep TCGF were examined: the biochemical properties, the interspecies activity, and the susceptibility of sheep TCGF activity to inhibition by monosaccharides. To facilitate the characterization studies, the optimal conditions for sheep TCGF were established and were used as a standard method to prepare crude TCGF supernatants (Sn's). This standard method consisted of a 48 hour culture of peripheral blood lymphocytes in RPMI 1640 at 5 x 10^6 cells/ml in the presence of 2% fetal calf serum, 4 µg/ml Concanavalin A and 0.1 mg/ml polyethylene glycol 6000. The interspecies activity of sheep TCGF was determined by culturing human, sheep, rat and mouse activated T cells in the presence of sheep Sn. TCGF supernatants from the other species were also tested to provide a comparative study. It was found that sheep TCGF could only support the growth of activated sheep cells, but sheep activated cells could proliferate in response to human, rat and sheep TCGF's. In contrast, it was found that human TCGF could support the growth of activated cells from all 4 species, but human cells could only respond to human TCGF. The biochemical studies of sheep TCGF revealed that sheep TCGF was precipitated by 60-80% saturation of ammonium sulfate, was eluted between 0-0.15 NaCl in

DEAE-cellulose chromatography, possessed an apparent molecular weight of 30,000d and possessed isoelectric points in the range of 7.1-7.9 and 5.1-5.6. Initial studies were conducted to determine if the charge heterogeneity of sheep TCGF was due to residual ConA or was due to different molecular species of sheep TCGF. It was demonstrated that the addition of 0.1M alpha methyl mannoside (α-MM) resulted in a significant inhibition of sheep TCGF activity. Experiments were conducted which confirmed that α-MM inhibition was not due to residual ConA. This study provides strong evidence that α-MM interferes with the binding of sheep TCGF to the TCGF receptor on the surface of the activated T cells.

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Finally, I thank my wife, Lydia, and my family for all of their support. I give Lydia my deepest gratitude for the long hours she spent typing drafts and final copy and for all of the understanding she gave me during this difficult year.

DEDICATION

This thesis is dedicated with love to the memory of my grandfather, Harold Frank Latta, Sr.

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B cell	bone marrow derived lymphocyte
BCGF	B Cell Growth Factor
BSA	bovine serum albumin
CHF	Cytotoxic Helper Factor
CTL	cytotoxic T cell lines
d	daltons
DEAE	diethylaminoethyl
FCS	fetal calf serum
IEF	isoelectric focussing
IL1	Interleukin 1
IL2	Interleukin 2
KHF	Killer Helper Factor
KLH	keyhole limpet hemocyanin
2-ME	2-mercaptoethanol
α-MM	alpha-methyl mannoside
PAGE	polyacramide gel electrophoresis
PBS	phosphate buffered saline
PEG	polyethylene glycol
PMA	phorbol myristic acid
SCIF	Secondary Cytotoxic Inducing Factor
SEM	standard error of the mean
Sn	crude TCGF supernatant
T cell	thymus derived lymphocyte
TCGF	T Cell Growth Factor

T_{DTH}	delayed hypersensitivity effector T cell
T_H	helper T cell
TRF	T cell Replacing Factor
T_S	suppressor T cell
TSF	Thymocyte Stimulating Factor

INTRODUCTION

The response of the whole animal to a majority of infectious agents or foreign bodies (antigens) is dichotomous in that they induce both a cell-mediated immune response and a humoral response. The former represents activation of the T cell population alone and results in expanded clones of delayed hypersensitivity effector T cells (T_{DTH}) whereas the latter activates the B cell population resulting in the expansion of antigen-specific B cell clones and the production of antibody by plasma cells. For either class of response to occur, there is a stringent requirement for a complex series of cell interactions involving the antigen-specific responding B or T cell with helper T cells and macrophages. It is now known that many of these cellular interactions are mediated through intercellular messengers called lymphokines, also known as soluble factors. These lymphokines may be antigen-specific or nonspecific and are principally the products of helper T cells. These factors may be further divided into helper factors which act at various stages of the response to facilitate activation, proliferation or differentiation of the responding cell clones or suppressor factors which regulate the magnitude of the immune response. The subject of this thesis, T cell growth factor (TCGF) is one of these lymphokines, being produced by T cells and acting nonspecifically to support the expansion of helper T cell clones or clones of T_{DTH} cells.

The requirement for cellular collaboration in the immune response

About 25 years ago, it was shown that removal of the Bursa of Fabricius in chickens resulted in the elimination of the humoral response component but had little effect on the cell-mediated immune response (Glick, 1955). A few years later, it was reported that removal of the thymus in neonatal mice resulted in gross deficiencies in the humoral response and complete abrogation of the cell mediated response (Miller, 1964). These studies were followed by attempts to determine the nature of the cells which were required for the induction of the humoral response. Mice were depleted of responding cells by irradiation and were reconstituted with thymus cells, bone marrow cells or both from syngeneic donors. Only the mice reconstituted with both thymus and bone marrow cells responded to a subsequent injection of sheep red cells and produced anti-sheep red cell antibodies (Claman, Chaperon and Triplett, 1966), thus suggesting that cells derived from both of these sources were required for the induction of humoral immunity. It was later shown that the T cell did not produce antibody (Mitchell and Miller, 1968) but that this was derived from bone marrow cells (Nossal, Cunningham, Mitchell and Miller, 1968). Further work demonstrated the requirement for macrophages in the response (Mosier, 1967) and for the interacting cells to be genetically histocompatible (Katz, Hamaoka, Dorf and Benacerraf, 1973; Rosenthal and Shevach, 1974) for an optimal response to occur. These and other studies showed that there were two main classes of lymphocytes, one derived from the thymus (T helper cells), the other class from the bone marrow (B cells) and that these

cells acted antigen-specifically in the response. The macrophage appeared to be responsible for processing of antigen although it was later shown that this cell appeared to present antigen to the B cell resulting in the activation of the latter (Rosenthal and Shevach, 1974; Rosenthal, 1978).

Subpopulations of regulatory T cells

Subpopulations of T cells have now been defined by the use of T cell specific alloantisera which has been raised by cross-immunizing mice identical in H-2 with thymocytes (Boyse and Old, 1978). One series of alloantigens which have been defined by this method is the Lyt series which is restricted to expression on T cells. There are three well characterized loci: Lyt 1 on chromosome 19 and Lyt 2 and Lyt 3 on chromosome 6. Helper T cells bear the alloantigen Lyt 1+ whereas suppressor and cytotoxic T cells express Lyt 2 and Lyt 3 antigens (Cantor and Boyse, 1975; Huber, Cantor, Shen and Boyse, 1976). The use of monoclonal antibodies through cell fusion (Kohler and Milstein, 1975) should result in more precise definition of cell populations (Williams, Galfre and Milstein, 1977) operating in immunoregulatory circuits in the immune response. Although it is generally assumed, based on evidence derived from man and mouse, that sheep possess similar subpopulations of helper, suppressor and cytotoxic cells, such cells have not to date been characterized.

Cell-cell interactions are mediated by lymphokines.

Antigen-specific lymphokines bind antigen and usually possess I region determinants of the H-2 complex. The majority of antigen-

specific lymphokines studied have a molecular weight in the range of 40,000 to 70,000 daltons (d) (Altman and Katz, 1980). Antigen-specific helper factors are produced by T_H cells upon antigen stimulation. These helper factors include factors specific for keyhole limpet hemocyanin (KLH) (Feldmann and Basten, 1972); the synthetic peptide (T,G)-A--L (Howie and Feldmann, 1977); and sheep erythrocytes (Taussig and Munro, 1977). The mechanism by which antigen-specific factors augment the antibody response is not known (Feldmann and Kontiainen, 1981). Antigen-specific suppressor factors are also produced by T cells upon antigen stimulation and have been shown to be produced to the following antigens: KLH (Tada, Taniguchi and David, 1976; Kontiainen and Feldmann, 1977); the synthetic peptide GAT, GT (Theze, Kapp and Benacerraf, 1977); sheep erythrocytes (Taussig, Corvalan, Binns and Holliman, 1979); picrylchloride (Zembala and Asherson, 1977; Greene, Pierres, Dorf and Benacerraf, 1977); H-2 antigen (Kindred and Corley, 1977); and tumor specific antigens (Kilburn, Talbot, Teh and Levy, 1979).

The first suggestion of a role for antigen nonspecific lymphokines in the antibody response was reported in 1971 when it was shown that mixed lymphocyte culture supernatants could enhance antibody responses in B cell enriched populations to T cell dependent antigens (Dutton, Falkoff, Hirst, Hoffman, Kappler, Kettman, Lesley and Vann, 1971). Subsequent studies established that supernatants derived from antigen-stimulated (Rubin and Coons, 1971), alloantigen-stimulated (Ekpaha-Mensah and Kennedy, 1971), or lectin-stimulated (Watson, 1973; Rich and Pierce, 1974) T cell cultures could mediate antibody responses in a

nonspecific fashion due to soluble factors. It was later discovered that nonspecific factors present in supernatants prepared by these methods could augment the induction of cytotoxic T cells (Altman and Cohen, 1976; Plate, 1976). It is now known that stimulation of T cell cultures as described above results in a multitude of different factors (Altman and Katz, 1980). These crude culture supernatants will either augment or suppress an immune response depending on the type and quantity of the stimulus and the culture conditions (Altman and Katz, 1980). Problems encountered in the study of nonspecific lymphokines include difficulties in isolation and biochemical characterization due to minute quantities produced in culture (Watson, Frank, Mochizuki and Gillis, 1982) and the confusion which has resulted from assay systems which measure more than one factor (Aarden et al., 1979; Leibson, Marrack and Kappler, 1981; Garman and Fan, 1983). The presence of inhibitory molecules in stimulated T cell cultures may also lead to inaccuracies in assaying lymphokines (Tadakuma and Pierce, 1978). Recent advances include the use of T cell hybridomas and T cell lymphoma and leukemia cell lines which are enabling workers to obtain higher yields of some of these factors (Watson et al., 1982; Schrader, Clark-Lewis and Barlett, 1982). Through the use of cloned cell lines in many of the biological assays, the contaminating effects from other lymphokines are often reduced (Swain and Dutton, 1982; Rao, Mizel and Cantor, 1983). The nonspecific factors studied have been found to lack MHC determinants with the exception of Allogeneic Effect Factor (Armending and Katz, 1974) and Immunoglobulin Binding Factor (Gisler and Fridman, 1975).

T cell growth factor

In 1976, it was discovered that supernatants from mitogen-stimulated human T cells could support the long term growth of antigen-activated T cells (Morgan, Ruscetti and Gallo, 1976). The following year, it was discovered that mitogen stimulated murine T cells possessed a similar activity due to a factor known as T cell growth factor (TCGF) (Gillis and Smith, 1977). During the same time period, two factors were discovered which had a synergistic effect with T cell mitogens on the mitotic response of resting T cells. These factors were called Thymocyte Stimulating Factor (Chen and Di Sabato, 1976) and Costimulator (Shaw, Monticone, Mills and Paetkau, 1978). Concurrent studies also revealed a factor which could augment the induction of cytotoxic T cells termed Killer Helper Factor (KHF) or Secondary Cytotoxic Inducing Factor (SCIF) (Wagner and Rollinghoff, 1978; Wagner, Rollinghoff, Schawaller, Hardt and Pfizenmaier, 1979). Biochemical studies revealed that TCGF, TSF, Costimulator, KHF, and SCIF cochromatographed suggesting that they belonged to a class of closely related molecules (Watson, Gillis, Marbrook, Mochizuki and Smith, 1979a). It was also found that the partially-purified factors possessed T cell-replacing factor activity (TRF) by supporting the development of plasma cells in T cell-depleted spleen cell cultures (Farrar, Simon, Koopman, and Fuller-Bonar, 1978; Watson, Aarden, Lefkovits, Shaw and Paetkau, 1979b). These biochemical studies led Aarden et al., (1979) to suggest the term Interleukin-2 (IL2) for the group of molecules previously known as TCGF, TSF, Costimulator, KHF, and SCIF. IL2 was defined as having the following

properties:

1. IL2 has a synergistic effect with T cell mitogens (TSF and Costimulator activities).
2. IL2 maintains the long-term growth of antigen activated T cells (TCGF activity).
3. IL2 augments the induction of cytotoxic T cells (KHF or SCIF activity).
4. IL2 enhances the *in vitro* antibody response in B cell enriched cultures (TRF activity).

Recent evidence has shown that some of the activities previously ascribed to IL2 are in fact due to other factors, some of which may synergize with or require IL2 for their release (see below).

The fourth activity of IL2 described above is now known to be the property of another factor, T cell replacing factor (TRF) which acts late in the immune response (Schimpl and Wecker, 1972) and is biochemically distinct from IL2 (Hubner, Muller, Schimpl and Wecker, 1978; Leibson *et al.*, 1981). TRF induces antibody formation in highly purified B cells because it is a differentiation signal which is required by antigen activated B cells to differentiate into antibody forming cells (Hubner *et al.*, 1978; Swain and Dutton, 1982; Osawa and Naruo, 1982). TRF has further been shown to be distinct from IL2 through the use of T cell hybridomas (Schrader *et al.*, 1982; Osawa and Naruo, 1982), T cell clones (Rao, Mizel and Cantor, 1983) and the ability of IL2 dependent cells to absorb IL2 activity but not TRF activity from samples containing both factors (Kaieda, Okada, Yoshimura, Kishimoto, Yamamura and Kishimoto, 1982). However, TRF activity can be absorbed with activated B cells (Kaieda *et al.*, 1982). It appears that the ability of IL2 to reconstitute an antibody response is not due to direct action on B cells but to the activity of IL2 on residual T cells present in these

cultures (Watson et al., 1979a; Rao et al., 1983). This is supported by the fact that IL2 does not induce antibody formation in highly purified B cell cultures but TRF does (Leibson et al., 1981; Rao et al., 1981). In the presence of T cells, IL2 synergizes with TRF due to its ability to act on activated T cells thus amplifying T cell help through clonal expansion. It has recently been postulated that IL2 is required to induce TRF production (Raulet, Hunig and Parker, 1982). In addition to TRF, there is another B cell helper factor produced by activated T cells which is important in the antibody response. This is a factor which promotes proliferation of antigen activated B cells without inducing differentiation and is known as B cell growth factor (Howard, Farrar, Hilfiker, Johnson and Paul, 1982; Muraguchi, Kasahara, Oppenheim and Fauci, 1982).

Recent studies have also demonstrated that the induction of cytotoxic T cells is dependent on factors distinct from IL2. Experiments have shown that purified IL2 alone was not sufficient for the induction of cytotoxic T cells from pre-cytotoxic T cells (Reddehase, Suessmuth, Meyers, Falk and Droege, 1982; Finke, Scott, Gillis and Hilfiker, 1983; Garman and Fan, 1983). It has now been shown that there are cytotoxic helper factors distinct from IL2 which synergize with IL2 to augment the cytotoxic T cell response (Garman and Fan, 1983). These cytotoxic helper factors have been separated from IL2 through biochemical studies (Garman and Fan, 1983; Finke et al., 1983). In addition, Garman and Fan (1983) demonstrated that optimal production of a cytotoxic helper factor (CHF) occurred after four days in culture compared with the

optimal IL2 production at two days, and that IL2 activity could be absorbed with IL2-dependent cells whereas CHF could not. Finke et al. (1983) have studied a cytotoxic helper factor distinct from IL2 which is produced by macrophages. The ability of IL2 to synergize with cytotoxic helper factors appears to be through clonal expansion of mature cytotoxic T cells (Garman and Fan, 1983). There is also evidence that IL2 can act in the cytotoxic response by inducing T cell subsets to produce immune interferon (Kasahara, Hooks, Dougherty and Oppenheim, 1983).

These recent studies have established that activities previously ascribed to IL2 (the ability to induce antibody formation in B cell enriched cultures and to induce formation of cytotoxic T cells) are dependent on other lymphokines as well as IL2. The only property unique to IL2 is its ability to maintain long term growth of activated T cells (TCGF). The synergistic effect with T cell mitogens or costimulator activity is also shared with the macrophage product Interleukin-1 (IL1). It is ironic because the term IL2 was derived to simplify lymphokine nomenclature, but it has instead added to the confusion (Larsson, 1982a). The earlier name of IL2, T cell growth factor, is a more accurate description since it refers to a unique property of the molecule. Therefore, IL2 will be referred to as TCGF in this work.

TCGF production

Antigen or mitogen activation is essential for T cells to produce or respond to TCGF. The classical definition for lymphocyte activation is the binding of an antigen through cell surface receptors on resting

lymphocytes which results in several rounds of division followed by differentiation into antibody secreting cells, T regulatory cells and T effector cells (Wendel and Parker, 1976). The T cell mitogens Concanavalin A (ConA) and Phytohemagglutinin (PHA) are plant lectins which induce nonspecific polyclonal activation of lymphocytes. TCGF is produced by a subpopulation of regulatory T helper cells (Lyt 1+23- in mouse, OKT4+ in man) although there is some dispute as to whether non-helper T cells also produce TCGF (Andrus, Prowse and Lafferty, 1981; Silva, Alvarez, Bonnard and De Landazuri, 1981; Meurer, Hussey, Penta, Fitzgerald, Stadler, Schlossman and Reinherz, 1982). In order for TCGF production to occur, TCGF producers require antigen and the presence of histocompatibility determinants (Ia^+ mouse, $HLA\text{-}DR^+$ man) on accessory cells (Palacios, 1982). These activated cells are then responsive to IL1 (Gery and Waksman, 1972; Mizel, Oppenheim and Rosenstreich, 1978) a macrophage product which is an essential requirement for IL2 production (Smith, Gillis, Baker, McKenzie and Ruscetti, 1979; Farrar, Mizel and Farrar, 1980; Larsson, Iscove and Coutinho, 1980). After the appropriate stimuli, the cells produce TCGF. A recent study has suggested that a non TCGF producing T_H cell is also required for TCGF production (Larsson, 1982b). Antigen activation of T cells results in expression of TCGF receptors as shown through absorption studies (Bonnard, Yasaka and Jacobson, 1979; Coutinho, Larsson, Gronuik and Andersson, 1979; Hilfiker and Farrar, 1981; Robb, Munck and Smith, 1981). Resting T cells (Bonnard et al., 1979 and activated B cells (Smith et al., 1979) do not absorb TCGF. A cell surface antigen (TAC)

has been defined on human activated T cells, but not resing T cells, through monoclonal antibodies. Anti-TAC antibodies block absorption and action of human TCGF suggesting that the TAC antigen is the TCGF receptor (Leonard, Depper, Uchiyama and Waldmann, 1982; Miyawak, Yackie, Uwadana, Olfzeki, Nagaoki and Taniguchi, 1982; Malek, Robb and Shevach, 1983). The binding of TCGF to the receptor results in clonal expansion of these activated T cells.

Figure 1 illustrates how TCGF production is induced in response to antigen activation followed by IL1 binding, and how TCGF receptors are induced through activation. This figure also illustrates the present concepts of the roles TCGF, IL1 and TRF may play in regulation of the antibody response to a specific antigen.

T cell leukemia and lymphoma lines have been found which produce TCGF constitutively and in response to lectin stimulation (Watson et al., 1982). In addition, T cell hybridomas which produce TCGF have been described (Harwell, Skidmore, Marrack and Kappler, 1980; Schrader and Clark-Lewis, 1981). These products exhibit some heterogeneity but appear to be biochemically similar to TCGF obtained from normal cells (Watson et al., 1982). One notable exception to this is a TCGF species obtained from a cutaneous T cell lymphoma cell line (Gootenberg, Ruscetti and Gallo, 1982).

Kinetics of TCGF production

Experiments with mice have shown that maximum TCGF production occurs within the first 24 hours after mitogen stimulation (Gullberg, Ivous, Coutinho and Larsson, 1981) or after anti-Thy 1 treatment

FIGURE 1

Regulation of the antibody response by immunoregulatory factors.

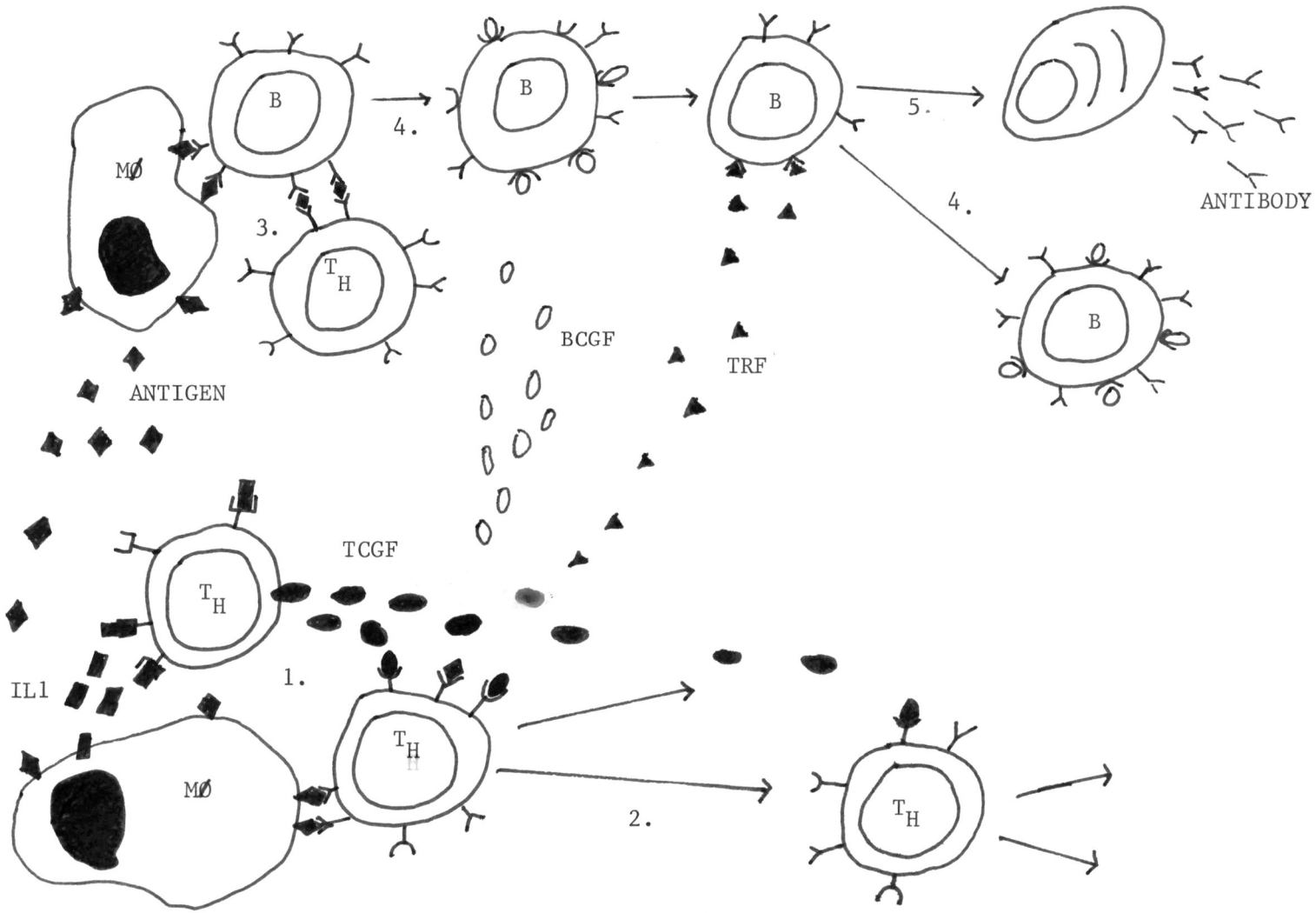
1. Antigen presentation to TCGF producing cells by (Ia^+ mouse, DR^+ man) macrophages (MØ) results in IL1 receptor expression. Upon binding with IL1 these cells produce TCGF. Antigen activation of T cells results in expression of TCGF receptors.

2. The binding of TCGF to TCGF receptors results in the clonal expansion of activated helper T cells (T_H). It can be postulated that BCGF, TRF and antigen specific helper factor (not shown) production by T_H cells is induced by TCGF.

3. Antigen presentation to B cells, mediated by macrophages, helper T cells, or by antigen specific helper factor, results in B cell activation.

4. Antigen activation of B cells results in BCGF responsive cells. The binding of BCGF induces clonal expansion of antigen specific B cells.

5. The binding of TRF to TRF responsive cells results in differentiation of these cells to plasma cells (terminally differentiated antibody producing cells).


B
MØ
3.
T_H
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ANTIBODY
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BCGF
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ANTIGEN
TCGF
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IL1
1.
MØ
T_H
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T_H

(Konaka, Norcross, Maino and Smith, 1981) in vitro and coincides with the expression of receptors for IL2 on activated T cells (Konaka et al., 1981). In man, the kinetics of TCGF production in vitro is dependent on the cell concentration and the presence of low amounts of serum (Alvarez, Silva and de Landazuvi, 1979).

The production of TCGF is terminated by suppressor T (T_S) cells which appear in the in vitro culture 24 hours after initiation (Gullberg and Larsson, 1982). Further regulation of TCGF activity might be due to a 50,000d inhibitor in sera of normal mice (Hardt, Rollinghoff, Pfizenmaier, Mosmann and Wagner, 1981). In addition, a non-specific suppressor factor of 10,000d has been found which selectively inhibits TCGF production (Kramer and Koszinowski, 1982). Macrophages secrete a prostaglandin (PGE_2) which is a potent inhibitor of TCGF activity and TCGF production (Tilden and Balch, 1982). Recently a non-specific inhibitor of TCGF production has been reported which is induced by an antigen specific suppressor factor (Malkovsky, Asherson, Stockinger and Watkins, 1982).

Examination of TCGF production in vivo is a more difficult task, but preliminary evidence indicates that TCGF is released from resting lymph nodes in sheep prior to antigenic challenge and the factor disappears within 6 hours of the initiation of an in vivo response (English and Whitehurst, submitted for publication). This is the only instance of TCGF reported in vivo.

Biochemical properties of TCGF

Partial purification of mouse, human and rat TCGF has been accomplished by a sequence of steps beginning with precipitation with 60-80% ammonium sulphate followed by ion exchange chromatography (DEAE cellulose, carboxymethyl cellulose) and then molecular exclusion chromatography (Sephadex G100). These steps resulted in biologically active fractions with molecular weights of approximately 35,000d in the mouse (Watson et al., 1979a), 20,000d in the rat (Gillis, Smith and Watson, 1980) and 25,000d (Mier and Gallo, 1980) or 15,000d (Gillis, Smith and Watson, 1980) in man. The discrepancy in apparent molecular weights in man appears to be due to heterogeneity in TCGF molecular species which is dependent on the manner of preparation (Welte, Wang, Mertelsmann, Venuta, Feldman and Moore, 1982). Further purification techniques include polyacrylamide gel electrophoresis (PAGE) and isoelectric focussing. The molecular weights of the major peaks obtained from PAGE are 25,000d in the mouse (Watson et al., 1982), 13,000d in man (Watson et al., 1982) and 16,000d in rat (Di Sabato, 1982). Treatment of mouse TCGF with sodium dodecyl sulfate has resulted in a biologically active peak of 15,000d in PAGE suggesting that murine TCGF may be a dimer (Caplan, Gibbs and Paetkau, 1981). Isoelectric focussing results in some heterogeneity of TCGF possessing up to three moieties of different isoelectric points. It has been demonstrated that heterogeneity in mouse TCGF (Watson et al., 1982) and human TCGF (Robb and Smith, 1981) is due to post synthetic glycosylation since treatment with neuraminidase results in a homogenous peak in isoelectric focussing for both

species. Mouse TCGF has been determined to be a glycoprotein consisting of 10% carbohydrate as determined from CsCl bouyant density (Shaw et al., 1978; Watson et al., 1982). This glycoprotein nature explains the heterogeneity in isoelectric focussing and PAGE. Through the use of monoclonal antibodies to TCGF, which are now becoming available, a higher degree of purification of TCGF should be possible (Gillis and Henney, 1981; Stadler and Oppenheim, 1982).

Assay for TCGF activity

After each purification step, the biological activity of each fraction can be tested using a standard TCGF assay (Gillis et al., 1978). Human TCGF has been reported to be biologically active at below one nanomolar concentration (Mier and Gallo, 1982). The standard assay for TCGF utilizes TCGF dependent cytotoxic T cell lines (CTL) which are maintained in growth by regular addition of TCGF. These cells express receptors for TCGF and show a stringent requirement for TCGF. To assay TCGF activity in a sample, the crude or partially purified product is serially diluted and added to cultures of CTL cells. After a 24 hour culture period, growth is determined by pulsing the cells with ^{3}H-thymidine for five hours. Radiolabelled incorporation is then determined to measure TCGF induced proliferation. The murine CTL cells are effective for assaying mouse and human TCGF, but due to species restrictions, they will not demonstrate sheep TCGF activity (English, Latta and Whitehurst, submitted for publication). Lectin activated T cells can also be used for TCGF assays (Gullberg et al., 1981). ConA activated T cells, cultured for 3 days followed by washing to remove residual

lectin, can be used to assay TCGF samples. In the absence of TCGF, ^{3}H-thymidine incorporation by these activated cells approaches background levels.

This project deals with the partial characterization of sheep TCGF.

At present, very little is known about sheep TCGF. Sheep TCGF produced *in vivo* has been reported to possess a molecular weight of 15,000d (English and Whitehurst, submitted for publication). This project examines the optimal conditions for production, the interspecies activity, and the biochemical properties of sheep TCGF produced *in vitro*.

To determine the optimal production conditions, the optimal T cell mitogen concentration, cell concentrations and incubation periods were established. In addition, the effects of various supplements on TCGF production were studied. The effect of residual lectin in crude or partially purified TCGF samples was also examined.

Sheep TCGF was tested for its ability to support the growth of activated T cells from other species. Previous studies have shown that TCGF from swine, guinea pigs, rat and human cells exhibited TCGF activity on mouse activated cells, but human activated cells were only responsive to human TCGF (Lindsay, Schwulera and Sonneborn, 1982).

The biochemical properties of sheep TCGF were determined through the use of protein separation techniques. These included ammonium sulphate precipitation, DEAE-cellulose chromatography, molecular exclusion chromatography and isoelectric focussing. These procedures have allowed partial purification and characterization of sheep TCGF.

TCGF studies are important because they provide information on the role TCGF plays in the regulation of the immune response. TCGF appears to have great potential medically through its ability to clone antigen specific T_H cells or cytotoxic T cells. The study of sheep TCGF should provide information on the phylogeny of the TCGF molecule since TCGF has been partially characterized in humans, rats and mice. The study of *in vitro* produced sheep TCGF is also valuable because it allows for comparative studies with sheep TCGF produced *in vivo* (English and Whitehurst, submitted for publication).

MATERIALS AND METHODS

Lymphocyte Processing

Sheep of the Suffolk and Dorset breeds were bled from the jugular vein into heparinized tubes. The blood was mixed 1:1 with phosphate buffered saline (PBS) and 10 ml aliquots were layered on to 5 ml of Ficoll-hypaque (70% v/v of 9% Ficoll (Sigma, St. Louis) and 30% v/v of 34% hypaque (Winthrop Laboratories, New York, N.Y.)) in sterile screw capped 15 ml Pyrex tubes. The tubes were spun at 600g for 30 minutes, and then the mononuclear cell layers were removed. The lymphocytes were washed 3 times with 3 volumes of PBS. Prior to the last wash, 0.1 ml of the cell suspension was removed and diluted in PBS for a cell count. Cells were counted in a hemacytometer (American Optics, Buffalo, N.Y.) using the 40x objective on a Zeiss microscope (West Germany).

Human lymphocytes obtained from volunteers were extracted from peripheral blood in an identical manner. The only difference in processing was that human lymphocytes required less time (15 minutes at 600g) on the Ficoll-hypaque gradients.

Murine spleen cells were obtained from C57Bl/6, BALB/c and CBA-J strains, and Wistar rats were used as a source of rat spleen cells (Charles River Breeding Labs, Wilmington, Mass.). The spleens were removed aseptically and spleen cell suspensions were prepared by passing the spleens through fine wire mesh in RPMI 1640. The cells were washed twice in RPMI 1640 by centrifugation and the viable cells were counted using dye exclusion (Trypan Blue). After the lymphocytes were

processed for each species, they were used to prepare TCGF supernatants and activated cells for TCGF assays.

Preparation of TCGF supernatants

The lymphocytes were resuspended at 3-5 x 10^6 cells/ml in TCGF supernatant culture media. TCGF supernatant media was prepared with RPMI 1640 media (M.A. Bioproducts, Walkersville, Md.) supplemented with 2% heat-inactivated fetal calf serum (FCS), 25 mM HEPES buffer, 2mM glutamine, 2 x 10^{-5} M 2-mercaptoethanol, streptomycin (400 µg/ml, Gibco, Grand Island, N.Y.), penicillin (400 units/ml, Gibco, Grand Island, N.Y.), and 6 µg/ml Concanavalin A (ConA) (Sigma, St. Louis, Mo.). Polyethylene Glycol 6000 (PEG) (Sigma, St. Louis, Mo.) was added (0.1 mg/ml) to improve TCGF recovery (Mier and Gallo, 1980). The supernatants were cultured at 37°C for 36-48 hours in 75 cm^2 Corning flasks (Corning, N.Y.) in a humidified atmosphere of 5% CO_2 in air. The supernatants were harvested by high speed centrifugation, filter sterilized with Nalgene 100 ml 0.2 µm filters (Rochester, N.Y.) and frozen at -20°C.

This method was defined as the standard method for the preparation of crude TCGF supernatants from sheep, mouse and rat lymphocytes. The study of the optimal conditions (Results I) for sheep TCGF production aided in defining these culture conditions. Unless otherwise stated, crude TCGF supernatants used in subsequent studies (Results II, III, IV) were prepared in this fashion. Crude TCGF supernatants are referred to in this work as Sn's.

TCGF Assay

Lymphocytes were cultured at 3-5 x 10^6 cells/ml in 25 cm^2 Corning flasks in RPMI 1640 supplemented with antibiotics, glutamine, 2-mercaptoethanol, 10% FCS and 6 µg/ml ConA for a 72 hour culture period at 37°C in a humidified atmosphere of 5% CO_2 in air. The ConA activated cells were washed 3 times in RPMI 1640 and the viable cells were resuspended at 1 x 10^6 cells/ml in RPMI 1640 supplemented with glutamine and antibiotics.

Two fold serial dilutions of the TCGF test samples in RPMI 1640 were prepared to give 1 ml samples from 100% to 6% of the original crude supernatant concentrations. The activated cells (0.1 ml) and FCS (0.1 ml) were then added to give 1 x 10^5 cells/ml and 10% FCS, respectively. Each cell suspension was then dispensed into 4 wells (0.2 ml per well) in Costar U-Bottomed 96 well microplates (Cambridge, Mass.). The plates were cultured for 72 hours at 37°C in a humidified atmosphere with 5% CO_2 in air. Five hours before harvesting, the cultures were pulsed with 25 µl RPMI 1640 containing 0.5 µCi ^{3}H-thymidine (ICN, Irvine, Ca.). The cells were harvested with a multiple cell harvester (Skatron, Norway), placed in scintillation vials (Owens Illinois, Toledo, Ohio) containing 5 ml of Betafluor scintillation counting fluid (National Diagnostics, Somerville, N.J.), and counted on a Beckman LS 7000 counter. The mean count for each of the quadruplicate cultures and the standard error of the mean were determined. The standard error was omitted on some of the graphs to retain clarity, but in all cases,

the standard error was within 15%. The student T test was used to determine statistical significance ($p < 0.05$) between means.

Concentration of large volume sheep TCGF supernatants

Large volume sheep TCGF supernatants were prepared as previously described from 100-120 ml of peripheral blood. This amount usually yielded from 100-200 ml of crude TCGF supernatants. The supernatants were harvested as previously described. Five to ten ml were set aside for assay and the remainder was concentrated by ammonium sulfate precipitation, pressure dialysis or by lyophilization. Throughout these studies, PEG was added to all buffers, solutions and dialysis media at 0.1 mg/ml to improve TCGF recovery (Mier and Gallo, 1980).

Ammonium sulfate precipitation was performed by slowly adding ammonium sulfate (Sigma, St. Louis, Mo.) directly to the supernatants at 4°C while stirring to reach 80% saturation (54.4 g/100 ml). The supernatants were then stirred for 2 hours at 4°C followed by centrifugation for 15 minutes at 10,000 g. The supernatants were removed, and the precipitates were redissolved in a minimal amount of PBS (3-5 ml).

Amicon 350 ml ultrafiltration units (Lexington, Mass.) with PM10 filters (molecular cutoff-10,000d) were used to concentrate large volume TCGF supernatants through pressure dialysis. The supernatants were concentrated at 4°C with 60 lb. pressure to 6-8 ml volumes.

Supernatants to be lyophilized were placed in Spectrapor dialysis bags (6,000-8,000d molecular weight cutoff, Los Angeles, Cal.), and dialyzed against two volumes of 0.2M ammonium bicarbonate plus PEG.

Following lyophilization, the samples were redissolved in a minimum amount of PBS (3-5 ml).

Ammonium Sulfate Fractionation

Ammonium sulfate fractionation was carried out in a similar manner to ammonium sulfate concentration as described above, but instead of adding ammonium sulfate initially to a concentration of 80%, it was added stepwise beginning with 40% followed by 60% and finally 80%. This allowed 0-40%, 40-60%, and 60-80% precipitates to be collected. The precipitates were redissolved in distilled water and extensively dialyzed against H_2O + PEG, followed by PBS + PEG and finally RPMI 1640 + PEG. The fractions were then filter sterilized, serially diluted and tested for TCGF activity.

Ion Exchange Chromatography

Ion exchange chromatography was performed with diethylaminoethyl (DEAE) cellulose. Twenty grams of DEAE-cellulose (Sigma, St. Louis, Mo.) were placed in several volumes of water which were decanted after settling to remove fine particles. The DEAE was then placed in an acid buffer (0.3 M NaH_2PO_4) overnight, washed through aspiration with the acid buffer and distilled water, placed in 0.5N NaOH, extensively washed with H_2O, and finally washed twice with the starting buffer (0.05 M NaCl, 0.01M TRIS-HCl, PEG, pH 8).

The DEAE was titrated to pH 8 and was poured into a 2cm x 40cm column containing the starting buffer to reach a height of 25 cm. The column was allowed to settle overnight. The TCGF sample was carefully layered on top of the DEAE. The NaCl concentration was increased step-

wise with 4 buffers containing 0.1M TRIS-HCl/PEG and the following NaCl concentrations: 0.05M, 0.10M, 0.15M, and 0.20M. The buffers were added in 600 ml volumes at a flow rate of 3 ml/minute. Four fractions were collected corresponding to the eluant for each buffer. These fractions were concentrated to 6 ml volumes using Amicon ultrafiltration units. The four fractions were extensively dialyzed against H_2O, followed by PBS and finally RPMI 1640. They were then sterilized and tested for TCGF activity.

Sephadex G100 Chromatography

Molecular exclusion chromatography was performed using Sephadex G100 (Sigma, St. Louis, Mo.) which was pretreated by soaking in PBS for 3 days or by boiling for 6 hours and then degassing for 5 minutes with a vacuum pump. The slurry was then poured into a 2cm x 100cm Pharmacia column. The column was allowed to settle for 24 hours and was attached to a metering pump at a flow rate of 22 ml/hour. The TCGF sample was mixed with 2 ml of saturated sucrose and was layered on the column. Phosphate buffered saline plus PEG (350 ml) was eluted through the column and 4 ml fractions were collected by upward displacement. The fractions were then extensively dialyzed against PBS and RPMI 1640 before being filter sterilized and tested for TCGF activity.

Isoelectric Focussing

Isoelectric focussing (IEF) was performed on a LKB 8100 column (Stockholm, Sweden). A cathode solution containing NaOH and 70% sucrose (weight/volume) was placed at the base of the column. A 110 ml sucrose gradient from 50%-5% (weight/volume) was prepared from the mixing of a

light gradient solution and a dense gradient solution. The dense gradient solution contained the TCGF sample which was extensively dialyzed to remove all salt ions. Both gradient solutions contained the ampholines (LKB, Stockholm, Sweden) which were added to form a pH gradient from 3 to 10. The column was electrophoresed at 1000 volts for 48 hours. The electrode solutions were then removed and 4 ml fractions were collected. The pH of each fraction was determined as soon as it was collected. The fractions were dialyzed against 2 x 100 volumes of water to remove sucrose and ampholines followed with PBS and the RPMI 1640. The fractions were then filter sterilized and tested for TCGF activity.

RESULTS

I. Optimal Conditions for Sheep TCGF Production and for Sheep TCGF Bioassays

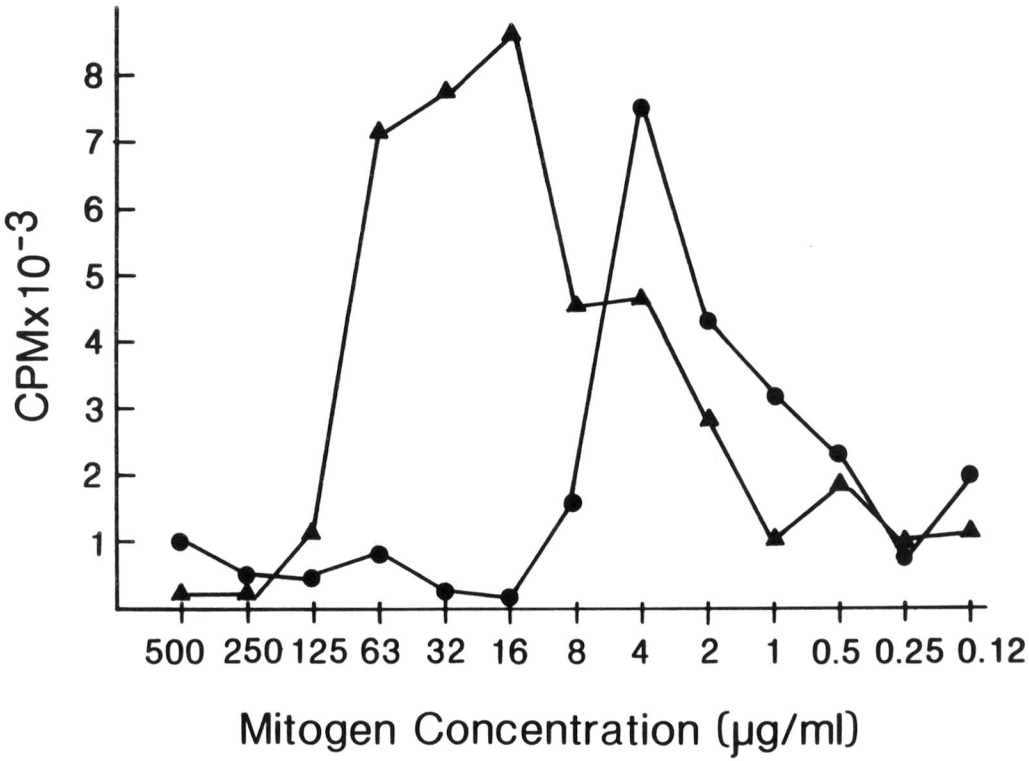
To facilitate the characterization of sheep TCGF, the optimal conditions for sheep TCGF production were established. These optimal conditions defined the standard method for preparing crude sheep TCGF supernatants (sheep Sn). In addition, the culture conditions for the preparation of lectin activated sheep T cells and the culture conditions for these activated cells in the presence of TCGF were examined to establish a sensitive assay for sheep TCGF.

T cell mitogen dose response

Peripheral lymphocytes were tested for their ability to respond to the T cell mitogens ConA and phytohemaglutinin (PHA) in order to establish the optimal lectin concentration to induce sheep TCGF production and to activate T cells for TCGF assays. Two fold serial dilution were made for each lectin from 500 µg/ml to 0.12 µg/ml. Cells and FCS were added to each dilution to give a final cell concentration of 1×10^6 cells/ml and 10% FCS concentration. The samples were cultured in quadruplicate (0.2 ml each) for 3 days and pulsed with ^{3}H-thymidine in a manner identical to activated cell cultures (TCGF assays). The mitotic response as determined by ^{3}H-thymidine incorporation was optimal for PHA at 16 µg/ml and ConA at 4 µg/ml (Figure 2). The optimal mitogen concentrations were then used to prepare TCGF supernatants and activated T cells for TCGF assays. ConA was the mitogen of choice to minimize lectin contamination since it evoked an optimal mitotic response at a lower concentration.

FIGURE 2

Mitogen dose response. Serial dilutions of ConA (●—●), and PHA (▲—▲) were tested against sheep peripheral blood lymphocytes. Each point represents the mean incorporation of ^{3}H-thymidine by quadruplicate cultures of 2 x 10^{5} cells. The response of the cells + media was 463 $\pm$ 123 cpm.


CPMx10^{-3}
8
7
6
5
4
3
2
1
500 250 125 63 32 16 8 4 2 1 0.5 0.25 0.12
Mitogen Concentration (µg/ml)

Variability of sheep TCGF production

Throughout this study, it has been my experience that the production of TCGF varies within a population of sheep. A typical example shows that some sheep appear to be low producers, whereas other sheep produce high titers of TCGF (Figure 3). The three TCGF supernatants shown here were prepared from different sheep under identical culture conditions.

Effect of cell concentration and incubation on sheep TCGF production

To determine the optimal incubation and cell concentration and the kinetics for sheep TCGF production, supernatants were prepared at 1 x 10^6 cells/ml, 5 x 10^6 cells/ml, and 1 x 10^7 cells/ml for 12, 24, 36, and 48 hours. The four supernatants at a concentration of 1 x 10^6 cells/ml did not demonstrate any significant difference in TCGF activity. At 5 x 10^6 cells/ml, TCGF activity was shown to be lowest at 12 hours, and it increased throughout the culture period, demonstrating maximal activity at 48 hours. A higher cell density (1 x 10^7 cells/ml) exhibited maximal TCGF activity at 12 hours and steadily declined thereafter. The cell density and incubation time for maximal TCGF production was determined to be 5 x 10^6 cells/ml for 48 hours since the sheep Sn prepared under these conditions contained the highest TCGF activity (Figure 4).

Effect of protein supplements on TCGF production

Sheep TCGF supernatants were prepared in the presence of 2% FCS, 2% dialyzed FCS, 2% bovine serum albumin (BSA) and 2% sheep lymph to determine their effect on TCGF production. A supernatant without a protein supplement was used as a control. It appeared that the greatest

FIGURE 3

The variability of sheep TCGF production. Three sheep Sn (SIL1 ●—● , SIL2 ■—■ , SIL3 ▲—▲) were prepared from different sheep and were tested against sheep activated T cells for growth supportive activity (TCGF activity (TCGF activity). Each point represents the mean isotope incorporation of 2×10^4 cells. Background incorporation of activated cells + media was 337 ± 78.

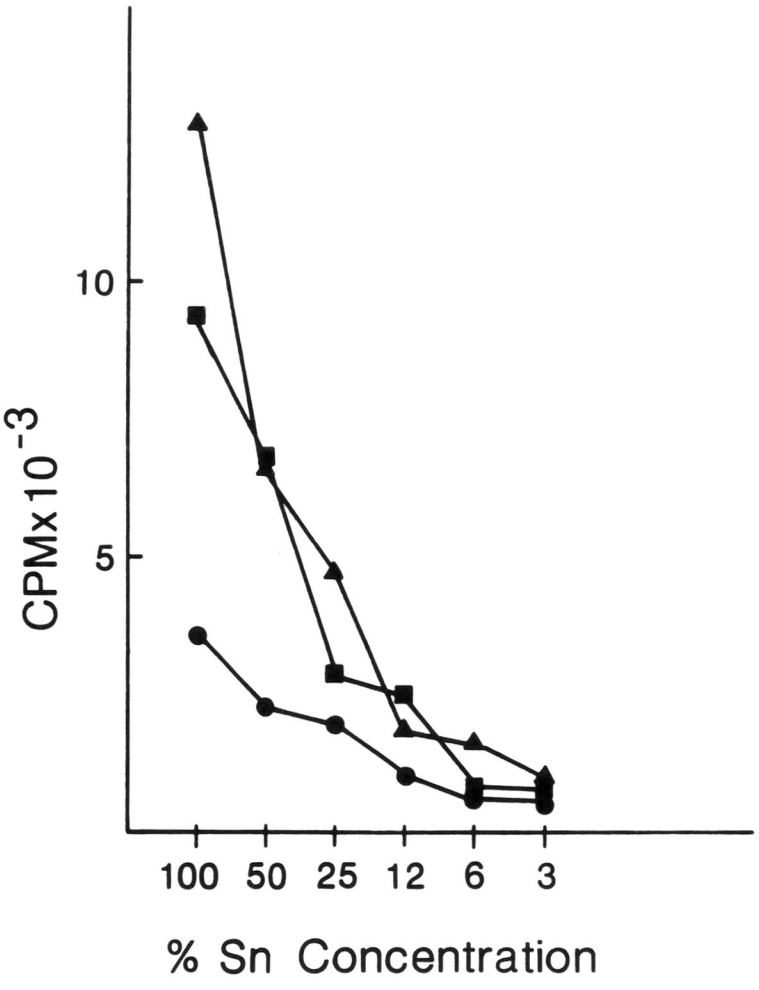
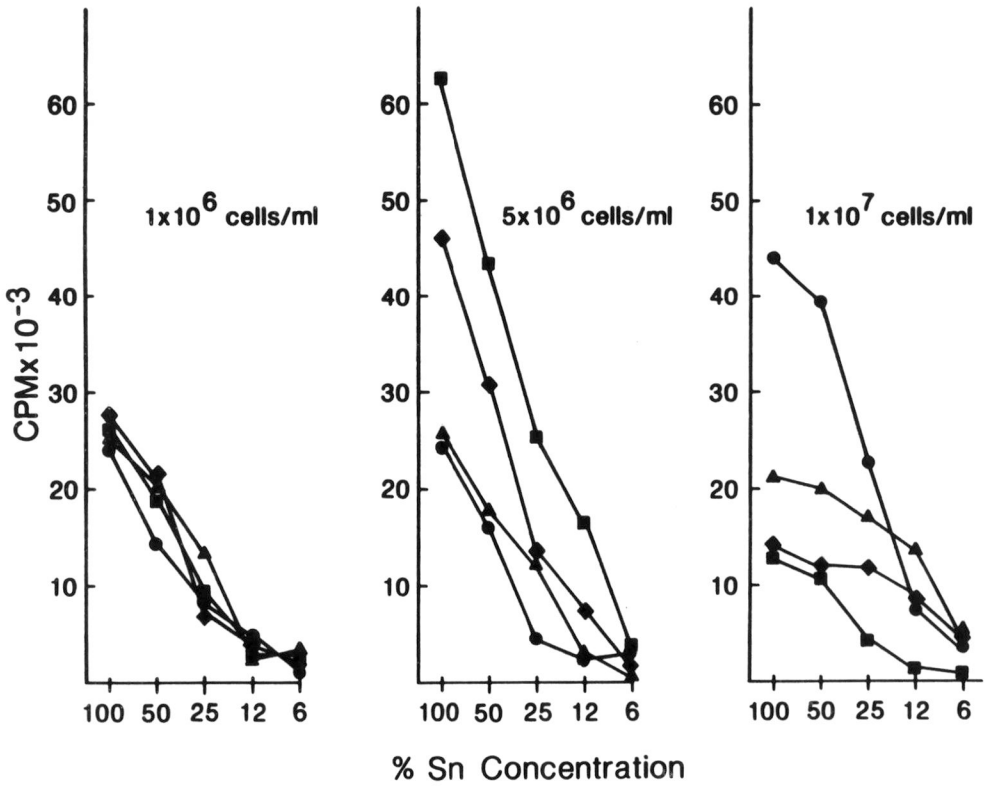

CPMx10^{-3}
10
5
100 50 25 12 6 3
% Sn Concentration

FIGURE 4

The effects of cell concentrations and incubation time on TCGF production. Lymphocytes were aliquoted to prepare sheep Sn's with cell concentrations of 1 x 10^6, 5 x 10^6 and 1 x 10^7 cells/ml for the following culture periods: 12 hours (●—●), 24 hours (▲—▲), 36 hours (◆—◆), and 48 hours (■—■). The resulting sheep Sn's were then assayed against activated T cells for TCGF activity. Each point represents isotope incorporation of 2 x 10^4 cells. The background incorporation of activated cells + media was 637 $\pm$ 119 cpm. The 48 hour sheep Sn prepared from 5 x 10^6 cells/ml was demonstrated to be significantly higher in TCGF activity than the 36 hour sheep Sn prepared from 5 x 10^6 cells/ml ($p < 0.05$) and the 12 hour sheep Sn prepared from 1 x 10^7 cells/ml ($p < 0.05$).


CPMx10-3
60
50
40
30
20
10
1x10^6 cells/ml
5x10^6 cells/ml
1x10^7 cells/ml
100 50 25 12 6
% Sn Concentration

TCGF activity was in the supernatant supplemented with 2% FCS (Figure 5). In this supernatant, TCGF activity was significantly higher than the control. A subsequent experiment comparing TCGF supernatants prepared with 2% FCS and 2% sheep serum demonstrated that sheep serum was the better supplement (Figure 6). However, since high titers of TCGF were present in FCS supernatants, 2% FCS was preferentially used as a supplement for standard prepared sheep Sn due to its availability.

Phorbol Myristic Acid (PMA) has been reported to synergize with ConA to induce TCGF production (Fuller-Farrar, Hilfiker, Farrar and Farrar, 1981). Polyethylene glycol (PEG) has been reported to help prevent loss of TCGF activity through adherence of the molecule due to its hydrophobic nature (Mier and Gallo, 1982). Three sheep Sn's were prepared in the presence of PEG (0.1 mg/ml), PMA (0.1 µg/ml), and PEG and PMA. No significant difference in TCGF activity could be demonstrated between these supernatants (Figure 7).

The concentrated TCGF samples were then dialyzed against media, filter sterilized, and diluted back to 100% concentration. The results indicated that there was over 80% recovery after concentration in the TCGF supernatant which contained PEG, but there was only 60% recovery in the supernatant which contained PMA (Figure 8).

Based on these results, it was determined that there was no benefit in adding PMA to TCGF supernatants. This was further supported by recent evidence which has shown that PMA binds to serum proteins and is therefore nondialyzable (Krakauer, Mizel and Oppenheim, 1982) and can be detrimental to TCGF assays (see Discussion). To improve TCGF recovery

FIGURE 5

Effects of protein supplements on TCGF production. Five sheep Sn's were prepared with the following supplements: 2% FCS (▲—▲), 50 mg/ml BSA (○—○), 2% dialyzed FCS (●—●), control-no supplement (△—△), and 2% sheep lymph (■—■). The Sn's were tested against sheep activated T cells for TCGF activity. Each point represents the mean isotope incorporation of 2 x 10^4 cells. The background incorporation for activated cells + media was 3515 $\pm$ 109 cpm. TCGF activity in the 2% FCS was significantly higher than the control TCGF activity ($p < 0.05$).

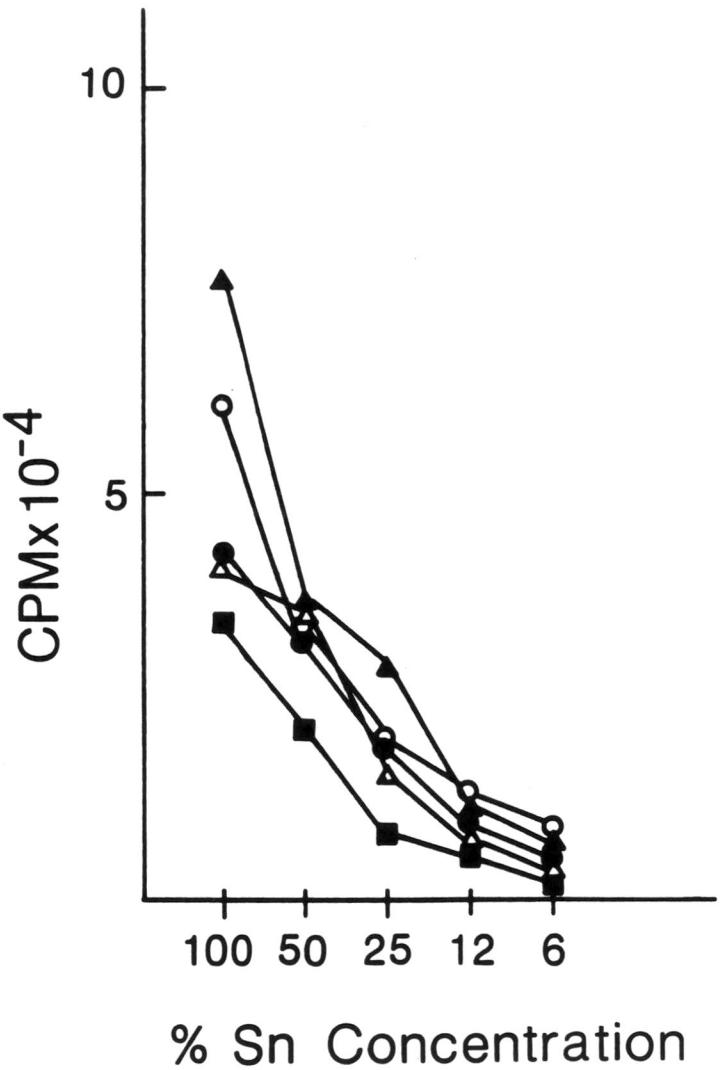

10
5
CPMx10⁻⁴
100 50 25 12 6
% Sn Concentration

FIGURE 6

Comparison between two protein supplements. Two sheep Sn's were prepared with 2% sheep serum (●—●) and 2% FCS (■—■) and were tested for TCGF activity. Each point represents the mean isotope incorporation of 2×10^4 cells. The background incorporation for activated cells + media was 1533 $\pm$ cpm. Statistical significance was shown between these sheep Sn's ($p < 0.05$).

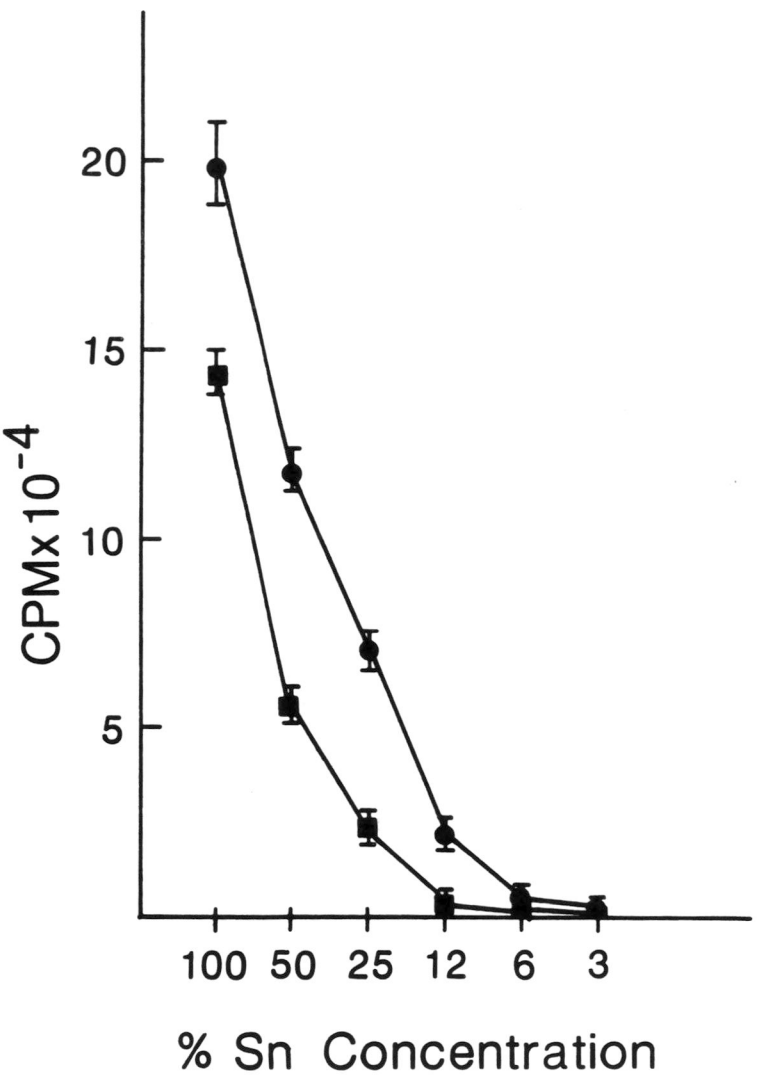

20
15
10
5
CPMx10-4
100 50 25 12 6 3
% Sn Concentration

FIGURE 7

The effects of PMA and PEG on TCGF production and recovery in sheep Sn's. Sheep Sn's were prepared with PEG (0.1 mg/ml) (■—■), PMA (100 ng/ml) (●—●), and PEG and PMA (▲—▲) and were tested for TCGF activity. Each point represents the mean isotope incorporation of 2 x 10^4 cells. The background incorporation for activated cells + media was 250 $\pm$ 85 cpm. No statistical significance was evident between these three supernatants ($p > 0.05$).

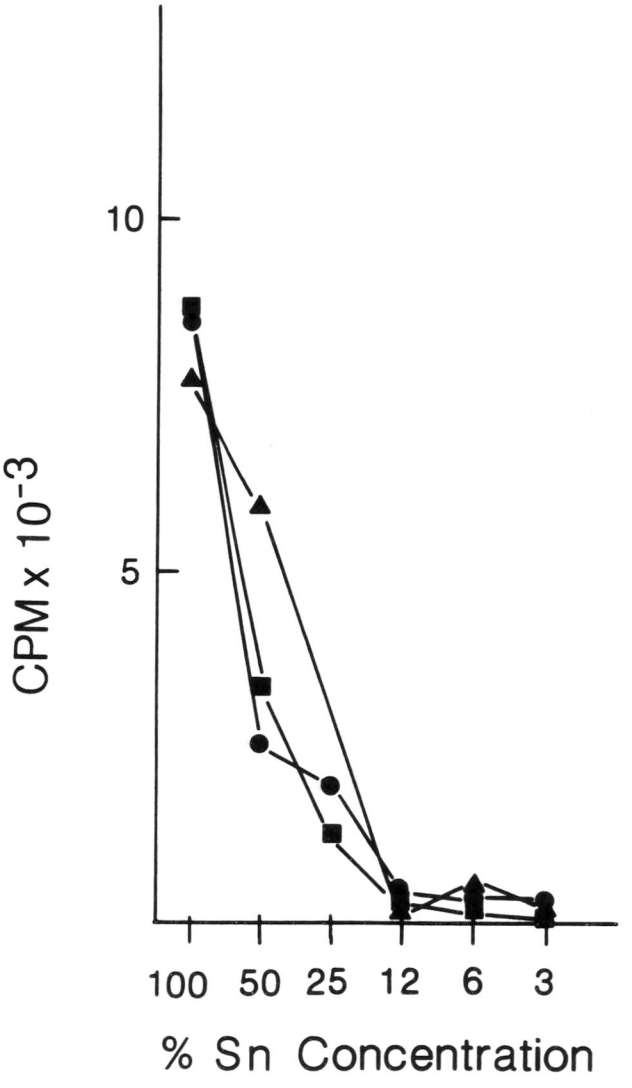

10
5
CPM x 10^{-3}
100 50 25 12 6 3
% Sn Concentration

FIGURE 8

The effects of PEG and PMA on TCGF recovery following concentration. Two bulk sheep Sn's were prepared (one with PEG and the other with PMA). Each supernatant was tested for TCGF activity at 100% Sn concentration and at 100% Sn concentration following ultrafiltration concentration. (The supernatants were concentrated 8x and diluted back to 100% to determine TCGF recovery.)

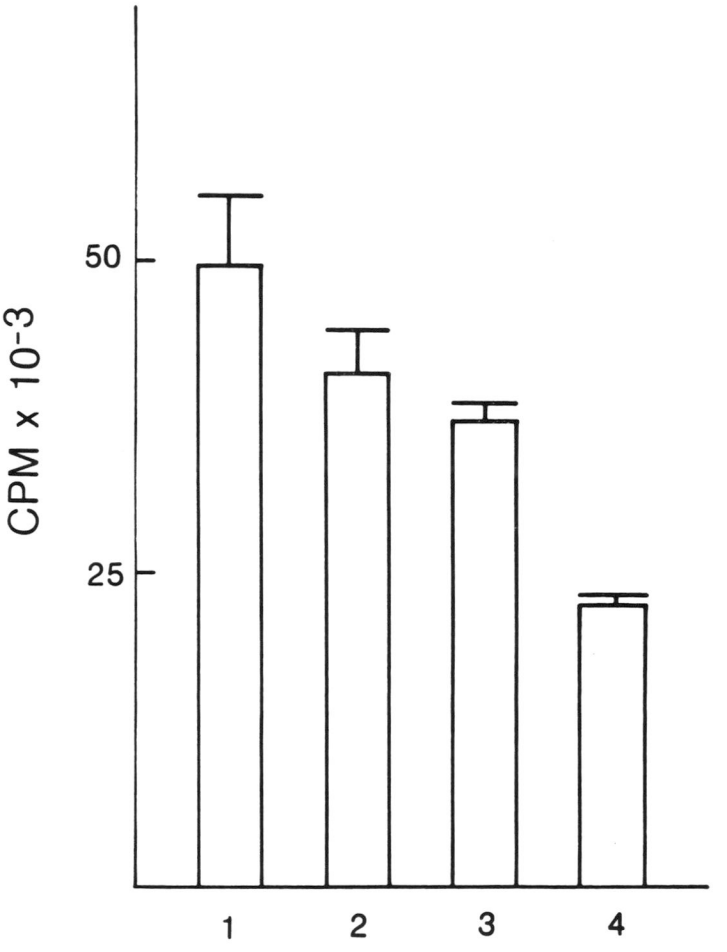
Sample 1 PEG Sn at 100% Sn conc.

Sample 2 PEG Sn-concentrated 8x and diluted back to 100% conc.

Sample 3 PMA Sn at 100% Sn conc.

Sample 4 PMA Sn-concentrated 8x and diluted back to 100% Sn conc.

The value for each sample was the mean isotope incorporation for 2×10^4 cells. The background incorporation for activated cells + media was 775 ± 119 cpm.


CPM x 10-3
50
25
1
2
3
4

during purification techniques, PEG was added to all TCGF supernatants, chromatography buffers and dialysis media.

The effect of preculturing on TCGF supernatants

It has been reported that preculturing human lymphocytes in the absence of ConA prior to resuspending the cells with ConA increases TCGF production by reducing the activity of ConA induced suppressor T cells (Northoff, Carter and Oppenheim, 1980). An experiment was conducted to analyze the effects of preculturing on sheep TCGF production. Sheep peripheral lymphocytes were divided evenly into two aliquots. A sheep Sn (SIL9) was prepared by resuspending one aliquot at 3×10^6 viable cells/ml for a 48 hour culture in the presence of ConA. The second aliquot was resuspended at 3×10^6 viable cells/ml for 48 hours without lectin. SIL10 was then prepared by resuspending the precultured cells with ConA at 3×10^6 viable cells/ml for 48 hours. A 45% loss of cells was observed due to cell death in the preculture period. The results demonstrated that TCGF activity/1.5×10^6 viable cells appeared to be higher in SIL10 than in SIL9, but the difference was not significant. The TCGF production/original 1.5×10^6 cells was lower in SIL10 than in SIL9 due to cell death during the preculture period; therefore, the total yield of TCGF activity was higher in SIL9 than SIL10. (Table 1, Exp. 1).

In a subsequent experiment, two sheep Sn's were prepared as described above with an equal number of lymphocytes; however, the precultured Sn was precultured in the presence of 30% rat Sn. In this experiment, only a 30% loss of cells was observed following preculturing.

TABLE 1

The Effect of Preculturing on TCGF Productivity

Experiment 1	*TCGF Prod./1.5×10^6 orig. cells	*TCGF Prod./1.5×10^6 viable cells
Normal Sn (SIL9)	40,081	40,081
Precult. Sn (SIL10)	28,420	51,673
Experiment 2		
Normal Sn (SIL34)	47,503	47,503
Precult. with 30% Rat Sn (SIL35)	48,767	69,667

*The TCGF productivity for each Sn was calculated from the mean isotope incorporation at 50% Sn concentration. For the precultured Sn's, the original cells were defined as the viable cells at the initiation of the preculture period and the viable cells were defined as the live cells following the preculture period. For the normal Sn's, the original cells and viable cells were identical, being defined as the live cells at the start of the cultures. Background incorporation of activated cells + media was 3758 $\pm$ 297 cpm for exp. 1 and was 922 $\pm$ 156 cpm for exp. 2.

The precultured supernatant (SIL35) was significantly higher than the normal Sn (SIL34) in TCGF activity/3 x 10^6 viable cells. The TCGF production/original 3 x 10^6 cells was equivalent in both supernatants which suggests the total yield of TCGF activity was equivalent (Table 1, Exp. 2). Since cells precultured with rat Sn produced sheep Sn's with high activities in smaller volumes than normal sheep Sn's, these precultured Sn's were used in some of the biochemical studies.

The effects of preculturing lymphocytes before lectin activation

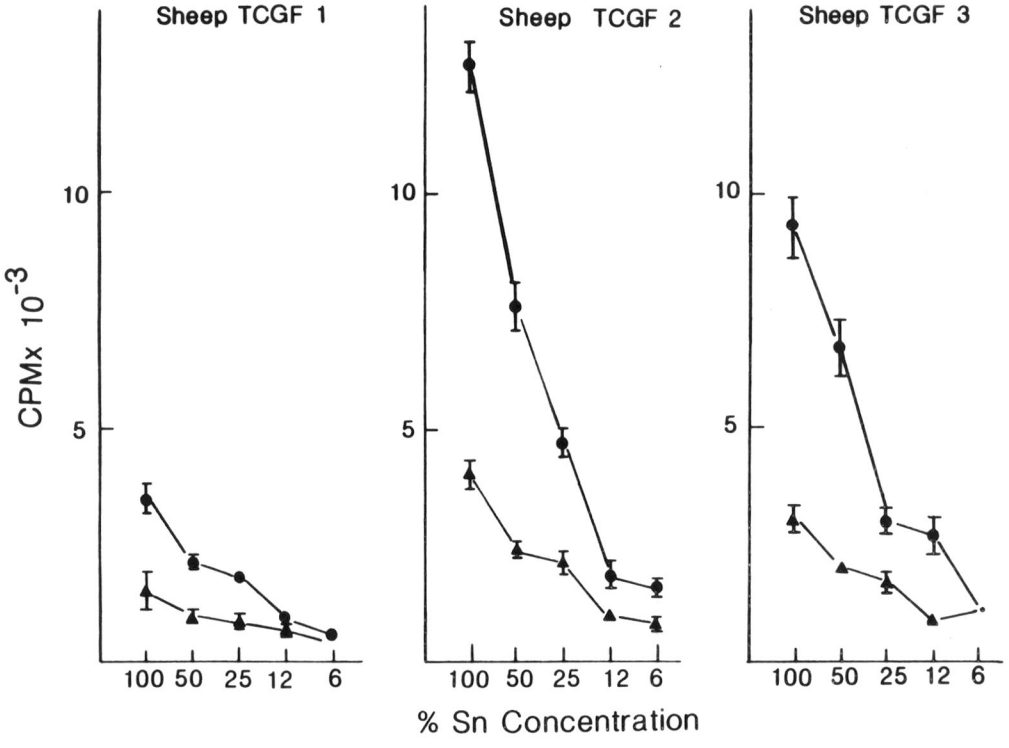
Since it has been suggested that preculturing lymphocytes inhibits the activity of suppressor T cells (Northoff et al., 1980), the effects of such treatment on the TCGF responsiveness of lectin activated sheep T cells were studied. Peripheral lymphocytes were obtained and divided into two aliquots. One aliquot was cultured in the presence of ConA for 72 hours under the culture conditions described in the standard method for preparing activated sheep cells. The second aliquot was precultured for 48 hours under the same conditions in the absence of ConA; the cells were then counted, and the viable cells were resuspended with ConA for a 72 hour culture under identical conditions to the standard method (SIL1, SIL2, SIL3). The results demonstrated that the activated cells prepared by the standard method were more responsive than the precultured activated cells to the sheep TCGF present in each supernatant (Figure 9).

The optimal incubation period for sheep TCGF assays

As previously described, lectin activated sheep T cells are used to assay for TCGF activity. An experiment was conducted to establish

FIGURE 9

The effects of preculturing before lectin activation. Lectin activated cells were prepared by the standard method and were prepared by preculturing the cells for 48 hours prior to addition of lectin. The cells were then tested against 3 sheep Sn's (Sheep TCGF 1, Sheep TCGF 2, and Sheep TCGF 3), Each point represents the mean isotope incorporation of 2 x 10^4 cells (precultured cells (▲—▲) and standard prepared cells (●—●)). The background incorporation of activated cells + media was 337 $\pm$ 78 cpm for the standard prepared cells and 768 $\pm$ 51 cpm for the precultured cells.


Sheep TCGF 1
Sheep TCGF 2
Sheep TCGF 3
CPMx 10^{-3}
10
5
100 50 25 12 6
% Sn Concentration

the culture period for assaying sheep Sn's which would allow optimal sensitivity for detecting sheep TCGF activity. Replicate cultures of activated cells in the presence of sheep Sn (SIL5) and in the presence of a commercial human TCGF preparation were prepared. The cultures were harvested on days 1-5. SIL5 exhibited the highest TCGF activity on day 3, while the human TCGF preparation demonstrated the highest activity on day 5. It was concluded that a 3 day culture period was optimal for assaying sheep Sn. The lower TCGF activity on day 4 and day 5 for SIL5 can be attributed to TCGF depletion. The human TCGF preparation exhibited higher TCGF activity than SIL5 and was able to support the growth of activated cells for 5 days in culture (Figure 10).

This experiment provided useful information on the culture conditions required for the long term growth of sheep activated cells. Long term cultures of sheep activated cells are now being used to assay sheep TCGF (English, personal communication). This experiment demonstrated that the commercial human TCGF which was partially purified and delectinated was superior to crude sheep TCGF prepared by the standard method in maintaining long term cultures of sheep activated cells. This data suggested that long term cultures should be subcultured every 3 to 5 days to provide fresh media, fresh TCGF and lower cell densities.

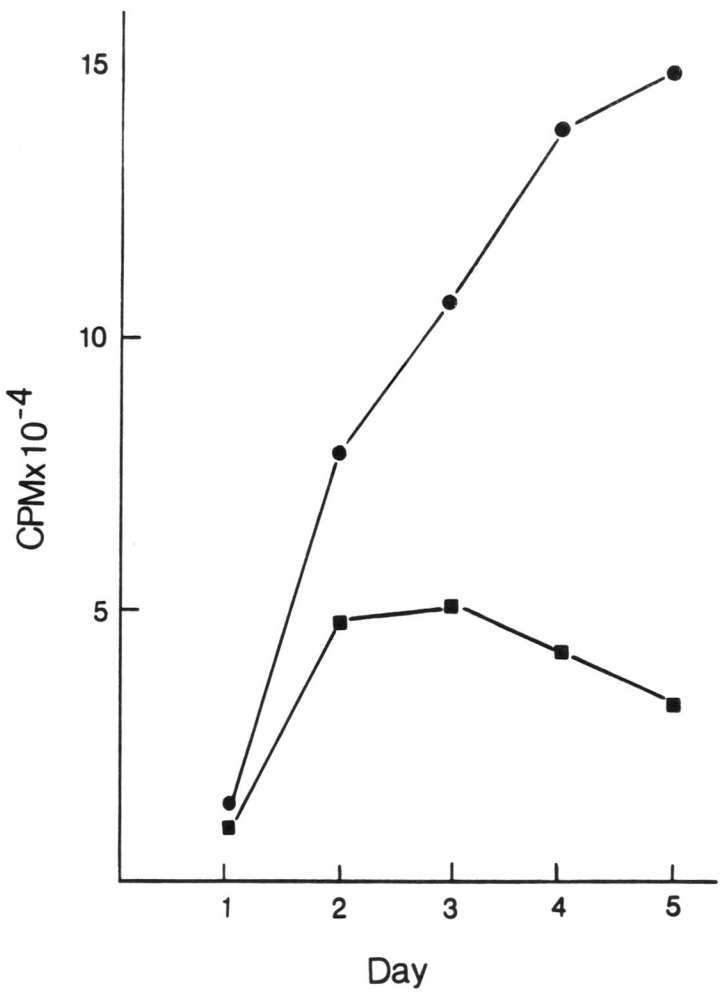
Summary

The conclusions reached from this study of optimal conditions are as follows:

1. The optimal ConA concentration for preparing sheep Sn and for activating lymphocytes for TCGF assays was 4 μg/ml.

FIGURE 10

The optimal incubation period for TCGF assays. Activated cells were cultured in replicate cultures in the presence of two fold serial dilutions of a sheep Sn and a commercial human TCGF preparation and were harvested on days 1-5. Each point represents the mean incorporation of 2×10^4 cells at 100% concentration of sheep Sn (■—■) and at 25% concentration of the human TCGF preparation (●—●). The background incorporation of activated cells + media was 3386 $\pm$ 575 cpm at day 1, 7447 $\pm$ 60 cpm at day 2, 3515 $\pm$ 119 cpm at day 3, 2037 $\pm$ 178 cpm at day 4, and 1486 $\pm$ 30 at day 5.


15
10
5
CPMx10^{-4}
1
2
3
4
5
Day

2. Sheep TCGF production varied within a population of sheep examined.

3. The kinetics of sheep TCGF production were dependent on cell concentration. The optimal incubation period and cell density was found to be 48 hours at 5×10^6 cells/ml.

4. Protein supplements augment TCGF production. PMA did not enhance sheep TCGF production. PEG improved sheep TCGF recovery during concentration procedures.

5. Preculturing lymphocytes before lectin addition improved TCGF production per viable cell. Lymphocytes precultured with rat Sn yielded Sn with equivalent total TCGF activity to normal cultured control Sn.

6. Preculturing did not improve the TCGF responsiveness of lectin activated cells.

7. The optimal culture period for assaying serial dilutions of sheep Sn was 3 days.

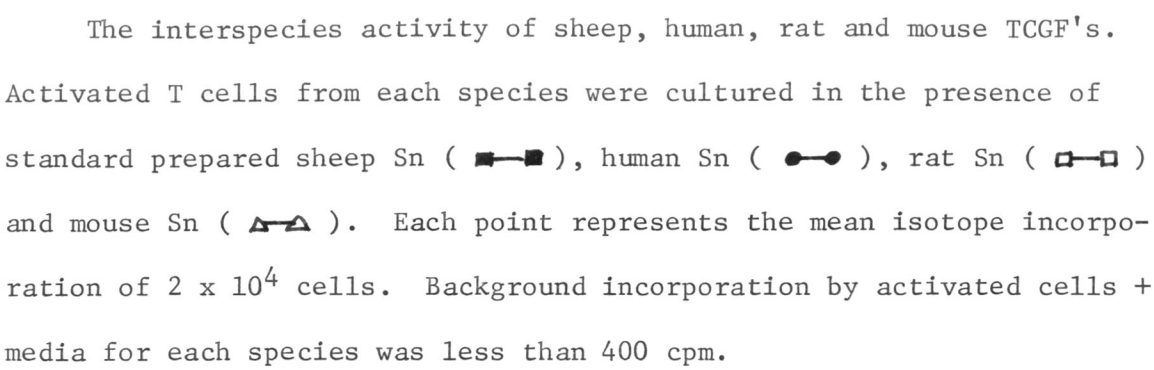
II. The Interspecies Activity of Sheep TCGF

Sheep Sn's prepared by the standard method were serially diluted and assayed against lectin activated cells prepared from human and sheep peripheral lymphocytes and mouse and rat splenocytes to determine the interspecies activity of sheep TCGF. Human TCGF (commercial), rat Sn, and mouse Sn were also tested for xenogeneic activity to provide a comparative study. Commercial human TCGF was diluted with media 1:1 before being serially diluted. Rat and mouse Sn's were prepared as previously described.

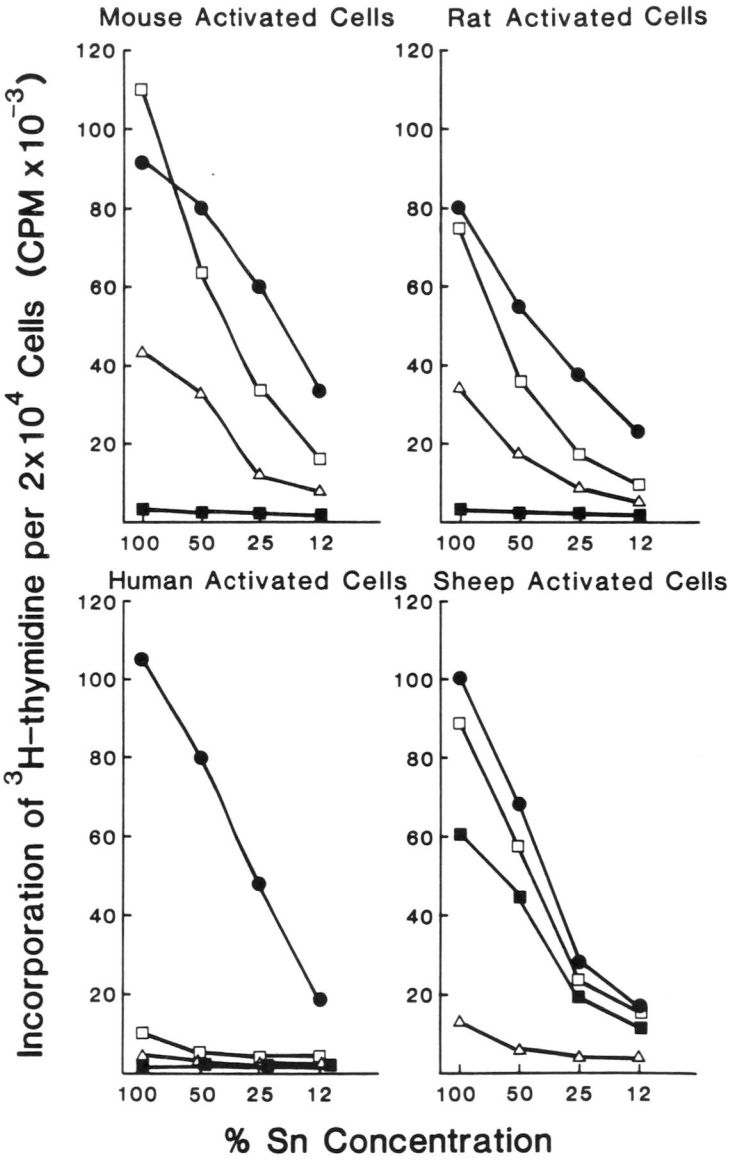
The results showed that sheep TCGF prepared under the standard conditions could only maintain the growth of activated sheep cells. In contrast to this, human TCGF could support the growth of all 4 species; however, human activated cells were responsive only to human TCGF. Rat TCGF supported the growth of sheep, rat and mouse cells and mouse TCGF only supported rat and mouse cells (Figure 11). These results demonstrated that sheep TCGF was the most restricted in xenogeneic activity, but sheep activated cells could grow in the presence of human, rat, and sheep TCGF.

The investigation of alternate methods of sheep TCGF production led to a sheep TCGF preparation (SIL4) which exhibited optimal growth supportive activity on mouse, human, and sheep cells (English *et al.*, submitted for publication). This suggests that the method by which TCGF production is induced affects the interspecies reactivity (see Discussion).

FIGURE 11



The interspecies activity of sheep, human, rat and mouse TCGF's. Activated T cells from each species were cultured in the presence of standard prepared sheep Sn (■—■), human Sn (●—●), rat Sn (□—□) and mouse Sn (△—△). Each point represents the mean isotope incorporation of 2 x 10^4 cells. Background incorporation by activated cells + media for each species was less than 400 cpm.


Mouse Activated Cells
Rat Activated Cells
Human Activated Cells
Sheep Activated Cells
Incorporation of ^{3}H-thymidine per $2x10^{4}$ Cells ($CPM \times 10^{-3}$)
120
100
80
60
40
20
100 50 25 12
% Sn Concentration

In summary, the results demonstrated that standard prepared sheep TCGF was the most restricted in xenogeneic activity, but sheep activated cells could grow in the presence of human, rat and sheep TCGF. Human TCGF was unique in supporting the growth of all 4 species, but human activated cells would only proliferate in response to human TCGF.

III. The Biochemical Properties of Sheep TCGF

The high loss of TCGF activity following purification techniques did not allow for sequential purification steps. For this reason, ammonium sulfate fractionation, DEAE-cellulose chromatography, G100 chromatography and IEF were performed on separate sheep TCGF preparations in order to study the biochemical characteristics of sheep TCGF.

Ammonium sulfate fractionation of sheep TCGF

A sheep TCGF supernatant prepared by the standard method was fractionated by ammonium sulfate precipitation to obtain 0-40%, 40-60%, and 60-80% fractions. After extensive dialysis followed by filter-sterilization, these fractions were assayed for TCGF activity. The 60-80% fraction was found to contain the majority of the TCGF activity (Table 2).

DEAE-cellulose chromatography

Sheep TCGF supernatants were prepared, immediately concentrated, dialyzed against the starting buffer, and layered on DEAE cellulose columns. The samples were eluted stepwise with NaCl buffers (pH 8, 0.01M Tris-HCl, PEG, NaCl 0.05M-0.20M). Fractions were collected representing the following buffers: 0.0-0.05M, 0.05-0.10M, 0.10-0.15M, and 0.15-0.20M NaCl. The fractions were concentrated by pressure dialysis and were tested for TCGF activity following extensive dialysis and filter sterilization. Each of these three supernatants demonstrated considerable heterogeneity following DEAE chromatography. The data from one of these supernatants is shown in Table 3. The data suggests that the majority of the sheep TCGF activity is eluted between 0.0-0.15M NaCl.

TABLE 2

Ammonium Sulfate Fractionation of Sheep TCGF

Fraction	$\%(NH_4)_2SO_4$ Saturation	*TCGF Activity
1	0-40	1246 ± 218
2	40-60	1108 ± 154
3	60-80	4268 ± 320

*TCGF Activity is expressed as CPM (^{3}H-Thymidine Incorporation) The values given are the means of 4 cultures at 100% conc. ± the SEM. The background incorporation of activated cells + media was 1536 ± 50.

TABLE 3

DEAE-Cellulose Chromatography

Fraction	NaCl Concentration	*TCGF Activity
1	0-0.05M	49149 ± 608
2	0.05-0.10M	31567 ± 2507
3	0.10-0.15M	38955 ± 899
4	0.15-0.20M	3510 ± 763

*The values given are the mean isotope incorporation of 2 x 10^4 cells at 50% conc. ± the SEM. The background incorporation of activated cells + media was 568 ± 84 cpm.

The significant amount of TCGF activity present in these three fractions suggests that there are at least two peaks representing different species of sheep TCGF with differing isoelectric points. Part of this TCGF activity may also be due to residual ConA.

Apparent molecular weight of sheep TCGF

A large volume of precultured sheep TCGF supernatant was concentrated, dialyzed against PBS plus PEG and layered on a G100 column and was eluted with 400 ml of PBS plus PEG. Fractions (4.0 ml) were collected, extensively dialyzed against PBS and RPMI 1640, filter-sterilized, and assayed for TCGF activity. The major peak of TCGF activity was contained in fractions corresponding to an apparent molecular weight of 30,000d (Figure 12). This molecular weight corresponds closely to the apparent molecular weight of standard prepared sheep TCGF (English, personal communication).

Isoelectric points of sheep TCGF

Isoelectric focussing (IEF) was performed on both sheep TCGF prepared by the standard method and by the preculturing method. The results demonstrated two major peaks for both samples with isoelectric points in the following pH ranges: 7.1-7.9, 5.1-5.6 (Figure 13, 14). The presence of two peaks for sheep TCGF agrees with the data obtained from DEAE-cellulose chromatography. Recent data has shown that ConA cochromatographs with peak 1 (pH 7.1-7.9), suggesting that part or all of the TCGF activity in this range may be due to ConA.

FIGURE 12

Molecular exclusion chromatography of sheep TCGF. A precultured sheep Sn was fractionated on a G100 column. Following dialysis, the even numbered 4 ml fractions from 44-80 were tested against activated sheep cells for sheep TCGF activity (●—●). Each point represents the mean isotope incorporation of 2 x 10^4 cells. The background incorporation of activated cells + media was 830 $\pm$ 115 cpm. The graph indicated where albumin 68,000d, ovalbumin 43,000d and chymotrypsinogen 25,000d are eluted.

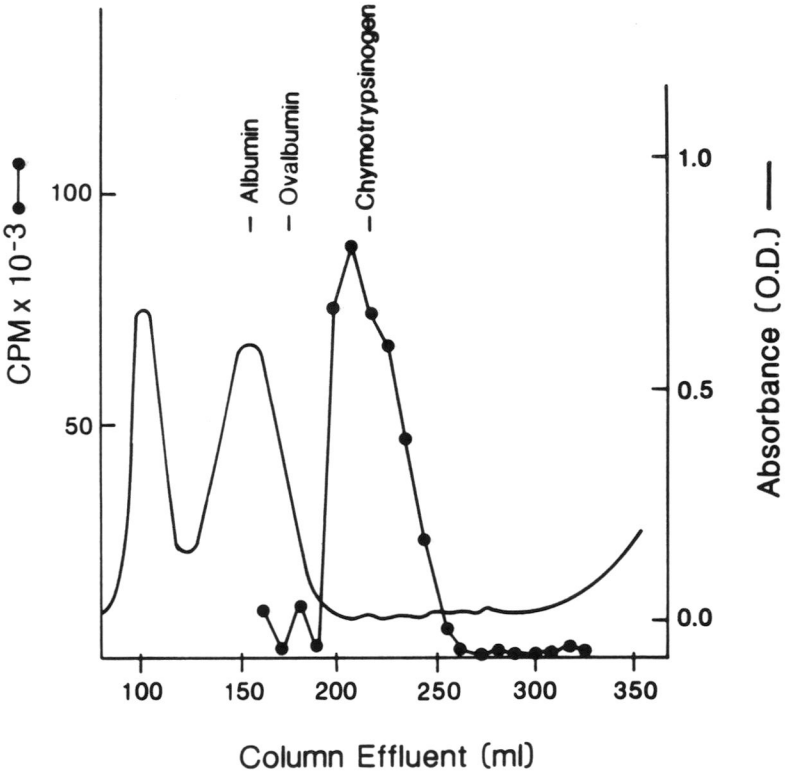

CPM x 10-3
100
50
Albumin
Ovalbumin
Chymotrypsinogen
1.0
0.5
0.0
Absorbance (O.D.)
100
150
200
250
300
350
Column Effluent (ml)

FIGURE 13

Isoelectric focussing of sheep TCGF. A standard prepared sheep Sn was electrophoresed for 48 hours over a sucrose stablized pH gradient from 3-10. The pH (——) was determined from the resulting IEF fractions before they were dialyzed and tested for TCGF activity (●—●). Each point represents the mean isotope incorporation of 2 x 10^4 cells. The background incorporation of activated cells + media was 398 $\pm$ 107 cpm.

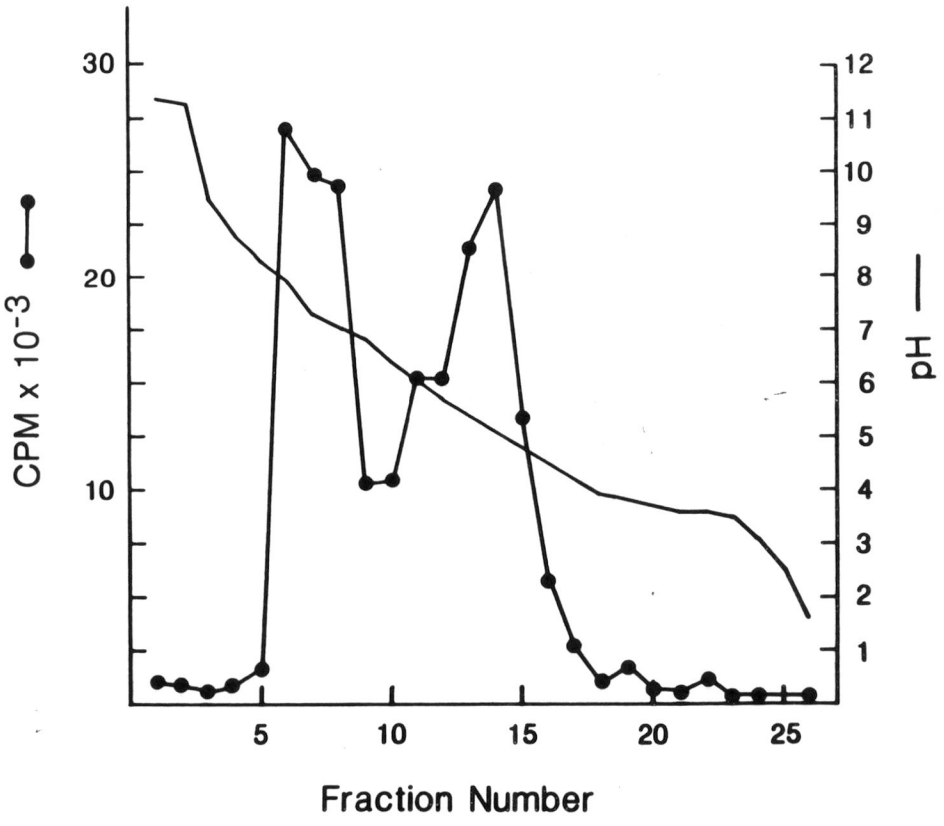
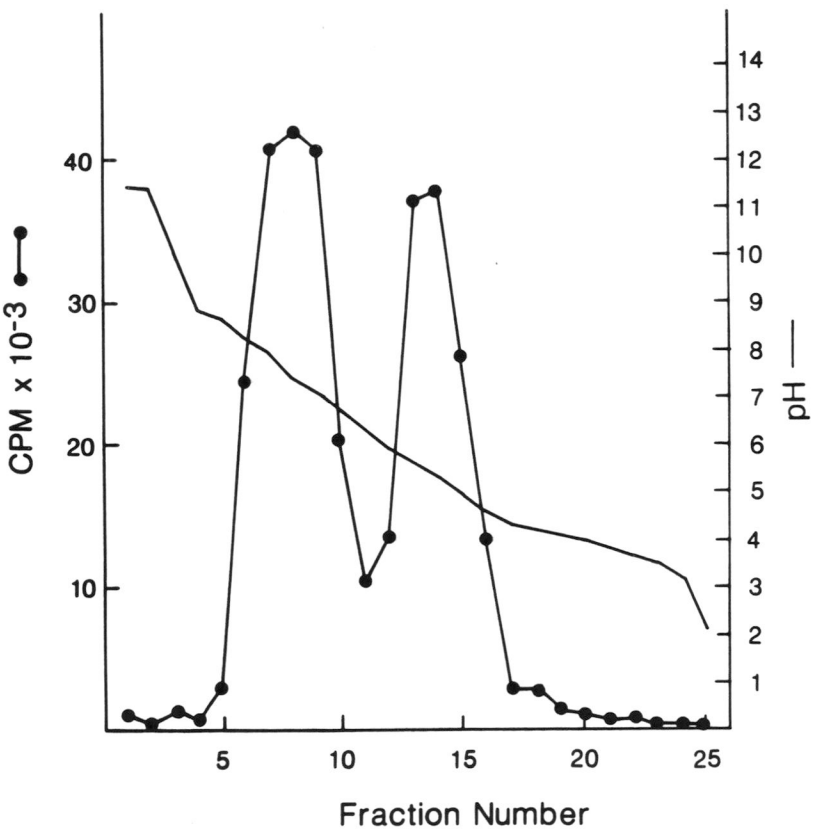

CPM x 10^{-3}
30
20
10
12
11
10
9
8
7
6
5
4
3
2
1
pH
5
10
15
20
25
Fraction Number

FIGURE 14

Isoelectric focussing of sheep TCGF. A precultured sheep Sn was electrophoresed on a pH gradient (3-10) as previously described. After pH determination (——) and dialysis, each fraction was tested against activated T cells for TCGF activity (●—●). Each point represents the mean incorporation of 2 x 10^4 cells. The background incorporation of activated cells + media was 663 ± 135 cpm.


CPM x 10-3
40
30
20
10
5
10
15
20
25
Fraction Number
14
13
12
11
10
9
8
7
6
5
4
3
2
1
pH

Summary

In summary, the biochemical properties of sheep TCGF derived from sheep Sn are as follows:

1. Sheep TCGF is precipitated by 60-80% saturation of $(NH_4)_2SO_4$.

2. Sheep TCGF is eluted from DEAE cellulose with NaCl concentration from 0-0.15M. This broad range is due to charge heterogeneity.

3. The apparent molecular weight of sheep TCGF determined by molecular exclusion chromatography is 30,000d.

4. Isoelectric focussing yields two activity peaks with pI values in the pH range of 7.1-7.9 (IEF peak 1) and 5.1-5.6 (IEF peak 2).

IV. Inhibition of Sheep TCGF by alpha-Methyl Mannoside

The effect of different sugars on sheep TCGF activity

The effects of 0.1M concentrations of alpha-methyl mannoside (α-MM), l-fucose, l-rhamnose and N-acetylglucosamine on sheep TCGF induced proliferation of activated sheep cells were examined. The only sugar which significantly inhibited sheep TCGF activity was α-MM (Figure 15).

α-MM inhibition of sheep TCGF is not due to residual lectin.

ConA binds specifically to α-MM and this sugar inhibits ConA mitotic activity (Sharon and Lis, 1972). One possible explanation for the inhibitory activity of α-MM on sheep TCGF may be due to residual ConA or due to a synergistic effect of lectin with TCGF (costimulator activity) (Shaw et al., 1978). Subsequent experiments have contradicted this proposal. Commercial human TCGF prepared from PHA stimulated lymphocytes was inhibited with α-MM to a similar magnitude as sheep TCGF (Figure 16,17). PHA was not inhibited with α-MM. Another experiment demonstrated that the addition of mitogenic amounts of lectin to sheep activated cells resulted in an insignificant increase in cell growth and did not exhibit a synergistic effect in the presence of wh sheep TCGF (Figure 18). Recent studies have supported that α-MM inhibition of sheep TCGF activity is not due to residual lectin. It has been shown that crude mouse TCGF containing the same level of residual ConA as sheep TCGF is not inhibited by α-MM (English et al., submitted for publication). In vivo produced TCGF was prepared in the absence of

FIGURE 15

The effect of various sugars on sheep TCGF activity. Two fold serial dilutions of a sheep Sn (SIL27) were tested against activated sheep cells in the absence (⊙—⊙) and presence of 0.1M concentrations of various sugars (l-fucose (◆—◆), l-rhamnose (▲—▲), n-acetyl glucosamine ●—● , and α-MM ■—■). Each point represents the mean incorporation of 2 x 10^4 cells. The difference between the TCGF activity in the control (SIL27 alone) and in SIL27 + α-MM was significant ($p < 0.05$). No significant difference could be demonstrated between SIL27 + the other sugars and the control ($p > 0.05$). The background incorporation of activated cells + media was 944 ± 153 cpm.

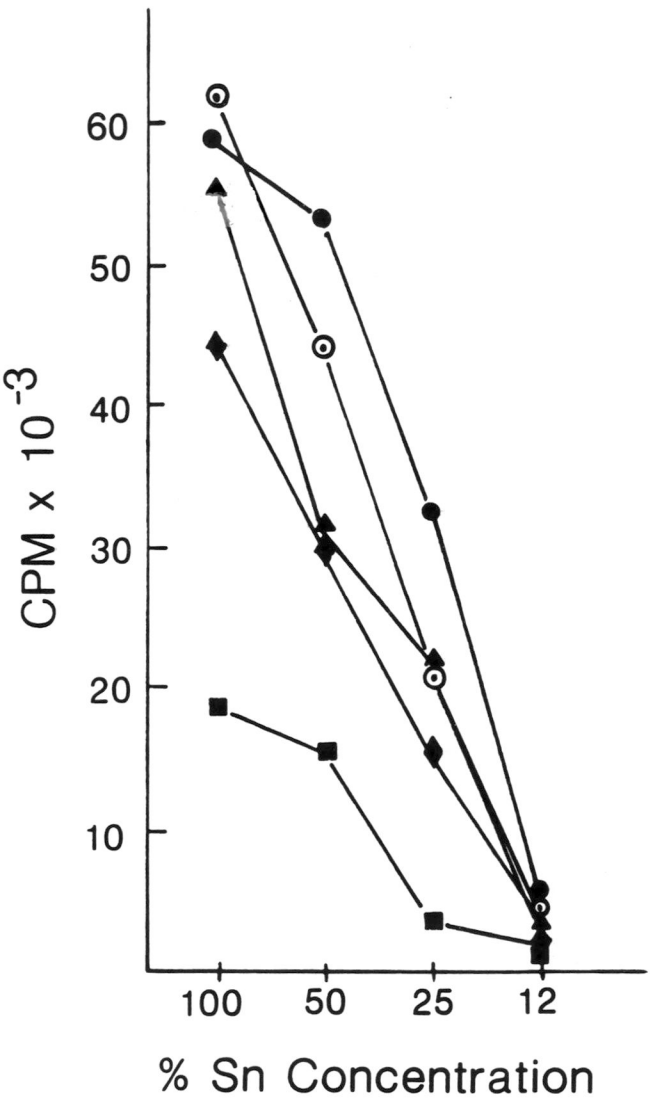

60
50
40
30
20
10
CPM x 10^{-3}
100
50
25
12
% Sn Concentration

FIGURE 16

α-MM inhibition of human TCGF. Two fold serial dilutions of a commercial human TCGF preparation (diluted 1:1 with media) were tested against activated sheep cells in the presence (▲—▲) and absence (●—●) of 0.1M concentrations of α-MM. Each point represents the mean incorporation of 2 x 10^4 cells. The background incorporation of activated cells + media was 772 ± 208 cpm.

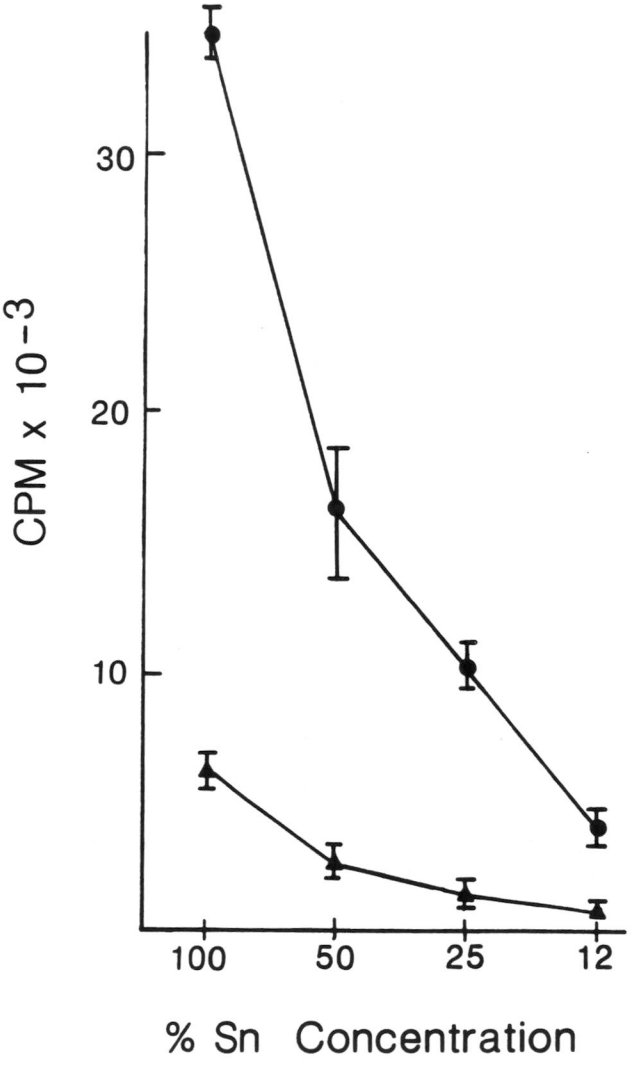

CPM x 10−3
30
20
10
100
50
25
12
% Sn Concentration

FIGURE 17

α-MM inhibition of sheep TCGF. Two fold serial dilutions of a sheep Sn were tested against activated sheep cells in the presence (▲—▲) and absence (●—●) of 0.1M concentrations of α-MM. Each point represents the mean incorporation of 2×10^4 cells. The background incorporation of activated cells + media was 531 ± 129 cpm.

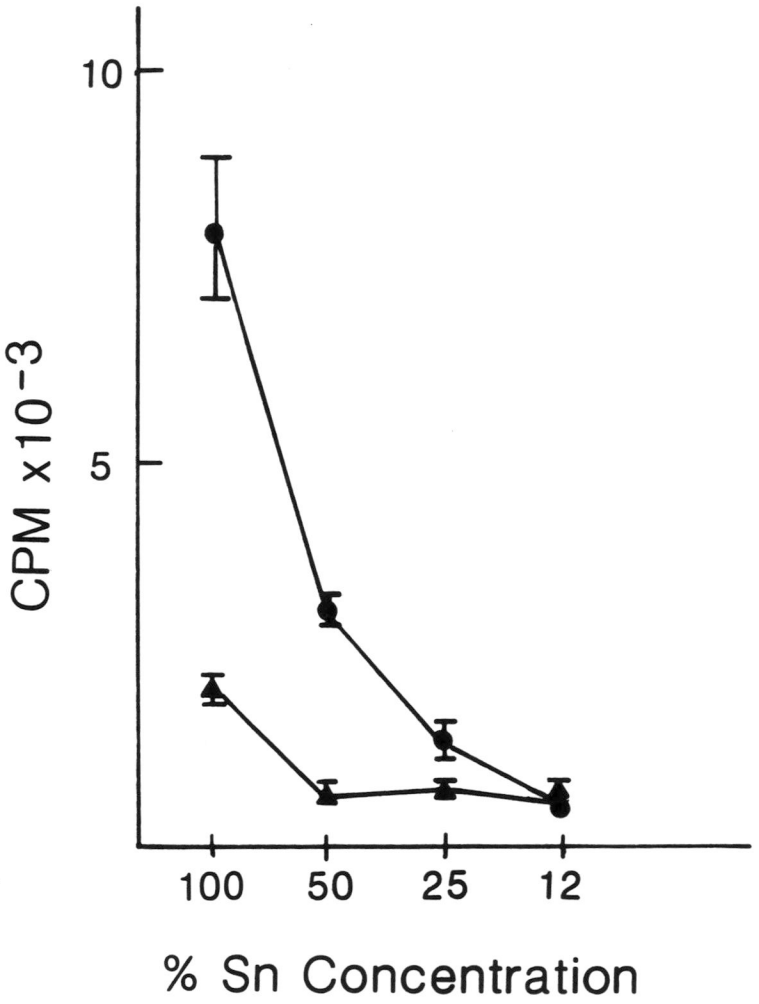
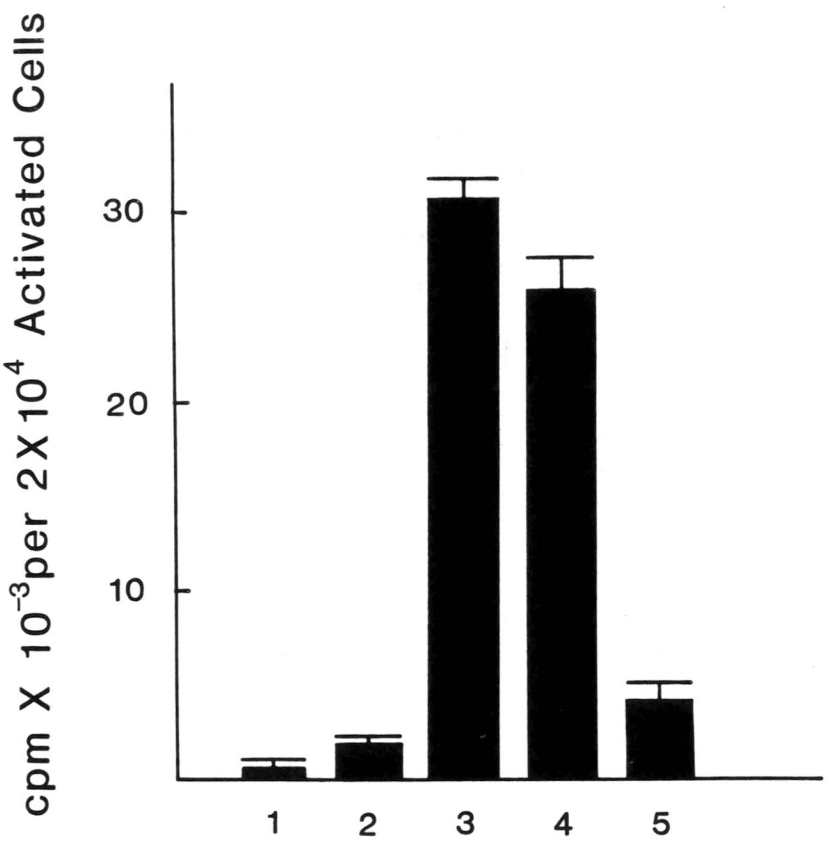

10
5
CPM x10-3
100
50
25
12
% Sn Concentration

FIGURE 18

The effect of ConA on the growth of activated sheep T cells. The activated cells were cultured in media alone (1), with 0.5 μg/ml ConA (2), in the presence of 50% sheep Sn (3), sheep Sn + ConA (4), and with sheep Sn, ConA and 0.1M α-MM (5). Each value was the mean isotope incorporation of 2 x 10^4 cells.


cpm X 10^{-3}per 2X10^4 Activated Cells
30
20
10
1
2
3
4
5

lectin and has been shown to be completely inhibited in the presence of α-MM (English et al., submitted for publication).

The effects of α-MM on purified sheep TCGF

A pooled IEF peak of sheep TCGF (peak 1, pH 7.1-7.4) was tested in the presence and absence of α-MM and was also tested ± α-MM after being passed through a Sephadex G10 column to remove ConA (Rosenberg, Schwarz, Spiess and Brown, 1980). There was no significant difference between the normal IEF peak and the G10 treated peak without ConA. The addition of α-MM abrogated the TCGF activity in both samples (Figure 19). These results suggest that α-MM was inhibiting TCGF and not residual ConA. However, recent information has shown that ConA cochromatographs with this peak and that certain concentrations of ConA can evoke a mitotic response on activated cells as well as peripheral cells (English, personal communication).

To determine if IEF peak 1 consisted entirely of ConA, it was tested against sheep activated cells and human activated cells. If the TCGF activity of peak 1 was only due to contaminating lectin, then it should evoke a response in both human and sheep cells; however, if the activity was due to sheep TCGF, it should only evoke a response on the activated sheep cells due to species restrictions. As Table 4 demonstrates, only the sheep cells demonstrated TCGF activity. This data suggests that the residual ConA in this sample was not enough to cause a mitotic response to activated cells alone, but it may synergize with sheep TCGF.

The effects of α-MM on IEF peak 2 (pH 5.1-5.6) were also examined. Based on isoelectric focussing data obtained from ConA, this peak

FIGURE 19

The effect of ∝-MM on sheep TCGF IEF peak 1 before and after Sephadex G10 treatment. The activated cells were cultured in the presence of 60% IEF peak 1 (1), IEF peak 1 + 0.1M ∝-MM (2), G10 treated IEF peak 1 (3) and G10 treated IEF peak 1 + ∝-MM (4). Each value was the mean isotope incorporation of 2 x 10^4 cells. No significant difference was evident between (1) and (3) ($p > 0.05$). The background incorporation of activated cells + media was 568 $\pm$ 91 cpm.

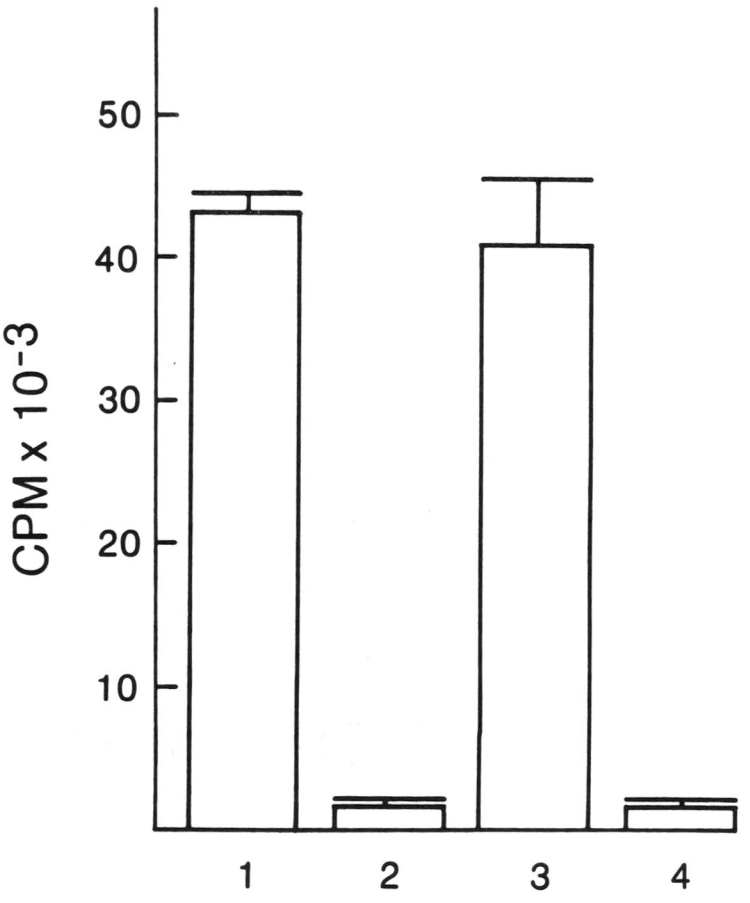

CPM x 10-3
50
40
30
20
10
1
2
3
4

TABLE 4

Inhibition of Sheep TCGF IEF Peaks by ∝-MM

	*TCGF Activity	% Inhibition
IEF Peak 1	16496 ± 3650	-
IEF Peak 1 + ∝-MM	667 ± 144	96%
IEF Peak 2	3914 ± 287	-
IEF Peak 2 + ∝-MM	1427 ± 291	64%

*The values given are the mean isotope incorporation of 2 x 10^4 cells at 100% conc. ± the SEM. The concentration of ∝-MM was 0.1M. The background incorporation of activated cells + media was 772 ± 208 cpm.

should be relatively free of residual ConA (English, personal communication). As the data demonstrates, peak 2 was also inhibited by α-MM though not to the same degree as peak 1 (Table 5).

Summary

Five sugars were tested with sheep TCGF, and α-MM was the only sugar that inhibited TCGF activity. Two experiments were conducted to determine if residual lectin was responsible for α-MM inhibition. The first experiment demonstrated that PHA prepared human TCGF was inhibited by α-MM, and the second experiment showed that the addition of mitogenic amounts of ConA did not affect TCGF activity or affect the growth of activated cells in the absence of TCGF. These two experiments, plus other studies discussed above, provided strong evidence that α-MM inhibition of crude TCGF supernatants is not due to residual lectin.

Two IEF peaks were also examined for α-MM inhibition. Peak 1 was completely inhibited by α-MM. It is now known that this peak was contaminated with ConA, but interspecies studies suggested that TCGF activity was also present. Peak 2, which did not cochromatograph with ConA was also inhibited by α-MM but to a lesser extent than peak 1.

TABLE 5

IEF Peak 1 Tested Against Activated Human and Sheep Cells

IEF Peak 1 (% Conc.)	*Sheep Activated T Cells TCGF Activity
100	36245 ± 682
50	29023 ± 524
25	8246 ± 944
12	3359 ± 589

IEF Peak 1 (% Conc.)	*Human Activated T Cells TCGF Activity
100	551 ± 117
50	503 ± 191
25	270 ± 118
12	606 ± 146

*The values given are the mean isotope incorporation of 2 x 10^4 cells ± the SEM. The background incorporation of activated cells + media was 768 ± 144 cpm for the human cells and 772 ± 208 cpm for the sheep cells.

DISCUSSION

Optimal conditions for sheep TCGF production and for sheep TCGF assays

Lectin induced T cell proliferation is not due to a direct mitogenic effect of the lectin but to its ability to induce TCGF production (Stadler and Oppenheim, 1982); therefore, the lectin concentration which stimulates a maximal mitotic response of T cells is the lectin concentration which induces maximal TCGF production. Other studies have shown that maximal TCGF production coincides with maximal TCGF receptor expression (Konaka et al., 1981). For these reasons, mitogen dose responses of PHA and ConA were utilized to determine the optimal lectin concentration for sheep TCGF production and for the activation of sheep T cells for TCGF responsiveness (TCGF receptor expression). The optimal lectin concentration for sheep determined in this study (4 µg/ml ConA or 16 µg/ml PHA) agree closely with the ConA and PHA concentrations routinely used to induce TCGF production in mouse, rat, and man (Lindsay et al., 1982; Di Sabato, 1982; Mier and Gallo, 1982).

This study revealed that there is variability in the in vitro TCGF production within a population of sheep. This is in agreement with studies of TCGF production among normal human subjects (Alvarez et al., 1979) which demonstrates that while some individuals consistently produce high titers of TCGF, others are low producers. Although there is accepted variability in human TCGF production, it has recently been shown that several immunodeficiency syndromes appear to be related to extreme deficiencies in TCGF production (Welte et al., 1982).

Excessive TCGF production appears to be linked to human lymphoblastic leukemia (Gillis, Mertelsmann, Clarkson and Moore, 1980).

In this study, it was shown that the kinetics of sheep TCGF production was dependent on cell density. It was demonstrated that at 1×10^7 cells/ml, sheep TCGF production was maximal within the first 12 hours of the culture period, but at 5×10^6 cells/ml, TCGF production was maximal within the final 12 hours of a 48 hour culture. I have shown that TCGF production at 1×10^6 cells/ml was not sufficient to differentiate between the various incubation periods; however, other studies have suggested that at this cell concentation, TCGF production was maximal during the final 24 hours of a 72 hour culture period (English, personal communication). Similar results were reported in a study of TCGF production by human cells (Alvarez et al., 1979). It appears that TCGF utilization by activated T cells which is dependent on cell density was primarily responsible for these observed differences in TCGF production. At high cell densities, TCGF would be rapidly depleted early in the culture, while at low cell density, TCGF utilization is less pronounced, and the TCGF concentration would increase throughout the culture period.

It was demonstrated that 2% sheep serum and 2% FCS significantly enhanced TCGF production. This finding is in agreement with a study of human TCGF where it was reported that low concentrations of serum enhanced TCGF production (Alvarez et al., 1979). In addition, investigators have shown that bovine serum albumin helps to stabalize human TCGF (Mier and Gallo, 1982).

In addition to lectin and protein supplements, other additives routinely used in the preparation of crude TCGF supernatants include 2-mercaptoethanol (2ME), PMA and PEG. Mercaptoethanol is a reducing agent which is thought to improve lymphocyte culture conditions by neutralizing toxic metabolites (Golub, 1981). The addition of 2ME to culture media has been demonstrated to enhance TCGF production (Schook, Kristensen, Otz, Lazary and de Weck, 1982). PMA is a tumor promoter which has been reported to act synergistically with ConA to induce TCGF production (Fuller-Farrar et al., 1981). In this study, initial investigations suggested that PMA did not synergize with ConA in the production of sheep TCGF. Recent studies have shown that PMA binds to serum proteins and is therefore nondialyzable (Krakauer et al., 1982 and Orosz, Roopernian and Bach, 1982). In addition, it has been shown that PMA enhances TCGF production in the absence of macrophages and it synergizes with lectin in inducing lymphocyte proliferation (Krakauer et al., 1982). Based on this information, PMA was not added to sheep TCGF culture supernatants. PEG has been shown to improve TCGF recovery by preventing its adherence to culture vessels and membranes due to its hydrophobic nature (Mier and Gallo, 1982). This finding would explain why in the present study the recovery of TCGF was significantly increased during the purification procedures.

The effects of preculturing lymphocytes on sheep TCGF production were examined since it has been reported that preculturing human lymphocytes before lectin stimulation resulted in increased TCGF production (Northoff et al., 1980). These investigators found evidence that

preincubation resulted in the depletion of suppressor T cells or the loss of their functional ability. The loss of suppressor T cell activity was thus responsible for the increased TCGF production since many of the mechanisms which regulate TCGF production are under direct control of suppressor T cells or their products (Hardt et al., 1981; Gullberg and Larsson, 1982; Malkovsky et al., 1982). Additionally, it has been shown that ConA induced suppressor T cells are responsible for inhibiting murine TCGF production within 24 hours in culture (Gullberg et al., 1981). This study provided evidence that preculturing sheep lymphocytes led to suppressor T cell depletion since the production of TCGF per viable cell was greater in a precultured sheep Sn than in the control sheep Sn prepared from equal numbers of cells, thus suggesting that in addition to suppressor T cell depletion, some TCGF producing cells were also depleted.

The effects of preculturing cells in the presence of rat Sn were investigated. This resulted in a sheep Sn with higher TCGF productivity per viable cell than the normal control sheep Sn and resulted in a TCGF yield equivalent to the control sheep Sn. This suggested that the presence of rat Sn was effective in maintaining the viability of TCGF producing cells. How this rat Sn acts is not clear since the precultured cells do not express receptors for TCGF (Bonnar et al., 1979) and hence the mechanism by which viability is maintained is likely to differ from that which operates in the maintenance of cell growth. These results on preculturing have been confirmed by a similar study (English, personal communication).

Many investigators have employed lectin activated T cell blasts to assay for TCGF activity (Mier and Gallo, 1980; Gullberg et al., 1981). In the absence of TCGF dependent sheep cell lines, lectin activated sheep cells provided an effective assay for sheep TCGF. In this study, it was found that a three day culture of activated sheep cells was an optimal culture period for assaying sheep TCGF present in sheep Sn. Shorter culture periods demonstrated lower sheep TCGF activity and were not sufficient to quantitate TCGF activity in a dose dependent fashion. Longer culture periods showed lower TCGF activity due to TCGF utilization. Commercial human TCGF was more potent than a majority of sheep TCGF preparations and supported growth for 5 days in culture.

Interspecies activity of sheep TCGF

The results demonstrated that sheep TCGF present in Sn exhibited the highest degree of species restrictions since it only supported the growth of activated sheep T cells. The TCGF's prepared from mouse and rat also demonstrated some species restrictions; however, human TCGF supported the growth of activated T cells derived from all 4 species. The xenogeneic activity of human TCGF has been reported in other works (Lindsay, et al., 1982; Ruscetti and Gallo, 1980; Gillis et al., 1980). Sheep activated cells continue to grow in the presence of sheep TCGF, rat TCGF and human TCGF, but human activated cells only grow if supplied with human TCGF.

A recent work demonstrated a sheep TCGF species (SIL4) which provided optimal growth supporting activity for human, mouse and sheep activated T cells (English et al., submitted for publication). This

TCGF supernatant was prepared from a mixed culture of efferent lymphocytes from the popliteal node and allogeneic peripheral blood lymphocytes in the presence of lectin. The xenogeneic activity of SIL4 could not be attributed to endotoxin or residual lectin since SIL4 did not have a mitogenic effect on resting lymphocytes. It appears that the activity of SIL4 was not due to the presence of high titers of sheep TCGF since concentrated sheep Sn's prepared by the standard method do not exhibit xenogeneic activity. It appeared that this method of induction generated a molecular species of TCGF which exhibited xenogeneic activity and was not present in standard prepared sheep Sn. Other workers have also suggested that the method of induction determines the molecular forms of TCGF produced (Welte et al., 1982).

The conclusion that can be reached from this study is that there are significant differences in the interspecies activity of sheep, human, rat and mouse TCGF. These differences can be attributed to a number of variables which include the affinity of the different TCGF's to the TCGF receptors for each species and the levels of TCGF produced from each species. It appears that standard prepared sheep TCGF has low binding affinity with receptors on xenogeneic cells since concentrated sheep Sn's exhibited the same species restrictions (English et al., submitted for publication). In contrast, it appears that human TCGF has a high binding affinity for the receptors for each species and was present in sufficient concentrations to support the growth of activated cells from each species. As described above, modifying the method of TCGF production may influence the molecular forms of TCGF produced and

the interspecies activity. It is possible that post synthetic glycosylation affects interspecies activity since this study provides evidence that the binding of TCGF to the TCGF receptor involves glycosyl residues.

The biochemical properties of sheep TCGF

This work demonstrated considerable differences between the biochemical properties of sheep TCGF and those reported for TCGF's from other species. For example, DEAE-cellulose chromatography of sheep Sn resulted in 3 active fractions eluted with the following salt concentrations (0-0.5M; 0.05-0.10M; 0.10-0.15M). Murine TCGF activity can be removed from DEAE-resins through stepwise elution with 0.15-0.20M NaCl (Watson et al., 1979a). Human TCGF does not bind to DEAE and therefore would be present in a 0-0.5M fraction (Gillis et al., 1980). The IEF results obtained from sheep Sn provide additional evidence for charge heterogeneity of sheep TCGF.

This work showed that sheep TCGF activity was precipitated by 60-80% saturation with $(NH_4)_2SO_4$. This finding is consistent with the salt concentration used to precipitate human TCGF (Gillis et al., 1980) and mouse TCGF (Watson et al., 1979a).

Molecular exclusion chromatography of sheep Sn resulted in a single peak of TCGF activity with an apparent molecular weight of 30,000d. This is in contrast to in vivo produced sheep TCGF which has an apparent molecular weight of 10-20,000d (English and Whitehurst, submitted for publication). There are two possible explanations for this discrepency in molecular weights. The first is that TCGF found in Sn may be in a

dimeric form while *in vivo* produced TCGF is monomeric. It has been reported that sodium dodecyl sulfate treatment of mouse TCGF (30,000d) resulted in a biologically active fraction possessing a molecular weight of 15,000d, suggesting that mouse TCGF is dimeric (Caplan *et al.*, 1981). Another possibility is that these two molecular weights correspond to two different molecular species of TCGF. It has been suggested that the molecular heterogeneity of TCGF is dependent on the method of production (Welte *et al.*, 1982). Molecular exclusion chromatography of human, mouse and rat TCGF yield apparent molecular weights of 15,000d (Gillis *et al.*, 1980), 30,000d (Watson *et al.*, 1979a) and 15,000d (Gillis *et al.*, 1980), respectively.

Isoelectric focussing of sheep Sn and precultured sheep Sn resulted in two major peaks with isoelectric points in the following pH ranges: 7.1-7.9 (peak 1) and 5.1-5.6 (peak 2). Human TCGF demonstrates a major activity peak with an isoelectric point of pH 6.8 and a minor peak at pH 7.1 (Gillis *et al.*, 1980; Ruscetti and Gallo, 1980). Other workers have reported a third activity peak for human TCGF with a pI value of 8.1 (Stadler and Oppenheim, 1982). Isoelectric focussing of murine TCGF has resulted in activity peaks at pH 4.3-4.5, pH 4.9-5.1 and a variable peak at pH 5.2-5.6. The acidic pI values reported for mouse TCGF and the more basic values reported for human TCGF are in agreement with the reported affinity of these lymphokines to DEAE-resins as described above. The reason for charge heterogeneity of sheep TCGF may be due to post synthetic glycosylation as has been demonstrated for mouse TCGF (Watson *et al.*, 1982) and human TCGF (Robb and Smith, 1981).

Based on these findings, it is not surprising that the interspecies activity of sheep TCGF differs from the TCGF's from other species.

The recent finding that the isoelectric point of ConA coincides with the pI of one of the sheep TCGF species (IEF peak 1) suggests that part or all of this TCGF activity is due to ConA (English, personal communication). However, there is strong evidence that TCGF activity in peak 1 is primarily due to sheep TCGF and not ConA. If ConA alone was responsible for the high TCGF activity in IEF peak 1, then similar activity peaks corresponding to ConA should be found in G100 chromatography and ammonium sulfate fractionation of sheep Sn. This was not the case since there was no significant TCGF activity corresponding to the molecular weight of ConA (55,000d) in G100 fractions or in the ammonium sulfate fraction (0-40% saturation) where ConA is found. In addition, if peak 1 consisted entirely of ConA, then it would be expected to exhibit xenogeneic activity on human activated cells in a similar manner; however, it has been demonstrated that sheep TCGF from sheep Sn does not support the growth of activated human cells. The active fractions from peak 1 were pooled and tested against human activated cells and sheep activated cells. Only the activated sheep cells proliferated in response to peak 1, suggesting that TCGF activity in peak 1 was due to sheep TCGF and not contaminting lectin. However, it should be noted that some workers have reported that peripheral human lymphocytes do not respond well to low doses of ConA (Farrar, Benjamin, Hilfiker, Howard, Farrar and Fuller-Farrar, 1982), but it has been my experience that identical

doses of ConA are equally effective in activating human and sheep lymphocytes.

It has also been demonstrated that mitogenic amounts of ConA did not significantly increase the mitotic response of activated cells in the absence of TCGF and did not have a synergistic effect in the presence of TCGF. This agrees with a similar study which demonstrated that lectin activated human T cells became TCGF dependent after a few days in culture and were no longer responsive to lectin (Mier and Gallo, 1980). The only instances in which activated sheep cells responded significantly to ConA alone were when the cells exhibited a high mitotic activity in the control culture in the absence of TCGF (English, personal communication). In most cases, the ^{3}H-thymidine incorporation in the control culture for standard prepared activated cells approached background levels (approximately 500 cpm) and these cells were responsive to TCGF but not ConA as described above.

Finally, passing pooled peak 1 over Sephadex G10 to remove ConA did not cause a significant change in TCGF activity. This also suggested that the activity in peak 1 was due to sheep TCGF and that ConA did synergize with this lymphokine.

Based on this information, I postulate that TCGF activity present in IEF peak 1 is due primarily to sheep TCGF and is not affected by contaminating lectin. In order to confirm the exact relationship between ConA and sheep TCGF in IEF peak 1, further studies should include isoelectric focussing of lectin free sheep Sn, partially purified TCGF samples, and sheep Sn prepared with PHA. One method to prepare lectin

free Sn is to expose lymphocytes to lectin for several hours followed by extensive washing of the cells. The cells are then resuspended in the absence of lectin for a normal culture period. The resulting supernatants have been shown to contain high titers of TCGF (Spiess and Rosenberg, 1981). Another method is to use ConA-sepharose to stimulate lymphocytes. This immobilized lectin can be removed from culture supernatants through centrigugation. This study suggests that partial purification through ammonium sulfate fractionation or molecular exclusion chromatography are effective in separating ConA from TCGF activity, thus minimizing the effects of residual lectin. Due to the different pI values of PHA and ConA, isoelectric focussing of PHA lymphocyte stimulation induces production of the same molecular species of TCGF found in ConA sheep Sn.

Long term growth cultures of TCGF dependent activated sheep T cells are now being developed, and they should facilitate the further characterization of sheep TCGF (English, personal communication). It has been demonstrated in this work that short term cultures of lectin activated cells are effective in assaying the unique property of TCGF (the ability to support the growth of activated cells). However, the ability of TCGF samples to support the long term growth of TCGF dependent sheep T cells would provide an unequivocal assay for sheep TCGF.

α-MM inhibits sheep TCGF activity

There is a great deal of controversy on whether TCGF's from different species are glycosylated. An early study of mouse TCGF failed to produce evidence for glycosylation of mouse TCGF (Watson et al.,

1982); however, in a more recent study, it was shown that neuraminidase treatment resulted in charge homogeneity, confirming the presence of sialic acid moieties (Watson et al., 1982). In addition, it has been shown that murine TCGF binds to a variety of lectin columns providing further evidence that the molecule is a glycoprotein (Clark-Lewis and Schrader, 1982; Altin and Di Sabato, 1980). Although some investigators have reported that there is no evidence for the glycosylation of human TCGF (Mier and Gallo, 1982), others have reported variable glycosylation of human TCGF based on enzyme treatments (Robb and Smith, 1981). It has been suggested that rat TCGF is not glycosylated based on its failure to bind to lectins (Di Sabato, 1982). It is important to realize that the binding of a molecule to lectins in contingent on the lectin specific carbohydrates being readily accessible.

In order to investigate whether sheep TCGF was glycosylated, various sugars were added to sheep Sn to determine their effects on sheep TCGF activity. The results demonstrated that sheep TCGF activity was only inhibited by α-methyl mannoside. Since ConA binds specifically to α-MM, it was possible that the α-MM inhibition of TCGF activity was due to residual lectin; however, evidence presented in this study eliminated this possibility. It was shown that α-MM also inhibited human TCGF activity which was prepared in the presence of PHA, a lectin that is not specific for α-MM, and that addition of ConA to cultures of activated cells in the presence or absence of TCGF did not result in an enhanced mitotic response. It was demonstrated that α-MM inhibited the sheep TCGF activity of the two pooled IEF peaks. Based on the isoelect-

ric point of ConA, IEF peak 2 should have been relatively free of lectin. IEF peak 1 was treated with Sephadex G10 to remove ConA and was then tested for α-MM. The results suggested that sheep TCGF present in IEF peak 1 was completely inhibited by α-MM.

English et al. (submitted for publication) demonstrated further evidence which suggested that α-MM inhibition of sheep TCGF was not due to residual lectin. In this work, it was demonstrated that mouse Sn's prepared from lymphocytes stimulated with the same ConA concentration as sheep Sn's were not affected by α-MM. In addition, in vivo produced sheep TCGF, which was lectin free, was completely inhibited by α-MM. Another possibility was that α-MM inhibition of sheep TCGF was due to lectin absorbed on the surface of the activated T cells. To investigate this possibility, lectin activated cells were washed in media with α-MM to remove absorbed ConA, followed by extensive washing in media alone. This treatment did not affect the TCGF responsiveness of these cells in the absence of α-MM which suggests that α-MM inhibition was not due to absorbed lectin (English, personal communication).

Based on this information, it can be postulated that the binding of TCGF to the TCGF receptor on activated T cells involves carbohydrate moieties (English et al., submitted for publication). This finding is in agreement with a recent study which shows that absorption of TCGF by murine lymphocytes can be inhibited with a specific monosaccharide (Palladino, Ranges, Scheid and Oettgen, 1983). It can be theorized that there is a functional relationship between the binding of lectin

to and the binding of TCGF to the TCGF receptor since both appear to involve glycosyl residues (English et al., submitted for publication).

In summary, the optimal conditions for sheep TCGF production in vitro were established and were used to produce sheep Sn's for TCGF characterization studies. It was demonstrated that standard prepared sheep TCGF differed considerably from human, mouse and rat TCGF's in interspecies activity. Biochemical studies of sheep TCGF also revealed significant differences from the molecular characteristics reported for human, mouse and rat TCGF's which could account for the differences in interspecies activity. Additionally, it was demonstrated that α-MM inhibits TCGF activity which suggests that the binding of sheep TCGF to TCGF receptors involves glycosyl residues. Long term growth cultures of activated sheep T cells are now being developed, and they should aid in the further molecular characterization of sheep TCGF.

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