THE UNIVERSITY OF MANITOBA

# THE FUNCTIONAL CHARACTERIZATION OF IN VIVO ACTIVATED INTERLEUKIN-2 RESPONSIVE T CELLS IN RHEUMATOID ARTHRITIS BY CELL CLONING TECHNIQUES

by

William A. Ofosu-Appiah

A Thesis

submitted to the Faculty of Graduate Studies in

partial fulfillment of the requirements for the

Degree of Doctor of Philosophy

Department of Immunology,

Winnipeg, Manitoba

May, 1988

ISBN 0-315-47924-8

# THE FUNCTIONAL CHARACTERIZATION OF IN VIVO ACTIVATED INTERLEUKIN-2 RESPONSIVE T CELLS IN RHEUMATOID ARTHRITIS BY CELL CLONING TECHNIQUES

BY

WILLIAM A. OFOSU-APPIAH

A thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

DOCTOR OF PHILOSOPHY

# TABLE OF CONTENTS

Page No.

Page No.

Page No.

## ACKNOWLEDGEMENTS

I wish to express my sincere gratitude to my supervisor, Dr. Richard Warrington for his excellent guidance and support during the course of these investigations. I thank him for providing me with the opportunity for independent work in the midst of encouragement and direction. I am also grateful to Dr. John Wilkins who provided critical analysis of the experiments and their design as well as constructive criticisms. I was fortunate to pursue this project in an atmosphere which was friendly, scientific and thought-provoking. I would also like to thank Ms. Patty Sauder, Mr. John Rutherford, Mr. Keng Wong, Ms. Lorraine Mischuk and Ms. Joan Cook who faithfully imparted to me their technical skills and also analyzed some samples for me. I am particularly indebted to the physicians of the Rheumatic Diseases Unit who provided the samples for the studies. A special acknowledgement is given to Mercy Deh and my parents for their support, encouragement and understanding throughout the course of these studies. I also wish to thank Drs K. Morgan, University of Manchester, England and A.R. Poole, Shriner's Hospital, McGill University, Montreal, Canada for providing me with purified connective tissue antigens.

I am grateful to Mrs. Theresa Coulson, who expertly typed and organized this thesis.

Finally, the financial support of the Arthritis Society is gratefully acknowledged.

## ABSTRACT

Cloned T cell lines were established by cloning in the presence of interleukin (IL)-2, *in vivo* activated, IL-2 responsive T cells obtained from Rheumatoid Arthritis (RA) peripheral blood (PB), synovial fluid (SF), synovial tissue (ST) and Normal (N) PB, using a limiting dilution technique developed in this laboratory. These T cells have been maintained in tissue culture for up to 8 months.

The precursor frequency of such *in vivo* activated, IL-2 responsive T cells were higher in RA SF (1/60 - 1/400), than autologous PB (1/108 - 1/657), ST (1/202 - 1/1751) and N PB (1/324 - 1/1200). The clonal burst sizes of the ST clones were very small and all efforts to expand and maintain them in long-term culture failed. SF T cells responded with equal cloning efficiency in the presence of SF cells (SFC) or PB cells (PBC) used as filler, although the clonal burst sizes were larger using SFC as irradiated filler. In contrast, PB T cells grew poorly on SFC used as filler. A prevalence of CD8+ (67%) T cell clones were generated from SF while CD4+ (83% and 92%) predominated in PB from RA and normal subjects respectively. The functional analysis of the clones demonstrated that all the clones released interferon-gamma (IFN-$\gamma$) following PHA-P stimulation but none produced detectable levels of IL-2. IL-2 production after PHA and Phorbol myristate acetate (PMA) stimulation was similar in RA PB and N PB but decreased in SF clones. All the clones produced a fibroblast-activating factor (FAF) upon PHA stimulation, with SF clones producing higher levels than PB clones. A few SF T cell clones constitutively produced low levels of FAF. The clones exerted cytotoxic effects upon K562 (NK-sensitive),

Raji (NK-resistant) colo (solid tumor cells), synovial fibroblasts, autologous or allogeneic PHA-blast targets but not fresh PBL , while the clones themselves lacked NK antigens such as CD11, CD16, Leu 7 (or HNK-1) but expressed CD3, T101, HLA-DR (Ia) antigens suggesting a T cell lineage. Thus, the clones are more compatible with lymphokine-activated killer (LAK); non-MHC restricted cytotoxic cells or autologous mixed lymphocyte reaction (AMLR) generated cells.

Connective tissue antigens (CTA) such as collagen and proteoglycan, stimulated the clones either to release IFN-γ or proliferation. RA clones showed increased CTA reactivity compared to Normal clones. The clones were capable of presenting CTA to each other to cause proliferation in the absence of APC, although the proliferative responses were higher in the presence of APC. The proliferative responses were markedly inhibited by anti-Ia AB suggesting that the HLA-DR antigens are critically involved in the antigen presentation. The responsiveness of the clones to soluble CTA was strictly fibonectin (Fn)-dependent, while with polymerised CTA, Fn served to enhance IFN production under serum-free conditions. Addition of a synthetic peptide (Gly-Arg-Asp-Ser-Pro) which comprises a cell attachment determinant of the Fn molecule did not inhibit CTA-induced IFN production. Anti-CD3 AB and AB to the framework determinants of the T cell receptor β chain abrogated the CTA-induced IFN production. None of the clones convincingly expressed an Fn-binding activity by indirect immunofluorescence or immnoperoxidase using purified human Fn and rabbit anti-human Fn or by cell attachment studies utilizing immobilized Fn-coated plates.

The conclusion drawn from these findings are: (1) IL-2 responsive T cells derived from RA SF and PB exhibit differences in both surface phenotypes and growth requirements suggesting that either SF T cells may have been selected for their ability to grow in the joint environment or that a selective stimulating activity is exerted by the synovial filler on synovial T cells in contrast to PB T cells which grow poorly.

(2) RA clones expressed greater reactivity to CTA than did N PB clones. The fact that all clones, irrespective of origin, reacted to CTA suggests that the responses to CTA was mediated by a similar mechanism, with the CTA expressing a common or shared epitopes. The recognition of CTA by the T cell clones was not mediated by an Fn receptor on the surface of T cells but rather by a receptor linked to or closely associated with the CD3 antigen receptor complex. Thus, the responses to CTA is mediated via the T cell antigen receptor pathway. Taken together, these results provide further evidence about the possible pathogenetical role of activated T cells in RA.

## OBJECTIVE OF THE INVESTIGATION

In the last few years, considerable evidence has been provided that T cell-mediated processes play a major role in the pathogenesis of RA. Such evidence is based on T cells being the predominant cells infiltrating the synovium (Abrahamsen et al, 1975, Van Boxel and Paget, 1975), the majority of which are activated (Burmester et al, 1981; Forre et al, 1982). An increased proportion of T cells present in PB and SF (Kluin-Nelemans et al, 1984; Burmester et al, 1984) are also activated as defined by the expression of HLA-DR (Ia), Tac and Transferrin antigens. In addition, lymphokines, presumably from activated T cells, have been found in synovial fluid (Statsny et al, 1975) reinforcing the concept of ongoing immune response in the joint. It is however, unknown whether the infiltrating cells proliferate in situ or were recruited to the site of inflammation from the circulation. The rationale for performing these studies is that such in vivo activated T cells may be actively involved in the pathogenesis of the disease in that, DR antigens which they express on their surface play an important role in the induction and maintenance of immune response (Forre et al, 1982).

T cell abnormalities in RA are probably related to both regulatory and effector functions. A defect in regulatory function will allow autoreactive T cells to react with autoantigens implicated in the pathogenesis of RA leading to inflammatory reactions. There is considerable evidence from animal models that T cell reactions with antigens in joints initiate inflammatory polyarthritis. Animals whose

T cells are sensitised to autoantigens such as collagen (Trentham et al, 1979; Ofosu-Appiah et al, 1983) or proteoglycan (Van Eden et al, 1985; Champion et al, 1982) develop arthritis. If T cells expressing such autoreactivity are present in vivo, it should be possible to isolate, clone and expand them in vitro using IL-2 and subsequently characterize them further. Previous work in this department (Wilkins et al, 1983) and others (Clark et al, 1984) have demonstrated that T cell lines can be established from RA synovial fluids. However, a better understanding of the pathogenetic role of activated T cells in RA requires the direct analysis of T cells infiltrating the tissue, fluid and present in PB. For this purpose, a number of studies focused on surface phenotype analysis and functional properties. On the contrary, only a few studies (Schlesier et al, 1984) have been concerned with the clonal analysis of the activated T cells in SF and PB. The purpose of this investigation was to study the role of in vivo activated, IL-2 responsive T cells in PB and the joint play in the pathogenesis of RA. In an effort to determine the functional properties (such as cytolytic activities, lymphokine production and antigenic specificity) of such activated T cells, I have undertaken to clone T cells from SF, ST and PB and establish stable T cell lines using IL-2. Such an approach would hopefully give an insight into the nature of the immune reaction occuring in the joint as well as the mechanism(s) responsible for the perpetuation of the inflammation and ultimately help in the design of regimens for immune intervention.

## LIST OF FIGURES

Page No.

Page No.

## LIST OF TABLES

Page No.

Page No.

# LIST OF ABBREVIATIONS

| | |
|---|---|
| AET | Aminoethylthiouronium-bromide |
| AMLR | Autologous mixed lymphocyte reaction |
| APC | Antigen presenting cell |
| ARA | American rheumatism association |
| BCDF | B cell differentiation factor |
| BCGF | B cell growth factor |
| BSA | Bovine serum albumin |
| CHY-A | Chymotrypsinogen A |
| CM | Conditioned medium |
| CMI | Cell-mediated immunity |
| CPM | Counts per minute |
| CR | Chromium-51 |
| CSA | Cyclosporin A |
| CTA | Connective tissue antigens |
| CYTO-C | Cytochrome C |
| DI | Denatured type I collagen |
| DII | Denatured type II collagen |
| EBV | Epstein-barr virus |
| EDTA | Ethylenediamine tetraacetic acid |
| ELISA | Enzyme-linked immunosorbent assay |
| FAF | Fibroblast activating factor |
| FCS | Fetal calf serum |
| FITC | Fluorescein isothiocyanate |
| FN | Fibronectin |

| | |
|---|---|
| GAR | Goat anti-rabbit immunoglobulin |
| HBSS | Hanks Balanced salt solution |
| H-TdR | Tritiated thymidine |
| HUS | Human serum |
| ICM | Interleukin -2 containing medium |
| IFN- | Interferon-gamma |
| IL-1 | Interleukin-1 |
| IL-2 | Interleukin-2 |
| KD | Kilodalton |
| LAK | Lymphokine activated killer cell |
| LDA | Limiting dilution analysis |
| LYS | Lysozyme |
| M | Moles |
| MAB | Monoclonal antibody |
| MW | Molecular weight |
| NI | Native type I collagen |
| NII | Native type II collagen |
| NK | Natural killer cell |
| NP-40 | Non-ionic detergent P-40 |
| OVA | Ovalbumin |
| PB | Peripheral blood |
| PBL | Peripheral blood lymphocytes |
| PBS | Phosphate buffered saline |
| PFC | Plaque forming cell |
| PHA-P | Phytohemagglutinin-P |
| PG | Proteoglycan |

PMA Phorbol myristic acetate

PPD Purified protein derivative

PWM Pokeweed mitogen

RA Rheumatoid arthritis

RFC Rosette forming cell

RHP Reverse hemolytic plaque assay

RNASE Ribonuclease

RPMI 1640 Rosewell park memorial institute medium 1640

SF Synovial fluid

SFL Synovial fluid lymphocytes

ST synovial tissue

# INTRODUCTION

Rheumatoid Arthritis (RA) is defined as a systemic and chronic disease of unknown aetiology, affecting mainly peripheral synovial joints, leading to the breakdown of joint structures (Gardner, 1972). Immunological factors have been implicated in the pathogenesis of the disease. In Fig. 1, I have sketched the events following stimulation of the cellular arm of the immune response by a putative antigen (connective tissue antigens, viruses, bacteria etc.) resulting eventually in generalised inflammation and tissue damage characteristic of RA. The first pathological changes of RA are observed in the synovial tissue and therefore a better understanding of the structure and functions of the synovium is relevant to this investigation and thus, will be reviewed in detail.

# CHAPTER 1: LITERATURE REVIEW

## Normal Synovial Tissue and Fluid

### 1:1 Structure and Functions:

The joints of the body possess a cavity which is surrounded by a fibrous capsule and articular cartilage. The capsule comprises an outer layer of dense fibrous tissue and an inner layer of loose, vascularised connective tissue, which is called synovial tissue. The synovial tissue, or synovium, differs from the lining of other body cavities in that, it has no basement membrane and does not form a continuous layer with the articular cartilage surface (Castor et al, 1960). The intercellular ground substance however, is in continuity with the synovial cavity. Thus, the joint space is simply a large

extracellular space of loose connective tissue.

The area of the synovial membrane facing the synovial cavity consists of one to three layers thick of synovial lining cells (SLC). Electron microscopy has revealed that the SLC layer contained a macrophage-like cell termed (type A), fibroblast-like cell (type B) and an intermediate or (type C) populations (Barland et al, 1964). Beneath the SLC is vascularized loose connective tissue interspersed with fibroblasts, macrophages, mast cells, a few lymphocytes and leukocytes. Collagen fibres provide structural support for the synovial tissue cells. These collagen fibres are more abundant in the outer layers of the synovial tissues.

The type A cell is characterized by a Golgi apparatus, many vacuoles and vesicles with few endoplasmic reticulum, suggesting that it is not a secretory cell but rather phagocytic cell (Edwards et al, 1982). These cells express both surface Ia (HLA-DR) antigen and receptors for the Fc portion of the immunoglobulin molecule (Fc receptors). Their cytoplasm stains positive for non-specific esterase which is an enzyme marker for macrophages (Edwards et al, 1982). Type B cells are secretory cells and are characterized by abundant rough endoplasmic reticulum and ribosomes and contains far less vacuoles and vesicles than type A cells. Type B cells are the predominant cells in normal synovial lining of most species (Ghadially and Roy, 1968).

The synovial cells synthesize and secrete connective tissue elements which maintain the structural integrity of the joint. They also secrete other macromolecules which endow normal synovial fluid with the lubrication function in the joint.

Synovial fluid fills the joint space and is a filtrate of plasma

(Ropes et al, 1939) mixed with hyaluronic acid produced by the SLC. The functions of hyaluronic acid are numerous, but the most important one is to lubricate the joint but the exact method whereby lubrication is accomplished is still not fully understood. Normal synovial fluid contains few cells. Of these, monocytes predominate. Lymphocytes occur in small numbers while polymorphonuclear leukocytes occur occasionally (McCarty, 1962). Turnover of synovial fluid components occurs through synovial tissue (Hamerman et al, 1969).

1:1:1 Rheumatoid Synovial Tissue and Fluid:

There is consensus among investigators that the earliest histological signs of RA occurs within the synovial tissue. These are characterised by proliferation of SLC, congestion and oedema of the connective tissue stroma, fibrin deposition and cellular infiltration of the synovium by inflammatory cells such as macrophages, T and B lymhocytes and plasma cells (Gardner, 1972). Polymorphonuclear neutrophils have recently been shown to be present at the pannus-cartilage junction (Mohr et al, 1981). It has been suggested by Ziff (1973) that different types of immune reactions occur in the joint and that delayed-type hypersensitivity reaction takes place in the sublining of the synovium, whereas immunoglobulin and rheumatoid factor production occur in other layers. In the joint cavity, immediate hypersensitivity or (Arthus-like) reactions occur, which are characterised by the predominance of neutrophils in the synovial fluid. As the inflammation proceeds to the chronic phase, villous hypertrophy of the synovial membrane occurs. At the margins of the articular cartilage, the pannus, a hypertrophic synovium, invades and destroys the cartilage (Kobayashi and Ziff; 1975). The destruction of articular cartilage

involves the degradation of collagen and proteoglycan, the two major components of connective tissue by collagenases and other enzymes secreted by the cells in the pannus. Indeed, increased amounts of collagenases have been demonstrated by immunofluorescent technique at the pannus-cartilage junction (Wooley et al, 1977). The degradation of proteoglycan appears to be mediated by both leukocyte elastase (Menninger et al, 1980) and Cathepsin D (Dingle, 1971; Poole et al, 1976) which are also present at the cartilage-pannus junction.

The rheumatoid synovial fluid reflects the changes that occur within the synovial tissue and hence it is usually used as an index of the severity of the arthritis. These include increased concentrations of plasma proteins (Pruzanski et al, 1973) and decreased oxygen and glucose concentrations (Roberts et al, 1967). Decreased complement levels (Pekin and Zvaifler, 1964), the presence of rheumatoid factors (Rodnan et al, 1963), collagen-anti-collagen immune complexes (Clague and moore, 1984) and the presence of inflammatory cells in rheumatoid synovial fluids suggest that immune mechanisms underlie the disease.

1:1:2 Aetiology:

The aetiology of RA is still unknown but several theories proposing that infectious agents (bacteria, viruses and mycoplasma) are important in the pathogenesis of RA have been described (Hammerman, 1975). Unfortunately none of these theories have stood the test of time (Zvaifler, 1979). This is because, to date, no bacterial, viral antigens and mycoplasmas have been isolated from synovial fluid or tissue of RA patients. Recently, Epstein-Barr virus (EBV) has become an attractive candidate in the autoimmune disease because of its ubiquity, persistence and ability to alter immune responses. Although

Rheumatoid patients cannot handle EBV infection compared with normals as demonstrated by the high titres of antibodies to EBV antigens (Alspaugh et al, 1981) and the inability of RA T lymphocytes to control the outgrowth of EBV-infect B cell (Tosato et al, 1981), there is no evidence that EBV gets into the joint space. The EBV antigen has not been demonstrated on cultured synovial cells or extracted synovial membranes (Fox et al, 1986).

Because of the failure to isolate infectious agents from RA synovial fluid and tissue samples, attention turned towards immune response directed against a normal or altered tissue components. Thus, another hypothesis emerged that lymphocytes capable of reacting with normal tissue components such as collagen exist in healthy individuals (Solinger and Stobo, 1981; Kammer and Trentham, 1984 ) but such autoreactive T cells are held in check by a complex immunosurveillance mechanisms. In RA however, such immunosurveillance is defective, thereby allowing autoreactive T cells to proliferate to antigens in the joint, releasing soluble products such as interferon and interleukins which could contribute to the production of the chronic inflammation seen in RA. The demonstration that native type II collagen, a major component of articular cartilage can cause an inflammatory polyarthritis in rats which is histologically similar to the chronic proliferative synovitis seen in RA has given credence to the hypothesis that immunity to autoantigens may represent one of the complex processes involved in the disease.

Concepts of Immunopathogenesis:

1:1:3 Immune-complexes:

Initially, RA was perceived as immune-complex (IC) disease based on

the profound decrease of complement in synovial fluid (Marcus and Townes, 1971; Zubler et al, 1976). ICs in the form of aggregated immunoglobulins and complement components have been demonstrated in rheumatoid articular collagenous tissues (Cooke et al, 1975; Ohno and Cooke, 1978). IgG immune-complexes containing RF could be eluted from RA synovial tissue (Munthe and Natvig, 1971). Rheumatoid factor (RF), which is an autoantibody that reacts with the Fc portion of autologous Immunoglobulin (Ig)G (Goodman, 1961), was implicated in the pathogenesis of RA, because it was the predominant constituent of immune-complexes in sera (Franklin et al, 1957) and synovial fluids (Vaughan et al, 1968; Winchester et al, 1970). The interest in RF waned when it was later discovered that a variety of non-articular diseases such as tuberculosis (Singer et al, 1962), parasitic infections (Houba and Allison, 1966), and pulmonary diseases (Tomasi et al, 1962) were also associated with elevated levels of serum RF. Furthermore, RF has been demonstrated in normal adult subjects with elderly subjects having higher levels than young adults (Wernick et al, 1981; Rodriguez et al, 1982). These findings led to the suggestion that RF is merely a by-product of the disease process; being a response to the causative agent, but not involved in the pathogenesis of RA (Christian, 1961). The presence of RF in sera of normal subjects suggests that it may have a physiological role, possibly in normal immune homeostasis (Rodriguez et al, 1982). Collagen-anti-collagen immune complexes have been demonstrated in RA synovial fluid (Menzel et al, 1976; Cague and Moore, 1984) and it has also been implicated in the chronic inflammation seen in RA. In those earlier concepts of the disease, T cells were considered to play little, if any, role in the

pathogenesis of RA. However, the finding of Paulus et al,( 1977) that the joint inflammation subsided in RA patients after thoracic-duct drainage, and also the selective suppression of T cell but not B cell functions by total lymphoid irradiation resulting in disease improvement (Kotzin et al, 1981; Field et al, 1983; Brahn et al, 1984) the predominance of T cells in RA synovium (Van Boxel and Paget, 1975; Bankhurst et al, 1976) and the demonstration of lymphokines in synovial fluid (Stasny et al, 1975; Wood et al, 1983; Wilkins et al, 1983) established that T cells may play a fundamental role in the disease processes.

Since then, there has been a spectacular progress in understanding of the pathogenesis of joint inflammaion. Although, infectious agents still continue to be attractive concept of the disease, there is an overwhelming evidence that RA is an immunologically-mediated disease.

1:1:4 Autoimmunity to Joint Antigens:

The current concept of the pathogenesis of RA (Trentham et al., 1985) is that it is a T cell dependent immune response to a restricted antigen(s) within the joint, with the inflammation being a consequence of secondary recruitment of inflammatory cells. Immunohistochemical studies by (Klareskog et al, 1982) on RA synovium supported the concept of T cell-mediated immune response in that, antigen-presenting macrophages were seen in close proximity to activated T cells. Thus, the lesion in its entirety resembles a self-perpetuating delayed-type hypersensitivity to the putative antigen challenge. It is also possible that some of the complications of RA such as vasculitis may be B-cell and complement-mediated. Although cell-mediated immunity to collagen (Trentham et al, 1978b; Smolen et al, 1980; Stuart et al,1980)

and proteoglycan (Glant et al, 1980) in RA patients has been detected by using assays of lymphocyte transformation and of production of leukocyte migration inhibition factor or leukocyte-derived monocyte chemotactic factor, the significance of these findings in the context of the aetiology and pathogenesis of the disease is still not resolved. The future direction will be the identification of the important critical epitopes on the autoantigens in joint playing a crucial role in the disease.

## T CELL ACTIVATION:

### 1:1:5 Recognition of antigen by T cells:

T cell activation is a complex process, involving the recognition of foreign antigens in the context of the appropriate MHC antigens (Bevan, 1975; Zinkernagel and Doherty, 1975). This T cell recognition of antigen in association with self MHC antigen, the precise mechanisms of which is still not fully understood, could occur in one of three ways namely: (a) T cells use distinct receptors to recognize antigen and self-MHC product, referred to as the "dual receptor model" (b) a single T cell receptor recognizes antigen associated with self MHC, termed the "associative recognition model" (c) the antigen modifies the self MHC antigen on the antigen-presenting cell (APC) which the T cell recognizes as altered self, referred to as the "altered self model." The latter idea was based on experimental data which showed that T cells recognize and react to non-self MHC on foreign cells very efficiently. All the models have some experimental evidence but this MHC antigen association is not the theme of this review so it will not be discussed in detail.

1:1:6 Activation of T cells by Antigen-Presenting Cells (APC):

It is generally accepted that macrophages and monocytes are the classical APCs required for T cell activation (Rosenthal and Shevach, 1973; Treves et al, 1981; Alpert et al, 1981). These cells present antigen and also release hormonal signals such as IL-1, important to T cell activation. It has become increasingly clear both in human and murine systems that several different cell types such as dendritic cells (Nussenzweig et al, 1980) and B cells (Glimcher et al, 1982; Issekutz et al,1982) are able to present antigen to T cells. The wealth of data in the literature suggest that antigen processing, which involves degradation of native complex proteins (Ziegler and Unanne, 1981; Grey et al, 1982 ; Scala and Oppenheim, 1983) by possibly a proteolytic process in the cytoplasm of the cell, is a prerequisite for T cell activation whether the presenting cells are B cells or monocytes.

There is some controversy as to whether IL-1 is necessary for T cell activation. Studies by Walker et al (1982) using a T cell hybridoma which can respond to antigen in the absence of IL-1, showed that this T-cell hybridoma proliferated when antigen was presented to it on fixed macrophages or B cells. This study suggested, that the events leading to T cell activation is related to the antigenic display rather than in the production of IL-1. More studies will be required to resolve this controversy. The precise mechanisms and the extent of antigen processing required for presentation are not known and it is apparent that various cells differ in their ability to process and display a particular antigenic eiptope for stimulation of T cells (Malek et al, 1983; Allen and Unanue, 1984).

Recently, a number of studies have shown that human T cells, when activated by antigen or mitogen express Ia antigens (Ko et al, 1979). These Ia+ T cells but not resting Ia- T cells, were potent stimulators of both autologous and allogeneic MLR in the absence of APC (Gerrard et al, 1985). In addition, the activated T cells can present antigens such as insulin (Ben-Nun et al, 1985), tetanus toxoid (Triebel et al, 1986; Gerrard et al, 1986) to each other to cause proliferation. However, the autopresentation required a critical mimimal cell number. The activated T cells presented denatured antigen more effectively than soluble, native antigen (Gerrard et al, 1986). Since denatured antigen may represent a form of processed antigen, it suggests that activated T cells can present antigen but may not be able to process antigen as effectively as other presenting cells. The ability of activated T cells to present antigen may have important implications in vivo, especially in autoimmune disease such as RA where activated T cells predominate in the synovium. This aspect will be commented upon in the discussion section.

T cell activation process involves blastogenesis, replication and expression of new cell surface antigens. Many of these activation antigens are receptors for growth factors such as Tac (IL-2 receptor) (Uchiyama et al, 1981a). Some of the antigens appear early following antigen recognition and have relatively short half-lives. These include 4F2 which occurs in 4 hrs (Haynes et al, 1981), transferrin receptor in 8 hrs (Sutherland et al, 1981), IL-2 receptor in 8-16 hrs (Uchiyama et al, 1981a, 1981b) and insulin receptor, within 24 hrs (Helderman and Strom, 1978).

HLA-Dr antigen is a late activation antigen which is expressed on

T cells 2-4 days after stimulation by antigen or mitogen. It is these HLA-DR antigens that mediate MHC-restricted interactions between T cells, macrophages and B cells and consequently serve as the major stimulator of MLR and as targets for some human allogeneic cytolytic T cells. The functional role of T cell HLA-Dr which is structurally and serologically identical to B cell HLA-Dr, has also been shown to be capable of initiating AMLR, MLR as well as being able to present antigen. These activation markers have been and will continue to be important in both diagnostic and therapeutic regimens.

# CHAPTER 2: MATERIALS AND METHODS

## 2:1 Patients and Normal Controls:

I studied 46 adult patients (24 females, 22 males) who fulfilled the ARA criteria for definite or classical RA (Ropes et al, 1959). Their mean age was 51 ± 18 years (range 18-83) and 50 ± 17 years (range 24-87) for the female and male patients respectively. These patients were used exclusively for PB and SF studies. In addition, 28 adult RA patients (18 females, mean age 64±15 yrs range 34-84 yrs; 10 males, mean age 60 ±5 yrs range 43-81 yrs) undergoing hip replacement surgery were used as a source of synovial tissue lymphocytes. Two patients with Reiter's disease and 1 patient with reactive arthritis were also included in the study. The patients were considered as having active disease based on the number of tender and swollen joints, morning stiffness and by laboratory parameters such as increased ESR and reduced C3 levels in serum. All the patients were being treated with a variety of non-steroidal anti-inflammatory drugs. Peripheral blood (PB) was obtained by venipuncture and synovial fluid (SF) by aspiration of the inflamed knee joints. PB samples were also obtained from 31 normal healthy subjects (15 females, 16 males), who served as controls. Their mean age was 31 ± 8 years (range 24-40).

## CELL SEPARATION

### 2:1:1 Peripheral blood (PB) and synovial fluid (SF):

Mononuclear cells (MNC) were obtained from heparinized blood by Ficoll-diatrizoate density gradient centrifugation (LSM, Litton Bionetics, Kensington, MD) (Boyum, 1968). The blood was diluted 1:1 with physiological saline and layered onto Ficoll-diatrizoate gradient

at a ratio of 2 mls of blood to 1 ml of the gradient and centrifuged at room temperature for 20 minutes. at 400g. The MNC from the interface were washed three times in Hank's balanced salt solution (HBSS) (Gibco, Grand Island, NY, USA) and resuspended in RPMI 1640 containing 2 mM L-glutamine, 25 mM Hepes (KC Biological, Lenexa, KS, USA), 100 g/ml penicillin G and 100 g/ml streptomycin (Gibco, Grand Island, NY, USA) and 50 g/ml gentamycin (Schering Corporation, Kenilworth, NJ, USA). Heparinised SF was treated with hyaluronidase (50 g/ml, Number H2376; Sigma, St. Louis, MO) for 30 mins at 37 $^{o}$C to reduce the viscosity of the fluid. It has been shown that hyaluronidase treatment does not affect the functional properties of the MNC (Silver et al, 1982). The SF was centrifuged at 1000 xg for 10 minutes and the supernate removed and stored at -20 $^{o}$C. The cell deposit was suspended in 6 mls of saline and layered on 3 mls of Ficoll-diatrizoate and processed as described for blood.

2:1:2 Synovial Tissue

Elution and purification of ST lymphocytes was carried out by a method of (Abrahamsen et al, 1975). Synovial tissue was obtained from patients undergoing hip replacement surgery. The tissue was minced with a pair of scissors and transferred to a spinner flask containing 50 ml of 1 mg/ml bacterial collagenase (Type IV, No. C-5138, Sigma, St. Louis, MO, USA) and 0.15 mg/ml deoxyribonuclease (Type I, No. 260912, Sigma, MO, USA) and 50 g/ml hyaluronidase in HBSS containing 5 mM CaCl. The suspension was stirred magnetically and digested for 1 hr at 37 $^{o}$C and then filtered through a steel gauze, pore size 200 m to remove debris. The cell suspension was washed thrice with HBSS and suspended in RPMI 1640.

The cells were counted and seeded at 1 x $10^6$/ml in 5 ml of RPMI 1640 + 10% FCS in 75 cm tissue culture flasks and incubated overnight at 37°C in 5% $CO_2$ in a humidified incubator. The non-adherent cells were removed by gentle shaking and washing of the plastic surface of the tissue culture flask with a pipette. The adherent cells, comprised a mixture of fibroblasts and dendritic cells. They were grown in RPMI 1640 containing 10% FCS and split every 3-4 weeks. The fibroblasts were used for the detection of fibroblast activating factor production by the T cell clones.

The non-adherent cells were purified on Ficoll-diatrizoate density gradient similar to the procedure described for the isolation of PB and SF MNC. Viability assessed by trypan blue dye exclusion was routinely >85%.

2:1:3 IL-2 Production and Partial Purification:

Tonsils were obtained from children undergoing tonsilectomy operations because of chronic tonsilitis. The tissue was rinsed well in HBSS to get rid of all blood and minced with scissors. The minced tissue was pressed against a steel gauze to obtain a single cell suspension. The suspension was allowed to stand for 15 minutes for the debris to settle. The supernate was collected and washed 3 times in HBSS and cultured at 2 x $10^6$/ml in RPMI 1640 containing 1% FCS and pulsed with 0.1% PHA-P (Difco, Detroit, MI, USA) in 225 cm flasks for 4 hrs. The cells were washed in lectin-free RPMI 1640 containing 1% FCS and incubated for a further 36 hrs. The supernatants were harvested by centrifugation, sterilised, pooled and aliquoted at 2 litre volumes and stored at -20°C until used at 6-8% to supplement media as crude IL-2 for cloning work.

An attempt was made to partially purify the IL-2 present in the 2 litre tonsil supernatants using the method of (Ruscetti et al, 1980) with some modifications. The supernatants were first centrifuged to remove all debris and filtered through 0.45 μm Millipore filter and saturated with 80% ammonium sulfate . The mixture was incubated for 24 hrs at 4°C and the precipitate collected after centrifugation at 10,000 xg for 30 minutes at 4°C. The deposit was suspended in 5 mls of 10 mM Tris-HCl, pH 8.0 and dialyzed exhaustively against the previous buffer. The dialysate was then applied to DEAE-Sephadex column equilibrated with 10 mM Tris-HCl, pH 8.0. The IL-2 was eluted from the column with an increasing NaCl gradients, commencing at 0.05 M to 0.45 M in 10 mM Tris-HCl, pH 8.0. All the fractions were assayed for IL-2 activity using the murine, IL-2 dependent cell line CTLL-2. The details of the technique will be described later. The IL-2 activity was recovered from the 0.05M - 0.15 M NaCl fractions but there was a considerable loss of IL-2, only 30% being recoverable. Thus, for the cloning work, the crude IL-2 supernatant was used and then the clones expanded and maintained on a partially purified human IL-2 obtained from a commercial source (Electro-Nucleonics, Fairfield, NJ, USA).

2:1:4 IL-2 Responsive T Cell Cloning by LDA

Spontaneously activated, IL-2 responsive lymphocytes from PB and SF were cloned by limiting dilution analysis (LDA) using various responder cells at 12-1000 cells per well in the presence of either $10^4$ autologous irradiated (50 Gy) PB or SF MNC in RPMI 1640 containing 10% FCS or human serum (HuS) and the crude IL-2 at 8% final concentration. The LDA was performed in 96-well round-bottom microtitre plates in a

total volume of 200 µl. In some experiments, the lymphocytes were first stimulated with PHA-P 0.1% for 48 hrs followed by cloning either in the presence or absence of filler cells. When filler cells were used, the responder cells varied from 1.5 - 200 cells per well.

Cyclosporin A, a drug which inhibits T cell proliferation but not pre-activated T cells, was also included in some experiments to ascertain whether only IL-2 responsive cells were limiting in the experimental conditions or some non-specific stimulation of the T cells by the FCS occurred simultaneously. In all the experiments, 24 replicate microcultures were set up for each cell concentration and for irradiated filler cells alone as a control and incubated at 37°C in 5% $CO_2$ for 14 days. The cultures were fed on day 7 by removing 100 1 of the medium and replenishing them with 100 µl of fresh IL-2 containing medium and incubating the cultures for a further 7 days. Usually proliferation of the responder cells were clearly evident at day 14 and thus, all the cultures were scored for growth at this time either microscopically (i.e. visually) or by $^{3}$H-Tdr uptake. These 2 methods of scoring for growth were of comparable sensitivity; however, the $^{3}$H-Tdr uptake method was tedious , time consuming and expensive so the visual scoring method was routinely used. Occasionally, when the cultures were difficult to score visually, the $^{3}$H-Tdr uptake method was used to confirm the visual score. It has the advantage of being objective.

2:1:5 Expansion of Clones:

The proliferative cultures at a limiting dilution (Lefkovits, 1972) with >95% probability of being clonal, were subcultured and expanded in RPMI 1640 containin 10% FCS and 3.5% partially purified human IL-2 to

96-round bottom plates. The cultures were fed every 3-4 days and given $10^4$ irradiated autologous or pooled MNC and PHA-P 0.1% every 4 weeks. Under these conditions, cultures showed a strong proliferation, generating a number of cells suitable for functional studies (average 2 x $10^6$ cells) in 14 days. By these repeated stimulations, clones have been maintained up to 8 months with no detectable loss of their functional repertoire or surface phenotypes. Overall, SF clones survived longer in cultures than PB clones. Various cell types including K562, Raji, U937 and an EBV transformed cell line termed JR-2 were used as filler cells to see whether they will increase the cell yield of the clones, but they were no better than the pooled MNC + PHA. Supernatants from various cell lines, MLR, and recently, a synergistic factor, produced by MNC stimulated with staphylococcal enterotoxin A (Mills et al, 1986) believed to sustain clones up to 1 yr without the need for repeated stimulation, was also tried for the expansion of the clones. The results were not convincing i.e. in long term culture studies, there was no significant increase in absolute numbers as compared to the conventional IL-2 medium. Thus, throughout this project, the expansion and maintenance of the IL-2 dependent T cell clones were achieved through either autologous or pooled irradiated MNC and PHA-P 0.1% in the IL-2 containing medium.

## IDENTIFICATION OF CELL SURFACE ANTIGENS

### 2:1:6 Indirect Immunofluorescence:

Antigenic phenotype of the clones was determined by indirect immunofluorescence. Clones ($10^5$) were treated with 50 µl of the appropriate dilutions in RPMI 1640 containing 2% FCS and 0.1% azide (RPSA) of the CD primary series monoclonal antibodies (mAb): CD3

(1:50), CD4 (1:15) CD8 (1:10) purchased from ( Ortho Pharmaceuticals, Don Mills, Ontario, Canada) T65 mAb (1:10) directed against T101 antigen present on human T cells (Royston et al, 1984), HLA-DR (anti-Ia) mouse alloantiserum (1:10) (Cedarlane Laboratories, Hornby, Ontario, Canada) which detects determinants common to mouse Ia and human DR antigens, CD16 (1:40), HNK-1 or Leu 7 (1:10), CD11 (1:10) and anti-Leu 19 (1:10) (Becton Dickinson, Mountain view, CA). The cells were incubated on ice for 30 minutes, washed twice in RPSA and subsequently labelled with 50µl of the antibody which is 1:10 dilution of rhodamine conjugated $F(ab')_2$ rabbit anti-mouse IgG (Cappel, Cochranville, PA, USA) and the mixture incubated on ice for a further 30 minutes. The control consisted of omitting the primary monoclonal antibody during the first incubation period and subsequently exposure to secondary antibody. All the samples were washed twice and examined with a Leitz UV microscope equipped with epi-illumination.

2:1:7 Rosetting with immunobeads:

Recently, a rosetting techniques using CD4/CD8, sIg, T65 monoclonal antibodies coated polyacrylamide beads in a Quantigen System developed by (Bio-Rad, Mississauga, Ontario, Canada) were used. In pilot studies performed in this laboratory, it was shown that this Quantigen System gave comparable numbers of antigen reactive cells to those obtained using immunofluorescence. Because this technique is rapid and cheaper with stable rosettes which can be enumerated the following day, it superseded the immunofluorescence method. For this procedure, $10^5$ clonal cells were treated with 10 µl of the CD4/CD8 bead in a v-shaped microtited plate and spun at 50 xg for 3 minutes. The cells were incubated at 37 °C for 30 minutes. After incubation, 5 µl of

erythrosin B vital stain was added to distinguish between the live and dead cells, the pellet gently resuspended, with a pasteur pipette and a drop applied to a slide and 200 cells counted. The CD4 cells show a pink rosette, CD8 cells yellow rosette and null cells have no rosettes. Macrophages and phagocytes ingest the beads which can be seen in the cytoplasm. Cells having three or more beads were regarded as positive.

For other monoclonal antibodies, a polyacrylamide beads coated with rabbit anti-mouse Ig (a gift from Bio-Rad Diagnostics) was preloaded with the appropriate antibody at 4°C for 30 minutes, washed and added to the clones in the rosetting conditions described for the Quantigen System.

2:1:8 Analysis of Surface Receptor for SRBC:

SRBC obtained commercially from the Ross Animal Services (Dugald, Manitoba, Canada) in Alsever's solution were washed five times in physiological saline at room temperature. Aminoethylthiouronium-bromide (AET, Sigma, St. Louis, MO, USA) was prepared according to the method of Kaplan and Clark (1974). The AET was prepared as 0.14 M solution in distilled water, pH 9.0. To 1 ml of washed, packed SRBC, 4 mls of AET solution was added, the suspension mixed thoroughly and incubated at 37°C for 15 minutes, shaking gently every 5 minutes. After incubation, cold saline was added and the cells centrifuged at 100 xg for 10 minutes. The pellet cells were washed 4 times with cold saline and twice with RPMI 1640. RPMI 1640 containing 10% FCS was added to yield a 4% cell suspension. These AET-coupled SRBC (SRBC-A) were used immediately or stored at 4°C for 5 days without deterioration in their rosetting activity.

To detect rosette forming cells (RFC), 200 µl of $10^5$ T cell

clones in RPMI 1640 containing 10% FCS, was mixed with 100 µl of a 4% SRBC-A suspension in RPMI 1640 + 10% FCS in screw capped tubes (12 x 75 mm). The tubes were placed in a 37 °C water bath and incubated for 15 minutes, shaking every 5 minutes, followed by centrifugation at 100 xg for 10 minutes at 20 °C. The cell pellets were incubated on ice for 1 hr and suspended by gentle shaking. A drop of gentian violet was added to stain the nuclei of the T cells and thus facilitate counting. A drop of the suspension was placed on a clean slide, coverslip applied and the edges sealed with nail polish. A total of 200 cells were counted and the results expressed as percent of RFC. T cells with three or more adherent SRBC-A were considered positive.

2:1:9 Preparation of Connective Tissue Antigen (CTA):

Native bovine type II collagen was extracted from nasal septum by pepsin solubilization after previous treatment with 2M Magnesium chloride (Trelstad et al, 1977). Acid soluble native type I collagen was extracted from fetal calf skin and purified by the method of (Jackson and Cleary, 1967). The collagen types appeared pure on polyacrylamide gel electrophoresis and the type II collagen was negative for uronic acid, suggesting that there was no proteoglycan contamination (Bitter and Muir, 1962). Bovine nasal proteoglycan was prepared by extraction in 4M Guanidine hydrochloride followed by dissociative Caesium chloride density gradient centrifugation (Roughley and White, 1980). The proteoglycan preparation appeared free of contaminating proteins by Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis. The CTA were lyophilized and stored in a dessicator at -20 °C until used.

For *in vitro* studies, the CTA were dissolved in 0.5M acetic acid

at a concentration of 2 mg/ml and dialyzed against 0.01 M acetic acid/0.15 M NaCl, pH 3.0 for 24 hrs. The collagens were sterilized by ultra-violet irradiation in an open petri dish surrounded by moist paper towels overnight in a hood. Any loss of buffer was replaced as necessary. Some of the collagens were diluted to 500 g/ml and further sterilized by millipore (0.8$\mu$ ) filtration. This constituted the working solution for the 96 hr proliferative responses. The inclusion of 0.01 M acetic acid kept the helical collagens in solution for the filtration to be achieved. Denatured collagens were prepared by heating the collagens at 56 $^{o}$C for 30 minutes to unwind the alpha ( $\alpha$ ) chains and expose the hidden determinants of the collagen molecule. In preliminary studies, various CTA concentrations (10-400 $\mu$g/ml) were exposed to T cell clones, to determine the optimum concentration for IFN production. It was seen that 50-100 $\mu$g/ml gave the maximum stimulatory activity with 400 $\mu$g/ml CTA being inhibitory. Thus, 50 $\mu$g/ml final concentration of CTA was used in all subsequent studies. Control cultures received 0.01M acetic acid/0.15M NaCl buffer alone. The use of antigens in 0.01 M acetic acid/0.15 M NaCl buffer did not alter the pH of the medium or affected cell growth and proliferation.

2:2 Production of Lymphokines by T Cell Clones:

T cell clones were stimulated with native type I (NI), native type II (NII), denatured type I (DI), denatured type II (DII) and proteoglycan (PG) at a concentration of 50 $\mu$g/ml as well as irrelevant or control antigens such as purified protein derivative (PPD), ovalbumin (OVA), lysozyme (LYS) in RPMI 1640 containing 10% FCS at 5 x $10^{4}$ per 200 microlitres ( $\mu$l). Positive control cultures were stimulated with 0.1% PHA-P (a T cell mitogen). Lymphokine production was also done under

serum-free conditions with the RPMI 1640 supplemented with fibronectin at 30 µg/ml. After incubating the cultures for 24 hrs at 37$^0$C, the time of optimum lymphokine production, they were spun down, the supernatants removed and assayed for the presence of gamma ( $\gamma$ ) IFN and IL-2 production. In some studies, phorbol myristic acetate (PMA) and PHA were used for IL-2 production. In this case, the clones were pulsed PMA + PHA for 4 hrs, washed three times in RPMI 1640 containing 10% FCS, and then incubated for a further 20 hrs. The supernatants were collected and assayed for IL-2 activity.

For fibroblast activating factor (FAF) production, 5 ml volumes of the T cell supernatants were prepared since more supernatants were required for the assays.

2:2:1 Interferon Anti-viral Assay:

Antiviral activity in the supernatants was measured by a modification of the method of (McManus, 1976) which quantitates the ability of IFN to protect A549 cells (human lung carcinoma) from lysis by murine encephalomyocarditis virus. For the microassay, 1.5 x $10^4$ A549 cells (human lung carcinoma) were seeded in 0.1 ml in each of the 96 wells of the flat-bottomd microtitre plates and the plates incubated for 4 hrs at 37$^o$C for the cells to adhere. The interferon samples to be titrated were diluted in the first well of a row in the microtitre plate to a final volume of 0.2 ml. Subsequent wells in the row contained 0.1 ml of RPMI 1640 containing 10% FCS and thus two fold dilutions of the samples were made by transferring 0.1 ml serially to the end of the row. On each plate 2 rows of 8 wells were used as virus and cell controls. The plates were incubated at 37$^o$C for 24 hrs, followed by the inoculation of 20 µl of a standardized (1/164) murine

encephalomyocarditis (EMC) virus to all wells except the cell control wells. The plates were incubated for a further 24 hrs. The medium from each well was decanted onto an absorbent pad and the cell monolayers were stained with 0.1 ml of 0.5% (weight/volume) crystal violet (5gm/litre in 70% methanol) for about 1 minute. After decanting the stain, the plates were rinsed gently with tap water and left on the absorbent pad to dry. The interferon titre was taken as the reciprocal of the dilution represented in the well in which 50% of the cell monolayer was protected from the cytopathic effects (CPE) of the virus. All the titrations were done in duplicate and a standard IFN was included in all assays so the units of IFN in the samples can be calculated. The standard IFN was prepared by titration against NIH alpha ( $\alpha$ ) IFN reference standard. The level of sensitivity of this assay is 0.6 unit/ml. The IFN was characterised as $\gamma$ by its instability at pH 2.0 for 1 hr, heat lability at 56 $^{o}$C for 1 hr and also blocking of the activity with monoclonal anti-IFN-$\gamma$ .

2:2:2 IL-2 Microassay:

The amount of IL-2 in the culture supernatant was quantitated using a modification of the technique originally described by (Gillis et al, 1978). This procedure is based on the ability of IL-2 to support the proliferation of the IL-2 dependent mouse cytotoxic T cell line, CTLL-2 (Baker et al, 1979). The CTLL-2 cells were washed twice in RPMI 1640 containing 10% FCS to remove existing IL-2, resuspended at $5 \times 10^4$ cells/ml and dispensed in triplicate into flat bottom microtitre plates in 100 µl volume. Two fold serial dilutions of the T cell clone supernatants were made and 100 µl was added to each microtitre well. Control wells comprised CTLL-2 cells incubated with medium alone or

with a known concentration of laboratory IL-2 standard which was partially purified human IL-2 (3%) obtained from (Electronucleonics, Fairfield, NJ, USA). The cells were incubated for 24 hrs at 37°C in 5% $CO_2$, pulse labelled with 0.2 µCi of $^3$H-thymidine (Amersham, Oakville, Ontario, Canada) and harvested 6 hrs later. The samples were harvested onto glass-fibre filters using an automated PHD cell harvester (PHD harvester, Cambridge, MA, USA) and the incorporation of the radiolabel determined by liquid scintillation spectrometry. The results were expressed as the mean of the total cpm of $^3$H-thymidine incorporated by triplicate cultures, since the amount of IL-2 in the supernatants were too low to convert to units.

2:2:3 IL-1 assay: (IL-1 Dependent LBRM-33 Production of IL-2):

Human IL-1 is usually assayed by its ability to directly stimulate the proliferation of mouse thymocytes (Lachman, 1983). Although the thymocyte assay is relatively easy to perform, it lacks specificity in that proliferation can be due to either IL-1 or IL-2. Recently, a more sensitive assay for IL-1 (Gillis and Mizel, 1981) has been developed which measures the production of IL-2 by the murine T lymphocyte clone LBRM-33 stimulated with PHA and IL-1.

Cultures were established in flat bottom microtitre plates with each well containing a 100 µl aliquot of LBRM-33 cells ($5 \times 10^4$ cells per well). A 100 µl of various dilutions of IL-1 supernatant to be tested was added to each well together with 0.1% PHA-P. A positive control contained 100 µl of recombinant IL-1 and PHA, while the negative control contained medium and 0.1% PHA. The cultures were incubated for 24 hrs at 37°C in 5% $CO_2$. IL-2 activity was determined

by removing 100 µl samples from the wells and testing such supernatants in an IL-2 microassay using 5 x $10^3$ CTLL-2 cells/well. After 24 hrs incubation, the cultures were pulsed with $^3$H-Tdr for 6 hrs, harvested and the radioactivity incorporated by the CTLL-2 cells measured by liquid scintillation spectroscopy. The data were expressed as a mean of $^3$H-Tdr incorporated in triplicate cultures ± SD because the counts were too low to convert to units/ml.

2:2:4 Antigen-Induced Proliferation Assay:

T cell clones were examined for antigen-specific proliferation in a 96 hr assay by using the $^3$H-Tdr assay. Prior to testing, the cloned T cell cultures were "rested" for 14 days (i.e. they had not received irradiated filler cells), and washed to remove all IL-2. The purity of the clones was checked by staining cytocentrifuge smears for non-specific esterase (Yam et al, 1971). No esterase-positive accessory cell contamination was seen in the "rested" T cell clones. T cell clones (5 x $10^4$/well) were cultured with CTA and irrelevant antigens such as PPD, OVA, LYS as well as PHA in the presence of irradiated (35 Gy) autologous MNC ( $10^4$/well) as APC or in the absence of APC in 96-well round-bottomed microtitre plates in 200 µl of RPMI 1640 medium containing 10% FCS in the absence of IL-2. A positive control of the clones response to 3% IL-2 and a negative control of medium (RPMI 1640-10% FCS) was included in each assay. Cell-free synovial fluid was examined under light microscope to confirm the absence of cells; then 0.1ml aliquots at 5%, 10% and 20% with or without 3% IL-2 added to T cell clones at 5 x $10^4$/well. Following 96 hr incubation at 37$^o$C in 5% $CO_2$, the cultures were pulsed with 0.2 µCi of $^3$H-Tdr and harvested 16 hrs later using an automatic multiple sample harvester (PHD, Cambridge,

MA, USA). The level of radioactive uptake was assessed by liquid scintillation spectroscopy. The results were expressed as mean counts per minute (cpm) ± SD of triplicate cultures. In some experiments, to check that the proliferative response detected was due to a typical antigen-induced proliferation and not a mitogenic response, the effect of anti-HLA-DR (Ia) antibodies in the proliferative response was determined. The anti-Ia was present throughout the culture period.

2:2:5 Cytotoxicity Assay:

Mycoplasma-free K562, Raji and Colo target cells were labelled by incubation of $5 \times 10^6$ cells in 0.5 ml of RPMI 1640-10% FCS containing 300 Ci of sodium chromate-51 (New England Nuclear, Boston, MA) for 1 hr at 37°C, followed by two washings with RPMI 1640 medium and the cells resuspended a $5 \times 10^4$/well.

To obtain the precursor frequency estimates of cytotoxic cells, cultures from the limiting dilution assays described earlier were tested for cytotoxic activity 14 days after culture. The cultures were washed twice in RPMI 1640-10% FCS, resuspended in 200 1 of the same medium and 100 1 of the suspension added to $5 \times 10^3$ labelled target cells in 100 1 in a V-bottomed microtitre plate. In some experiments, PHA 0.1% was added to the system to detect lectin-dependent cytotoxicity of the cultures. The plates were centrifuged at 80 xg for 3 minutes and incubated for 4 hrs at 37°C in a humidified atmosphere plus 5% $CO_2$. After incubation, the plates were centrifuged at 400 xg for 10 minutes and 100 µl of supernatant was removed and assessed for radioactivity in a gamma-counter. Positive wells were defined as those in which $^{51}Cr$ release exceeded three standard deviations from the mean of the release value obtained for control cultures which had received

only irradiated filler cells and IL-2 during the limiting dilution assay. Spontaneous release (<10% of maximum) was determined by incubating the targets with RPMI 1640-10% FCS alone.

Since the cultures derived from the limiting dilution analysis (LDA) assays may contain more than 1 precursor cell per well, cultures were picked from wells in which the probability of being clonal origin was >95% and the cells expanded in IL-2 so as to analyze in detail their cytotoxic properties. The clones were tested for cytotoxicity against K562, Raji, PHA-blast autologous or allogeneic and fresh PB labelled targets at various effector to target ratios. To label synovial fibroblasts with $^{51}Cr$, 5000 cells/well were cultured in round-bottomed microtitre plates for 48 hrs to allow fibroblasts time to re-synthesize their surface antigens after trypsinization, and then labelled with $^{51}Cr$ as previously described. The labelled cells were then used as targets in the cytotoxic assay. In some experiments, a panel of MABS was used to define further the cell surface structures on the T cell clones that play a role in the lysis of the targets. In this assay, the clones at $2 \times 10^6$ /ml in ICM was divided into 4 aliquots. Saturating doses of CD3, CD4 and CD8 MABS were added to 3 aliquots, while the 4th served as a control. All 4 tubes were incubated at 37°C for 24 hrs. The clones were washed with RPMI 1640+10% FCS and prior to performing the cytotoxic assay, the level of the CD3, CD4 and CD8 antigens remaining on all 4 cell aliquots was determined by indirect immunofluorescence. To analyze the specificity of and to determine whether separate or multiple receptors for target structures were present on the cytotoxic clones, cold-target inhibition assay, which involves adding 5 fold excess of unlabelled targets to a mixture of

effector cells and labelled target cells were also carried out. In this assay, the maximum isotope release was determined by incubation of targets with 0.5% NP-40. All samples were run in triplicates. The maximum and spontaneous release were measured in sextuplets. The percentage specific lysis was calculated as:

$$\% \text{ specific lysis} = \frac{\text{Experimental cpm} - \text{spontaneous cpm}}{\text{Maximum cpm} - \text{spontaneous cpm}} \times 100$$

2:2:6 Fibroblast Proliferation Assay:

The preparation and maintenance of synovial fibroblasts have been described elsewhere (Abrahamsen et al, 1975). In preliminary experiments, the cell density and kinetics of the optimum proliferation of synovial fibroblasts were determined, and it was found that $2 \times 10^4$ cells/well and 72 hrs of incubation gave good responses hence this protocol was used in all subsequent assays.

Fibroblast proliferation was monitored by using a modification of $^3$H-Tdr incorporation assay described by (Wahl and Gately, 1983). Trypsinized primary (1-3 passages) of synovial fibroblasts were dispensed at $2 \times 10^4$ cells/well into flat-bottomed microtitre plates in 100 µl RPMI 1640 containing 10% FCS. After 4 hrs incubation at 37°C to allow adherence, the medium was removed and the adherent cells washed to remove all serum and cultured overnight in serum-free medium. After removing the media, control supernatants (generated in the absence of PHA or CTA but reconstituted with these stimuli at the end of culture) and test supernatants diluted (1:4 vol/vol) were added to the fibroblast monolayer and the cultures incubated for 72 hrs at 37°C in 5% $CO_2$. All experiments were done in triplicate in final volume of

200 µl. After 72 hrs incubation, the cultures were pulsed for 18 hrs with 0.2 µCi of $^{3}$H-Tdr. Before harvesting, the cultures were exposed to 0.25% Trypsin-EDTA for 40 minutes to detach the monolayers and harvested with an automated cell harvester. In some experiments, 1 µg/ml of indomethacin was added during the initiation of cultures or after culture to the stimulated T cell supernatants and assayed for fibroblast proliferation. The $^{3}$H-Tdr uptake was assessed by liquid scintillation spectroscopy and the data expressed as counts per minute (CPM) ± SD of triplicate cultures. The viability of fibroblasts assessed by Trypan blue dye exclusion at the end of culture was routinely >95%.

## PHYSICOCHEMICAL CHARACTERIZATION OF FIBROBLAST ACTIVATING FACTOR

### 2:2:7 AcA 54 Chromatography of Supernatants:

After dialysis against phosphate buffered saline (PBS, 0.15 M NaCl/0.02 M phosphate, pH 7.2), the samples were concentrated to 0.5 ml by ultrafiltration (Amicon Corp., Lexington, MA). The concentrated samples were cleared of insoluble material by centrifugation at 2000 xg for 10 minutes at 4°C, before they were applied to a 1.8 x 100 cm column of AcA 54 (Ultrogel, France) which has been equilibrated with RPMI 1640 and gel filtration performed at 4°C. The void volume was determined with blue dextran (BD) and the column calibrated with proteins of known molecular weight such as BSA, OVA, CYTO-C and CHY-A. The sample was eluted at a flow rate of 10 mls/hr and 1 ml fractions collected. The protein concentration was estimated by ultraviolet light (280 nm) absorption using spectrophotometer. The samples were filter sterilised and assayed individually.

### 2:2:8 Heat and Acid (pH 2.0) Stability Studies:

1 ml aliquots of purified, pooled active fractions were heated to 56 °C for 30 minutes and 60 minutes and assayed for the ability of fractions to stimulate synovial fibroblast proliferation. Another 1 ml aliquot of the active fraction was also reduced to pH 2.0 using 6 M HCl and incubated at room temperature for 1 hr. The samples were neutralized with Hepes buffered RPMI 1640 and the pH raised to 7.2. The samples were then diluted 1:4 in RPMI 1640 and tested for FAF activity.

2:2:9 Trypsin-sensitivity studies:

Trypsin (10x concentrated, Flow Labs, Mississauga, Canada) was coupled to Affi-Gel 10 (Ultrogel, France) using 0.1M Hepes buffer, pH 6.0 at 4 °C for 4 hrs according to the manufacturer's instructions in the (Chromatography and Immunochemistry Manual of Bio-Rad, page 46, 1985). After coupling, all unreactive sites of the beads were blocked with 1 M ethanolamine-HCl, pH 8.0 for 1 hr, washed in HBSS. The trypsin binding to Affi-Gel 10 was monitored by measuring the protein concentration using Bio-Rad protein assay.

1 ml aliquots of pooled fractions with FAF activity was incubated at room temperature (25 °) for 16 hrs with either the beads alone or Trypsin-Affi-Gel 10 conjugate (2 mg). The suspension was spun down and the supernatants collected and assayed for FAF activity.

2:3 Cell Attachment Assay

This assay was performed according to the method of Cardarelli and Pierschbacher, (1986). Flat-bottomed microtitre plates untreated for tissue culture were coated with 50 µg/ml in 100 1 volume of BSA, Fn, NII collagen or NII-Fn mixture at room temperature for 2 hrs. Unbound proteins were removed from the wells by washing three times with PBS. During the coating procedure, care was taken to prevent dryness.

In order to ensure even distribution of the clones in the wells, 100 µl of RPMI 1640 was added to each well followed by 100 µl of $10^5$/ml of clones in RPMI 1640. The plates were incubated for 4 or 24 hrs at 37°C in 5% $CO_2$. After incubation, the unadhered clones were removed by decanting the medium washing three times by flooding the wells with PBS. The adherent cells were then fixed with 3% paraformaldehyde in PBS, washed once in PBS, stained with 0.2% crystal violet, dried and photographed.

# CHAPTER 3: RESULTS

## 3:1 Comparison of IL-2 Responsive T Cell Precursor Frequency:

A representative sample (N = 5) of precursor frequencies of IL-2 responsive T cells derived from PB, SF and ST are shown in (Fig. 1). It can be seen that the profiles obtained in the LDA were linear in a semi-logarithmic plot of fraction of non-responding cultures (fnr) against cell input per well. The lines intercepted the ordinate at Fnr = 1.0 which is in agreement with the predicted Poisson distribution and thus, compatible with a single hit phenomenon (Lefkovits and Waldmann, 1979). This means that under the experimental conditions used in this study, only IL-2 responsive T cells were limiting. The precursor frequency of IL-2 responsive T cells was estimated from the line corresponding to the Fnr = 0.37 which represents the mean value of one precursor cell per culture (Lefkovits and Waldmann, 1979). The LDA data were analyzed by a computer program kindly provided by Dr. Rachel McKenna, (Transplant Unit, Univ. of Manitoba, Canada). This program estimates the frequency of responding cells by the $X^2$ minimization and likelihood maximization according to the method of Taswell, (1981) In preliminary experiments it was found that visual examination of the plates gives a clear-cut discrimination between proliferating (positive) and negative wells. To determine the reliability of this method, $^3$H-Tdr uptake was used to score cultures previously scored visually. Enumeration of positive (i.e. mean cpm of irradiated filler cells + 3X SD) and negative wells allowed the estimation of the precursor frequency. The results were comparable to the visual or microscopic method (Fig. 2). The rest of the cumulative precursor

Figure 1: Frequency analysis of the precursors of proliferating IL-2 responsive T cells derived from RASFL, RAPBL and NPBL. The responder cells were cultured in the presence of IL-2 and $10^4$ autologous irradiated (50Gy) PBL as filler. After 14 days of culture, each microculture was scored microscopically for growth. Each point is based on a group of 24 replicate cultures of 5 RA patients and normals. Linearity of the data from each set of limiting dilution microcultures was tested by $X^2$ minimization analysis by the method of Taswell (1981).

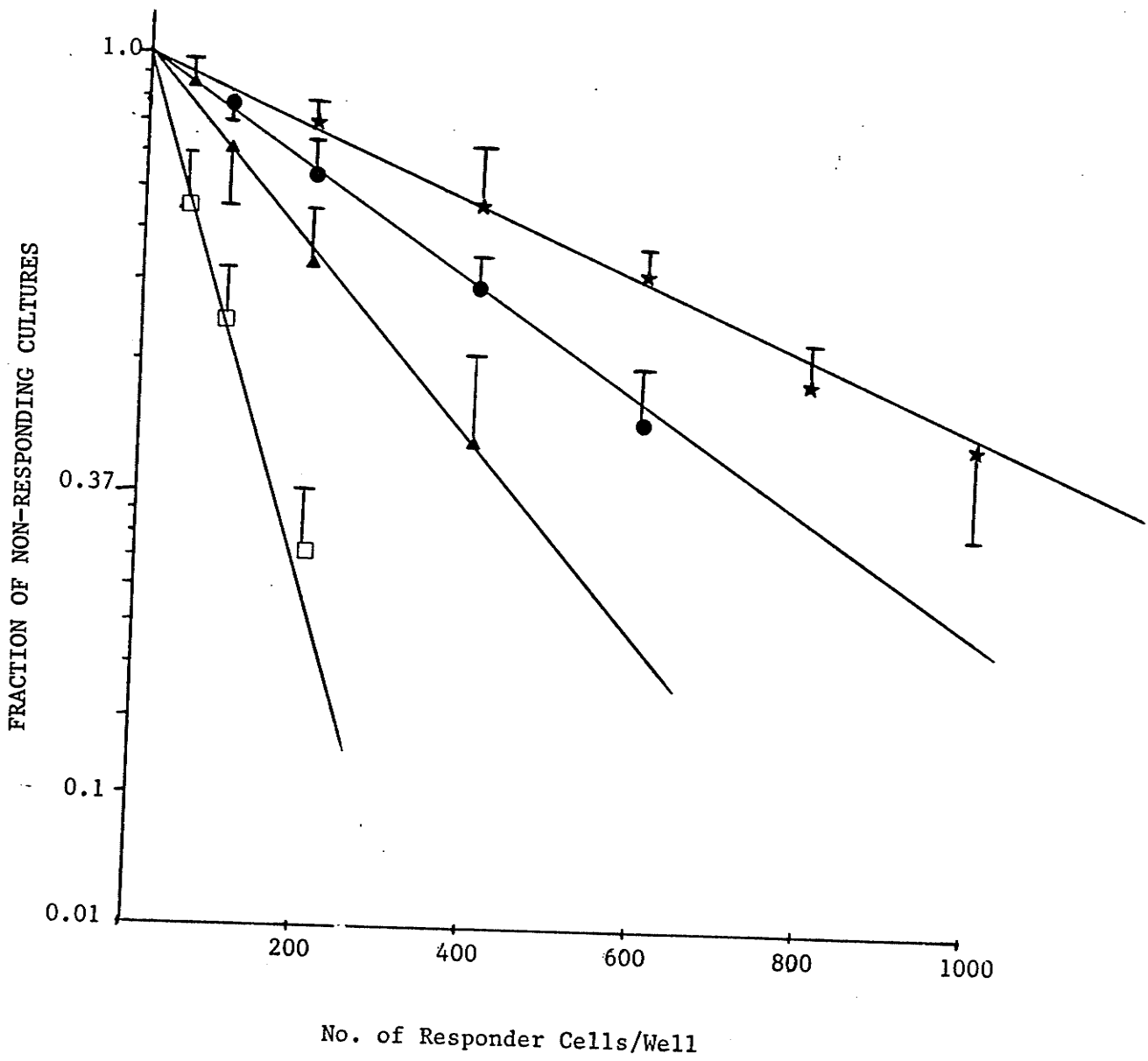
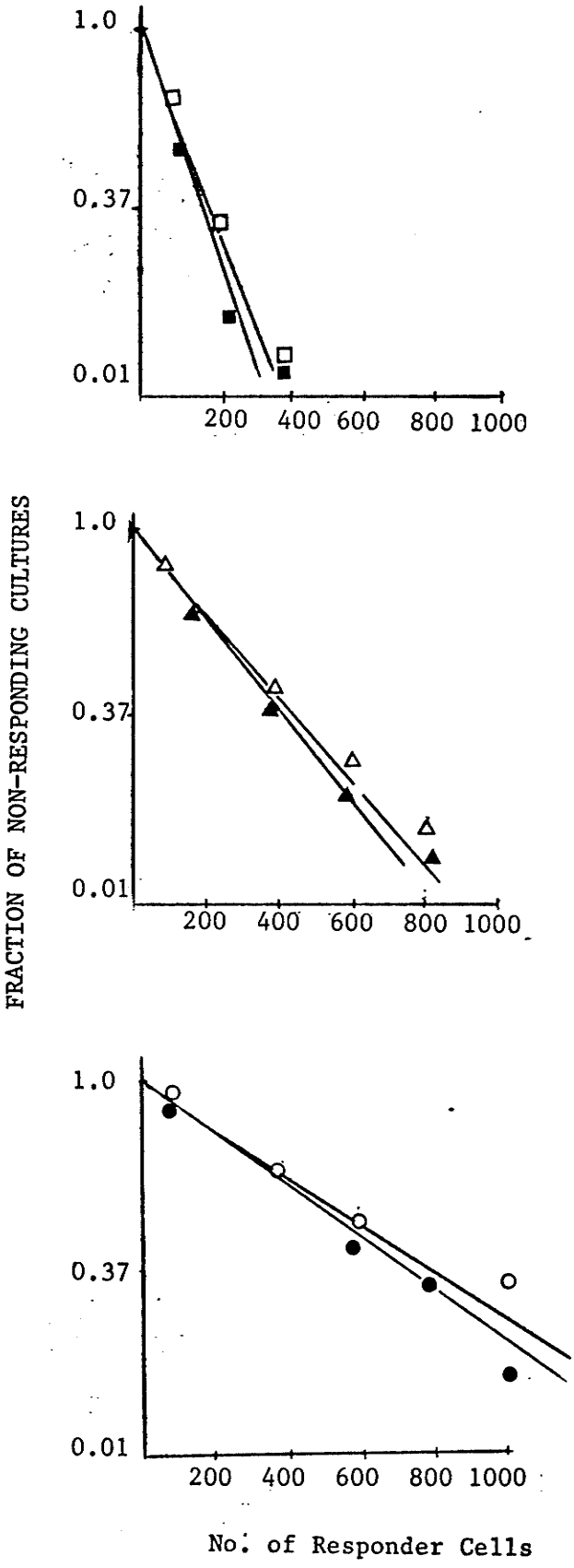

RA SF
RA PB
N PB
RA ST
1.0
0.37
0.1
0.01
FRACTION OF NON-RESPONDING CULTURES
200
400
600
800
1000
No. of Responder Cells/Well

Figure 2: Comparison between $^{3}$H-TdR incorporation (closed symbols) and microscopic scoring (open symbols) of wells positive for proliferation in limiting dilution analysis (LDA): LDA of I1-2 responsive cells from RASFL, RAPBL and NPBL were scored after 14 days in culture and then pulsed with $^{3}$H-TdR for 6 hrs. Microcultures were considered positive for growth if the $^{3}$H-TdR incorporation exceeds the mean ±3SD of $^{3}$H-TdR incorporated by 24 replicate wells of irradiated filler alone. Panel (A) IL-2 responsive cells from RASFL . Panel (B) IL-2 responsive cells from RAPBL. Panel (C) I1-2 responsive cells from NPBL. The two methods gave comparable percursor frequencies.


A
1.0
0.37
0.01
200 400 600 800 1000
B
1.0
0.37
0.01
200 400 600 800 1000
C
1.0
0.37
0.01
200 400 600 800 1000
FRACTION OF NON-RESPONDING CULTURES
No. of Responder Cells

frequency data for IL-2 responsive T cells in SF, PB and ST are shown in (Table 1). The precursor frequency of IL-2 responsive T cells was higher in SF 1/151 (range 1/60 - 1/400) than autologous PB 1/300 (range 1/108 - 1/657), N PB 1/491 (range 1/324 - 1/1200), ST 1/716 (range 1/202 - 1/1751). It should be noted that the burst sizes for the ST IL-2 responsive T cells were smaller than SF and PB T cell clones. Also, different batches of conditioned medium (CM) varied the precursor frequency of SF T cells but did not influence PB T cells appreciably. A plausible explanation for this observation will be given in the discussion section. The effect of PHA on the precursor frequency of IL-2 responsive T cells was examined. The precursor frequency of IL-2 responsive T cells from both RA and normal PB significantly increased when the blast cells were cultured in the presence of IL-2 and $10^4$ autologous irradiated filler cells. The precursor frequency values were 1/5.5 for normal subjects, 1/5.8 for active RA and 1/10 for inactive RA patients. There was a considerable overlap of frequencies, with a tendency for lower IL-2 responsive T cell frequency in the inactive RA group while the active and control groups had similar values. In contrast, the SF IL-2 responsive T cells showed the lowest frequency values (Table 2) which suggests that majority of the SF T cells are pre-activated or exhausted from exposure to materials in the fluid and therefore are hyporesponsive to PHA. After cloning and resting the cells in IL-2 containing medium, the SF T cells exhibit normal responsiveness to PHA.

3:1:1 Effect of Serum on Precursor Frequency:

The cloning experiments were performed in FCS because preliminary experiments had indicated that FCS supported cell growth better than

TABLE 1

CUMULATIVE DATA ON PRECURSOR FREQUENCY OF IL-2 RESPONSIVE CELLS

| SOURCE OF LYMPHOCYTES | SOURCE OF FILLER | MEAN PRECURSOR FREQUENCY (RANGE) |
|---|---|---|
| RAPBL (N=41) | PBL | 1/300 (1/108 - 1/657) |
| RASFL (N=38) | PBL | 1/151 (1/60 - 1/400) |
| RASFL (N=15) | SFL | 1/136 (1/50 - 1/398) |
| RAST (N=15) | SYNOVIAL CELLS | 1/716 (1/202 - 1/1751) |
| REITER'S PBL (N=2) | PBL | 1/410 (1/370 - 1/450) |
| REITER'S SFL (N=2) | PBL | 1/288 (1/201 - 1/375) |
| REACTIVE ARTHRITIS | | |
| PBL (N=1) | PBL | NT |
| SFL (N=1) | SFL | 1/372 |
| NPBL (N=26) | PBL | 1/491 (1/324 - 1/1200) |

Limiting dilution analysis for the Precursor frequency of IL-2 responsive T cells from Normal, RA and non-RA subjects. PBL and SFL IL-2 responsive T cells from RA and non-RA patients had similar precursor frequencies, although there was a tendency for RA patients to have higher frequencies. It should be noted that the clonal burst sizes of synovial tissue (ST) cells were very small in comparison to normal and RA burst sizes.

NT= Not Tested

TABLE 2

FREQUENCIES OF PHA-INDUCED LYMPHOBLAST CLONED BY LIMITING DILUTION

| SOURCE OF LYMPHOCYTES | CULTURED WITH AUTOLOGOUS FILLER | MEAN FREQUENCY | RANGE |
|---|---|---|---|
| CONTROL PBL (N=10) | - | 1/364 | 1/260-1/420 |
| | + | 1/5.5 | 1/4-1/6 |
| ACTIVE RAPBL (N=10) | - | 1/328 | 1/210-1/430 |
| | + | 1/5.8 | 1/4-1/8 |
| ACTIVE RASFL (N=10) | - | NT | |
| | + | 1/30 | 1/12-1/56 |
| INACTIVE RAPBL (N=6) | - | 1/380 | 1/340-1/800 |
| | + | 1/10 | 1/5-1/19 |

Mononuclear cells (1 x $10^6$/ml) were stimulated with PHA-P (0.1%) in RPMI 1640+ 10% FCS for 48 hrs at 37°C. After culture, the supernatants were saved for IL-2 measurement and the blast cells washed, viability tested and cloned by limiting dilution analysis with or without irradiated autologous PBL filler at $10^4$cells/well in the presence of 3% IL-2 for 14 days. Each microculture was scored microscopically for growth and the precursor frequency determined as previously described.

+ = Presence of filler  - = Absence of filler

NT = Not tested

human serum (HuS). FCS has been shown to be blastogenic for human PBL (Johnson and Russell, 1965; Lakhanpal and Handwerger, 1986). Thus, it is possible that FCS might activate some resting (Go) T cells to an IL-2 responsive (G1) phase of the cell cycle. To explore that possibility, normal resting PBL were cultured in the presence of IL-2 containing medium supplemented with either FCS or HuS and the precursor frequencies of IL-2 responsive T cells compared.

There was a higher frequency of IL-2 responsive cultures in the FCS-containing medium than those in HuS-containing medium (Table 3). However, this increase was less than 15%, indicating that FCS stimulation was not responsible for the majority of the clones generated in response to IL-2. The proportion of CD4 and CD8 clones derived from FCS and HuS did not differ.

3:1:2 Influence of Different Filler Cells on T Cell Cloning Efficiency:

Experiments were performed to examine the possible influence of SF and PB filler cells on the cloning efficiency and the types of clones generated. (Table 4) shows that there was a slightly higher frequency of IL-2 responsive T cells if SF IL-2 responsive cells were cultured in the presence of irradiated autologous SF filler cells. The CD4:CD8 ratios did not change irrespective of the source of fillers used. However, it was noted that clones often appeared to have a higher growth rate when they were cultured in the presence of SF filler cells. The frequency of PB cells responding to IL-2 in the presence of PB filler was found to be lower than that of SF cells. However, when PB cells were cultured with SF filler, there was a marked decrease in the frequency of clones responding from 1/260 - 1/563 to less than 1/1000

TABLE 3

EFFECT OF SERUM SUPPLEMENT ON T CELL PRECURSOR FREQUENCY

| EXPERIMENT | SOURCE OF CELLS | SERUM | FREQUENCY |
|---|---|---|---|
| I | NPBL | FCS | 1/760 |
| | | HUS | 1/900 |
| II | NPBL | FCS | 1/580 |
| | | HUS | 1/800 |
| III | NPBL | FCS | 1/424 |
| | | HUS | 1/509 |
| IV | RAPBL | FCS | 1/243 |
| | | HUS | 1/337 |
| | RASFL | FCS | 1/116 |
| | | HUS | 1/139 |
| V | RAPBL | FCS | 1/375 |
| | | HUS | 1/422 |
| | RASFL | FCS | 1/105 |
| | | HUS | 1/117 |
| VI | RAPBL | FCS | 1/208 |
| | | HUS | 1/317 |
| | RASFL | FCS | 1/52 |
| | | HUS | 1/80 |

Normal and RA lymphocytes were cloned under limiting conditions in the presence of IL-2 and 10% V/V of the indicated serum. Precursor frequencies for IL-2 responsive clones were determined on day 14

TABLE 4

EFFECT OF DIFFERENT FILLER CELLS ON THE GROWTH OF IL-2 RESPONSIVE CELLS

| EXPERIMENT | RESPONDER CELLS | FILLER | PRECURSOR FREQUENCY |
|---|---|---|---|
| I | PBL | PBL | 1/480 |
| | PBL | SFL | 1/1000 (1/24)* |
| | SFL | PBL | 1/300 |
| | SFL | SFL | 1/230 |
| II | PBL | PBL | 1/260 |
| | PBL | SFL | 1/1000 (0/24)* |
| | SFL | PBL | 1/375 |
| | SFL | SFL | 1/260 |
| III | PBL | PBL | 1/302 |
| | PBL | SFL | 1/3137 (12/24)* |
| | SFL | PBL | 1/193 |
| | SFL | SFL | 1/183 |
| IV | PBL | PBL | 1/563 |
| | PBL | SFL | 1/5484 (4/24)* |
| | SFL | PBL | 1/273 |
| | SFL | SFL | 1/214 |

Limiting dilution analysis for IL-2 responsive T cells in the presence of $10^4$ irradiated (50Gy) PBL or SFL and IL-2, as determined on day 14.

* Represents the proportion of proliferative cultures obtained at the highest concentration of responders tested i.e. 1000 cell/well.

to 12/1000 responding cells. These experiments were set up at the same time with the same PB and SF as were used in the SF cell cloning experiment. It would therefore appear that SF cells either lack the capacity to support PB IL-2 responsive cells or they are inhibitory to such PB clones. To explore the latter possibility further, indomethacin, an inhibitor of prostaglandin synthesis (Ford-Hutchinson et al, 1976) was added during the cloning experiments and its effect on the cloning efficiency of PB IL-2 responsive cells in the presence of SF filler cells studied. It can be seen in (Table 5) that the addition of indomethacin did not significantly increase the IL-2 responsive T cell precursor frequency indicating that prostaglandins, released by the non-lymphoid synovial cells were not responsible for the decreased PB IL-2 responsive precursor frequency. This finding further supports the notion that SF T cells have been subjected to selective pressures within the RA joint in being able to grow in the presence of SF filler cells.

3:1:3 Effect of Cyclosporin A on the Cloning Efficiency:

These studies were based on the premise that only IL-2 responsive cells are limiting under the cloning conditions. However, it has recently been shown that FCS could stimulate resting cells to IL-2 responsive state (Lakhanpal and Handwerger, 1986). In order to rule out the possibility of generating FCS-induced IL-2 responsive T cells, experiments were undertaken to determine if Cyclosporin A (CSA), an inhibitor of lymphocyte activation but not of proliferation of pre-activated cells (Miyawaki et al, 1983) would affect the precursor frequencies seen in the LDA.

The effect of CSA on both PHA-induced proliferation of PB

TABLE 5

EFFECT OF INDOMETHACIN ON PRECURSOR FREQUENCY OF IL-2 RESPONSIVE CELLS

| RESPONDER CELLS | SOURCE OF FILLER | INDOMETHACIN (1ug/ml) | MEAN PRECURSOR FREQUENCY (RANGE) |
|---|---|---|---|
| RAPBL (N=5) | PBL | - | 1/240 (1/194-1/366) |
| RAPBL | PBL | + | 1/234 (1/191-1/361) |
| RAPBL | SFL | - | 1/3548 (1/2316-1/4332) |
| RAPBL | SFL | + | 1/2569 (1/1705-1/3164) |
| RASFL (N=5) | PBL | - | 1/136 (1/58-1/215) |
| RASFL | PBL | + | 1/133 (1/58-1/212) |
| RASFL | SFL | - | 1/104 (1/51-1/185) |
| RASFL | SFL | + | 1/100 (1/51-1/180) |

The influence of synovial fluid filler cells on the cloning of IL-2 responsive T cells in the presence or absence of indomethacin. In these experiments, the use of synovial fluid filler cells greatly suppressed the PBL IL-2 responsive T cells but were marginally reversed by indomethacin. Addition of indomethacin to the other fillers did not significantly alter the precursor frequency.

+ = with Indomethacin - = without Indomethacin

lymphocytes and the precursor frequency is shown in (Table 6). CSA at a final concentration of 2.5 g/ml abrogated the PHA-induced PB lymphocytes proliferation but did not influence the precursor frequencies of IL-2 responsive cells observed in three donors. These findings suggest that under the cloning conditions employed in these studies, in vitro generation of IL-2 responsive cells was insignificant.

3:1:4 Surface Antigens of the Clones:

To obtain a profile of surface antigen expression of the clones a panel of monoclonal antibodies associated with T and B lymphocytes, NK and macrophages was tested on the clones. The cumulative results are shown in (Table 7). All the clones expressed receptors for AET-treated SRBC, T101 antigen, CD3 antigen and either CD4 or CD8 (i.e. the expression of the latter antigens was mutually exclusive). The total number of clones examined to date is 783 for CD4/CD8 and sIg/T101 expression, 200 for CD3 and 100 for rosette forming cells. None of the clones were phagocytic or expressed surface immunoglobulin (sIg).

The expression of HLA-Dr antigen was determined using a monoclonal antibody to human HLA-DR. In some cases, a cross-reacting murine alloantigera to Ia was used. Regardless of which antibody was used 57-94% of all cells within a given clone reacted with this antibody and 48-68% of clones expressed the Tac (IL-2 receptor) antigen. All clones tested to date expressed these activation antigens.

The clones (N =100) were examined for the expression of CD11, CD16, Leu 19 and HNK-1 antigens which are associated with NK and macrophages. None of the clones showed reactivity with the monoclonal antibodies to these antigens whereas 12-15% of normal PBL reacted with

TABLE 6

EFFECT OF CYCLOSPORIN A ON THE PRECURSOR FREQUENCY OF IL-2 RESPONSIVE CELLS AND PHA-INDUCED PROLIFERATING LYMPHOBLASTS

| SOURCE OF CELLS | CYCLOSPORIN A(CSA) | PRECURSOR FREQUENCY |
|---|---|---|
| NPBL | - | 1/418 |
| | + | 1/420 |
| NPBL | - | 1/385 |
| | + | 1/390 |
| RAPBL | - | 1/224 |
| | + | 1/228 |
| RASFL | - | 1/97 |
| | + | 1/100 |

| | | $^{3}$H-TdR UPTAKE (CPM X $10^{-3}$) |
|---|---|---|
| NPBL | UNSTIMULATED | 334 ± 35 |
| | PHA STIMULATED | 57,643 ± 4237 |
| | PHA + CSA (2.5ug/ml) | 220 ± 48 |

IL-2 responsive cells from NPBL, RAPBL and RASFL were cultured with IL-2 in the limiting dilution assay in the presence or absence of CSA.Precursor frequencies for proliferating cells were determined on day 14 of culture. In a separate experiment, normal PBL were stimulated with PHA-P 0.1% in the presence or absence of CSA. After 48 hrs incubation, the cultures were pulsed with 0.2uCi of $^{3}$H-TdR for 6 hrs and the amount of radioactivity incorporated by the cells assessed by liquid scintillation spectroscopy.

CD11, CD16 and HNK-1 in our test system. None of the clones reacted with Leu 19 AB, while 7% of normal PBL recated with the AB.

These results indicate that the phenotype of the clones are: CD3+, T101+, CD4+ or CD8+, SRBC receptor+, HLA-Dr+, CD11-, CD16- , HNK-1- and Leu 19-

Comparison of SF and PB Derived Clones:

Normal PB derived clones were predominantly CD4+ (92%) while RA PB had a slightly lower proportion of CD4+ (83%) cells. There was a tendency for a higher proportion of CD8+ (67%) clones in RA SF. However, this increased CD8+ clones in SF was not a unique feature of RA in that, SF clones generated from 2 Reiter's disease patients and 1 reactive arthritis patient displayed a similar predominance of CD8+ clones to that obtained with RA SF (Table 7).

DISCUSSION

These findings indicate that the predominant IL-2 responsive cells in SF are CD3+, CD8+, HLA-Dr+, T101+. In contrast, this phenotype was expressed by about 10% and 20% of normal and RA PB cells respectively. Thus, RA SF cell population differed phenotypically from PB which suggest the need to study cells from both the local site of inflammation and PB in order to obtain an accurate assessment of pathogenic events in RA. The increased proportion of CD8+ cells in SF was not unique to RA as three non-RA SF samples gave rise to comparable proportions of CD8+ cells. However, it should be noted that not all RA SF displayed this pattern, for two SF samples gave a clonal distribution pattern which more closely resembled that of PBL. Indeed, Nilsson and Biberfeld (1984) have shown that in acute (i.e. less than 1 month) onset of non-septic synovitis CD4+ cells predominated in SF

TABLE 7

CELL SURFACE PHENOTYPE OF IL-2 RESPONSIVE T CELL CLONES DERIVED FROM RA PATIENTS AND NORMALS

| CELL SOURCE | MEAN % REACTIVITY WITH MAB | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | CD3 | CD4 | CD8 | IA | TAC | CD11 | CD16 | HNK-1 | LEU 19 | SRBC RECEPTOR | FCR-IgG |
| NPBL | 95 (90-99) N=200 | 92 (80-99) | 8 (0-20) N=211 | 73 (57-89) N=100 | 51 (48-60) | 0 (0-1) | 0 (0-1) | 0 (0-1) N=100 | 0 (0-1) | 95 (92-100) N=100 | 0 |
| RAPBL | 92 (89-98) N=200 | 83 (80-98) | 17 (0-25) N=290 | 76 (59-90) N=100 | 52 (49-64) | 0 (0-1) | 0 (0-1) | 0 (0-1) N=100 | 0 (0-1) | (90-98) N=100 | 0 |
| RASFL | 94 (91-100) N=200 | 33 (20-74) | 67 (20-80) N=282 | 78 (64-92) N=100 | 55 (52-68) | 0 (0-1) | 0 (0-1) | 0 (0-1) N=100 | 0 (0-1) | (90-100) N=100 | 0 |
| REITER'S DISEASE | NT | 25 (20-30) | 75 (65-80) N=20 | (66-94) | NT | NT | NT | NT | NT | 93 (90-100) N=20 | 0 |
| REACTIVE ARTHRITIS | NT | 30 (25-35) | 70 (68-84) N=10 | 74 (61-90) | NT | NT | NT | NT | NT | 90 (89-100) N=10 | 0 |

The cell surface phenotypes of the clones was determined by indirect immunofluorescent or using monoclonal antibodies directed against membrane antigens with subsequent rosetting using anti-mouse Immunoglobulin-coated polyacrylamide beads(see material and methods for details)
( ) = Range of values observed N=Number of clones examined.

whereas in chronic synovitis in both RA and non-RA CD8+ represented an increased proportion of cells in SF as compared to PB cells. This finding suggests that the phenotype of T cells in SF may be related to the duration of the synovitis rather than to its etiology or stimuli. Nevertheless, it is also conceivable that the decreased proportion of CD8+ cells in RA PB and increased proportion in SF may reflect a homing of the CD8+ cells to the joint in response to antigens or other stimuli. This hypothesis may be consistent with the finding of (Janossy et al, 1980) who showed that human T lymphocytes of helper and suppressor types have characteristic recirculation patterns and occupy different microenvironments.

The antigenic phenotype of the clones and their strict IL-2 dependency indicate that they are of T cell lineage. These data are in accordance with the findings of (Burmester et al, 1981; Fox et al, 1982) that an increased proportion of activated synovial T cells are found in the CD8+ population. A similar finding was obtained by Schlesier et al, (1984) using limiting dilution of analysis of SF lymphocytes. It should be emphasized that the IL-2 responsive T cells derived from SF in this study by no means represent the whole spectrum of T cells in the inflamed joint. The culture conditions will certainly select for a subpopulation of activated T cells able to grow well under the culture conditions previously described. Several reports indicate that large granular lymphocytes (LGL) or NK cells can be expanded in culture in IL-2 containing medium and constitute the bulk of spontaneously IL-2 responsive cells in PB (Timonen et al, 1982; Vose & Bonnard, 1983; Suzuki et al, 1983) and that this situation might also apply to synovial T lymphocytes. The fact that none of the clones to date (N =

100) express the CD16 antigen which is expressed on 85-90% of NK cells (Perussia et al, 1983) suggests that the clones are indeed T cells. Furthermore it has been reported that when bulk PB lymphocyte cultures are stimulated with cloned recombinant IL-2 (rIL-2) both T cell and CD16+ fractions of the cultures contain DNA synthesizing cells in comparable frequencies (Trinchieri et al, 1984). However, recent studies (Lanier et al, 1986) have shown that CD16+ IL-2 responsive cells require much higher levels of IL-2 (500-1000 units/ml) for growth than were used in our culture system which facilitate T cells but not NK cells being grown out under these conditions. The inability of SF cells to function as effective fillers for autologous PB lymphocytes was a surprise since there have been several reports indicating that SFL can act as efficient accessory cells for mitogen induced proliferative responses (Petersen et al, 1983). The basis for this effect may be due to active inhibition of PB lymphocytes by the increased proportion of activated CD8+ cells while SF lymphocytes are resistant to this inhibitory effect. Alternatively, the non-lymphoid synovial cells may be secreting factor(s) such as prostaglandins which is a potent inhibitor of T cell proliferation (Goodwin et al, 1977). To further clarify the mechanism of this suppression, indomethacin was added to the culture system to see whether the inhibitory effect can be reversed. The inhibitory effect of SFC on PB cells was not due to the action of prostaglandins since there was no statiscally significant difference between the precursor frequencies of cells cultured with or without indomethacin. Studies by Burmester et al. (1984) showed that low-density preparations of synovial cells containing Ia+ macrophages and dendritic cells are less stimulatory than peripheral blood non-T

cells in the induction of MLR or AMLR. These cell types may be responsible for the observed inhibition of PBL LDA cultured with SF filler cells.

In an earlier study, the precursor frequencies of spontaneously IL-2 responsive cells from RA PB and SF were similar although there was a tendency for the precursor frequencies of SF cells to be slightly higher than PB cells . This finding was rather surprising since several reports have indicated an increased proportion of T cells in SF are activated as defined by the expression of HLA-Dr and Tac antigens (Burmester et al, 1981; Fox et al, 1982). It has been suggested that HLA-Dr expression per se is not indicative T cells capable of responding to IL-2, since there appears to be a dissociation between the IL-2 receptor detected by the Tac antibody and HLA-Dr expression (Burmester et al, 1984). Studies by Robb et al. (1984) suggested that expression of anti-Tac reactivity may be a poor indicator of IL-2 responsiveness since this antibody detects both high and low affinity IL-2 receptors and only the former appear to be of physiological relevance to IL-2 dependent proliferation.

However, when the old batch of IL-2 containing medium (ICM) ran out and a new batch was used in subsequent studies, clear differences between SFC and PBC precursor frequencies were obtained. The most plausible explanation for the change in frequency appears to relate to the IL-2 sources used for each group of experiments as these were performed with different IL-2 batches. Prior to using the new batch of ICM, the IL-2 level was determined using the IL-2 dependent cell line CTLL-2. Dilutions of ICM containing equivalent amounts of IL-2 (30 units/ml) were used in the LDA.

In preliminary experiments, when the old and new batches of ICM, recombinant human IL-2 and partially purified Electronucleonics IL-2 were used on normal PBL in LDA, comparable precursor frequencies were obtained suggesting that IL-2 activity in the various preparations was the only determining factor in these assays.

The finding that SF cells could be cloned with equal efficiency in the presence of ICM and either irradiated SFL or PBL fillers while PB cells grew poorly on SF filler cells suggest differences in growth requirements of SFC and PBC. Such differences may be reflected in the enhanced responsiveness of SFC to the new batch of ICM used in the rest of the studies. A closer examination of the new batch of ICM indicated that some of the supernatants obtained from tonsil lymphocytes have been stimulated with PHA + an EBV lymphoblastoid cell line (LBL). Recent studies by ( Abdel-Nour et al,1986), have shown that autologous SF stimulated the proliferation of SF T cells efficiently in the presence of LBL , possibly through the release of growth factors which augmented the proliferation of SF T cell. It is conceivable that the LBL released additional growth factors into the ICM, which perhaps synergized with IL-2 to increase the proliferative response to SF T cells in the cloning system. The data reported here suggest that under the cloning conditions, only <u>in vivo</u> activated, IL-2 responsive T cells were limiting and that no <u>in vitro</u> activation was occurring. Comparable precursor frequencies were obtained from normal PBL using FCS and HuS to supplement the growth medium indicating that the majority of clones were not FCS-induced. Also, the addition of concentrations of cyclosporin A which inhibited PHA-induced proliferation to cultures did not alter the precursor frequencies of

IL-2 responsive cells generated in media containing FCS thus, further supporting the notion that in vitro activation by FCS components was not responsible for the observed IL-2 responsive cells. Taken together, these results suggest that the clones obtained in the limiting dilution analysis were not generated in response to serum factors or residual mitogens in the IL-2 containing medium and thus, the CD8+ predominance in the SFC clones reflects increased IL-2 responsiveness in that population at the initiation of culture confirming the previous finding with bulk lymphocyte cultures, a selective expansion of CD8+ cells (Wilkins et al, 1984).

RESULTS

3:1:5 Mitogen and Antigen-induced IFN , IL-1 and IL-2 Production by T Cell Clones:

The T cell clones were examined for their capacity to secrete lymphokines upon mitogenic and antigenic stimulation. None of the clones constitutively produced detectable levels of IFN or IL-2. However, with PHA stimulation, all the clones tested released increased amounts of IFN (Fig. 3). The time course studies with PHA-stimulated T cell clones and bulk culture of normal and RA PB lymphocytes demonstrated that IFN levels were higher for both RA and normal bulk cultures at 48-72 hrs. In contrast, the T cell clones exhibited maximum IFN release after 24 hrs in culture and demonstrated a decline in activity when evaluated at 48-96 time intervals (Fig. 4). Characterization of the interferon released by the PHA-stimulated T cell clones demonstrated that (1) its activity was abrogated by acid (pH 2.0) treatment (2) its activity was lost by incubation at 56 $^{o}$C for 30 minutes (3) it was inhibited by treatment with anti-IFN-$\gamma$

Figure 3: PHA-P induced release of IFN-γ by RA and normal T cell clones. All clones were cultured at 5 x $10^4$ cells/200ul in RPMI1640+10% FCS and stimulated with 0.1% PHA-P for 24 hrs at 37°C. The supernatants were harvested and assayed for IFN-γ . Data is expressed as a single symbol for each individual clone.

N = number of clones examined.

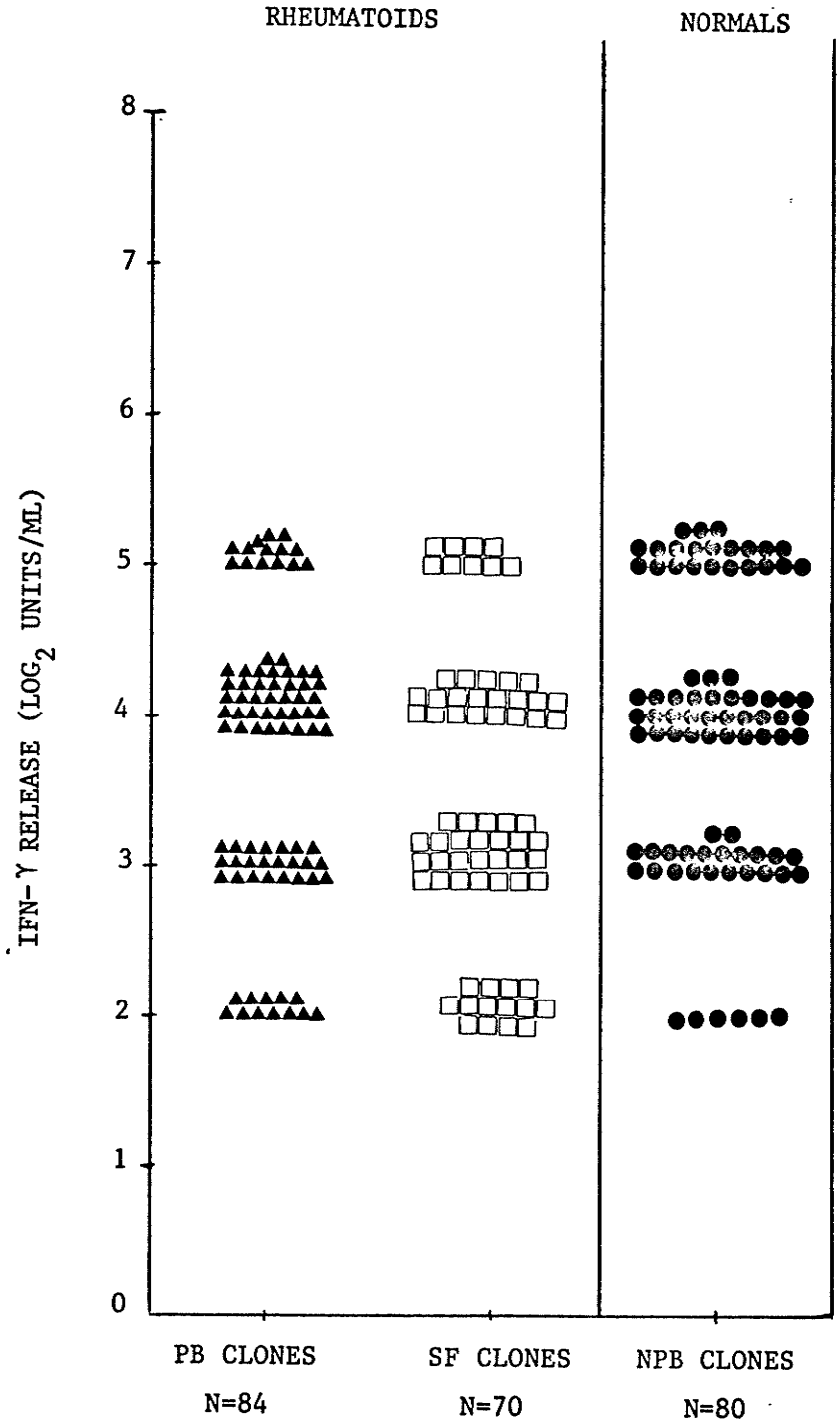
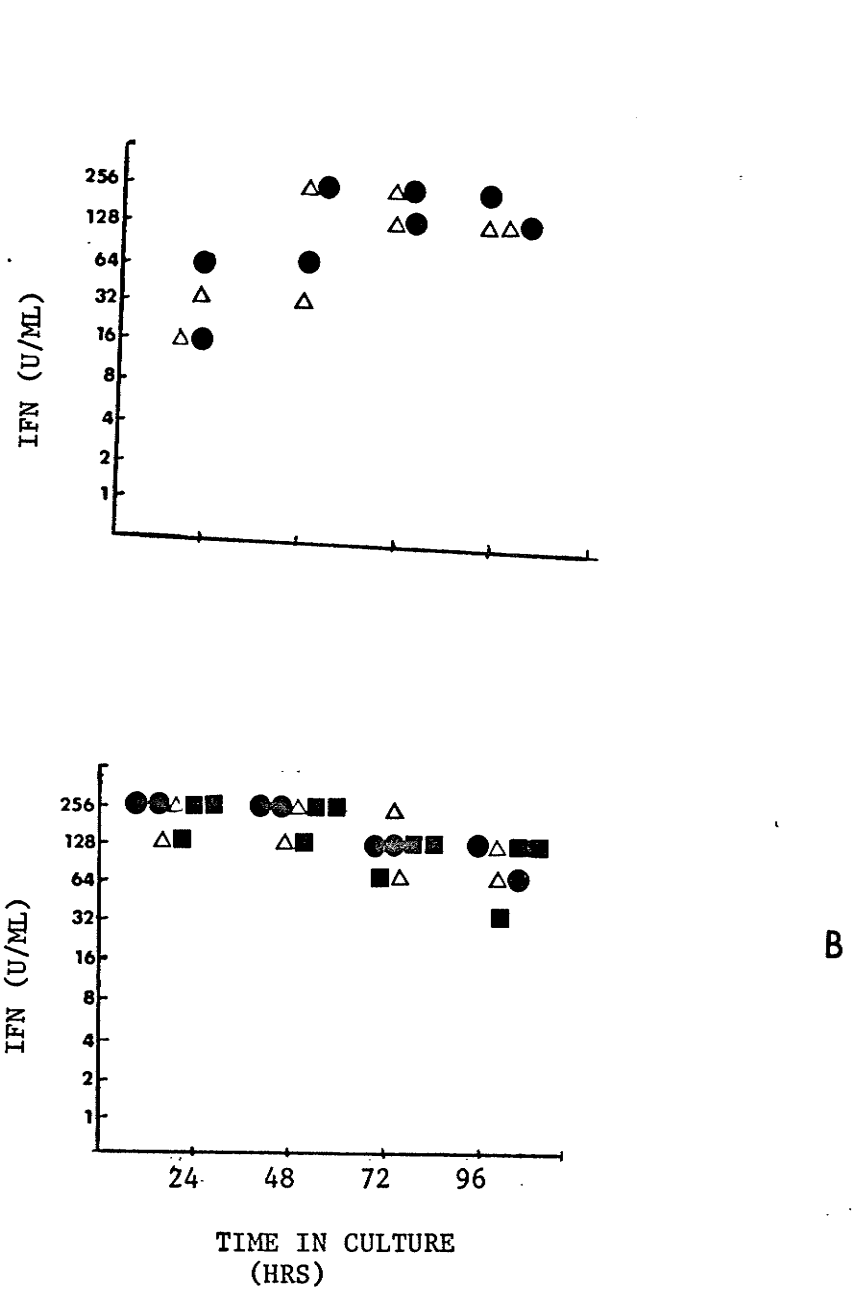

RHEUMATOIDS
NORMALS
IFN-γ RELEASE ($LOG_2$ UNITS/ML)
8
7
6
5
4
3
2
1
0
PB CLONES
N=84
SF CLONES
N=70
NPB CLONES
N=80

Figure 4: Kinetics of IFN-γ release by bulk RA and normal PBL and cloned T cells. High levels of IFN-γ were detected within 24 hrs of stimulating T cell clones, irrespective of origin, with PHA-P (Panel B). In contrast, maximal levels of IFN-γ production by bulk RA and normal PBL stimulated with the same mitogen was not reached until 48 - 72 hrs in culture (Panel A).


RASF CLONE
RAPB CLONE
NPB CLONE
A
B
IFN (U/ML)
256
128
64
32
16
8
4
2
1
24
48
72
96
TIME IN CULTURE
(HRS)

monoclonal antibody (Table 8). These data strongly suggest that almost all of the IFN released by the T cell clones was IFN-γ . The fact that all clones, irrespective of origin, released similar levels of IFN- indicate that there was no qualitative differences between normal and RA T cell clones in their ability to produce IFN-γ .

No significant IL-2 activity could be detected in the supernatants of the T cell clones in the presence or absence of PHA. The inability to detect IL-2 in the supernatants was not due to the presence of inhibitors that interfered with the IL-2 assay, since a standard IL-2 preparation added to the supernatants retained full activity compared to the standard IL-2 preparation assayed alone (Table 9). However, stimulation of the clones with PMA+PHA produced IL-2 with higher levels from N PB nd RA PB clones while SF clones produced decreased levels (Fig. 5). Interestingly, CD4+ SF T cell clones produced comparable levels of IL-2 to CD4+ PB clones. The PMA+PHA-induced supernatants from SF CD8+ clones were not toxic to the CTLL-2 cells as assessed by Trypan blue dye exclusion. Thus, the diminished IL-2 production by RA SF may reflect qualitative differences in cells from the two compartments, in that SF clones grow faster than PB clones and therefore may utilize more IL-2. Irradiation of CD8+ SF T cell clones did increase the IL-2 production to the same level as PB CD8+ cells, the previous differences being no longer significant.

IL-1 activity in culture supernatants has traditionally, been measured by its capacity to enhance thymocyte proliferation in response to suboptimal concentrations of T cell mitogens (Maizel et al, 1981). However, given the fact that the thymocytes can also respond to IL-2, this assay is a less specific method for detecting IL-1. In order to

TABLE 8

CHARACTERIZATION OF INTERFERON (IFN) PRODUCED BY T CELL CLONES

| SUPERNATANTS | TREATMENT WITH | IFN(U/ML) |
|---|---|---|
| RAPBL CLONE | NONE | 160 |
| | ANTI-IFN-$\gamma$ | 2 |
| | ACID(pH 2.0) | 2 |
| | HEAT | 4 |
| RASFL CLONE | NONE | 80 |
| | ANTI-IFN-$\gamma$ | 0 |
| | ACID(pH 2.0) | 0 |
| | HEAT | 2 |
| NPBL CLONE | NONE | 160 |
| | ANTI-IFN-$\gamma$ | 0 |
| | ACID(pH 2.0) | 0 |
| | HEAT | 2 |

T cell clone supernatants were treated with anti-IFN-$\gamma$ (1:2,10,000U/ml) for 24 hrs or reduced to pH 2.0 with 6M HCL for 1 hr and neutralised with Hepes buffered RPMI 1640 and the pH raised to 7.2 or heated at 56$^{o}$C for 30 mins. The controls were untreated. All supernatants were tested for their anti-viral activity. All treatments destroyed the anti-viral activity of the supernatants, thus confirming their $\gamma$ nature.

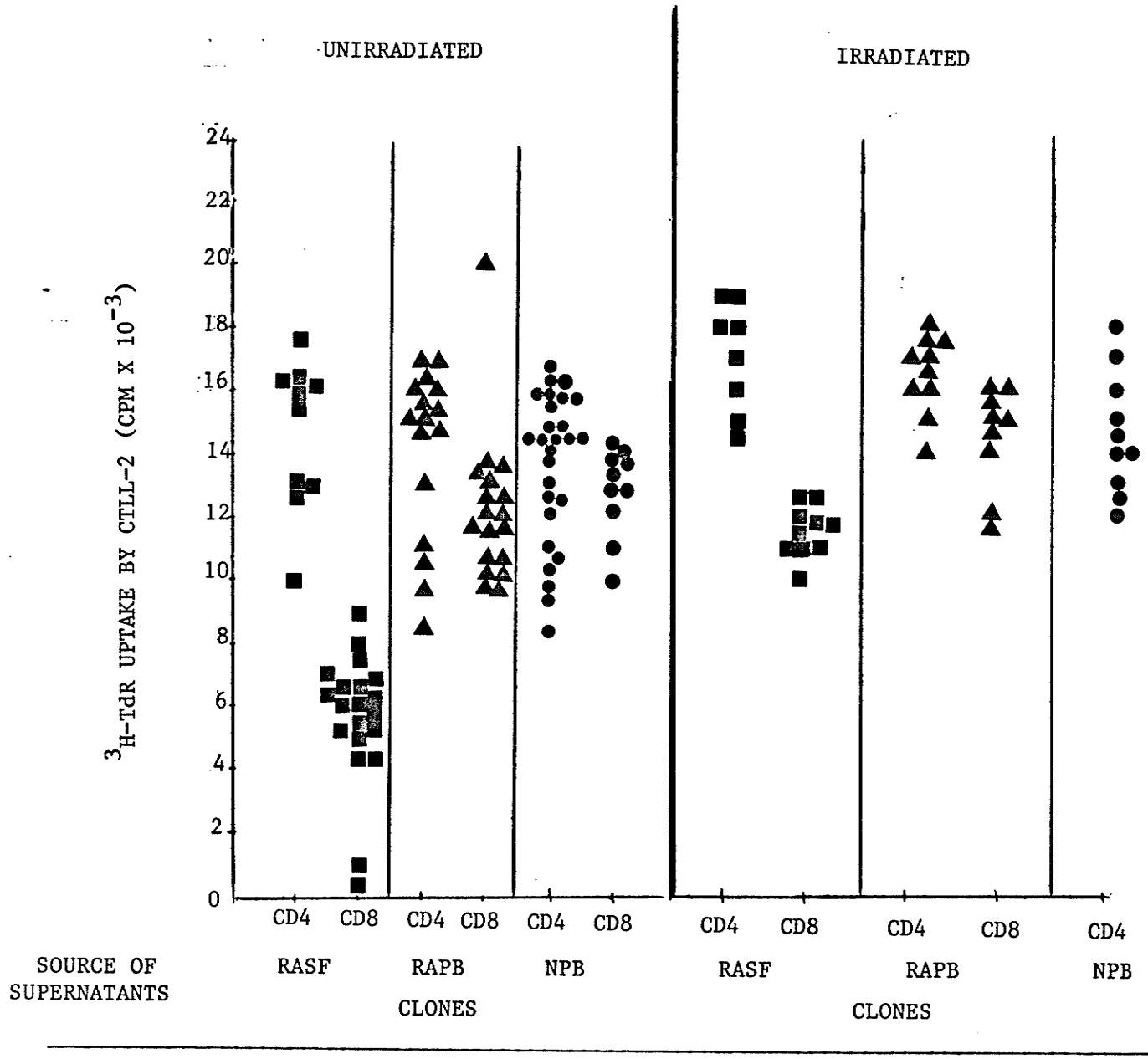
TABLE 9

LACK OF INHIBITOR OF IL-2 ACTIVITY IN SUPERNATANTS OF T CELL CLONES

| SUPERNATANTS FROM CLONES | ADDITION OF IL-2 | PROLIFERATION ($^3$H-TdR UPTAKE BY CTLL-2 CELLS) |
|---|---|---|
| RAPBL | - | 101 ± 10 |
| | + | 9853 ± 388 |
| RAPBL | - | 197 ± 16 |
| | + | 9664 ± 846 |
| RASFL | - | 480 ± 282 |
| | + | 9442 ± 369 |
| RASFL | - | 381 ± 97 |
| | + | 9560 ± 497 |
| NPBL | - | 178 ± 58 |
| | + | 9747 ± 399 |
| NPBL | - | 151 ± 77 |
| | + | 9678 ± 287 |
| CTLL-2 | - | 122 ± 10 |
| | + | 9987 ± 984 |

T cell clones were stimulated with 0.1% PHA for 24 hrs. The supernatants were reconstituted with or without IL-2 at a final dilution of (1:2, 1.5%) in flat-bottomed microtitre plates containing 5 x $10^3$ CTLL-2 cells. The cultures were incubated for 24 hrs at 37$^o$C, pulsed labelled with $^3$H-TdR for 6 hrs, harvested and counted. Data represent means and standard deviations of triplicate cultures.

Figure 5: IL-2 levels in culture supernatants of PMA+PHA-pulsed T cell clones from RA patients and normal subjects. T cell clones were pulsed with PMA (20ng/ml) + PHA (0.1%) for 4 hrs, washed thrice in RPMI 1640+10% FCS and incubated at 37°C for a further 20 hrs in a fresh medium. The cells sedimented by centrifugation and IL-2 activity of the supernatants measured by $^{3}$H-TdR uptake by CTLL-2, an IL-2 dependent cell line. The bars show the mean of triplicate cultures. Each symbol represents an individual clone. IL-2 activity of culture supernatants derived from PMA alone, PHA alone or unstimulated cultures were less than 100 CPM. The maximum $^{3}$H-TdR uptake by CTLL-2 cells in response to 3% IL-2 was 34,218 CPM.


UNIRRADIATED
IRRADIATED
$^{3}$H-TdR UPTAKE BY CTLL-2 (CPM X $10^{-3}$)
24
22
20
18
16
14
12
10
8
6
4
2
0
CD4
CD8
CD4
CD8
CD4
CD8
CD4
CD8
CD4
CD8
CD4
SOURCE OF SUPERNATANTS
RASF
RAPB
NPB
RASF
RAPB
NPB
CLONES
CLONES

accurately quantitate IL-1 activity in the supernatants of the T cell clones, the tumour cell line, LBRM-33 (an IL-1 dependent, IL-2 producer) was used. This cell line secretes IL-2 only in the presence of IL-1 and PHA (Gillis and Maizel, 1981) thus, making this approach a very sensitive and reliable method for IL-1 detection.

As shown in (Table 10), no detectable levels of IL-1 can be obtained from the T cell clones supernatants as measured by CTLL-2 $^{3}$H-Tdr uptake, but a significant level of uptake occured in the positive control which contained rIL-1 + PHA. In contrast, the LBRM-33 cells cultured with PHA alone did not cause CTLL-2 proliferation, confirming the strict requirement for IL-1 before the cells could secrete IL-2.

Reactivity of T Cell Clones to Soluble and Immobilized Connective Tissue Antigens (CTA):

Lymphokine production (IFN-γ and IL-2) was used as a measure of reactivity of the T cell clones to CTA which comprises collagen and proteoglycan. The optimum concentration of soluble denatured type II collagen was determined for the T cell clones. It can be seen from (Fig. 6) that concentrations of 50-100 μg/ml of the antigen induced maximum release of IFN-γ . However, there was a significant reduction in IFN-γ production at CTA concentrations greater than 100 μg/ml. A concentration of 50 μg/ml of CTA was used in all subsequent studies. The results were rather surprising in that each clone reacted with both native types I and II collagen and to a lesser extent with proteoglycans suggesting that there might be a common determinant on the interstitial collagens and proteoglycan or that the clones were induced to produce IFN due to non-specific interactions with the CTA. A larger number of clones was tested for responses to soluble native

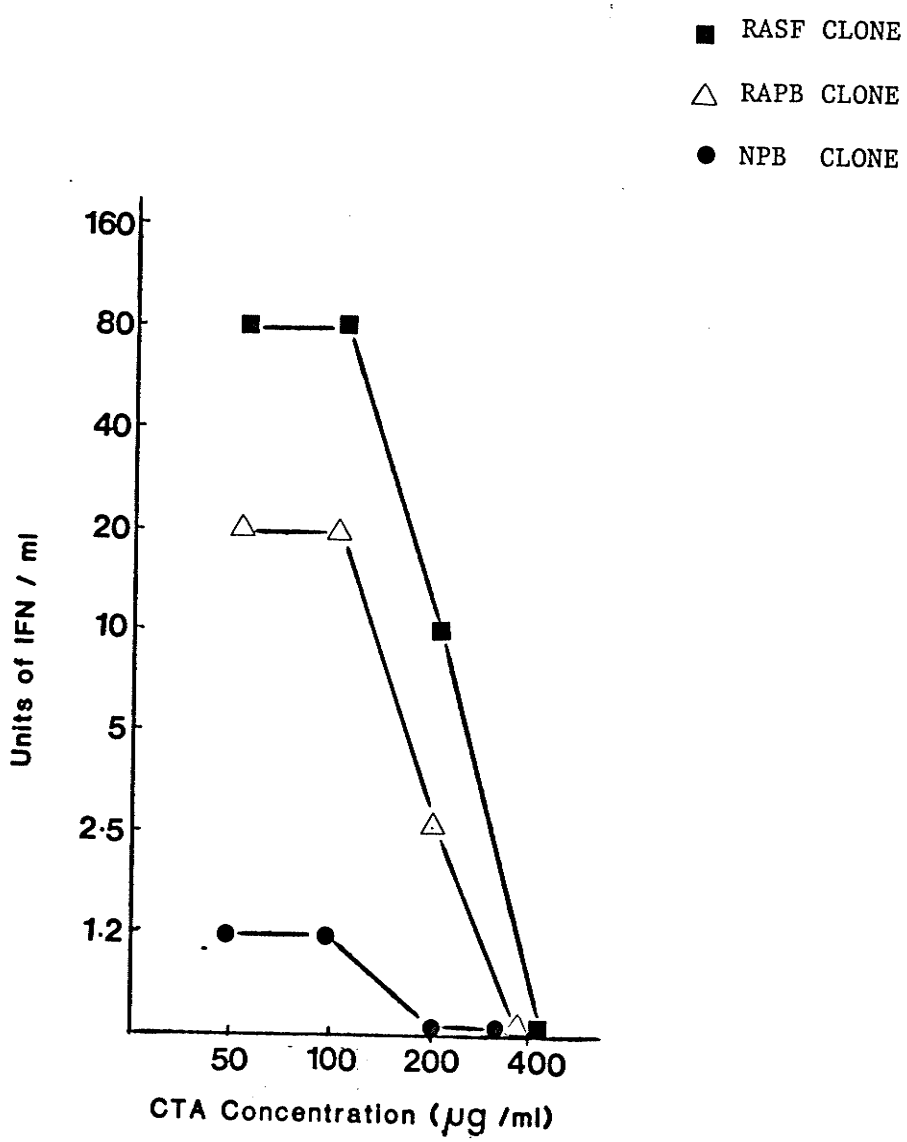
TABLE 10

$^{3}$H-TdR INCORPORATION IN THE DIRECT ASSAY FOR IL-1 DEPENDENT IL-2 PRODUCTION BY THE LBRM-33 CELL LINE

| CLONES | STIMULI | CPM ± SD |
|---|---|---|
| RAPBL | 25% SUPN. + 0.1% PHA | 352 ± 36 |
| RAPBL | " | 304 ± 44 |
| RASFL | " | 319 ± 10 |
| RASFL | " | 498 ± 90 |
| NPBL | " | 306 ± 37 |
| NPBL | " | 387 ± 51 |
| RAPBL | 10% SUPN. + 0.1% PHA | 238 ± 58 |
| RAPBL | " | 324 ± 38 |
| RASFL | " | 277 ± 26 |
| RASFL | " | 312 ± 43 |
| NPBL | " | 315 ± 40 |
| NPBL | " | 366 ± 21 |
| | CONTROLS | |
| | MEDIUM | 117 ± 20 |
| | PHA | 120 ± 19 |
| | rIL-1 + PHA | 4451 ±61 |
| | CTLL-2 + IL-2 | 18,289 ± 3589 |

LBRM-33 cells (5 x $10^{4}$/well) were cultured with T cell clone supernatants at 10% and 25% final concentration for 24 hrs. The supernatants were removed and then assayed for IL-2 activity using CTLL-2 cells. Data represent mean counts per minute (CPM) ± standard deviation (SD) of triplicate cultures.

Figure 6: Optimization of soluble denatured type II collagen (DII) induced IFN production. T cell clones ($5 \times 10^4$/200ul) were stimulated with various concentrations of soluble DII collagen for 24 hrs, the supernatants collected and tested for IFN activity.


RASF CLONE
RAPB CLONE
NPB CLONE
160
80
40
20
10
5
2·5
1·2
Units of IFN / ml
50
100
200
400
CTA Concentration (µg /ml)

and denatured type I or II collagen, proteoglycan and a various correlative control antigens such as ovalbumin, PPD and lysozyme. These experiments were performed in the absence of autologous APC as previous studies indicated that the clones themselves can function as APC in the CTA-induced IFN productions. The absence of APC makes the system clean for monitoring T cell-derived IFN production, as irradiated APC can also secrete IFN. The cumulative data from these studies indicated that all of the clones examined (N=90) responded to both native and denatured types I and II collagens and proteoglycan (Fig. 7). In general, denatured types I and II collagens induced increased IFN production by RA SF clones compared to PB clones. In contrast, the control antigens did not induce IFN production. This finding is indicative of a CTA restricted response, for the control antigens did not stimulate IFN production. If an antigen specific response was occurring, then the CTA must share a common epitope(s). However the fact that all the clones , irrespective of origin, reacted to CTA suggests that the responses were mediated by a similar mechanism, with the CTA having a common or shared epitopes. The ability of anti-CD3 and anti-TcR antibodies (Becton Dickinson, Mountain view, CA) which recognizes the CD3/ antigen receptor complexes, to abrogate CTA-induced IFN production suggest that the recognition of CTA by the T cell clones may be mediated by a receptor linked to or closely associated with the CD3 antigen-receptor complex (Table 11). This indicated that the responses to CTA are antigen-specific, occuring through the antigen-receptor pathway.

3:1:6 Effect of Fibronectin (Fn) on IFN and IL-2 Production:

An alternative plausible explanation for the polyspecificity of CTA-

Figure 7: CTA-Induced IFN Production In Serum-containing medium by IL-2 responsive T cell clones. T cells ($5 \times 10^4$) were cultured with CTA for 24 hrs and the culture supernatants assayed for IFN. The CTA were used at a final concentration of 50ug/ml and comprised Native type I collagen (NI), Denatured type I collagen (DI), Native type II collagen (NII), Denatured type II collagen (DII), Proteoglycan (PG), Purified protein derivative of Mycobacterium tuberculosis (PPD), Ovalbumin (OA), Lysozyme (LYS). Each point represents the mean ± standard error of mean of 30 SF, 30 PB and 30 NPB T cell clones.

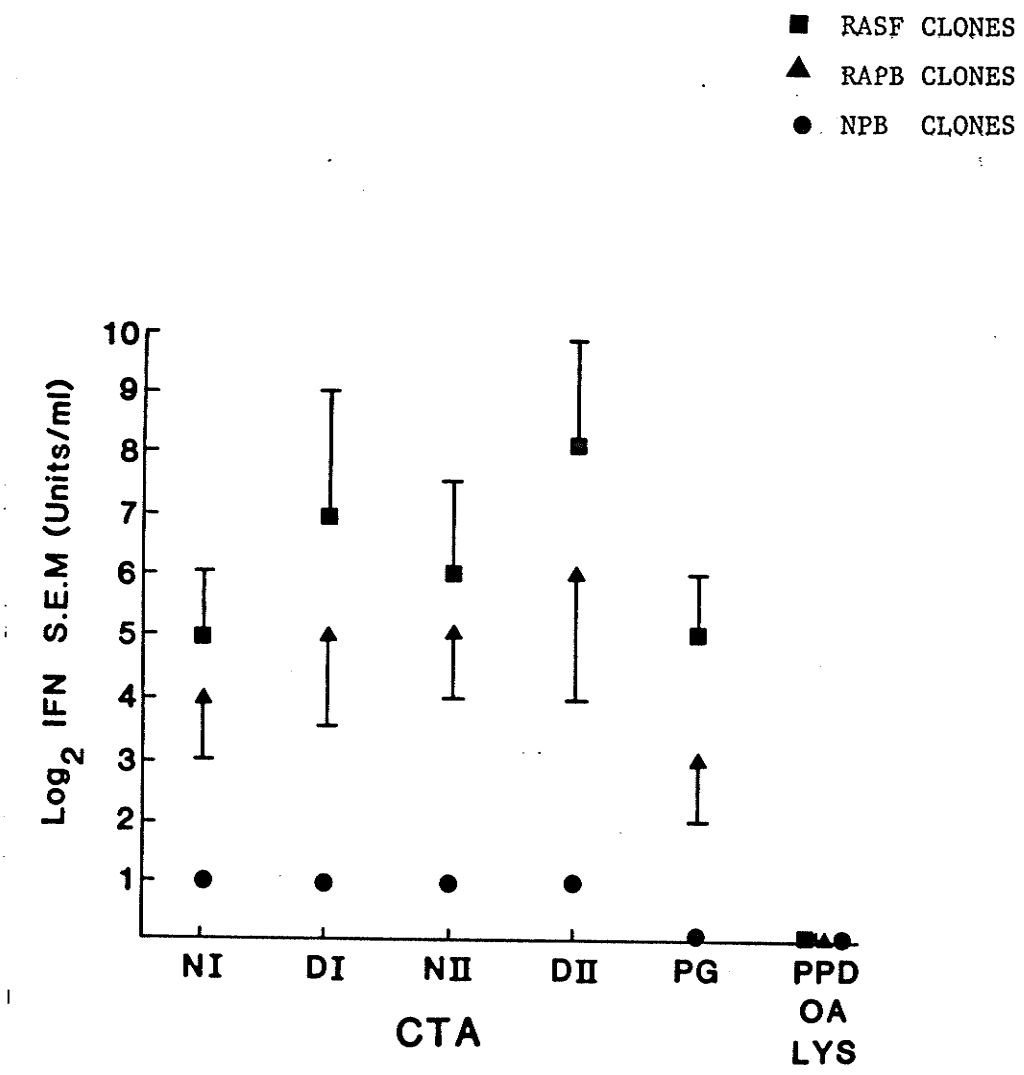

RASF CLONES
RAPB CLONES
NPB CLONES
Log2 IFN S.E.M (Units/ml)
10
9
8
7
6
5
4
3
2
1
NI
DI
NII
DII
PG
PPD
OA
LYS
CTA

TABLE 11

EFFECT OF MODULATION OF T CELL SURFACE ANTIGENS ON DENATURED TYPE II COLLAGEN (DII)-INDUCED IFN PRODUCTION

| | | ANTIBODY TREATMENT | | | | |
|---|---|---|---|---|---|---|
| CLONES | MEDIUM | CD3 | CD4 | CD8 | TcR* | HLA-DR/IA |
| RASFA8 | 80+ | 2.5 | 80 | 80 | 2.5 | 40 |
| RASFA10 | 80 | 1.25 | 80 | 80 | 2.5 | 40 |
| RAPBG3 | 20 | 0 | 20 | 20 | 2.5 | 10 |
| RAPBG6 | 20 | 1.25 | 20 | 20 | 2.5 | 10 |

T cell clones ($5 \times 10^4$ cells/200ul) were incubated with saturating doses of the indicated antibodies for 4 hrs at 37°C in serum-containingmedium, followed by addition of DII collagen (50ug/ml) and incubated for a further 20 hrs. The supernatants were harvested and assayed for IFN activity.

+ = Units of IFN/ml

* = Ab to the $\beta$ chain of the T cell receptor

induced IFN production by the clones was that they were reacting with a molecule that could bind to CTA in the culture system. Fn is one of the molecules which has a strong affinity for collagen expecially denatured collagen and thus it is a potential candidate. To test the hypothesis that Fn may be involved in the CTA responses, IFN induction was performed under serum-free conditions to exclude Fn from the culture system. None of the clones tested produced IFN i.e. under serum-free conditions all responses to CTA were abolished (Fig. 8). However, stimulation with PHA under identical conditions did result in IFN production (40-100 units/ml) indicating that the clones remained functional under these culture conditions. These results suggest that a serum component, possibly Fn, is required for stimulation of the clones by CTA.

The addition of purified human plasma Fn to serum-free cultures of clones containing 50 µg/ml of soluble type II collagen resulted in a dose dependent recovery of IFN production (Fig. 9). A concentration of Fn above 30 µg/ml did not increase the IFN production so a concentration of 30 µg/ml was used in all serum-free studies. The Fn alone failed to induce a response. A total of 90 clones were tested for their response to CTA and control antigens under serum-free conditions in the presence of 30 µg/ml of Fn. In these experiments neither Fn nor CTA induced IFN production while a mixture of the CTA and Fn resulted in the full restoration of IFN production relative to that seen in the presence of serum (Fig. 8).It should be noted that occasionally, some clones produce low levels of IFN (2 units/ml) to CTA under serum-free conditions.

Figure 8: CTA-Induced IFN Production In Serum-free medium supplemented with Fibronectin by IL-2 responsive T cell clones.

Clones (N=30) of RASF, RAPB and NPB were cultured under serum-free conditions in the absence (open symbols) or presence (closed symbols) of 30ug/ml of purified human plasma fibronectin and the CTA for 24 hrs. The supernatants were collected and tested for IFN activity.

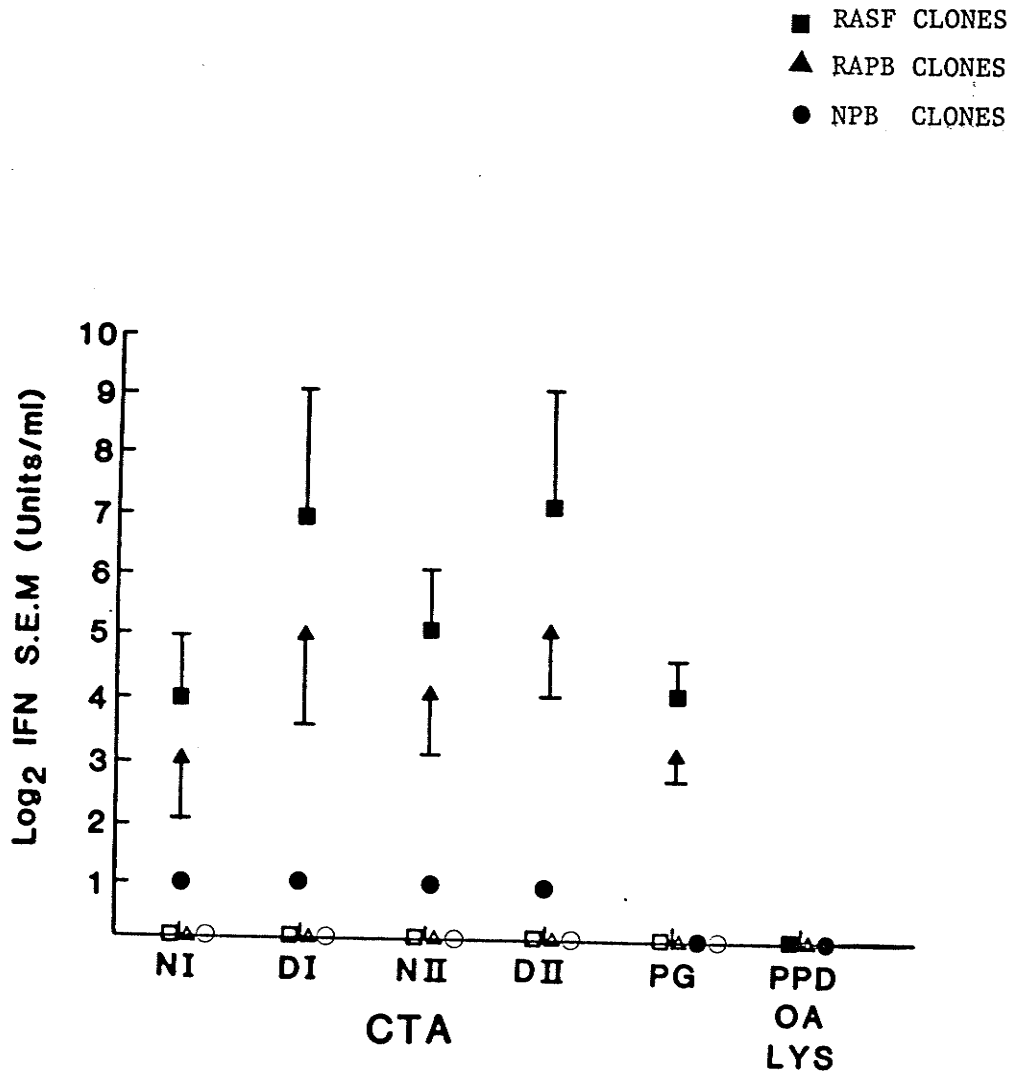
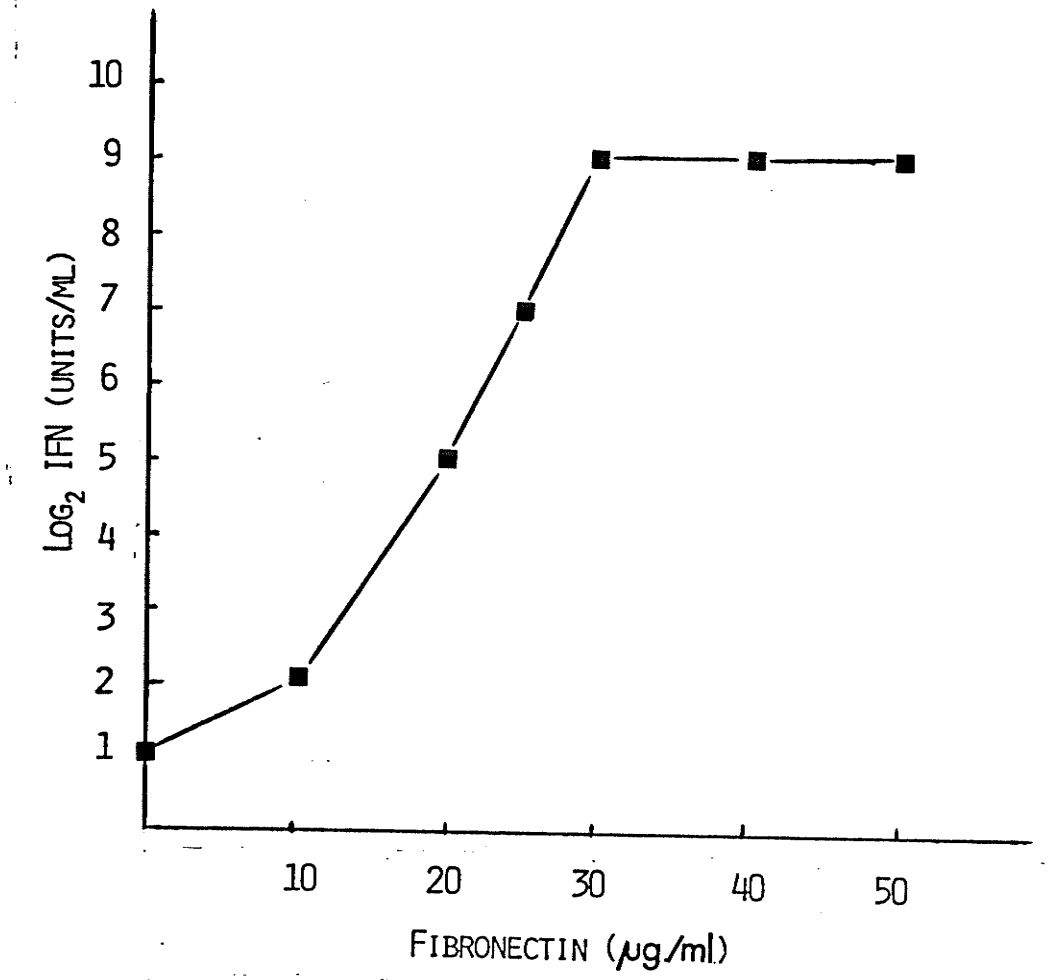

RASF CLONES
RAPB CLONES
NPB CLONES
Log2 IFN S.E.M (Units/ml)
10
9
8
7
6
5
4
3
2
1
NI
DI
NII
DII
PG
PPD
OA
LYS
CTA

Figure 9: Enhancement Of Soluble Denatured type II collagen (DII)-Induced IFN Production By Fibronectin (Fn). SF T cell clone was stimulated under serum-free conditions with DII collagen (50μg/ml) and various doses of Fn to determine the optimum dose of Fn required for enhanced IFN production. Fn doses of 30 - 50μg/ml gave the best enhancement of DII-induced IFN production. A dose of 30μg/ml was subsequently used in all assays.


10
9
8
7
6
5
4
3
2
1
LOG2 IFN (UNITS/ML)
10
20
30
40
50
FIBRONECTIN (µg/ml)

Collagen Requirement for IFN Production:

The previous results did not distinguish between the relative roles of collagen and Fn in the activation or the possibility that collagen-Fn interactions merely increased the avidity of interactions between the clones and the CTA. It is conceivable that Fn or collagen alone was the stimulus for IFN production or that the Fn-collagen interactions in some way, possibly by cross linking the relevant molecular moieties, resulted in a complex with a strong avidity for the clones. If this hypothesis is correct, then immobilization of collagen or the Fn separately to culture wells should provide an array of antigenic determinants capable of multi-point binding to the clones. Flat bottomed microtitre wells were coated overnight with either Fn or collagen washed free of unbound proteins followed by either media alone or Fn or collagen for a further 24 hrs. The plates were washed free of unbound protein and the clones added to the wells. The supernatants were collected after 24 hrs and assayed for IFN activity.

Those wells receiving media or Fn alone did not produce detectable IFN. The cultures receiving collagen alone had significant levels of IFN. However, the addition of Fn to the cultures with collagen enhanced this response comparable to the levels observed with soluble collagen (Table 12). Immobilized control antigens (PPD, OVA and LYS) did not induce IFN production. Optimization of the dose response for immobilized CTA indicated that, like soluble collagen, 50-100 µg/ml was required for IFN production. In contrast to soluble CTA, high doses of CTA were not inhibitory, rather exhibiting a plateau effect. (Fig. 10). These results suggested that Fn could enhance the collagen responses but in contrast to collagen, Fn is incapable of inducing IFN

TABLE 12

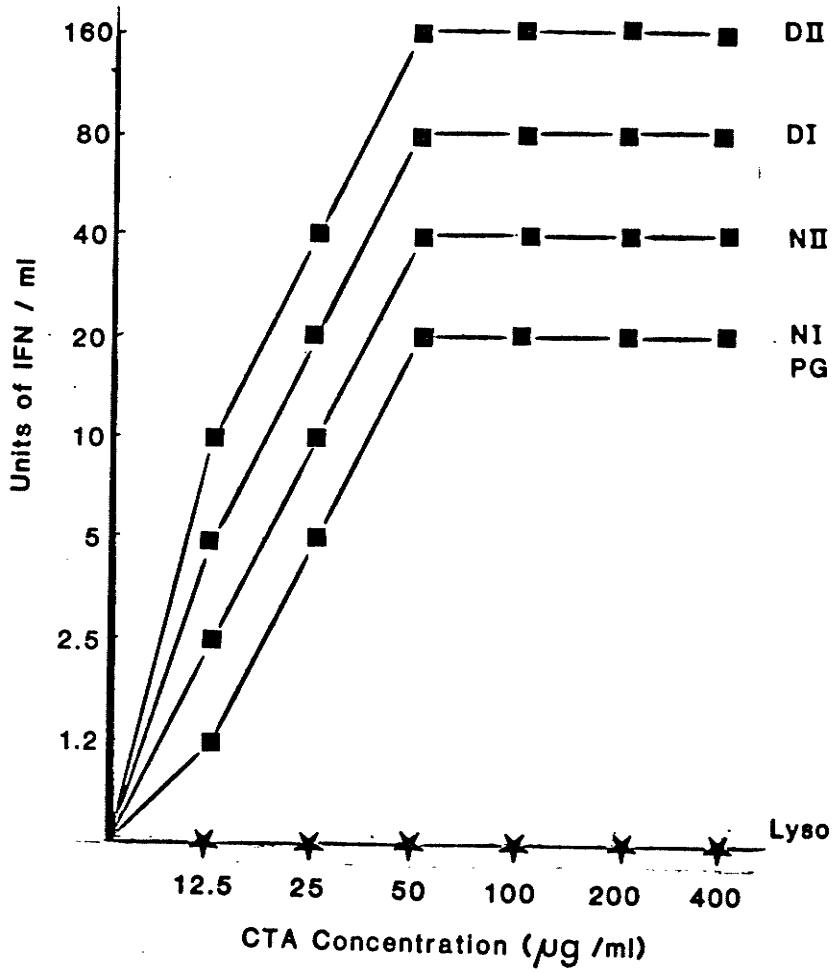
FIBRONECTIN (FN) ENHANCEMENT OF IFN PRODUCTION BY IMMOBILIZED DII COLLAGEN

| CLONES | MEDIUM | FN | DII | DII + FN |
|---|---|---|---|---|
| RASFA8 | 0 | 0 | 20* | 80 |
| RASFA10 | 0 | 0 | 20 | 80 |
| RAPBG3 | 0 | 0 | 5 | 20 |
| RAPBG6 | 0 | 0 | 5 | 20 |

T cell clones were cultured in the presence of either serum-free medium alone or with immobilized DII collagen (50ug/ml) for 24 hrs, the supernatants collected and tested for IFN activity.

* = units of IFN/ml

Figure 10: Optimization Of Immobilized CTA-Induced IFN Production. SF T cell clones ($5 \times 10^4$) were exposed to various doses of CTA immobilized in flat-bottomed microtitre plates for 24 hrs. The supernatants were aspirated and assayed for IFN activity.


160
80
40
20
10
5
2.5
1.2
Units of IFN / ml
DII
DI
NII
NI
PG
Lyso
12.5
25
50
100
200
400
CTA Concentration (μg /ml)

production. A further plausible explanation for the polyspecificity of the T cell clones to CTA is that, although the T cells are statistically clonal, there may be cells with different specificities in the population. An obvious approach to resolving that question would be to subclone the cell at 0.3 cells/well. Unfortunately, all attempts to subclone at that cell concentration failed. Therefore an alternative approach was used based on the observation that high dose (i.e 400 µg/ml) of collagen can tolerize clones resulting in no IFN production. Thus, the rationale is that, if the cultures contained cells with different specificities, then tolerization of one cell population with high dose to one antigen, should not affect the ability of the other cells to respond to another antigen. If, on the other hand, all cells in the culture were specific to the CTA, then tolerization with one antigen would abrogate the responsiveness to the other antigens. While this approach does not conclusively prove the clonality of the cloned T cells, it does offer an indirect way of understanding the nature of the specificities within the T cell clone populations. As shown in (Table 13), treatment of 2 clones with a tolerizing dose (400 µg/ml) of CTA and subsequently challenged with an optimal dose (50 µg/ml) of the respective CTA did not result in IFN production. This finding suggests that only one cell population is recognizing a common or shared antigenic determinants on the CTA.

The tolerization protocol also markedly inhibited the PHA-induced IFN production by the T cell clones. It is possible that the high dose CTA may be toxic to the cells and thus, inhibition of the IFN production by the clones may not represent a genuine antigen specificity. To rule out that possibility, cloned allo-specific

TABLE 13

EFFECT OF HIGH DOSE TOLERIZATION ON THE REACTIVITY OF T CELL CLONES TO CONNECTIVE TISSUE ANTIGENS (CTA)

| RASFA8 | CHALLENGED WITH : | | | | | |
|---|---|---|---|---|---|---|
| TOLERIZED WITH : | NI | DI | NII | DII | PG | PHA |
| MEDIUM | 5 | 20 | 20 | 80 | 2.5 | 160* |
| NI | 0 | 0 | 0 | 0 | 0 | 2.5 |
| DI | 0 | 0 | 0 | 0 | 0 | 0 |
| NII | 0 | 0 | 0 | 0 | 0 | 1.25 |
| DII | 0 | 0 | 0 | 0 | 0 | 0 |

| RASFA10 | CHALLENGED WITH : | | | | | |
|---|---|---|---|---|---|---|
| TOLERIZED WITH : | NI | DI | NII | DII | PG | PHA |
| MEDIUM | 10 | 40 | 20 | 80 | 5 | 160 |
| NI | 0 | 0 | 0 | 0 | 0 | 1.25 |
| DI | 0 | 0 | 0 | 0 | 0 | 0 |
| NII | 0 | 0 | 0 | 0 | 0 | 1.25 |
| DII | 0 | 0 | 0 | 0 | 0 | 0 |

T cell clones ($5 \times 10^4$/ 200ul) were cultured with or without 400ug/ml of CTA for 24 hrs and subsequently challenged with 50ug/ml of the respective CTA for a further 24 hrs. The supernatants were harvested and tested for IFN levels.

* = Units of IFN/ml

collagen, was stimulated with the same high dose of DII collagen in the presence of PHA or PHA alone and the supernatants tested for IFN activity. (Table 14) shows that tolerization of the cell line with high dose DII collagen, did not affect the ability of the clone to respond to PHA; the same level of IFN being produced as in the control. This finding strongly suggest that inhibition of the PHA responses of the T cell clones by high dose CTA was not due to toxic effects, but rather may be due to the modulation off or blockade of the part of the antigen receptor complex recognized by PHA.

This polyspecificity of responses to CTA is not a property of all IL-2 responsive T cells, in that, allo-specific T cell clones, generated in response to an EBV cell line, had no reactivity to CTA, while normal uncloned LAK cells expressed low levels of reactivity (Table 15). To establish conclusively that the clones do not react with fibronectin, the clones were either incubated with or pretreated with 1-5 mg/ml of a synthetic peptide (Gly-Arg-Gly-Glu-Ser-Pro) which comprises a cell attachment determinant of the Fn molecule for 24 hrs followed by a mixture of collagen and Fn, collagen alone or Fn for a further 24 hrs. The supernatants were collected and assayed for IFN activity. The peptide had no effect on IFN production (Fig. 11). This result suggests that Fn does not bind to Fn-like structure(s) on the clones but rather to the CTA. Furthermore, none of the clones convincingly expressed a fibronectin-binding activity by indirect immunofluorescence using purified human plasma Fn and rabbit anti-human Fn or by immunoperoxidase method using a monoclonal anti-FN AB (037, Sera Lab, UK) which recognizes a putative 150/50 KD T cell surface antigen.

TABLE 14

EFFECT OF HIGH DOSE DII COLLAGEN TOLERIZATION OF ALLO - SPECIFIC T CELL CLONE RESPONSE TO PHA

| STIMULUS | | |
|---|---|---|
| DII | PHA | IFN PRODUCTION (UNITS/ML) |
| - | - | 0 |
| + | - | 0 |
| - | + | 80 |
| + | + | 80 |

An allo-specific T cell clone, 274-1 ($5 \times 10^4$/200ul) was stimulated with PHA and DII collagen (400ug/ml) simultaneously or PHA or DII collagen alone for 24 hrs. The supernatants were then assayed for IFN activity.

- = without stimulus + = with stimulus

TABLE 15

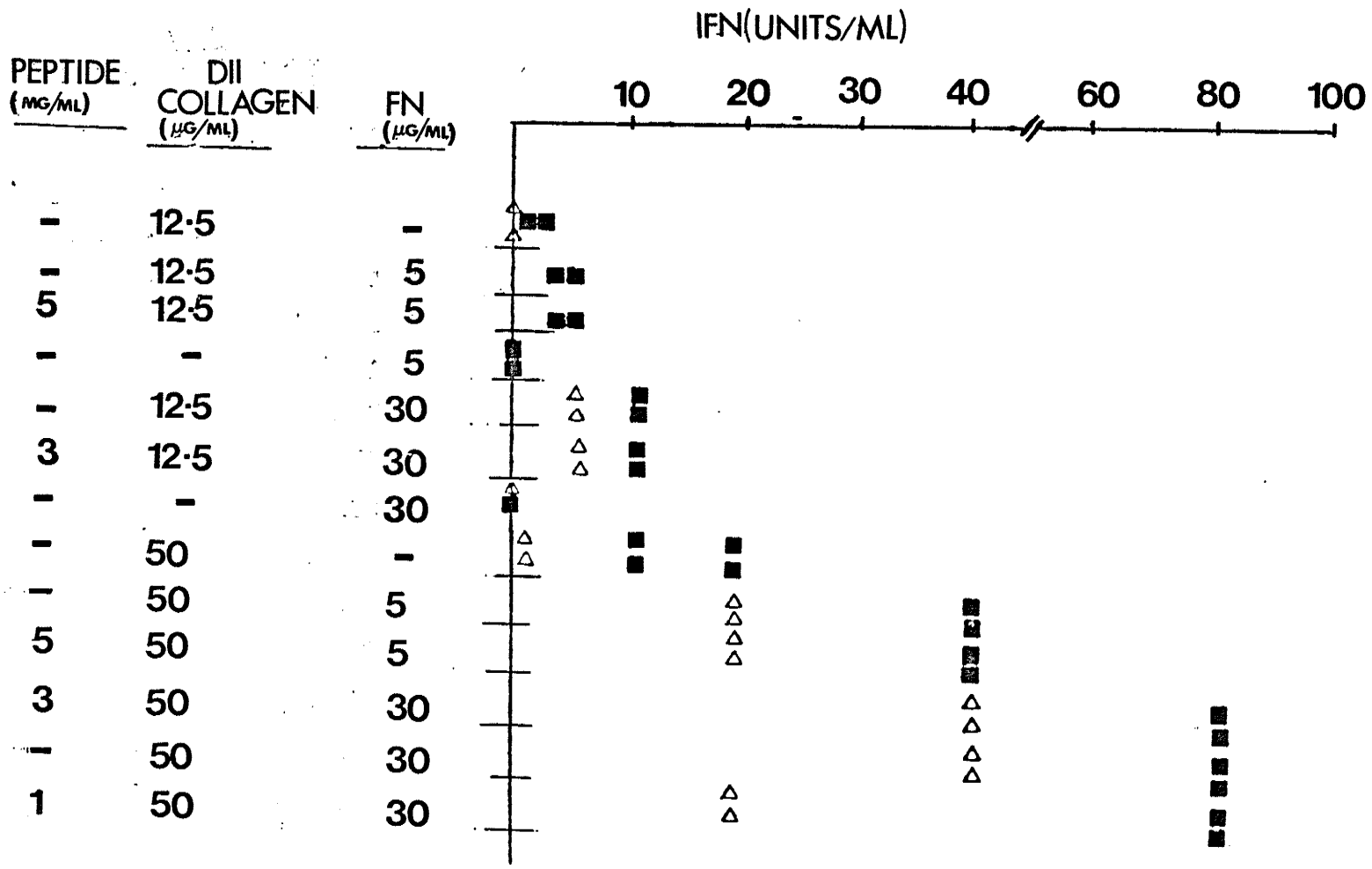
RESPONSE OF ALLO - SPECIFIC CYTOTOXIC T CELL CLONES AND NORMAL UNCLONED LYMPHOKINE-ACTIVATED KILLER CELLS (LAK CELLS) TO CTA

| | STIMULUS | | | | | | |
|---|---|---|---|---|---|---|---|
| LINES | MEDIUM | NI | DI | NII | DII | PG | PHA |
| 274-1 | 0 | 0 | 0 | 0 | 0 | 0 | 80* |
| 10B2 | 0 | 0 | 0 | 0 | 0 | 0 | 80 |
| 3D9 | 0 | 0 | 0 | 0 | 0 | 0 | 40 |
| LAK-11 | 0 | 2.5 | 2.5 | 2.5 | 2.5 | 0 | 160 |
| LAK-17 | 0 | 2.5 | 2.5 | 2.5 | 2.5 | 0 | 160 |
| LAK-18 | 0 | 2.5 | 2.5 | 2.5 | 2.5 | 0 | 160 |

T cell clones and lines were stimulated with or without PHA 90.1%) or CTA (50ug/ml) for 24 hrs, the culture supernatants harvested and assayed for IFN activity.

* = Units of IFN/ml

Figure 11: Effect Of Synthetic Peptide On DII-induced IFN Production. T cell clones were pre-incubated with 1-5mg/ml of synthetic peptide (Gly-Arg-Ser-Pro), comprising the cell attachment determinant of the Fn molecule for 24 hrs in serum-free medium containing 3% IL-2, 5 or 30µg/ml of Fn added to the cell suspension and subsequent exposure to 12.5µg/ml or 50µg/ml immobilized DII collagen. After a further 24 hrs incubation at 37°C, the supernatants were assayed for IFN activity. SF clones (■) PB clones ( △ )


IFN(UNITS/ML)
10
20
30
40
60
80
100
PEPTIDE (MG/ML)
DII COLLAGEN (µG/ML)
FN (µG/ML)
- 12·5 -
- 12·5 5
5 12·5 5
- - 5
- 12·5 30
3 12·5 30
- - 30
- 50 -
- 50 5
5 50 5
3 50 30
- 50 30
1 50 30

3:1:7 Cell attachment assay

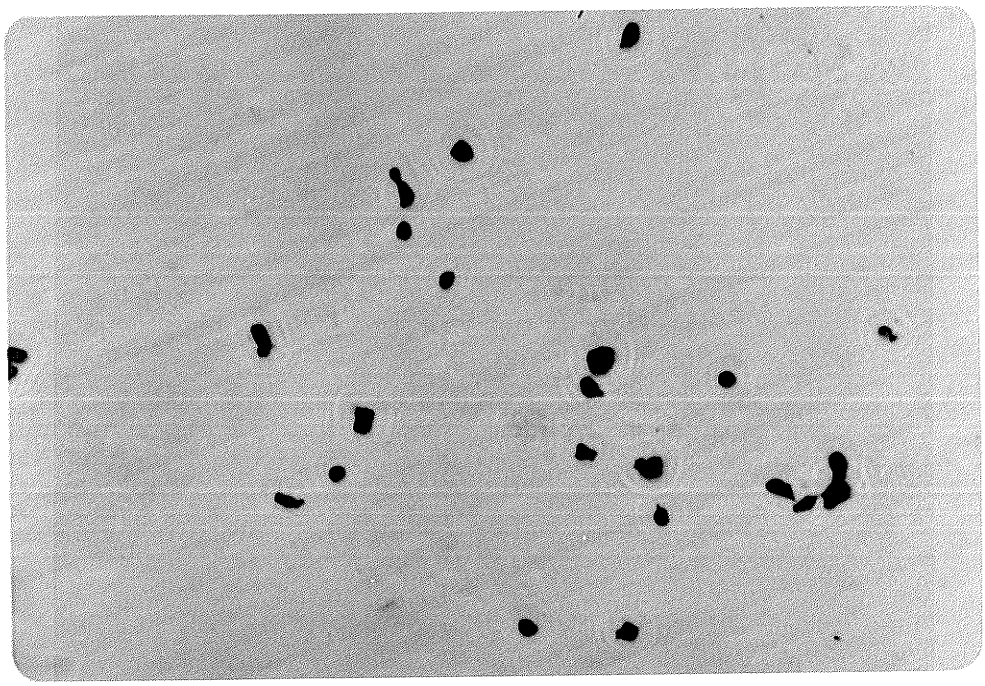
To further assess whether the clones possess a Fn-like receptor, which mediated their interaction with CTA, the clones were incubated on flat-bottomed microtitre plates (untreated for tissue culture), coated with Fn, NII collagen, NII collagen-Fn mixture and BSA as a control. As shown in (Fig. 12 A, B), a significant number of clones adhered to both NII collagen and NII collagen-Fn coated plates, with the latter showing increased level of adhesion. In contrast, Fn or BSA coated plates had little, if any, clones attached (Fig. 12 C,D) respectively.

These results strongly suggest that the clones do not possess a Fn-like receptor and that their interaction with CTA may be via a specific receptor for CTA.

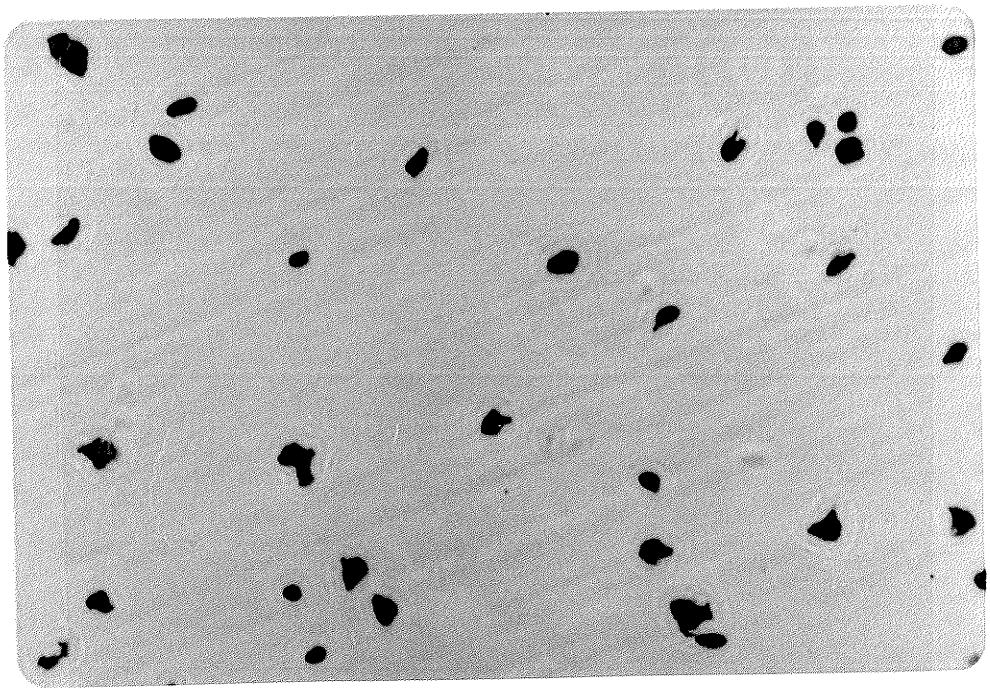
3:1:8 Proliferative Responses to CTA, Control Antigens and Synovial Fluid :

The kinetics of the CTA proliferation peaked at 96 hrs Fig. 13a, hence all the proliferation studies was assayed at 96hrs in culture.The proliferative responses to CTA was polyspecific, which is consistent with the presence of a common or shared epitopes on the CTA to which the clones recognized. The control antigens and PHA did not cause proliferation, indicating a CTA restricted response (Fig. 13b) This CTA proliferative response is unique to RA clones as it was not present in normal clones. Fresh RA PB and SF MNC also showed CTA reactivity Fig. 13c, indicating the presence of CTA-reactive cells in the starting cell population. Normal clones, however, do respond to CTA with elaboration of IFN. The lack of proliferative response to PHA was due to the kinetics of the response, the optimum being 24-48 hrs. Thus, by 96 hrs, the cells may have completed the growth cycle and returned

Figure 12: Cell attachment assay showing the binding of T cell clones to plates coated with NII Collagen (A), Collagen and Fibronectin (B), Fibronectin (C) and BSA (D) which served as control.

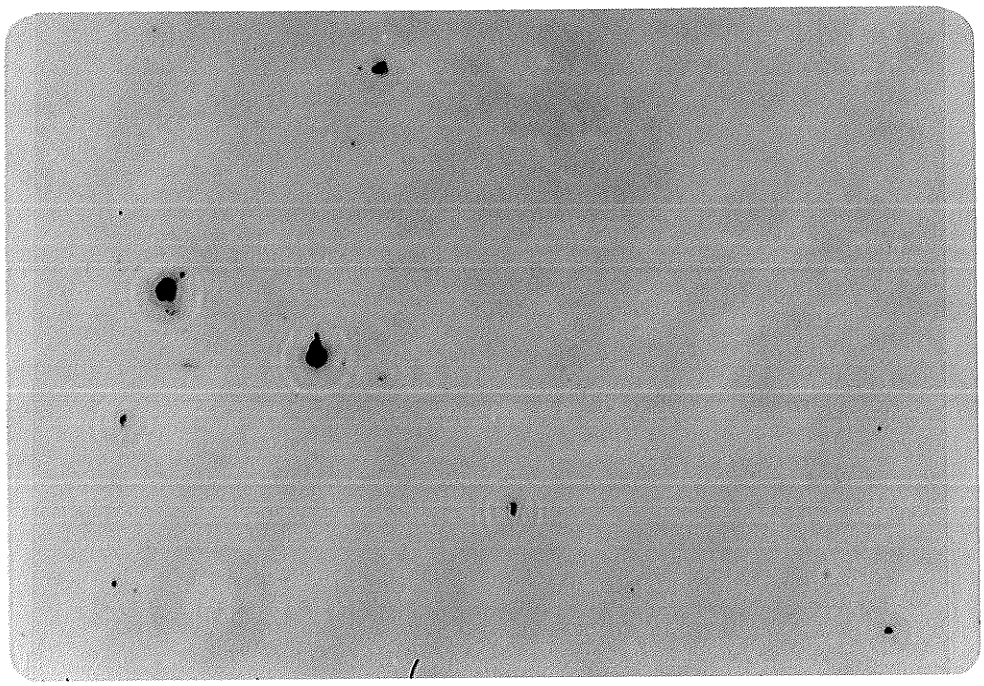


A

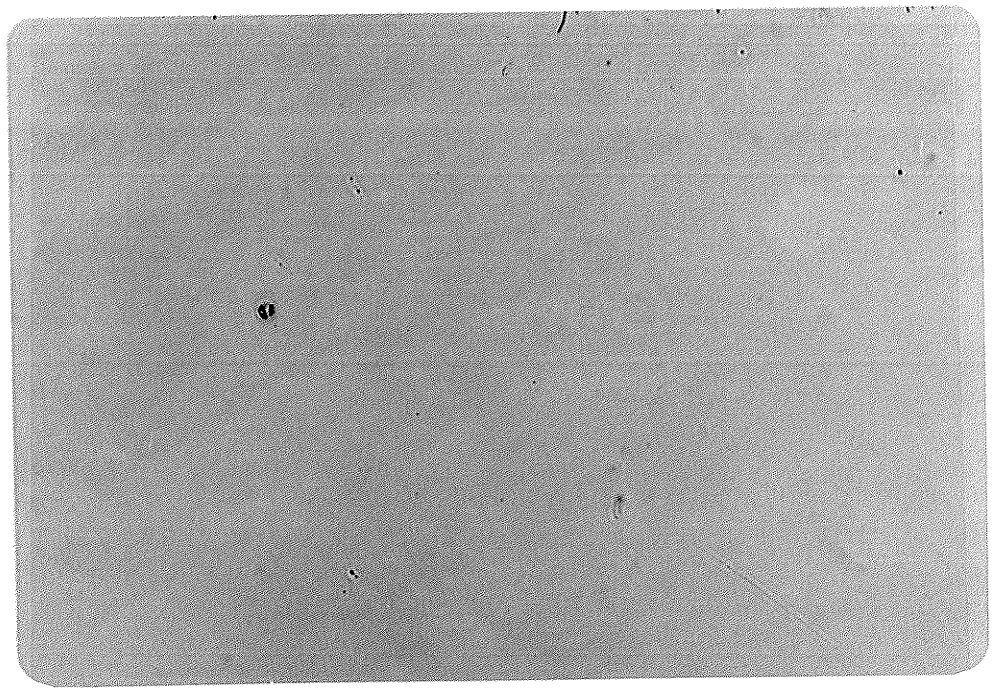


B

Figure 12: Cell attachment assay showing the binding of T cell clones to plates coated with Fibronectin (C) and BSA (D).



C



D

Figure 13a: Kinetics Of CTA Proliferation.

Triplicate cultures of T cell clones ($5 \times 10^4$) were stimulated with soluble native type II collagen in the absence of APC in 200µl total volume. The cultures were harvested after 24, 48, 72, 96, 120 and 144 hours in culture proir to labelling with 0.2µCi of $^3$H-TdR for 18 hrs. The cultures were counted and the results expressed as mean counts per minute (CPM). Controls comprised T cell clones cultured in medium alone or in 3% IL-2. RASF clone (■) RAPB Clone ( △ ). Medium control CPM was between 196-436.

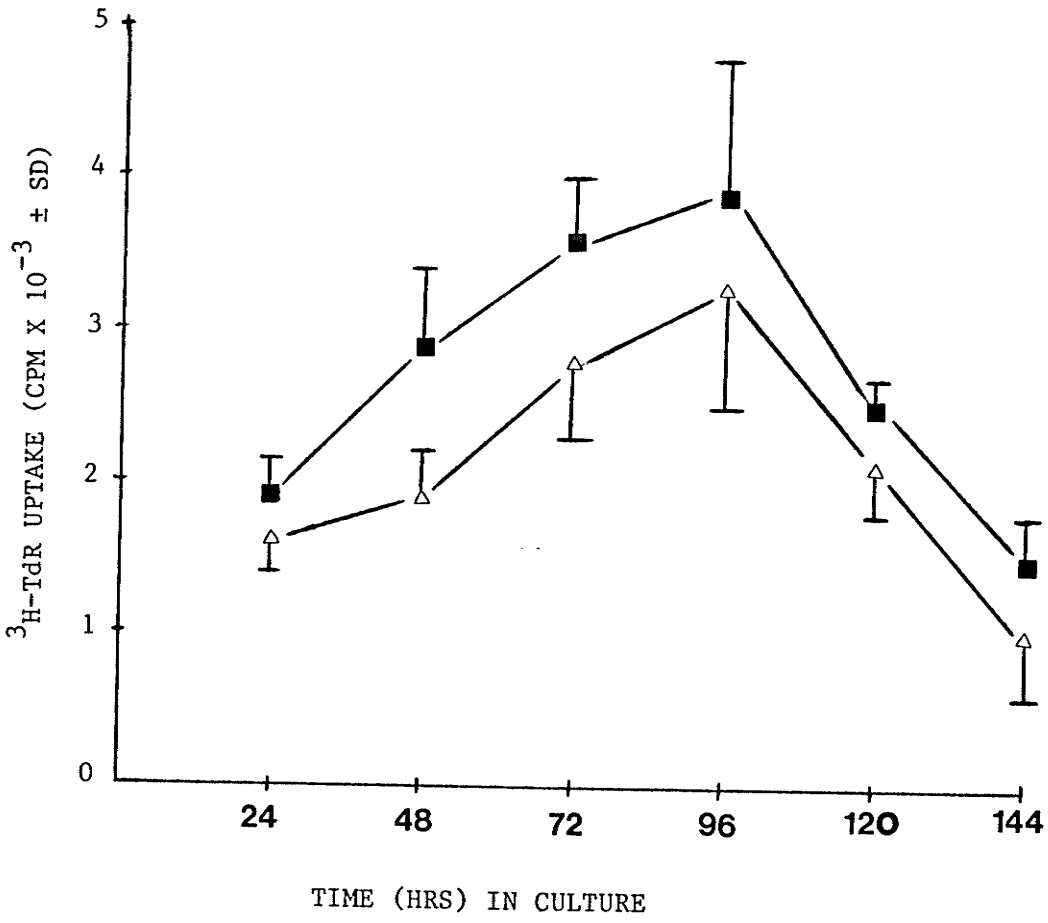

5
4
3
2
1
0
$^{3}$H-TdR UPTAKE (CPM X $10^{-3}$ ± SD)
24
48
72
96
120
144
TIME (HRS) IN CULTURE

Figure 13b: CTA-Induced Proliferation By IL-2 Dependent T Cell Clones Without (Panel A) or with (Panel B) Autologous Antigen-Presenting Cells Triplicate cultures of T cell clones ($5 \times 10^4$) were stimulated with soluble CTA in the presence of autologous irradiated (50Gy, $10^4$/well) PB MNC as source of APC or absence of APC in 200µl total volume. Mouse Ia alloantiserum, 1:50 final dilution was added to some cultures. After 96 hr incubation, the cells were pulsed-labelled with 0.2µCi of $^3$H-TdR for 18 hrs, harvested and counted. The results are expressed as mean counts per minute (CPM). Controls comprised T cell clones cultured in medium alone, normal mouse serum or 3% IL-2.
SF clones (■) RAPB clones (△) and NPB clones (●).

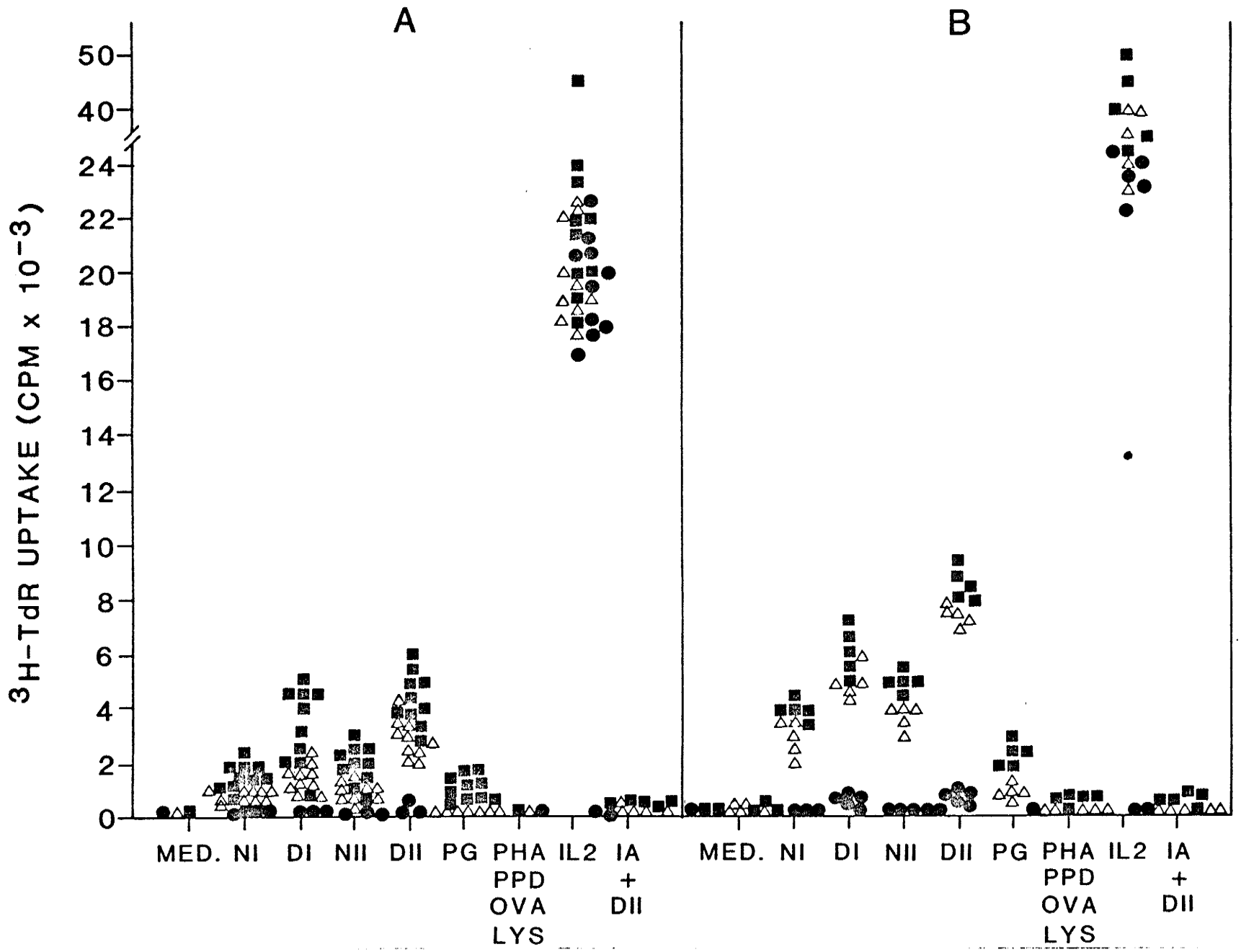
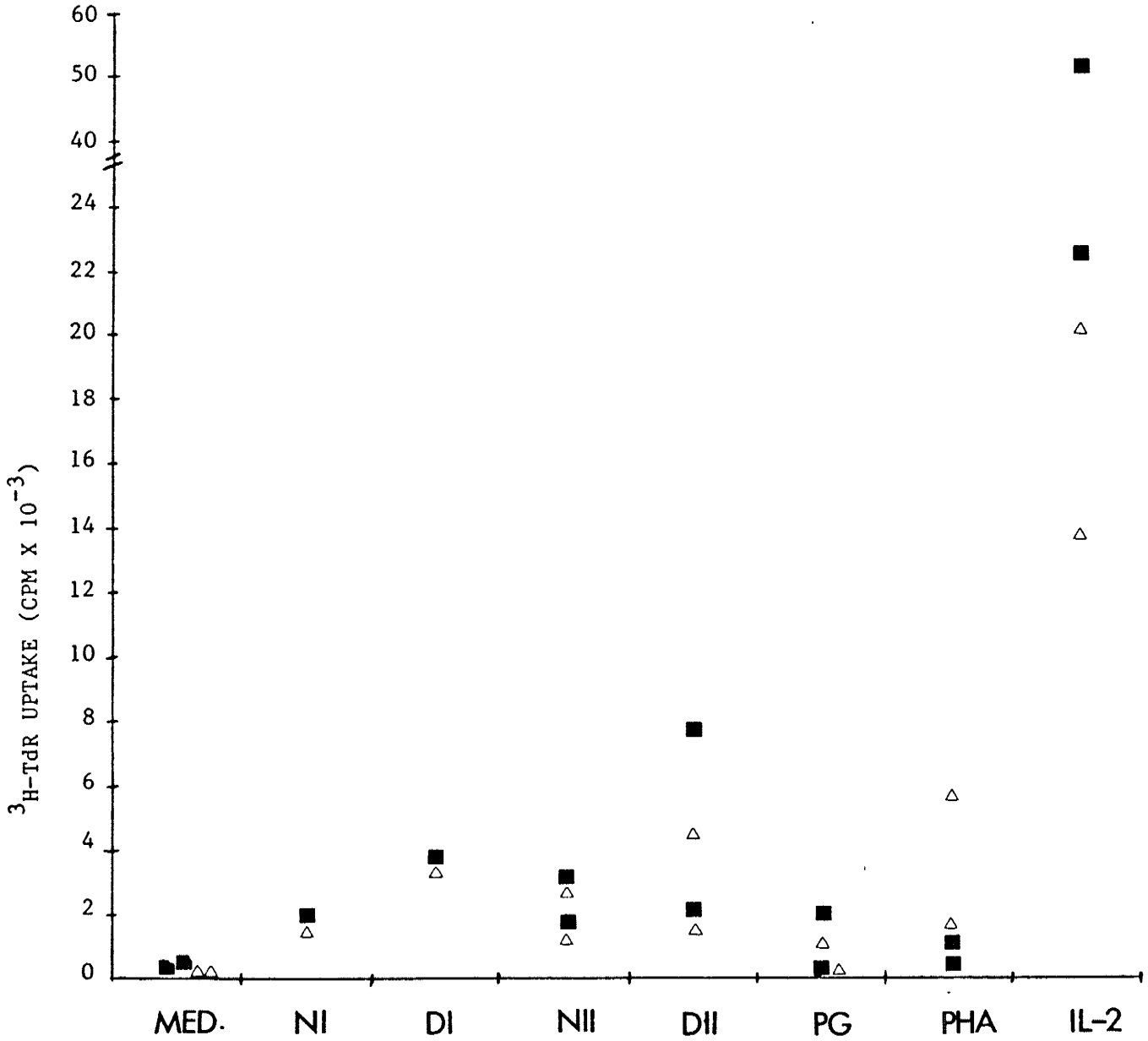

A
B
$^3$H-TdR UPTAKE (CPM x $10^{-3}$)
50
40
24
22
20
18
16
14
12
10
8
6
4
2
0
MED.
NI
DI
NII
DII
PG
PHA
PPD
OVA
LYS
IL2
IA
+
DII

Figure 13c: CTA-Induced Proliferation By Fresh RA PB And SF MNC. MNCs ($10^5$/well) were stimulated with CTA (50µg/ml) in RPMI 1640 containing 10% FCS in 200µl total volume. The cultures were incubated at 37°C for 96 hrs, pulsed-labelled with 0.2µCi of $^3$H-TdR for 18 hrs, harvested and counted. The results were expressed as mean counts per minute (CPM). Controls consisted of MNC cultured in medium alone or in 3% IL-2.

SF clone (■) RAPB clone (△)


60
50
40
24
22
20
18
16
14
12
10
8
6
4
2
0
$^{3}$H-TdR UPTAKE (CPM X $10^{-3}$)
MED.
NI
DI
NII
DII
PG
PHA
IL-2

to the resting (Go) phase (Table 16).

Interestingly, the Ia+, IL-2 responsive T cell clones were able to present both native and denatured antigens to themselves to cause proliferation, but the latter antigen induced the highest proliferative responses. The ability of the activated T cell clones to stimulate proliferative responses to both native and denatured antigens was always less than that of mononuclear APC under identical conditions.

To verify that the proliferative responses to the CTA were the result of a typical antigen-induced proliferation and not a mitogenic response, the effect of anti-Ia antibody on the proliferative response to denatured type II collagen was examined. As shown in Fig. 13, the proliferative response induced by DII collagen was abrogated by anti-Ia antibody both in the presence and absence of APC. Thus, it would appear that antigen presentation by the activated T cell clones or APC is linked to the expression of class-II molecules, confirming a typical antigen-induced phenomenon. If the CTA was acting as a mitogen, then the normal clones should have proliferated. The observation that RA but not normal clones proliferated in the presence of CTA suggests that during culture, the CTA-responsive cells from RA are expandable while those from normal are not.

Proliferative Response to Autologous SF:

In order to determine whether SF contains materials that could induce T cells to proliferate, RA T cell clones were incubated with autologous, cell-free SF diluted to 5%, 10% and 20% supplemented with or without 3% IL-2 and examined for their ability to induce stimulation. The results, shown in Table 17 indicated a modest level of $^{3}$H-Tdr uptake by RA T cell clones exposed to autologous SF.

TABLE 16

KINETICS OF PHA RESPONSE OF T CELL CLONES

$^{3}$H-TdR UPTAKE (CPM X $10^{-3}$ ± SD)

TIME IN CULTURE

| | 24 HRS | | 48 HRS | | 72 HRS | | 96 HRS | |
|---|---|---|---|---|---|---|---|---|
| CLONES | MEDIUM | PHA | MEDIUM | PHA | MEDIUM | PHA | MEDIUM | PHA |
| RASFA8 | 0.8±0.5 | 4.5±0.2 | 0.5±0.02 | 10.7±0.7 | 0.2±0.03 | 2.1±0.2 | 0.5±0.4 | 0.4±0.2 |
| RASF10 | 0.7±0.1 | 5.9±0.4 | 0.4±0.01 | 13.4±0.2 | 0.1±0.01 | 2.5±0.2 | 0.2±0.01 | 0.2±0.09 |
| RASF11 | 1.1±0.02 | 7.1±0.4 | 0.7±0.06 | 14.8±1.4 | 0.2±0.02 | 2.5±0.1 | 0.1±0.08 | 0.2±0.04 |
| RAPBG3 | 0.9±0.1 | 13.2±0.6 | 0.4±0.1 | 9.5±3.5 | 0.1±0.03 | 2.3±0.5 | 0.5±0.2 | 0.4±0.1 |
| RAPBG6 | 0.8±0.05 | 11.5±0.2 | 0.3±0.05 | 10.8±2.6 | 0.2±0.06 | 1.7±0.1 | 0.08±0.05 | 0.3±0.09 |
| RAPBG8 | 0.7±0.08 | 10.3±0.8 | 0.3±0.04 | 10.5±5.6 | 0.1±0.04 | 2.2±0.3 | 0.2±0.02 | 0.5±0.2 |
| NPBC5 | 1.5±0.3 | 9.1±0.4 | 1.1±0.08 | 13.8±0.3 | 1.3±0.1 | 2.5±1.0 | 0.2±0.08 | 0.1±0.04 |
| NPBC6 | 0.7±0.08 | 9.8±1.0 | 0.7±0.04 | 16.0±1.4 | 0.3±0.06 | 3.0±0.4 | 0.1±0.01 | 0.09±0.02 |
| NPBC7 | 0.9±0.4 | 7.5±1.1 | 1.6±0.8 | 15.2±0.4 | 0.3±0.06 | 2.9±0.1 | 0.09±0.02 | 0.2±0.1 |

T cell clones (5 x $10^{4}$/200ul) were cultured in the presence of 0.1% PHA-P in round-bottomed microtitre plates. On the indicated hours of culture, the cultures were pulsed-labeled with 0.2uCi Of $^{3}$H-TdR for 6 hrs, harvested and counted. Data are expressed as Mean Counts per minute (CPM) ± standard deviation (SD) of triplicate cultures.

TABLE 17

EFFECT OF SYNOVIAL FLUIDS ON T CELL CLONES PROLIFERATION

$^{3}$H-TdR INCORPORATION (CPM X$10^{-3}$ ± SD)

| EXPT. 1: | SYNOVIAL FLUID DILUTION (% V/V) | | | | | | CONTROLS | |
|---|---|---|---|---|---|---|---|---|
| CLONES | 5% | 5%±IL-2 | 10% | 10±IL-2 | 20% | 20%±IL-2 | +IL-2 | -IL-2 |
| RASFB5 | 0.3±0.01 | 1.7±0.3 | 0.4±0.06 | 2.0±0.02 | 0.6±0.2 | 1.7±0.3 | 6.3±1.1 | 0.3±0.02 |
| RASFB6 | 0.9±0.4 | 6.3±0.09 | 0.5±0.03 | 1.4±0.8 | 1.6±0.07 | 1.8±0.3 | 8.5±0.05 | 0.3±0.01 |
| RAPBC2 | 0.2± 0.02 | 1.8±0.4 | 0.3±0.01 | 1.8±0.1 | 0.1±0.09 | 1.5±0.06 | 7.7±0.2 | 0.3±0.05 |
| RAPBC7 | 0.1±0.01 | 1.7±0.2 | 0.2±0.03 | 1.6±0.2 | 0.1±0.02 | 1.3±0.05 | 8.0±0.2 | 0.4±0.07 |
| EXPT.2 : | | | | | | | | |
| RASFC6 | 11.7±0.7 | 368±5.8 | 8.8±2.2 | 150±3.7 | 12.4±5.8 | 60.8±8.2 | 356±37 | 0.3±0.1 |
| RASFC8 | 5.6±0.2 | 277±2.5 | 10.4±3.9 | 154±10.2 | 16.2±0.9 | 0.9±0.1 | 344±4 | 0.2±0.01 |
| RAPBD1 | 3.0±0.2 | 138±17 | 2.6±0.6 | 135±20 | 2.7±0.6 | 0.3±0.04 | 252±7 | 0.3±0.1 |
| RAPBD2 | 0.6±0.06 | 150±5 | 7.1±4.9 | 138±9.5 | 3.4±1.9 | 0.9±0.08 | 267±12 | 0.2±0.1 |

T cell clones (5 X $10^{4}$/200ul) were cultured in the presence of various dilutions of autologous synovial fluid with or without IL-2 at 3% final concentration. After 48 hrs in culture, the cells were labeled with $^{3}$H-TdR and then counted. Data are expressed as Mean Counts per minute (CPM) ± standard deviation of triplicate cultures.

However, addition of exogenous IL-2 to the 5% and 10% but not 20% SF increased the $^{3}$H-Tdr uptake.

In one patient, the SF induced a high level of $^{3}$H-Tdr uptake by the autologous T cell clones. Analysis of the fluid demonstrated the presence of high levels of IL-2-like activity (66.3 units/ml). SF from other arthritides (Reiter's and reactive arthritis) did not cause increased proliferation. These results suggests that a few RA SF, in contrast to non-RA SF, can induce a modest level of proliferation by T cell clones *in vitro*; the demonstration of this response being critically dependent upon appropriate dilution of the SF. This, in turn, is indicative of the presence of inhibitor(s) in SF.

3:1:9 FAF Production by T cell Clones

PHA and CTA-stimulated T cell clones supernatants were tested for their ability to cause synovial fibroblasts to proliferate as assessed by $^{3}$H-Tdr incorporation. It can be seen in Figs. 14, 15 that supernatants obtained from SF T cell clones demonstrated a significant enhancement of fibroblast proliferation when compared to supernatants from autologous PB and normal PB clones. The control unstimulated T cell clone supernatants prepared in the absence of PHA or CTA but reconstituted with these stimuli at the end of culture as well as supernatants from clones stimulated with irrelevant antigens (PPD, OVA and LYS) did not cause proliferation. About 30% of the SF T cell clones constituitively produced the factor that caused a minimal level of fibroblast proliferation. Thus, PHA-stimulated T cell clones released a factor into the culture medium that induced fibroblast proliferation under serum-free conditions. However, for CTA-induced FAF production, the response was serum dependent. The fact that the control cultures

Figure 14: FAF Activity In Supernatants Of T Cell Clones Stimulated With PHA. Tcell clones were stimulated with 0.1% PHA for 24 hrs in serum-free RPMI 1640 medium. The cell-free supernatants were dialyzed against RPMI 1640, filter sterilized, diluted 1:4 and assayed for their capacity to support synovial fibroblast proliferation. The control supernatants reconstituted with PHA and were also dialyzed before tested.

SF clones (■) RAPB clones (△) and NPB clones (●).

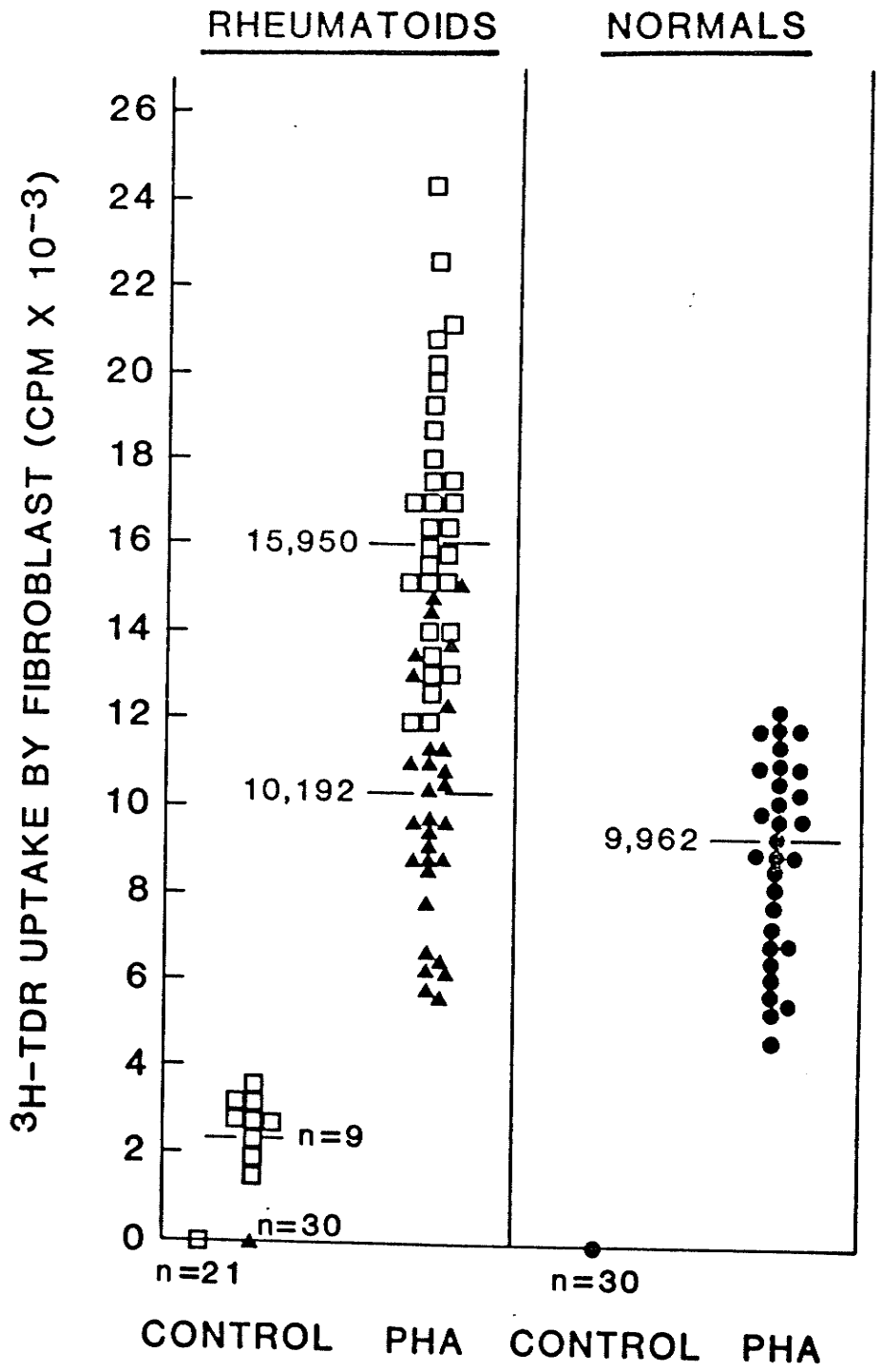
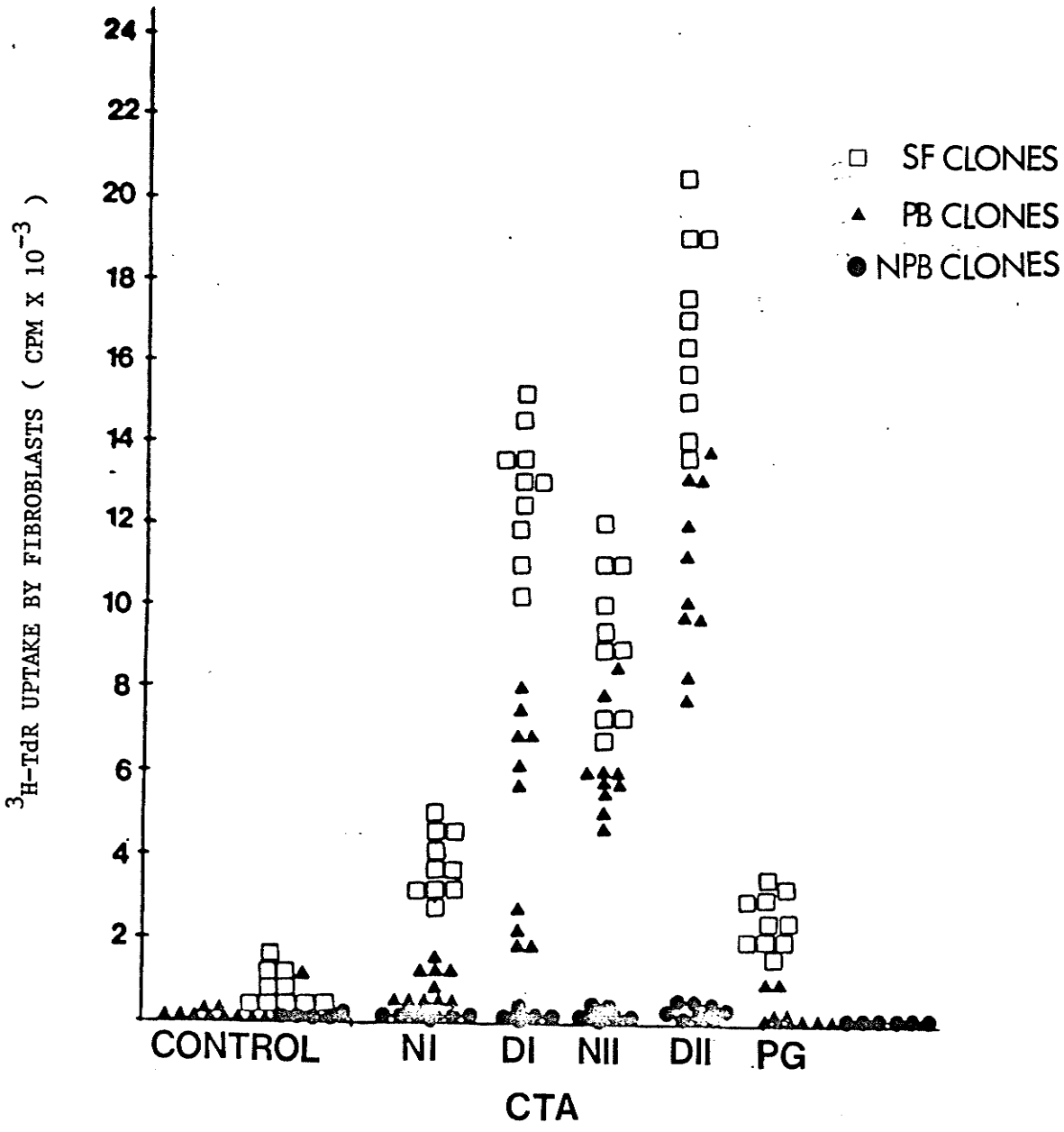

RHEUMATOIDS
NORMALS
$^3$H-TDR UPTAKE BY FIBROBLAST (CPM X 10$^{-3}$)
26
24
22
20
18
16
14
12
10
8
6
4
2
0
15,950
10,192
9,962
n=9
n=30
n=21
n=30
CONTROL
PHA
CONTROL
PHA

Figure 15: FAF Activity In Supernatants Of T Cell Clones Stimulated with CTA. T cell clones were stimulated with CTA in RPMI 1640 containing 10% FCS for 24 hrs. The supernatants were collected, dialyzed against RPMI 1640, diluted 1:4 and aasayed for their ability to induce synovial fibroblast proliferation. Controls (Medium alone, Medium + NI, DI, NII, DII and PG) dialyzed against RPMI 1640 gave CPM values between 20 - 134.


$^{3}$H-TdR UPTAKE BY FIBROBLASTS ( CPM X $10^{-3}$ )
24
22
20
18
16
14
12
10
8
6
4
2
SF CLONES
PB CLONES
NPB CLONES
CONTROL
NI
DI
NII
DII
PG
CTA

reconstituted with PHA did not possess FAF activity indicates that the proliferative responses of the fibroblasts were not a consequense of the PHA. Furthermore, addition of PHA directly to the fibroblasts did not cause proliferation.

Both dialyzed and non-dialyzed supernatants induced fibroblast proliferation *in vitro* but dialyzed supernatants stimulated much greater increase in $^{3}$H-Tdr incorporation by the fibroblasts (Table 18). This finding indicated that serum-free PHA stimulated T cell clones supernatants contained non-dialyzable substances that stimulated fibroblast proliferation and that these supernatants also contained dialyzable material that interfered with the FAF activity. Addition of indomethacin to the test system during initiation of culture did not enhance fibroblast proliferation in the non-dialyzed supernatants (Table 19), suggesting that the inhibitor present in the non-dialyzed supernatants was not prostaglandin.

Kinetics of Fibroblast Proliferation:

After 20 hrs of incubation in the absence of serum, fibroblasts were exposed to a 1:4 dilution of the T cell clone supernatants and cultured for 24 to 96 hrs at 37$^{o}$ in 5% $CO_2$ and subsequently pulsed labelled with $^{3}$H-Tdr (Fig. 16). Maximal fibroblast proliferation proliferation was found to occur at 72 hrs. Although the supernatants from PHA-stimulated clones induced greater fibroblast proliferation, there was significant proliferation of fibroblasts in supernatants from unstimulated control cultures suggesting that the clone spontaneously released low levels of FAF. By 96 hrs, the proliferation decreased presumably due to either depletion of the FAF, increased cell density or released of an inhibitor.

TABLE 18

EFFECT OF DIALYSIS OF T CELL CLONE SUPERNATANT ON PROLIFERATION OF SYNOVIAL FIBROBLASTS

| CONDITION | FINAL CONC.(%) | $^{3}$H-TdR UPTAKE (CPM±SD) |
|---|---|---|
| MEDIUM | - | 161 ± 60 |
| NON-DIALYZED SUPERNATANT | 10 | 1883 ± 97 |
| | 25 | 5074 ± 228 |
| | 50 | 904 ± 69 |
| DIALYZED SUPERNATANT | 10 | 8520 ± 170 |
| | 25 | 11507 ± 672 |
| | 50 | 2071 ± 211 |

T cell clone(RASF) was stimulated with 0.1% PHA-P in RPMI1640 containing 10% FCS for 24 hrs. The supernatant was divided into two 1 ml aliquots. One aliquot was dialyzed against RPMI 1640 for 24 hrs and the other aliquot served as control. Both supernatants were filter sterilized and tested at 10%, 25% and 50% final concentration for fibroblast proliferation. The results are expressed as Mean Counts Per Minute (CPM) ± Standard Deviation (SD) of triplicate cultures.

TABLE 19

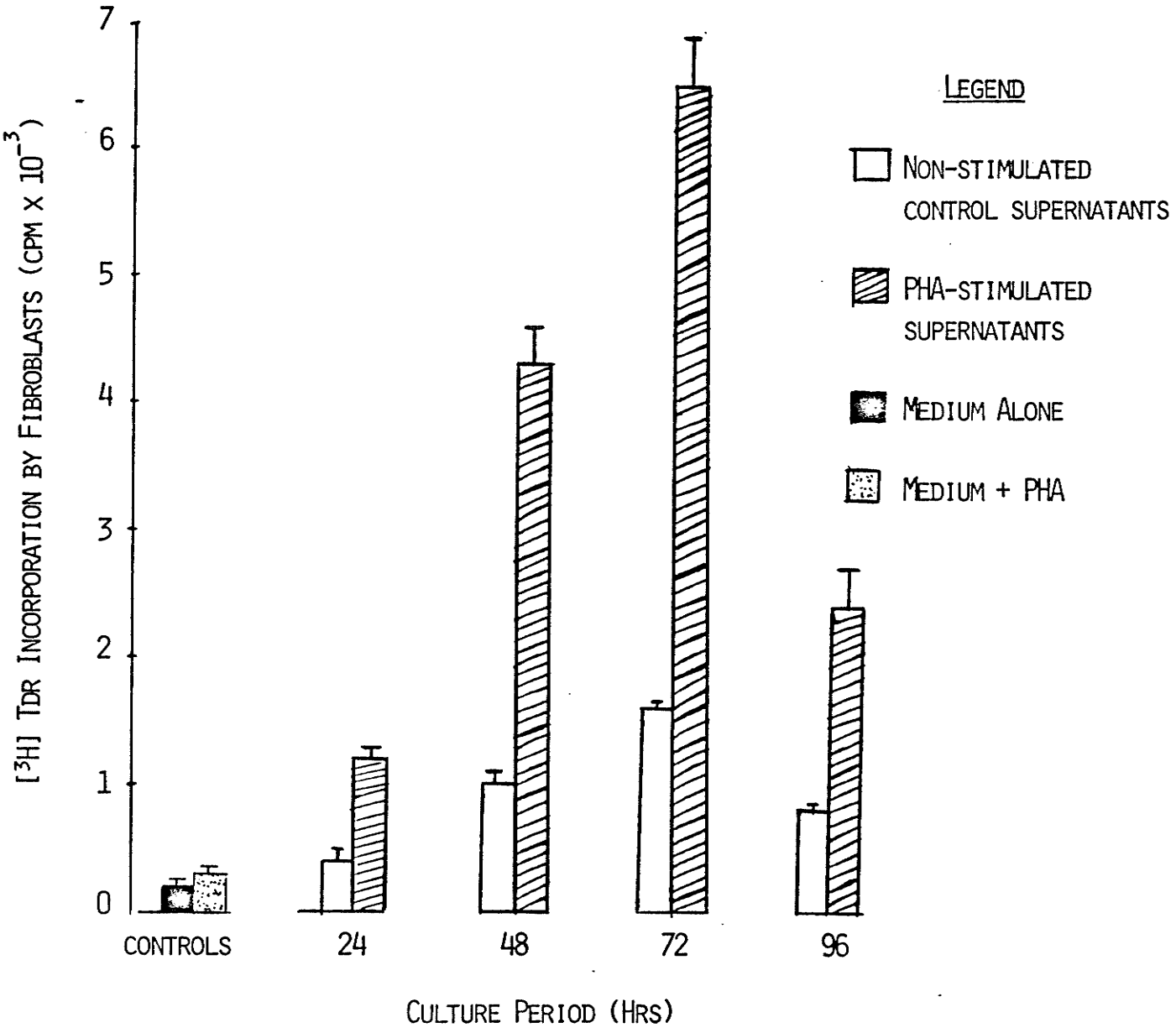
EFFECT OF INDOMETHACIN ON T CELL CLONES SUPERNATANTS ON SYNOVIAL FIBROBLASTS PROLIFERATION

$^{3}$H-TdR UPTAKE (CPM ± SD)

| CLONES | MEDIUM | SUPERNATANT ALONE | SUPERNATANT ± INDOMETHACIN |
|---|---|---|---|
| RASF | 1167 ± 123 | 11360 ± 291 | 11809 ± 808 |
| RAPB | 373 ± 31 | 10047 ± 825 | 10138 ± 831 |
| NPB | 189 ± 34 | 8744 ± 592 | 8391 ± 1873 |

T cell clones (2.5 X $10^{5}$/ml) were stimulated with 0.1% PHA-P under serum-free conditions with and without indomethacin (1ug/ml) at the initiation of cultures. After 24 hrs, the supernatants were dialyzed against RPMI 1640, filter sterilized, diluted 1:4 and tested for their ability to induce synovial fibroblast proliferation.

Figure 16: Kinetics Of The In Vitro Proliferative Response Of Fibroblasts To T Cell Supernatants. Triplicate cultures of synovial fibroblasts, 2 x $10^4$/well were treated with 1:4 dilution of undialyzed supernatant of RASF T cell clone prepared in RPMI 1640 containing 10% FCS. At 24, 48, 72 and 96 hrs, the cultures were pulsed-labeled for 18 hrs with $^3$H-TdR, trypsinized and harvested. The amount of radioactivity incorporated by the fibroblasts was assessed by liquid scintillation spectroscopy. Control samples (Medium + 10% FCS and Medium + PHA) were also diluted 1:4 and assayed for their effect on fibroblast proliferation. Data represent mean CPM ± SD.


7
6
5
4
3
2
1
0
[3H] TdR Incorporation by Fibroblasts (cpm x 10-3)
CONTROLS
24
48
72
96
Culture Period (Hrs)
Legend
Non-stimulated control supernatants
PHA-stimulated supernatants
Medium Alone
Medium + PHA

Cell Density Studies:

The effect of cell density on the proliferative response is shown in (Fig. 17). It can be seen that maximal fibroblast proliferation occured at $2 \times 10^4$ cells/well. Fibroblasts at a concentration of $0.1 \times 10^4$ cells/well incorporated less $^3$H-Tdr. The inhibition seen at $4 \times 10^4$ cells/well could be due to overcrowding. Thus, cell density is critical in the fibroblast proliferative response.

Mechanism of Suppression:

Viability assays were performed on the fibroblasts after 96 hrs in culture, using trypan blue dye exclusion to determine if direct cytotoxicty played a role in the decreased fibroblast proliferation. The viability of fibroblasts incubated with supernatants from PHA-stimulated T cell clones was comparable to the fibroblasts incubated in unstimulated T cell clone supernatants reconstituted with PHA, being 82% and 79% respectively. Thus, the activated supernatants did not contain a cytotoxic factor which could interfere with fibroblast growth.

However, addition of 1 µg/ml of indomethacin to the stimulated supernatants and cultured on synovial fibroblasts for 96 hrs partially reversed the inhibitory effect (Table 20). In medium with indomethacin alone, no effect on fibroblast viability was seen. Thus, the fibroblast inhibition seen at 96 hrs of culture was in part, at least due to the production of prostaglandin E by the fibroblasts, which suppressed the proliferation. This is in accordance with the work of Korn, (1983) who showed that PHA-stimulated MNC supernatants exposed to fibroblasts, induced them to secrete prostaglandin E.

Figure 17: Effect Of Cell Density On Synovial Fibroblasts Proliferation. PHA-activated RAPB T cell clone supernatant prepared in RPMI 1640 +10% FCS for 24 hrs, was dialyzed against RPMI 1640 and tested at 1:4 dilution for the effect on synovial fibroblasts proliferation using a fibroblast cell concentration ranging from 0.1 - 4 x $10^4$/well. The cultures were harvested after 72 hrs. Control supernatant was reconstituted with PHA, dialyzed and tested at the same dilution. The data represent the mean of triplicate cultures ± SD.

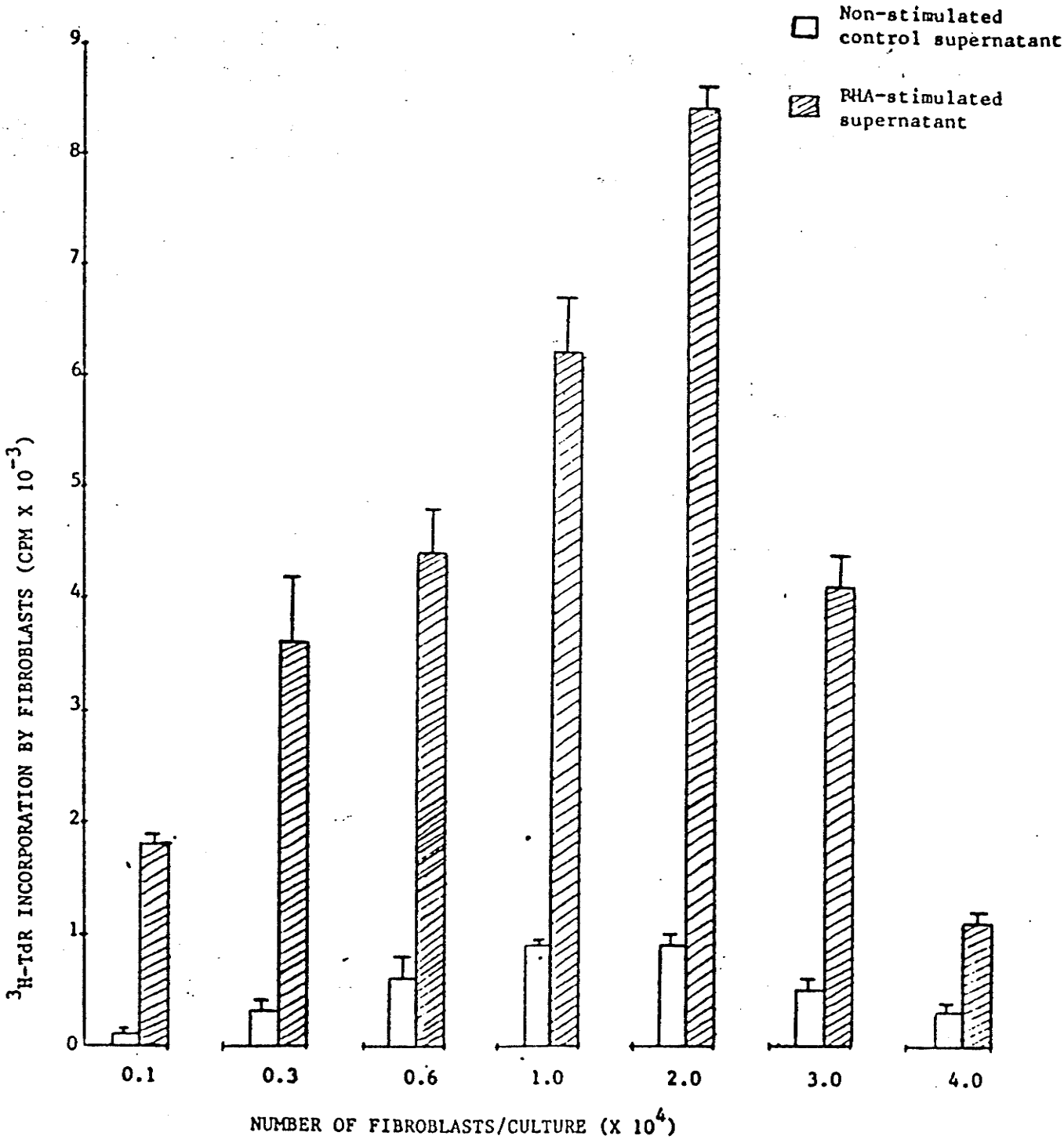

Non-stimulated control supernatant
PHA-stimulated supernatant
$^3$H-TdR INCORPORATION BY FIBROBLASTS (CPM X $10^{-3}$)
0
1
2
3
4
5
6
7
8
9
0.1
0.3
0.6
1.0
2.0
3.0
4.0
NUMBER OF FIBROBLASTS/CULTURE (X $10^4$)

TABLE 20

EFFECT OF INDOMETHACIN ON SYNOVIAL FIBROBLAST PROLIFERATION

| SUPERNATANTS | ADDITION OF INDOMETHACIN | $^{3}$H-TdR UPTAKE (CPM ± SD) |
|---|---|---|
| RASF | - | 5959 ± 33 |
| | + | 7372 ± 721 |
| RAPB | - | 4215 ± 179 |
| | + | 5497 ± 115 |
| NPB | - | 3351 ± 47 |
| | + | 4481 ± 451 |

Addition of Indomethacin (1ug/ml) to the T cell clones supernatants after 24 hrs, partially reversed their inhibitory activity after 96 hrs in culture. In this experiment, the activity of the supernatants was tested with or without indomethacin. The results are expressed as Mean counts per minute (CPM) ± standard deviation (SD) of triplicate cultures.

+ = with Indomethacin - = without indomethacin

Physicochemical Characterization of FAF found in T cell Clone Supernatants:

AcA 54 gel filtration was used to determine the molecular weight (M.W.) of the factor in the supernatants responsible for inducing synovial fibroblast proliferation. The 24 hr PHA-stimulated serum-free supernatants (5 mls) were dialyzed against PBS and concentrated 10 fold to (0.5 ml) and chromatographed. When the individual fractions were assayed on a sub-confluent, serum-free fibroblasts, a single peak of stimulatory activity was found in the M.W. region of 40 KD (Fig.18), with SF T cell clone supernatants giving the highest stimulatory activity. The T cell clones used to generate the FAF supernatants were devoid of monocytes, as determined by non-specific esterase, so the 40KD FAF was unequivocally of T cell origin. Monocytes are also capable of releasing factors causing fibroblast growth (Schmidt et al, 1982).

Heat and pH (Acid) Stability Studies:

The heat stability of the 40KD factor was determined by heating the factor at 56 $^{o}$C for 30 and 60 minutes. The heated and unheated factors were tested for their ability to induce synovial fibroblast proliferation. As can be seen in (Table 21), heated and unheated factors from both SF and PB clones retained their activities but after 60 minutes there was a slight drop in activity. The factor also showed stability at pH 2.0.

Trypsin-Sensitivity Studies:

To determine whether the factor is protein in nature, the factors were exposed to immobilized trypsin and then assayed for FAF activity. Exposure of the factor to trypsin resulted in a marked loss of activity, thus confirming its protein-like nature (Table 21).

Figure 18: ACA 54 Elution Profile of FAF.

5 mls of RA SF and PB T cell clones PHA-stimulated supernatants were dialyzed and concentrated to 0.5 ml by ultra-filtration and applied onto ACA 54 column. 1 ml fractions were collected and tested individually at 1:4 dilution as described in materials and methods (2:2:7). The proliferative response of synovial fibroblasts (CPM) to individual fraction is shown by dots (-•-•-•-) in panels A and B for SF and PB T cell supernatants respectively. Elution positions of M.W. standards BD, blue dextran (2000 KDa), BSA, bovine serum albumin (68 KDa), OVA, ovalbumin (43 KDa), CHY A, chymotrypsinogen A (25 KDa) and CYTO C, cytochrome C (13 KDa) are shown.

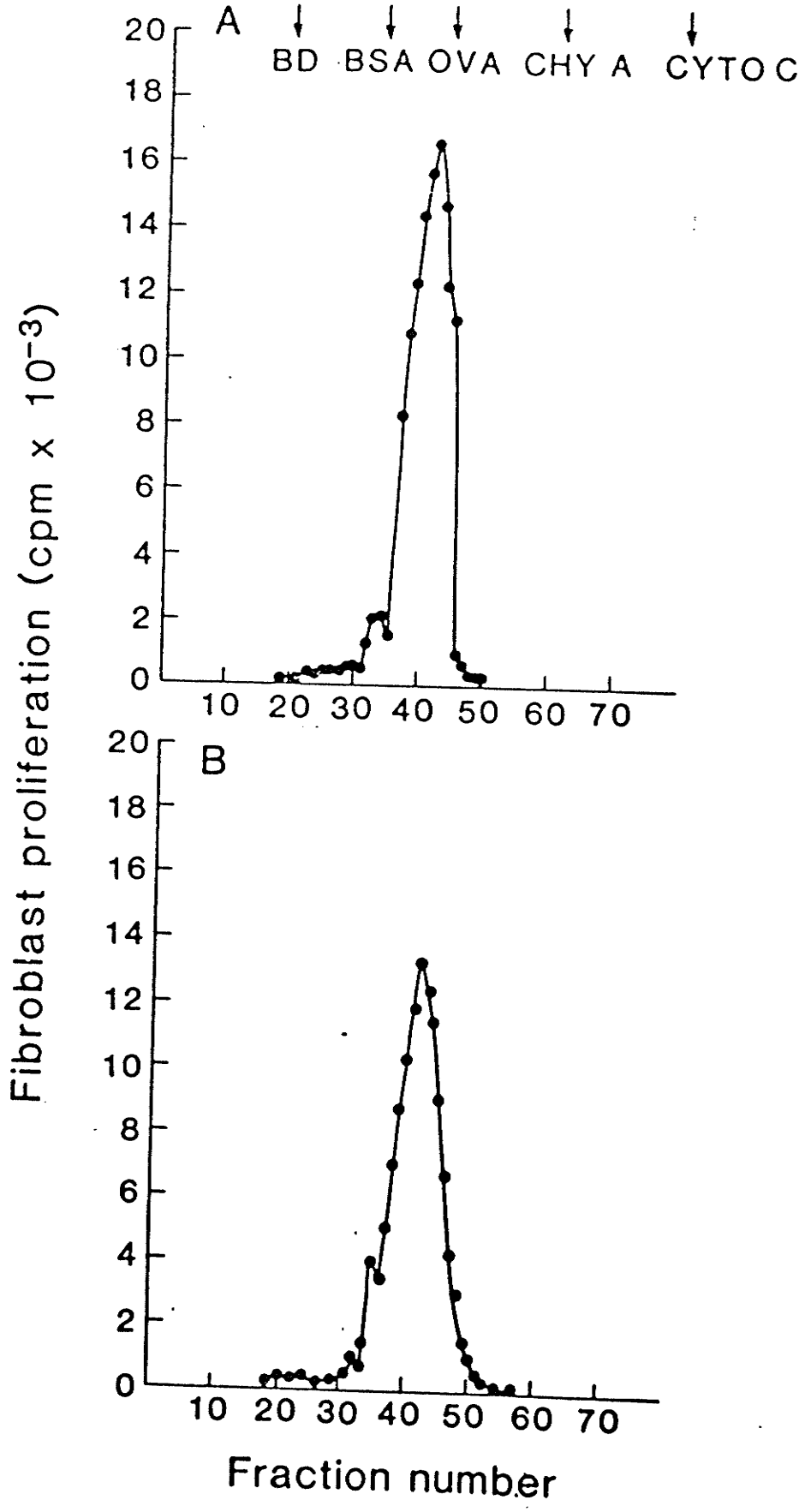

A
BD
BSA
OVA
CHY A
CYTO C
B
Fibroblast proliferation (cpm x 10⁻³)
Fraction number
0
2
4
6
8
10
12
14
16
18
20
10
20
30
40
50
60
70

TABLE 21

THE PHYSICOCHEMICAL CHARACTERIZATION OF PB AND SF T CELL CLONES INDUCED FAF

| | FAF ORIGIN | |
|---|---|---|
| | PB T CELL CLONE | SF T CELL CLONE |
| EXPT. 1 : | | |
| CONTROL | 95 ± 22 | 98 ± 17 |
| UNTREATED | 12470 ± 322 | 14746 ± 353 |
| 56°C 30 mins. | 12244 ± 132 | 14579 ± 684 |
| 56°C 60 mins. | 11430 ± 596 | 13351 ± 191 |
| EXPT.2 : | | |
| UNTREATED | 9701 ± 311 | 12142 ± 882 |
| pH 2.0 60 mins. | 9591 ± 735 | 11909 ± 715 |
| EXPT. 3 : | | |
| UNTREATED | 10332 ± 226 | 12310 ± 26 |
| AFFI-GEL 10 BEADS | 9822 ± 266 | 11927 ± 854 |
| AFFI-GEL 10-TRYPSIN | 1727 ± 56 | 2236 ± 100 |

1 ml aliquots of PHA-stimulated RAPB AND SF T cell clones fractionated peaks were pooled, concentrated(5x) and dialyzed against RPMI 1640. The fractions were either heated at 56°C for 30 and 60 mins., reduced with 6M HCL or incubated with Affi-gel 10 beads alone and Affi-gel 10-Trypsin for 16 hrs at room temperature. All the fractions were diluted 1:4 and tested for fibroblast proliferation using $^{3}$H-TdR uptake.(See materials and methods for details)

The Effects of rIFN-γ and rIL-2 on Synovial Fibroblast Proliferation:

Previous studies had shown that stimulation of the T cell clones with PHA elaborated IFN-γ and possibly IL-2. All efforts to detect IL-2 in the culture supernatants proved futile. However, the fact that the PHA can stimulate the T cell clones to proliferate under the right kinetic conditions, suggest that the clones can produce some IL-2 which may be of low levels or are being utilized by the clones for growth.

An attempt was therefore made to differentiate the FAF from these T cell lymphokines. It is conceivable that these lymphokines are either directly stimulatory for fibroblasts or synergized with the FAF to augment the proliferative responses. As shown in Table 22, neither rIFN-γ nor rIL-2 was able to induce synovial fibroblast proliferation at the concentrations tested. These concentrations were chosen because they reflected the maximal levels of these lymphokines which would be expected to be in the supernatants.

Furthermore, an anti-IFN-γ monoclonal antibody which blocked IFN-activity did not affect the FAF activity in the supernatants, suggesting that IFN-γ was not involved in the synovial fibroblast proliferative response.

Although IL-2 activity was not detectable in the supernatants, there was a possibility that very low levels of this lymphokine might synergize with the FAF to enhance fibroblast proliferation. Absorption of the FAF-containing supernatants with sufficient CTLL-2 to remove detectable levels of IL-2 activity from IL-2 containing supernatants did not influence the FAF activity (Table 22). In addition, anti-IL-1 antibody treatment of the supernatants had no effect on fibroblast proliferation (Table 23).

TABLE 22

EFFECTS OF ANTI-IFN-γ ANTIBODY, RECOMBINANT IFN-γ AND RECOMBINANT IL-2 ON SYNOVIAL FIBROBLAST PROLIFERATION

| EXPT.1 : STIMULUS | ANTI-IFN-γ ANTIBODY TREATMENT | $^{3}$H-TdR UPTAKE (CPM ± SD) |
|---|---|---|
| MEDIUM | - | 142 ± 78 |
| PHA-SUPERNATANT | - | 14660 ± 420 |
| PHA-SUPERNATANT | + (1 HR) | 14464 ± 500 |
| PHA-SUPERNATANT | + (24HR) | 13195 ± 346 |
| EXPT. 2 : | | |
| MEDIUM | - | 171 ± 70 |
| IFN-γ 1 UNIT/ML | - | 243 ± 32 |
| 10 UNITS/ML | - | 118 ± 14 |
| 20 UNITS/ML | - | 112 ± 10 |
| 100 UNITS/ML | - | 97 ± 15 |
| PHA-SUPERNATANT | - | 12591 ± 904 |
| IL-2 30 UNITS/ML | - | 137 ± 27 |
| 50 UNITS/ML | - | 97 ± 23 |
| CTLL-2 ABSORBED SUPERNATANT | - | 12170 ± 366 |

1 ml aliquot of supernatants was incubated at 37°C for 1 hr and 24 hrs with anti-IFN-γ antibody (1:2 dilution; 10000units/ml) and then tested for the ability to induce synovial fibroblast proliferation. In preliminary experiments, 1:2 dilution of the anti-IFN- antibody blocked 160 units/ml of IFN- . These supernatants contained 20 units/ml of IFN-γ . CTLL-2 ($10^5$/ml) was used to absorb IL-2 . Control supernatant containing 30 units/ml IL-2 was successfully removed by the cell concentration.

TABLE 23

EFFECT OF ANTI-IL-1 ANTIBODY ON SYNOVIAL FIBROBLAST PROLIFERATION

| STIMULUS | ANTI-IL-1 ANTIBODY TREATMENT [a] | $^{3}$H-TdR UPTAKE (CPM ± SD)[b] |
|---|---|---|
| MEDIUM | - | 700 ± 249 |
| PHA-SUPERNATANT(SF) | - | 10307 ± 1818 |
| PHA-SUPERNATANT(SF) | + | 10995 ± 1439 |
| MEDIUM | - | 325 ± 18 |
| PHA-SUPERNATANT(PB) | - | 6972 ± 597 |
| PHA-SUPERNATANT(PB) | + | 7140 ± 509 |
| MEDIUM | + | 300 ± 100 |

[a] SF and PB T cell clone supernatants were treated with or without anti-IL-1 antibody (Genzyme, Boston) at a final dilution of 1:2 ($10^4$ units/ml) and the suspension incubated at 37°C for 24 hrs. The supernatants were diluted 1:4 and assayed for their ability to induce synovial fibroblast proliferation.

[b] Counts per minute ± standard deviation

The anti-IL-1 antibody inhibited IL-1 induced proliferation of LBRM-33 cells indicating that the antibody works.

## DISCUSSION

IFN-γ is a potent mediator that is thought to play a major role in immune processes by virtue of its ability to regulate a variety of lymphocyte and macrophage actions. IFN-γ has been shown to enhance a variety of lymphocyte functions, including the stimulation of the expression of IL-2 receptor on T cells (Johnson and Farrar, 1983), augmentation of NK cell activity (Claeys et al., 1982) and inhibition of suppressor T cell function (Knop et al., 1982). It induces the expression of HLA-DR determinants on human monocytes (Basham and Merigan, 1983), and synovial fibrolasts (Geppert and Lipsky, 1987). It also has a stimulatory activity on synovial fibroblasts (Brinckerhoff and Guyre,1985), suggesting that IFN may play a role in the inflammatory process in RA.

The present study demonstrates that RA and normal T cell clones stimulated with PHA released similar amounts of IFN-γ. Thus, there was no qualitative differences between RA and normal T cell clones in their ability to respond to mitogenic stimulus. None of the clones constitutively produced IFN-γ which suggests that although these T cell clones can produce IFN-γ when stimulated, they are relatively quiescent when the source of the stimulus is removed. Alternatively, the clones may have switched-off spontaneous IFN-γ release during the transition from *in vivo* to *in vitro* culture.

The time course for IFN-γ release in RA T cell clones demonstrated maximum levels at 24 hrs as opposed to resting T cells which required 48-72 hrs for maximum IFN-γ production. This observation suggests that if T cells are pre-activated then, exposure to

the appropriate stimulus, release increased amounts of IFN-γ in a relatively short time. It is widely known that the major IFN-producer is CD4+ helper cell (Cunningham and Merigan, 1984; Martinez-Maza et al, 1984) However, in this study, both CD4+ and CD8+ T cell clones released similar amounts of IFN-γ . Thus, no correlation seems to exist between the cell surface phenotype of a T cell clone and the level of IFN-γ production.

Various human disorders have been described in which there have been defective IFN-γ production by peripheral blood mononuclear cells including SLE (Neighbour and Grayzel, 1981); Leprosy (Nogueira et al., 1983) which support the concept that release of IFN-γ by T cells can be used to monitor the status of CMI function in humans (Epstein and Cline, 1974).

In RA, there are reports of impaired IFN-γ production in AMLR with RA PB lymphocytes (Hasler et al., 1983), by stimulation of PB lymphocytes with mitogens (Seitz et al, 1987), after treatment of RA PB and SF lymphocytes with IL-2 (Combe et al., 1985), stimulation of SF lymphocytes with mitogens (PHA or CON A) (Stratton and Peter, 1978) and absent IFN-γ production in supernatants of RA synovium cultured explants (Chin et al., 1983). These findings suggest that defective IFN-γ production may contribute to some aspects of the pathogenesis of the disease. It has been suggested that the decreased IFN-γ production in AMLR may be due to the inhibitory effects monocytes generated prostaglandins (Hasler et al , 1983). However, (Combe et al., 1985) showed that removal of the adherent cells did not increase the IFN-γ production suggesting that excessive production of prostaglandin was not responsible for the defect observed in their

study. Thus, it appears multiple mechanisms may account for abnormalities of IFN-γ production in RA patients. The defective IFN production by RA lymphocytes may reflect the stage of the disease in that, only RA patients with highly active disease showed impaired IFN production when their lymphocytes were stimulated with mitogens (Seitz et al, 1987). Other investigators (Neighbour and Grayzel, 1981) found a normal IFN-γ production by RA PB lymphocytes. Our data are in agreement with that of the latter authors. We were unable to detect any abnormalities in the ability of RA T cells to release IFN-γ ; a finding which confirms our previous work (McKenna et al., 1987)

IFN-γ has been detected in serum and SF of RA patients (Cesario et al., 1983; Degre et al., 1983). The latter authors demonstrated that IFN-γ titres in SF were higher than serum, suggesting a local production of IFN-γ in the inflamed joint. They also found no correlation between IFN-γ titres and disease activity measured by laboratory parameters.

The increased IFN-γ titres in SF reported by Degre et al., (1983) may possibly result from interaction of sensitized T cells to an unknown antigen. Thus, the finding in this study that CTA could stimulate T cell clones in vitro to release IFN-γ , raises the possibility of such interaction occurring in vivo. Overall, a low level of IFN- was released by normal T cell clones on exposure to CTA while RA T cell clones released increased amounts. The demonstration of reactivity to CTA in normal subjects was not surprising since collagen reactivity has been described in normal subjects and may be linked to the possession of the HLA-DR4 antigen (Smolen et al., 1980; Solinger et al., 1981). The pattern of CTA reactivity by the T cell

clones was rather unexpected in that, all clones, irrespective of origin, reacted polyspecifically to both soluble and immobilized CTA. There are several explanations for this observation. Firstly, this pattern of reactivity suggests that there might be a common or cross-reacting epitopes on the complex multideterminant CTA to which the clones recognize. Secondly, it is also possible that the clones were interacting with a molecule in the tissue culture system that could associate with or has affinity for CTA. Fibronectin (Fn), a large glycoprotein of plasma, tissue fluids with binding sites for cells, collagen and other CTA (Yamada and Olden, 1978; Ruoslahti et al., 1981) is a prime candidate. Thirdly, although the T cells used in this study are statistically clonal, there may be cells with two specificities within the clonal population. To further explore and elucidate the mechanisms and the nature of the CTA polyspecificity, experiments were conducted under serum-free conditions to exclude Fn. All responses to soluble CTA were abrogated indicating that a serum factor(s) is required for CTA-induced IFN production. The production of IFN by PHA under identical conditions, argued against the T cells not being functional. However, supplementation of the medium with Fn resulted in the full restoration of IFN production relative to that seen in the presence of serum. Fn alone failed to induce IFN production.

However, when immobilized CTA alone was used under serum-free conditions, significant levels of IFN was produced and when Fn was added increased levels of IFN was produced. Thus, with immobilized CTA, Fn served to enhance IFN production. These results suggests that immobilization of CTA displayed an array of epitopes for multipoint interaction with the clones to induce IFN production and that

presumably, Fn served to cross-link the relevant epitopes in the CTA resulting in either a complex with a higher avidity or conformational change in the CTA molecule, leading to a stronger stimulatory signal.

Recenty, a 150/55 kd labile antigen complex which is detectable by anti-Fn monoclonal antibody has been demonstrated on human T lymphocytes cultured on collagen matrix gels (Sundquist and Otteskog, 1987). Fn-receptor has been detected on a subpopulation of murine thymocytes by their ability to adhere to Fn-coated plates and inhibition of the binding by a peptide to the cell attachment determinant in the Fn molecule (Cardarelli and Perschbaker, 1983). Thus, it still remained possible that the T cell clones interacted with CTA via a Fn-like receptor. When a saturating dose of a synthetic peptide (Gly-Arg-Asp-Ser-Pro) which comprises the cell attachment determinant of the Fn molecule was added to the test system, it did not inhibit CTA-induced IFN production, suggesting that the clones did not interact with CTA via Fn receptor on their surface. Furthermore, none of the clones convincingly expressed a Fn-binding activity by indirect immunofluorescence or immunoperoxidase using purified Fn and rabbit anti-human Fn. It was demonstrated in a cell attachment assay that very few, if any, clones adhered to FN-coated plates while a significant proportion adhered to both collagen and collagen-Fn coated plates. Thus, the clones can directly bind collagen. This binding of collagen by the clones is in agreement with a recent finding by (Myers et al, 1987) who demonstrated in the rat model of collagen-induced arthritis that a population of spleen T cells, non-adherent to tissue culture plates but adherent to type II collagen, can suppress the arthritis when injected in small numbers $< 1 \times 10^6$ /animal, suggesting that active

cell-mediated mechanisms might be responsible for the antigen-specific suppression of collagen-induced arthritis. The wealth of data in the literature indicate that T cells do not express Fn-binding activity using either immobilized Fn (Bianco, 1983) or soluble Fn (Lause et al, 1984; Cseh et al, 1985; Klingerman et al, 1986), although very recent studies indicate that fresh or Con A and/or TPA blast T cells do adhere to immobilized substratum (Kurki et al, 1987). These descrepancies may reflect subtle differences in epitopes of the cell attachment domain of the human plasma fibronectin molecule since all these studies utilized human plasma fibronectin. To determine whether the T cell clones reactivity to CTA is mediated by the classical (i.e. antigen-receptor) pathway or alternative (i.e. non-antigen receptor) pathway, anti-CD3 and anti- TcR monoclonal antibodies were added to the culture system and resulted in the abolition of the CTA-induced IFN production suggesting that the recognition of CTA by the T cell clones may be mediated by a receptor linked to or closely associated with the CD3 antigen receptor complex.Thus, the reactivity to CTA is mediated via the T cell receptor pathway. None of the other T cell associated antigens such as CD4 and CD8 had any effect on CTA-induced IFN production. Anti-Ia antibody had a marginal (i.e. one-fold reduction in IFN titres) effect, indicating that HLA-DR antigens may play a role in the processes leading to IFN production.

Another plausible explanation for the observed CTA polyspecifity was that cells with different specificities may be present in the clonal populations. An obvious approach to answering that question would be to subclone the cells at 0.3 cells/well. However, all attempts to subclone at that cell concentration failed. An indirect

approach was therefore used to resolve the issue, based on the premise that if there are cells with different specificities in the clonal population, then tolerisation with one specific antigen should not affect the response to another specific antigen. If, on the other hand, there are cells with one specificity to the antigen, then tolerisation with one antigen would lead to unresponsiveness to the other antigen. Indeed, we found that tolerisation of the T cell clones to one CTA, abolished all responses to other CTA, suggesting that there may be one cell population reacting with CTA. Since the tolerisation protocol markedly affected PHA-induced IFN production, it is conceivable that the CTA was toxic to the T cell clones. When the viability of the T cells were examined using Trypan blue dye exclusion, all the cells were viable. Furthermore, when allo-specific T cell clones, which do not respond to CTA, were tolerised with CTA and challenged with PHA, the same level of IFN was produced when compared to their untolerised counterparts. This suggested that the diminished PHA responses of the RA CTA-responsive T cell clones after tolerization was not due to toxic effects but rather due to the modulation of the CD3 portion of the antigen receptor complex recognized by PHA (Fleischer, 1984).

The findings reported here suggest that T cells that are immunologically responsive to CTA are present in the peripheral blood and joint of RA patients and normal subjects. The persistent exposure of CTA either as native or denatured antigens alone or complexed with Fn to the sensitized T cells may result in increased IFN production. Fn is significantly elevated in SF as compared to plasma in RA (Scott et al., 1982; Carsons et al., 1981) and its binding to all

CTA suggest that it could be important in the pathogenesis of RA. It is possible that CTA-interactions with the sensitized T cells in the joint are of low affinity and that Fn would serve to increase the affinity thereby generating a stronger stimulus and increased IFN production. Such increased IFN-γ production within the joint, will cause increased expression of HLA-DR (Ia) antigens on synovial cells (Geppert and Lipsky, 1987). This expression of HLA-DR antigens on synovial cells would enable them to present antigens to T cells to proliferate or initiate AMLR, culminating in a heightened cellular immune processes and tissue damage. At present, we can only speculate on the role of IFN-γ in the disease, but given the plethora of activities of IFN-γ on several immune functions, it should play an important role in the pathogenesis of RA.

No IL-2 activity was detected in the culture supernatants from PHA-stimulated T cell clones. The inability to detect IL-2 in the culture supernatants of both normal and RA T cell clones was not due to production of soluble suppressor material that interfered with the IL-2 assay, because addition of optimal concentration of standard IL-2 to the supernatants gave comparable IL-2 activity relative to the standard IL-2. Very recent studies have shown that IL-2 inhibitors are secreted by human macrophages and endothelial cells (Kashiwado et al, 1987). Since the RA T cell clones proliferated in response to CTA or PHA in the absence of exogenous IL-2, it seems possible that these clones produce some IL-2 upon antigenic or mitogenic stimulation, which is rapidly utilized by the clones themselves because of their strict IL-2 dependency, in order to proliferate and thus, would not be detected in the IL-2 bioassay. Stimulation of the clones with PHA cause

the expression of mRNA for IL-2 (unpublished observation), suggesting that the clones have the potential to secrete IL-2.

It has been reported that following PMA + PHA or Con A stimulation, both CD4+ and CD8+ T cells release equivalent amounts of IL-2 (Luger et al., 1982; Meuer et al., 1982b). Indeed, we found that both normal and RA PB CD4+ and CD8+ T cell clones produced equivalent amounts of IL-2 when stimulated with PMA + PHA. In contrast, SF CD8+ T cell clones produced decreased levels of IL-2 while CD4+ clones produced IL-2 levels comparable to their PB counterparts. The PMA + PHA-induced supernatants from CD8+ SF T cell clones was not toxic to the CTLL-2, the IL-2 dependent T cell line used to measure the IL-2 activity, as assessed by Trypan blue dye exclusion. In addition, no inhibitory factor was detected when standard IL-2 was added to the supernatants. The diminished IL-2 production may not reflect a defect per se, but rather qualitative differences in cells from the two compartments in that SF CD8+ T cells grow faster and better than PB clones in long-term cultures and thus are more likely to be excessive consumers of IL-2. Irradiation (35 Gy) of the CD8+ T cell clones to reduce the utilization of the IL-2 produced, indicated that the clones produced IL-2 comparable to CD4+ clones, thus supporting the notion that SF CD8+ T cells utilize more IL-2 for growth.

IL-2 plays a central role in the regulation of CMI and clonal expansion of antigen or mitogen stimulated T cells. A number of studies have demonstrated impaired IL-2 production by both RA PB and SF T cells to mitogens (Emery et al., 1984; Combe et al., 1984; Combe et al., 1987) and also during AMLR (Pope et al., 1984; Miyasaka et al., 1984) indicating that IL-2 abnormalities may underlie the pathogenesis

of RA. In the present study, we did not detect any defect in the ability of RA T cells to secrete IL-2, but rather a normal IL-2 production. This is in agreement with our previous studies (McKenna et al., 1986), where it was shown in a cellular interleukin (CILA) assay that in active RA there was a normal or increased IL-2 production relative to that produced by inactive RA and normal controls at lower cell densities. Tan et al., (1984) also reported a normal IL-2 production in RA but they used a mouse thymocyte assay which is not specific for IL-2 and can detect IL-1 as well. Recently, Lemm and Warnatz (1986) demonstrated that RA SF T cells stimulated with Con A secreted increased levels of IL-2 as compared to autologous PBL. Thus, the conflicting data between hypo and hyperproduction of IL-2 by RA T cells may reflect differences in methodology, patients selection and disease activity (active or inactive). It is difficult to reconcile how defective IL-2 production by RA T cells could maintain autoreactive IL-2 responsive T cells in the joint. A tenable explanation would be normal or hyperproduction of IL-2 in RA. More controlled studies are urgently needed to resolve the two conflicting viewpoints on IL-2 production in RA before IL-2 therapy in modulation of the disease process can be contemplated.

IL-2-like activity has been demonstrated in the SF of RA patients (Wilkins et al., 1983; Nouri et al., 1984). This lymphokine would be important in the maintenance and proliferation of the activated T cells present in the joint. To examine this possibility, the ability of autologous SF to support the proliferation of IL-2 dependent T cell clones was studied. It was found that dilution of the SF to 5-10% but not 20% induced a moderate level of proliferation of SF T cell clones

while autologous PB showed a weak proliferation. In one patient, the SF supported both PB and SF T cell clones with the latter having the highest proliferative index. Analysis of this particular SF demonstrated a high level of IL-2-like activity (66.3 units/ml). In all these experiments, addition of exogenous IL-2 to the SF showed only a slight increase in proliferation of the T cell clones relative to that of the T cells cultured in standard IL-2 alone; suggesting the presence of an inhibitor of IL-2 in the SF. The presence of an inhibitor of IL-2 activity in SF has already been suggested by (Wilkins et al., 1983; Warrington et al., 1985). The interpretation of this finding is that SF contain a growth factor which can support the proliferation of activated T cells. SF T cell clones proliferated better than autologous PB to SF suggesting that SF T cells are less sensitive to the inhibitory material than PB, thus reinforcing the concept of qualitative differences and growth requirements between cells of the two compartments. We have recently identified and characterised the IL-2-like activity in SF and demonstrated that the activity is abrogated by anti-IL-2 monoclonal antibody indicating presence of IL-2 in SF (Warrington et al., submitted for publication). Cell-mediated autoimmunity generally plays a fundamental role in chronic inflammation. To date, no causative viral or bacterial agents are known to be directly involved in the pathogenesis of RA. One way in which inflammation could be initiated and/or subsequently perpetuated is by the development of autoimmune response to connective tissue antigens. The demonstration that injection of native type II collagen, a component of articular cartilage, induced an experimental arthritis in rats (Trentham et al., 1977) and mice (Courtenay et al.,

1980) raises the possibility that similar immune responses to cartilage or synovial components could contribute to the joint inflammation in RA. Since collagen, might be one of the autoantigens implicated in the pathogenesis of the disease and CMI to types II and III (Trentham et al., 1978), types I and III (Endler et al., 1978) collagens and proteoglycans (Glant et al., 1980) has been detected in RA, by lymphocyte transformation as well as by production of leukocyte migration inhibition factor, we tested connective tissue antigens (CTA) for their ability to stimulate T cell clones in the presence and absence of autologous accessory cells. CTA stimulated the T cell clones to proliferate, and as were the case in IFN-γ production, the responses were polyspecific. The control antigens PPD, OVA, LYS and PHA did not induce proliferation, indicating a CTA restricted response. This CTA response was unique to RA as it was not present in normal clones. The lack of proliferation of normal clones to CTA indicate that the few CTA-reactive clones from RA are expandable during culture while the CTA-reactive clones from normals are not. This suggests that with RA lymphocytes, we are detecting immunologic memory to the autoantigens that presumably developed as a result of the joint inflammation or tissue destruction. Fresh PB and SF mononuclear cells also showed CTA reactivity indicating the presence of CTA-reactive cells in the starting population. The normal T cell clones did show immune reactivity to CTA by producing IFN-γ . The lack of proliferation of normal T cell clones to CTA suggest that antigen-induced proliferation may either be a less sensitive measurement of CTA reactivity than lymphokine production or that the two processes do not necessarily reflect the same activation pathway.

Alternatively, it is also possible that functional activation of T cells, such as the release of lymphokines and proliferation may relate to the number of signals required.

Reactivity of HLA-DR4+ positive normal cells to collagen is usually detected by a lymphokine but not transformation assays (Smolen et al., 1980; Kammer et al., 1984). These results are in agreement with that of Klareskog et al, (1982) who demonstrated increased reactivities of synovial T cells against type II collagen, whiles PBL from normals had no reactivity to type II collagen as measured by antigen-induced proliferation in vitro. The lack of PHA response was due to a kinetic effect in that the optimum time of proliferation occurred at 48 hrs. Interestingly, there was no requirement for conventional APC for proliferation to occur, indicating that the Ia+ T cell clones can function as APC, being able to present antigen to themselves. The CTA polyspecificity might be due to the presence of common or cross-reacting epitopes on the complex multideterminant CTA to which the clones recognize. Alternatively, the maintenance of the clones in long-term culture in the presence of IL-2 may have resulted in specificity degradation of their T cell receptor complex, hence the T cells were not able to exhibit exquisite specificity for CTA. This degeneration of the T cell receptor specificity has been observed in both murine cytolytic (Shortman et al, 1983; Wilde et al, 1984) and human cytolytic T cells maintained on IL-2 in long-term culture. Mycoplasma contamination of the T cell clones could contribute to the polyspecificity of the CTA responses by altering the functional capabilities of such T cell clones. However, testing of the clones for mycoplasma by both fluorescent DNA staining technique and by culture,

the most sensitive and reliable method up to 4 weeks, revealed no signs of Mycoplasma infection.

These results extend previous findings on the autoantigenicity of native and denatured types I, II and III collagens (Trentham et al., 1978b; Endler et al., 1980, Smolen et al., 1980) in RA patients. The observation that immune reactivity to collagen was usually greater to denatured than native collagen (Stuart et al., 1980) is in accordance with our data and suggests that the response may develop secondarily to tissue degradation. These results indicate that immune reactivity to collagen in RA is not specific for a particular type of interstitial collagen. Since bulk PBL cultures were used in these studies, it is not known whether different lymphocytes were responding to the different types of collagen or one lymphocyte recognize and respond to a common or cross-reacting antigenic determinants on the collagens.

Although there have been several reports of specificity of CMI to the various types of collagen in animals (Trentham et al, 1978a; Stuart at al, 1979), recent studies demonstrate that collagen-immune T cells are capable of recognizing antigenic determinants shared by genetically distinct types of collagen. For example, MacKel et al, (1982) showed that mice sensitized to native or denatured type I collagen mount a significant delayed type hypersensitivity (DTH) to challenge with native or denatured type I collagen as well as native type IV collagen (derived from basement membrane). Similarly, Champion and Poole, (1982) also demonstrated that rabbits immunized with type III collagen or after partial medial menisectomy mount CMI to to types I and II collagens as well as their peptides which they attributed to cross-reaction with type III peptides. It has been documented that most

macromolecules display an array of epitopes that potentially can be recognized by T cells. Moreover, the same or very similar epitopes may be displayed by apparently unrelated macromolecules. Thus, epitope-specific cross-reactivity may occur between the same or different molecules in similar or different tissues of the same individual, different individuals of the same species and even different species (Rowley and Jenkin, 1962). Thus, T cells may recognize the same epitope or dissimilar molecules. Indeed, work by Sredni and Schwartz, (1980) has shown that antigen-specific T cell clones exhibit cross-reactivity and can be activated by apparently dissimilar antigenic determinants. Furthermore, Van Eden et al, (1985) have demonstrated that T cell clones derived from adjuvant arthritic rats (AA), cross-react both in vitro and in vivo with components of purified protein derivative of Mycobacterium tuberculosis and certain components of joints such as chondroitin sulfate, proteoglycan extracted from pig and rat cartilage. These experiments indicate that epitope-specific cross-reactive autoimmunity resulting from molecular mimicry may underlie the immunopathology of AA. It is also conceivable that the IL-2 responsive T cells derived from the joints of RA patients may be analogous to the AA model, whereby such T cells clones have the capacity to recognize and respond to cross-reactive epitopes on the various CTA. It could be such small functionally active cell subsets in the joint that initiate and perpetuate the chronic inflammatory process. There is an emerging consensus among the investigators of the immunology of collagen, as reviewed by Stuart et al, (1984) that, at least in the mouse model of arthritis, the immune responses to collagen are highly cross-reactive with other collagens, leading to the generation and expansion of

collagen-reactive T cells that lack species or collagen type specificity, i.e cells from mice immunized with either native or denatured chick type II collagen respond equally well to chick native or denatured type II collagen, bovine type II collagen and rat type I collagen. No cells have been identified that react exclusively with native type II collagen. The T cell clones were able to present denatured collagens better than native collagens. Denaturation of native collagen may mimic antigen processing in that sites on the molecule may be unmasked that were previously hidden on the native collagen. Indeed, denatured antigen has been shown to by-pass the need for processing for antigen presentation for some T cell clones (Allen and Unanue, 1984). Conformational changes in antigens as a result of denaturation, have been shown to be critical in determining the need for native antigen processing (Streicher et al, 1984). Recently, (Gerrard et al. 1986) showed that activated human T cells are able to present both denatured keyhole limpet hemocyanin (KLH) and Tetanus toxoid (TT) to themselves much more efficiently than the native forms. In the studies reported here, with both native and denatured CTA, the ability of T cell clones to stimulate proliferative responses was always less than the ability of APC under identical conditions. This finding suggests that either denaturation of CTA may only expose a limited number of epitopes to which the clones respond or that the APC can secrete additional factor(s) that the T cell clones cannot produce. This suggests that activated T cell clones can present antigen but may not be able to process antigen as efficiently as APC. Overall, the CTA reactivity of the T cell clones may be consistent with the findings of (Allen and colleagues, 1987), who examined the processing and

presentation of human fibrinogen, a large globular protein with similar M.W. to collagen, 340,000 and 300,000 KD respectively. Surprisingly, they found that fibrinogen did not need to be processed in that pre-fixed or chloroquine-treated APC could present native fibrinogen to a panel of T cell hybridomas or clones. Thus, a determinant recognized by fibrinogen-specific T cells, localized to the carboxy terminal portion of the alpha chain of native fibrinogen, did not appear to need any processing and also proteases on the surface of the fixed APC appear not to be involved in further degradation of fibrinogen. While it is difficult to prove that proteolysis does not occur during the recognition phase (e.g by proteases leaking from fixed APC or dying T cells), the results are consistent with recognition of the native fibrinogen. It is therefore, reasonable to suggest that in those studies and probably in the CTA proliferation described here that, proteases associated with T cells could be degrading the antigen. The inhibition of proliferative responses to CTA with anti-Ia antibody indicate that the response detected was a typical antigen-induced proliferation and not a mitogenic effect. Thus, it would seem that HLA-DR molecules are critically involved in antigen presentation. Indeed, Heuer and Holsch, (1985) have shown a regulatory role of T cell Ia molecules in the transmission of antigen-induced signals. Antigen presentation by activated T cells is well documented. Ben-Nun et al, (1985) showed that irradiated Ia+ T cell clones functioned effectively to stimulate Beef insulin-specific T cell clones. Similarly, Triebel et al. (1986) also demonstrated that diphtheria toxoid (DT)-specific T cell clones expressing HLA-Dr, DQ, DP and the Tac antigens can present antigen in DT-specific proliferative responses. The proliferative

responses were inhibited by pre-incubation of the T cells clones with anti-DR MABS, suggesting that the stimulatory molecules were predominantly DR molecules.

These T cells, upon activation and expression of DR antigens, could themselves present antigen thereby amplifying the inflammatory response in the joint. Studies have shown that the majority of non-lymphoid synovial cells, despite the presence of large amounts of Ia antigens on their cell surfaces are not potent inducers of T cell proliferation but rather strong suppressors of polyclonal T cell activation (Burmester et al., 1984). Thus, Ia+ T cells may complement the non-lymphoid synovial cells in the inflammatory process in the joint, possibly via a self-perpetuation of HLA-DR dependent delayed-type cell reaction as suggested by (Klareskog et al. 1982).

The T cell clones were also examined for their ability to generate mediator(s) that can modulate fibroblast growth. Fibroblast activating factor (FAF) is a cytokine, thought to play a major role in connective tissue diseases by virtue of its ability to enhance fibroblast recruitment and proliferation in vivo (Postlethwaite et al., 1976; DeLustro and LeRoy, 1982). In the rheumatoid synovium, lymphocytes, macrophages, granulocytes and fibroblasts all participate in the inflammatory process making it difficult to determine a role for soluble mediators. The cloning of IL-2 responsive T cells from the joint fluid and PB has helped in the analysis of T cell products which may contribute to the inflammatory process. These studies demonstrate that stimulation of RA T cell clones with PHA or connective tissue elements in either serum-free medium or serum containing medium results in release of a factor that induces synovial fibroblasts to

proliferate. RA SF T cell clones secreted higher levels of FAF than autologous PB and N PB clones and a few SF T cell clones spontaneously produced low levels of FAF.

The FAF has an apparent m.w. of 40,000 daltons, it is heat and acid stable, trypsin-sensitive and thus appears to be a protein. Similar characteristics have been reported for FAF produced by human T cells and the acute lymphoblastic leukemia (HSB2) T cell line (Wahl and Gately, 1983). The T cell clones used in this study also produced IFN-for which there are conflicting reports with respect to its effect on the proliferation of fibroblasts. Brinckerhoff and Guyre (1985) reported that recombinant IFN-γ (rIFN-γ ) caused both dermal and synovial fibroblasts to proliferate. However, Duncan and Berman (1985) using the same dermal fibroblasts, and the same rIFN-γ and similar doses with identical media, found an inhibitory effect. It is possible that different culture conditions may account for this discrepancy. In the first study, the fibroblasts were grown to confluency on 35 mm cluster plates while in the latter studies, microtitre plates with subconfluent cells were used.

Several lines of evidence suggest that the FAF activity in our assays is not the result of IFN-γ . Recombinant IFN-γ over a dose range of 0.1-1000 units/ml did not stimulate fibroblast proliferation. Furthermore, addition of anti-IFN-γ monoclonal antibody which has been shown to block the effect of IFN-γ in viral neutralization assays, had no suppressive effect on the proliferation of fibroblasts. In addition, the activity of FAF was not destroyed by heat (56 $^{o}$C for 1 hr) and acid (pH 2.0) treatments. IFN-γ has been shown to be heat (Epstein, 1977) and acid (Younger and Salin, 1973) labile. The lack of

synovial fibroblast proliferation by recombinant IL-2 and the demonstration of FAF activity after absorption of supernatants with CTLL-2, a murine IL-2 dependent cytotoxic T cell line, suggests that the FAF is distinct from IL-2. This failure of IL-2 to induce fibroblast proliferation is in agreement with the finding of (Schmidt et al. 1982). It was also found that the T cell supernatants did not contain detectable levels of IL-1 nor was their activity inhibited by anti-IL-1 antibody. Taken together, these findings suggest that the FAF is distinct from both IFN- and IL-2. The addition of indomethacin to the stimulated supernatants containing FAF partially restored the synovial fibroblast proliferation, suggesting that fibroblast prostaglandin synthesis may play a role in mediating the FAF's suppressive effect after 96 hrs in culture. Prostaglandins are known to be regulators of fibroblast proliferation ( Korn et al., 1980).

Recent evidence suggests that both T cells and macrophages in synovial fluid and tissues but not peripheral blood of RA patients spontaneously secrete FAF (Malone et al., 1984; Wahl et al., 1985). The spontaneous FAF production by SF T cell clones but not PB T cell clones is in accordance with the findings of (Wahl et al., 1985). In contrast to our study, where we consistently obtained enhanced FAF production with PHA, attempts to enhance FAF production with Con A and/or LPS failed, which they interpreted to mean that the mononuclear cells are already maximally activated in vivo. There are a number of possible explanations for the difference. Firstly, Con A has been shown to activate suppressor cells (Rice et al., 1979) and it is therefore possible that such activated cells and their soluble products

could inhibit the response of the cells secreting FAF. Secondly, since bulk cultures were used, it is conceivable that other regulatory cells could release mediators which would simply render the responder cells unresponsive to further stimulus. When cloned cultures are used, as in the present study, such regulatory constraints are curtailed and consequently, the cells can respond to further stimuli. It is also possible that during long-term culture of T cells, they lose their ability to spontaneously secrete FAF and may require mitogenic or antigenic stimulation for increased FAF production.

The cells spontaneously secreting FAF in synovial tissue were phenotyped as HLA-DR+, CD3+, CD4+ T cells and CD11+, CD15+ macrophage-like cells (Wahl et al., 1985). Both populations secreted mediators that promoted fibroblast growth. However, the phenotype of cells spontaneously secreting FAF in synovial fluid was not established. In previous studies (Ofosu-Appiah et al., 1986) we have reported that HLA-Dr+, CD3+, CD8+ cells were predominant in SF whereas HLA-Dr+, CD3+ and CD4+ cells were predominant in PB. In the present studies, both SF and PB CD8+ and CD4+, HLA-Dr+, CD11-, CD16- clones secreted FAF.

A number of studies have shown that activated T cells and monocytes produce factors that mediate fibroblast proliferation (DeLustro et al., 1980; Wahl and Wahl, 1981), recruitment of fibroblasts (Tsukamoto et al., 1981) and enhance collagen and proteoglycan synthesis (Castor, 1975; Johnson and Ziff, 1976). Although the mechanisms of <u>in vivo</u> activation of the mononuclear cells is unknown, it is believed that delayed-type hypersensitivity reactions are involved (Klareskog et al., 1982; Postlethwaite and Kang, 1983). In synovial fluid, mononuclear cell factor (MCF), which is probably

identical to IL-1, has been isolated (Fontana et al., 1982) and shown to enhance secretion of collagenases and prostaglandins. Thus, activated cells within the SF and ST not only generate mediators of fibroblast growth but also enhance secretion of cartilage degradation enzymes. The degradation of articular cartilage would release connective tissue antigens (CTA), which can stimulate RA mononuclear cells to secrete leukocyte inhibitory factor and thus, CTA has been implicated in the pathogenesis of RA. That CTA can stimulate CTA-responsive T cells in the joint or peripheral blood to elaborate FAF, is supported by the finding that exposure of RA T cell clones to CTA in serum containing medium released FAF which induced synovial fibroblasts to proliferate in vitro. Collagen and collagen peptides (Fisher et al., 1982) and native types I, II and III (Biswas and Dayer, 1979) can stimulate fibroblasts and adherent synovial cells to synthesize and secrete collagenase. Thus, it is conceivable that the CTA could directly stimulate the fibroblasts to proliferate without the need for FAF. However, when CTA alone were added to synovial fibroblasts, no proliferation occurred, indicating that the FAF produced by the T cell clones in response to CTA, was responsible for the fibroblast proliferation.

These observations are of potential significance in the pathogenesis of chronic inflammation in RA in that autosensitization to CTA may well exacerbate the lesion. The local production of FAF by CTA-responsive T cells could expand the fibroblast population adjacent to cell-mediated reactions. The chronic exposure of fibroblasts to activated cell products might allow for selective, uncontrolled proliferation of fibroblast populations. These selected cells, in

turn, might prove resistant to normal regulation of growth suppression. Indeed, (Korn, 1983) has shown that short term exposure of normal fibroblasts to mononuclear cell products results in the persistence of abnormal fibroblasts.

These data raise the possibility that, in vivo, this T cell-CTA interaction could release FAF and other cellular mediators that are capable of expanding the local fibroblast population and/or recruiting of fibroblasts. Thus, FAF may be important in the perpetuation of the chronic inflammation seen in RA.

## RESULTS

### Cytotoxic Activity of the Clones

#### 3:2 Frequency of Cytotoxic Precursors of in vivo activated IL-2 Responsive Cells:

The LDA assay was used to measure in parallel, the precursor frequencies of IL-2 responsive and cytotoxic cells. To estimate the precursor frequency of cytotoxic cells, individual wells from LDA cultures were split and simultaneously assayed for lytic activity against both K562 and Raji cell targets. (Table 24) show the results of such experiments. It is evident that the frequency of cytotoxic precursors was identical to the frequency of the IL-2 responsive cells, suggesting that all cells which proliferated were also cytotoxic for both K562 and Raji.

#### 3:2:1 Analysis of Specificity of Cytotoxicity by the Clones:

It was demonstrated in the LDA assay that the IL-2 responsive cytotoxic cells showed promiscuous killing of targets. This could be interpreted to mean that those cultures not at a limiting dilution, probably possess more than one cytotoxic precursor cell and that the cytotoxic

TABLE 24

CYTOTOXIC CELL AND INTERLEUKIN-2 (IL-2) RESPONSIVE PRECURSOR FREQUENCIES

| EXPERIMENT | IL-2 RESPONSIVENESS | PRECURSOR FREQUENCY FOR : K562 CYTOTOXICITY | RAJI CYTOTOXICITY |
|---|---|---|---|
| 1 RAPBL | 1/202 | 1/202 | NT |
| RASFL | 1/101 | 1/101 | NT |
| 2 RAPBL | 1/200 | 1/180 | 1/180 |
| RASFL | 1/80 | 1/70 | 1/70 |
| 3 RAPBL | 1/201 | 1/201 | 1/201 |
| RASFL | 1/60 | 1/50 | 1/50 |
| 4 RAPBL | 1/203 | 1/203 | 1/203 |
| RASFL | 1/90 | 1/80 | 1/80 |
| 5 NPBL | 1/401 | 1/360 | NT |
| 6 NPBL | 1/390 | 1/360 | 1/360 |
| 7 NPBL | 1/340 | 1/300 | 1/300 |

Individual wells from limiting dilution cultures were split and simultaneously assayed for lytic activity against both K562 and Raji cell targets. Positive wells were defined as those in which $^{51}Cr$ release exceeded three standard deviations from the mean of the release value observed for control cultures which had received only irradiated filler cells and IL-2 during the limiting dilution assay (See 2:2:5 for details)

NT = Not Tested

activities against the two targets merely reflected different clones of effector cells in the same well. To rule out that possibility, cultures were expanded from wells in which there is a greater than 95% probability of being clonal, and tested for the pattern of cytotoxicity against a variety of targets such as tumor targets (K562, Raji and Colo), autologous or allogeneic PHA-blast and fresh PBL targets. As shown in Figs.19, 20, the clones exhibited a pattern of broad reactivity. It is apparent that all clones, irrespective of origin, lysed NK sensitive K562 and NK insensitive (Raji and Colo) targets as well as autologous or allogeneic PHA-blast targets. None of the clones killed fresh PBL targets. The level of target cell killing was always greater against Raji than K562 or Colo cells. This may be a reflection of the relative sensitivities of the targets to the action of the clones in that, addition of PHA to the cytotoxic assay did not increase the level of kill against both K562 and Raji targets (Table 25), suggesting that cross-linking or "bridging" of carbohydrate moieties, resulting in target cell recognition was not the critical event in the cytotoxicity assay. To examine whether the cytotoxic activity of the clones was stable in culture, repeated cytotoxic assays were performed. Table 26 shows the levels of cytotoxicity of some representative clones tested 14-28 days after first assay. All the clones maintained the same profile of cytotoxicity as seen in the first assay i.e. higher level of cytotoxicity against Raji than K562 targets.

The ability of the clones to kill autologous or allogeneic blast cells but not fresh PBL suggest that the blast cells may express a determinant recognized by the non-MHC restricted cytotoxic cells which is not expressed or present in low numbers on fresh PBL. The moderate

Figure 19 : Cytotoxic Activity Of T Cell Clones.

A comparison of the relative cytotoxic activities of T cell clones derived from normal PBL ( ○ ● ), RAPBL ( ▲ X ) and RASFL ( □ ■ ). The clones were assayed for cytotoxicity against K562 (Panel A) or Raji (Panel B) at the indicated effector : target ratios. The standard error for the assay was less than or equal to 10% of the means in all groups.

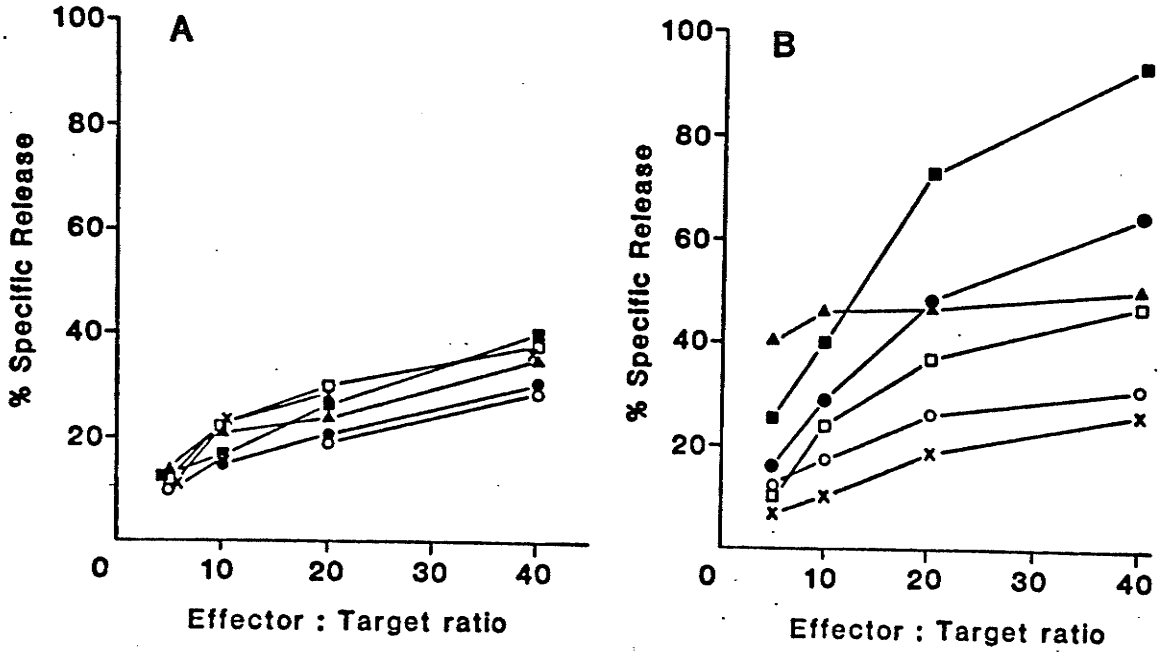

A
B
% Specific Release
Effector : Target ratio
100
80
60
40
20
0
10
20
30
40

Figure 20: Promiscuous Cytotoxic Activity Of IL-2 Dependent T Cell Clones. Comparison of cytotoxic activity of T cell clones against autologous PHA-activated PB lymphoblasts, allogeneic lymphoblasts, colo (solid tumor cell line) and fresh PBL targets. The cytotoxic assay was performed using the conventional 4 hr $^{51}Cr$-release assay. Note that clones lysed autologous PBL better than allogeneic targets but had little, if any, cytotoxicity against fresh PBL targets.

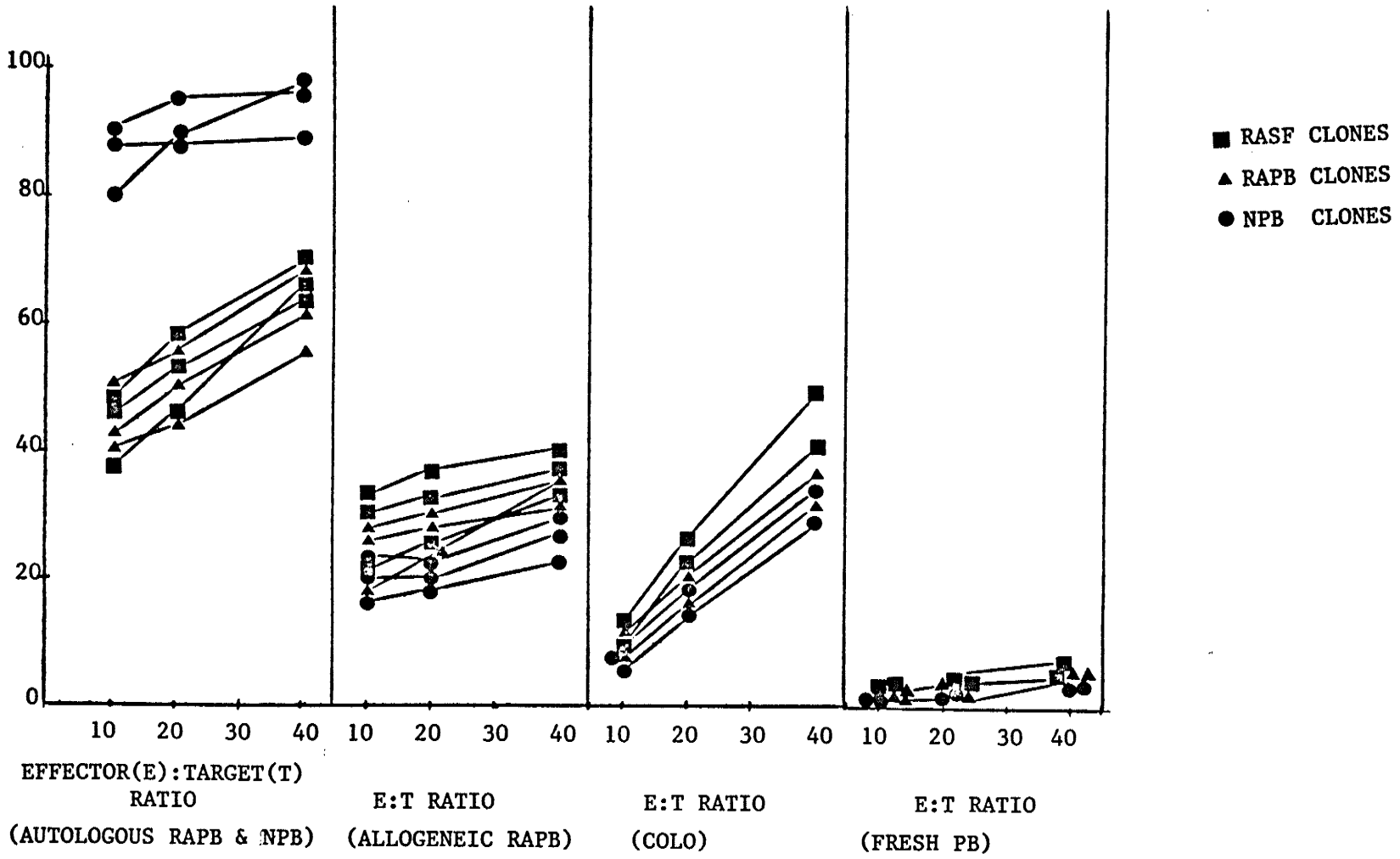

% SPECIFIC $^{51}$CR-RRLEASE
100
80
60
40
20
0
10 20 30 40 10 20 30 40 10 20 30 40 10 20 30 40
EFFECTOR(E):TARGET(T) RATIO
(AUTOLOGOUS RAPB & NPB)
E:T RATIO
(ALLOGENEIC RAPB)
E:T RATIO
(COLO)
E:T RATIO
(FRESH PB)
RASF CLONES
RAPB CLONES
NPB CLONES

TABLE 25

EFFECT OF PHA-P ON CYTOTOXIC ACTIVITIES OF T CELL CLONES

| | % SPECIFIC $^{51}$CR-RELEASE | | | |
|---|---|---|---|---|
| | EFFECTOR : TARGET RATIO = 40:1 | | | |
| | K562 | | RAJI | |
| CLONES | -PHA | +PHA | -PHA | +PHA |
| RAPBG6 | 37 | 34 | 42 | 39 |
| RAPBG8 | 35 | 32 | 50 | 47 |
| RAPBG10 | 41 | 38 | 49 | 46 |
| RASFB9 | 40 | 39 | 66 | 64 |
| RASFG4 | 38 | 36 | 47 | 44 |
| RASFG6 | 43 | 40 | 52 | 49 |
| NPBD3 | 39 | 37 | 46 | 43 |
| NPBD4 | 42 | 40 | 54 | 51 |
| NPBD5 | 34 | 32 | 65 | 62 |

PHA does not enhance cytotoxic activity of T cell clones. T cell clones were assayed for their cytotoxic activity against K562 and Raji targets in the presence or absence of 0.1% PHA-P. The PHA-P was present throughout the 4 hr-cytotoxicity assay. The results represent the mean of triplicate cultures.

TABLE 26

STABILITY OF THE CYTOTOXIC ACTIVITY OF IL-2 RESPONSIVE T CELL CLONES

| CLONES | DAYS AFTER TESTING | % SPECIFIC CYTOTOXICITY EFFECTOR : TARGET RATIO = 40:1 K562 | RAJI |
|---|---|---|---|
| RAPBG3 | 14 | 48 | 66 |
| | 21 | 37 | 49 |
| | 28 | 26 | 37 |
| RAPBG4 | 14 | 45 | 58 |
| | 21 | 33 | 49 |
| | 28 | 23 | 36 |
| RASFA8 | 14 | 41 | 68 |
| | 21 | 43 | 50 |
| | 28 | 22 | 36 |
| RASFA10 | 14 | 46 | 56 |
| | 21 | 42 | 51 |
| | 28 | 28 | 40 |
| NPBC5 | 14 | 43 | 52 |
| | 21 | 40 | 45 |
| | 28 | 25 | 39 |

T cell clones were tested for cytotoxicity against K562 and Raji targets after days 14, 21 and 28 in culture, to examine whether the clones exhibited stable cytotoxic properties. The cytotoxic assay was performed in a conventional 4 hr-$^{51}$Cr-release assay. The data represent the mean of triplicate cultures.

level of cytotoxicity against RA as opposed to OA synovial fibroblasts Fig. 21, suggest that RA T cells may be sensitized to synovial cell antigens *in vivo*. SF T cell clones showed greater lytic activity than PB clones. There was also low but significant cytotoxic activity against OA synovial cells. It is possible that, for OA synovial cells, 48 hrs incubation may not be sufficient for the re-expression of the full array of determinants recognized by the clones after trypsinization.

3:2:2 Cold Target inhibition and Specificity of Recognition of the Targets:

The broad specificity of cytotoxicity against a wide variety of targets might indicate that all the targets possessed a single, common structure recognized by the clones, or alternatively that the clones had multiple recognition structures, either one for each target or group of targets. In an attempt to discriminate between the two hypotheses, cold target inhibition experiments were carried out, using unlabelled targets as inhibitors of the lysis of the same labelled targets and effectors. It can be seen in Fig. 22 that there was a cross-inhibition of lysis by the cold targets. This finding of cross-inhibition by the cold targets suggests that the two tumor cells presented the same or similar structure(s) for recognition by the non-MHC restricted cytotoxic cells. A lack of cross-inhibition by the cold targets in this study would have suggested that multiple receptors for target structures were probably present on the clones.

3:2:3 The Role of CD3, CD4 and CD8 Antigens in Non-MHC Restricted Cytotoxic Cell Function:

It has been shown that classical cytotoxic T cells (CTL) utilize the

Figure 21: Cytotoxic Activity Of T Cell Clones Against Allogeneic RA OA (Osteoarthritic) Synovial Cells. The cytotoxicity was performed in a 4 hr $^{51}Cr$-release assay with effector to target ratios of 40:1. Each symbol represent the value for each clone.

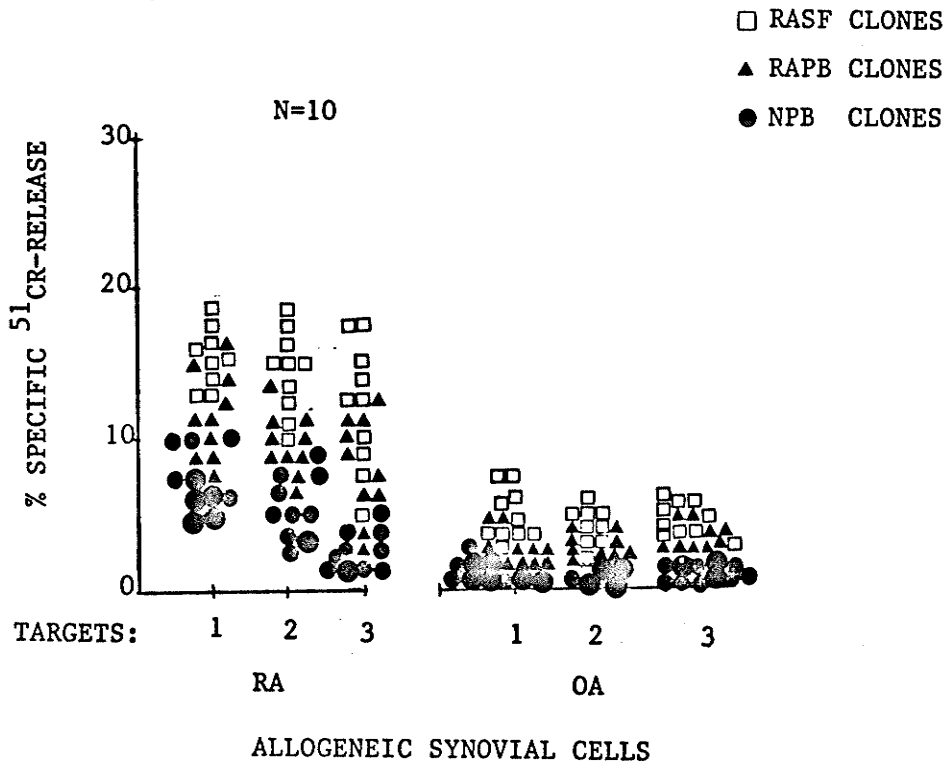
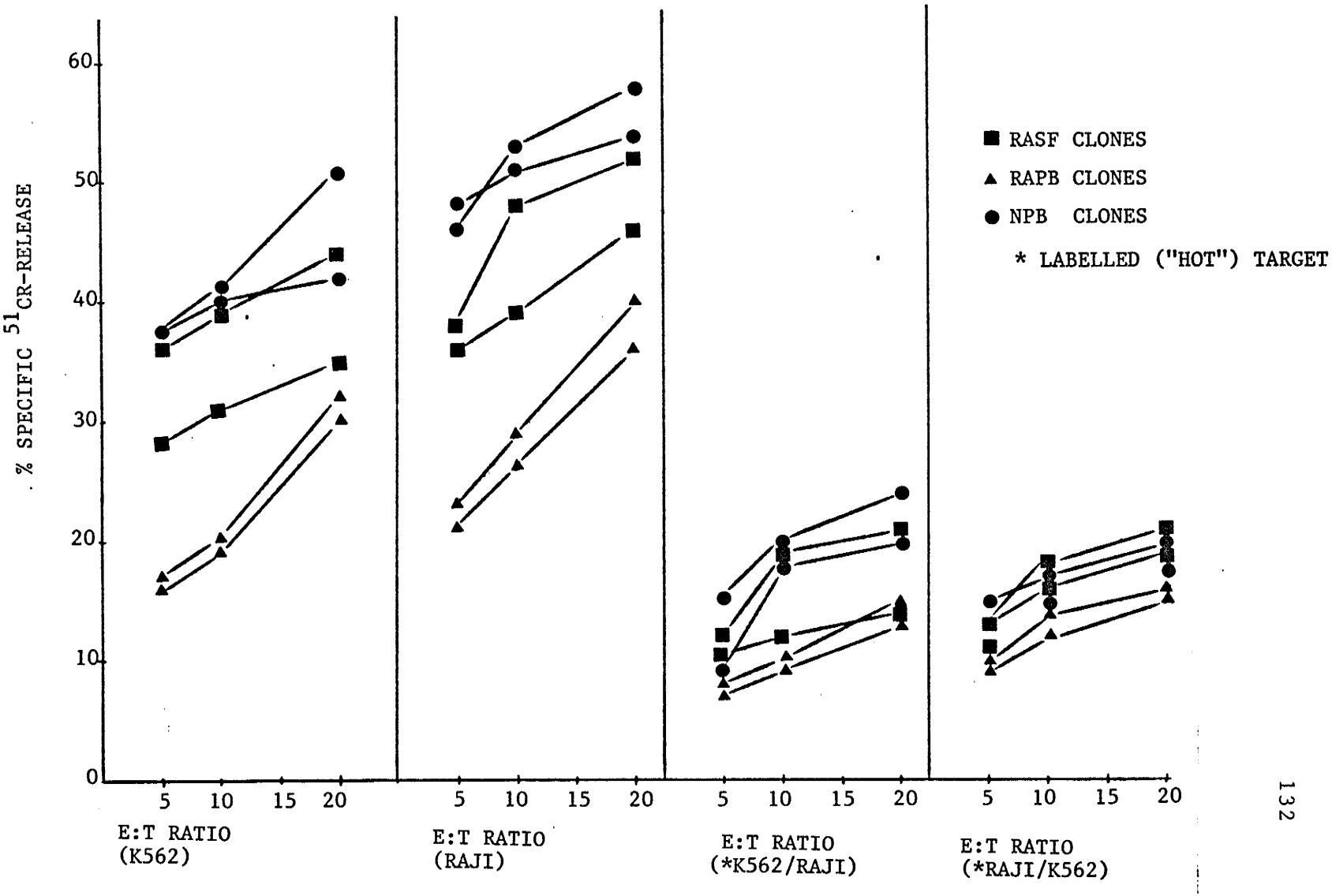

□ RASF CLONES
▲ RAPB CLONES
● NPB CLONES
N=10
% SPECIFIC 51CR-RELEASE
30
20
10
0
TARGETS: 1 2 3 1 2 3
RA
OA
ALLOGENEIC SYNOVIAL CELLS

Figure 22: Cold Target Inhibition Analysis Of Non-MHC Restricted Cytotoxic T Cell Clones Specificities. The clones were incubated with $^{51}Cr$-labelled K562 or Raji targets, either in the presence of 5 fold excess unlabelled (cold) K562 or Raji. The percentage target cell lysis was calculated in each group.


% SPECIFIC $^{51}$CR-RELEASE
60
50
40
30
20
10
0
5 10 15 20
E:T RATIO (K562)
E:T RATIO (RAJI)
E:T RATIO (*K562/RAJI)
E:T RATIO (*RAJI/K562)
RASF CLONES
RAPB CLONES
NPB CLONES
* LABELLED ("HOT") TARGET

CD3/Ti antigen receptor complex as well as the CD4 or CD8 antigens in the lysis of targets (Meuer et al., 1982), suggesting a role for these molecules in the recognition and/or activation mechanisms of CTL. To delineate whether CD3, CD4 or CD8 antigens were involved in the recognition or subsequent events leading to lysis of the targets, the effect of modulating off these antigens from the surface of the clones was evaluated. As Table 27 shows, a marked reduction in the level of either CD3, CD4 or CD8 antigen expressed on the surface of the clones had no significant effect on the cytotoxicity of these clones to both K562 and Raji targets. Furthermore, either antibody when left in the culture system throughout the experiment did not affect the cytotoxic activity of the clones. These data strongly suggest that neither of these antigens is involved in the lytic process of non-MHC restricted cytotoxic cells. The HLA-DR (Ia)+ clones expressed CD3 and T101, Tac antigens and either CD4 or CD8+ but lacked NK markers CD11, CD16 and HNK-1. These cells, which most closely resemble non-MHC restricted cytotoxic T cells, are present with increased frequency in RA synovial fluids.

## DISCUSSION

The principal finding of this study was that SF had increased precursor frequencies of IL-2 responsive cytotoxic cells compared to autologous PB or normal PB. The precursor frequency obtained for normal PB was in close agreement with the findings of Vie et al, (1986) who used autologous serum and irradiated non-adherent filler cells and recombinant IL-2 in an effort to reduce the possibility of non-specific activation and obtained frequencies of (1/407-1/1098). The LDA

TABLE 27

EFFECT OF CD3, CD4 AND CD8 ANTIBODIES ON CYTOTOXIC ACTIVITIES OF T CELL CLONES

| | BEFORE MODULATION | | | % $^{51}$CR-RELEASE | | AFTER MODULATION | | | % $^{51}$CR-RELEASE | |
|---|---|---|---|---|---|---|---|---|---|---|
| CLONES | CD3 | CD4 | CD8 | K562 | RAJI | CD3 | CD4 | CD8 | K562 | RAJI |
| RAPBG1 | 82* | 64 | 0 | 32 | 48 | 4 | 2 | 0 | 30 | 46 |
| RAPBG3 | 91 | 58 | 1 | 38 | 46 | 6 | 3 | 0 | 37 | 44 |
| RASFA8 | 86 | 1 | 67 | 45 | 52 | 3 | 0 | 3 | 42 | 49 |
| RASFA10 | 89 | 0 | 64 | 39 | 46 | 4 | 0 | 2 | 36 | 41 |
| NPBC5 | 86 | 71 | 0 | 47 | 54 | 3 | 2 | 0 | 44 | 50 |
| NPBC6 | 92 | 68 | 1 | 43 | 48 | 5 | 2 | 0 | 40 | 45 |

The effect of antibodies to T cell surface antigens on cytotoxicity of T cell clones were examined. The clones were pre-incubated with antibodies at a saturating dose (1:2 final dilution) for 24 hrs. Prior to washing the cells, the level of CD3, CD4 and CD8 antigens remaining on the surface of the clones were evaluated by indirect immunofluorescence. The clones were then washed and specific lysis against K562 and Raji tested in a 4 hr $^{51}$Cr-release assay

* = % Reactivity with antibodies

analysis also showed that the frequencies of IL-2 responsive i.e. proliferating cells and the cytotoxic precursors were identical in all experiments, suggesting that both precursors were derived from the same cell. It was rather surprising to find a high proportion of CD4+ cytotoxic clones. It is not clear whether the differentiation of CD4+ cells to cytotoxic cells is an intrinsic capacity of the CD4+ cells or a process regulated by other cells or factors. Support for the latter possibility comes from the recent finding of Chen et al, (1986) that proliferative non-cytotoxic CD4+ clones acquire specific cytotoxic activity after treatment with rIFN-$\gamma$ or $\alpha$ . Since the IL-2 conditioned medium used for the cloning contained IFN-$\gamma$ , it is conceivable that a large proportion of non-cytotoxic CD4+ clones may have differentiated into non-MHC restricted cytotoxic clones.

Evaluation of the cytotoxic activity of clones derived from the IL-2 responsive cells displayed a wide range of cytotoxic activity against a spectrum of tumor and non-tumor targets in vitro. These IL-2 responsive clones are therefore, more compatible with the non-MHC restricted cytotoxic T cells described by (Rayner et al. 1985). The wealth of data in the literature indicate that the effectors mediating this activity are difficult, if not impossible, to distinguish from the LGL fraction of PBL (Lanier et al., 1986). However, it has been suggested that while the bulk of this cytotoxic activity is associated with NK cells (CD3-, CD16-, Leu 19+) in fresh isolates and short term cultures, there is also a minor T cell component (CD3+, CD16-, Leu 19+) which becomes the dominant non-MHC restricted effector cell in long term cultures (Lanier et al., 1986). It is the latter cell type that the cloning conditions employed in this study may have selected for

since virtually all the clones phenotyped to date were (CD3+ CD16-) while the major (CD16+ IL-2) responsive population requires higher levels of IL-2 (500-1000 units/ml) than were used in our system (30 units/ml). Lymphokine-activated killer (LAK) cells, designated non-MHC restricted cytotoxic cells by (Lanier et al., 1986), have been reported to derive from (CD3- CD2-) precursors that are (CD16+ and CD11-) (Grimm et al., 1983) indicating that they may not be of thymic lineage. Following stimulation by IL-2, the non-MHC restricted cytotoxic may remain (CD3-), although reports concerned with the cells acquiring T cell markers are available (Timonen et al., 1982; Abo et al., 1983; Allavera et al. 1984). Although purified LGL preparations were used in these studies, it is possible that a few activated T cells may have contaminated the preparations, which can respond to IL-2.

There is an emerging consensus that lysis of NK-resistant tumor targets can be mediated by populations of IL-2 activated CD3+ CD2+ lymphocytes (Kurnick et al., 1986; Lanier et al., 1986; Ortaldo et al., 1986). This observation is in accordance with our findings and indicates that cytolytic activities may be widely shared by phenotypically diverse precursor pool of lymphoid cells.

Another interesting finding of this study is that despite the expression of CD3, CD4 and CD8 antigens in the cloned T cells, the cytotoxic activity of these non-MHC restricted effectors was not affected by either the addition of CD3, CD4 or CD8 mAbs to the cytotoxicity assay or the modulation of the antigens prior to performing the cytotoxic assay. These findings suggest that the IL-2 responsive T cell clones used in this study differed from classical CTL, the cytotoxic activity of which is abrogated by modulation of

either the CD3 determinant or the clonotypic structure (Ti) and also the CD4 or CD8 antigens (Meuer et al., 1982; Hercend et al., 1983). This finding is in agreement with that of Van de Grind et al., (1984) who demonstrated that the presence of CD3 or CD8 markers on IL-2 activated killer cells was not a prerequisite for the lysis of target cells. Similarly, Moretta et al., (1984) showed that cytolytic activity of CD3+ NK-like clones is generally not inhibited by anti-CD3 antibodies. The inability of CD4 or CD8 antibodies to inhibit the cytotoxic activity of the T cell clones in this investigation is not surprising since a number of studies (McDonald et al., 1982; Marrack et al., 1983; Biddison et al., 1984) have provided evidence that these molecules strengthen the binding between CTL and targets in cases where the interaction is of low avidity. The data also further suggest that in addition to the presence of T cell antigens on the clones, another molecule(s) may be responsible for the interaction of the clones with targets leading to the lysis of the targets. It has been demonstrated that T200 and LFA-1 molecules are involved in adhesive interactions between cells (Moller et al., 1982).

The cold target inhibition studies indicated that the targets may express the same or similar structures for recognition by non-MHC restricted cytotoxic cells (NMRCC). Recently, Werkmeister et al., (1985) have described an antibody, reactive with melanoma target cell glycolipids that blocked promiscuous killing by activated killer cells, suggesting that a receptor for a carbohydrate determinant may be involved in the killing process. Although, NMRCC are generally thought to be incapable of lysing fresh lymphoid cells (Grimm et al., 1985), recently Sondel et al., (1986) reported a modest lysis of normal

lymphoid cells with NMRCC. However, in this study, the NMRCC did not lyse fresh PBL but lysed autologous PBL blast cells. This is in accordance with the findings of (Schlesier et al, 1984) that RA SF T cells kill autologous blast cells. There was no correlation between the phenotype of the clones and cytotoxic activity in that both CD3+ CD4+ and CD3+ CD8+ clones killed to the same degree. The ability of SF T cell clones to lyse allogeneic synovial fibroblasts suggest that cytotoxic activity against fibroblasts in vivo may contribute to the disease activity in the joint. This finding may be in agreement with the work of Hedberg and Kallen, (1964) who demonstrated a cytopathic effect of RA and some non-RA SF T cells on human fetal fibroblasts in a visual assay after 15-20 hrs incubation. Similarly, Griffiths et al, (1976) using $^{51}$Cr-labelled synovial cells as targets also showed that RAPB MNC lyse synovial cells. However, in contrast to this study, where a moderate level of cytotoxicity was obtained, they observed increased cytotoxicty, which is explainable by the fact that MNC used in their studies contain monocytes which may be capable of killing synovial cells. Furthermore, these authors used 18 hr cytotoxic assay instead of the conventional 4 hr assay used in the investigations reported here. Pearson et al, (1975), using a microcytotoxicity assay, demonstrated that cytotoxic activity of RA lymphocytes against allogeneic synovial cells was greater than lymphocytes from normal subjects; and also that the cytotoxic activity of RA lymphocytes was greater than when RA but not non-RA synovial cells were used as target cells. This finding is in agreement with the data from this work, where it has been shown that RA T cell clones expressed greater cytotoxic activity against allogeneic RA than OA synovial fibroblasts. The fact that cytotoxic activity was

seen against both RA and OA fibroblasts by RA T cell clones argues against the presence of antigens unique to RA synovial cells. Since there was no autologous RA fibroblasts to compare, it is reasonable to suggest that the cytotoxic assay may not be measuring pre-sensitization of RA T cells to synovial tissue specific antigens, but rather detecting a response to alloantigens present on the synovial cell membranes. Nevertheless, the data suggest that RA T cells may be sensitized to synovial antigens and that such autoimmunity may develop secondary to joint destruction or as a consequence of the inflammatory process. Further work is required to establish whether chronic inflammation in RA may result from local immune responses to antigens present in the synovial tissues.

In RA, there have been conflicting reports on the status of NK activity. A number of studies (Combe et al, 1984; Karsh et al, 1985) demonstrated impaired NK function in peripheral blood of RA patients while Neighbour et al. (1982) reported a normal NK activity in the PB of RA patients. The impaired NK activity reported in the first study correlated with disease activity and when the RA patients were in remission, the NK activity was normal. The bulk of evidence, however, suggests that NK activity is normal in the PB of RA patients.

The NK activity in SF from RA patients is even more controversial. In previous studies, (Silver et al., 1982; Armstrong and Panayi, 1983) employing whole mononuclear cells observed decreased NK activity in SF as compared to RA PB. Other studies reported either a normal (Dobloug et al., 1982) or increase in NK activity in SF (Reinitz et al., 1982). The latter study, however, used conjugate-binding assay whereas the others employed a $^{51}$Cr-release assay. Thus, the conflicting reports

mentioned above may be due in part, to differences in protocols for cell isolation or cytotoxic assay procedure, and the concentration of macrophages and other accessory cells. It was demonstrated by Silver et al., (1982) that overnight culture of SF with or without pronase treatment resulted in some increase in cytotoxicity, suggesting that SF contained an inhibitor of NK activity; a concept supported by the fact that incubation of control PBL in SF resulted in a decrease in NK activity. Immune-complexes present in SF could be responsible for the decrease in NK activity.

It has also been shown that SF NK activity can be augmented by addition of indomethacin or removal of glass-adherent cells, suggesting that prostaglandin synthesis by the adherent cells may be partly responsible for the deceased NK activity in SF (Combe et al, 1984). Our study found a normal NK activity in SF. Perhaps cloning of the effector cells and studying their cytotoxic properties will be the best method of resolving the conflicts of decreased or normal NK activity in SF.

Goto and Zvaifler (1985) have recently reported that overnight cultured synovial fluid cells from RA patients express non-MHC restricted cytotoxic activity resembling that observed with LAK or AMLR generated killers. The AMLR has been shown to enhance NK-like activity, altering the phenotype of cytotoxic cells and increasing the repertoire of targets capable of being lysed (Goto and Zvaifler, 1983). These authors Goto and Zvaifler, (1985) subsequently showed that IgG-Fc-cells that were (CD2+ HNK-1-) mediated the non-MHC restricted cytotoxicity in SF. There was however, the presence of typical HNK-1+ cells also in SF. Treatment of the effector populations with

complement and monoclonal antibodies CD2 or 4F2 removed the bulk of this activity. In contrast, treatment with CD4, CD8, OKIaI, CD11, or Leu 7 and complement did not affect the cytotoxicity of the SF cells, thus, differentiating these effectors from the clones described in this study. These differences in surface phenotype may be indicative of differences in the sensitivies of the assay system used to demonstrate the surface markers or the culture methods employed may select for a subpopulation of cells in the SF.

There was no consistent functional differences demonstrable between RA PB and SF non-MHC restricted cells except for the expression of CD8 antigen on cells isolated from SF. It is intriguing to speculate about the reason for high non-MHC restricted cytotoxic precursors in SF, observed in this study. The influx could be related to a reaction to a putative viral agent, an antigen localised in the joint, aberrations in immunoregulation or ongoing tissue destruction. At present, we can only characterize and describe the phenomenon but we cannot define their importance or role in the disease process.

## GENERAL DISCUSSION

In the past years, studies of cellular immune abnormalities in RA have, by necessity, utilized peripheral blood cells because of the small numbers of lymphoid cell that can be obtained from the joint fluid. It is possible that cell-mediated immune events of pathogenetic importance may be restricted to compartments showing pathological involvement and not in the peripheral blood. Indeed, studies have demonstrated both phenotypic (Fox et al, 1982) and functional (Silver et al, 1982) differences between synovial fluid and peripheral blood cells emphasizing the need to study cells from the pathological sites.

Recently, our laboratory (Wilkins et al., 1983) and others (Clark et al., 1984) have successfully generated long term IL-2 dependent T cell lines from synovial fluids of RA patients. This ability to generate large numbers of T cells from the SF compartment in the form of stable lines offers a better way to study the role of such T cells in the disease process. Since the T cell lines were generated from bulk cultures and are heterogeneous in nature, the functional repertoire of the T cells with respect to antigen specificity, cytotoxic activity and production of immunoregulatory molecules cannot be fully resolved.

The development of T cell cloning techniques has given a new approach to investigating T cell functions. Using T cell cloning techniques, it was possible to generate long term IL-2 dependent T cell clones from SF and PB of RA patients. Such cloned T cell lines are invaluable in evaluating the properties of putative disease-specific or autoreactive T cells and thus help to elucidate the nature of

possible autoantigens and their role in initiating and/or perpetuating the chronic inflammation seen in RA patients.

We demonstrated that the IL-2 responsive clones derived from SF and PB were indeed T cells by their strict IL-2 dependency and the expression of CD3, CD4 and CD8+ surface antigens. The T cell clones were not generated in response to FCS components, which have been shown to be capable of activating resting T cells to IL-2 responsive state (Lakhanpal and Handwerger, 1986) because addition of cyclosporin A, which inhibits antigen or mitogen induced proliferation did not alter the precursor frequency. It should be emphasized that the T cell lines established in this study are unlikely to reflect the whole spectrum of T cells in the joint but rather a small functionally active cell subpopulation. CD8+ T cell clones were generated with a higher frequency than CD4+ clones. The prevalence of CD8+ cells in SF is unlikely to be due to selection pressure in our cloning assays since a high proportion of CD4+ T cell clones were generated from SF of 2 RA patients. The predominance of CD8+ T cell clones in SF is consistent with the results of (Burmester et al., 1981; Fox et al., 1982; Wilkins et al., 1983) who showed in bulk cultures that RA patients have increased proportion of CD8+ cells in SF when compared to autologous PB. Schlesier et al. (1984) also showed in a cloning assay that the majority of T cells generated from RA SF were of the CD8+ phenotype. These findings suggest that some selective pressures may exist within the joint.

Further evidence supporting the existence of selective pressures within the joint was also obtained from limiting dilution assays of synovial fluid and peripheral blood T cells grown on irradiated

peripheral blood or synovial fluid mononuclear cell filler. It was found in these studies that the average precursor frequency of IL-2 responsive peripheral blood cells cloned in the presence of PBL filler was 1/260 but in the presence of synovial fluid filler, the precursor frequency was <1/1000; whereas for synovial fluid cells, the precursor frequencies on PB filler and SF filler were the same being 1/200. This observation suggested that PB IL-2 responsive cells were inhibited by the SF mononuclear cell filler while SF IL-2 responsive cells were not. It was possible that the non-lymphoid synovial cells secreted prostaglandins which are potent suppressors of T cell growth. However, addition of indomethacin, an inhibitor of prostaglandin synthesis to the culture system, had no statistically significant effect on the precursor frequency of PB IL-2 responsive T cells grown on SF filler cells suggesting that prostaglandin was not responsible for the suppressive effect seen. Phenotypically, there was no difference between CD4 and CD8+ PB clones, in that both grew less well on SF filler cells.

The problem inherent in assessing the functional properties of SF T cells rather than T cells from synovial tissue, the real battleground, is whether SF cells accurately reflect the actual lymphocytic infiltration in the synovial tissue. Since T cells found in the synovial fluid migrated from the tissue, and both CD4+ and CD8+ T cells may be found in the tissue with either increased CD4+ T cells (Meijer et al, 1982), decreased CD4+ T cells (Koch et al, 1984) or normal CD8+ T cells (Forre et al, 1982), depending on the area of the tissue sampled; this suggests that SF T cells may be representative of the T cell population in the tissues and thus, can be used to give an

insight into the functional properties of T cells from the synovial compartment. Nevertheless, care should be taken in interpreting SF data alone. All attempts to expand and maintain synovial tissue T cells failed. The failure to maintain ST T cells may be partly due to technical problems, but more importantly reflect the nature of the samples. The tissue samples were obtained from RA patients undergoing hip replacement surgery and therefore represent "burnt-out" cases with few T cells. Future studies with synovectomy samples may be the best source for generating T cell clones but it was not possible to obtain such specimens in this study.

The ability of the clones to produce other lymphokines such as IFN- , IL-2 and FAF were also examined since these mediators influence the function and recruitment of other cell types in the joint. IFN- release by RA T cells was found to be normal when compared to normal T cells. This is in agreement with other published reports. The regulation of IL-2 production and responsiveness is a subject of great controversy as to whether there is impaired or hyperproduction of IL-2. The results obtained in this study suggest a normal IL-2 production and responsiveness by RA T cell clones. It is conceivable that in bulk cultures other regulatory cells may modulate the activity of the IL-2 secreting cells. Indeed, (Combe et al., 1987) reported a decreased PHA-stimulated IL-2 production by both RA PB and SF lymphocytes and that addition of indomethacin or irradiation of suppressor cells increased the IL-2 release of SF and PB cells.

The clones also displayed non-MHC restricted cytotoxicity (NMRC) in being capable of killing both NK sensitive (K562), NK insensitive (Raji and Colo) targets, autologous or allogeneic blast cells and

synovial cells. It was found that RA PB T cell clones had a normal NMRC activity. This is in agreement with most studies which evaluated NK function in RA PBL. In contrast, a number of studies reported a decreased cytotoxic activity in SF T cells. In this study, SF NMRC cells were found to be efficient and potent killers. This observation calls for caution in the interpretation of bulk culture data where other regulatory cells or molecules can modulate SF NMRC. The decreased cytotoxic activity in RA SF cells noted by other investigators, may reflect accessory cell function rather than defect in the T cells per se. The ability of the clones to kill autologous PBL blast targets is in agreement with results that T cell clones derived from RA SF kill autologous blast cells (Schlesier et al, 1984).

This study also demonstrated that RA SF contained increased levels of NMRC compared to autologous PB as demonstrated by the cytotoxic precursor frequencies. The important question is whether the NMRC T cells in the joint have any relevance in the pathogenesis of RA?. At present, the significance of the increased NMRC cells in the joint is unknown. It is however, reasonable to suggest that these cells may be playing a pathogenetic role in the ongoing tissue destruction and inflammation. It has been suggested that T-cell associated serine proteinases play an important role in cell killing by cytotoxic T cells (Redelman and Hudig, 1980; Chang and Eisen, 1980). There are reports that these secreted serine proteinases can degrade basement membrane-like extracellular matrices (Sheela and Barret, 1982; Kramer et al., 1985). This raises the possibility that these NMRC cells in joint might contribute to the cartilage destruction in RA or that these enzymatic activities may facilitate the migration of T cells into the

inflamed tissue.

With regard to the patho-etiology of RA, the detection and estimation of antigen-specific or reactive T cells from the joint and PB to antigens such as CTA implicated in the pathogenesis of RA will be of great help in showing association of specific reactivity pattern with RA. In RA to date, no causative viral or bacterial agents are implicated in the pathogenesis of RA. The role of autoimmunity to CTA has generated considerable interest and thus, has been the area of intense investigation in RA. The animal (rat or mouse) models of collagen type II-induced arthritis have suggested that immune response to collagen might be of significance in the pathogenesis of RA. In RA patients, similar immune reactivity to native and denatured collagens have been found (Trentham et al., 1977; Smolen et al., 1980; Stuart et al., 1980) suggesting that immune response to collagen may initiate and/or perpetuate the chronic inflammatory state.

To determine whether such autoreactive T cell clones exist in the joint and PB, CTA were tested for their ability to stimulate T cell clones in the presence or absence of autologous accessory cells. Surprisingly, the CTA-induced responses were polyspecific as measured by either lyphokine production or proliferation; suggesting that the CTA may share a common epitope which the clones recognize. It is also conceivable that the maintenance of T cells in long-term culture in the presence of IL-2 may have resulted in specificity degradation of their T cell receptor complex (Shortman et al,1983; Wilde et al, 1984), with the T cells not being able to exhibit exquisite specificity for CTA. Another possibility is that the T cell clones recognized CTA via Fn-like receptors on the their cell surface, which is regulated by the

antigen-receptor pathway, even though all efforts to prove such Fn-CTA interaction using various synthetic peptides comprising the cell attachment determinant on the Fn molecule have consistently been unrewarding. The ability of anti-Ia antibodies to inhibit CTA-induced proliferation indicated that the responses observed was a typical antigen-induced proliferation and not a mitogenic effect. The finding that anti-CD3 or anti-TcR antibody can abrogate CTA-induced IFN production indicates that the recognition of CTA by the T cell clones is mediated by a receptor linked to or closely associated with the CD3/antigen receptor complex. Of great interest is the finding that the Ia+ IL-2 dependent T cell clones could present antigen to each other to cause proliferation, even though the proliferative responses were always less than classical APC under identical conditions. A number of studies have shown that T cell lines easily lose their antigenic specificity if they are not regularly re-stimulated with the relevant antigen (Sredni et al., 1981; Friedman et al., 1982). Since the antigen of pathogenic significance in RA is still unknown, a stronger antigenic stimulus comprising pooled irradiated MNC and PHA were used to expand and maintain the clones. This procedure enabled the clones to retain their apparent antigen-reactivity. The exact mechanism(s) whereby either APC alone or in conjunction with CD3 antibody could maintain antigen-specificity is not well understood. It has been postulated that APC maintains antigen-specificity of T cells via AMLR-like mechanism, with the CD3 antibody reacting with the CD3 antigen of the T cell antigen-receptor to maintain antigen-responsivenesss presumably through the maintenance of the expression of the antigen receptor (Clark et al, 1985). Thus, in our system, MLR-like

reaction and stimulation of CD3 antigen by PHA may be responsible for the maintenance of antigen-reactivity of the T cell clones. This approach offers an opportunity to investigate the specificity of autoantigens implicated in autoimmune diseases.

This ability of APC and IL-2 alone and/or CD3 antibody to maintain antigen reactivity and specificity of T cells is well documented in other systems, involving PPD-specific IL-2 dependent T cells derived from PB and cerebrospinal fluid (CSF) of tuberculosis patients (Clark et al, 1985) and Tetanus toxoid-specific IL-2 dependent T cell lines (Padula et al, 1985). Attempts to use SF as a source of possible antigenic material in the expansion of the T cell clones were not advantageous compared to the pooled irradiated filler cells alone.

The ability of the clones to present antigen to each other has a in vivo implication and suggests that these activated T cells could further amplify the immune responses in the joint.

In conclusion, cumulative data suggest that in vivo activated, IL-2 responsive T cells may play an important role in the local immune responses in the inflamed joint, possibly in responses to CTA and/or via release of soluble mediators or as a consequence of abnormally regulated immune responses. Furthermore, the direct demonstration that RA SF T cell clones can release increased levels of FAF upon exposure to CTA, which cause fibroblast proliferation and recruitment, provides further evidence for the pathogenetic involvement activated T cells in RA. If RA is a T cell-mediated disease and should collagen autoimmunity be involved in the pathogenetic process, then trials involving antigen-specific suppression as demonstrated in experimental arthritis (Holoshitz et al., 1983; Cremer et al., 1983; Brahn and

Trentham, 1984) would be the ultimate tests of collagen involvement in the disease process in RA.

It is likely that future investigations will concentrate on the generation of T cell clones from synovial tissue using the T cell cloning techniques developed in this study, to examine antigen-specific cell-mediated immune response of possible pathogenetic significance and also to ascertain whether autoimmunity to CTA or synovial cell antigens is an effector mechanism in RA. More importantly, such clones may also allow for the development of specific immunomodulating reagents targeted towards the T cell receptor which recognize the CTA. This approach would hopefully, lead to selective inactivation or elimination of the autoreactive T cells for CTA, providing the means of abrogating the undesirable autoimmune process.

## REFERENCES

Abo, T.; Miller, C. A.; Gartland, G. L.; Balch, C. M. (1983) J. Exp. Med. 157:273.

Abrahamsen, T. G.; Froland, S. S.; Natvig, J. B.; Pahle, J. (1975) Scand. J. Immunol. 4:823.

Allavana, P. and Ortaldo, J. R. (1984) J. Immunol. 132:2363.

Allen, P. M. and Unanue, E. R. (1984) J. Immunol. 132:1077.

Allen, P.M. (1987) Immunol. Today, Vol. 8:270.

Alpert, S. D.; Jonsen, M. E.; Broff, M. D.; Schnecberger, E.; Geha, R. S. (1981) J. Clin. Immunol. 1:21.

Alspaugh, M. A.; Henle, G.; Lenette, E. T.; Henle, W. (1981) J. Clin. Invest. 67:1134.

Armstrong, R. D. and Panayi, G. S. (1983) Clin. Rheumatol. 2:243.

Baker, P. E.; Gillis, S.; Smith, K. A. (1979) J. Exp. Med. 149:273.

Bankhurst, A. D.; Husby, G.; Williams, R. C. (1976) Arth. Rheum. 19:555.

Barland, P.; Novikoff, A. B.; Hamerman, D. (1964) Am. J. Pathol. 44:853.

Basham, T. Y. and Merigam, T. C. (1983) J. Immunol. 130:1492.

Ben-Nun, A.; Strauss, W.; Leeman, S.A.; Cohn, L.E.; Murre, C.; Duby, A.; Seidman,J.G; Glimcher, L.H. (1985) Immunogenetics 22:123

Bevan, M. J. (1975) J. Exp. Med. 142:1349.

Bianco, C. (1983) Ann. N.Y. Acad. Sci. Vol.408:603

Biddison, W. E.; Rao, P. E.; Talle, M. A.; Goldstein, G.; Shaw, S. (1984) J. Exp. Med. 159:783.

Biswas, C. and Dayer, J. M. (1979) Cell 18:1305.

Bitter, T. Muir, H.M. (1962). Anal. Biochem. 4:330.

Boyum, A. (1968) Scand. J. Clin. Lab. Invest. 21:(Suppl. 97):7.

Brahn, E.; Helfogott, S. M.; Belli, J. A.; Anderson, R. J.; Reinherz, E. L.; Schlossman, S. F.; Austen, K. F.; Trentham, D. E. (1984) Arth. Rheum. 27:481.

Brahn, E. and Trentham, D. G. (1984) Cell. Immunol. 86:421.

Brinckerhoff, C. E. and Guyre, P. M. (1985) J. Immunol. 134:3142.

Burmester, G. R.; Yu, D. T.; Irani, A. M.; Kunkel, H. G.; Winchester, R. J. (1981) Arthr. Rheum. 24:1370.

Burmester, G. R.; John, B.; Gramataki, M.; Zacher, J.; Kalden, J. R. (1984) J. Immunol. 133:1230.

Cardarelli, P. M. and Pierschbaker, M. D. (1983) Proc. Natl. Acad. Sci (USA) 83:2647.

Carsons, S.; Mosesson, M. W.; Diamond, H. S. (1981) Arthr. Rheum. 24: 1261.

Castor, C. W. (1960) Arthr. Rheum. 3:140.

Castor, C. W. (1975) Ann. N.Y. Acad. Sci. 256:304.

Cesario, T. C.; Andrews, B. S.; Martin, D. A.; Jason, M.; Treadwell, T.; Friou, G.; Tilles, J. G. (1983) J. Rheum. 10:647.

Champion, B. R. and Poole, A. R. (1981) Coll. Relat. Res. 1:453.

Champion, B.R. and Poole, A.R. (1982) Arthr. Rheum. 25:274

Chang, T. W. and Eisen, H. N. (1980) J. Immunol. 124:1028.

Chen, L-K.; Mathieu-Mahul, D.; Sasporte, M.; Degros, L.; Bensussan, A. (1986) Human Immunol. 17:214.

Chin, R.; Chiou, S.; Multz, C. V.; Allison, A. C. (1983) Fed. Proc. 42:947.

Christian, C. L. (1961) Arthr. Rheum. 4:86.

Claeys, H.; Van Damme, J.; Deley, M.; Vermylen, C.; Billiau, A. (1982) Br. J. Hematol. 50:85.

Clague, R. B. and Moore, L. J. (1984) Arthr. Rheum. 27:1370.

Clark, R. B.; Muirhead, S. P.; Pollard, M. K. (1984) Clin. Immunol. Immunopathol. 33:287.

Clark, R. B.; Ligenheld, E. G.; Donaldson, J. O.; Pollard, M. K. (1985) Clin. Immunol. Immunopathol. 36:176.

Combe, B.; Pope, R. M.; Darnell, B.; Kincaid, W.; Talal, N. (1984) J. Immunol. 133:709.

Combe, B.; Pope, R. M.; Fischbach, M.; Darnell, B.; Bann, S.; Talal, N. (1985) Clin. Exp. Immunol. 59:520.

Combe, B.; Andary, M.; Klein, B.; Clot, J.; Sany, J. (1987) J. Rheum. 14:226.

Cooke, T. D.; Hurd, E. R.; Jasin, H. E.; Bienenstock, J.; Ziffin, M. (1975) Arthr. Rheum. 18:541.

Courtenay, J. S.; Dallman, M. J.; Dayan, A. D.; Martin, A.; Mosedale, B. (1980) Nature 283;666.

Cseh, K.; Jacob, L.; Torok, J.; Kalabay, L.; Marticsek, J.; Pozonyi, T.; Benedek, S. (1985) Immunol. Letters 9:301.

Cunningham, A. L. and Merigam, T. C. (1984) J. Immunol. 321:197.

Degre, M.; Melibye, O. S.; Clark-Jenssen, O. (1983) Ann. Rheum. Dis. 42:672.

DeLustro, F.; Sherer, G. K.; LeRoy, E. C. (1980) J. Reticuloendothel. Soc. 28:519.

DeLustro, F. and LeRoy, E. C. (1982) J. Reticuloendothel. Soc. 31:295.

Dingle, J. T. (1971) In Tissue Proteinases (Eds. Barrett, A. J. and

Dingle, J. T.) page 313. Amsterdam, North Holland Publishing Co.

Doblong, J. H.; Forre, O.; Kvien, T. K.; Egeland, T.; Degre, M. (1982) Ann. Rheum. Dis. 41:490.

Duncan, M. R. and Berman, B. (1985) J. Exp. Med. 162:516.

Edwards, J. C.; Sedgwick, A. D.; Willougby, D. A. (1982) Ann. Rheum. 41:282.

Emery, P.; Panayi, G. S.; Nouri, M. E. (1984) Clin. Exp. Immunol. 57:123.

Epstein, L. and Cline, M. J. (1974) Clin. Exp. Immunol. 16:553.

Epstein, L. B. (1977) Tex. Rep. Biol. Med. 35:42.

Field, E. H.; Strober, S.; Hoppe, R. T.; Calin, A.; Engelman, E. G.; Kotzin, B. L.; Tanay, A. S.; Calin, H. J.; Terrell, C. P.; Kaplan, H. S. (1983) Arthr. Rheum. 26:937.

Fisher, W. D.; Golds, E. E.; Van Der Rest, M.; Cooke, T. D.; Lyons, H. E.; Poole, A. R. (1982) J. Bone and Joint Surg. 64:546.

Fleischer, B. (1984) Eur. J. Immunol. 14:748.

Fontana, A.; Hengartner, H.; Weber, E.; Fehr, K.; Gardo, P. J.; Cohen, G. (1982) Rheumatol. Int. 2:49.

Ford-Hutchinson, A. W.; Smith, M. J.; Walker, J. R. (1976) Br. J. Pharmacol. 57:4678.

Forre, O.; Doblong, J. H.; Natvig, J. B. (1982) Scand. J. Immunol. 15:227.

Fox, K. I.; Chilton, T.; Rhodes, G.; Vaughan, J. H. (1986) J. Immunol. 137498.

Franklin, E. C.; Holman, H. R.; Muller-Ederhard, H. J.; Kunkel, H. G. (1957) J. Exp. Med. 105:425.

Gardner, D. L. (1972) Ann. Rheum. Dis. 31:235.

Geppert, T.D. and Lipsky, P.E. (1987) J. Rheum. 14(Suppl. 13):59.

Gerrard, T. L.; Volkman, D. J.; Jurgensen, C. H., Fauci, A. S. (1985) Cell. Immunol. 95:65.

Gerrard, T. L.; Volkman, D. J.; Jurgensen, C. H.; Fauci, A. S. (1986) Human Immunol. 17:416.

Ghadially, F. N. and Roy, S. (1967) Ann. Rheum. Dis. 26:426.

Gillis, S.; Ferm, M. M.; Ou, W.; Smith, K. A. (1978) J. Immunol. 120:2027.

Glant, T.; Csongor, J.; Szucs, T. (1980) Scand. J. Immunol. 11:247.

Glimcher, L. H.; Kim, K. J.; Green, I.; Poul, W. E. (1982) J. Exp. Med. 155:445.

Goodman, J. W. (1961) Proc. Soc. Exp. Biol. of Med. 106:822.

Goodwin, J. S.; Bankhurst, A. D.; Messner, R. P. (1977) J. Exp. Med. 146:1719.

Goto, M. and Zvaifler, N. J. (1983) J. Exp. Med. 157:1309.

Goto, M. and Zvaifler, N. J. (1985) J. Immunol. 134:1483.

Grey, H. M.; Colon, J. M.; Chesnut, R. W. (1982) J. Immunol. 129:2389.

Grimm, E. A.; Ramsey, K. M.; Mazunder, A.; Wilson, D. J.; Djeu, J. Y.; Rosenberg, S. A. (1983) J. Exp. Med. 157:884.

Grimm, E. A. and Wilson, D. J. (1985) Cell. Immunol. 94;568.

Hammerman, D.; Barland, P.; Janis R. (1969) The Biological Basis of Med. (Eds. Bittar, E. E. and Bittar, N.) Acad. Press N.Y. page 209.

Hamerman, D. (1975) Ann. N.Y. Acad. Sci. 256:25.

Hasler, F.; Bluestein, H. G.; Zvaifler, N. J.; Epstein, L. B. (1983) J. Exp. Med. 157:173.

Haynes, B. F.; Hemler, M. E.; Mann, D. L.; Eisenbark, G. S.; Shelhamer, J.; Mostowski, H. S.; Thomas, C. A.; Strominger, J. L.; Fauci, A. S. (1981) J. Immunol. 126:109.

Hedberg, H. and Kallen, B. (1964) Acta. Path. Microbiol. Scand. 62:177.

Helderman, J. H. and Strom, T. B. (1978) Nature 274:62.

Hercend, T.; Meuer, S.; Brennan, A.; Edson, M. A.; Acuto, O.; Reinherz, E. L.; Schlossman, S. F.; Ritz, J. (1983) J. Exp. Med. 158:1547.

Heuer,J. and Kolsch, E. (1985) J. Immunol. 134:4031.

Holoshitz, J.; Naparstek, Y.; Ben-Nun, A.; Cohen, I. R. (1983) Science 219:56.

Houba, V. and Allison, A. C. (1966) Lancet 1:848.

Issekutz, T.; Chu, E.; Geha, R. S. (1983) J. Immunol. 129:1446.

Jackson, D.S; Cleary, E.G. (1967) In: Glick, D. (Ed.) Methods of Biochem. Anal. N.Y: Interscience Publishers, 15:32.

Janossy, G.; Tidman, N.; Selby, W. S.; Thomas, J. A.; Granger, S. (1980) Nature 288:81.

Johnson, H. M. and Farrar, W. L. (1983) Cell. Immunol. 75:154.

Johnson, G. J. and Russell, P. S. (1965) Nature 208:343.

Johnson, R. L. and Ziff, M. (1976) J. Clin. Invest. 58:240.

Kammer, G. M. and Trentham, D. E. (1984) Arthr. Rheum. 27:489.

Kaplan, M. E. and Clark, C. (1974) J. Immunol. Methods 5:131.

Karsh, J.; Dorval, G.; Osterland, C. K. (1981) Clin. Immunol. Immunopathol. 19:43.

Kashiwado, T.; Oppenheimer-Marks, N.; Ziff, M. (1987) ARA Abstract C103 page 591.

Klareskog, L.; Forsum, U.; Scheynius, A.; Kalelitz, D.; Wigzell, H. (1982) Proc. Natl. Acad. Sci (USA) 79:3632.

Klingermann, H.G.; Ebert, J.; Deeg, J. (1986) J. Leukocyte Biol. 40:

Kluin-Nelemans, H. C.; Van der Linden, J. A.; Gmelig-Meyling, F. H.; Schurerman, H. J.; (1984) J. Rheumatol. 11:372.

Knop, J. R.; Stemmer, C.; Neumans, C.; DeMaeyer, E.; Macher, E. (1982) Nature 296:757.

Ko, H. S., Fu, S. M.; Winchester, R. J.; Yu, D. T.; Kunkel, H. G. (1979) J. Exp. Med. 150:246.

Koch, B; Locher, P; Burmester, G.R; Mohr, W; Kalden, J.R. (1984) Rheumatol. Int. 4:79.

Kobayashi, I. and Ziff, M. (1975) Arthr. Rheum. 18:475.

Korn, J. H.; Halushka, P. V.; LeRoy, E. C. (1980) J. Clin. Invest. 65:543.

Korn, J. H. (1983) J. Clin. Invest. 71:1240.

Kotzin, B. L.; Strober, S.; Engleman, E. G.; Calin, A.; Hoppe, R. T.; Kansas, G. S.; Terwell, C. P.; Kaplan, H. S. (1981) N. Engl. J. Med. 305:969.

Kramer, M. D.; Benninger, L.; Schirrmacher, V.; Moll, H.; Pester, M.; Nerz, G.; Simon, M. M. (1986) J. Immunol. 136:4644.

Kurki, P; Vartio, T; Virtanen, I. (1987) Scand. J. Immunol. 26:645.

Kurnick, J. T.; Kradin, R. L.; Blumberg, R.; Schneeberger, E. E.; Boyle, L. A. (1986) Clin. Immunol. Immunopathol. 38:367.

Lachman, L. B. (1983) Fed. Proc. 42:2639.

Lakhanpal, S. and Handwerger, B. S. (1986) J. Immunol. Methods 94:197.

Lanier, L. L. and Phillips, J. H. (1986) Immunol. Today 7:5.

Lause, D.B.; Beezhold, D.H.; Doran, J.E. (1984) J. Immunol. 132:1294

Lefkovits, I. (1972) Eur. J. Immunol. 2:360.

Lefkovits, I. and Waldmann, T. (1979) Cambridge Univ. Press. Cambridge, U.K.

Lemm, G. and Warnatz, H. (1986) Clin. Exp. Immunol. 64:71.

MacDonald, H. R.; Glasebrook, A. L.; Bron, C.; Kelso, A.; Cerottini, J. C. (1982) Immunol. Rev. 68:89.

Mackel, A.M.; DeLustro, F.; LeRoy, E.C. (1982) J. Exp. Med. 156:1042.

Malek, T. R.; Clement, L. T.; Schevach, E. M. (1983) Eur. J. Immunol. 132:1077.

Malone, D. G.; Wahl, S. M.; Wilder, R. L. (1984) Arthr. Rheum. 27: (Suppl.):535 (Abstract).

Marrack, P.; Endres, R.; Shimonkevitz, R.; Zlotnik, A.; Dialynas, D.; Fitch, F.; Keppler, J. (1980) J. Exp. Med. 158:1077.

Marcus, R. L. and Townes, A. S. (1971) J. Clin. Invest. 50:282.

McCarty, D. J. (1962) Am. J. Med. Sci. 243:288.

McKenna, R. M.; Ofosu-Appiah, W.; Warrington, R. J.; Wilkins, J. A. (1986) J. Rheum. 13:28.

McKenna, R. M.; Wilkins, J. A.; Warrington, R. J. (1987) J. Rheum. (In press).

McManus, N. H. (1976) Appl. Environ. Microbiol. 31:35.

Meijer, C.J.L.M; deGraaff-Reitsma, C.B; Lafeber, G.J.M; Cats, A. (1982) J. Rheumatol. 9:359.

Menninger, H.; Putzier, R.; Mohr, W. (1980) Z. Rheumatol. 39:145.

Menzel, J.; Steffen, C.; Kolarz, G.; Eberl, R.; Frank, O.; Thumb, N. (1976) Ann. Rheum. Dis. 35:446.

Meuer, S. C.; Hussey, R. E.; Penta, A. C.; Fitzgerald, K. A.; Stadler, B. M.; Schlossman, S. F.; Reinherz, E. L. (1982b) J. Immunol. 129:1076.

Meuer, S. C.; Fitzgerald, K. A.; Hussey, R. E.; Hodgdon, J. C.; Schlossman, S. F.; Reinherz, E. G. (1983) J. Exp. Med. 138:1194.

Mills, B. J.; Shively, J. E.; Mitsky, P. S.; Hawke, D. H.; Forman, D. H.; Wright, C. L.; Todd, C. W. (1986) Immunol. 59:57.

Miyasaka, N.; Nakamura, T.; Russell, I. J.; Talal, N. (1984) Clin. Immunol. Immunopathol. 31:109.

Miyawaki, T.; Yachie, A.; Ohzeki, S.; Nagaoki, T.; Taniguchi, N. (1983) J. Immunol. 130:2737.

Mohr, W.; Westerhellweg, H.; Wessinghage, D. (1981) Ann. Rheum. Dis. 40:396.

Moller, G. (1982) Immunol. Rev. 68 (Ed.)

Moretta, A.; Pantaleo, G.; Mingari, M. C.; Melioli, G.; Moretta, L.; Cerottini, J. C. (1984) Eur. J. Immunol. 14:121.

Munthe, E. and Natwig, J. B. (1971) Clin. Exp. Immunol. 8:249.

Myers,L.K.; Stuart, J.M.; Kang, A.H. (1987) ARA meeting, Absrtact 56 page 519.

Neighbour, P. A. and Grayzel, A. I. (1981) Clin. Exp. Immunol. 45:576.

Nilsson, E. and Biberfeld, G. (1984) Clin. Exp. Rheum. 2:57.

Nogueira, N.; Kaplan, G.; Levy, E.; Sarno, E.; Kushner, P.; Granelli-Piperno, L.; Vieira, L.; Gould, V.; Levis, W.; Steinman, R.; Yip, Y.; Cohn, Z. (1983) J. Exp. Med. 158:2165.

Nouri, A. M. E.; Panayi, G. S.; Goodman, S. M. F. (1984) Clin. Exp.

Immunol. 58:402.

Nussenzweig, M. C.; Steinman, R. M.; Gutchinov, B.; Cohn, Z. A. (1980) J. Exp. Med. 152:1070.

Ofosu-Appiah, W. A.; Morgan, K.; Lennox-Holt, P. J. (1983) Ann. Rheum. Dis. 42:331.

Ofosu-Appiah, W. A.; McKenna, R. M.; Warrington, R. J.; Wilkins, J. A. (1986) Clin. Exp. Immunol. 64:555.

Ohno, O. and Cooke, T. D. (1978) Arthr. Rheum. 21:516.

Ortaldo, J. R.; Mason, A.; Overton, R. (1986) J. Exp. Med. 1193.

Padula, S.J.; Pollard, M.K.; Lingenheld, E.G.; Clark, R.B. (1985) Am. Soc. Clin. Invest. 75:788.

Paulus, H. E.; Machleder, H. I.; Levine, S.; Yu, D. T. Y.; McDonald, N. S. (1977) Arthr. Rheum. 20:1249.

Pekin, J. J. and Zvaifler, N. Y. (1964) J. Clin. Invest. 43:1372.

Perussia, B.; Starr, S.; Abraham, S.; Fanning, V.; Trinchieri, G. (1983) J. Immunol. 130:2133.

Petersen, J.; Andersen, V.; Ingemann-Hansen, T.; Halkjaer-Kristensen, J.; Wilk, A.; Thyssen, H. (1983) Scand. J. Rheum. 12:299.

Poole, A. R.; Hembry, R. M.; Dingle, J. T. (1976) Arthr. Rheum. 19:1295.

Postlethwaite, A. E.; Synderman, R.; Kang, A. H. (1976) J. Exp. Med. 144:1188.

Postlethwaite, A. E. and Kang, A. H. (1983) Arthr. Rheum. 26:22.

Pruzanski, W.; Russell, M. L.; Gordon, D. A.; Ogryzlo, M. A. (1973) Am. J. Med. Sci. 265:483.

Rayner, A. A.; Grimm, E. A. Lotze, H. T.; Wilson, D.; Rosenberg, S. A. (1985) J. Natl. Cancer Inst. 75:67.

Redelman, D. and Hudwig, D. (1980) J. Immunol. 124;870.

Reinitz, E.; Neighbour, P. A.; Grayzel, A. I. (1982) Arthr. Rheum. 25:1440.

Rice, L.; Laughter, A. H.; Twoney, J. J. (1979) J. Immunol. 121:1540.

Roberts, J.E.; McLees, B. D.; Kerby, G. P. (1967) J. Lab. Clin. Med. 70:503.

Robb, R. J.; Green, W. C.; Rusk, C. M. (1984) J. Exp. Med. 160:1126.

Rodnan, G. P.; Eisenbeis, C. H.; Creighton, A. S. (1963) Am. J. Med. Sci. 35:182.

Rodriguez, M. A.; Ceuppens, J. L.; Goodwin, J. S. (1982) J. Immunol. 128:2422.

Ropes, M. W.; Bennett, G. A.; Bauer, W. (1939) J. Clin. Invest. 18:351.

Ropes, M. W., Bennett, G. A.; Cobb, S.; Jacox, R.; Jessar, R. A. (1959) Ann. Rheum. Dis. 18:49.

Rosenberg, S. A.; Spiess, P.; La Freniere, R. (1986) Science 233:1318.

Rosenthal, A. S. and Schevach, E. M. (1973) J. Exp. Med. 138:1194.

Rowley, D. and Jenkin, C.R. (1962) Nature 193:151.

Royston, I.; Majada, J. A.; Yamamoto, G. Y.; Baird, S. M.; Meserve, B. L.; Griffiths, J. C. (1980) J. Immunol. 125:725.

Ruoslahti, E.; Engvall, E.; Hayman, E. G. (1981) Coll. Relat. Res. 1:95.

Scala, G. and Oppenheim, J. J. (1983) J. Immunol. 131:1160

Schlesier, M.; Ramb-Lindhauer, C.; Gartner, M.; Peter, H. H. (1984) Rheumatol. Int. 4 (Suppl.):1.

Schmidt, J. A.; Mizel, S. B.; Cohen, D.; Green, I. (1982) J. Immunol. 128:2177.

Scott, D. L.; Farr, M.; Crockson, A. P.; Walton, K. W. (1972) Clin. Sci.

62:71.

Seitz, M; Napierski, I; Augustin, R; Hunstein, W; Kirchner, H. (1987) Scand. J. Rheumatol. 16:257.

Sheela, S. and Barret, J. C. (1982) Carcinogenesis 3:363.

Shortman, K.; Wilson, A.; Scollay, R. (1983) J. Immunol. 132:584.

Silver, R. M.; Redelman, D.; Zvaifler, N. J.; Naides, S. (1982) J. Immunol. 128:1758.

Singer, J. M.; Plotz, C. M.; Peralta, F. M.; Lyons, H. C. (1962) Ann. Intern. Med. 56:545.

Smolen, J. S.; Menzel, E. J.; Scherak, O.; Kojer, M.; Kolarz, G.; Steffen, C.; Mayr, W. R. (1980) Arthr. Rheum. 23:424.

Solinger, A. M. and Stobo, J. D. (1981) Arthr. Rheum. 24:1057.

Sondel, P. M.; Hank, J. A.; Kohler, P. C.; Chen, B. P.; Minkoff, D. Z.; Molenda, J. A. (1986) J. Immunol. 137:502.

Sredni, B. and Schwartz, R.H. (1980) Nature 287:855.

Sredni, B.; Tse, H. Y.; Chen, C.; Schwartz, R. J. (1981) J. Immunol. 126:341.

Stratton, J.A; Peter, J.B. (1978) Clin. Immunol. Immunopathol. 10:233

Streicher, H.Z.; Berkower, I.J.; Busch, M.; Gurd, F.R.N.; Berzofsky, J.A. (1984) Proc. Natl. Acad. Sci. 81:6831.

Stuart, J. M.; Postlethwaite, A. E.; Townes, A. S.; Kang, A. H. (1980) Am. J. Med. 69:13.

Stuart, J. M.; Townes, A. S.; Kang, A. H. (1982) J. Clin. Invest. 69:673.

Stuart, J.M.; Townes, A.S.; Kang, A.H. (1984) Ann. Rev. Immunol. 2:199

Sundquist, K. G. and Otteskog, P. (1987) J. Immunol. 138:825.

Sutherland, R.; Delig, D.; Schneider, E.; Newman, R.; Kemshead, J.; Greaves, M. (1981) Proc. Natl. Acad. Sci. (USA) 78:A515.

Suzuki, R.; Honda, K.; Itoh, K.; Kumagai, K. (1983) J. Immunol. 130:981.

Tan, P.; Shore, A.; Leary, P.; Keystone, E. C. (1984) J. Rheum. 11:593.

Thomas, Y.; Rogozinski, L.; Irigoyen, H.; Friedman, S. M., Kung, P. C.; Goldstein, G.; Chess, L. (1981) J. Exp. Med. 154:459.

Timonen, T.; Ortaldo, J. R.; Stadler, B. M.; Bonnard, G.D.; Sharrow, S. O.; Herberman, R. B. (1982) Cell. Immunol. 72:178.

Tomasi, T. B.; Fudenberg, H. H.; Finby, N. (1962) Amer. J. Med. 33:243.

Tosato, G.; Steinberg, A. D.; Blaese, R. M. (1981) N. Engl. J. Med. 305:1238.

Trelstad, R.L; Rubin, D; Gross, J. (1977) Lab. Invest. 36:501.

Trentham, D. E.; Townes, A. S.; Kang, A. H.; David, J. R. (1978a) J. Clin. Invest. 61:89.

Trentham, D. E.; Dynesius, B. A.; Rocklin, B. E.; David, J. R. (1978b) N. Engl. J. Med. 299:327.

Trentham, D. E. (1985) J. Rheum. (Suppl. 12) 12:7.

Treves, A. J.; Tal, T.; Barak, V.; Fuks, Z. (1981) Eur. J. Immunol. 11:487.

Triebel, F.; Roquefeuile, S.D.; Blanc, C.; Charron, D. J.; Debre, P. (1986) Human Immunol. 15:302.

Trinchieri, G.; Matsumoto-Kobayashi, M.; Clark, S. C.; Seehra, J.; London, L.; Perussia, B. (1984) J. Exp. Med. 160;1147.

Tsukamoto, Y.; Helsel, W. E.; Wahl, S. M. (1981) J. Immunol. 127:673.

Uchiyama, T.; Nelson, D. L.; Fleisher, T. A.; Waldmann, T. A. (1981a) J. Immunol. 126:1348.

Uchiyama, T.; Broder, S.; Waldman, T. A. (1981b) J. Immunol. 126:1393.

Van Boxel, J. A. and Paget, S. A. (1975) N. Engl. J. Med. 293:517.

Van de Griend, R. J.; Van Krimpen, B. A.; Ronteltap, C. P. M.; Bolhuis, R.C. H. (1984) J. Immunol. 132:3185.

Van Eden, W.; Holoshits, J.; Nevo, Z.; Frenkel, A.; Klajman, A.; Cohen, I. R. (1985) Proc. Natl. Acad. Sci. (USA) 82:5117.

Vaughan, J. H.; Jacox, R. J.; Noell, P. (1968) Arthr. Rheum. 11:135.

Vie, H.; Bonneville, M.; Sondermeyer, P.; Moreau, J. R.; Soulillou, J. P. (1986) Immunol. 57:351.

Vose, B. M. and Bonnard, G. D. (1983) J. Immunol. 130:687.

Wahl, S. M. and Wahl, L. M. (1981) Lymphokines 2:279.

Wahl, S. M. and Gately, C. L. (1983) J. Immunol. 130:1226.

Wahl, S. M.; Malone, D. G.; Wilder, R. L. (1985) J. Exp. Med. 161:210.

Walker, E.; Warner, N. L.; Chesnut, R.; Kappler, J.; Marrck, P. (1982) J. Immunol. 128:2164.

Warrington, R. J.; McKenna, R.; Ofosu-Appiah, W.; Wilkins, J. A. (1985) Arthr. Rheum. 20:1318.

Werkmeister, J. A.; Triglia, T.; Andrews, P.; Burns, G. F. (1985) J. Immunol. 135:689.

Wernick, R.; Lospalluto, J. J.; Fink, C. W.; Ziff, M. (1981) Arthr. Rheum. 24:1501.

Wilde, D. B.; Prystowsky, M. B.; Ely, J. M.; Vogel, S. N.; Dialynas D. P.; Fitch, F. W. (1984) J. Immunol. 133:636.

Wilkins, J. A.; Warrington, R. J.; Sigurdson, S. L.; Rutherford, W. J. (1983) J. Rheum. 10:109.

Wilkins, J. A.; Olivier, S. L.; Warrington, R. J. (1984) Clin. Exp.

Immunol. 58:1.

Winchester, R. J.; Agnello, V.; Kunkel, H. G. (1970) Clin. Exp. Immunol. 6:689.

Wood, D.D; Ihrie, E.J.; Dinarello, C.A.; Cohen, P.L. (1983) Arthr. Rheum. 26:975.

Wooley, D. E.; Crossley, J. M.; Evanson, J. M. (1977) Arthr. Rheum. 20:1231.

Yam, L.T.; Li, C.Y.; Crosby, S. (1971) Amer. J. Pathol. 55283.

Yamada, K. M. and Olden, K. (1978) Nature 275:179.

Younger, J. S. and Salvin, S. B. (1973) J. Immunol. 111:1914.

Ziegler, K. and Unanue, E. R. (1981) J. Immunol. 127:1869.

Ziff, M. (1973) Fed. Proc. 32:131.

Zinkernagel, R. M. and Doherty, P. C. (1975) J. Exp. Med. 141:1427.

Zubler, R. H.; Nydegger, U.; Perrin, L. H.; Fehr, K.; McCormick, J.; Lambert, P. H.; Miescher, P. A. (1976) J. Clin. Invest. 57:1308.

Zvaifler, N. J. (1979) Etiology and Pathogenesis of RA (Ed. McCarty, D. J.) In Arthritis and Allied Conditions (Edition 9), Philadelphia, Lea and Febiger, page 28.