

INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps. Each original is also photographed in one exposure and is included in reduced form at the back of the book.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

UMI

A Bell & Howell Information Company
300 North Zeeb Road, Ann Arbor MI 48106-1346 USA
313/761-4700 800/521-0600



PERMISSION DE REPRODUIRE ET DE DISTRIBUER LA THÈSE - PERMISSION TO REPRODUCE AND DISTRIBUTE THE THESIS

NOM DE L'AUTEUR - NAME OF AUTHOR	
CANNONS, Jennifer	
ADRESSE POSTALE - MAILING ADDRESS	
92 Stewart,	
Ottawa, Ontario K1H 1J6	
GRADE-DEGREE	ANNEE D'OBTENTION - YEAR GRANTED
M.Sc. (Microbiology and Immunology)	1997
TITRE DE LA THESE - TITLE OF THESIS	
AN INCREASE IN THE FREQUENCY OF hprt MUTANT T LYMPHOCYTES IN THE	
PERIPHERAL BLOOD, SYNOVIAL FLUID, AND SYNOVIAL TISSUE OF RA PATIENTS	

L'AUTEUR PERMET, PAR LA PRESENTE, LA CONSULTATION ET LE PRÊT DE CETTE THESE EN CONFORMITE AVEC LES REGLEMENTS ETABLIS PAR LE BIBLIOTHECAIRE EN CHEF DE L'UNIVERSITE D'OTTAWA. L'AUTEUR AUTORISE AUSSI L'UNIVERSITE D'OTTAWA, SES SUCCESEURS ET CESSIONNAIRES, A REPRODUIRE CET EXEMPLAIRE PAR PHOTOGRAPHIE OU PHOTOCOPIE POUR FINS DE PRÊT OU DE VENTE AU PRIX COÛTANT AUX BIBLIOTHEQUES OU AUX CHERCHEURS QUI EN FERONT LA DEMANDE.

LES DROITS DE PUBLICATION PAR TOUT AUTRE MOYEN ET POUR VENTE AU PUBLIC DEMEURERONT LA PROPRIÉTÉ DE L'AUTEUR DE LA THESE SOUS RÉSERVE DES RÉGLEMENTS DE L'UNIVERSITÉ D'OTTAWA EN MATIÈRE DE PUBLICATION DE THÈSES.

THE AUTHOR HEREBY PERMITS THE CONSULTATION AND THE LENDING OF THIS THESIS PURSUANT TO THE REGULATIONS ESTABLISHED BY THE CHIEF LIBRARIAN OF THE UNIVERSITY OF OTTAWA. THE AUTHOR ALSO AUTHORIZES THE UNIVERSITY OF OTTAWA, ITS SUCCESSORS AND ASSIGNEES, TO MAKE REPRODUCTIONS OF THIS COPY BY PHOTOGRAPHIC MEANS OR BY PHOTOCOPYING AND TO LEND OR SELL SUCH REPRODUCTIONS AT COST TO LIBRARIES AND TO SCHOLARS REQUESTING THEM.

THE RIGHT TO PUBLISH THE THESIS BY OTHER MEANS AND TO SELL IT TO THE PUBLIC IS RESERVED TO THE AUTHOR, SUBJECT TO THE REGULATIONS OF THE UNIVERSITY OF OTTAWA GOVERNING THE PUBLICATION OF THESES

June 2/97
DATE

Jennifer Cannons
(AUTEUR) SIGNATURE (AUTHOR)



Université d'Ottawa • University of Ottawa

UNIVERSITÉ D'OTTAWA



UNIVERSITY OF OTTAWA

ÉCOLE DES ÉTUDES SUPÉRIEURES
ET DE LA RECHERCHE

SCHOOL OF GRADUATE STUDIES
AND RESEARCH

CANNONS, Jennifer

AUTEUR DE LA THÈSE-AUTHOR OF THESIS

M.Sc.(Microbiology and Immunology)

GRADE-DEGREE

Microbiology and Immunology

FACULTÉ, ÉCOLE, DÉPARTEMENT-FACULTY, SCHOOL, DEPARTMENT

TITRE DE LA THÈSE-TITLE OF THE THESIS

An Increase in the Frequency of hprt Mutant T
Lymphocytes in the Peripheral Blood, Synovial Fluid,
and Synovial Tissue of RA Patients

R. Goldstein and H.C. Birnboim

DIRECTEUR DE LA THÈSE-THESIS SUPERVISOR

EXAMINATEURS DE LA THÈSE-THESIS EXAMINERS

J.R. Dillon

W. Ross

M. Freedman

J.-M. De Koninck, Ph.D.

(LE DOYEN DE L'ÉCOLE DES ÉTUDES SUPÉRIEURES
ET DE LA RECHERCHE)

SIGNATURE

A handwritten signature in dark ink, appearing to read "J.-M. De Koninck".

(DEAN OF THE SCHOOL OF GRADUATE STUDIES
AND RESEARCH)

**AN INCREASE IN THE FREQUENCY OF *hprt* MUTANT T LYMPHOCYTES IN THE
PERIPHERAL BLOOD, SYNOVIAL FLUID, AND SYNOVIAL TISSUE OF RA PATIENTS**

A thesis submitted to the School
of Graduate Studies
University of Ottawa

In partial fulfillment of the requirement for the degree of
Master's of Science
Department of Microbiology and Immunology
Faculty of Medicine

By Jennifer Cannons



National Library
of Canada

Acquisitions and
Bibliographic Services

395 Wellington Street
Ottawa ON K1A 0N4
Canada

Bibliothèque nationale
du Canada

Acquisitions et
services bibliographiques

395, rue Wellington
Ottawa ON K1A 0N4
Canada

Your file Votre référence

Our file Notre référence

The author has granted a non-exclusive licence allowing the National Library of Canada to reproduce, loan, distribute or sell copies of this thesis in microform, paper or electronic formats.

The author retains ownership of the copyright in this thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without the author's permission.

L'auteur a accordé une licence non exclusive permettant à la Bibliothèque nationale du Canada de reproduire, prêter, distribuer ou vendre des copies de cette thèse sous la forme de microfiche/film, de reproduction sur papier ou sur format électronique.

L'auteur conserve la propriété du droit d'auteur qui protège cette thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

0-612-28406-9

ABSTRACT

Rheumatoid arthritis (RA) is a chronic systemic autoimmune disease in which synoviocytes, macrophages, and T lymphocytes participate in the disease process. CD4⁺ T lymphocytes extravasate and infiltrate the synovial tissue due to the upregulation of adhesion molecules on the synovium, endothelium, and the T cells themselves. The milieu of the inflamed synovium, is mitogenic for T cells due to the presence of antigen presenting cells and genotoxic due to the presence of reactive nitrogen and oxygen species released by inflammatory cells. The X-linked hypoxanthine guanine phosphoribosyl transferase (*hprt*) gene can be implemented as a marker for estimating *in vivo* mutations in T lymphocytes. I used the *hprt* clonal assay to determine the *in vivo* frequency of mutant T cells (FMC) from the peripheral blood, synovial fluid, and synovial tissue of RA patients and controls. The results demonstrate that there is an increased FMC in the peripheral blood of RA patients compared to controls. There was also a significant elevation in the corrected FMC (cFMC), which takes in to account the cloning efficiency of the T cells, in the peripheral blood of RA patients compared to controls. There is an elevated FMC and cFMC in synovial fluid of RA patients compared to the peripheral blood of controls. However, the FMC and cFMC from the peripheral blood of unselected RA patients from the outpatient clinic is not significantly different than from the synovial fluid of RA patients suggesting that the synovial fluid does not contain the necessary mitogenic and mutagenic factors to induce T cell genetic damage. The FMC and cFMC from RA and osteoarthritis (OA) synovium is approximately ten fold greater than the FMC and cFMC from the peripheral blood of the same patients which suggests that the mitogenic and genotoxic environment of the inflamed synovium is capable of inducing T cell mutations. There was no significant difference in the cloning efficiency of T cells (CE), FMC, and

cFMC from the peripheral blood of RA patients with 'active' or 'inactive' disease. No correlation between the cFMC from the peripheral blood of RA patients and clinical disease parameters and patient medication was found.

The mutant T lymphocyte clones from the peripheral blood of RA patients displayed a phenotype similar to the RA and OA synovial tissue T cell lines. The RA and OA synovial tissue T cell lines and the RA peripheral blood mutant T cell clones bound to fibronectin with similar affinity and this interaction was inhibited by anti-CD29 monoclonal antibody and soluble fibronectin containing the CS-1 binding domain but not anti-IgG₁, anti-CD7, or anti- α 4 β 7 monoclonal antibodies. The RA peripheral blood non-mutant and the control mutant and non-mutant clones did not adhere to fibronectin. The RA peripheral blood mutant T cell clones and the RA synovial tissue T cell lines also expressed similar levels of CD29. The RA peripheral blood non-mutant T cell clones displayed a low level of CD29 expression compared to the mutant T cell clones. This data suggests that the RA peripheral blood T cell clones and RA synovial tissue T cells lines have a similar phenotype and the RA peripheral blood mutant clones may have acquired *hprt* mutations while in the inflamed synovium.

There is an increase in the frequency of *hprt* mutant T lymphocytes from the peripheral blood, synovial fluid, and synovial tissue of RA patients compared to controls. The RA peripheral blood mutant T cell clones and RA synovial tissue T cell lines display a similar phenotype with respect to CD29 expression and fibronectin adherence. Thus, the RA peripheral blood mutant T cell clones may have passed through the inflamed environment of the subsequently re-entered the circulation or have the capacity to infiltrate the inflamed synovium.

ACKNOWLEDGMENTS

I wish to thank my supervisors Dr. J. Karsh, Dr. H. C. Birnboim, and Dr. R. Goldstein who made this project possible. I am grateful for their encouragement, expertise, and helpfulness. I would further like to thank the other members of my thesis committee Dr. J. Dillon and Dr. A. MacKenzie for their critical comments.

I would like to thank Dr. Fillion and Gina Graziani-Bowering for their expertise and patience in flow cytometric analysis. Their help was very much appreciated. Much thanks to graduate students Ken Pelkey, Gina Graziani-Bowering, Julie Stone, Theresa Podrebarac, and Rob Pon for their camaraderie and encouragement.

Many thanks to Nancy Biggs and Lu Ming for their technical assistance. Finally, a special thanks to Dr. A. Cranney for her help with the clinical data and statistical evaluations.

This thesis is dedicated to

my boyfriend Ken Pelkey and to my parents

who have been very supportive in the past two years.

TABLE OF CONTENTS

	PAGE
TITLE PAGE	i
ABSTRACT	ii
ACKNOWLEDGMENTS	iv
DEDICATION	v
TABLE OF CONTENTS	vi
LIST OF TABLES	xi
LIST OF FIGURES	xii
LIST OF ABBREVIATIONS	xiv
CHAPTER 1: INTRODUCTION	1
1. Rheumatoid Arthritis	1
1.1 Clinical Background	1
1.2 Epidemiology	2
1.3 Description of a Normal Synovium Compared to the Rheumatoid Synovium	2
2. Etiology	6
2.1 Evidence for Genetic Association	6
2.2 Evidence for T Lymphocyte Involvement	7
2.3 Infectious Etiology of RA	10
2.3i Bacterium	11
2.3ii Viruses	11
2.3iii Superantigens	12

3. Cellular and Biochemical Aspects of Rheumatoid Arthritis	13
3.1 Adhesion Molecules Involved in the Inflamed Synovium	13
3.1 <i>i</i> Selectins	13
3.1 <i>ii</i> CD44	14
3.1 <i>iii</i> Integrins	15
3.2 Reactive Nitrogen and Oxygen Species and Their Involvement in RA	18
4. Frequency of Mutant T Cells from the Peripheral Blood of Patients with Other Autoimmune Diseases	19
5. Statement of Hypothesis	21
6. Specific Objectives	23
CHAPTER 2: METHODS	24
1. Description of Patients and Healthy Controls	24
2. Measures of Clinical Disease Parameters	24
3. Cloning of T Lymphocytes	25
3.1 Assay for <i>hprt</i> Mutant T Lymphocytes	25
3.2 Synovial Fluid T Lymphocytes	26
3.3 Synovial Tissue T Lymphocytes	26
3.4 Determining the Cloning Efficiency and the Frequency of Mutant T Lymphocytes	28
3.5 Radiolabelling Method to Validate Visual Scoring of T Cell Clones	28
3.6 The Frequency of Mutant T Cells in the Absence or Presence of Catalase	29
3.7 Expansion of T Lymphocyte Clones	29

4. Flow Cytometry	30
5. Cell Adhesion	31
5.1 Fibronectin Binding Assay	31
5.2 Specificity and Inhibition Assay	34
6. Statistical Analysis	34
CHAPTER 3: RESULTS	36
1. Patient Characteristics	36
2. The Frequency of Mutant T Cells from the Peripheral Blood of RA	
Patients and Controls	38
2.1 Proliferation Assay	38
2.2 The Cloning Efficiency of Peripheral Blood T Cells from RA	
Patients and Controls	38
2.3 The Frequency of Mutant T Lymphocytes from the Peripheral	
Blood of RA Patients and Controls	40
2.4 The Frequency of Mutant T Lymphocytes in the Presence or	
Absence of Catalase	44
2.5 <i>In Vitro</i> Genotoxic Effect	46
3. Clinical Disease	48
3.1 Disease Activity	48
3.2 Clinical Disease Parameters	48
3.3 Patient Medication	51

4. The Frequency of Mutant T Cells from the Synovial Fluid of RA Patients	50
4.1 The Cloning Efficiency of Synovial Fluid T Lymphocytes	56
4.2 The Frequency of Mutant T Cells from the Synovial Fluid of RA Patients	56
5. The Frequency of Mutant T Cells from RA and OA Synovial Tissue	58
5.1 The Cloning Efficiency of T Cells from the Synovial Tissue and Peripheral Blood of RA and OA Patients	58
5.2 The Frequency of Mutant T Cells from the Synovial Tissue and Peripheral Blood of Surgical RA and OA Patients	60
6. Cell Adhesion	61
6.1 Fibronectin Adhesion Assay	61
6.2 Inhibition Assay	63
7. Flow Cytometric Analysis of the RA Peripheral Blood T Cell Clones and RA Synovial Tissue T Cell Lines	66
7.1 Expression of Cell Surface Markers	66
7.2 Integrin Expression	66
CHAPTER 4: DISCUSSION	70
1. T Lymphocyte Involvement in RA	70
2. The Frequency of Mutant T Cells from the Peripheral Blood of RA Patients and Controls	70
3. The Frequency of Mutant T Cells from the Synovial Fluid of RA Patients	72
4. The Frequency of Mutant T Cells from the Synovial Tissue and Peripheral Blood of Surgical RA Patients	73

5. The Increase in the Frequency of Mutant T Cells Is Not an <i>In Vitro</i> Phenomenon	76
6. Correlation of Clinical Disease with the Frequency of Mutant T cells from the Peripheral Blood of RA Patients	77
6.1 Disease Activity	77
6.2 Clinical Disease Parameters	77
6.3 Patient Medication	78
7. Phenotype of the Peripheral Blood RA and Control T Cell Clones and RA and OA Synovial Tissue T Cell Lines	79
8. Mutation Frequency Versus Mutation Rate	83
9. Reactive Nitrogen and Oxygen Species	85
9.1 RA Synovium	85
9.2 Nitric Oxide Can Cause DNA Damage	86
9.3 Can Sustained Levels of Genotoxic Species Result in Neoplasm?	87
10. Summary and Conclusion	88
11. Future Direction	90
11.1 T Lymphocytes Important in RA Pathogenesis	90
11.2 Reactive Nitrogen and Oxygen Species and Their Involvement in the FMC in RA	91
REFERENCES	93
APPENDICES	xvii

LIST OF TABLES

	PAGE
1. The characteristic of the RA patients, OA patients, and controls used in the study.	37
2. The cFMC from the peripheral blood of RA patients with a minimum period of two months between samples.	42
3. The cFMC from the peripheral blood of controls with a minimum period of two months duration between samples.	43
4. The relationship between the CE of T lymphocytes from the peripheral blood of RA patients and clinical disease parameters.	52
5. The relationship between the cFMC from the peripheral blood of RA patients and clinical disease parameters.	53
6. The relationship between the CE of T cells from the peripheral blood of RA patients and current patient medication.	54
7. The relationship between the cFMC from the peripheral blood of RA patients and current patient medication.	55

LIST OF FIGURES

1. Depiction of a normal healthy synovium and a rheumatoid synovium.
2. Proliferation assay of the *hprt* and wildtype T cell clones from the peripheral blood of RA patients.
- 3A. The CE of T lymphocytes from the peripheral blood of RA patients and controls.
- 3B. The FMC from the peripheral blood of RA patients and controls.
- 3C. The cFMC from the peripheral blood of RA patients and controls.
- 4A. The CE of T lymphocytes from the peripheral blood of RA patients and controls for the catalase assay.
- 4B. The FMC from the peripheral blood of RA patients and controls in the absence and presence of catalase.
- 4C. The cFMC from the peripheral blood of RA patients and controls in the absence and presence of catalase.
- 5A. The CE of T lymphocytes from the peripheral blood of controls plated on day 0 or on day 2.
- 5B. The FMC from the peripheral blood of controls plated in selection medium on day 0 or 2.
- 5C. The cFMC from the peripheral blood of controls plated in selection medium on day 0 and 2.
- 6A. The CE of T lymphocytes from the peripheral blood of RA patients with 'active' and 'inactive' disease.
- 6B. The FMC from the peripheral blood of RA patients with 'active' or 'inactive' disease.
- 6C. The cFMC from the peripheral blood of RA patients with 'active' or 'inactive' disease.
7. The correlation between the cFMC from the peripheral blood of RA patients with clinical disease parameters.
- 8A. The CE of T lymphocytes from the synovial fluid of RA patients and the peripheral blood of controls.
- 8B. The FMC from the synovial fluid of RA patients and the peripheral blood of controls.
- 8C. The cFMC from the synovial fluid of RA patients and the peripheral blood of controls.
- 9A. The CE of T lymphocytes from the synovial tissue and peripheral blood of RA and OA patients.
- 9B. The FMC from the synovial tissue and peripheral blood of RA and OA patients.
- 9C. The cFMC from the synovial tissue and peripheral blood of RA and OA patients.
- 10A. A correlation between cFMC from the peripheral blood with synovial tissue of RA patients.
- 10B. A correlation between the cFMC from the peripheral blood with synovial tissue of OA patients.

- 11A. Binding to fibronectin by RA and OA synovial tissue T cell lines and RA mutant and non-mutant T cell clones in the absence and presence of various antibodies.
- 11B. Binding to fibronectin by RA and control mutant and non-mutant T cell clones in the absence and presence of various antibodies.
12. Binding to fibronectin is mediated by CD29 interaction with the CS-1 fibronectin fragment.
13. Immunophenotype of the RA peripheral blood mutant and non-mutant T cell clones: expression of CD3, CD4, and CD8.
14. CD29 expression on the RA synovial tissue T cell lines and the RA peripheral blood mutant and non-mutant T cell clones.
15. IL-2 stimulation of 1 MNC/well in non-selection medium.
16. PHA stimulation of $2 \times 10^4 \text{ MNC/well}$ in selection medium.
17. Synovial tissue T cell adherence to fibronectin and poly L-lysine coated wells.

LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
BMDP	Bio-medical data processing
BSA	Bovine serum albumin
CE	Cloning efficiency
cFMC	Corrected frequency of mutant T cells
cNOS	Constitutive nitric oxide synthase
CRP	C-reactive protein
Cy 5	carboxymethylindocyanine succinimide ester 5
ELISA	Enzyme linked immunosorbant assay
ESR	Erythrocyte sedimentation rate
FITC	Fluorescein isothiocyanate
FBS	Fetal bovine serum
FMC	Frequency of mutant T cells
H ⁺	Hydrogen
HEL	Hen egg lysozyme
HEV	High endothelial venules
HLA	Human leukocyte antigen
H ₂ O ₂	Hydrogen peroxide
<i>hprt</i>	Hypoxanthine guanine phosphoribosyl transferase gene
HPRT	Hypoxanthine guanine phosphoribosyl transferase protein
HSFM	Hybridoma serum free medium

IgA	Immunoglobulin A
IgG	Immunoglobulin G
IL-1	Interleukin 1
IL-2	Interleukin 2
IFN-gamma	Interferon gamma
iNOS	Inducible nitric oxide synthase
KDa	KiloDalton
NMMA	NG ^G -monomethyl-L-arginine
MAb	Monoclonal antibody
MHC	Major histocompatibility complex
MNC	Mononuclear cell
MR	Mutation rate
NO	Nitric oxide
O ₂	Oxygen
OA	Osteoathritis
OH·	Hydroxyradical
ONOO-	Perioxynitrite
PB	Peripheral blood
PBS	Phosphate buffered saline
PE	Phycoerythrin
PHA	Phytohemagglutinin
PMN	Polymorphonuclear cell

RA	Rheumatoid arthritis
RF	Rheumatoid factor
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SCID	Severe combined immunodeficient
SD	Standard deviation
SF	Synovial fluid
ST	Synovial tissue
TCR	T cell receptor
6-TG	6-thioguanine
TNF- α	Tumor necrosis factor-alpha
TRI	PE conjugated to Cy 5

CHAPTER 1: INTRODUCTION

1. RHEUMATOID ARTHRITIS

1.1 Clinical Background

Rheumatoid arthritis (RA) is classified as an autoimmune disease. The mechanisms which trigger the disease and the exact target of the immune response remain elusive. RA is characterized by a chronic inflammation of the synovial joints. Histological examination of the synovial tissue reveals the infiltration by blood derived mononuclear cells (MNC), predominately T cells, macrophages, mast cells, and plasma cells. All of these cells demonstrate signs of activation (Harris 1993; Firestein 1994). In time, rheumatoid disease usually results in the progressive destruction of cartilage and bone. The damage is mediated mainly by cytokine induction of destructive enzymes, chiefly matrix metalloproteinases (Zvaifler and Firestein 1994). There is also prominent neovascularization of the hyperplastic synovium, and evidence for systemic inflammation including the upregulation of acute phase proteins (Firestein 1994). In particularly severe disease, the disease can spread to involve the blood vessels and other organs. RA is associated with serious morbidity and even increased mortality (Pincus and Callahan 1986). RA patients have an increased risk for the development of hematopoietic cancers of B cells and myelocytes, including lymphoma, and multiple myeloma (Gridley *et al.* 1993; Laakso *et al.* 1986).

1.2 Epidemiology

RA has a world-wide distribution and involves all racial and ethnic groups. The prevalence of RA is approximately 1% (Zvaifler 1989). Women are affected 2 to 3 times more often than men in their third to fifth decade. The disease can occur at any age and generally increases in incidence in the advancing years with the peak incidence in women appearing in the fourth decade. The incidence of the disease is virtually the same in both sexes after the age of 60 (Wordsworth *et al.* 1992).

1.3 Description of Normal Synovium Compared to the RA Synovium

Synovium is the connective tissue lining of the diarthrodial (synovial) joints, tendons, and bursae which does not extend over the articular cartilage. The space enclosed by synovium contains small amounts of fluid rich in hyaluronan. The synovium allows deformable packing which permits movement of the adjacent non-deformable tissue. The difference between synovium and other soft connective tissue is that synovium is cavitated to allow movement to occur between tissues rather than within tissue. The primary functions of the synovium are to maintain an intact non-adherent tissue surface, to control the volume and composition of the synovial fluid, to lubricate the cartilage, and to provide nutrients to the chondrocytes (Edwards 1994).

The synovial membrane of healthy diarthrodial joints consists of a layer of 1 to 3 cells. The sublining is relatively acellular containing scattered blood vessels, fat cells, fibroblasts, and an extracellular matrix. Two main types of synovial lining cells have been identified. Type A synoviocytes have a phagocytic capacity with endocytic vacuoles, and are derived from a monocytic lineage. Type B synoviocytes display secretory functions, have an active Golgi, and are believed to be specialized mesenchymal cells of fibroblast origin (Edwards 1994; Zvaifler and

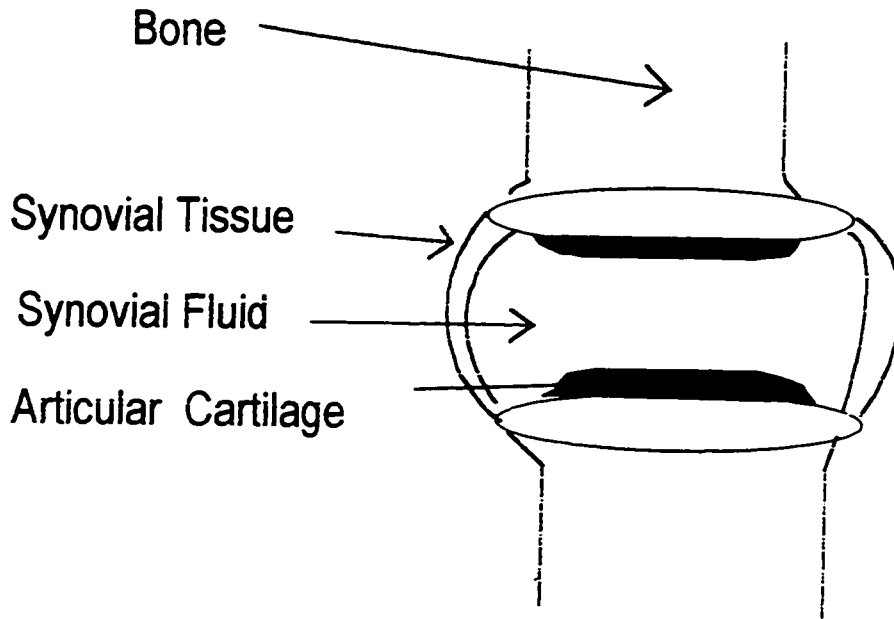
Firestein 1994). Figure 1A depicts a normal healthy synovium. Within the inflamed RA joint, the synovial lining increases to 5 to 10 layers, primarily by the accumulation of type A synoviocytes. The synovium becomes hyperplastic and assumes features of a transformed aggressive locally destructive granulation tissue. Under the influence of cytokines and growth factors, type B synoviocytes change into proliferating invasive cells that express oncogenes, secrete metalloproteinases, display features of a transformed phenotype and eventually invade the cartilage and bone (Zvaifler and Firestein 1994). Simultaneously, T cells, macrophages, mast cells, dendritic cells, and plasma cells accumulate in the sub-lining region and deep within the tissue (Zvaifler and Firestein 1994; Firestein 1994). Figure 1B depicts the destructive environment of the inflamed rheumatoid synovium.

Both joint inflammation and destruction are hallmarks of RA. One of the critical destructive elements is the hyperplastic synovium, referred to as pannus. Some researchers refer to pannus as the entire inflamed synovium, while others restrict the term to the vascularized connective tissue at or near the bone-synovium border. Pannus is usually viewed as an extension of the synoviocytes from the joint lining expanding under the influence of an immunologically mediated process (Zvaifler and Firestein 1994). In 1994, Zvaifler and associates named cells from the pannus, as pannocytes. Pannocytes are not typical fibroblast-synoviocytes: they are much more homogeneous, have a rhomboid shape, with a hypochromic nucleus and a prominent nucleolus. These cells were observed in a large erosion from a patient with long-standing disease, and the authors concluded that they may be important in the induction and perpetuation of solid tissue injury. However, the cells may have arisen or been transformed due to the aggressive mitogenic and toxic environment of the RA synovium.

Figure 1







A) Depiction of a normal healthy synovium. The space enclosed by the synovium contains the synovial fluid. The synovial tissue consists of a layer of 1 to 3 cells. The synovium does not extend over the articular cartilage. B) Diagram of a rheumatoid synovium. The synovial tissue lining cells have increased in size and number. Mononuclear cells from the peripheral blood have infiltrated the synovial fluid and tissue. Matrix metalloproteinases secreted by macrophages and type A and B synoviocytes can degrade the extracellular matrix. Reactive nitrogen and oxygen species secreted by type A synoviocytes, macrophages, fibroblasts, and chondrocytes can cause cellular damage. There is also increase expression of cytokines such as TNF- α , IL-1, and IFN-gamma.

A) Normal Synovium

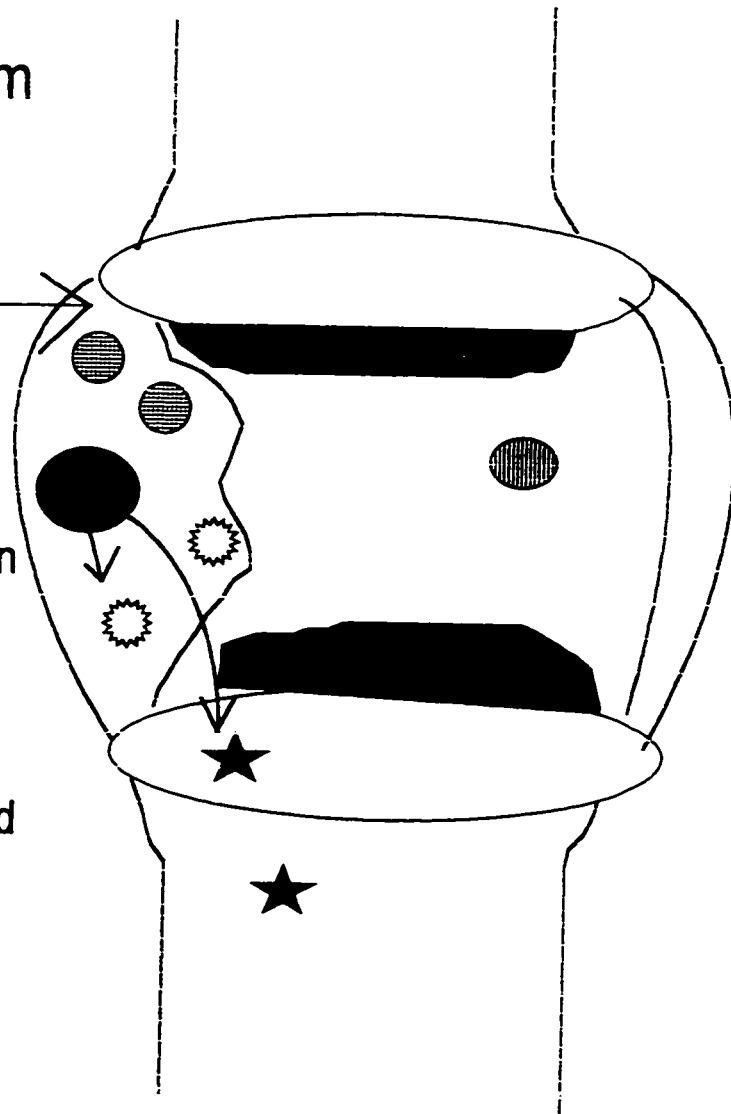


B) Rheumatoid Synovium

LEGEND

-  CD4+ T cell infiltration
-  CD8+ T cells
-  Macrophages
-  Reactive Nitrogen and Oxygen Species
-  Metalloproteinases
-  Pattern of Secretion

Pannus



In 1994 Zvaifler and Firestein postulated that the earliest changes in rheumatoid cartilage occurs when cells of unknown origin arise from the perichondral synovial membrane at the joint margin and dissect under the layer of type B synoviocytes. Immediately below the layer of invading cells, the cartilage matrix is depleted of proteoglycan and the chondrocytes have enlarged empty lacuna. Soon, blood vessels proliferate and the pannus becomes a granulation tissue containing a variety of cell types, including T cells, B cells, macrophages, and mast cells to sustain the disease process (Zvaifler and Firestein 1994). In a similar view of pannus formation, Fassbender believed that the initial invasion of cartilage matrix and subchondral bone occurs at the site of synovial attachment and is mediated by clusters of homogeneous, immature, polyhedral cells with large folded nuclei. Only after vascularization is the cartilage invaded by fibroblast-like synoviocytes and macrophages. The transition to a permanent fibrous pannus occurs as the fibroblasts become spindle-shaped and newly synthesized collagen is deposited (Fassbender 1983). Both of these hypotheses are based on the assumption that the initial synovial proliferation is a T-cell-independent process; however, the subsequent and sustained inflammation resulting in tissue damage is T cell mediated. What causes the change in the synoviocytes? Possible mechanisms involve the expression of various oncogenes (Muller-Lander *et al.* 1995), a viral infection resulting in the genetic alteration of synoviocytes (Zvaifler 1989), T lymphocyte stimulation and cytokine secretion (Goronzy *et al.* 1995), or a spontaneously occurring abnormality in a stem cell from which the synoviocytes arose (Kuzmich *et al.* 1995; Hirohata *et al.* 1996).

2. ETIOLOGY

2.1 Evidence For Genetic Association

Immunohistochemical studies demonstrated that T cells are a dominant component of the infiltrating cells in rheumatoid synovium (Goronzy *et al.* 1995). In the 1978, Stastny noted that a high frequency of RA patients shared HLA class II molecules. Immune recognition and response by T lymphocytes requires the formation of a trimolecular complex along with costimulation (Kuby 1994). One school of thought postulates that disease-associated HLA class II molecules selectively bind a particular self or foreign processed peptide. The recognition of the MHC antigen peptide complex by CD4⁺ T cells expressing a selected TCR initiates and sustains the synovial inflammation (Todd *et al.* 1988; Gregersen *et al.* 1987).

Twin and other cohort genetic studies have demonstrated that a major genetic contribution to disease predisposition resides in the HLA-DRB1 locus. More than 80% of Caucasian RA patients express DR1 or DR4 subtypes which share an epitope mapping to amino acids 70 to 74 of the DR β chain, in the polymorphic region that surrounds the peptide binding cleft (Gregersen *et al.* 1987). This is the most important evidence to support the concept that T cell recognition is important at some stage in the pathogenesis of RA.

In the peptide selection model of RA, a single copy of the disease-associated HLA-DR molecule would be a sufficient risk factor, but this is not the case. There is an increased number of individuals with RA who have two disease-associated HLA-DRB1 alleles (Wordsworth and Pile 1992). In 1992, Nepom and Nepom suggested that the risk of developing clinical RA among Caucasians is approximately 5 times higher in individuals expressing 2 disease-associated HLA-DRB1*04 alleles than individuals with 1 allele (Nepom and Nepom 1992). These data show that

the simple model of a dominant susceptibility gene is not sufficient to explain the HLA-DR association with RA.

The majority of patients express 2 different disease-associated alleles and truly homozygous RA patients are rare. To examine the nature of the synergistic action of 2 disease-associated alleles, Goronzy and Weyand compared patients with a double dose RA-associated allele to patients with a single dose. Their results revealed that RA patients with a double dose associated allele had more aggressive and destructive disease. Sixty one percent of patients with a disease linked HLA-DRB1*04 allele on both haplotypes required joint replacement surgery in the first decade of disease compared to 25% of patients with a single dose allele. In addition, all of the patients in the study that were homozygous for HLA-DRB1*04 developed major organ involvement (Goronzy and Weyand 1993). These data imply that there is a correlation between HLA-DRB1 genotype and joint destruction as well as extra-articular manifestations in RA patients.

There is no convincing evidence that the onset of synovitis is a HLA-DRB1 dependent phenomenon (Goronzy and Weyand 1995; Weyand *et al.* 1992). It has been hypothesized that the biologic effect of the HLA genes is on the formation of the TCR repertoire. Therefore, the range of T cell receptors that an individual has may determine the disease manifestations and disease pattern after the initiating event.

2.2 T Lymphocyte Involvement

In spite of the increasing knowledge concerning the basic immunology of T lymphocyte function, the role of T cells in RA remains elusive. T lymphocytes comprise 30 to 50% of the synovial tissue cells (Firestein 1994). The T lymphocytes within the synovium are predominantly TCR $\alpha\beta$ ⁺, CD4⁺, CD45RO^{hi}, and CD29^{hi}; however, a small percentage of activated TCR $\alpha\beta$ ⁺ CD8⁺

and TCR gamma delta cells are also found (Firestein 1994). There are two basic histologic patterns of the T cell infiltrates in the RA synovium. Aggregates of the lymphocytes, which can contain up to several hundred T cells, collect in the sublining areas. The size and number of aggregates varies between patients. A second pattern is a diffuse T cell infiltration without obvious organization. In this pattern, T cells are located below the lining and extend into the sublining (Firestein 1994).

The majority of T cells are HLA-DR⁺ CD45RO⁺, CD29^{hi} which suggests previous exposure to antigen followed by a post-activational state (Firestein 1994). Within the synovium, plasma cells secrete IgG and IgA, thus there is strong evidence for T cell activation because T cells are required for class switching (Williams 1994). Although the majority of synovial membrane T cells are activated, only a minority are CD25⁺ (Goronzy and Weyand 1995). CD25 is upregulated on T cells upon antigenic stimulation, thus the low expression might imply that these cells were either previously activated, have been activated by TCR-independent pathways such as ligation with a costimulatory molecule CD28, or they are functionally anergic. In addition, analysis of CD25⁺ T cells might involve selection of a small subpopulation of T cells that are involved in the pathogenesis of the disease. T cells within the synovium produce other cytokines, such as IL-4, and IFN-gamma (Feldman *et al.* 1996), yet investigators have not fully analyzed the cytokine receptor expression on synovial T cells. Although there is evidence of T cell activation, there is little evidence for T cell proliferation (Lia *et al.* 1995).

Many investigators continue to support the concept that RA is a T-cell-mediated disease (Goronzy and Weyand 1995; Gregersen *et al.* 1987). These researchers propose a model of an antigen recognition event in the synovial tissue whereby an initial number of autoreactive T cells recognize an arthritogenic processed antigen in context with MHC. Several techniques have been

used to probe for T cell clonality in the synovial fluid and tissue. Lymphocyte oligoclonality has been found in some studies; however, the frequency of the clonally expanded T cell was not high. The low frequency of clonal proliferation is consistent with a model of an antigen-specific response. For example, Doherty and coworkers determined the frequency of influenza specific T cells in different target tissues of virus-infected mice. They reported that although 50% of the locally infiltrating cells secreted lymphokines, only 1% of the T cells were antigen-specific (Doherty *et al.* 1992). Therefore, all of the T cells within synovium may not proliferate and differentiate in response to a mitogenic stimulation within the synovium but have the ability to produce inflammatory cytokines. Such cytokines may stimulate antigen presenting cells to secrete matrix metalloproteinases as well as increase the expression of adhesion molecules on the vascular endothelium. Many of the T cells in the joint may have been drawn in due to the inflammatory process and a subset of these T lymphocytes may be antigen specific or autoreactive.

Another approach to analyze the TCR repertoire in RA patients has compared the V_{α} and V_{β} segment usage in the peripheral blood, synovial fluid, and tissue T cells. This approach is based on animal model studies, where the animal is immunized with an antigen that closely resembles a self-antigen. The immune response generated is often restricted, selecting particular V_{α} and V_{β} TCR gene segments (Houry and O'Sullivan 1995). Studies to determine the frequency of V_{α} and V_{β} usage in all 3 compartments have yielded conflicting results (Goronzy and Weyand 1995; Jenkins *et al.* 1993; Krawinkel and Pluschke 1992; Khazali *et al.* 1995; Lunardi *et al.* 1992; Fischer *et al.* 1996), and the studies essentially do not support the view that the synovial TCR repertoire of RA patients is dominated by select T cells. Recently, Ignatowicz and coworkers (1996) determined that thymuses expressing a single MHC/peptide combination continued to permit large numbers of thymocytes with different TCRs to mature. They described a transgene-encoded

protein in mice lacking endogenous class II and invariant chain. All the detectable class II in these mice carried the transgene encoded peptide and the CD4⁺ T cells were efficiently positively selected. The selected CD4⁺ T cells reacted with the same class II protein, carrying an array of other self-peptides with extraordinarily high frequency. The cells also reacted with foreign class II with high frequency (Ignatowicz *et al.* 1996). From these results one can speculate, that the T lymphocytes infiltrating the inflamed joint may express various TCRs and still be able to interact with the same processed antigen in context with MHC but with a different affinity, or that one specific TCR can interact with a variety of processed peptides with varying affinity. The infiltrating T cells with various TCRs may reflect an immune dysregulation in the thymus. Alternatively, the T cell may be secondary to the disease initiation and be responsible for sustaining the disease. The T lymphocytes may react with a variety of self-antigens that were previously hidden prior to the cartilage and bone destruction by enzymes and oxygen radicals.

There is unequivocal evidence that T lymphocytes are present in RA synovium. The genetics of the HLA-DRB1 association as well as the TCR studies in RA are consistent with the model that multiple T cell specificities are involved in the progression of the disease. However, it has not been conclusively determined if T lymphocytes contribute to the disease initiating event(s).

2.3 Infectious Etiology of RA

I have just recently discussed the roles that T lymphocytes and HLA genetics may play in the pathogenesis of rheumatoid arthritis. Interestingly, RA has been proposed to be caused by an infectious agent. Although there has never been substantial evidence to support this claim, polyarthritis or monoarthritis can occur during many bacterial and viral infections (Wordsworth and Pile 1994).

2.3i Bacterium:

Despite 50 years of extensive study, there is a lack of evidence implicating a bacterial pathogen as the cause of RA. *Mycoplasma* has been thought to be the cause of RA because it can induce experimental arthritis in rabbits (Cole 1977). Reports have appeared that *Mycoplasma* was isolated from the synovial fluid and membrane in humans, only for a short time after the inception of the inflammatory synovitis and then subsequently becomes undetectable (Harris 1993). It has been observed that RA patients have large amounts of *Clostridium perfringenes* in their feces; however, *Clostridium* outgrowth has also been documented in other chronic inflammatory rheumatic diseases and in individuals without disease (Zvaifler 1989). Attempts to isolate bacterial cell wall components in the biopsies of RA synovium also have proved to be futile (Pritchard *et al.* 1980). Adjuvant induced arthritis in rats uses Freund's complete adjuvant containing antigenic components of *Mycobacterium tuberculosis*. Reactivity against mycobacterial antigens has been demonstrated by T cells from RA patients, although this reactivity could be due to the lysis of cells and upregulation of heat shock protein on the cell surface (Holoshitz *et al.* 1986). The RA patient may produce antibodies to *Mycobacterium tuberculosis* heat shock proteins which may cross-react with the heat shock proteins expressed on self cells. It is unlikely that *Mycobacterium tuberculosis* is responsible for RA, but cross reaction between environmental antigens and self-proteins is an interesting hypothesis.

2.3ii Viruses:

There is an interesting hypothetical link between the RA-associated HLA-DR4 and DR1 molecules and Epstein Barr Virus (EBV). The EBV glycoprotein gp110 resembles HLA-DR4 and

DR1 molecules, thus patients with an EBV infection could develop antibodies that can cross-react with the RA susceptibility cassette on HLA-DR4 and DR1. Investigators have demonstrated EBV genome in RA synovial tissue; however, EBV genome has also been found in normals (Roudier *et al.* 1988).

Hepatitis B infection is complicated by arthritis in 10 to 30% of the cases (Duffy *et al.* 1976), but only once has the subsequent development of classic RA been reported (Morris and Stevens 1978). When injected, rubella virus can localize in cartilage and inflammatory polyarthritis has been noted after natural infection or immunization. Rubella can elicit a broad spectrum of articular responses, but there is insufficient evidence that it can induce RA (Grahame *et al.* 1983). The human B19 strain of parvovirus causes a common exanthematous disease in children and was the most frequently identified etiologic agent in patients with early synovitis (White *et al.* 1984). However human B19 parvovirus association with classical RA is yet to be verified (Simpson 1984).

2.3iii Superantigens

Exogenous stimuli such as vaccination, infection, or superantigens can lead to changes in the TCR repertoire over time. Superantigens are proteins produced by microorganisms which specifically bind to the CDR4 region of the V β element of the TCR and the MHC β chain leading to an expansion of T cells which share the same TCR, thus skewing the repertoire. It has been proposed that superantigens initiate a hyperresponsive state to self-antigens and may be responsible for the loss of T cell tolerance resulting in the synovial infiltration. The superantigen hypothesis has not gained acceptance because investigators have shown that repeated stimulation of T cells with superantigens leads to their death and thus would not be able to survive within the synovium (Goronzy and Weyand 1995; Choi *et al.* 1989). A skewing of the TCR repertoire, which

would be expected if a superantigen is present, has also not been conclusively demonstrated (Goronzy *et al.* 1995; Jenkins *et al.* 1993; Krawinkel and Pluschke 1992).

3. CELLULAR AND BIOCHEMICAL ASPECTS OF RHEUMATOID ARTHRITIS

3.1 Adhesion Molecules Involved in the Inflamed Synovium

The presence of infiltrating leukocytes is a fundamental feature of RA. Circulating leukocytes adhere to post-capillary high endothelial venules (HEV) in the synovium. After the leukocytes have infiltrated the tissue, they produce an array of cytokines including, IL-2, TNF α , IL-1, IFN-gamma, GM-CSF, and IL-6, which perpetuate the inflammatory cycle (Liao and Haynes 1995; Feldman *et al.* 1996).

3.1*i* Selectins

The selectin family of adhesion molecules are involved in the initial shear resistance adhesion of leukocytes to endothelial cells to recruit leukocytes to sites of inflammation. Selectins are highly glycosylated molecules which contain a short cytoplasmic region, a single membrane spanning domain, and an extracellular portion. The extracellular domain is characterized by a lectin-like domain, epidermal growth factor-like domain, and a variable number of complement regulatory domains. Selectins bind through Ca²⁺ dependent recognition of carbohydrate moieties (Amaout 1993; Liao and Haynes 1995).

L-selectin (CD62L) is expressed on leukocytes and is involved in the initial stages of leukocyte attachment to HEV. CD62L interacts with its ligand Glycam-1 on endothelial cells.

Binding leads to leukocyte rolling, CD62L shedding, and upregulation of other adhesion molecules to consolidate attachment (Oppenheimer-Marks and Lipsky 1994; Varani *et al.* 1994).

E-selectin (CD62E) is upregulated on endothelial cells following TNF α , IL-1, or IFN-gamma stimulation (Abbot *et al.* 1992). The peak expression of CD62E occurs within 4 hours and usually disappears from the cell surface after 24 hours, despite the continual presence of stimulus (Amaout 1993). The ligand for CD62E is a carbohydrate moiety, sialyl Lewis X. CD62E is involved in leukocyte binding to endothelium and is upregulated in the synovial microvasculature (Abbot *et al.* 1992; Koch *et al.* 1991).

P-selectin (CD62P) is normally stored in the α granules of platelets and Weibel-Palade bodies of endothelial cells. CD62P binds to P-selectin glycoprotein ligand. Stimulation of endothelial cells with proinflammatory agents such as thrombin, histamine, or oxygen radicals results in a transient expression of CD62P on the surface of the cells (Liao and Haynes 1995; Varani *et al.* 1994). P-selectin only binds activated T cells. T lymphocytes isolated from RA synovial fluid bind well to P-selectin. Studies evaluating leukocyte binding to frozen sections have demonstrated the CD62P-dependent adhesion of monocytes is 3 to 20 fold greater on RA synovium than on skin. In this assay, the specific binding of monocytes to RA synovial tissue venules could be blocked by 90% with anti-CD62P, 20 to 50% by anti-CD62E, and 30 to 40% by anti-CD62L MAb (Damle *et al.* 1992).

3.1ii CD44

CD44 is a lymphocyte-homing receptor mediating specific adhesion of lymphocytes to mucosal HEV and hyaluronate (Goldstein *et al.* 1989; Aruffo *et al.* 1990). CD44 is found on T cells, B cells, monocytes, macrophages, endothelial cells, and fibroblasts (Liao and Haynes 1995).

CD44 ligation of T cells results in homotypic adhesion of T cells, IL-2 production, and T cell costimulation (Haynes *et al.* 1991a; Haynes *et al.* 1991b). CD44 ligation of monocytes results in IL-1 release, and monocyte-T cell aggregation (Haynes *et al.* 1991a). T lymphocytes derived from the synovial fluid have demonstrated an upregulated expression of CD44 (Kelleher *et al.* 1995).

3.1iii Integrins

The integrins are heterodimeric surface membrane receptors mediating divalent cation dependent cell-cell and cell-matrix interactions. Each integrin is composed of an α chain non-covalently associated with a β subunit. This association is required for cell surface expression. Each α subunit consists of a short C-terminal cytoplasmic domain, a membrane spanning region, and a large extracellular region consisting of 7 tandem repeats (Amaout 1993). The β subunit is smaller than the α (except for $\beta 4$). The β chain is composed of a large N-terminal extracellular region containing in its C-terminal half a characteristic cysteine rich motif repeated 4 times. The β subunit spans the membrane once, and has a short cytoplasmic tail (Amaout 1993).

The integrins can be divided into several sub-families based on the nature of their associated β subunit. The largest family contains the $\beta 1$ integrin (CD29) and the $\beta 1$ integrin is highly expressed on RA synovial fluid and tissue T lymphocytes (Rodriguez *et al.* 1992; Elices *et al.* 1994). $\beta 1$ integrin can associate with 10 different α chains. The $\alpha 4\beta 1$ integrin mediates transendothelial migration through contact with vascular cell adhesion molecule-1 (VCAM-1) which is expressed on endothelial cells and on the synovium (Chan *et al.* 1992; Johnston *et al.* 1996; Morales-Ducret *et al.* 1992). VCAM-1 is upregulated by cytokine stimulation: IL-1, TNF- α , and IFN- γ , all of which are released by inflammatory cells in the synovium (Elices *et al.* 1990).

Several integrins can mediate binding to a single ligand; for example, $\alpha 4 \beta 1$, $\alpha 4 \beta 7$ and, $\alpha 5 \beta 1$ can bind to fibronectin (Elices *et al.* 1990; Hemler 1990). Fibronectin is a dimer with a molecular weight of 550 KDa whose 2 chains are linked by 2 disulphide bonds near the COOH terminus. Fibronectin is a multifunctional protein capable of homotypic and heterotypic interactions with collagens, heparin, and cell surface receptors (Damell *et al.* 1990). The diversity of fibronectin is generated by alternative splicing of a primary fibronectin RNA transcript. The fibronectin gene contains 3 separate exons that are subject to alternative splicing, termed EIIIA, EIIIB, and IIICS. The presence of additional acceptor and donor splice signals within the IIICS region allows generation of increased diversity in fibronectin by virtue of multiple IIICS polypeptide variants (Elices *et al.* 1994). Despite the apparent redundancy of the integrins, binding of different receptors to the same ligand is usually mediated by 1 or more distinct sites on the ligand. For example, the integrin $\alpha 5 \beta 1$ binds to the RGD (arginine-glycine-aspartic acid) fibronectin domain (Pytela *et al.* 1985), and $\alpha \beta 1$ binds to the CS-1 domain containing a 25 amino acid sequence (Humphries *et al.* 1986; Wayner *et al.* 1989). This characteristic probably permits different signals to be transmitted by the same matrix component through more than one integrin receptor. In 1994, Elices and associates reported that CS-1 fibronectin fragment is selectively expressed on RA but not normal synovium. In contrast to the extracellular matrix forms of fibronectin, CS-1 expression was upregulated on the RA synovial vasculature and intimal lining. CS-1 on the RA synovium specifically localizes to the lumenal surface of endothelial cells and is capable of mediating intravascular attachment of T cells that express a functional $\alpha 4 \beta 1$ integrin (Elices *et al.* 1994).

Signaling through the integrin receptor $\alpha 4 \beta 1$ is implicated in costimulation of T lymphocytes (Maguire *et al.* 1995). Actin-binding proteins that co-localize with integrins in focal

adhesion include α -actinin, talin, vinculin, and tensin. These structural proteins are believed to play important roles in stabilizing cell adhesion and regulating cell shape, morphology, and mobility. They may also serve as a framework for the association of signaling proteins that regulate signal transduction pathways leading to integrin-induced changes in behavior (Clark and Brugge 1995).

The $\alpha_4\beta_7$ integrin is found on leukocytes that home to the Peyer's Patches and intestinal epithelium (Shyjan *et al.* 1996) as well as on RA synovial tissue T cells (Lazarovits and Karsh 1993). The $\alpha_4\beta_7$ protein interacts with mucosal addressin adhesion molecule (MAdCAM-1) expressed on the intestinal epithelium (Shyjan *et al.* 1996; Erle *et al.* 1994), fibronectin expressed in the extracellular matrix (Chan *et al.* 1992), and VCAM-1 expressed on endothelial cells (Chiu *et al.* 1995).

The β_2 integrins generally mediate homotypic and heterotypic cell-cell interactions. The β_2 chain (CD18) is expressed on leukocytes and is found in association with 3 different α chains (CD11a, b, c) (Amaout 1990). Lymphocyte Functional Associated Antigen-1, LFA-1, (CD11a/CD18) binds to ICAM-1, 2, and 3 and helps to mediate endothelial migration of leukocytes in synovium (Oppenheimer-Marks *et al.* 1990). Synovial tissue T cells express an increased level of LFA-1 compared to peripheral blood T lymphocytes (Takahashi *et al.* 1992).

It appears that many adhesion molecules on vascular endothelium are upregulated in the RA synovium. This upregulation may be due to an intrinsic change in the endothelium or secondary to the release of cytokines such as IL-1, TNF- α , and IFN-gamma by synoviocytes and the infiltrating MNC (Liao and Haynes 1995; Feldman *et al.* 1996). The MNC infiltrating the synovium also appear to have unregulated various adhesion molecules.

3.2 REACTIVE NITROGEN AND OXYGEN SPECIES AND THEIR INVOLVEMENT IN RA

RA is characterized by actively inflamed joints. Macrophages, lymphocytes, and mast cells are located in the synovial tissue. Polymorphonuclear cells (PMN) are found in the synovial fluid, and deposits of fibrin and PMN infiltration are located on the synovial membrane (Zvaifler 1989). The activated leukocytes would be expected to release toxic oxygen metabolites and there is a great deal of evidence suggesting that reactive nitrogen and oxygen species (RNS and ROS) may play a role in the inflamed synovium (Kaur and Halliwell 1994; Sakurai *et al.* 1995).

Recently, nitric oxide (NO) a reactive nitrogen and oxygen species has been identified as a potent and pleotropic mediator. The function of NO as an intracellular messenger is enhanced by its low molecular weight, high diffusibility, and lipid solubility while its cellular effects are due to its reactivity as a free radical (Stuehr and Griffith 1992). NO is synthesized by a constitutive Ca^{2+} -calmodulin-dependent NO synthase (cNOS) within macrophages, endothelial cells, neurons, adrenal cells, and other cells. NO is also generated in macrophages, PMN, lymphocytes, and hepatocytes by an inducible nitric oxide synthase (iNOS). iNOS is not Ca^{2+} -calmodulin-dependent, is induced by cytokines, and is inhibited by glucocorticoids. Its expression results in the sustained release of NO in amounts that far exceed those produced by the constitutive pathway (Stuehr and Griffith 1992).

Recently, there has been a great deal of evidence to suggest that reactive nitrogen and oxygen species play a role in the pathogenesis of RA. Lunec and Blake (1985) found that greater than 90% of synovial fluid and serum of the RA patients tested had evidence of free radical oxidation products. Several investigators have also reported that the serum antioxidant level, such as vitamins E and A, reduced in RA patients (Halliwell *et al.* 1988; Heiovaara *et al.* 1994). Farrell and coworkers were able to measure the concentration of nitrite, a breakdown product of NO, in the

serum and synovial fluid of RA and OA (osteoarthritic) patients, and controls. The concentrations of serum nitrite from the RA and OA patients were significantly greater than controls. As well, synovial fluid nitrite levels were significantly elevated in RA compared to OA patients (Farrell *et al.* 1992). Recently, Sakurai and associates have shown that iNOS was predominantly expressed in CD14⁺ synoviocytes, endothelial cells, chondrocytes and to a lesser extent macrophages and synovial fibroblasts (Sakurai *et al.* 1995). The cartilage from OA patients was reported to spontaneously release micromolar concentrations of NO and this production was influenced by the presence of inflammatory cytokines (Amin *et al.* 1995). Stimulated macrophages can produce IL-1, which can act to stimulate PMN, synoviocytes, and chondrocytes to produce NO and metalloproteinases (Farrell *et al.* 1995; Rosen *et al.* 1995; Krane 1989).

NO production is upregulated in OA and RA synovium and may serve as a pathogenic mediator of connective tissue destruction in arthritis. Thus, in states of chronic inflammation, NO and its break-down products, could cause severe genetic damage to the surrounding cells which could be a mechanism for genetic damage in the rheumatoid synovium. The genetic damage of infiltrating lymphocytes may be then used as a marker of RNS and ROS within the inflamed joint.

4. AN INCREASE IN THE FREQUENCY OF MUTANT T CELLS IN THE PERIPHERAL BLOOD OF PATIENTS WITH AUTOIMMUNE DISEASES

Hypoxanthine guanine phosphoribosyl transferase (*hprt*) encodes an enzyme involved in the purine salvage pathway. The *hprt* T cells lack a functional HPRT protein, thus can convert non-toxic 6-thioguanine (6-TG) to a toxic nucleotide. Cells that lack the HPRT function are able to grow in the presence of 6-TG, while non-mutant cells do not survive (Albertini *et al.* 1982; Albertini

et al 1985). The hemizygous nature of the *hprt* locus permits the recovery of mutations primarily affecting the function of the single gene (Fusco *et al.* 1992b). Therefore, by comparing the growth of T cells in the presence and absence of 6-TG it is possible to estimate the *in vivo* frequency of mutant T cells.

An elevation in the frequency of *hprt* mutant T lymphocytes has been reported in the peripheral blood of patients with Multiple Sclerosis (MS) (Allegretta *et al.* 1990; Lodge *et al.* 1994; Sriram 1994) and Systemic Lupus Erythematosus (SLE) (Dawisha *et al.* 1993; Gmelig-Meyling *et al.* 1992; Theocharis *et al.* 1995). In MS patients, the increased frequency of *hprt* mutant T cells correlated with patients who had chronic progressive disease and severe disease. Interestingly, the mutant T cell clones from MS patients were more frequently reactive to myelin basic protein, a protein implicated in the pathogenesis of the disease, than non- mutant clones or control clones. DNA blot analysis revealed that the mutant T cell were not derived from a single cell but expressed different TCR (Allegretta *et al.* 1990). There also was a correlation between frequency of mutant T cells derived from the peripheral blood of SLE patients and cumulative active disease and disease duration (Dawisha *et al.* 1993). Further investigations utilizing a PCR strategy demonstrated that the increase in the frequency of *hprt* mutant T cells was not due to clonal expansion (Theocharis *et al.* 1995). These studies hypothesized that mutation occurs preferentially in dividing T cells, presumably as a result of errors of replication, temporally insufficient DNA repair, or fixation of mutation. However, no definitive experiments were completed to verify if indeed the T lymphocytes from either MS or SLE patients displayed enhanced proliferative capacity, errors in DNA repair enzymes, or errors in the replication machinery.

5. STATEMENT OF HYPOTHESIS

T lymphocytes represent a dominant proportion of the MNC infiltrating the rheumatoid synovium and are important in the pathogenesis of RA. Most of these T cells are CD4⁺, CD29^{hi}, CD45RO⁺, and HLA-DR⁺, indicating a memory cell phenotype. Many T cells enter and remain within the synovium for an undefined time period and subsequently may re-enter the peripheral circulation. Evidence suggests that a subset of T lymphocytes within the inflamed rheumatoid synovium may be autoreactive or antigen-specific.

An elevation in the frequency of hypoxanthine guanine phosphoribosyl transferase (*hprt*) mutant T cells has been reported in the peripheral blood of MS (Allegretta *et al.* 1990; Lodge *et al.* 1994; Sriram 1994) and SLE (Dawisha *et al.* 1993; Gmelig-Meyling *et al.* 1992; Theocharis *et al.* 1995). patients. This increase in mutation frequency has been attributed to a postulated increase in errors in replication or insufficient DNA repair associated with a disease-related enhancement of T cell proliferation. Since T lymphocytes also appear to be important in RA, I examined the *hprt* mutant frequency in the peripheral blood, as well as the synovial fluid, and synovial tissue of these patients. There is little evidence for T cell proliferation within the synovial tissue but activation markers are expressed on synovial tissue T lymphocytes (Lia *et al.* 1995). There is insufficient evidence that proliferation alone is associated with genetic mutation, nevertheless, proliferating T cells may have an increased probability of mutation depending on the environment. Thus, I hypothesized that proliferation alone is insufficient to induce an elevated mutation frequency in T lymphocytes from RA patients. The rheumatoid synovium is a mitogenic and mutagenic factors environment, and both of these factors are required for the induction of certain types of mutations. Mitogenic factors for T cells identified in the joint include antigen presenting cells such as

macrophages and dendritic cells, and various cytokines including IL-4, IFN-gamma, and IL-2; although, the latter cytokine is found at low levels in the synovium (Goronzy *et al.* 1995; Panayi *et al.* 1992). Potentially genotoxic and cytotoxic RNS and ROS have been found in rheumatoid synovium. These lines of evidence support the notion that the inflamed joint of RA patients contains the necessary mitogenic and mutagenic factors that could cause T cell mutations.

Therefore, I have utilized a strategy to isolate a rare subset of T lymphocytes that may have potentially been mitogenically stimulated and genetically damaged. Using the *hprt* clonal assay that has been previously described, I have analyzed a population of mutant T lymphocytes from RA patients. The hemizygous nature of the *hprt* locus permits the recovery of mutations primarily affecting the function of the single gene (Fusco *et al.* 1992b). By comparing the growth of T cells in the presence and absence of 6-TG it is possible to estimate the *in vivo* frequency of mutant T cells (Albertini *et al.* 1982; Albertini 1985).

With the insight that the inflamed synovium is a potentially mitogenic and mutagenic environment, I hypothesize that there will be an elevated frequency of *hprt* mutant T cells within the peripheral blood, synovial fluid, and synovial tissue of RA patients compared to controls. The RA peripheral blood mutant T lymphocytes may have acquired this mutation in the inflamed synovium and then re-entered the peripheral circulation, and thus may display a similar phenotype as the RA synovial tissue derived T lymphocytes.

6. SPECIFIC OBJECTIVES

1. To determine the frequency of *hprt* mutant T lymphocytes in the peripheral blood, synovial fluid, and synovial tissue of RA patients and controls.
2. To verify that the increase in the frequency of mutant T cells in the peripheral blood of RA patients is not due to the *in vitro* culture conditions.
3. To correlate the corrected frequency of mutant T lymphocytes from the various compartments of the RA patients with clinical disease activity, disease parameters, and current disease treatment.
4. To clone the mutant and non-mutant T cells from RA patients and controls, and to characterize the cell surface markers: CD3, CD4, and CD8, using flow cytometry.
5. To define the functional significance of the mutant and non-mutant peripheral blood T cell clones through a fibronectin adhesion assay and flow cytometric analysis.

CHAPTER 2: MATERIALS AND METHODS

1. DESCRIPTION OF PATIENTS AND HEALTHY CONTROLS

The patient group consisted of 90 RA and 10 OA patients. The patients were followed at the Ottawa General Hospital Rheumatic Disease Unit. Patients fulfilled the diagnostic criteria of the American College of Rheumatology for the classification of RA of at least 6 months duration (Arnett *et al.* 1988) or a clinical diagnosis of OA of the knee or hip. A peripheral blood sample was obtained from 80 unselected RA patients from the outpatient clinic. A synovial tissue and peripheral blood sample was obtained from 8 RA and 8 OA patients following joint replacement surgery. Synovial fluid was obtained from 2 RA patients. The 20 controls were from available hospital and university personnel. The protocol for obtaining peripheral blood, synovial fluid, and synovial tissue samples from patients and controls was approved by the institutional review board.

2. MEASURES OF CLINICAL DISEASE PARAMETERS

The clinical evaluation of each RA patient included the patient's age in years, duration of RA in years, duration of morning stiffness in minutes, the patient's assessment of pain on a 100 mm anchored horizontal visual analogue scale, the patient's assessment of disease activity on a 100 mm anchored horizontal visual analogue scale, present medication, presence of rheumatoid nodules, presence of joint erosions on X-rays, the number of tender joints, and smoking status. Evidence has arisen that smoking and increasing age can increase the frequency of mutant T cells in the peripheral blood (Jones *et al.* 1993; Jones *et al.* 1995). The clinical laboratory tests included the presence of rheumatoid factor (RF) using latex fixation, erythrocyte sedimentation rate (ESR) using the Westergren method, and the level of C-reactive protein (CRP). The ESR and CRP levels

are used to determine systemic inflammation. 'Active' RA was defined by the presence of 3 of the following criteria: ≥ 9 tender joints, ≥ 6 swollen joints, ≥ 45 minutes of morning stiffness, and ESR ≥ 28 mm/hour. The ESR for a healthy individual is approximately 20 mm/hour. This is the definition of 'active' RA patients for inclusion in clinical trials (Felson *et al.* 1993).

3. CLONING OF T LYMPHOCYTES

3.1 Assay For *hprt*⁻ T Lymphocytes

T lymphocytes were cultured to establish the frequency of *hprt*⁻ mutant T cells (Albertini *et al.* 1982; Albertini 1985). Peripheral blood MNC were isolated from a 20 ml heparinized venous blood sample from RA patients and controls by gradient centrifugation over Ficoll-Hypaque (Pharmacia Biotech Inc., Piscataway, NJ). A standard limiting dilution protocol was used to determine the T lymphocyte cloning efficiency by seeding 1, 5, and 10 MNC/well. MNC were plated in a 96-well round bottom plate (Diamed Laboratories) containing 200 μ l of RPMI-1640 pH 7.3 (Canadian Life Technologies, Burlington Ont.) supplemented with 10% Fetal Bovine Serum (Canadian Life Technologies), 20% Hybridoma Serum Free Medium (Canadian Life Technologies), 30 IU/ml of IL-2 (Sigma Chemicals Ltd. St.-Louis MO.) (Figure 15), and 2 mM L-glutamine (Canadian Life Technologies). This medium is referred to as complete medium. Figure 15 in the appendix describes an IL-2 titration used to determine the appropriate concentration of IL-2 to be used. The plated MNC were stimulated with 1.0 μ g/ml of phytohemagglutinin (PHA) (Sigma Chemicals Ltd.). PHA is a commonly lectin commonly implemented to stimulate T lymphocytes. This lectin is able to bind to the TCR and costimulatory molecules to provide to appropriate signal for T cell activation and proliferation. Figure 16 in the appendix describe a titration to determine the

necessary amount of PHA to use. The T cells were also stimulated with 1.0×10^4 TK6 *hprt*⁻ feeder cells/well (from D. Wilkinson), inactivated with 80 Gy ¹³⁷Cs Gamma Cell-40. at the University of Ottawa. The dose rate was approximately 1.1 Gy/minute. TK6 cells are a lymphoblastic cell line and as feeder cells provide the necessary cognate interactions to insure adequate T cell growth. The TK6 cells had a deletion in the *hprt* gene, thus these T cell mutations could be examined later without the contamination of the TK6 cells. The *hprt*⁻ mutant T lymphocytes were isolated by plating 2.0×10^4 MNC/well in complete medium containing PHA, irradiated TK6 feeder cells, and 1×10^{-4} M 6-TG (2-amino-6-mercaptopurine from Sigma Chemicals Ltd.). Cultures were maintained in a 5% CO₂ / 95% O₂, 37°C incubator for 21 days.

3.2 Synovial Fluid

Synovial fluid was obtained from 2 RA patients in a sterile syringe. The fluid was diluted 5 times in PBS and the MNC were isolated by gradient centrifugation over Ficoll-Hypaque. The collected cells were plated and a *hprt* clonal assay was performed as previously described.

3.3 Synovial Tissue

Synovial tissue was obtained from 8 RA and 7 OA patients undergoing joint replacement surgery. The tissue was finely minced with sterile scissors and immediately submerged in a Petri dish [150 x 75 mm² or 10 x 20 mm² (Fisher, Nepean Ont.) depending on sample size], containing complete medium. The tissue was maintained in a 37°C, 5% CO₂ incubator. On day 2, MNC were isolated by gradient centrifugation over Ficoll-Hypaque and plated in a 10 x 20 mm² Petri dish

with CM. On day 4, the tissue was digested with 1 μ g/ml of collagenase type II (Sigma Chemicals Ltd.) while shaking in a sterile tube for 90 minutes and the MNC were collected, washed, and plated in CM. Collagenase is an enzyme that will help degrade the extracellular matrix and permit to release of synovial tissue T lymphocytes. Every 2 days, the cells were harvested from the tissue sample and plated in complete medium. On day 10, the collected cells were pooled, and cell viability was determined using trypan blue dye exclusion. When approximately 6×10^6 MNC had been isolated, the *hprt*⁻ clonal assay was performed, as described above. A peripheral blood sample was obtained from the surgical patients 2 to 10 days after receiving the synovial tissue. The *hprt* clonal assay was then complete using T cells from the peripheral blood and synovial tissue in parallel. This permitted us to compare the cloning efficiency and the frequency of mutant T cells in the peripheral blood and synovial tissue of the same patient.

Synovial tissue T cell lines were established from the synovium of RA and OA patients. The synovium was placed in a Petri dish containing complete medium. Every 2 days the T cells were isolated from the tissue and this subset of T cells were stimulated with fresh complete medium supplemented with PHA. When T cells could no longer be isolated, the collected cells were pooled and the cells were frozen in liquid nitrogen. Prior to using the cell lines for flow cytometric analysis or adhesion assay the synovial tissue T cell lines were thawed and stimulated with complete medium containing PHA. The synovial tissue T cells remained in CM for 14 to 21 days to allow maximal integrin expression.

3.4 Determining the Cloning Efficiency and the Frequency of Mutant T Lymphocytes

The growth of T lymphocyte clones was scored visually on day 21 of the assay using an inverted microscope. Cloning efficiency (CE) was calculated using the assumption of a Poisson distribution of clonable cells in limiting dilution studies (Eichmann *et al.* 1980; Albertini *et al.* 1982), from the following equation:

$$CE = -\ln \left(\frac{\text{fraction of negative wells}}{\# \text{ of MNC/well}} \right) \times 100\%$$

The CE in non-selection and selection medium was determined. The CE in the selection medium (containing 6-TG) is referred to as the frequency of mutant T cells (FMC). The corrected Frequency of Mutant T cells (cFMC) was calculated as (Albertini *et al.* 1982):

$$cFMC = \frac{\text{CE in selection medium containing 6-TG (FMC)}}{\text{CE in non-selection medium}}$$

Thus, the FMC represents the 'raw' data and the cFMC represents a comparison of the CE in selection versus non-selection medium.

3.5 Radiolabelling Method To Validate Visual Scoring of T Cell Clones

To ensure the validity of visual scoring, T cell clones were also assessed for their proliferative capacity by a radiolabelling method. Following the visual scoring, T cell clones were labeled with 1 μ Ci of [*methyl*-³H]-thymidine (Amersham Life Sciences Inc.) for 16 to 18 hours. Cells were harvested on glass fiber filters (Packard: A Canberra Company, Meriden CT.) using the Packard Filtermate 196 matrix harvester. The filters were cut out and placed into scintillation vials containing 1 ml 0.1% NaOH solution overnight. The following day, 9 ml of scintillation cocktail (BDH, Darmstadt Germany) was added and after 2 hours, the vials were counted using a scintillation counter.

To determine the appropriate concentration of PHA or IL-2 to stimulate the T cells a [*methy*-³H]-thymidine incorporation assay was also used. Cells were plated with various concentrations of either protein. The cells were harvested on glass fiber filters and the results were read using a β counter.

3.6 The Frequency of Mutant T Cells in the Absence or Presence of Catalase

Mononuclear cells may secrete genotoxic species including H₂O₂. H₂O₂ can penetrate the cell membrane and interact with transition metals bound to DNA to produce OH \cdot , which in turn can fragment the DNA. Catalase converts 2 H₂O₂ into 2 H₂O and O₂, thus preventing DNA damage. The assay for *hprt* lymphocytes was completed with 3 RA patients and 3 controls. Catalase (10 μ g/ml) (Sigma Chemicals Ltd.) was added to 4 of the 8 selection plates containing 6-TG. The clones were permitted to grow for 21 days in a 5% CO₂, 37°C incubator. The FMC and cFMC were calculated for the cells plated in the presence or absence of catalase.

3.7 Expansion of T Lymphocyte Clones

Following a 21 day incubation, mutant and non-mutant T lymphocyte clones from patients and controls were split and replated into fresh 96-well round-bottom plates containing 100 μ l of CM supplemented with 1.0 μ g/ml of PHA, and 1.0 x 10⁴ irradiated TK6 *hprt* feeder cells/well. The mutant clones were maintained in 1 x 10⁻⁴ M 6-TG. After a further 21 day incubation, the clones were split into 4 wells and restimulated once again. When a clone filled all 4 wells, the cells were transferred to a 24 well flat bottom plate. Approximately 1 ml of complete medium was added every 7 days to the growing clone and every 14 to 21 days the clones were stimulated with 1.0

ug/ml of PHA and 1.0×10^4 irradiated TK6 *hprt* feeder cells. When the cell number reached 1×10^6 , the clone was frozen or used for further studies.

4. FLOW CYTOMETRY

The fluorochromes to be detected by the argon laser of the flow cytometer must be excited at approximately 488 nm. The excited fluorochrome then emits its energy at a specific wavelength. A total of 2.5×10^5 T cells from control and RA patient peripheral blood mutant and non-mutant T cell clones were collected 14 to 21 days following PHA stimulation and the cell viability and number verified using trypan blue dye exclusion. The cells were washed twice in phosphate buffered saline (PBS) containing 0.1% NaN_3 and then the cells were incubated with 10 μl of gamma-globulin (Canadian Red Cross) for 10 minutes. Gamma-globulin was added to block non-specific binding: dying TK6 cells bind non-specifically and the TK6 have Fc receptors which may also bind the primary antibody. The cells were then incubated with 10 μl of BSA-Cy5 (Amersham Life Sciences) for 10 minutes on ice. BSA-Cy5 blocks carboxymethylindocyanine suinimide ester 5 (Cy5) receptors potentially present on the cell surface. To verify that the clones were indeed T lymphocyte clones, the cells were stained with anti-CD3 TRI (Sigma Chemicals Inc.), anti-CD4 phycoerythrin (PE) (Coulter Electronics, Hialeah Florida), and anti-CD8 fluorescein isothiocyanate (FITC) (Coulter) monoclonal antibodies (MAb) on ice for 20 minutes to avoid capping. The appropriate isotype controls for each MAb were included. The TRI compound is PE conjugated to Cy5. Thus, the fluorochrome can be excited by the argon laser because it contains PE. The energy is then transferred to the Cy5 molecule and emits energy at approximately 600 nm in the

FL-4 quadrant. The stained cells were collected and washed in PBS with 0.1% NaN₃ and 1% BSA (wash buffer).

To verify the integrin expression of the T cell clones, 2.5 x 10⁵ T cells from a clone were incubated with 10 µl of gamma-globulin for 10 minutes and then incubated with 10 µl of BSA-Cy5 (Amersham Life Sciences) for 10 minutes on ice. The cells were stained with anti-CD3 TRI (Sigma) and anti-CD29 PE (Coulter) MAb for 20 minutes. The cells were washed in the wash buffer. Stained cells were analyzed with an argon laser Epics XL-MCL Coulter Flow Cytometer (Coulter Electronics, Hialeah Florida) using Epics XL flow cytometry analysis software version 1.5. For all of the experiments, 10, 000 events were counted and the lymphocytes gated on viable cells based on forward and side scatter. Forward scatter is utilized to gate on the cell size and side scatter is used to gate on the cell granularity.

5. CELL ADHESION

5.1 Fibronectin Binding Assay

A colourimetric adhesion assay was used to quantify the number of cells adhering to fibronectin (Schulz *et al.* 1994; St. John *et al.* 1994). Wells of a 96-well flat-bottom microtitre ELISA plate (NUNC) were coated with either 120 KDa human plasma fibronectin, containing the RGD domain and the CS-1 sequence (20 µg/ml) (Canadian Life Technologies) or bovine serum albumin (BSA) (20 µg/ml) (Sigma Chemicals Ltd.) in 100 µl of an adhesion buffer overnight at 4°C. Adhesion buffer consisted of 150 mM NaCl, 1 mM CaCl₂, 1 mM MgCl₂, and 10 mM Hepes (pH 7.2). Wells of a separate positive control plate were coated with 100 µl of 0.01% polyL-lysine (Sigma

Chemicals Ltd.) for 1 hour at 4°C. The wells coated with BSA were used as a negative control and the wells coated with polyL-lysine were used as a positive control for binding.

Binding of the peripheral blood *hprt* mutant and non-mutant T cell clones from the same patient or control was compared to RA and OA synovial tissue T cell lines. Cells were collected 14 to 21 days following PHA stimulation and washed twice in the adhesion buffer. Following PHA stimulation, CD29 expression was minimal after 3 days and maintained maximal expression from 1 to 3 weeks (Hemler 1990; Rodriguez *et al.* 1992). The cell number and viability were determined by trypan blue dye exclusion. Approximately 1×10^5 cells were plated per well. Figure 17 in the appendix demonstrates synovial tissue T cell lines binding to wells coated with fibronectin in comparison to poly L-lysine. At the cell concentration of 1×10^5 cells/well, the synovial tissue T cells bound equally well to the fibronectin and poly L-lysine coated wells. The T cell clones from RA patients and controls and the RA synovial tissue T cell lines were incubated in adhesion buffer supplemented with 1 mM Mg^{2+} for 30 minutes. The T cells were incubated at 37°C, 5% CO_2 , in 0.5 ml polypropylene tubes either alone or with various MAb including anti-IgG₁ (Coulter), anti- $\alpha 4\beta 7$ donated by Dr. A Lazarovits), anti-CD7 (donated by Dr. A Lazarovits), and anti-CD29 (Sigma Chemicals Ltd.), for 1 hour. The anti-IgG₁ MAb was implemented as a negative control because all the antibodies used were of this isotype. Approximately 1×10^5 cells (Appendix Figure 17) were added to each of the wells and allowed to bind at 37°C, 5% CO_2 for 1.5 hours. T cells that were incubated with the various MAb were plated in the wells coated with fibronectin. Cells that were incubated without antibody were plated in wells coated with either fibronectin, BSA, or polyL-lysine.

Adherent cells were quantified as follows. Plates with wells coated with fibronectin or BSA were gently immersed in 2.5 litres of 0.85% NaCl to remove loosely adherent cells. The plates were submerged at an angle to ensure that the wells did not fill with air bubbles. The plates were inverted, raised, and allowed to float on the surface facing downward. The saline solution was stirred by a 30 mm stir bar at 300 rpm for 5 minutes. Prior to removing the plate from the saline solution, the plate was turned right side up and removed with the wells filled with saline solution. Adherent cells were fixed by transferring each plate to a container with 2.5 litres of 100% methanol (BDH). The plate was submerged slowly at an angle and rotated several times to allow the saline to mix with the methanol. After 5 minutes, the plate was removed from the container with the methanol in the wells to fix the adherent cells and maintained at room temperature for 1 hour. A control plate containing polyL-lysine coated wells was centrifuged at 2000 rpm for 5 minutes and fixed as described above. This will insure the maximal number of adherent cells. Excess methanol was gently removed from the wells by inverting the plates, and adherent cells were stained with 100 μ l of 0.1% amido black dye (Naphthol Blue Black dye from Sigma Chemicals Ltd.) for 1 hour. Excess stain was removed by washing the plate in a polypropylene container with 2.5 L of cold distilled water. Once again, the plate was submerged in the water on an angle to avoid trapping air bubbles and then the plate was turned gently in the water for 4 minutes. Water was removed from the wells by inverting the plates gently and 100 μ l of 10% sodium dodecyl sulfate was added to each well. Plates were kept overnight at room temperature to lyse cells and allow the dye to solubilize. The lysate was collected and centrifuged at 13,000 x g to remove cellular debris. The supernatant was collected and the colour intensity determined at a wavelength of 570 nm using an ELISA plate reader (Bio-Rad Model 3550 Microplate Reader, Richmond CA). The background values were established using wells that had been stained with the amido black dye but had not

received any cells. For comparison, the solution was also read by a spectrophotometer at 570 nm. Percentage adhesion was determined using the formula indicated below.

$$\% \text{ adhesion} = \frac{\text{OD}_{570\text{nm}} \text{ for cells adherent to experimental plate}}{\text{OD}_{570\text{nm}} \text{ for cells adherent to polyL-lysine}} \times 100$$

5.2 Specificity of Fibronectin Adherence

A fibronectin adhesion assay was implemented as previously described. The RA peripheral blood mutant T cell clones and RA synovial tissue T cell lines were incubated with various concentrations (0 to 50 $\mu\text{g/ml}$) of either a 40 KDa soluble fibronectin fragment containing the CS-1 (25 amino acid binding sequence) (Canadian Life Technologies) or with hen egg lysozyme (HEL) (Sigma Chemicals Ltd.), as a non-specific protein, prior to the exposure of the cells to the fibronectin coated wells. T cells were also incubated in wells coated with BSA as the negative control for binding and polyL-lysine as the positive control for binding.

6. STATISTICAL ANALYSIS

The (BMDP) statistical program was used. Comparison between the study groups was performed by the 2-tailed non-parametric non-paired Mann-Whitney test. Comparisons between peripheral blood and synovial tissue samples were analyzed by the 2-tailed non-parametric paired Wilcoxon test. A one way ANOVA was performed to ensure statistical significance using Levene's test. Correlation of measures of RA clinical disease activity and frequency of mutant T cells was

analyzed using the Spearman's rank analysis. P values less than 0.05 were considered to be statistically significant.

CHAPTER 3: RESULTS

1. PATIENT CHARACTERISTICS

The patient group consisted of 90 RA and 7 OA patients that were followed at the Ottawa General Hospital Rheumatic Disease Unit. Twenty hospital and university personnel were utilized as healthy sex-matched controls for the duration of the study. Table 1 describes the characteristics of the RA patients, OA patients, and controls used in the study. A peripheral blood sample was taken from 80 different unselected RA patients at the outpatient clinic. The average age of the patients was 57.1 +/- 12.7 years, which is not significantly different ($p>0.05$) than the average age of the controls, 32.1 +/- 17.2 years; although, the mean age of the controls is less than the RA patients. A synovial tissue sample was received from 8 different RA patients and 7 different OA patients. After 2 to 10 days following surgery, a peripheral blood sample was drawn from each patient. The mean age of the RA patients who underwent surgery was 55.2 +/- 14.4 years, which is also not significantly greater ($p>0.05$) than the mean age of controls. However, the mean age of the OA patients, 73.75 +/- 5.85 years, was significantly greater ($p<0.05$) than the RA patients and controls: 55.2 +/- 14.4 and 32.1 +/- 17.2 years, respectively. Older healthy age matched controls were not available. A synovial fluid sample was obtained from 2 RA patients with the mean age of 25.0 +/- 7.1 years. A peripheral blood sample was not taken from patients from whom a synovial fluid sample had been obtained.

Table 1
The characteristics of the RA patients, OA patients, and controls used in the study

	Control Peripheral Blood	RA Peripheral Blood	RA Synovial Fluid	RA Synovial Tissue	OA Synovial Tissue
Number	20	80	2	10	8
Sex	14 female 6 male	55 female 25 male	1 female 1 male	9 female 1 male	3 female 5 male
Mean Age ^a	32.1 +/- 17.2	57.1 +/- 12.7	25.0 +/- 7.1	55.2 +/- 14.4	73.8 +/- 5.9
Mean	Not	12.0 +/- 11.5	Not	13.5 +/- 14.4	Not
Duration ^a	Applicable		Available		Available

^a Mean (years) +/- SD

2. THE FREQUENCY OF MUTANT T CELLS FROM THE PERIPHERAL BLOOD OF RA PATIENTS AND CONTROLS

1. Proliferation Assay

The visual method for scoring T cell clones was validated using a [³H]-thymidine incorporation assay (Figure 2). An *hprt* clonal assay was completed on 5 RA patients and the scored wells were incubated in the presence of [³H]-thymidine to verify visual scoring. Irradiated TK6 *hprt* feeder cells were used as a negative control and represent the background levels of counts. Wells that had been visually scored as negative for both the selection (containing 6-TG) and non-selection plates had significantly lower counts compared to wells that had been visually scored as positive ($p < 0.008$). The wells that had been identified to contain a growing clone from either the non-selection or selection media did not have significantly different counts ($p > 0.05$). Also, the wells in the selection or non-selection media that were identified to contain a non-growing clone did not have significantly different counts ($p > 0.05$). Thus, the proliferating cells are likely to be a true clone. These results support the reliability of the visual scoring.

2. The Cloning Efficiency of Peripheral Blood T Cells from RA Patients and Controls

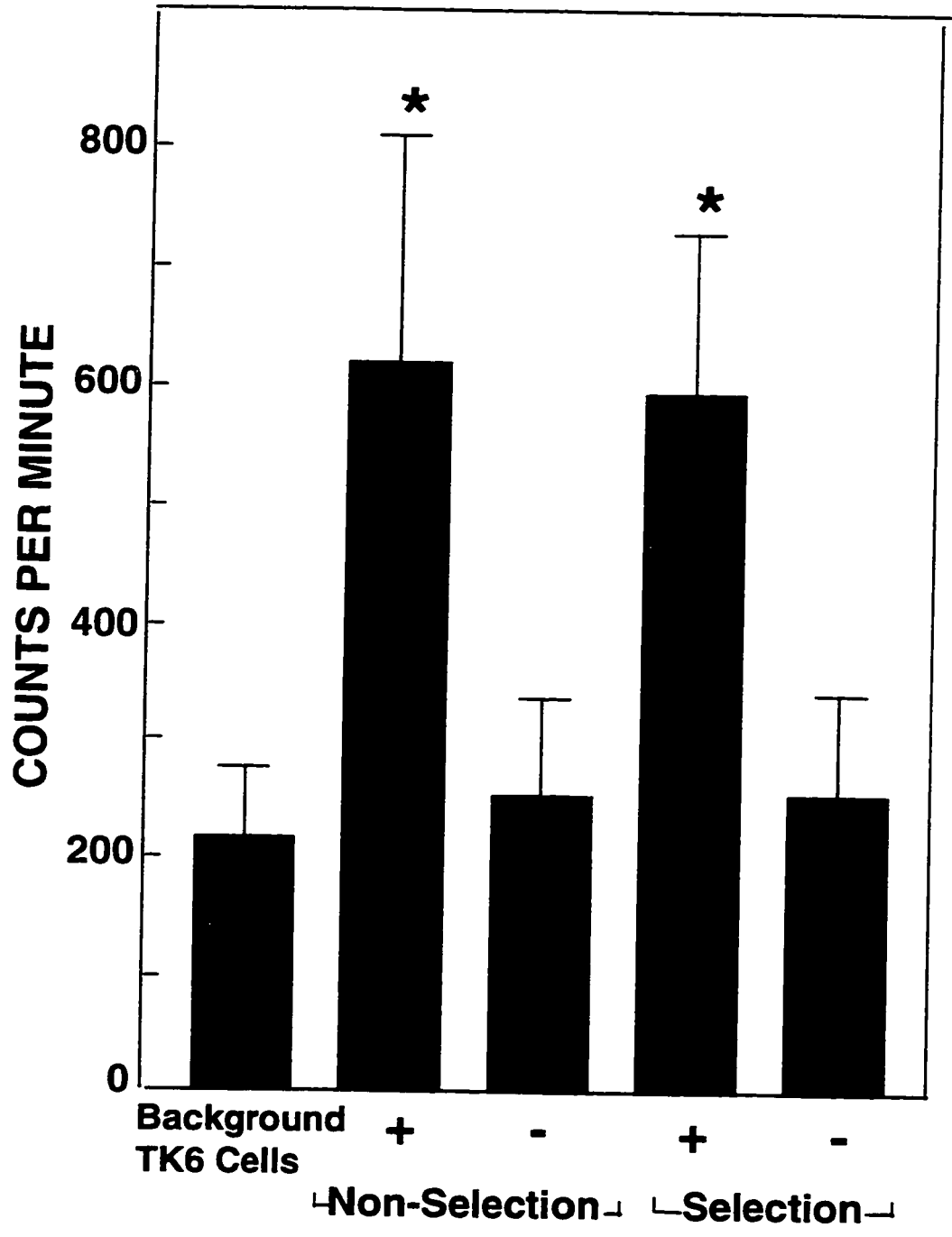
The T lymphocytes were scored visually under an inverted microscope. The CE of the T cells was determined using the assumption of a Poisson distribution of clonable cells in limiting dilution (Figure 3A). The CE of T lymphocytes from the peripheral blood of RA patients was not significantly less ($p > 0.05$) compared to the CE of control peripheral blood T cells: 25.7 +/- 12.4% and 37.1 +/- 19.9%, respectively.

Figure 2

Proliferation assay of *hprt* mutant and wild type T cell clones from RA patients. A [³H]-thymidine incorporation assay was used to verify the visual scoring of the mutant and non-mutant T cell clones. The background levels (BKG) represent the tritium uptake by TK6 *hprt* feeder cells after irradiation at 80 Gy and a 21-day incubation. Non-selection refers to clones that were derived from plating 1 MNC/well and selection refers to clones that were derived from plating 2×10^4 MNC/well in the presence of 6-TG. Plus (+) indicates wells that were scored as positive, containing a growing clone, and minus (-) indicates wells that were scored as negative that did not have a viable clone. Data represent n=5 (+/-SD). The wells that had been visually scored as positive had significantly greater counts than the wells that had been visually scored as negative for both the non-selection and selection conditions.

* Represents statistical significance when comparing positive versus negative wells.

■ RA PB T cells



3. The Frequency of Mutant T Lymphocytes in the Peripheral Blood of RA Patients and Controls

The frequency of mutant T lymphocytes (FMC) represents the CE of T cells in the presence of 6-TG. The cFMC is the CE of T cells in selection medium containing 6-TG, compared to the CE of T cells in the non-selection medium. The FMC and the cFMC, per 10^6 cells, from the peripheral blood of controls ranged from 0 to 1.04 and 0 to 1.78 respectively, while the FMC and cFMC from RA patients ranged from 0.17 to 6.29 and 0.89 to 18.36, respectively. The FMC and cFMC are described in terms of 10^6 cells. I have described both the FMC and cFMC because the FMC accounts for the 'raw' data, that is the number of mutant T cells per plate, and the cFMC takes into account the cloning efficiency of the T cells from the patient. If the CE of a particular patients T cells are very low then this will elevate the cFMC. The mean FMC in the peripheral blood of RA patients was significantly greater ($p < 0.0001$) than from controls: 1.55 ± 1.14 compared to 0.39 ± 0.23 (Figure 3B). The mean cFMC, was also significantly greater ($p < 0.0005$) in RA patients compared to controls: 6.26 ± 3.98 and 1.07 ± 0.71 , respectively (Figure 3C). The results clearly demonstrate that the FMC and cFMC were significantly greater in peripheral blood of RA patients compared to controls.

Several RA patients and controls were analyzed more than once throughout the duration of the study (Tables 2 and 3). There was a minimum period of two months between peripheral blood samples taken from the same subject. The mean cFMC from individual samples did not vary significantly throughout the duration of the study for both controls and RA patients. This data demonstrates that the mean cFMC in the peripheral blood of RA patients is consistently greater compared to controls.

Figure 3

A) The CE of T lymphocytes from the peripheral blood of RA patients and controls. The mean CE (+/- SD) of T cells from the peripheral blood of 80 RA patients (striped bar) was not significantly greater ($p>0.05$) than 20 controls (solid bar). B) The frequency of mutant T cells from the peripheral blood of RA patients and controls. The mean FMC (+/- SD) from the peripheral blood of 80 RA patients (striped bar) was significantly greater ($p<0.0001$) than 20 controls (solid bar). C) The corrected frequency of mutant T cells from the peripheral blood of RA patients and controls. The mean cFMC (+/- SD), which takes into account the CE of the T cells for each peripheral blood sample, in 80 RA patients (striped bar) was significantly greater ($p<0.0005$) compared to 20 controls (solid bar).

* Represents statistical significance.

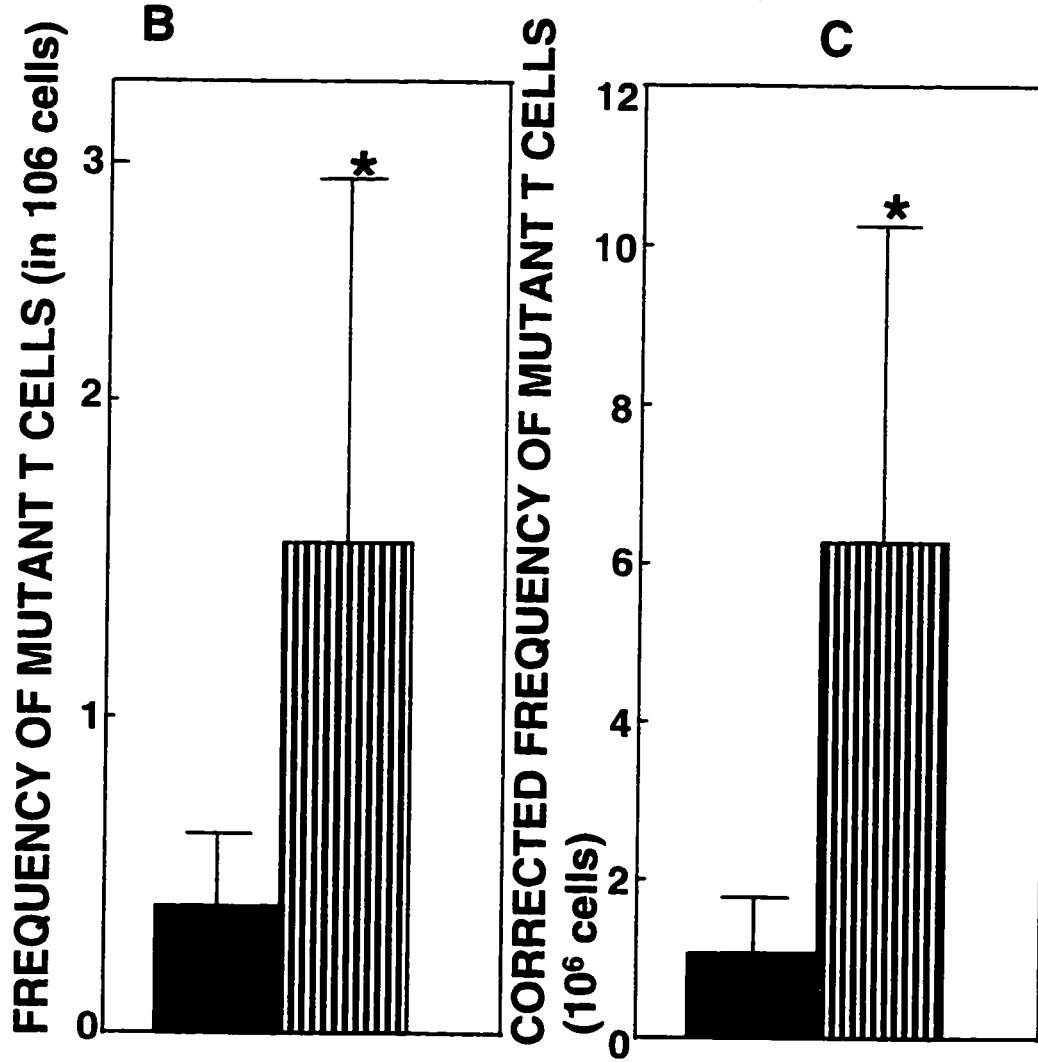
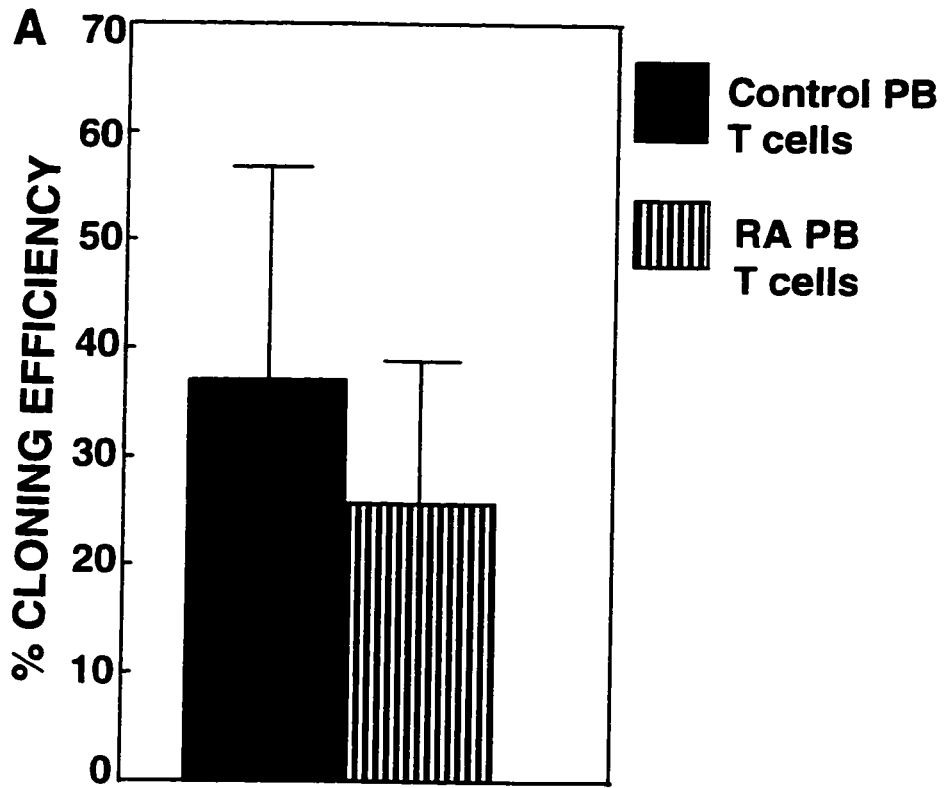


Table 2

The corrected frequency of mutant T cells in the peripheral blood of RA patients at various time intervals with a minimum of two months between samples

	cFMC1 ^a	cFMC2 ^a	cFMC3 ^a	cFMC4 ^a	cFMC5 ^a
Patient 01	3.06 +/- 1.21	3.28 +/- 4.19	7.00 +/- 4.41		
Patient 02	6.45 +/- 5.47	3.28 +/- 4.18			
Patient 03	4.28 +/- 1.73	6.18 +/- 1.93			
Patient 04	4.34 +/- 1.48	6.73 +/- 1.27	4.18 +/- 1.47		
Patient 05	8.40 +/- 3.16	3.49 +/- 4.02			
Patient 06	6.44 +/- 3.22	9.34 +/- 3.46	5.25 +/- 3.36	9.87 +/- 4.20	7.19 +/- 4.27
Patient 07	3.17 +/- 2.28	4.14 +/- 1.60			
Patient 08	5.81 +/- 5.54	5.52 +/- 4.99			
Patient 09	2.13 +/- 1.79	6.86 +/- 4.57			
Patient 10	5.81 +/- 5.54	3.86 +/- 1.92			
Patient 11	4.69 +/- 2.00	5.72 +/- 5.25			
Patient 12	4.05 +/- 3.50	5.78 +/- 4.74			
Patient 13	4.63 +/- 2.24	8.88 +/- 3.49			

^a Mean cFMC (per 10⁶ cells) +/- SD.

Table 3

The corrected frequency of mutant T cells from the peripheral blood of controls at various time intervals with a minimum of two months between samples

	cFMC1 ^a	cFMC2 ^a	cFMC3 ^a	cFMC4 ^a	cFMC5 ^a
Control 01	1.74 +/- 1.33	1.40 +/- 0.81			
Control 02	0.86 +/- 1.11	0.84 +/- 1.68	1.87 +/- 2.04	1.31 +/- 2.15	0.98 +/- 0.90
Control 03	0.35 +/- 0.61	1.67 +/- 1.19	2.34 +/- 2.37		
Control 04	0.93 +/- 1.07	3.21 +/- 4.11			
Control 05	0.57 +/- 0.84	0.64 +/- 0.74	2.38 +/- 1.95		
Control 06	0.70 +/- 1.03	1.42 +/- 0.84			
Control 07	0	1.38 +/- 1.06	1.59 +/- 1.27		

^a Mean cFMC (per 10⁶ cells) +/- SD.

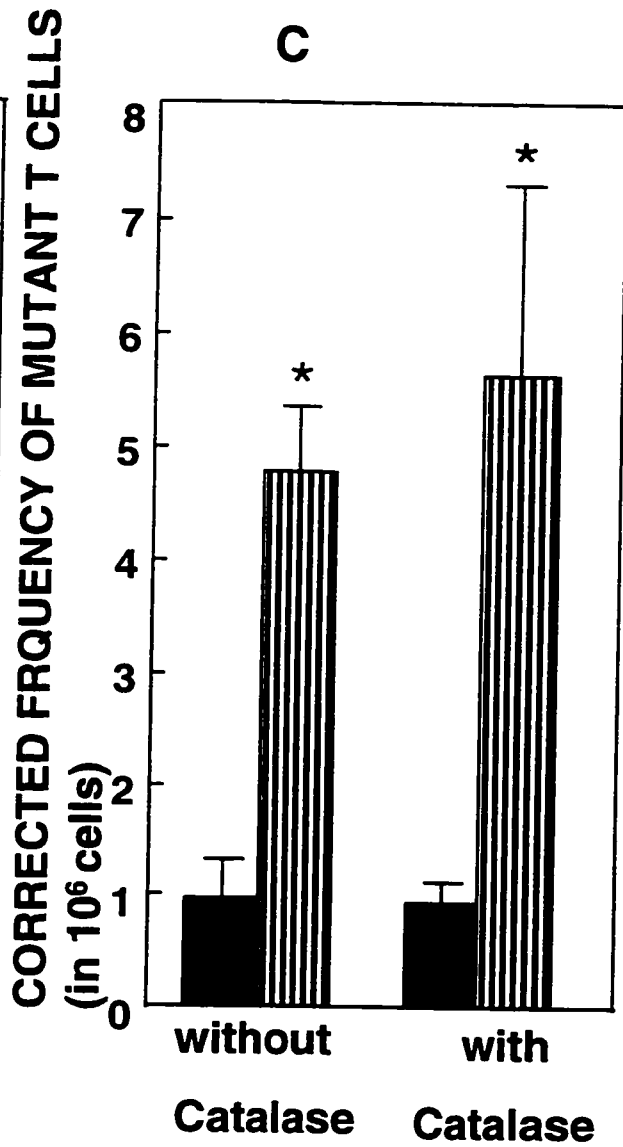
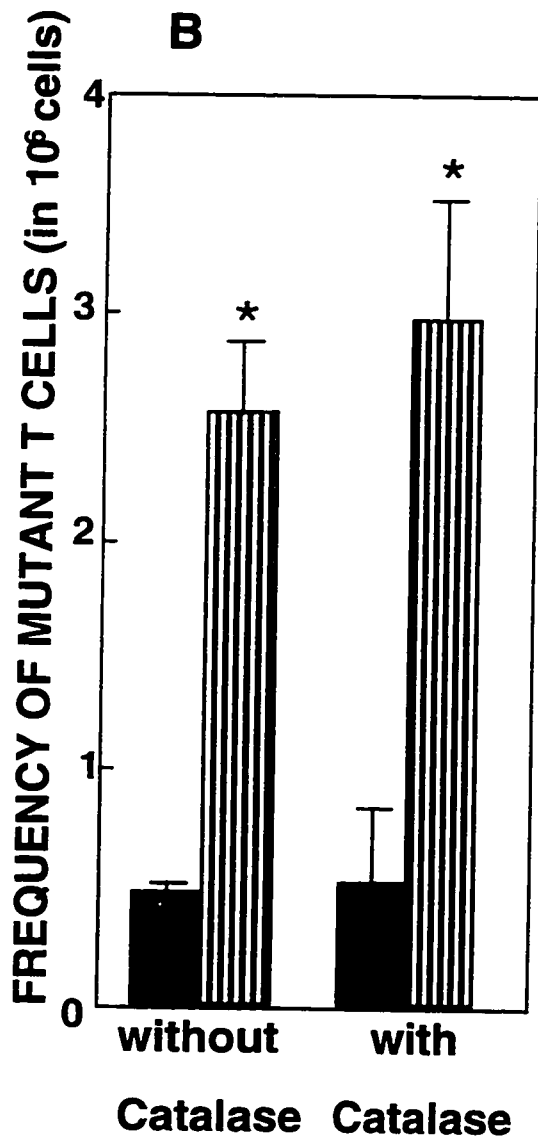
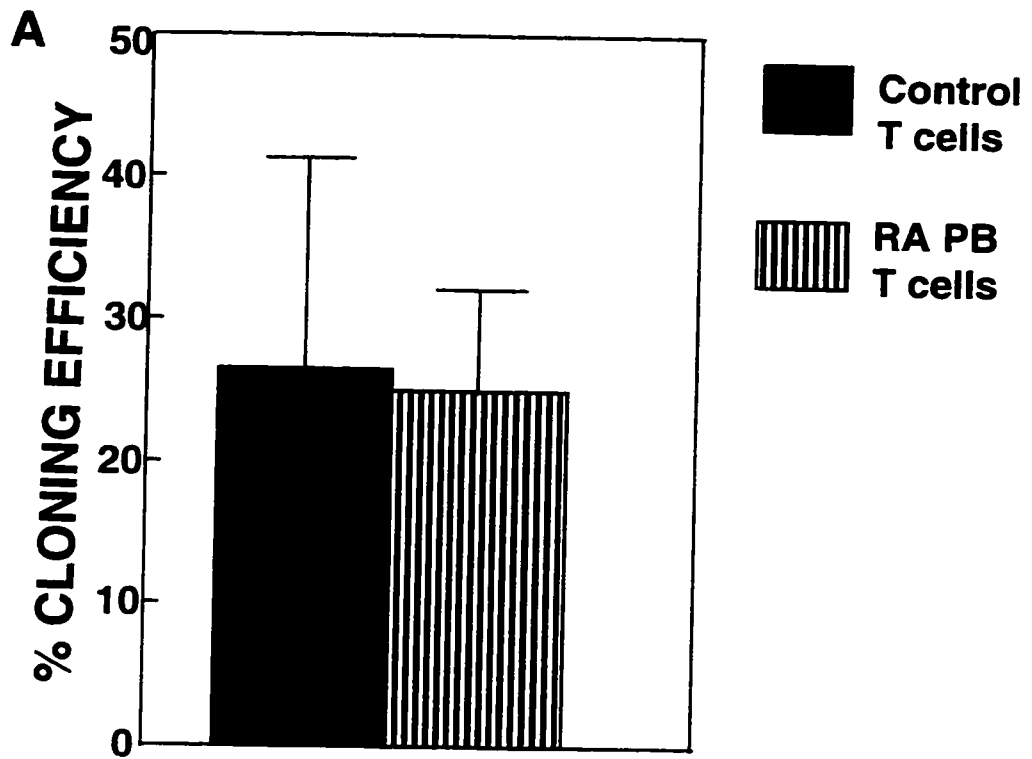
4. The Frequency of Mutant T Lymphocytes in the Presence or Absence of Catalase

When cells are cultured in the presence of 6-TG, it takes several hours for the chemical to enter the cell with a functional *hprt* gene, be converted into a toxic nucleotide, and kill the cell (Albertini 1982; Damell *et al.* 1990). Theoretically, among the isolated MNC, macrophages could release RNS and ROS, such as H_2O_2 , which may cause an *in vitro* increase in FMC. H_2O_2 causes DNA damage because it reacts with transition metals bound to DNA to produce hydroxy radical ($OH\cdot$) which immediately fragments the DNA molecule. To assess this possibility, the *hprt* clonal assay was performed on T cells isolated from the peripheral blood of RA patients and controls in the presence and absence of catalase. The enzyme catalase, converts 2 H_2O_2 into 2 H_2O and O_2 (Halliwell *et al.* 1988). The CE of T cells from the peripheral blood of these specific 3 RA patients was not significantly different ($p>0.05$) than 3 controls: 25.0 \pm 7.07 % and 26.5 \pm 14.75 %. (Figure 4A). The FMC did not vary significantly in the absence or presence of catalase ($p>0.05$) from the peripheral blood of RA patients: 2.61 \pm 0.31 and 3.02 \pm 0.52 (in 10^6 cells) respectively. The FMC from the peripheral blood of controls also did not vary significantly in the absence or presence of catalase ($p>0.05$): 0.49 \pm 0.04 and 0.54 \pm 0.33 (in 10^6 cells), respectively (Figure 4B). The FMC from the peripheral blood of RA patients was significantly greater ($p<0.05$) than controls in the absence or presence of catalase which is consistent with the data from the larger patient group. The cFMC also did not vary significantly ($p>0.05$) in the absence and presence of catalase in RA patients: 4.75 \pm 0.56 and 5.60 \pm 1.67, or in controls: 0.96 \pm 0.35 and 0.92 \pm 0.18, in 10^6 cells, respectively (Figure 4C). The cFMC was significantly greater ($p<0.05$) in the peripheral blood of RA patients compared to controls in both the absence and the presence of

Figure 4

A) The CE of T lymphocytes from the peripheral blood of RA patients and controls for the catalase assay. The data describes the CE of RA patients and controls prior to the addition of catalase and these RA patients and controls are not identical to the previously described sample. The mean CE (\pm SD) of peripheral blood T cells from 3 RA patients (striped bar) was not significantly different ($p > 0.05$) compared to 3 controls (solid bar). B) The frequency of mutant T cells from the peripheral blood of RA patients and controls in the absence and presence of catalase. The mean FMC (\pm SD) in the peripheral blood of 3 RA patients (striped bar) was not significantly different ($p > 0.05$) in the absence compared to the presence of catalase. The mean FMC (\pm SD) in the peripheral blood of 3 controls (solid bar) was not significantly different ($p > 0.05$) in the absence compared to the presence of catalase. The mean FMC from the peripheral blood of RA patients was significantly greater than 3 controls. C) The corrected frequency of mutant T cells in the peripheral blood of RA patients and controls in the absence and presence of catalase. The mean cFMC (\pm SD) in the peripheral blood of 3 RA patients (striped bar) was not significantly different ($p > 0.05$) in the absence versus the presence of catalase. The mean cFMC from the peripheral blood of 3 controls was not significantly different in the absence versus the presence of catalase. The mean cFMC from the peripheral blood of RA patients was significantly greater compared to the cFMC from control peripheral blood.

* Represents statistical significance versus controls.



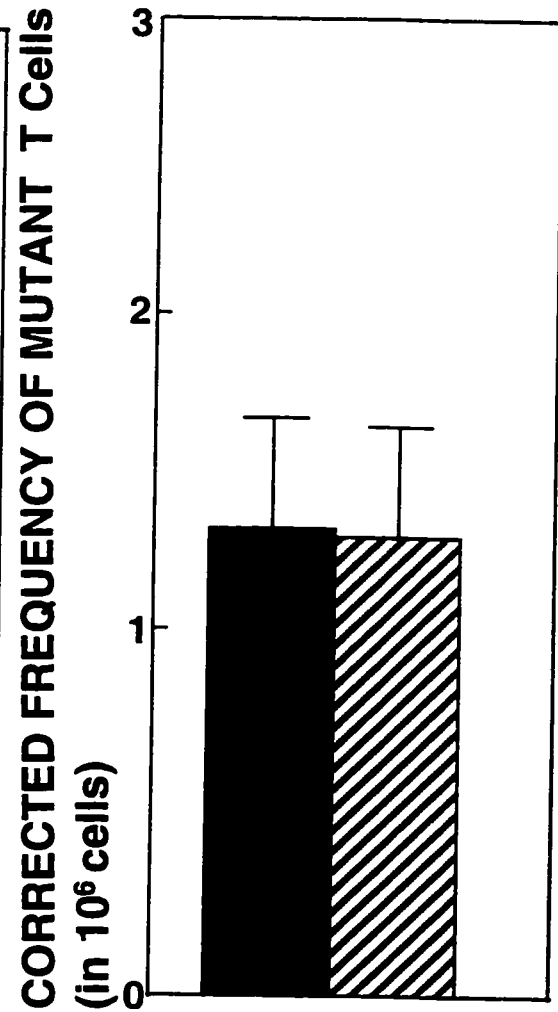
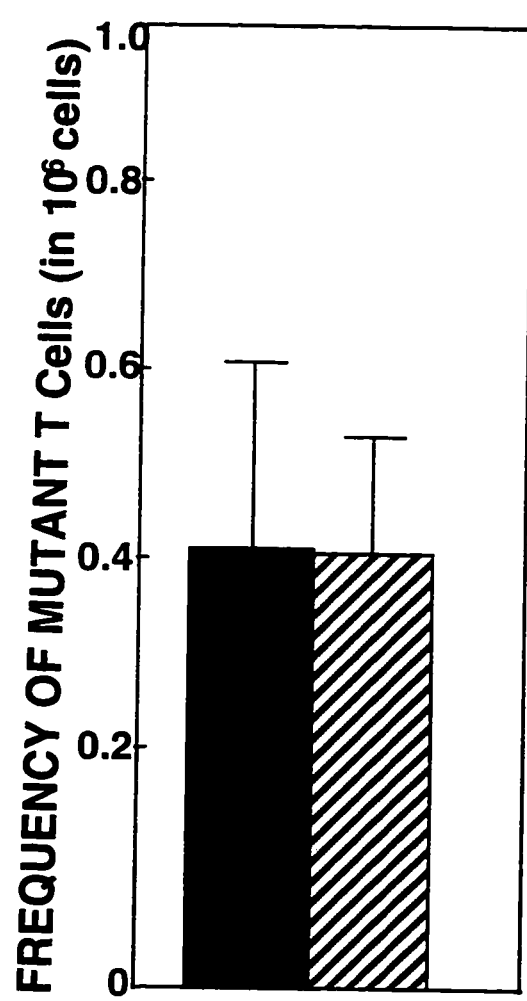
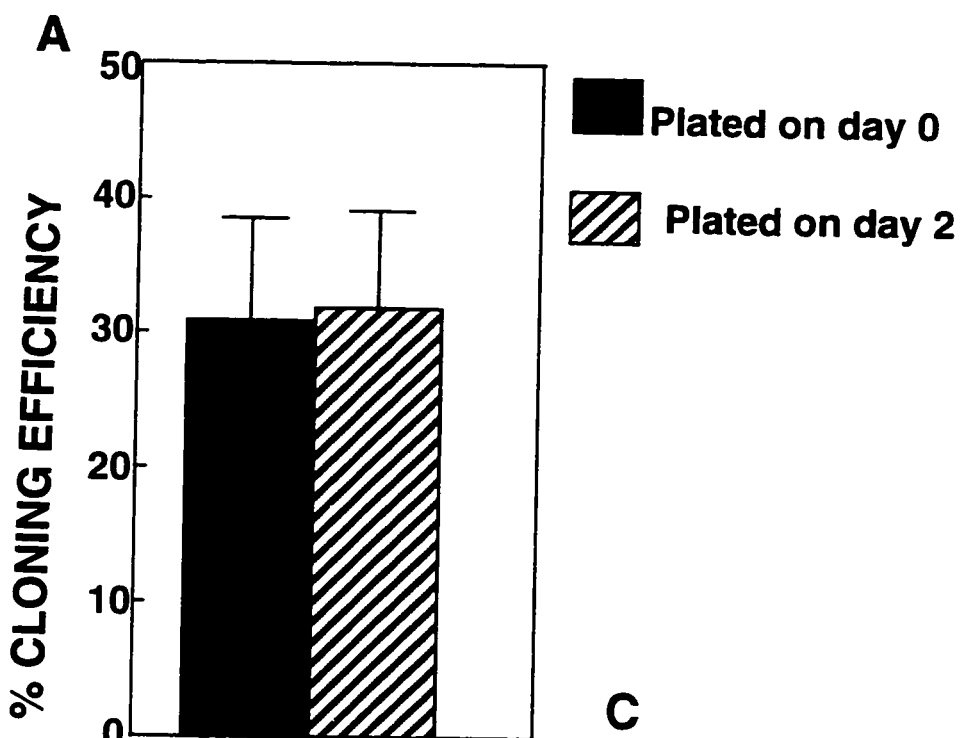
catalase. These results suggest that the mutant T cells are not an *in vitro* phenomenon caused by H₂O₂ in either RA patients or controls.

5. *In Vitro* Genotoxic Effect

Genotoxic species secreted by macrophages other than H₂O₂, such as ONOO⁻ and O₂⁻ may also cause genetic damage (Halliwell *et al.* 1988). To verify if these or other species caused an elevated frequency of mutant T cells, a peripheral blood sample was obtained from 3 healthy controls. The isolated MNC were either immediately plated in selection medium and a *hprt* clonal assay performed or the cells remained in complete medium for 2 days and then were plated in selection medium containing 6-TG. This 48-hour period may be sufficient time for the T cells to incur genetic damage and to be stimulated to proliferate by antigen presenting cells for the induction of mutation *in vitro*. The CE of T lymphocytes of either of the 2 subsets did not differ significantly ($p > 0.05$) (Figure 5A). The FMC was not significantly different ($p > 0.05$) in the T cells plated with 6-TG on day 0 compared to day 2: 0.46 +/- 0.19 and 0.45 +/- 0.12 (per 10⁶ cells), respectively (Figure 5B). The cFMC was also not significantly different ($p > 0.05$) in the T lymphocytes plated in 6-TG immediately or after a 2-day incubation: 1.44 +/- 0.33 and 1.41 +/- 0.34, in 10⁶ cells, respectively (Figure 5C).

Figure 5

A) The CE of T cells from the peripheral blood of controls plated on day 0 and day 2. The mean CE (+/- SD) of T cells from 3 controls plated in selection medium on day 0 (solid bar) was not significantly different ($p>0.05$) than the CE of T cells from the same 3 controls plated in selection medium on day 2 (striped bar). B) The frequency of mutant T cells from the peripheral blood of controls plated in selection medium on day 0 or 2. The mean FMC (+/- SD) from the peripheral blood of 3 controls plated in selection medium on day 0 (solid bar) was not significantly different ($p>0.05$) compared to the mean FMC from 3 controls plated on day 2 (striped bars). C) The corrected frequency of mutant T cells from the peripheral blood of controls plated on day 0 and 2. The mean cFMC (+/- SD) from the peripheral blood of 3 controls plated in selection medium on day 0 (solid bars) was not significantly greater ($p>0.05$) than the mean cFMC from the same 3 controls plated in selection medium on day 2 (striped bars).



3. CLINICAL DISEASE

3.1 Disease Activity

Several RA patients were divided into 2 categories based on their RA disease activity. Twenty patients were evaluated, 5 with 'active' and 15 with 'inactive' RA. There was no significant difference ($p>0.05$) in the CE of the T cells isolated from the active or inactive subset: 33.62 +/- 14.10 % and 40.80 +/- 14.2 %, respectively (Figure 6A). There was no significant difference ($p>0.05$) in the FMC from the RA patients with 'inactive' versus 'active' disease: 1.63 +/- 1.03 and 1.49 +/- 1.40, in 10^6 cells, respectively. There was not a significant difference ($p>0.05$) in the cFMC in the RA patients with 'active' disease compared to 'inactive' disease: 3.31 +/- 2.74 and 5.51 +/- 3.43 (per 10^6 cells), respectively (Figure 6 B and C). Therefore, the frequency of mutant T cells does not appear to correlate with current clinical disease activity.

3.2 Clinical Disease Parameters

The peripheral blood sample was drawn from the RA patients following a clinical evaluation. The patient was assessed for age, the duration of disease, the patient's assessment of pain, the patient's assessment of disease activity, joint count, and the length of morning stiffness. The clinical laboratory analysis provided levels of C-reactive protein and erythrocyte sedimentation rate. Using Spearman's rank analysis, none of the clinical disease parameters correlated with the cFMC in RA patients (Figure 7).

Figure 6

A) The CE of T lymphocytes from the peripheral blood of RA patients with 'active' or 'inactive' disease. The mean CE (\pm SD) of T cells from the peripheral blood of 5 RA patients with 'active' disease (solid bar) did not differ significantly ($p > 0.05$) compared to 15 RA patients with 'inactive' disease (striped bar). B) The frequency of mutant T cells from the peripheral blood of RA patients with 'active' and 'inactive' disease. The mean FMC (\pm SD) from the peripheral blood of 5 RA patients with 'active' disease (solid bar) was not significantly greater ($p > 0.05$) compared to 15 patients with 'inactive' disease (striped bar). C) The corrected frequency of mutant T cells from the peripheral blood of RA patient with 'active' and 'inactive' disease. The mean cFMC (\pm SD) from the peripheral blood of 5 RA patients with 'active' disease (solid bar) was not significantly different ($p > 0.05$) compared to 15 patients with 'inactive' disease (striped bar).

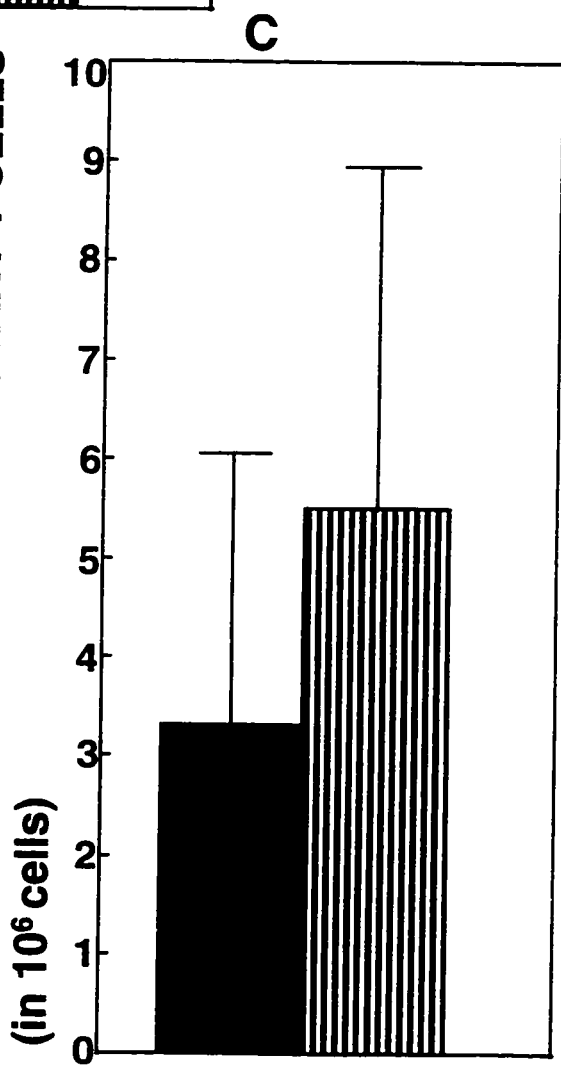
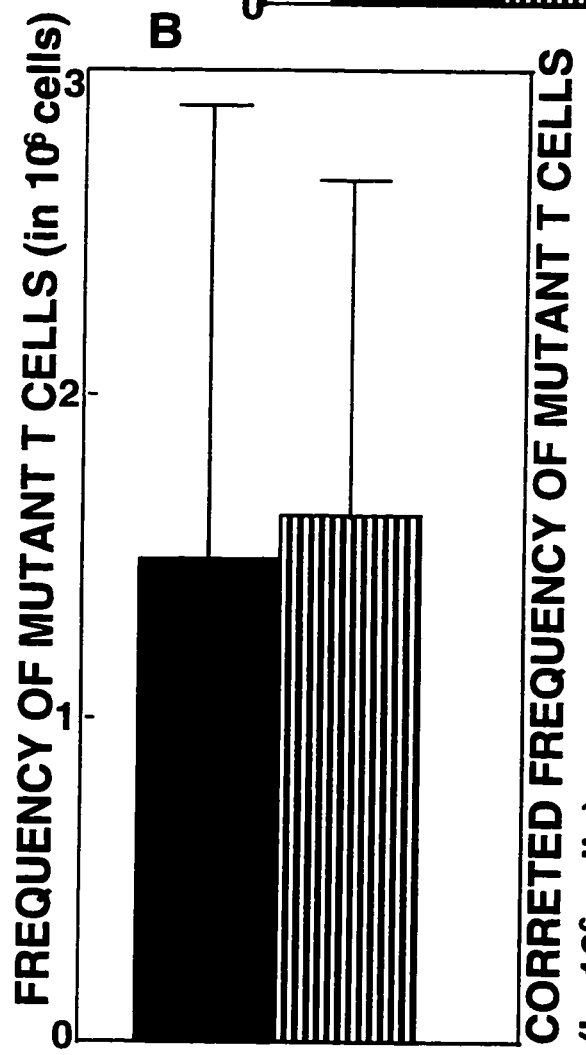
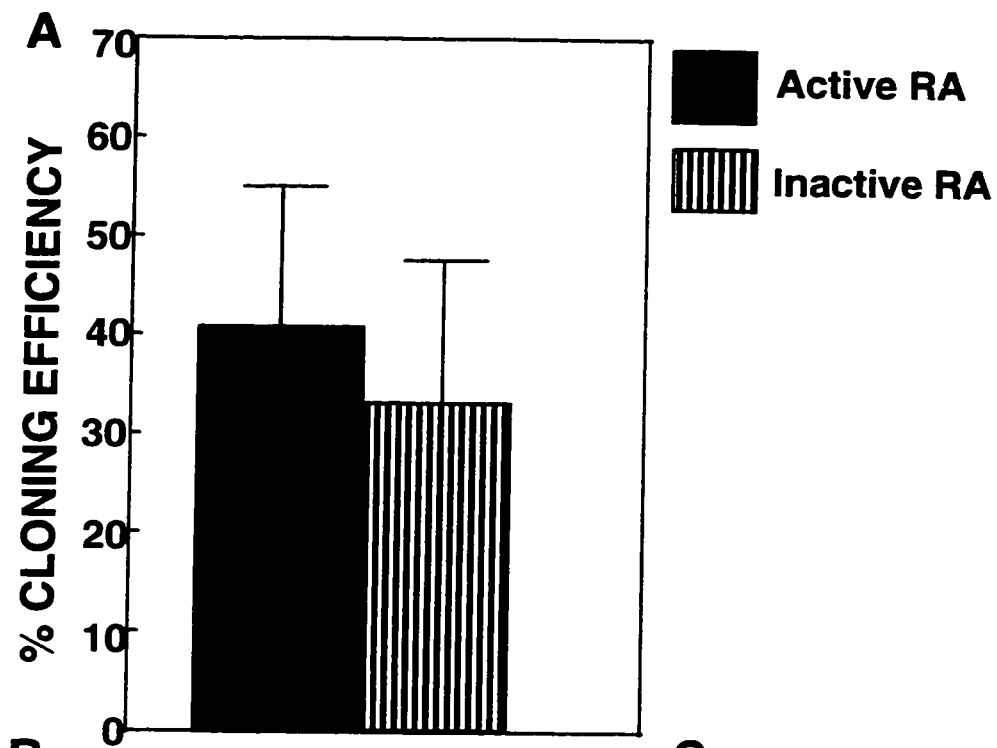
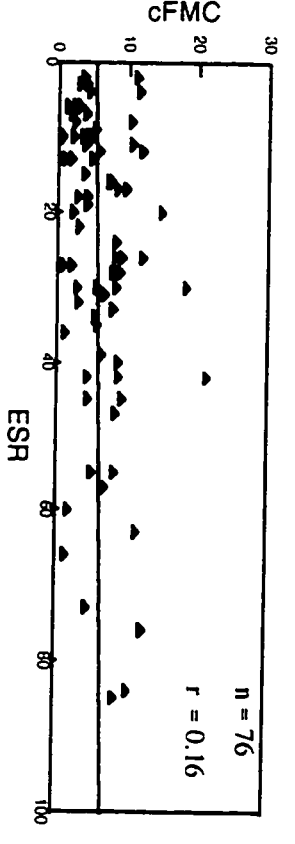
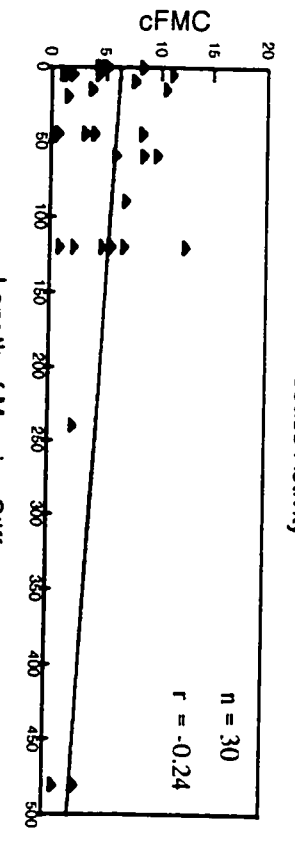
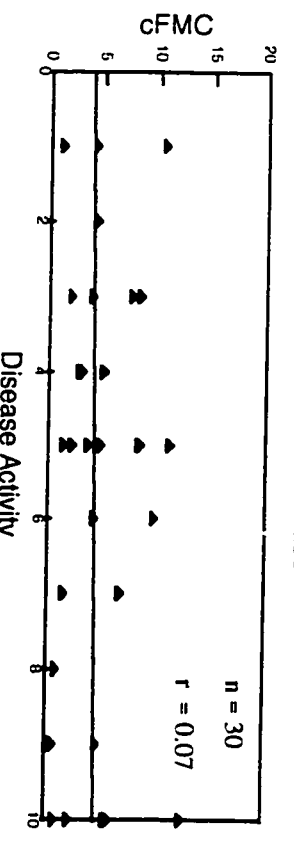
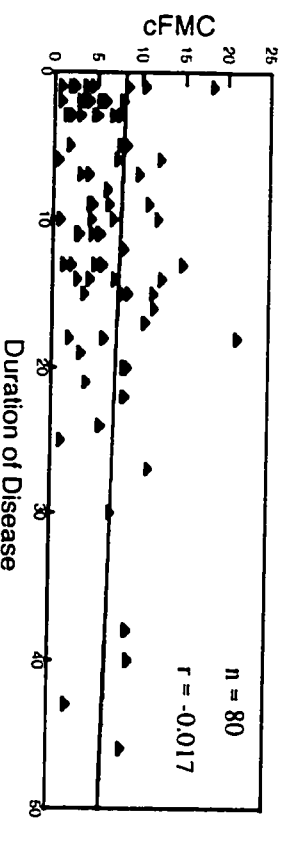
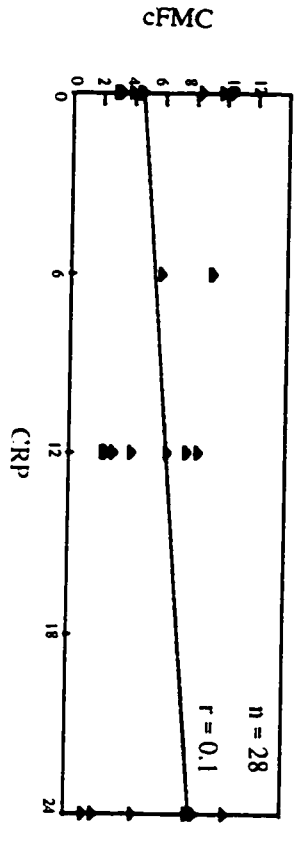
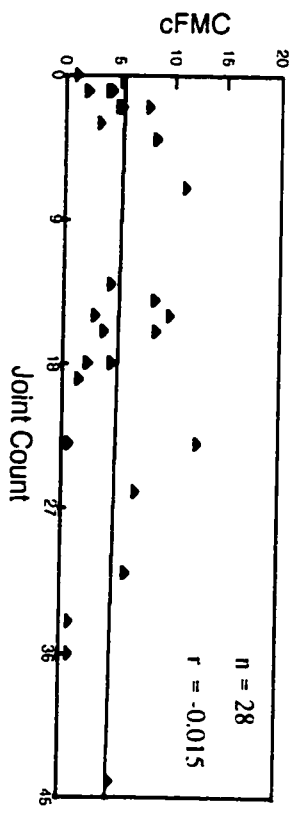
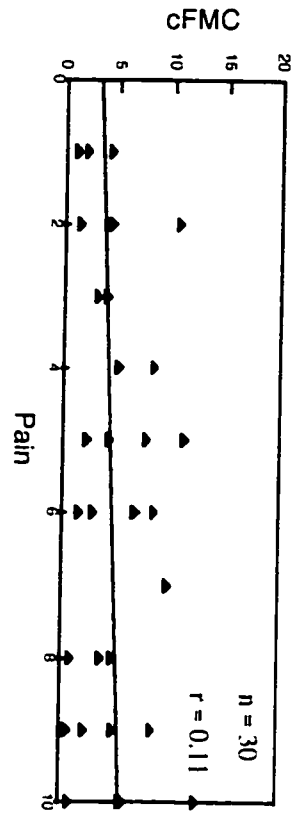
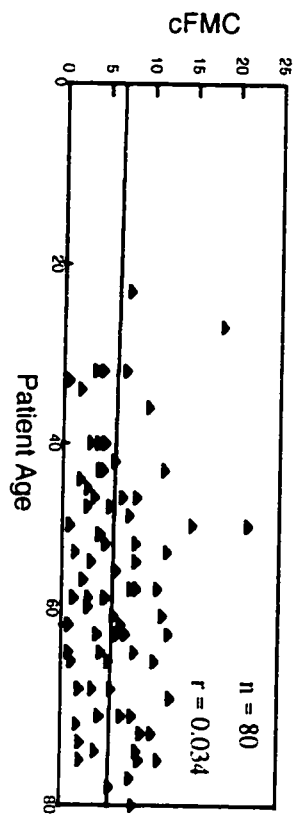


Figure 7

The correlation between the cFMC from the peripheral blood of RA patients and clinical disease parameters. Correlation coefficients, obtained from Spearman's analysis, and the number of patients analyzed for a particular criterion are shown in the upper right of each segment. None of these clinical disease parameters correlated with the cFMC in the peripheral blood of RA patients.



Many of the RA patients were also examined for the presence of erosions, rheumatoid nodules, rheumatoid factor (RF), and their cigarette smoking habits. The presence of the various clinical disease parameters and the CE of T cells from the RA patients did not correlate (Table 4). The presence of erosions, presence of RF, and smoking habits did not affect the cFMC significantly ($p>0.05$) (Table 5). There was a trend to increased cFMC with the presence of erosions, although it is not statistically significant. The presence of nodules correlated with a significant decline in the cFMC. Unfortunately, not all of the patients were analyzed for all four disease parameters leading to small sample sizes and statistical evaluations with low power. Further investigations need to be done prior to drawing a definitive conclusion.

3.3 Patient Medication

Patient medication at the time of the *hprt* clonal assay was also analyzed as a possible variable. Certain medications such as azathioprine, have the potential to induce genetic mutations (Connell *et al.* 1994). There was no significant difference ($p>0.05$) in the CE of T cells from the peripheral blood of RA patients that were being treated with RA disease-modifying medication compared to patients not currently taking this particular kind of medication (Table 6). There were also no significant associations found between the cFMC and various disease treatments (Table 7). Patients who were treated with gold did demonstrate an apparent decline, which was not statistically significant, in the cFMC compared to patients not currently taking the medication. Some patients were not currently taking any disease-modifying medication, yet the cFMC from these patients were still significantly elevated compared to controls.

Table 4

The relationship between the CE of T cells from the peripheral blood of RA patients and clinical disease parameters

	Erosions		Nodules		RF		Smoking Habits	
+/-^a	+	-	+	-	+	-	+	-
Number of Patients	52	16	27	21	53	18	9	19
CE^b	25.07 14.46	23.96 11.09	26.22 15.74	28.42 12.16	23.78 12.46	25.83 12.96	44.07 10.35	29.39 14.36
p^c	0.25		0.30		0.29		0.23	

^a Plus (+) indicates the patients with that disease parameter at the time of the *hprt* assay and minus (-) indicates the patients without that disease parameter at the time of the assay.

^b Mean (%) +/- SD.

^c ANOVA (Levene's test)

Table 5

The relationship between the corrected frequency mutant T cells from the peripheral blood of RA patients and clinical disease parameters

	Erosions		Nodules		RF		Smoking Habits	
	+	-	+	-	+	-	+	-
+/-^a	+	-	+	-	+	-	+	-
Number of patients	52	16	27	21	53	18	9	19
cFMC^b	6.85	4.57	4.72	6.57	6.27	5.79	5.21	5.32
	4.27	2.76	2.38	4.45	3.77	3.51	3.17	3.17
p^c	0.089		0.006		0.89		0.34	

^a Plus (+) indicates the patients that exhibit that particular disease parameter and minus (-) indicates the patients without that disease parameter.

^b Mean cFMC (per 10⁶ cells) +/- SD.

^c ANOVA (Levene's test)

Table 6

The relationship between the CE of T cells from the peripheral blood of RA patients with patient current medication

Medication	+/- ^a	Number of Patients	CE ^b	p ^c
Sulphasalazine	+	3	26.79 +/- 18.86	0.50
	-	75	25.03 +/- 13.13	
Gold	+	14	29.25 +/- 14.23	0.58
	-	64	24.19 +/- 12.94	
Azathioprine	+	5	26.09 +/- 11.70	0.48
	-	72	25.04 +/- 13.49	
Hydroxychloroquine	+	34	22.69 +/- 10.92	0.13
	-	44	26.97 +/- 14.61	
Methotrexate	+	23	24.89 +/- 12.97	0.89
	-	56	25.18 +/- 13.44	
Folate	+	7	19.69 +/- 7.60	0.29
	-	72	25.60 +/- 13.44	
Corticosteroids	+	23	18.13 +/- 10.43	0.40
	-	55	27.86 +/- 13.17	

^a Plus (+) indicates the patients currently taking that particular medication and minus (-) indicates the patients not taking that medication at the time of the *hprt* assay.

^b Mean (%) +/- SD.

^c ANOVA (Levene's test)

Table 7

The relationship between the corrected frequency of mutant T cells from the peripheral blood of RA patients and patient current medication

Medication	+/- ^a	Number of Patients	cFMC ^b	p ^c
Sulphasalazine	+	3	5.97 +/- 1.98	0.96
	-	75	6.06 +/- 3.67	
Gold	+	14	3.75 +/- 2.56	0.07
	-	64	6.57 +/- 3.64	
Azathioprine	+	5	9.50 +/- 3.20	0.54
	-	72	5.83 +/- 3.56	
Hydroxychloroquine	+	34	6.21 +/- 3.90	0.56
	-	44	5.95 +/- 3.43	
Methotrexate	+	23	6.18 +/- 4.12	0.53
	-	56	5.94 +/- 3.43	
Folate	+	7	6.74 +/- 4.66	0.31
	-	72	5.94 +/- 3.53	
Corticosteroids	+	23	7.28 +/- 3.54	0.95
	-	55	5.52 +/- 3.58	

^a Plus (+) indicates the patients currently taking that particular medication at the time of the *hprt* assay and minus (-) indicates the patients not taking that medication at the time of the assay.

^b Mean cFMC (per 10⁶ cells) +/- SD.

^c ANOVA (Levene's test)

4. THE FREQUENCY OF MUTANT T LYMPHOCYTES FROM THE RA SYNOVIAL FLUID

4.1 The Cloning Efficiency of Synovial Fluid T Lymphocytes

Equal proportions of CD4⁺ and CD8⁺ T lymphocytes are located in the synovial fluid of RA patients (Fox *et al.* 1982). An *hprt* clonal assay was performed on the T cells isolated from the synovial fluid of 2 RA patients. The mean CE of T lymphocytes from RA (n=2) synovial fluid T cells was not significantly different ($p>0.05$) compared to the CE of T cells from control (n=20) peripheral blood: 19.65 +/- 8.98 % and 37.1 +/- 19.9 %, respectively. (Figure 8A). A peripheral blood sample was not received from the patients providing the synovial fluid. The mean CE of T cells from the peripheral blood of unselected RA patients from the outpatient clinic versus the CE of RA synovial fluid T cells was not significantly different ($p>0.05$).

4.2 The Frequency of Mutant T cells from the Synovial Fluid of RA Patients

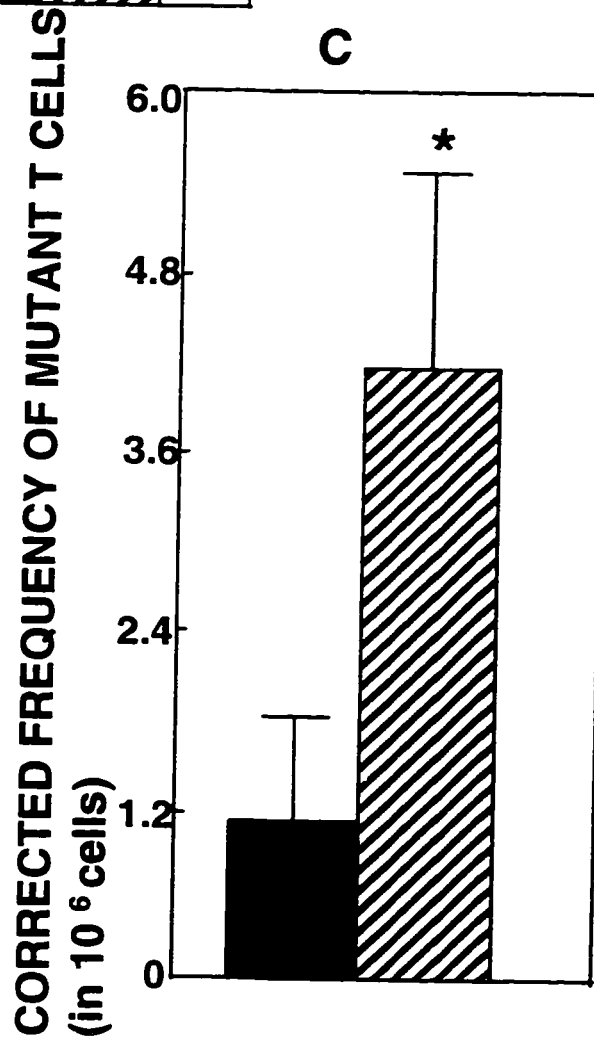
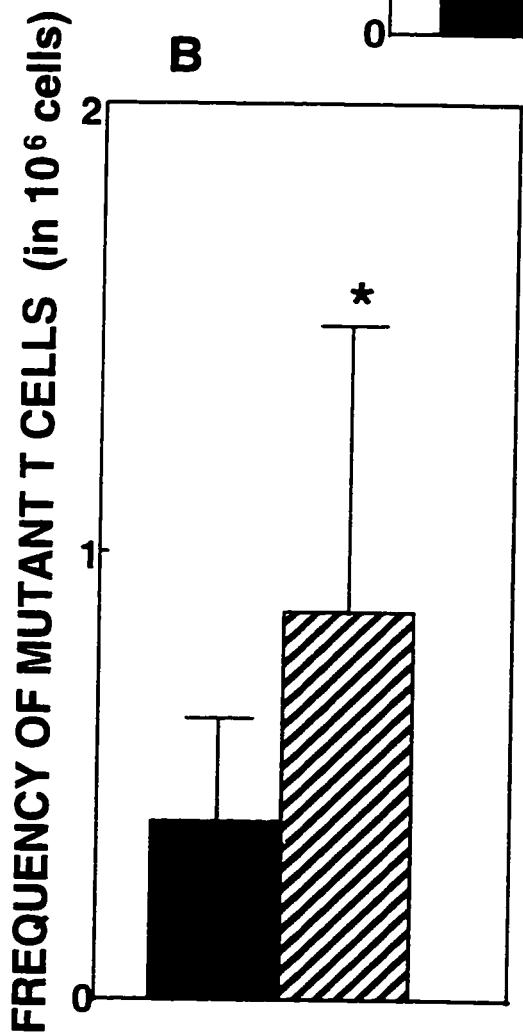
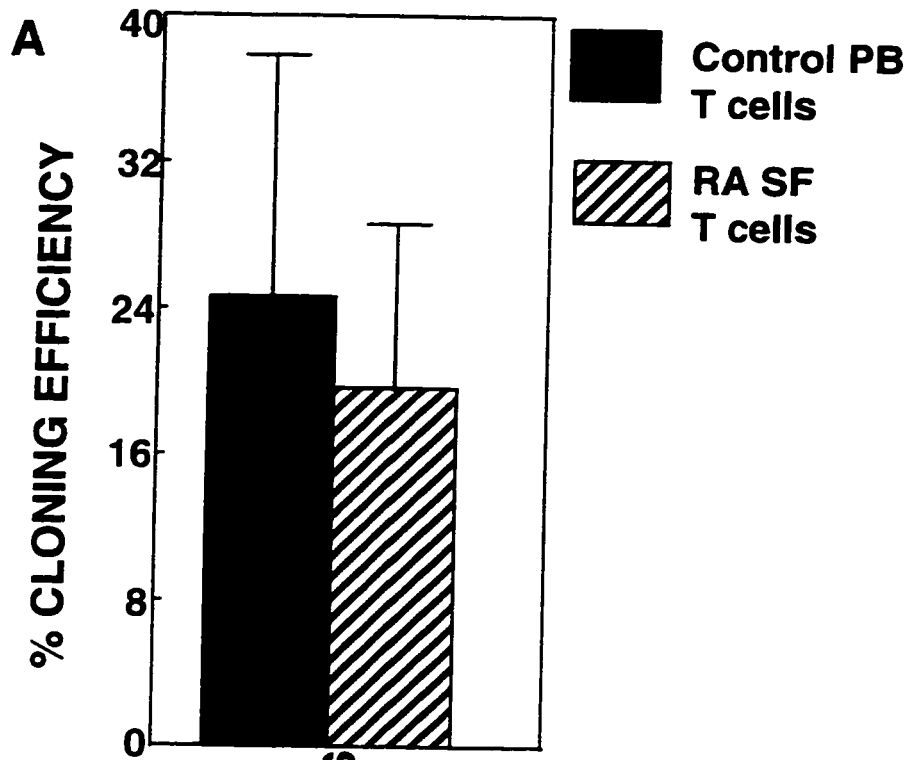
The mean FMC, per 10⁶ cells, from RA synovial fluid was significantly greater ($p<0.03$) compared to control peripheral blood: 0.87 +/- 0.64 and 0.39 +/- 0.23, respectively (Figure 8B). The mean cFMC from RA synovial fluid was also significantly greater ($p<0.02$) than the mean cFMC from control peripheral blood: 4.14 +/- 1.32 and 1.07 +/- 0.71 (per 10⁶ cells), respectively (Figure 8C).

The mean FMC (per 10⁶ cells) from RA peripheral blood and synovial fluid was not significantly different ($p>0.18$): 1.55 +/- 1.14 and 0.87 +/- 0.63, respectively. The mean cFMC from RA synovial fluid T cells was also not significantly different ($p>0.05$) compared to unselected

Figure 8

A) The CE of T lymphocytes from the synovial fluid of RA patients and the peripheral blood of controls. The CE (+/- SD) of T cells from the synovial fluid of 2 RA patients (hatched bar) did not differ significantly ($p>0.05$) compared to the CE of T cells from the peripheral blood of 20 controls (solid bar). B) The frequency of mutant T cells from the synovial fluid of RA patients and the peripheral blood of controls. The mean FMC (+/- SD) from the synovial fluid of 2 RA patients (hatched bar) was significantly greater ($p<0.03$) compared to the mean FMC from the peripheral blood of 20 controls (solid bar). C) The corrected frequency of mutant T cells from the synovial fluid of RA patients and the peripheral blood of controls. The mean cFMC (+/- SD) from the synovial fluid of 2 RA patients (hatched bar) was significantly greater ($p<0.02$) compared to the peripheral blood of 20 controls (solid bar).

* Represents statistical significance.



RA peripheral blood: 4.14 ± 1.32 and 6.26 ± 3.98 in 10^6 cells, respectively. Therefore, the FMC and cFMC in the synovial fluid is significantly elevated compared to control peripheral blood but not significantly different compared to the peripheral blood of unselected RA patients from the outpatient clinic. Although a peripheral blood sample was not obtained from the patients from whom I received a synovial fluid sample, these patients who would be expected to have similar disease activity as those RA patients from the outpatient clinic that donated a peripheral blood sample (Dr. R. Goldstein personal communication).

5. THE FREQUENCY OF MUTANT T LYMPHOCYTES IN RA AND OA SYNOVIAL TISSUE

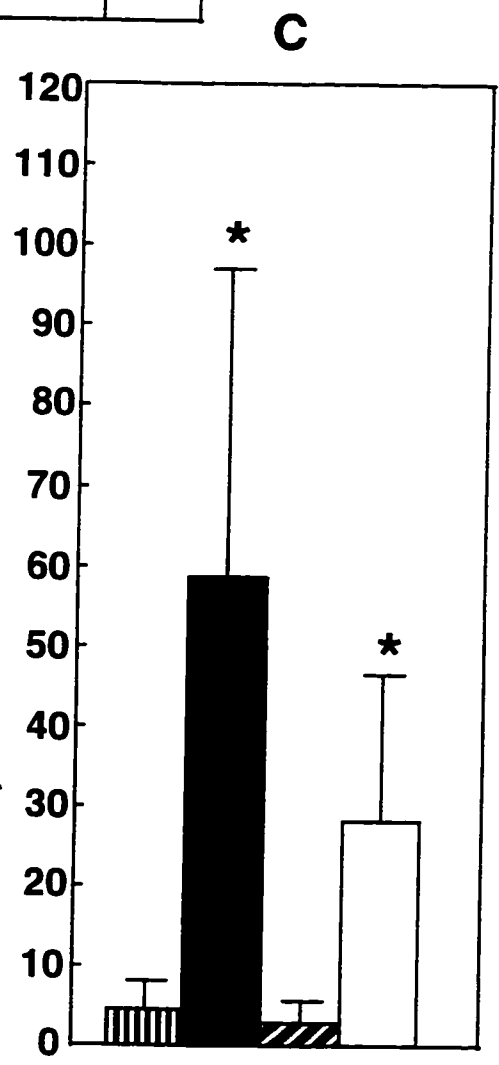
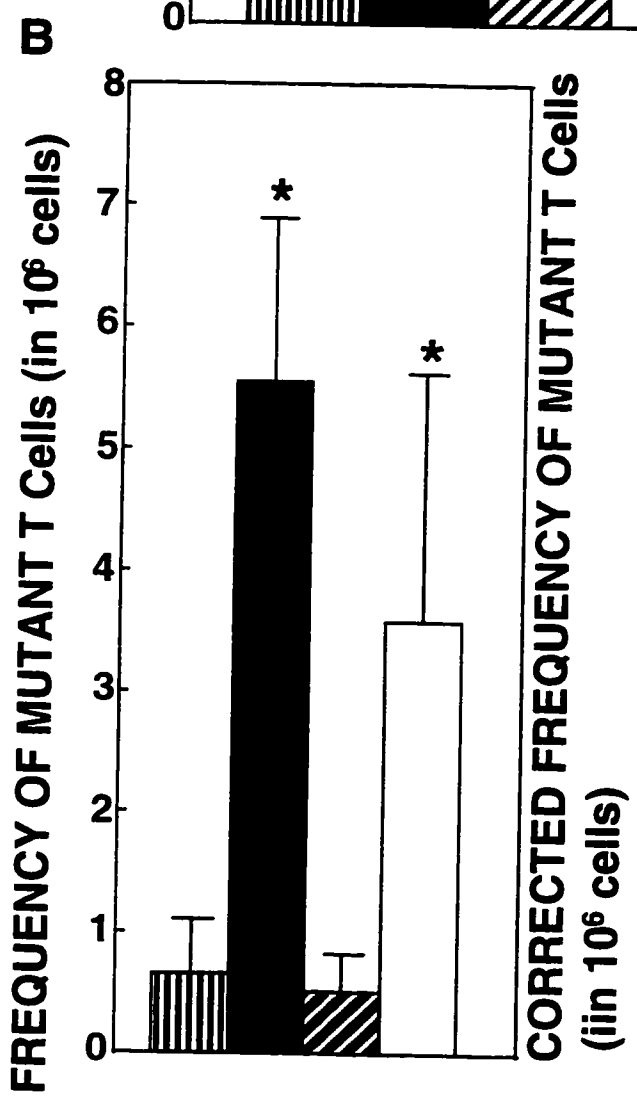
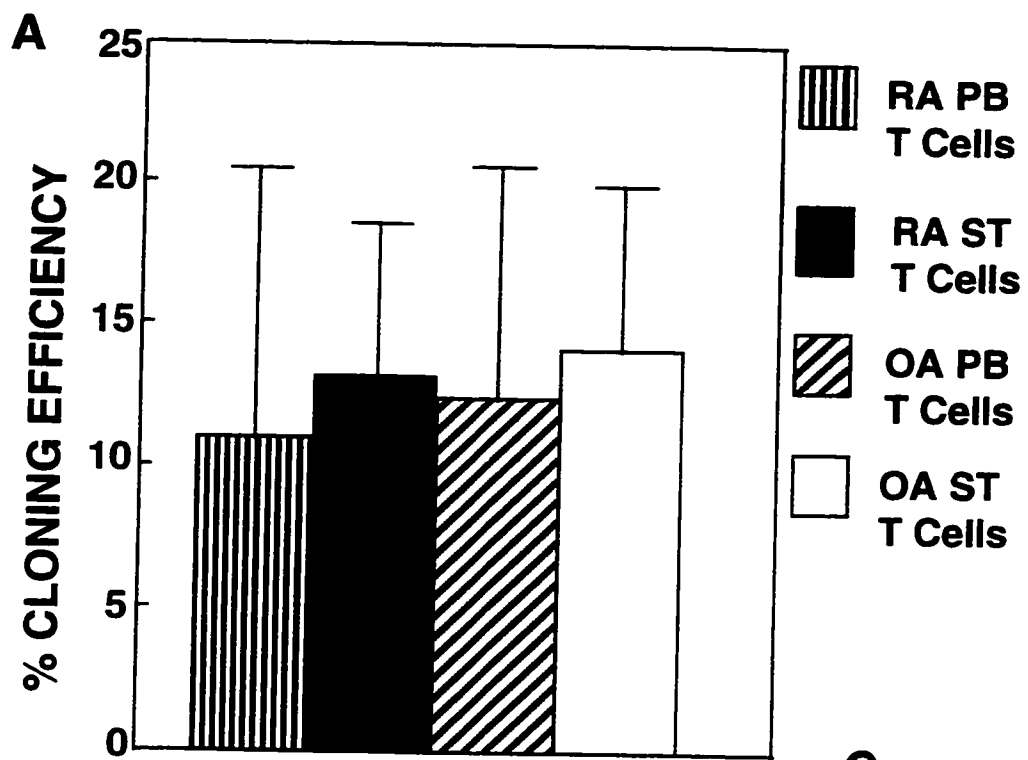
5.1 The Cloning Efficiency of T cells from the Synovial Tissue and Peripheral Blood of RA and OA Patients

$CD4^+ CD45RO^+$ T cells represent a dominant component of the MNC infiltrating the synovium and play a critical role in the pathogenesis of the disease (Nakao *et al.* 1990; Lasky *et al.* 1988). To analyze the critical T cell subsets of the synovium, the *hprt* clonal assay was performed on T cells from RA and OA synovial tissue and peripheral blood. OA is considered to be a non-inflammatory disorder of the joints characterized by deterioration of articular cartilage and the formation of new bone at the joint surfaces and margins (Brandt and Mankin 1993). An equal number of lymphocytes were isolated from both RA and OA synovial tissue samples. The CE of T lymphocytes from the synovium and peripheral blood of 8 RA patients, $12.59 \pm 5.19\%$ and $11.08 \pm 9.45\%$ respectively (Figure 9A), was significantly less ($p < 0.05$) than the CE of T cells derived from the peripheral blood of controls, $37.1 \pm 19.9\%$ (Figure 3). The CE of T cells from the synovium and peripheral blood of 6 OA patients, $14.2 \pm 5.76\%$ and $12.5 \pm 8.15\%$ (Figure 9A),

Figure 9

A) The CE of T lymphocytes from the synovial tissue and peripheral blood of RA and OA patients. There was not a significant difference ($p>0.05$) in the mean CE (\pm SD) of T cells from the peripheral blood (striped bar) and synovial tissue (solid bar) RA patients. The CE of T cells from OA synovial tissue (blank bar) and peripheral blood (hatched bar) was not significantly different ($p>0.05$). B) The frequency of mutant T cells from the synovial tissue and peripheral blood of surgical RA and OA patients. The mean FMC from the synovial tissue of RA patients (solid bar) was significantly greater ($p<0.05$) compared to the peripheral blood (striped bar) of the same patients. The mean FMC from the synovial tissue of OA (blank bar) was significantly greater ($p<0.05$) versus the peripheral blood of the same patients (hatched bar). C) The corrected frequency of mutant T cells from the synovial tissue and peripheral blood of RA and OA patients. The mean FMC from RA synovium (solid bar) was also significantly greater ($p<0.05$) than mean cFMC from the peripheral blood of the same patients (striped bars). The mean cFMC from OA synovium (blank bar) was significantly greater ($p<0.05$) compared to the mean cFMC from the peripheral blood of the same patients (hatched bar).

* Represents statistical significance.



respectively, was significantly less ($p < 0.05$) than controls. However, the CE of OA T cells can not reasonably be compared to the CE of controls because the OA patients are significantly older.

5.2 The Frequency of Mutant T Cells from the Synovial Tissue and Peripheral Blood of Surgical RA and OA Patients

The mean FMC from 8 RA synovium was significantly greater ($p < 0.01$) than from the peripheral blood of the same patients: 5.54 ± 1.34 and 0.66 ± 0.45 , per 10^6 cells, respectively (Figure 9B). The mean cFMC was also significantly greater ($p < 0.007$) in T lymphocytes derived from RA synovium compared to peripheral blood: 58.80 ± 38.22 and 4.56 ± 3.53 (in 10^6 cells) respectively (Figure 9C). Interestingly, the FMC and cFMC from the peripheral blood of surgical RA patients is significantly less ($p < 0.01$) than the FMC and cFMC from the peripheral blood of unselected RA patients derived from the outpatient clinic.

The mean FMC from 6 OA synovium was significantly greater ($p < 0.006$) than from the peripheral blood: 3.58 ± 2.03 and 0.51 ± 0.32 (per 10^6 cells) respectively (Figure 9B). The mean cFMC in 10^6 cells, is also significantly elevated ($p < 0.004$) in OA synovium compared to peripheral blood: 28.25 ± 18.36 and 2.78 ± 2.95 , respectively (Figure 9C). Therefore, the FMC and the cFMC in RA and OA synovium is significantly greater compared to peripheral blood of the same patients.

The mean FMC and cFMC from the peripheral blood of RA compared to OA patients did not differ significantly ($p > 0.05$). The mean FMC from RA synovium was significantly greater

($p < 0.03$) than OA synovium; however, the cFMC from RA versus OA synovium did not differ significantly ($p > 0.10$) (Figure 9B and C).

There was a positive correlation between the cFMC from RA synovium with cFMC from peripheral blood of the same patient (Figure 10A). This graph also demonstrates that the cFMC from the synovium is always consistently greater than from the peripheral blood. There was no correlation between the cFMC from synovium and peripheral blood of OA patients (Figure 10B). However, the cFMC from OA synovium was greater five out of six times than the cFMC from the peripheral blood.

6. CELL ADHESION

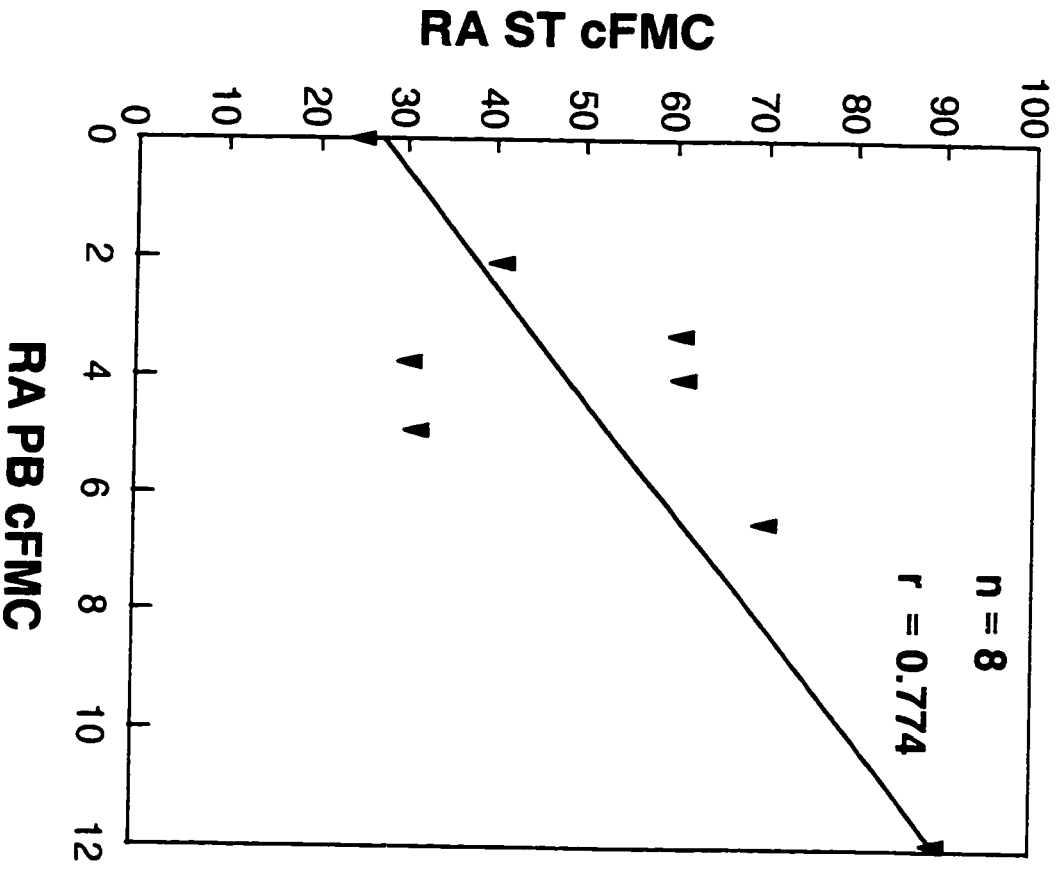
6.1 Fibronectin Adhesion Assay

Variation in the cell surface expression of cell adhesion molecules may explain the preferential retention of specific lymphocytes in rheumatoid synovium where they can function to mediate synovitis. The expression of CD29 ($\beta 1$ integrin) is fundamental in the rheumatoid process, mediating binding to the extracellular matrix components, most notably fibronectin (Hemler 1990). If the peripheral blood *hprt* mutant T cell clones sustained their genetic damage in the genotoxic synovial environment, then they may also display a phenotype similar to the synovial tissue T cell lines. The fibronectin adhesion assay clearly demonstrates that the RA and OA synovial tissue T cells specifically bind to fibronectin, following PHA stimulation. This interaction is inhibited with anti-CD29 MAb while anti-IgG₁, anti-CD7, and anti- $\alpha 4 \beta 7$ MAb did not have a significant effect on fibronectin binding ($p > 0.05$). The synovial tissue T lymphocytes did not bind to the BSA coated wells. The anti- $\alpha 4 \beta 7$ MAb was used because RA synovial tissue T cells have been

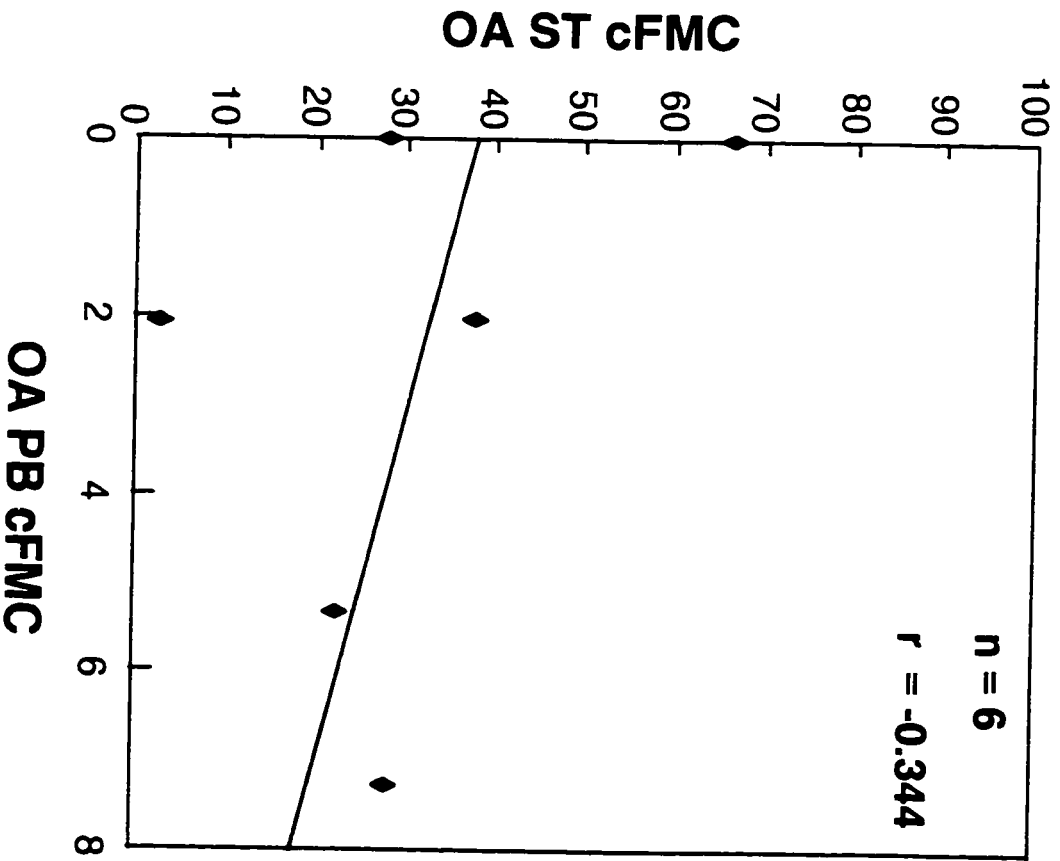
Figure 10

A) A correlation between the corrected frequency of mutant T lymphocytes from the peripheral blood with synovial tissue of RA patients. B) A correlation between the corrected frequency of mutant T lymphocytes from the peripheral blood with synovial tissue of OA patients. The number of patients examined and the Spearman's rank order analysis are in the top right hand corner of the graph.

A



B



shown to express high levels of this integrin. The $\alpha 4\beta 1$ and $\alpha 4\beta 7$ interact with the CS-1 domain of fibronectin suggesting that the anti- $\alpha 4\beta 7$ MAb may inhibit the binding to fibronectin by the synovial tissue T cell lines. However, the binding to fibronectin was not affected by anti-IgG₁, anti- $\alpha 4\beta 7$, or anti-CD7 MAb (Figure 11A).

Interestingly, the RA peripheral blood mutant T cell clones bound to fibronectin with similar affinity as the synovial tissue T cells and this interaction was only blocked by anti-CD29 MAb (Figure 11B). The peripheral blood T cell clones from RA patients and control were also stimulated 14 to 21 days prior to the adhesion assay. The addition of anti-IgG₁, anti-CD7 or anti- $\alpha 4\beta 7$ MAb did not have a significant ($p > 0.05$) effect on the ability of the mutant T cell clones to adhere to fibronectin. The RA peripheral blood non-mutant T cell clones, and the control mutant and non-mutant T cell clones did not bind to the fibronectin or BSA-coated wells (Figure 11B).

6.2 Inhibition Assay

To verify the specificity of the RA mutant T cell clones' ability to bind to fibronectin, an inhibition assay was performed (Figure 12). The RA mutant T cell clones and RA synovial tissue T cell lines bound to fibronectin and this binding was inhibited when the T cells were incubated with increasing amounts of a soluble fibronectin fragment containing the CS-1 binding sequence. The CS-1 25 amino acid sequence is the region of fibronectin that CD29 ($\beta 1$) specifically binds to. Binding to fibronectin was not affected by the addition of HEL, a non-specific protein. Therefore, the RA peripheral blood mutant T cell clones bound specifically to fibronectin and this interaction occurred between the CD29 receptor and the CS-1 domain of fibronectin.

Figure 11

A) Binding to fibronectin by RA and OA synovial tissue T cell lines and RA peripheral blood *hprt* mutant and non-mutant T cell clones in the absence and presence of various antibodies. Eight RA peripheral blood mutant T cell clones (striped bars) bound to fibronectin with equal affinity compared to the 6 RA (solid bars) and 6 OA (blank bars) synovial tissue T cell lines but the 8 non-mutant clones (hatched bars) did not bind. Binding to fibronectin by the RA and OA synovial tissue T cell lines and RA mutant T cell clones was significantly inhibited by anti-CD29 MAb ($p < 0.05$) while anti-IgG₁, anti-CD7, or anti- $\alpha 437$ MAb did not significantly inhibit fibronectin binding ($p > 0.05$). B) Binding to fibronectin by RA and control peripheral blood mutant and non-mutant T cell clones in the presence of various antibodies. The 8 RA mutant T cell clones (striped bars) bound to fibronectin. However, the 8 RA non-mutant (hatched bars) and the 6 control mutant (solid bars) and 6 non-mutant T cell clones (blank bars) did not bind to the fibronectin or the BSA coated wells. The addition of the various MAb did not have a significant effect on the RA non-mutant, and control mutant and non-mutant T cell clone fibronectin adherence.

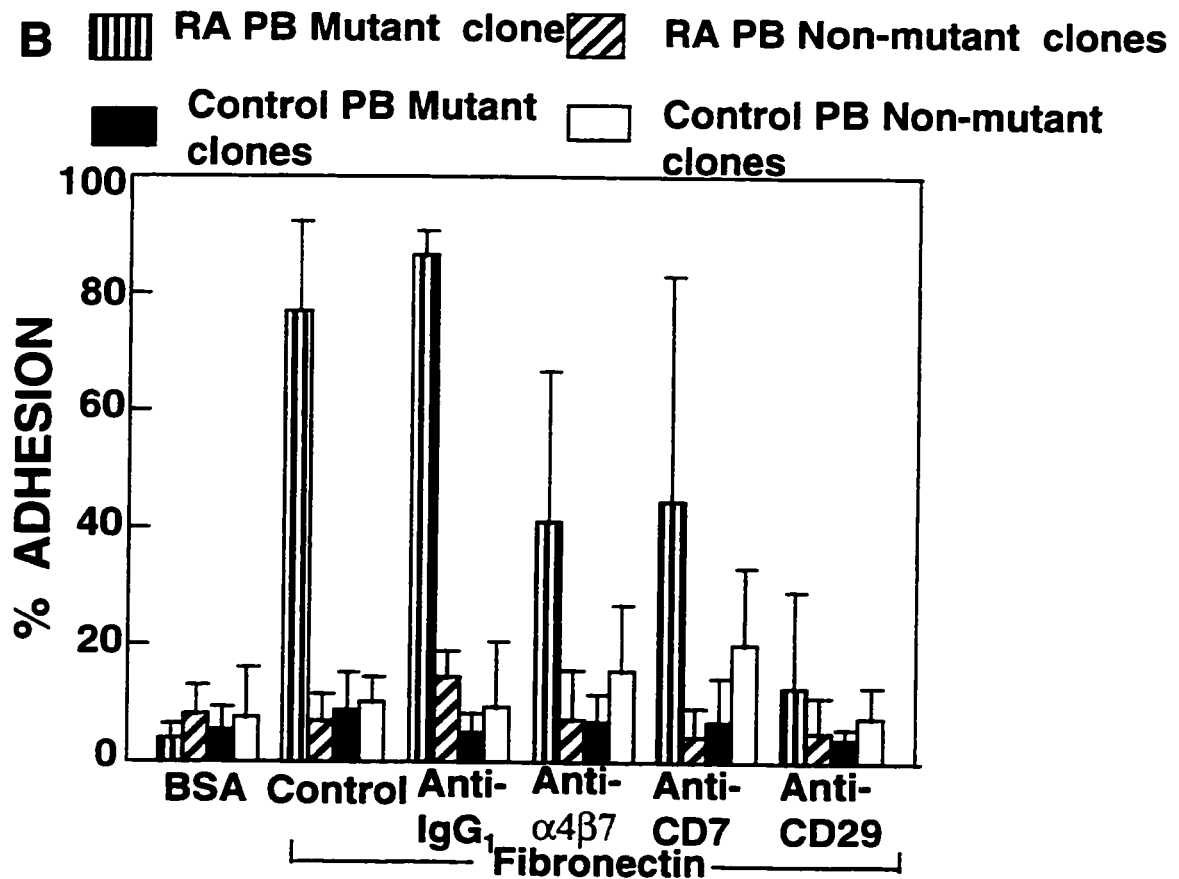
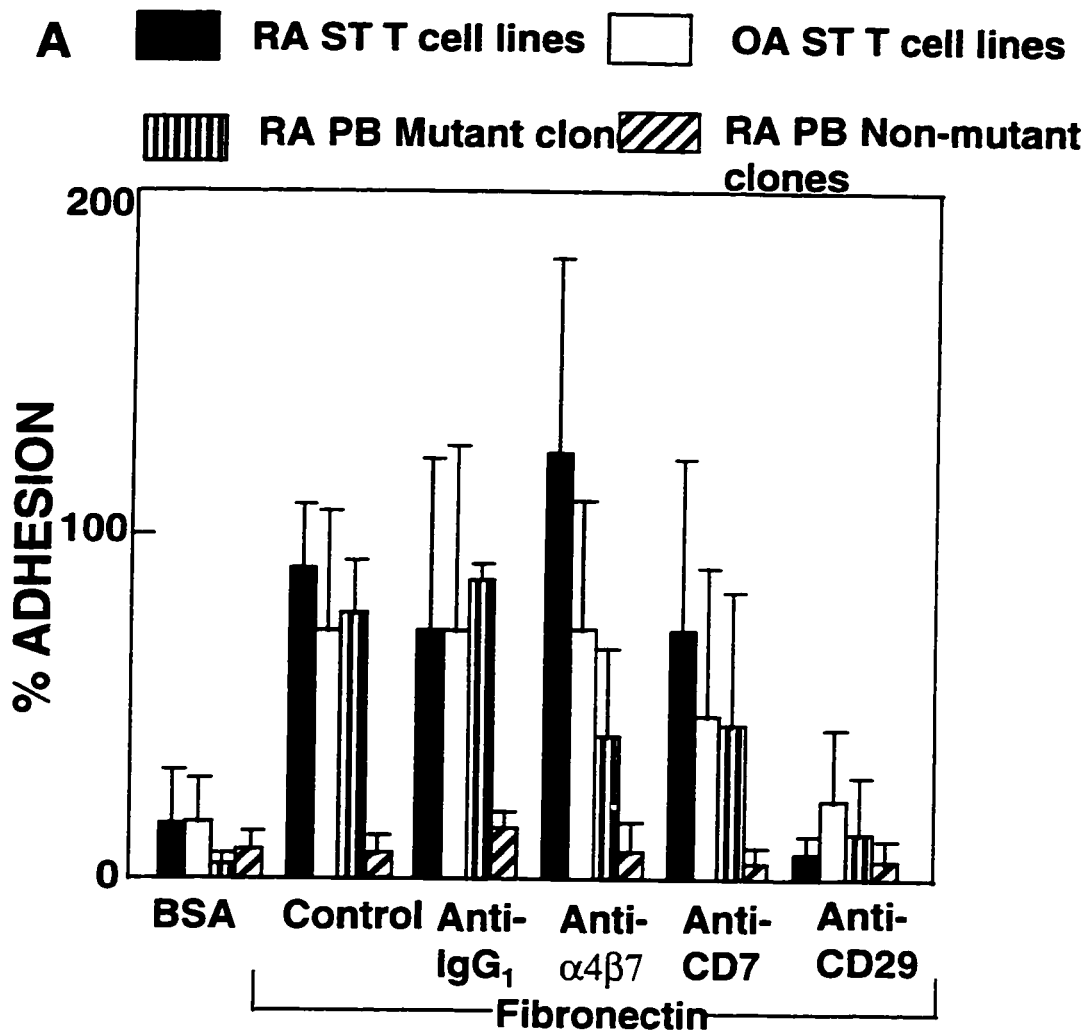


Figure 12

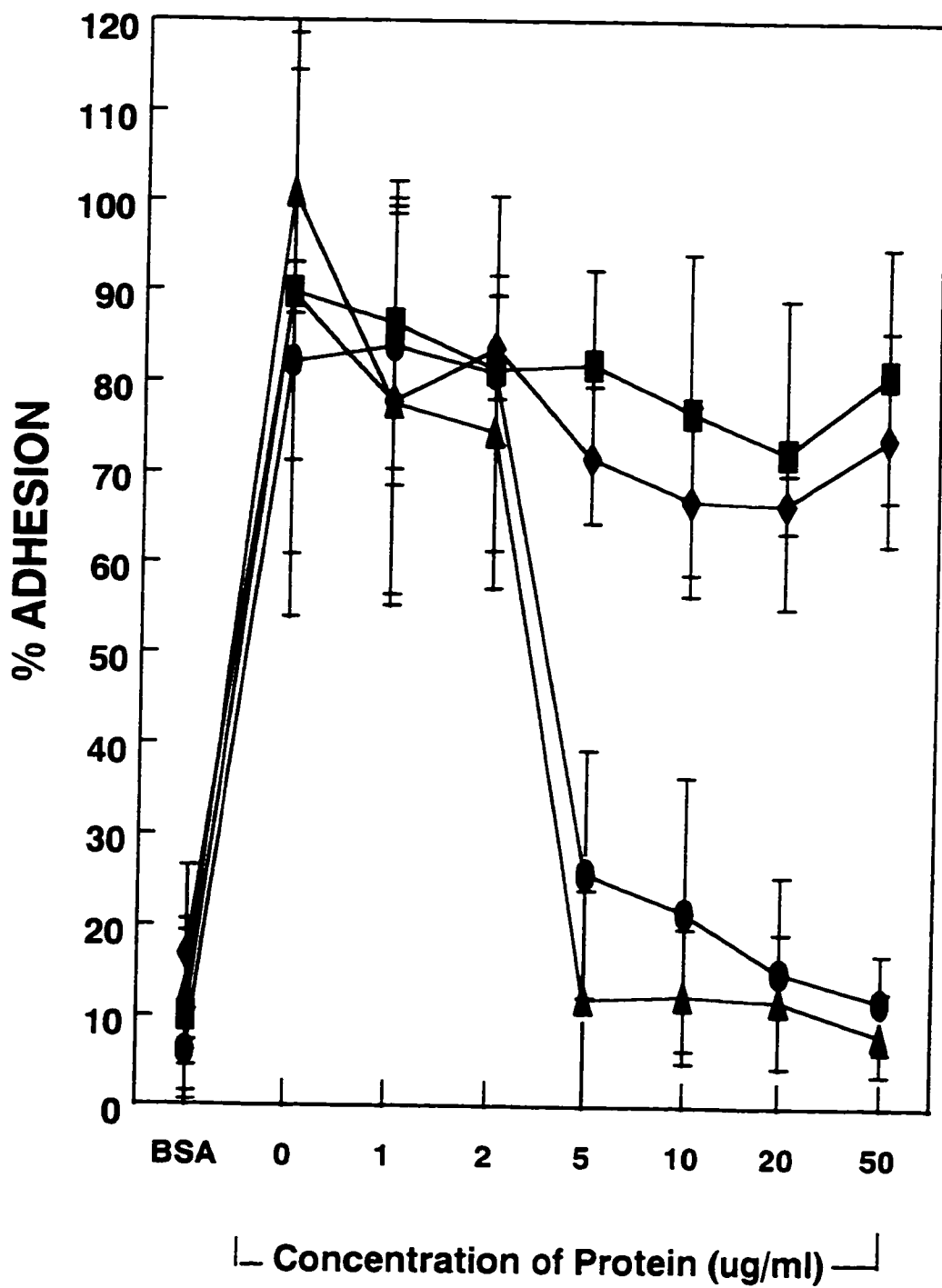
Binding to fibronectin is mediated through CD29 interactions with the CS-1 fibronectin fragment. The 3 RA synovial tissue T cell lines (closed triangle) and the 3 RA peripheral blood mutant T cell clones (closed circle) bound to fibronectin but in the presence of soluble fibronectin fragment containing the CS-1 domain the binding significantly declined. 3 RA synovial tissue T cell lines (closed square) and 3 RA peripheral blood mutant T cell clones (closed diamond) were incubated with a non-specific protein HEL, and binding to fibronectin was not significantly affected.

▲ RA ST, CS-1
n=3

◆ RA ST, HEL
n=3

● RA PB, CS-1
n=3

■ RA PB, HEL
n=3



7. FLOW CYTOMETRIC ANALYSIS OF THE RA PERIPHERAL BLOOD T CELL CLONES AND RA SYNOVIAL TISSUE T CELL LINES

7.1 Expression of T Cell Surface Markers

To verify the identity of the peripheral blood mutant and non-mutant T cell clones, these clones were immunophenotyped (Figure 13). Three colour flow cytometric analysis was used to determine the identity of the T cell clones. Eight RA peripheral blood mutant and 8 non-mutant clones containing 2×10^5 cells were analyzed. Mutant and non-mutant clones from the same patient were analyzed at the same time. RA synovial tissue T cell lines were implemented as positive controls. All of the mutant T cell clones were CD3⁺; however, these clones varied in the expression of CD4 and CD8. Some of the mutant clones appeared to be CD4⁻ CD8⁻ and others expressed low levels of CD4. The non-mutant clones were CD3⁺ and either CD4⁺ or CD8⁺.

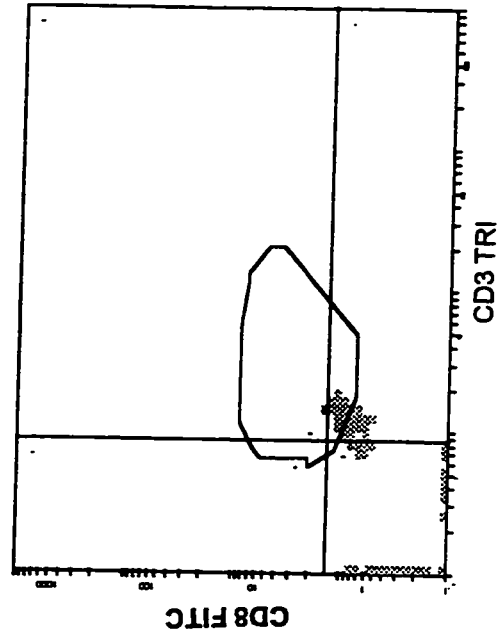
7.2 Integrin Expression

An increased adhesion to fibronectin may be explained by conformational changes in the integrins, an increased affinity of the integrin for fibronectin, or an increased level of integrin expression. To elucidate if the synovial tissue T cells and the RA mutant T cell clones express comparable levels of CD29, the expression of this integrin was investigated by flow cytometry (Figure 14). Ten mutant and 10 non-mutant clones containing 2×10^5 cells were analyzed 14 to 21 days following PHA stimulation. The mutant and non-mutant T cell clones from the same patient were analyzed at the same time. Hut 78 and RA synovial tissue T cell lines were used as positive control for CD29 expression. The 2 colour flow cytometric results indicate that the RA synovial tissue T cell lines and the RA peripheral blood mutant T cell clones were CD3⁺ and expressed comparable levels of CD29. The RA peripheral blood non-mutant T cell clones also expressed

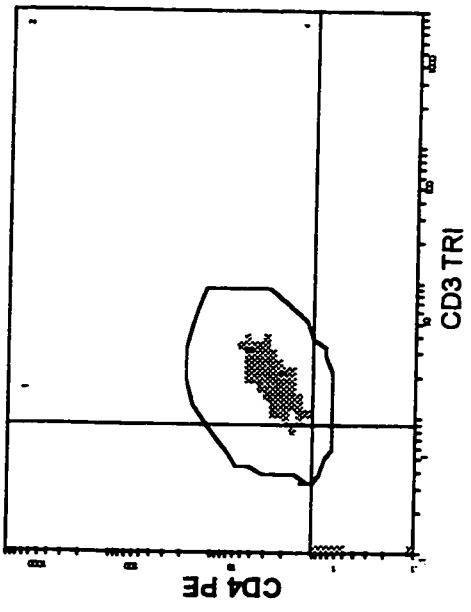
CD29 but at a much lower level than the mutant clones or the T cell lines. These results demonstrate that the fibronectin binding was mediated through CD29 and CD29 appears to be upregulated on T cells from the RA synovium and the RA peripheral blood mutant T cell clones.

Figure 13

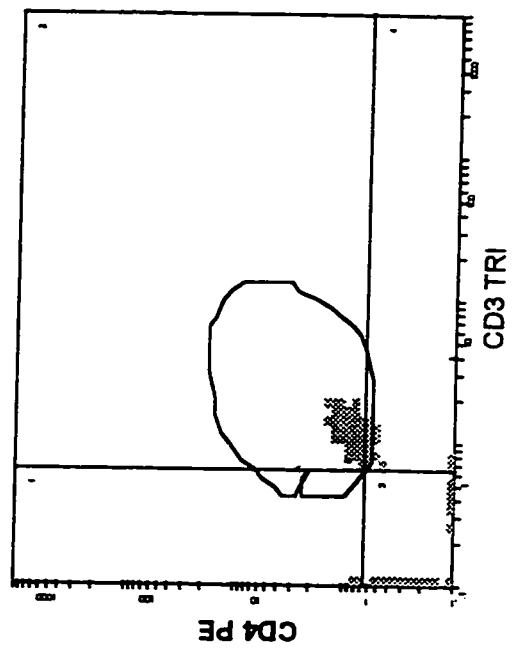
Immunophenotype of 8 RA peripheral blood mutant and 8 RA peripheral blood non-mutant T cell clones: expression of CD3, CD4, and CD8. The mutant and non-mutant T cell clones were from the same RA patients and the clones were collected and stained at the same time. The mutant T cell clones expressed CD3 and varying levels of either CD4 or CD8. A small portion of the mutant T cell clones immunophenotyped were CD4⁻ CD8⁻. A) RA peripheral blood mutant T cell clone stained with CD3 TRI and CD4 PE MAb. B) RA peripheral blood mutant T cell clone stained with CD3 TRI and CD8 FITC. C) RA peripheral blood non-mutant T cell clone stained with CD3 TRI and CD4 PE.



A



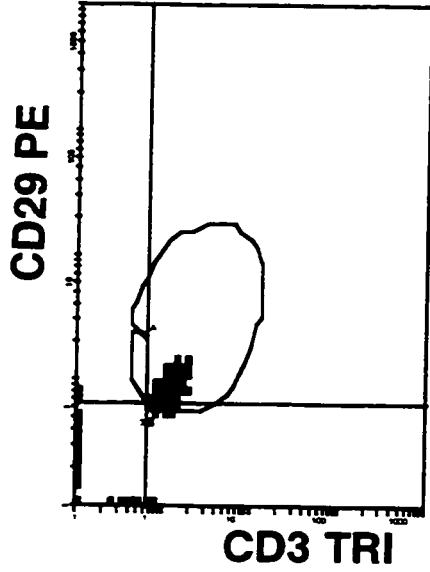
B



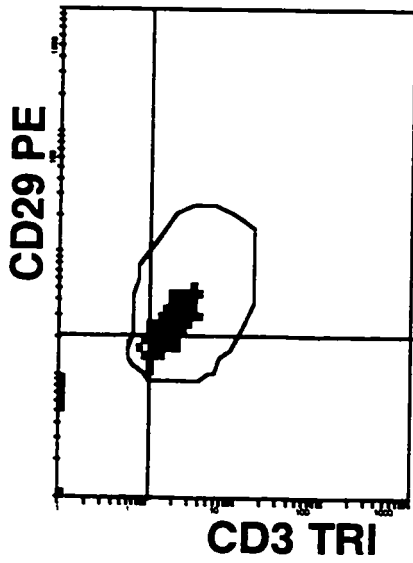
C

Figure 14

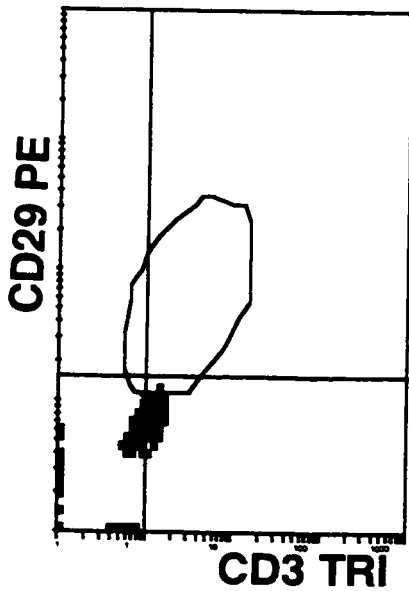
CD29 expression on the RA synovial tissue T cell lines and RA peripheral blood mutant and non-mutant T cell clones. The T cells were stained with CD3 TR1 and CD29 PE MAb. The mutant and non-mutant T cell clones were derived from the same patient and stained at the same time. A) The RA synovial tissue T cell lines (n=6) and B) the RA peripheral blood mutant T cell clones (n=10) express similar level of CD29 while the C) RA non-mutant T cell clones (n=10) usually expressed a lower level of CD 29.



A) RA Synovial Tissue T cells



B) RA Peripheral Blood Mutant T Cell Clone



C) RA Peripheral Blood Non-mutant T Cell Clone

CHAPTER 4: DISCUSSION

1. T LYMPHOCYTE INVOLVEMENT IN RA

The activation and proliferation of T lymphocytes plays an integral role in the pathogenesis of RA. T cells represent the dominant component of MNC infiltrating the synovium (Firestein 1994). The majority of these T cells are CD4⁺, and express CD45RO, CD29, and HLA-DR markers of activation (Firestein 1994; Harris 1993). These cell surface markers suggest that this subset of T cells are memory cells displaying features of post-activation. Although the majority of T cells within the inflamed RA synovial tissue are memory T cells there is very little evidence of T cell proliferation (Lia *et al.* 1995). There is also not very substantial evidence of T cell apoptosis: T cells upregulate bcl-2 and propidium iodine staining demonstrates viable non-dividing T cells (Panayi *et al.* 1992). Although many T lymphocytes infiltrate the synovium, it has been hypothesized that only a small subset of autoreactive and/or antigen-specific T cells are among the infiltrating cells. The identification and analysis of these autoreactive and/or antigen-specific T cells is particularly challenging. Using the *hprt* clonal assay, I have been able to select a subset of T lymphocytes that have more likely been stimulated and genetically damaged. The autoreactive and/or antigen-specific T cell subset may fall into this category.

2. THE FREQUENCY OF MUTANT T LYMPHOCYTES FROM THE PERIPHERAL BLOOD OF RA PATIENTS AND CONTROLS

The CE of controls in this study was similar to the CE of controls used in other *hprt* studies (Albertini *et al.* 1982). Although the assay to determine the appropriate concentration of PHA to stimulate T cell proliferation was not conclusive, the use of 1 µg/ml of PHA was able to stimulate

the T cells such that the CE of RA peripheral blood T cells was parallel to reported data. There was a decline, although not statistically significant, in the CE of T cells from the peripheral blood of RA patients compared to controls. This is consistent with earlier reports comparing the CE of T lymphocytes from the peripheral blood of patients with other autoimmune diseases such as MS (Allegretta *et al.* 1990; Sriram 1994) and SLE (Gmelig-Meyling *et al.* 1992; Theocharis *et al.* 1995) to healthy controls. The CE of T cells did not vary significantly in RA patients who were currently taking RA disease-modifying medication compared to those who were not. This suggests that disease treatment was not responsible for the slight non-significant decrease in CE. The CE of T cells also did not appear to be associated with a variety of clinical parameters. The decrease in CE of T cells from RA patients and other autoimmune diseases may be related to the disease process itself because T lymphocytes are chronically activated in all three of the autoimmune diseases mentioned.

There was a significant increase in the FMC in the peripheral blood of RA patients compared to controls. The cFMC, which takes into account the CE of the T cells from the individual samples, was also significantly elevated in the peripheral blood of RA patients compared to controls. The mean age of the RA patients examined were older than the mean age of controls, an increase in age alone is insufficient to account for the profound increase in the cFMC found in the peripheral blood of RA patients (Green *et al.* 1995; Jones *et al.* 1995). Green and coworkers (1995) determined that the mutation rate of the *hprt* locus in T lymphocytes was 5×10^{-7} which cannot explain the increase in the frequency of mutant T cells observed in the peripheral blood of RA patients. A similar increase in the frequency of mutant T cells has also been observed in the peripheral blood of patients with other autoimmune diseases including MS (Allegretta *et al.* 1990; Sriram 1994) and SLE (Gmelig-Meyling *et al.* 1992; Theocharis *et al.* 1995). These investigators

have hypothesized that the increase in the cFMC seen in the peripheral blood of MS and SLE patients was due to the enhanced proliferation of disease-related T cells and insufficient DNA repair mechanisms. However, in the RA synovium, T cell proliferation alone would appear to be an insufficient stimulus for the development of this increase in frequency of mutant T cells. The inflamed synovium does not contain very many proliferating T cells (Lai *et al.* 1995), thus I postulate that the mitogenic and mutagenic milieu of the RA synovium is an environment capable of inducing genetic damage to the infiltrating lymphocytes.

3. THE FREQUENCY OF MUTANT T CELLS FROM THE SYNOVIAL FLUID OF RA PATIENTS

The CE of T lymphocytes from the synovial fluid of RA patients was not significantly different from the CE of peripheral blood T cells from controls and unselected RA patients. Although there was a small sample size, the FMC and the cFMC from RA synovial fluid was significantly greater than the FMC and cFMC from the peripheral blood of controls. The FMC and cFMC from RA synovial fluid was not greater than from the peripheral blood of unselected RA patients from the outpatient clinic, who would be expected to be comparable. The T cells in the synovial fluid could have sustained genetic damage in the fluid due to the presence of RNS and ROS released by granulocytes (Stuehr and Griffith 1992). Although antigen-presenting cells such as macrophages are found within the synovial fluid, PMN predominate. Thus, the environment within the synovial fluid is likely not highly mitogenic and the cFMC observed in the synovial fluid of RA patients is in keeping with this. The synovial fluid T cells could have been damaged in the synovial tissue and entered the synovial fluid while recirculating.

4. THE FREQUENCY OF MUTANT T CELLS FROM THE SYNOVIAL TISSUE AND PERIPHERAL BLOOD OF SURGICAL RA PATIENTS

Synovial tissue was obtained from RA and OA patients undergoing joint replacement surgery. The CE of T cells from the peripheral blood and synovial tissue of RA patients compared to OA patients was similar, but significantly lower than the CE of T lymphocytes from controls. While the mean duration of RA disease was not different in the RA patients who did or did not undergo surgery, the decrease in the CE of T cells from the surgical RA patients may reflect the fact that these RA patients had a more aggressive, more severe, or more active disease for a longer period. The CE of T cells from the synovial tissue and peripheral blood of the OA patients was significantly less than the CE of control peripheral blood T cells. However, the OA patients were significantly older than the controls and age is associated with a decline in the CE of T cells in the healthy population (Jones *et al.* 1995). We were not able to study an age matched control group of elderly persons. Therefore, due to the disparity between the OA patients and controls, a conclusion can not be appropriately drawn.

The frequency of mutant T cells in RA and OA synovium was significantly greater compared to the peripheral blood of the same patients. The cFMC was 10-fold greater in the synovium of RA and OA patients compared to the peripheral blood. Within the RA synovium, T cells may be stimulated to proliferate by a variety of self or foreign processed-peptides presented by macrophages and dendritic cells in combination with costimulatory cytokines (Kuby 1994). The T cells can also be stimulated by TCR-independent pathways to release various cytokines and growth factors (Kuby 1994). Within this mitogenic environment, the T cells may suffer genetic damage due to the presence of genotoxic species released by macrophages, mast cells, synoviocytes, fibroblasts, and endothelial cells. The mutagenic and mitogenic environment of the

inflamed synovium may be one of the mechanisms which could explain the elevated cFMC in the synovial tissue of RA patients.

The FMC and cFMC from the peripheral blood of RA compared to OA patients did not differ significantly. The FMC from the RA synovium was significantly greater than from the OA synovium but there was no significant difference in the cFMC. A definitive conclusion can not be formulated because the mean age of the OA patients was significantly greater than the RA patients. The frequency of mutant T cells is known to increase with age (Green *et al.* 1995; Jones *et al.* 1995), suggesting that the elevated FMC and cFMC observed in OA patients may reflect in part the age of the patients as well as the disease process.

OA is considered to be a degenerative non-inflammatory disease of joints (Brandt and Mankin 1993). Upon histological examination, in early OA there is less synovial proliferation compared to RA. Nevertheless, lymphocytes can be seen during the late stages of the disease and an equal number of T cells were isolated from the synovium of RA and OA patients. As in RA, the T lymphocytes isolated from OA synovium were CD29⁺ and could adhere to fibronectin. These results imply that as osteoarthritis progresses, lymphocytes may infiltrate the synovium in a manner similar to what is thought occur in RA. Although the diseases may be initiated by two distinct, as yet undefined mechanisms, the damage sustained by the infiltrating synovial T lymphocytes may be caused by the mitogenic and mutagenic environment in both of these diseases. Once present in the RA or OA synovium, T lymphocytes may reside within the tissue for an undefined period and subsequently re-enter the peripheral circulation. Thus, the mutant T cells in the peripheral blood may represent the T cells that sustained damage in the synovium.

There was a positive correlation between the cFMC from RA synovium and peripheral blood from the same patient. These results suggest the T cells may incur genetic damage within the

mitogenic and mutagenic environment of the RA synovium and then re-enter the peripheral circulation. In the OA patients there was no correlation between the cFMC from synovium compared to peripheral blood of the same patient. This result suggests that the T cells from OA synovium may remain within the joint for a longer period, recirculate less frequently, or that the peripheral blood mutant T cells sustained genetic damage in a location other than the inflamed synovium.

Interestingly, the RA and OA mutant and non-mutant cells from the synovium could not be cloned in the long-term. The T lymphocytes grew to form a visible clone within 3 weeks and the cells died within the next 3 to 4 weeks in all cases except 1 RA patient. The T lymphocytes may have been overstimulated within the synovium due to the highly mitogenic environment and further inappropriate *in vitro* stimulation resulted in apoptosis. Therefore, the *in vitro* culture conditions may not have provided the necessary growth conditions to favour T cell growth, implying that with the appropriate conditions, the cFMC from RA and OA synovium may be even greater. T lymphocytes from the synovium appear to be chronically activated *in vivo*, thus stimulating the T cells *in vitro* may prove to be very difficult. Modulating the *in vitro* culture conditions such as PHA stimulation and cytokine addition may facilitate T cell growth. The activational state of T lymphocytes may vary from patient to patient due to the nature of the disease and the chronic stimulation of the T cells may vary due to the severity of disease within that particular joint in a specific patient.

5. THE INCREASE IN THE FREQUENCY OF MUTANT T CELLS IS NOT AN *IN VITRO* PHENOMENON

When the MNC are plated under selection condition with 6-TG, it takes several hours for the 6-TG to enter the *hprt*⁺ cells, be converted into a toxic nucleotide, and kill the cell (Albertini *et al.* 1982; Morley *et al.* 1983). Cells which contain a mutation in the *hprt* gene do not produce a functional HPRT protein and are able to survive in the presence of the purine analog, 6-TG. During this lag period, inflammatory cells such as macrophages and granulocytes are able to secrete a variety of ROS and RNS including H₂O₂. H₂O₂ is a lipid soluble molecule that is able to enter cell membranes. H₂O₂ can interact with transition metals within the cells including those associated with DNA, and produce OH⁻ which in turn can cleave the DNA molecule resulting in genetic damage (Halliwell *et al.* 1988). This event theoretically could result in an *in vitro* increase in the frequency of mutant T cells detected by this assay. Catalase was added to the cultures because this enzyme catalyzes the breakdown of 2 H₂O₂ into 2 H₂O and O₂. If the inflammatory cells were releasing sufficient H₂O₂ in culture to induce DNA damage, then the addition of catalase would prevent DNA damage via H₂O₂ and result in a decline in the frequency of mutant T cells detected by the *hprt* assay. There was no significant change in the FMC and the cFMC from the peripheral blood of RA patients and controls in the absence or presence of catalase. The argument that the elevated frequency of *hprt* mutant T cells is not an *in vitro* phenomenon, is further strengthened by the fact that the FMC and the cFMC from the peripheral blood of healthy controls did not vary significantly when the T lymphocytes were plated immediately in selection medium or remained in culture for 2 days and then were plated in the presence of 6-TG. Therefore, the 2-day culture duration may not provide sufficient time for T lymphocytes to suffer genetic damage from the

genotoxic species released by inflammatory cells and to be stimulated to proliferate due to the presence of MNC.

6. CORRELATION OF CLINICAL DISEASE WITH FMC FROM THE PERIPHERAL BLOOD OF RA PATIENTS

6.1 Disease Activity

RA patients were divided into 2 categories based on clinical disease parameters. I defined 'active' disease according to the clinical criteria for RA disease activity for the inclusion of patients in clinical trials (Felson *et al.* 1993). The CE of T lymphocytes from the peripheral blood of RA patients with 'active' or 'inactive' disease did not differ significantly. However, the CE of peripheral blood T cells from patients with 'active' or 'inactive' disease was significantly greater than the CE of T cells from the peripheral blood and synovial tissue of RA and OA patients who underwent joint surgery. These results suggest that the criteria for clinically active disease may not adequately reflect the amount of damage sustained during the course of the disease. The frequency of mutant T cells may reflect the total amount of 'active' disease in years and not current disease activity. I was not able to evaluate the total amount of 'active' disease or severity of 'active' disease for these patients in a retrospective manner.

6.2 Clinical Disease Parameters

There was no correlation between any of the clinical disease parameters evaluated and the cFMC from the peripheral blood of RA patients. The clinical disease parameters analyzed are useful measures of current disease activity and thus may not reflect long term genetic damage. Disease parameters which reflect the chronicity of disease include joint erosions. The presence of

erosions and RF, as well as the patient's cigarette smoking habits did not correlate with the cFMC from the peripheral blood. Although the result was not significant, there was a trend toward an increase in the cFMC with the presence of erosions which suggests that the cFMC may reflect disease chronicity. However, the patients with rheumatoid nodules had significantly lower cFMC compared to patients who did not have nodules. Rheumatoid nodules and joint erosions usually correlate with each other. Nodules tend to correlate with disease severity however, nodules can increase or decrease with the use of different medications. For example, methotrexate treatment correlates with increased nodule formation (Arnett *et al.* 1988; Felson *et al.* 1993). Thus, similar to current disease activity, the presence of nodules at one particular time point may be deceiving. The corrected frequency of mutant T cells may not be related to disease activity or severity, but with the degree of inflammation within a particular joint over the entire course of disease. Unfortunately, not all the patients had complete clinical data available. A definitive conclusion concerning the data of disease severity and chronicity can not be made until further patients are studied.

6.3 Patient Medication

Alkylating agents have been shown to increase the frequency of mutant cells (Branda *et al.* 1991). Azathioprine, an RA disease-modifying drug, and 6-mercaptopurine, the active *in vivo* metabolite of azathioprine, can also induce somatic mutations and potentially select for *hprt* deficient cells (Albertini *et al.* 1990). Interestingly, azathioprine is a medication used to treat RA and SLE patients that potentially can select *hprt* T cells. There was no correlation with an increase in the frequency of *hprt* mutant T cells from the peripheral blood of SLE patients and azathioprine

treatment (Gmelig-Meyling *et al.* 1992). To formally examine the possible contributions of the cytotoxic disease-modifying drugs the cFMC of subgroups of patients with or without a particular therapy were assessed. There was no significant association found between the cFMC and the various patient medications. These results suggest that the patient treatment did not cause the elevated FMC seen in the RA patients compared to controls. The results are strengthened by the fact that some patients who were not currently taking disease-modifying medication still had an increase in the cFMC in the peripheral blood compared to controls.

7. PHENOTYPE OF THE PERIPHERAL BLOOD RA AND CONTROL T CELL CLONES AND THE RA AND OA SYNOVIAL TISSUE T CELL LINES

The phenotype of the RA peripheral blood mutant T cell clones supports the concept that these T cells may have infiltrated the inflamed synovium. The RA synovial tissue T cell lines were predominantly CD4⁺ $\alpha\beta$ T cells which is consistent with earlier reports (Cush and Lipsky 1988). The RA and control peripheral blood T cell clones also demonstrated this pattern of expression: however, this is most likely due to the cloning process itself. The T lymphocytes derived from MS and SLE patients using the *hprt* clonal assay were also CD4⁺ suggesting that the cloning process may select CD4⁺ T cells (Ailegretta *et al.* 1990; Gmelig-Meyling *et al.* 1992). RA synovial tissue T lymphocytes exhibit a 2 to 3-fold increase in migratory capacity over normal and RA peripheral blood lymphocytes (Hauzenberger *et al.* 1994). Circulating T cells gain entry into the synovium first by binding to the vascular endothelium through LFA-1/ICAM-1 (Kavanaugh *et al.* 1994; Stewart *et al.* 1996; Yokoto *et al.* 1995) and $\alpha 4\beta 1$ /VCAM (Berlin *et al.* 1995; Johnston *et al.* 1996) interactions and then by infiltrating the synovial membrane (Elices *et al.* 1994). Variation in the surface expression of cell adhesion molecules may explain preferential retention of specific

lymphocytes in rheumatoid synovium. Cytokines such as IL-4 found in the synovium enhance VCAM expression and thus enhance T cell infiltration (Shimada *et al.* 1994). In the synovial fluid and tissue, T cells interact with fibronectin through $\alpha 4\beta 1$, $\alpha 5\beta 1$, (Rodriguez *et al.* 1992; Elices *et al.* 1994) and $\alpha 4\beta 7$ integrins (Chan *et al.* 1992).

The T lymphocytes derived from the synovial fluid and synovial tissue display an increased expression of CD29 ($\beta 1$ integrin) (Rodriguez *et al.* 1992; Elices *et al.* 1994). The RA and OA synovial tissue T cell lines and the RA peripheral blood mutant T cell clones bound to fibronectin with a similar affinity and this interaction was inhibited by anti-CD29 MAbs. Fibronectin adherence by RA synovial tissue T cell lines and RA peripheral blood mutant T cell clones was also inhibited when the cells were pre-incubated with soluble fibronectin containing the CS-1 sequence. The CS-1 inhibition assay was implemented to demonstrate the specificity of CD29 to the CS-1 fibronectin sequence. Previous investigators demonstrated that the CS-1 fibronectin sequence was exclusively restricted to inflamed RA endothelium and is a dominant fibronectin variant in the RA synovium compared to healthy tissue (Elices *et al.* 1994). An increase in CD29 expression was observed on the RA peripheral blood mutant T cell clones and the RA synovial tissue T cell lines. In contrast, the RA peripheral blood non-mutant T cell clones, and the control non-mutant and mutant T cell clones did not bind to fibronectin and expressed lower levels of CD29. Thus, the RA mutant T cell clones displayed a unique phenotype compared to the other T cell clones from both the RA patients and controls.

Arroyo and colleagues described enhanced expression of 15/7 epitope of $\beta 1$ integrin on synovial fluid T cells compared to peripheral blood T cells (Arroyo *et al.* 1995). This epitope was upregulated by IFN- γ , IL-2, and TNF- α all of which are found in the synovium (Feldman *et al.* 1996). Therefore, not only do the RA mutant peripheral blood T cells and synovial tissue T cells

express high levels of the $\beta 1$ integrin but they also might display the 15/7 epitope, which correlates with higher binding ability to fibronectin. However, I did not analyze this epitope expression. These results suggest that the RA peripheral blood mutant T cells and the RA synovial tissue T cells display a similar phenotype, thus I concluded that the mutant T cells may have acquired these characteristics while in the milieu of the inflamed synovium or now have the potential to enter the inflamed synovium due to the expression of various adhesion molecules.

Binding to fibronectin by the RA and OA synovial tissue T cells lines and RA peripheral blood mutant T cell clones was not significantly affected by the addition of anti- $\alpha 4\beta 7$ MAb. This integrin interacts with MAdCAM-1 and is found on lymphocytes that home to the Peyer's patches and intestinal epithelium (Erle *et al.* 1994; Shyjan *et al.* 1996). $\alpha 4\beta 7$ has also been found on RA synovial tissue T cells with various levels of expression (Lazarovits and Karsh 1993). This integrin can also interact with CS-1 domain of fibronectin (Chan *et al.* 1992) and VCAM-1 (Chiu *et al.* 1995). Using site-directed mutagenesis, Chiu and coworkers (1995) demonstrated that the amino acid residue requirements for VCAM-1 binding to $\alpha 4\beta 7$ and $\alpha 4\beta 1$ are similar but not identical. Chan and associates (1992) demonstrated that the B cell line, JY, expressed a high level of $\alpha 4\beta 7$ and 17 fold less of $\alpha 4\beta 1$ integrin. After 15 minutes of 12- σ -tetradecanoylphorbol-13-acetate (TPA) stimulation, which is a period too short to alter integrin expression, anti- $\beta 1$ MAb caused a 60 to 80% inhibition of JY cell adherence to CS-1 peptides and a 40 KDa fibronectin fragment. The investigators transfected some JY cells with the $\beta 1$ integrin, JY- $\beta 1$. On unstimulated JY- $\beta 1$ cells, anti- $\beta 1$ MAb caused a 100% inhibition of cell adhesion. Therefore, if $\alpha 4\beta 7$ is an adhesion receptor for the CS-1 domain of fibronectin or a region in the 40 KDa fragment, it is substantially less effective than $\alpha 4\beta 1$. Thus, I hypothesize that $\alpha 4\beta 7$ does interact with the CS-1 sequence

of fibronectin but with a lower affinity than $\alpha 4 \beta 1$. The mean binding to fibronectin by the synovial tissue T cell lines and peripheral blood mutant clones was not significantly inhibited by anti- $\alpha 4 \beta 7$ MAb; however, the results varied among patients and inhibition occurred in some individual RA patient samples. RA and OA synovial tissue T cell lines have been examined for the expression of $\alpha 4 \beta 7$ integrin and 30 to 100% of the cell lines expressed the integrin following PHA stimulation (Dr. Karsh, personal communication). This variability in expression of $\alpha 4 \beta 7$ by synovial tissue T cells following PHA stimulation may explain the variation in adhesion to fibronectin by synovial cell lines and T cell clones. Therefore, the RA and OA synovial tissue T cells and mutant T cell clones may express various levels of the $\alpha 4 \beta 7$ integrin, or $\alpha 4 \beta 7$ may mediate binding depending on the activation state of the cells. T lymphocytes that matured in the intra-epithelium of the gut also express high levels of this integrin, so the variability may also reflect T cell origin (Chan *et al.* 1992)

Anti-IgG₁ and anti-CD7 MAb were used as negative controls in the fibronectin binding assay. CD7 is a 40 KDa membrane glycoprotein expressed on T cells in association with CD3 and CD45. CD7 has been proposed to be involved in an alternative activation pathway for T cells (Carrera *et al.* 1988). Lazarovits and associates (1992) have described 2 subsets of T cells in RA synovium: CD7⁻ and CD7⁺. All the cells, within the 2 defined subsets are CD4⁺, CD29^{hi}, and CD45RO⁺. The presence of either anti-CD7 or anti-IgG₁ had no significant effect on mean fibronectin binding. Although, anti-CD7 MAb did have a variable effect on fibronectin binding from individual RA patient samples, which suggests that this ligand may be involved in cell adhesion or cell signaling to upregulate adhesion molecules. The variation in results may be explained through the differential expression of CD7 on T cells from the RA synovium.

8. MUTATION FREQUENCY VERSUS MUTATION RATE

The frequency of mutant T cells is defined as the proportion of mutant cells in a population and is readily estimated. The mutation rate (MR) is the rate at which mutational events occur. A T cell, in which a mutation has occurred, may divide to give rise to several descendants, so that a given FMC may reflect a considerably smaller number of mutational events (Green *et al.* 1995). Although, Fuscoe and colleagues isolated mutant colonies from T cell receptor gene rearrangement patterns and showed that most mutants tested were of independent origin rather than the offspring of a single mutation (Fuscoe *et al.* 1992a). Furthermore, Allegretta and coworkers determined TCR rearrangement of the *hprt* mutant and non-mutant clones from MS patients using DNA dot blot analysis revealed that there was not a clonal expansion of a single *hprt* mutant T cell (Allegretta *et al.* 1990). These results suggest that the increase in the frequency of mutant T cells observed in the peripheral blood, synovial fluid, and synovial tissue of RA patient is not due to a clonal expansion of a group of T cells.

Although the FMC is highly variable, when a large number of subjects was examined, it was clear that the FMC follows a well defined pattern. By following Lea and Coulson's methods (1949), it is possible to obtain estimates of MR. There is evidence for early selection against *hprt* cells and this would allow for the estimate of MR from a zero age intercept (McKeran *et al.* 1974). Green and associates (1995) determined that the mean cFMC of 80 healthy newborns was 0.93×10^{-6} . Assuming 10^{11} T lymphocytes to be present in the body and a 3 fold selection against *hprt* cells, they reached an estimate of MR of 0.25×10^{-6} . The investigators believed that this estimate should be reduced to take into account the likelihood that a proportion of mutants in newborns has arisen via V(D)J rearrangement related events which are likely to be specific to this stage in

development. To estimate the newborn cFMC, they took an estimate of a total potential T cell number in newborns of 1×10^{10} and a 10 fold selection against *hprt*⁻ cells, giving a MR of 0.9×10^{-6} . The investigators determined the FMC from people at various ages, and their results demonstrated that the MR assumes that the average T cell undergoes 0.26 divisions per year. If the average T cell undergoes 1 additional division per year then the estimate of MR will be 0.28×10^{-6} . The best estimate should reconcile the values obtained by these methods, thus the MR at the *hprt* locus is 5×10^{-7} . The precision of this estimate would be improved if more information on the rate of turnover of T cells were available.

The increase in the range of FMC with age reported by various investigators (Jones *et al.* 1995), can be accounted for by the bottle-neck effect, provided that the likelihood is high that a number of *hprt* mutants will expand to form substantial clones during the lifetime of the typical individual. The bottle-neck effect suggests that the mutant T cell will survive and give rise to progeny, thus increasing the overall number of mutant T cells within that given population (Green *et al.* 1995). However, even though an increase in FMC has been reported with age, factors such as the environment and smoking status also play a role (Jones *et al.* 1995 and Jones *et al.* 1993). Green and coworkers (1995) demonstrated that the FMC for healthy individuals between 30 and 50 years was 1.23×10^{-6} , which is very similar to the values obtained from controls in my *hprt* clonal assay and from other investigators (Robinson *et al.* 1994). Although the controls for the *hprt* assay are younger than the RA patients, the age of the patients alone can not account for the increase in the frequency of mutant T cells (Robinson *et al.* 1994; Albertini 1985). Thus, the increase in FMC seen in RA patients could be due to an expansion of *hprt*⁻ T cells which would result in a clonal

expansion of T cells in the peripheral blood, synovial fluid, and synovial tissue; however, there is inconsistent evidence to suggest clonal expansion of T cells occurs in the RA synovium.

9. REACTIVE NITROGEN AND OXYGEN SPECIES

9.1 RA Synovium

The *hprt* clonal assay has selected T lymphocytes from the peripheral blood, synovial fluid, and synovial tissue that have undergone mitogenic stimulation and genetic damage. Inflammatory cells infiltrate the synovium and in acutely activated joints, fibrin deposits and PMN are found (Firestein 1994; Harris 1993). The activated leukocytes can secrete RNS and ROS which can cause cellular and tissue damage (Bashir *et al.* 1993; Halliwell *et al.* 1988). Recently, there has been a great deal of evidence that reactive nitrogen and oxygen species may be involved in the pathology of RA (Farrell *et al.* 1992; Grabowski *et al.* 1996; Sakurai *et al.* 1995).

Macrophages infiltrating the OA or RA synovium can be stimulated to release IL-1 which can act on neutrophils (Rosen *et al.* 1995), synoviocytes (Strefanovic-Racic *et al.* 1992), and chondrocytes (Pelletier *et al.* 1993) to secrete NO and metalloproteinases (such as collagenase) which can genetically damage stimulated cells and degrade the extracellular matrix, respectively (Murrell *et al.* 1995; Krane 1989). A recent report indicated that NO could cause chondrocytes to undergo apoptosis (Blanco *et al.* 1995). Using a *Streptococcal* cell wall-induced arthritis, an animal model of RA, high levels of nitrite were found in the synovial fluid of the animal and the authors confirmed that the severity of disease declined when the animals were treated with NOS-inhibitor, N^G-monomethyl-L-arginine (NMMA) (McCartney-Francis *et al.* 1993). These data suggest that NO is present within the inflamed RA or OA synovium.

9.2 Nitric Oxide Can Cause DNA Damage

The *hprt* gene is approximately 44 kilobases in length and is composed of 9 exons encoding 800 nucleotides (Patel *et al.* 1986). Fuscoe and associates (1994) demonstrated that the hemizygous nature of the *hprt* locus permits the recovery of mutations primarily affecting the function of the single gene. In 1994, Nelson and colleagues demonstrated that X-rays induce total *hprt* gene deletion in 53% of the TK6 cells analyzed, compared to 16% spontaneous deletion. The X-ray total gene deletions were found to be significantly larger than those arising spontaneously. Only one spontaneous deletion encompassed any Xq26 marker other than *hprt*, while X-ray induced deletion extended at least 0.4 Mb beyond the *hprt*. Therefore, cells can tolerate *hprt* mutations and large scale gene deletion (Neison *et al.* 1995).

Recently, there has been increasing evidence that NO and its by-products can cause DNA strand breaks. In 1993, Fehsei and associates demonstrated that macrophages and the NO donor, sodium nitroprusside, caused DNA strand breaks in islet cells, while the addition of N^G-monomethyl-L-arginine (NMMA) prevented macrophage induced DNA damage. This finding provides evidence that NO produced by macrophages is capable of causing DNA strand breaks and possibly gene deletion but it does not exclude the contribution of other mutagenic species.

NO may play a key role in the maintenance of the inflammatory process and perhaps cause cellular as well as tissue damage in the RA or OA synovium. The damage caused by NO could reflect the elevated *hprt* mutation frequency observed in the RA and OA synovial tissue T lymphocytes, although I have not directly addressed this issue.

9.3 Can Sustained Levels of Genotoxic Species Result in Neoplasm?

Many cancers are characterized by aberrations in chromosome structure or number (Dameil *et al.* 1990). There is epidemiological data that has defined a relationship between autoimmune diseases and malignancy. The risk of colorectal cancer is significantly elevated in patients with Crohn's disease and ulcerative colitis (Greenstein *et al.* 1981). RA patients have an increased risk of hematopoietic cancers of B cells and myelocytes, including lymphoma and multiple myeloma (Gridley *et al.* 1993; Laakso *et al.* 1986). The elevated frequency of mutant T cells, the reported increase in serum and synovial fluid nitrite, the high level of expression of iNOS in inflammatory cells within the RA synovium, and the evidence that NO causes DNA strand breaks suggests a possible link between NO production, induction of genetic damage, and the development of neoplasm. While I did not examine the frequency of *hprt* mutant B cells, one could predict that the mutation frequency at this locus in B cells from RA peripheral blood and synovial tissue would also be elevated compared to controls. Therefore, the presence of mutant T cells within RA suggests a mechanism for the induction of malignancy.

The *hprt* clonal assay may be useful for the isolation and identification of a subset of T lymphocytes that are potentially relevant to the RA disease process. The *hprt* mutant T cells isolated from the peripheral blood of RA patients may represent T lymphocytes that were mitogenically stimulated and genetically damaged within the inflamed synovium and subsequently re-entered the peripheral circulation. Autoreactive and/or antigen-specific T lymphocytes may be among the mutant T cells subset; their further characterization may be useful in understanding the pathogenesis of RA.

10. SUMMARY AND CONCLUSION

- 1) I have utilized a strategy to identify and analyze a subset of T lymphocytes in RA patients.
- 2) There is an increase in the frequency of mutant T lymphocytes in the peripheral blood of RA patients compared to controls.
- 3) The increased frequency of mutant T cells from the peripheral blood of RA patients compared to controls is not an *in vitro* phenomenon as demonstrated by the catalase assay and the duration assay.
- 4) The increased frequency of mutant T cells from the peripheral blood of RA patients is not associated with RA disease activity as measured in this study.
- 5) No clear correlation was found between clinical disease parameters or current medication and the frequency of mutant T lymphocytes found in the peripheral blood of RA patients.
- 6) The frequency of mutant T cells from the synovial fluid was significantly greater compared to the peripheral blood of controls but not significantly greater compared to the peripheral blood of unselected RA patients from the outpatient clinic. This result suggests that the RA synovial fluid may not be a mitogenic and mutagenic environment.

7) The frequency of mutant T cells from the synovial tissue is increased approximately ten-fold compared to the peripheral blood of the same surgical RA or OA patient suggesting that the inflamed synovium may contain the necessary mitogenic and mutagenic factors to cause genetic damage to T lymphocytes. The peripheral blood mutant T cells may have sustained genetic damage in the inflamed synovium and then re-entered peripheral circulation.

8) The mutant T cell clones from the peripheral blood of RA patients and RA synovial tissue T cell lines have a similar phenotype: the cells bind to fibronectin equally well and express similar levels of CD29. The RA peripheral blood mutant T cell clones may have been genetically damaged in the inflamed synovium and re-entered the peripheral circulation or have the capacity to infiltrate the synovium.

11. FUTURE DIRECTION

11.1 T Lymphocytes Important in the Pathogenesis of RA

There is a low frequency of autoreactive or antigen-specific T cells in the peripheral blood, synovial fluid, and tissue of RA patients. These T cells may be among the mutant T cell population. Therefore, the characterization of this subset of T cells may provide a clue as to the role that T cells play in disease initiation and progression.

Autoreactive T lymphocytes in the peripheral blood and synovial tissue of RA patients may be among the mutant T cell population. Severe combined immunodeficiency (SCID) mice have recently been implemented to study the pathogenesis of RA (Hourii and O'Sullivan 1995). In 1995, Mima and associates demonstrated the induction of synovial hyperplasia, which mimicked histopathological changes observed in RA synovium at early stages of the disease, in SCID mice transferred with MNCs from rheumatoid joints by intraarticular injections. However, this induction did not occur with cells from all patients. In the affected mice, the TCR V β gene usage appeared to be skewed in the peripheral blood and synovial tissue. However, the predominance of T lymphocytes in the MNC injected into SCID mice suggests that the induction of synovial hyperplasia observed is T cell mediated. Thus, to verify the role of the mutant versus non-mutant T cell clones from the peripheral blood, synovial fluid, and synovial tissue, these cells could be injected into a SCID mouse. If the injected mutant T cell clones induce synovial hyperplasia, then this would strengthen my postulate that autoreactive T lymphocytes are part of the mutant T cell population. One could then speculate that the method of isolating the potentially autoreactive T cells is accurate and that the disease is T cell mediated.

11.2 Reactive Nitrogen and Oxygen Species and Their Involvement in the FMC in RA

Reactive nitrogen and oxygen species within the synovium may result in tissue, cellular, and genetic damage, and may contribute to the self-perpetuating nature of the rheumatoid process. To determine the extent that H_2O_2 could cause genetic damage T lymphocytes could be incubated with various concentrations of H_2O_2 in the absence and presence of catalase. The *hprt* clonal assay could be implemented to determine the frequency of *hprt* mutant T cells under these various culture conditions. However, other genotoxic species released from inflammatory cells such as NO and ONOO⁻ may damage the T cells (Wink *et al.* 1991; Nguyen *et al.* 1992). Furthermore, T lymphocytes could also be incubated with NO donors such as sodium nitroprusside in the absence and presence of inducible nitric oxide synthase inhibitors to determine the genetic damage incurred by T cells.

Synovial tissue samples obtained from RA patients could be used to determine the FMC, and the tissue could be stained for infiltrating macrophages, mast cells, lymphocytes, plasma cells, and PMN. The FMC could be correlated to the level of infiltration and the dispersion of cells. The infiltrating cells could be stained for iNOS expression and the results might explain the increase and variation in the FMC. It would be interesting to verify if the serum nitrite level is correlated with synovial tissue iNOS expression or with the FMC in the synovial tissue or peripheral blood of that patient.

Synoviocytes within the RA joint become hyperplastic and assume features of a transformed aggressively destructive tissue. The changes undergone by the synoviocytes should be analyzed and graded. The scores could then be compared to the cellular infiltration, the FMC, nitrite level, and iNOS expression. Type A and B synoviocytes may have undergone genetic damage and the *hprt* clonal assay could be modified to determine the mutation frequency of these cells.

With our current knowledge, we can hypothesize that reactive nitrogen and oxygen species caused *hprt* gene deletions in T cells from the synovial tissue. This postulate could be examined by cloning *hprt* mutant T cells from the peripheral blood and synovium of RA patients and controls and analyzing the *hprt* mutation via polymerase chain reaction and fluorescence *in situ* hybridization to define clonality and the molecular nature of the mutation. In parallel, one could obtain *hprt*⁺ T lymphocytes from the same patients and expose these cells to various NO-inducing chemicals, such as sodium nitroprusside and S-nitrosoacetylpenicillamine, to compare the kinds of mutations incurred within the synovium and via *in vitro* NO. By comparing the mutations occurring *in vivo* with spontaneous and chemical induced genetic events in T cells, our understanding of the mechanism of mutation in autoimmune disease will be strengthened.

REFERENCES

- Abbot, S.E., Kaul, A. and Stevens, C.R. (1992) Isolation and culture of synovial microvascular endothelial cells. *Arthritis Rheum.* **35**, 401-406.
- Aibertini, R.J. (1985) Somatic gene mutations *in vivo* as indicated by the 6-thioguanine-resistant T-lymphocytes in human blood. *Mutation Res.* **150**, 411-422.
- Aibertini, R.J., Castle, K.L. and Borchering, W.R. (1982) T-cell cloning to detect the mutant 6-thioguanine-resistant lymphocytes present in human peripheral blood. *Proc. Natl. Acad. Sci. USA* **79**, 6617-6621.
- Albertini, R.J., Nicklas, J.A. and O'Neill, P.J. (1990) Studies of DNA alterations in *in vivo* somatic cell mutations in humans. *Basic Life Sci.* **53**, 315-328.
- Allegretta, M., Nicklas, J.A., Sriram, S. and Albertini, R.J. (1990) T cells responsive to myelin basic protein in patients with multiple sclerosis. *Science* **247**, 718-721.
- Amin, A.R., Cesare, P.E., Vyas, P., Attur, M., Tzeng, E., Billiar, T.R., Stuchin, S.A. and Abramson, S.B. (1995) The expression and regulation of nitric oxide synthase in human osteoarthritis-affected chondrocytes: evidence for up-regulated neuronal nitric oxide synthase. *J. Exp. Med.* **182**, 2097-2102.
- Amaout, M.A. (1990) Structure and function of the leukocyte adhesion molecules CD11/CD18. *Blood* **75**, 1037-1050.
- Amaout, M.A. (1993) Cell adhesion molecules. In: Kelley, W.N., Harris, E.D., Ruddy, S. and Sledge, C.B., (Eds.) *Textbook of Rheumatology*, 4th edn. pp. 213-226. Philadelphia: W. B. Saunders Company.
- Amett, F.C., Jr., Edworthy, S.M., Bloch, D.A., McShane, D.J., Fries, J.F., Cooper, N.S., Healey, L.A., Kaplan, S.R., Liang, M.H., Luthra, H.S., Medsger, T.A., Jr., Mitchell, D.M., Neustadt, D.H., Pinals, R.S., Schaller, J.G., Sharp, J.T., Wilder, R.L. and Hunder, G.G. (1988) The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. *Arthritis Rheum.* **31**, 315-324.
- Arroyo, A.G., Garcia-Vicuna, R., Marazuela, M., Gonzalez-Amaro, R. and Sanchez-Madrid, F. (1995) Expression and functional significance of an activation-dependent epitope of the $\alpha 31$ integrins in chronic inflammatory diseases. *Eur. J. Immunol.* **25**, 1720-1728.
- Aruffo, A., Stamenkovic, I., Melnick, M., Underhill, C.B. and Seed, B. (1990) CD44 is the principal cell surface receptor for hyaluronate. *Cell* **61**, 1303-1313.
- Bashir, S., Gilmour, H., Denman, M.A., Blake, D.R. and Winyard, P.G. (1993) Oxidative DNA damage and cellular sensitivity to oxidative stress in human autoimmune diseases. *Ann. Rheum. Dis.* **52**, 659-666.

- Berlin, C., Bargatze, R.F., Campbell, J.J. von Andrian, U.H., Szabo, M.C. Hasslen, S.R., Nelson, R.D., Berg, E.L., Erlandsen, S.L., Butcher, E.C. (1995) α_4 integrin mediate lymphocyte attachment and rolling under physiological flow. *Cell* **80**, 413-422
- Blanco, F.J., Ochs, R.L., Schwarz, H. and Lotz, M. (1995) Chondrocyte apoptosis induced by nitric oxide. *Am. J. Pathol.* **146**, 75-85.
- Branda, R.F., O'Neill, P.J., Sullivan, L.M. and Albertini, R.J. (1991) Factors influencing mutation at the *hprt* locus in T-lymphocytes: women treated for breast cancer. *Cancer Res.* **51**, 6603-6607.
- Brandt, K.D. and Mankin, H.J. (1993) Pathogenesis of osteoarthritis. In: Kelley, W.N., Harris, E.D., Ruddy, S. and Sledge, C.B., (Eds.) *Textbook of Rheumatology*. 4th edn. pp. 1355-1365. Philadelphia: W.B. Saunders Company.
- Carrera, A.C., Rincon, M., Sanchez-Madrid, F., Lopez-Botet, M. and DeLandazuri, M.O. (1988) Triggering of co-mitogenic signals in T cell proliferation by anti-LFA-1 (CD18 CD11a), LFA-3, and CD7 monoclonal antibodies. *J. Immunol.* **141**, 1919-1924.
- Chan, B.M.C., Elices, M.J., Murphy, E. and Hemler, M.E. (1992) Adhesion to vascular cell adhesion molecule 1 and fibronectin. *J. Biol. Chem.* **267**, 8366-8370.
- Chiu, H.H., Crowe, D.T., Renz, M.E., Presta, L.G., Jones, S., Weissman, I.L. and Fong, S. (1995) Similar but nonidentical amino acid residues on vascular cell adhesion molecule-1 are involved in the interaction with $\alpha_4\beta_1$ and $\alpha_4\beta_7$ under different activity states. *J. Immunol.* **155**, 5257-5267.
- Choi, Y., Kotzin, B., Herron, L., Callahan, J., Marrack, P. and Kappler, J. (1989) Interaction of *Staphylococcal aureus* toxin "superantigens" with human T cells. *Proc. Natl. Acad. Sci. USA* **86**, 8941-8945.
- Clark, E.A. and Brugge, J.S. (1995) Integrins and signal transduction pathways: the road taken. *Science* **268**, 233-239.
- Cole, B.C. (1977) New models of chronic synovitis in rabbits induced by mycoplasma: Microbiological, histopathological, immunological observations in rabbits infected with *Mycoplasma arthritides* and *Mycoplasma pulmonis*. *Infect. Immunol.* **16**, 382-396.
- Connell, W.R., Kamm, M.A., Dickson, M., Balkwill, A.M., Ritchie, J.K. and Lennard-Jones, J.E. (1994) Long-term neoplasia risk after azathioprine treatment in inflammatory bowel disease. *Lancet* **343**, 1249-1252.
- Cush, J.J. and Lipsky, P.E. (1988) Phenotypic analysis of synovial tissue and peripheral blood lymphocytes isolated from patients with rheumatoid arthritis. *Arthritis Rheum.* **31**, 1230-1238.
- Damle, N.K., Klussman, K. and Dietsch, M.T. (1992) GMP-140 (P-selectin/CD62) binds to chronically stimulated but not resting CD4⁺ T lymphocytes and regulated their production of pro-inflammatory cytokines. *Eur. J. Immunol.* **22**, 1789-1793.

- Darnell, J., Lodish, H. and Baltimore, D. (1990) *Molecular Cell Biology*, 2nd edn. New York: Freeman and Company.
- Dawisha, S., Gmelig-Meyling, F. and Steinberg, A.D. (1993) Assessment of clinical parameters associated with increased frequency of mutant T cells in patients with systemic lupus erythematosus. *Arthritis Rheum.* **37**, 270-277.
- Doherty, P.C., Allan, W. and Eichelberger, M. (1992) Roles of alpha beta and gamma delta T cell subsets in viral immunity. *Ann. Rev. Immunol.* **10**, 123-151.
- Duffy, J., Lidsky, M.D. and Sharp, J.T. (1976) Polyarthritis, polyarteritis and hepatitis B. *Medicine* **55**, 19-37.
- Edwards, J. (1994) Synovium. In: Kipple, J.H. and Dieppe, P.A., (Eds.) *Rheumatoid arthritis*, (3rd edition) pp. 7.1-7.8. Hong Kong: Mosby-Year Book Europe Limited.
- Eichmann, K., Falk, I., Melchers, I. and Simon, M.M. (1980) Quantitative studies on T cell diversity. Determination of the precursor frequencies for two types of Streptococcus A-specific helper cells in nonimmune, polyclonally activated splenic T cells. *J. Exp. Med.* **152**, 477-492.
- Elices, M.J., Osborn, L. and Takada, Y. (1990) VCAM-1 on activated endothelium interacts with leukocyte integrin VLA-4 at a site distinct from the VLA-4/fibronectin binding site. *Cell* **60**, 577-584.
- Elices, M.J., Tsai, V., Strahl, D., Goei, S.A., Tollefson, V., Arrhenius, T., Wayner, E.A., Gaeta, F.C.A., Fikes, J.D. and Firestein, G.S. (1994) Expression and functional significance of alternatively spliced CS1 fibronectin in rheumatoid arthritis microvasculature. *J. Clin. Invest.* **93**, 405-416.
- Erle, D.J., Briskin, M.J., Butcher, E.C., Garcia-Pardo, A., Lazarovits, A.I. and Tidswell, M. (1994) Expression and function of the MAdCAM-1 receptor, integrin $\alpha 4\beta 7$, on human leukocytes. *J. Immunol.* **153**, 517-528.
- Farrell, A.J. and Blake, D.R. (1995) Nitric Oxide. *Biochem. Biophys. Res. Com.* **206**, 53-58
- Farrell, A.J., Blake, D.R., Palmer, R.M. and Moncada, S. (1992) Increased concentrations of nitrite in synovial fluid and serum samples suggest increased nitric oxide synthesis in rheumatic diseases. *Ann. Rheum.Dis.* **51**, 1219-1222.
- Fassbender, H.G. (1983) Histomorphological basis of articular cartilage destruction in rheumatoid arthritis. *Coll. Relat. Res.* **3**, 141-155.
- Fehsel, K., Jalowy, A., Qi, S., Burkart, V., Hartmann, B. and Kolb, H. (1993) Islet cell DNA is a target of inflammatory attack by nitric oxide. *Diabetes* **42**, 496-500.
- Feldman, M., Brennan, F.M. and Maini, R.N. (1996) Role of cytokines in rheumatoid arthritis. *Annu. Rev. Immunol.* **14**, 397-440.

- Felson, D.T., Anderson, J.J., Boers, M., Bombardier, C., Chernoff, M., Fried, B., Furst, D., Goldsmith, C., Kieszak, S., Lightfoot, R., Paulus, H., Tugwell, P., Weinblatt, M., Widmark, R., Williams, H.J. and Wolfe, F. (1993) The American College of Rheumatology preliminary core set of disease activity measures for rheumatoid arthritis clinical trials. *Arthritis Rheum.* **36**, 729-740.
- Firestein, G.S. (1994) Rheumatoid synovitis and pannus. In: Kipple, J.H. and Dieppe, P.A., (Eds.) *Rheumatoid arthritis*, 3rd edn. pp. 12.1-12.30. Hong Kong: Mosby-Year Book Europe Limited.
- Firestein, G.S. and Zvaifler, N.J. (1990) How important are T cells in chronic rheumatoid synovitis. *Arthritis Rheum.* **33**, 768-773.
- Fischer, D.C., Opalka, B., Hoffmann, A., Mayr, W. and Haubeck, H.D. (1996) Limited heterogeneity of rearranged T cell receptor V_{α} and V_{β} transcripts in synovial fluid T cells in early stages of rheumatoid arthritis. *Arthritis Rheum.* **39**, 454-462.
- Fox, R.I., Fong, S., Sabharwal, N., Cartstens, S.A., Kung, P.C. and Vaughan, J.H. (1982) Synovial fluid lymphocytes differ from peripheral blood lymphocytes in patients with rheumatoid arthritis. *J. Immunol.* **128**, 351-354.
- Fuscoe, J.C., Zimmerman, L.J., Harrington-Brock, K., Burnette, L., Moore, M.M., Nicklas, J.A., O'Neill, J.P. and Albertini, R.J. (1992a) V(D)J recombinase-mediated deletion of the *hprt* gene in T-lymphocytes from adult humans. *Mutation Res.* **283**, 13-20.
- Fuscoe, J.C., Zimmerman, L.J., Harrington-Brock, K. and Moore, M.M. (1992b) Large deletions are tolerated at the *hprt* locus of in vivo derived human T-lymphocytes. *Mutation Res.* **283**, 255-262.
- Fuscoe, J.C., Zimmerman, L.J., Harrington-Brock, K. and Moore, M.M. (1994) Deletion mutations in the *hprt* gene of T-lymphocytes as a biomarker for genomic rearrangements important in human cancers. *Carcinogenesis* **15**, 1463-1466.
- Gmelig-Meyling, F., Dawisha, S. and Steinberg, A.D. (1992) Assessment of *in vivo* frequency of mutated T cells in patients with systemic lupus erythematosus. *J. Exp. Med.* **175**, 297-300.
- Goldstein, L.A., Zhou, D.F.H., Picker, L.J., Minty, C.N., Bargatze, R.F., Ding, J.F. and Butcher, E.C. (1989) A human lymphocyte homing receptor, the hermes antigen, is related to cartilage proteoglycan core and link proteins. *Cell* **56**, 1063-1072.
- Goronzy, J.J., Bartz-Bazzanella, P., Hu, W., Jendro, M.C., Walser-Kuntz, D.R. and Weyand, C.M. (1995) Dominant clonotypes in the repertoire of peripheral CD4⁺ T cells in rheumatoid arthritis. *J. Clin. Invest.* **94**, 2068-2076.
- Goronzy, J.J. and Weyand, C.M. (1993) Interplay of T lymphocytes and HLA-DR molecules in Rheumatoid Arthritis. *Curr. Opin. Rheumatol.* **5**, 169-177.

- Goronzy, J.J. and Weyand, C.M. (1995) T cells in rheumatoid arthritis. Paradigms and facts. *Rheum. Dis. Clin. NA.* **21(3)**, 655-674.
- Grabowski, P.S., England, A.J., Dykuizen, R., Copland, M., Benjamin, N., Reid, D.M. and Ralston, S.H. (1996) Elevated nitric oxide production in rheumatoid arthritis: detection using the fasting urinary nitrate:creatinine ratio. *Arthritis Rheum.* **39**, 643-647.
- Grahame, R., Armstrong, R., Simmons, N., Wilton, J.M.A., Dyson, M., Laurent, R., Millis, R. and Mims, C.A. (1983) Chronic arthritis associated with the presence of intrasynovial rubella virus. *Ann. Rheum. Dis.* **42**, 2-13.
- Green, M.H.L., O'Neill, J.P. and Cole, J. (1995) Suggestions concerning the relationship between mutant frequency and mutation rate at the *hprt* locus in human peripheral blood T-lymphocytes. *Mutation Res.* **334**, 323-339.
- Greenstein, A.J., Sachar, D.B., Smith, H., Janowitz, H.D. and Aufses, A.H. (1981) A comparison of cancer risk in Crohn's disease and ulcerative colitis. *Cancer* **48**, 2742-2745.
- Gregersen, P.K., Silver, J. and Winchester, R.J. (1987) The shared epitope hypothesis. An approach to understanding the molecular genetics of susceptibility to rheumatoid arthritis. *Arthritis Rheum.* **30**, 1205-1213.
- Gridley, G., McLaughlin, J.K., Ekborn, Klareskog, L., Adami, H.O., Hacker, D.G., Hoover, R. and Fraumeni, J.F., Jr. (1993) Incidence of cancer among patients with rheumatoid arthritis. *J. Natl. Cancer Inst.* **85**, 307-311.
- Halliwell, B., Hoult, J.R. and Blake, D.R. (1988) Oxidants, inflammation, and anti-inflammatory drugs. *FASEB J.* **2**, 2867-2873.
- Harris, E.D. (1993) Etiology and pathogenesis of rheumatoid arthritis. In: Kelley, W.N., Harris, E.D., Ruddy, S. and Sledge, C.B., (Eds.) *Textbook of rheumatology*, 4th edn. pp. 833-873. Philadelphia: W. B. Saunders Company.
- Hauzenberger, D., Klominek, J. and Sundqvist, K.G. (1994) Functional specialization of fibronectin-binding β 1-integrins in T lymphocyte migration. *J. Immunol.* **153**, 960-971.
- Haynes, B.F., Hale, L.P. and Patton, K.L. (1991a) Measurement of an indicator of inflammatory disease activity: Up-regulation of the receptor for hyaluronate (CD44) in rheumatoid arthritis. *Arthritis Rheum.* **34**, 1434-1443.
- Haynes, B.F., Liao, H.X. and Patton, K.L. (1991b) The transmembrane hyaluronate receptor (CD44): multiple function, multiple forms. *Cancer Cells* **3**, 347-350.
- Heiovaara, M., Knekt, P., Aaran, R.K., Alfthan, G. and Aromaa, A. (1994) Serum antioxidants and risk of rheumatoid arthritis. *Ann. Rheum. Dis.* **53**, 51-53.

- Hemler, M.E. (1990) VLA proteins in the integrin family: structures, functions, and their role on leukocytes. *Annu. Rev. Immunol.* **8**, 365-378.
- Hirohata, S., Yanagida, T., Itoh, K., Nakamura, H., Yoshino, S., Tomita, T. and Ochi, T. (1996) Accelerated generation of CD14⁺ monocyte-lineage cells from the bone marrow of rheumatoid arthritis patients. *Arthritis Rheum.* **39**, 836-843.
- Holoshitz, J., Drucker, I., Yaretzy, K.A., van Eden, W., Kiajman, A., Lapidot, Z., Frenkel, A. and Cohen, I.R. (1986) T lymphocytes of rheumatoid arthritis patients show augmented reactivity to a fraction of mycobacteria cross reactive with cartilage. *Lancet* **2**, 305-309.
- Houri, J.M. and O'Sullivan, F.X. (1995) Animal models in rheumatoid arthritis. *Cur. Opin. Rheum.* **7**, 201-205.
- Humphries, M.J., Akiyama, S., Komoriya, K., Olden, K. and Yamada, K.M. (1986) Identification of an alternatively spliced site in human plasma fibronectin that mediates cell-type-specific adhesion. *J. Cell Biol.* **103**, 2637-2647.
- Ignatowicz, L., Kappler, J. and Marrack, P. (1996) The repertoire of cells shaped by a single MHC/peptide ligand. *Cell* **84**, 521-529.
- Jenkins, R.N., Nikaein, A. and Zimmermann, A. (1993) T cell receptor V β gene bias in rheumatoid arthritis. *J. Clin. Invest.* **92**, 2688-2697.
- Johnston, B., Issekutz, T.B. and Kubes, P. (1996) The α 4-integrin supports leukocyte rolling and adhesion in chronically inflamed postcapillary venules *in vivo*. *J. Exp. Med.* **183**, 1995-2006.
- Jones, I.M., Moore, D.H., Thomas, C.B., Thompson, C.L., Strout, C.L. and Burkhart-Schultz, K. (1993) Factors affecting HPRT mutant frequency in T-lymphocytes of smokers and nonsmokers. *Cancer Epidemiol. Biomarkers. Prev.* **2**, 249-260.
- Jones, I.M., Thomas, C.B., Tucker, B., Thompson, C.L., Pleshanov, P., Vorobtsova, I. and Moorell, D.H. (1995) Impact of age and environment on somatic mutations at the *hprt* gene of T lymphocytes in humans. *Mutation Res.* **3388**, 129-139.
- Kaur, H. and Halliwell, B. (1994) Evidence for nitric oxide-mediated damage in chronic inflammation: Nitrotyrosine in serum and synovial fluid from rheumatoid patients. *FEBS Letters* **350**, 9-12.
- Kavanaugh, A.F., Davis, L.S., Nichols, L.A., Norris, S.H., Rothlein, R., Scharschmidt, L.A. and Lipsky, P.E. (1994) Treatment of refractory rheumatoid arthritis with a monoclonal antibody to intracellular adhesion molecule 1. *Arthritis Rheum.* **37**, 992-999.
- Keilleher, D., Murphy, A., Hall, N., Omary, M.B., Kearns, G., Long, A. and Casey, E.B. (1995) Expression of CD44 on rheumatoid synovial fluid lymphocytes. *Ann. Rheum. Dis.* **54**, 566-570.

- Khazali, H.A., Lunardi, C. and So, A.K. (1995) CD4 T cells in the rheumatoid joint are oligoclonally activated and change during the course of disease. *Ann. Rheum. Dis.* **54**, 314-317.
- Koch, A.E., Burrows, J.C. and Haines, G.K. (1991) Immunolocalization of endothelial and leukocyte adhesion molecules in human rheumatoid and osteoarthritic synovial tissue. *Lab. Invest.* **64**, 313-320.
- Krane, S.M. (1989) Mechanisms of tissue destruction in rheumatoid arthritis. In: McCarty, D.J., (Ed.) *Arthritis and allied conditions*, 11th edn. pp. 698-714. Philadelphia: Lea and Febiger.
- Krawinkel, U. and Pluschke, G. (1992) T cell receptor variable region repertoire in lymphocytes from rheumatoid arthritis patients. *Immunobiology* **185**, 483-491.
- Kuby, J. (1994) *Immunology*, 2nd edn. New York: W.H. Freeman and Company.
- Kuzmich, P.V., Ecker, G.E. and Karsh, J. (1995) Rheumatoid manifestations in patients with myelodysplastic and myeloproliferative diseases. *J. Rheumatol.* **21**, 1649-1654.
- Laakso, M., Mutru, O., Isomaki, H. and Koota, K. (1986) Cancer mortality in patients with rheumatoid arthritis. *J. Rheumatol.* **13**, 522-526.
- Lai N.S., Lan J.L., Yu C.L., and Lin R.H. (1995) Role of tumour necrosis factor-alpha in the regulation of activated synovial T cell growth: down-regulation of synovial T cells in rheumatoid arthritis patients. *Eur. J. Immunol.* **12**, 3243-3248.
- Lasky, H.P., Bauer, K. and Pope, R.M. (1988) Increased helper inducer and decreased suppressor inducer phenotypes in the rheumatoid joint. *Arthritis Rheum.* **32**, 116-117.
- Lazarovits, A.I. and Karsh, J. (1993) Differential expression in rheumatoid synovium and synovial fluid of $\alpha 4\beta 7$ integrin. *J. Immunol.* **151**, 6482-6489.
- Lazarovits, A.I., White, M.J. and Karsh, J. (1992) CD7-T cells in rheumatoid arthritis. *Arthritis Rheum.* **35**, 615-624.
- Lea, D.E. and Coulson, C.A. (1949) The distribution of numbers of mutants in bacterial populations. *J. Genet.* **49**, 264-285.
- Liao, H., X. and Haynes, B.F. (1995) Roles of adhesion molecules in the pathogenesis of rheumatoid arthritis. *Rheum. Dis. Clin. NA.* **21**, 715-741.
- Lodge, P.A., Allegretta, M., Steinman, L. and Sriram, S. (1994) Myelin basic protein peptide specificity and T-cell receptor gene usage of *hprt* mutant T-cell clones in patients with multiple sclerosis. *Ann. Neurol.* **36**, 734-740.

- Lunardi, C., Marguerie, C. and So, A.K. (1992) An altered repertoire of T cell receptor V gene expression by rheumatoid synovial fluid T lymphocytes. *Clin. Exp. Immunol.* **90**, 440-446.
- Lunec, J., and Blake, D.R. (1985) The determination of dehydroascorbic acid and ascorbic acid in the serum and synovial fluid of patients with rheumatoid arthritis (RA). *Free Radic. Res. Commun.* **1**, 31-39.
- Maguire, J.E., Danahey, K.M., Burkly, L.C. and van Seventer, G.A. (1995) T cell receptor- and $\beta 1$ integrin-mediated signals synergize to induce tyrosine phosphorylation of focal adhesion kinase (pp125^{FAK}) in human T cells. *J. Exp. Med.* **182**, 2079-2090.
- McCartney-Francis, N., Allen, J.B., Mizel, D.E., Aibina, J.E., Xie, Q., Nathan, C.F. and Wahl, S.M. (1993) Suppression of arthritis by an inhibitor of nitric oxide synthase. *J. Exp. Med.* **178**, 749-754.
- McKeran, R., Howell, A., Andrews, T.M., Watts, R.W.E. and Ariett, C.F. (1974) Observation on the growth of myeloid progenitor cells and fibroblasts in culture, and their relevance to the pathogenesis of brain damage in the Lesch-Nyhan syndrome. *J. Neurol. Sci.* **22**, 183-195.
- Mima, T., Saeki, Y., Nishimoto, N., Matushita, M., Shimizu, M., Nomura, T. and Kishimoto, T. (1995) Transfer of Rheumatoid Arthritis into severe combined immunodeficient mice. The pathogenetic implications of T cell populations oligoclonally expanding in the rheumatoid joints. *J. Clin. Invest.* **96**, 1746-1758.
- Morales-Ducret, J., Wayner, E., Elices, M.J., Alvaro-Gracia, J.M., Zvaifler, N.J. and Firestein, G.S. (1992) $\alpha 4 \beta 1$ integrin (VLA-4) ligands in arthritis. Vascular cell adhesion molecule-1 expression in synovium and fibroblast-like synoviocytes. *J. Immunol.* **149**, 1424-1431.
- Morley, A.A., Trainor, K.J., Seshadri, R. and Ryall, R.G. (1983) Measurement of *in vivo* mutations in human lymphocytes. *Nature* **302**, 155-156.
- Morris, E.L. and Stevens, M.B. (1978) Rheumatoid arthritis- α sequel to HBsAg hepatitis. *Am. J. Med.* **64**, 859-862.
- Muller-Lander, U., Kriegsmann, J., Gay, R.E., and Gay, S. (1995) Oncogenes in rheumatoid arthritis. *Rheum. Dis. Clin. NA.* **21**, 675-689.
- Nakao, H., Eguchi, K., Kawakami, A., Migita, K., Otsubo, T., Ueki, Y., Shimomura, C., Tezuka, H., Matsunaga, M., Maeda, K. and Nagataki, S. (1990) Phenotypic characterization of lymphocytes infiltrating synovial tissue from patients with rheumatoid arthritis: Analysis of lymphocytes isolated from minced synovial tissue by dual immunofluorescent staining. *J. Rheumatol.* **17**, 142-148.
- Nelson, S.L., Fuscoe, J.C., Burkhart-Schultz, K. and Grosovsky, A.J. (1995) Mapping the end points of large deletions affecting the *hprt* locus in human peripheral blood cells and cell lines. *Radiat. Res.* **141**, 2-10.

- Nelson, S.L., Giver, C.R. and Grosovsky, A.J. (1994) Spectrum of X-ray-induced mutations in the human *hprt* gene. *Carcinogenesis* **15**, 495-502.
- Nepom, G.T. and Nepom, B.S. (1992) Predictions of susceptibility to Rheumatoid Arthritis by human leukocyte antigen genotyping. *Rheum. Dis. Clin. NA.* **18**, 785-92.
- Nguyen, T., Brunson, D., Crespi, C.L., Penman, B.W., Wishnok, J.S. and Tannenbaum, S.R. (1992) DNA damage and mutation in human cells exposed to nitric oxide *in vitro*. *Proc. Natl. Acad. Sci. USA.* **89**, 3030-3034.
- Oppenheimer-Marks, N., Davis, L.S. and Lipsky, P.E. (1990) Human T lymphocyte adhesion to endothelial cells and transendothelial migration: Alteration of receptor use relates to the activation status of both the T cell and the endothelial cell. *J. Immunol.* **145**, 140-148.
- Oppenheimer-Marks, N. and Lipsky, P.E. (1994) Transendothelial migration of T cells in chronic inflammation. *Immunologist* **2**, 58-64.
- Panayi, G.S., Lanchbury, J.S. and Kingsley, G.H. (1992) The importance of the T cell in initiating and maintaining the chronic synovitis of rheumatoid arthritis. *Arthritis Rheum.* **35**, 729-735.
- Patel, P.I., Framson, P.E., Caskey, T. and Chinault, A.C. (1986) Fine structure of the human hypoxanthine phosphoribosyl transferase gene. *Mol. Cell. Bio.* 393-403.
- Pelletier, J.P., DiBattista, J.A., Roughley, P., McCoullum, R. and Martel-Pelletier, J. (1993) Cytokines and inflammation in cartilage degradation. *Rheum. Dis. Clin. NA.* **19**, 545-568.
- Pincus, T. and Callahan, L.F. (1986) Taking mortality in Rheumatoid Arthritis seriously-Predictive markers, socioeconomic status and co-morbidity. *J. Rheumatol.* **14**, 240-251.
- Pritchard, D.G., Settine, R.L. and Bennett, J.C. (1980) Sensitive mass spectrometric procedures for the detection of bacterial cell wall components in rheumatoid arthritis. *Arthritis Rheum.* **23**, 608-610.
- Pytela, R., Pierschbacher, M.D. and Ruoslahti, E. (1985) Identification and isolation of a 140 kd cell surface glycoprotein with properties expected of a fibronectin receptor. *Cell* **40**, 191-198.
- Robinson, D.R., Goodall, K., Albertini, R.J., O'Neill, J.P., Finette, B., Trepal-Sala, M., Moustacchi, E., Bates, A.D., Beare, D.M., Green, M.H.L. and Cole, J. (1994) An analysis of *in vivo hprt* mutant frequency in circulating T-lymphocytes in the normal human population: a comparison of four datasets. *Mutation Res.* **313**, 227-247.
- Rodriguez, R.M., Pitzalis, C., Kingsley, G.H., Henderson, M.J., Humphries, M.J. and Panayi, G.S. (1992) T lymphocyte adhesion of fibronectin (FN): a possible mechanism for T cell accumulation in the rheumatoid joint. *Clin. exp. Immunol.* **89**, 439-445.

- Rosen, G.M., Pou, S., Ramos, C.L., Cohen, M.S. and Britigan, B.E. (1995) Free radicals and phagocytic cells. *FASEB J.* **9(2)**, 200-209.
- Roudier, J., Rhodes, G., Petersen, J., Vaughan, J.H. and Carson, D.A. (1988) The Epstein-Barr virus glycoprotein gp110, a molecular link between HLA DB4, HLA DR1, and rheumatoid arthritis. *Scand. J. Immunol.* **27**, 367-371.
- Sakurai, H., Kohsaka, H., Liu, F.M., Higashiyama, H., Hirata, Y., Kanno, K., Saito, I. and Miyasaka, N. (1995) Nitric oxide production and inducible nitric oxide synthase expression in inflammatory arthritides. *J. Clin. Invest.* **96**, 2357-2363.
- Schulz, J., Dettlaff, S., Fritzsche, U., Harms, U., Schiebel, H., Derer, W., Fesenig, N.E., Hulsen, A. and Bohm, M. (1994) The amido black assay: a simple and quantitative multipurpose test of adhesion, proliferation, and cytotoxicity in microplate cultures of keratinocytes and other cell types growing or in suspension. *J. Immunol. Methods* **167**, 1-13.
- Shimada, H., Eguchi, K., Ueki, Y., Nakashima, M., Izumi, Y., Kawabe, Y., Sakai, M., Aoyagi, T. and Nagataki, S. (1994) Interleukin 4 increases human synovial cell expression of VCAM-1 and T cell binding. *Ann. Rheum. Dis.* **53**, 601-607.
- Shyjan, A.M., Bertagnolli, M., Kenney, C.J. and Briskin, M.J. (1996) Human mucosal addressin cell adhesion molecule-1 (MAdCAM-1) demonstrates structural and functional similarities to the $\alpha 4 \beta 7$ -integrin binding domains of murine MAdCAM-1, but extreme divergence of mucin-like sequences. *J. Immunol.* **156**, 2851-2857.
- Simpson, R.W. (1984) Association of parvoviruses with rheumatoid arthritis of humans. *Science* **223**, 1425-1428.
- Sriram, S. (1994) Longitudinal study of frequency of *HPRT* mutant T cells in patients with Multiple Sclerosis. *Neurology* **44**, 311-315.
- St.John, J.T., Schroen, D.J. and Cheung, H.T. (1994) An adhesion assay using minimal shear force to remove nonadherent cells. *J. Immunol. Methods* **170**, 159-166.
- Stastny, P. (1978) Association of the B-cell alloantigen DRw4 with rheumatoid arthritis. *N. Engl. J. Med.* **298**, 869-871.
- Stewart, M.P., Cabanas, C. and Hogg, N. (1996) T cell adhesion to intercellular adhesion molecule-1 (ICAM-1) is controlled by cell spreading and the activation of LFA-1. *J. Immunol.* **156**, 1810-1817.
- Strefanovic-Racic, M., Stadler, J., Georgescu, H.I. and Evans, C.H. (1992) Nitric oxide production by cytokine stimulated synovial fibroblasts. *Trans. Orthop. Res. Soc. abstract* **17**, 228
- Stuehr, D.J. and Griffith, O., W. (1992) Mammalian nitric oxide synthases. *Advances in Enzymology and Related Areas of Molecular Biology* **65**, 287-346.

- Takahashi, H., Soderstrom, K. and Nilsson, E. (1992) Integrins and other adhesion molecules on lymphocytes from synovial fluid and peripheral blood of rheumatoid arthritis patients. *Eur. J. Immunol.* **22**, 2879-2885.
- Theocharis, S., Sfikakis, P.P., Lipnick, R.N., Klipple, G.L. and Steinberg, A.D. (1995) Characterization of *in vivo* mutated T cell clones from patients with systemic lupus erythematosus. *Clin. Immunol. Pathol.* **74**, 135-142.
- Todd, J.A., Acha-Orbea, H. and Bell, J.I. (1988) A molecular basis for the major histocompatibility complex class II-associated autoimmunity. *Science* **240**, 1003-1006.
- Varani, J., Mulligan, M.S. and Ward, P.A. (1994) The vascular endothelium and acute inflammation. In: Kipple, J.H. and Dieppe, P.A., (Eds.) *Rheumatoid arthritis*, pp. 11.1-11.12. (3rd edition) Hong Kong: Mosby-Year Book Europe Limited.
- Wayner, E.A., Garcia-Pardo, A., Humphries, M.J., McDonald, J.A. and Carter, W.G. (1989) Identification and characterization of the T lymphocyte adhesion receptor for an alternative cell attachment domain (CS-1) in plasma fibronectin. *J. Cell Biol.* **109**, 1321-1330.
- Weyand, C.M., Hicok, K.C., Conn, D. and Goronzy, J.J. (1992) The influence of HLA-DRB1 genes on disease severity in rheumatoid arthritis. *Ann. Intern. Med.* **117**, 801-806.
- White, D.G., Mortimer, P.P., Blake, D.R., Woolf, A.D., Cohen, B.J. and Bacon, P.A. (1984) Human parvovirus arthropathy. *Lancet* **1**, 419-421.
- Williams, D.G. (1994) Autoimmunity in rheumatoid arthritis. In: Kipple, J.H. and Dieppe, P.A., (Eds.) *Rheumatoid arthritis*, pp. 9.1-9.14. Hong Kong: Mosby-Year Book Europe Limited.
- Wink, D.A., Kasprzak, K.S., Maragos, C.M., Elespuru, R.K., Misra, M., Dunams, T.M., Cebula, T.A., Koch, W.H., Andrews, A.W., Allen, J.S. and Keefer, L.K. (1991) DNA deaminating ability and genotoxicity of nitric oxide and its progenitors. *Science* **254**, 1001-1003.
- Wordsworth, P. and Pile, K. (1994) Etiology. In: Kipple, J.H. and Dieppe, P.A., (Eds.) *Rheumatoid arthritis*, pp. 11.1-11.12. Hong Kong: Mosby-Year Book Europe Limited.
- Wordsworth, P., Pile, K. and Buckely, J.D. (1992) HLA heterozygosity contributes to susceptibility to Rheumatoid Arthritis. *Am. J. Hum. Genet.* **51**, 585-591.
- Yokoto, A., Murata, N., Saiki, O., Springer, T.A. and Kishimoto, T. (1995) High avidity state of leukocyte function-associated antigen-1 on rheumatoid synovial fluid T lymphocytes. *J. Immunol.* **155**, 4118-4124.
- Zvaifler, N.J. (1989) Etiology and pathogenesis of rheumatoid arthritis. In: McCarty, D.J., (Ed.) *Arthritis and Allied Conditions*, 11th edn. pp. 659-673. Philadelphia: Lea and Febiger.

Zvaifler, N.J. and Firestein, G.S. (1994) Pannus and pannocytes. Alternative models of joint destruction in rheumatoid arthritis. *Arthritis Rheum.* **37**, 783-789.

APPENDICES

Figure 15

IL-2 stimulation of T cells. IL-2 stimulation of 1 MNC/well in non-selection medium. MNC were plated at a concentration of 1MNC/well in non-selection medium for 21 days to permit clones to form. A [³H]-thymidine incorporation assay was completed and the results determined using a B counter. The cells were incubated with various concentrations of IL-2 to determine what concentration would maximally stimulate the T cell clones. 30 IU/ml of IL-2 initiated the peak stimulation of the T cell clones.

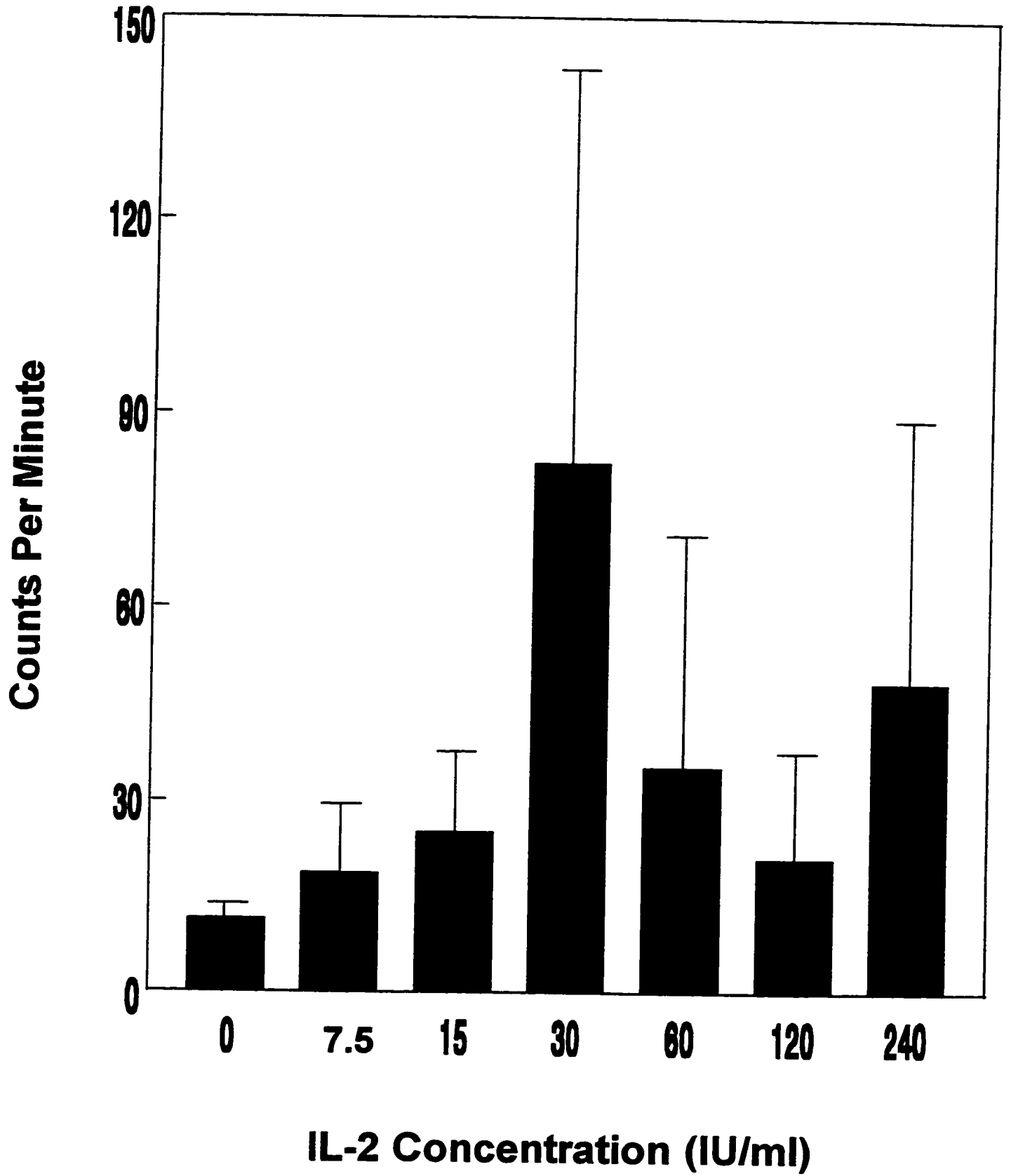


Figure 16

PHA stimulation of T cells. PHA stimulation of 2×10^4 MNC/well in selection medium containing 6-thioguanine. Following a 21 day incubation period, a [3 H]-thymidine incorporation assay was performed to determine what concentration of PHA could maximally stimulate T cells in the presence of 6-thioguanine. The results were read using a β counter and 0.5 to 2 μ g/ml of PHA produced the maximal stimulus.

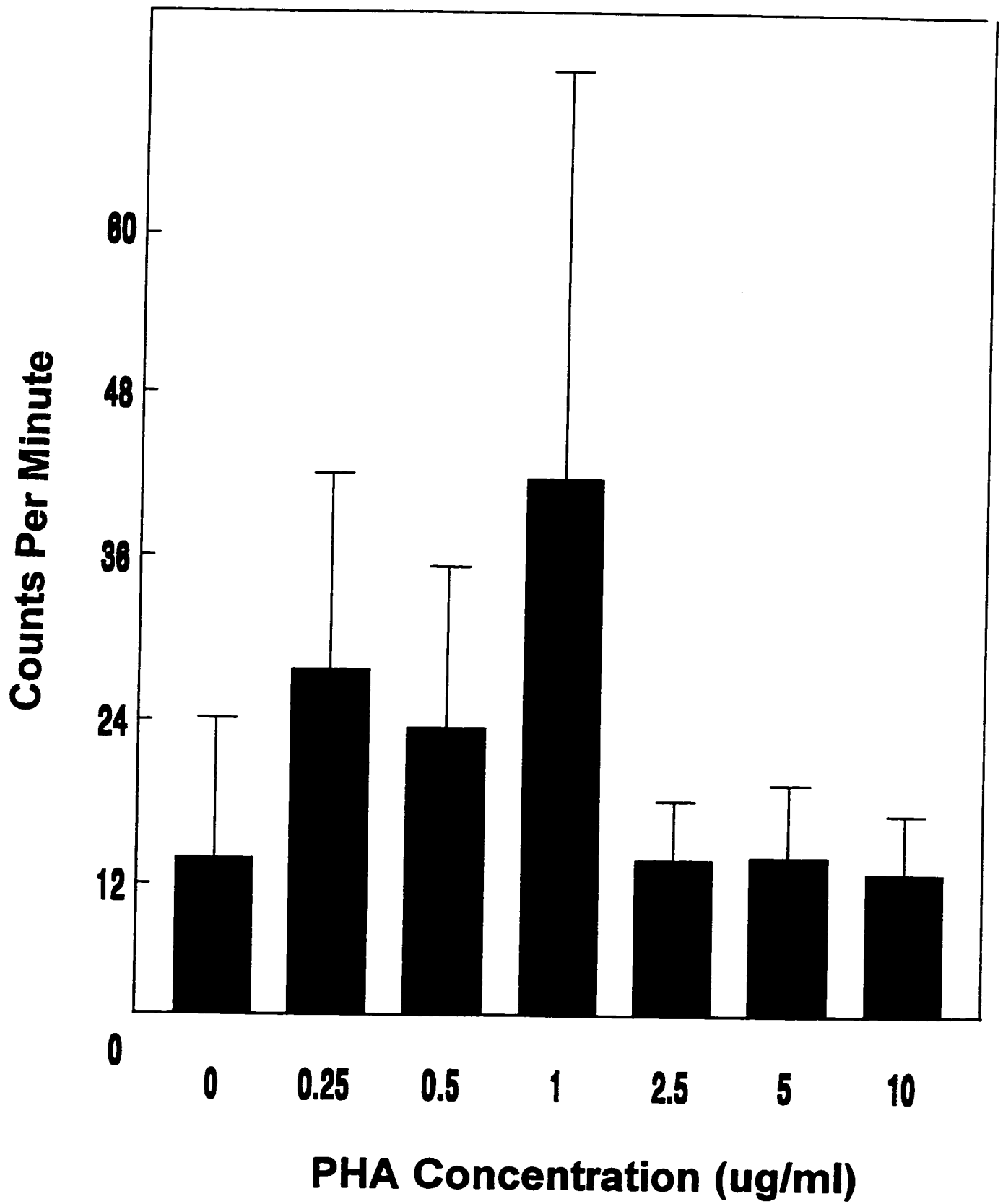


Figure 17

Synovial tissue T cell adherence to fibronectin coated wells. Wells of a 96-well flat bottom plate was coated with 10 $\mu\text{g/ml}$ of fibronectin (striped bar) and 0.01% poly L-lysine (solid bar). The synovial tissue T cell lines of various concentrations were incubated in the wells coated with fibronectin and poly L-lysine to determine which concentration of T cells would bind equally well to fibronectin and poly L-lysine coated wells. The absorbance is based on the readings obtained from the spectrophotometer.

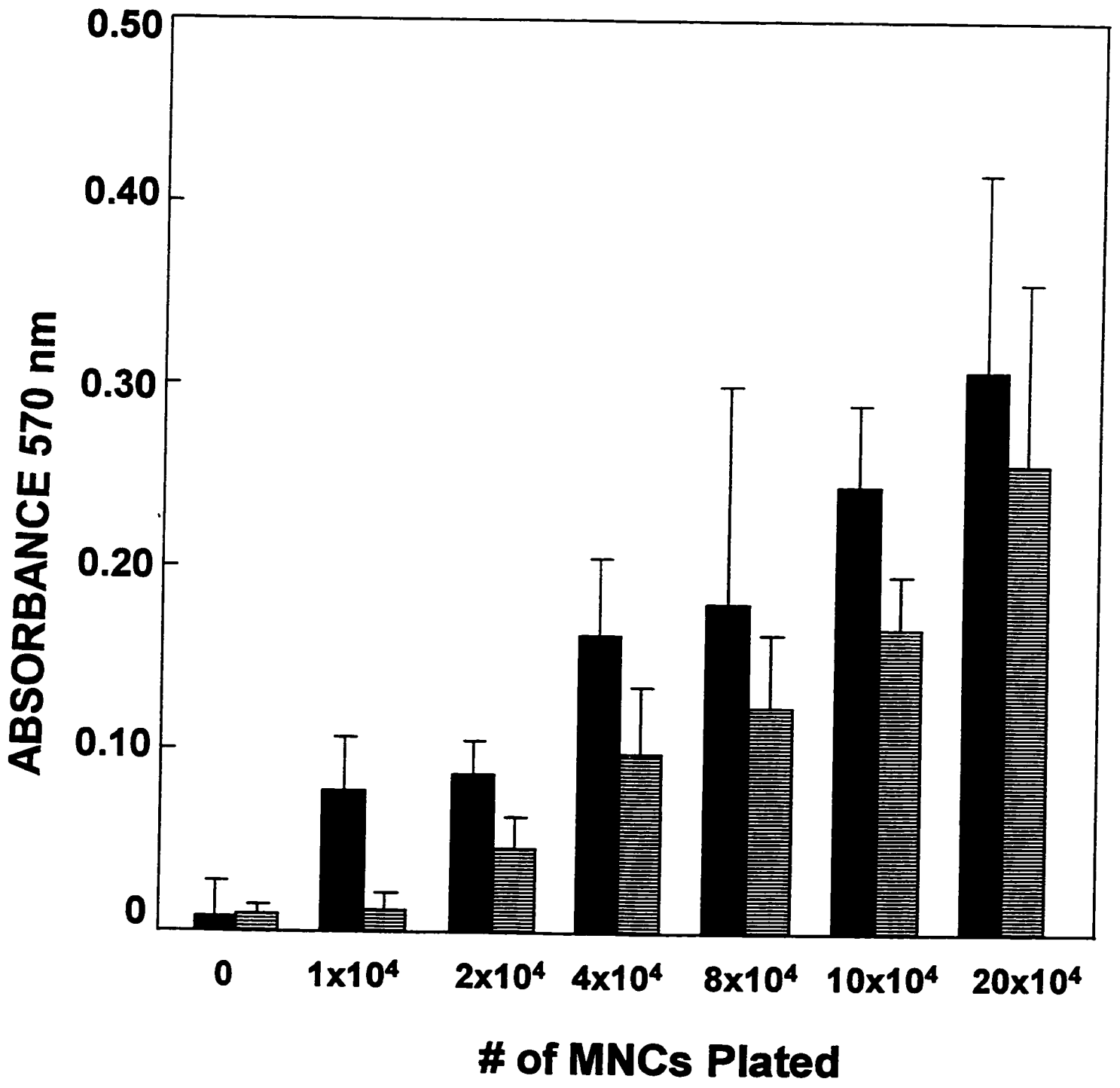
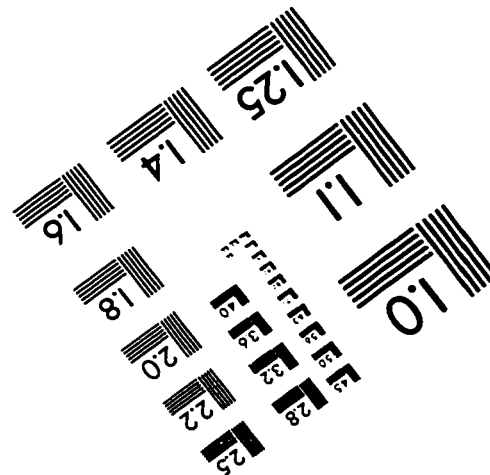
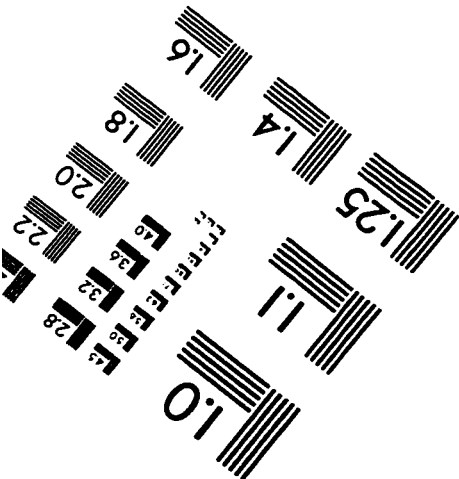
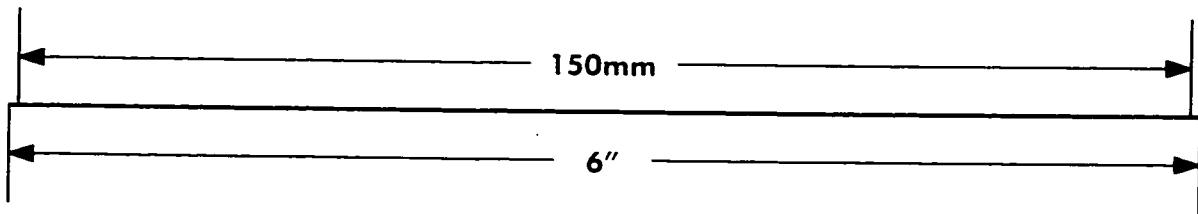
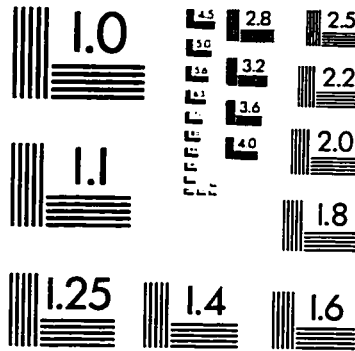
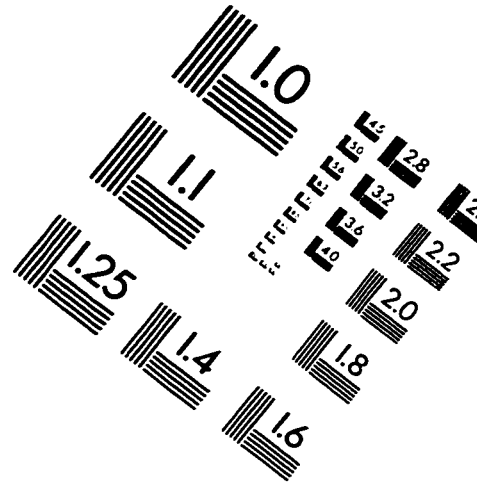
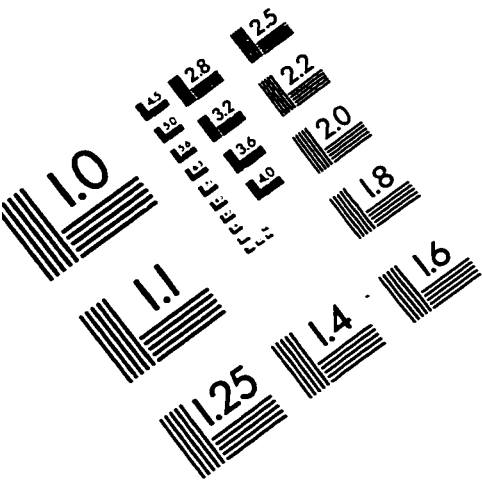


IMAGE EVALUATION TEST TARGET (QA-3)



APPLIED IMAGE, Inc
1653 East Main Street
Rochester, NY 14609 USA
Phone: 716/482-0300
Fax: 716/288-5989

© 1993, Applied Image, Inc., All Rights Reserved