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THE SEARCH FOR A SPECIFIC SYNOVIAL ANTIGEN IN RHEUMATOID ARTHRITIS.

Submitted by

J.E. MORGAN, B.Pharm.,MPS.

for the Degree of Doctor of Philosophy

1978

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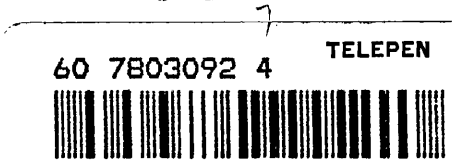

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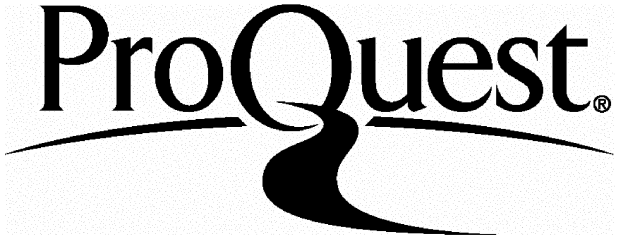

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ACKNOWLEDGEMENTS.

I would like to thank Professor R.J. Ancill for allowing me to work in his department. My thanks are also due to my supervisors, Dr. P.A. Bacon and Dr. A.J. Collins for support and encouragement.

I am indebted to Dr. N.D. Hall for all his help and advice during the course of this work.

I would like to thank the hospital administrator, Mr. C. Quinnell, for permitting me the use of the laboratory at the Royal National Hospital for Rheumatic Diseases.

Thanks are given to Mrs. J. Hayton for the patience she maintained in the typing of this thesis. I must thank my parents for their constant support and my husband for the continued inspiration.

I would like to thank all members of the "Min's" Staff who made my stay such a happy one but especially those who so freely gave of their blood and without whom this thesis would not have been possible.

TO MY HUSBAND
AND PARENTS

SUMMARY

A search has been made for the presence of an initiating antigen in rheumatoid arthritis to which rheumatoids would specifically respond *in vitro* tests of delayed hypersensitivity.

This has involved the testing of 25 synovial specimens prepared in a variety of ways. Crude synovial homogenates containing soluble and insoluble material have been tested in the leucocyte migration test (LMT) and the lymphocyte transformation test (LTT). Protein eluates and detergent solubilized protein have also been tested.

Results have been presented indicating that rheumatoid arthritis patients and age and sex-matched controls will respond to the same extent to an ubiquitous test antigen, streptokinase and to a non-specific cell membrane antigen from homogenized erythrocytes. However RA's do respond specifically to IgG in both native and aggregated forms to which controls do not respond in the LMT.

A protein has been eluted from inflammatory synovium which induces equal inhibition of migration in the LMT of both RA and control leucocytes and stimulation of RA and control lymphocytes in the LTT. The mechanism of action of this protein in the LMT and LTT has been discussed in the light of reports in the literature.

A role for this protein as an initiating factor in rheumatoid arthritis has not been established. A speculative role in the perpetuation of this chronic rheumatoid response has been presented in terms of the aetiology and pathogenesis of this disease.

CONTENTS

ACKNOWLEDGEMENTS		ii
SUMMARY		iii
ABBREVIATIONS		xi
CHAPTER 1	**INTRODUCTION**	
SECTION I	DEFINITION OF RHEUMATOID ARTHRITIS	1
SECTION II	THE PATHOLOGY OF JOINT INFLAMMATION	2
i	Synovial fluid	2
ii	Synovial cell changes	3
iii	Infiltration of synovium	4
iv	Pannus formation	5
v	Destruction of cartilage	5
SECTION III	INFLAMMATION	7
A.	Outcome of inflammation	7
B.	Chronic inflammation	7
SECTION IV	DELAYED HYPERSENSITIVITY	11
A.	T-lymphocytes	11
B.	Definition of delayed hypersensitivity	11
C.	Histology of delayed hypersensitivity reaction.	13
SECTION V	PATHOGENESIS	16
SECTION VI	ANIMAL ARTHRITIS	18
A.	Adjuvant arthritis of rats	18
B.	Experimental allergic arthritis of rabbits	19
C.	Other animal models	21
SECTION VII	DELAYED HYPERSENSITIVITY IN RHEUMATOID ARTHRITIS	22
A.	Putative antigens	24
a)	Infectious agents	24
i.	Bacterial infection	25
ii.	Viral infection	26
iii.	Immunoglobulins in rheumatoid arthritis	27
iv.	Joint fluid	29
v.	Cartilage components	31
vi.	Synovial tissue antigens	31

SECTION VIII	INTRINSIC IMMUNOLOGICAL DEFECT	34
SECTION IX	TESTS OF CELL-MEDIATED IMMUNITY	37
A	IN VIVO	37
B	IN VITRO	37
i.	Leucocyte migration test (LMT)	37
ii.	Correlation of IN VITRO with IN VIVO observations	40
iii.	Lymphocyte transformation test (LTT)	41
iv.	Correlation of IN VITRO with IN VIVO observations	42
SECTION X	DEFINITION	45

CHAPTER 2 EXPERIMENTAL

SECTION I	MATERIALS	46
SECTION II	LEUCOCYTE MIGRATION TEST	47
A	Materials	47
B	Method	47
C	Reading of migration areas	49
D	Cell viability	50
E	Preparation of apparatus	50
SECTION III	LYMPHOCYTE TRANSFORMATION TEST	51
A	Separation of lymphocytes from peripheral blood	51
i	Materials	51
ii	Method	51
B	Preparation of lymphocyte cultures	51
i.	Materials	51
ii.	Method	52
C	Calculating results	53
i.	Tritiated thymidine incorporation	53
ii.	Microscopic examination of cell cultures	53
D	Cell viability	54
E	Preparation of materials	54

CHAPTER 3 THE EFFECT OF STREPTOKINASE ON THE MIGRATION OF CONTROL AND RHEUMATOID LEUCOCYTES FROM PATIENTS RECEIVING VARIOUS DRUG REGIMES IN THE LMT.

SECTION I	INTRODUCTION	55

A	Drug effects	55
B	Streptokinase	56
SECTION II	METHODS	57
A	Streptokinase-streptodornase evaluation	57
i.	Removal of thiomersal from SK-SD	57
ii.	Results	57
B	Test Streptokinase evaluation	64
i.	Results	64
C	Comparision of patient groups	67
i.	Results	67
SECTION III	DISCUSSION OF RESULTS	70
A	Non-steroidal anti-inflammatory drugs	71
B	Penicillamine	71
C	Gold	72
D	Anti-malarials	73
E	Steroids	73
F	Cyclophosphamide	75
SECTION IV	CONCLUSION	76

CHAPTER 4 THE EFFECT OF CRUDE SYNOVIAL HOMOGENATES ON THE MIGRATION OF RHEUMATOID AND CONTROL LEUCOCYTES IN THE LMT.

SECTION I	INTRODUCTION	77
SECTION II	METHOD OF SYNOVIAL HOMOGENIZATION	78
SECTION III	RESULTS	79
SECTION IV	DISCUSSION	103
SECTION V	CONCLUSION	104

CHAPTER 5 THE EFFECT OF CEEL-MEMBRANE ELUATES ON RHEUMATOID AND CONTROL LEUCOCYTE MIGRATION IN THE LMT.

SECTION I	INTRODUCTION	105
SECTION II	EDTA/ME ELUATES	106
A	Method	106

SECTION III	CHOLATE SOLUBILIZATION	108
A	Materials	108
B	Method	108
SECTION IV	TRITON X100 SOLUBILIZATION	111
A	Materials	111
B	Method	111
SECTION V	COLUMN CHROMATOGRAPHY	113
A	Sephadex G 100	113
B	Ultragel ACA_{22}	113
C	Sepharose 6B	113
D	General information	113
SECTION VI	RESULTS	166
A	EDTA/ME elution	166
B	Cholate solubilization	167
C	TRITON X100 solubilization	168
SECTION VII	DISCUSSION	170
A	EDTA/ME elution	170
B	Cholate solubilization	171
C	TRITON X100 solubilization	172
SECTION VIII	CONCLUSION	173

CHAPTER 6 THE EFFECT OF NATIVE AND AGGREGATED IMMUNOGLOBULIN G ON RHEUMATOID AND CONTROL LEUCOCYTES IN THE LMT.

SECTION I	INTRODUCTION	174
SECTION II	PREPARATION OF IMMUNOGLOBULIN G	175
A	Native IgG	175
B	Aggregated IgG	175
C	Test of identity	175
SECTION III	RESULTS	180
SECTION IV	DISCUSSION	181
SECTION V	CONCLUSION	183

CHAPTER 7 THE EFFECT OF ERYTHROCYTE MEMBRANE HOMOGENATE ON THE MIGRATION OF CONTROL AND RHEUMATOID LEUCOCYTES IN THE LMT.

SECTION I	INTRODUCTION	184
SECTION II	METHODS	185
A	Homogenization	185
B	Preparation of Sephadex G200	185

SECTION III	RESULTS	195
SECTION IV	DISCUSSION	196
SECTION V	CONCLUSION	197

CHAPTER 8 THE EFFECT OF PUTATIVE ANTIGENS ON RHEUMATOID AND CONTROL LYMPHOCYTES IN THE LYMPHOCYTE TRANSFORMATION TEST.

SECTION I	INTRODUCTION	198
SECTION II	EXPERIMENTAL	199
A	Materials	199
B	Method	199
i.	Culture technique	199
ii.	Antigen preparation	199
SECTION III	RESULTS	220
A	PHA cultures	220
B	Streptokinase cultures	221
C	Combined eluates	221
D	Crude homogenates	221
SECTION IV	DISCUSSION	222
SECTION V	CONCLUSION	224

CHAPTER 9 DISCUSSION.

SECTION I	INTRODUCTION	226
SECTION II	CAUSES OF INHIBITION IN THE LMT	226
SECTION III	SIMILAR RESPONSES OF RHEUMATOID AND CONTROL LEUCOCYTES TO A STANDARD ANTIGEN.	226
SECTION IV	DISSIMILAR RESPONSES OF RHEUMATOID AND CONTROL LEUCOCYTES TO A TEST ANTIGEN :- IgG	227
A	Comparison of the results reported in Chapter 6 with those in the literature	227
SECTION V	SIMILAR RESPONSES OF RHEUMATOID AND CONTROL LEUCOCYTES TO ERYTHROCYTE HOMOGENATE	228

A	Comparison of results reported in Chapter 7 with those in the literature	228
B	Nature of inhibitory material	229
SECTION VI	SIMILAR RESPONSES OF RHEUMATOID AND CONTROL LEUCOCYTES TO CRUDE SYNOVIAL HOMOGENATES	229
A	Comparison of results reported in Chapter 4 with those in the literature	229
i.	Synovial homogenate	229
ii.	Other tissue homogenates	230
iii.	Discrepancy in methodology	230
B	Nature of inhibitory material in the crude synovial homogenates	231
C	Other sources of antigen in the rheumatoid joint	231
SECTION VII	EFFECT OF SYNOVIAL ELUATES IN THE LMT	232
A i.	EDTA/ME elution	232
ii.	Nature of inhibitory material	233
B	Detergent solubilization	234
C	Conclusion	236
SECTION VIII	CAUSES OF TRANSFORMATION IN THE LTT	236
SECTION IX	COMPARISON OF LMT WITH LTT	236
SECTION X	THE EFFECT OF CRUDE HOMOGENATES IN THE LTT	236
SECTION XI	THE EFFECT OF EDTA/ME ELUATES IN THE LTT	237
SECTION XII	POSSIBLE FUTURE LINES OF ENQUIRY	237
SECTION XIII	IMPLICATIONS OF THIS WORK	238
A	Assessment of test system	238
B	Assessment of antigen preparation	239
C	Conclusion	239
D	Implications for the aetiology and pathogenesis of RA	239
SECTION IV	CONCLUDING STATEMENT	240

REFERENCES

ABBREVIATIONS

Abbreviations used in this thesis are as follows:

AA	adjuvant arthritis of rats
ACTH	adrenocorticotrophic hormone
ME	2 mercaptoethanol
CFI	complete Freund's adjuvant
CMI	cell-mediated immunity
Con A	concanavalin A
CY	cyclophosphamide
DEAE-Sephadex A50	diethylaminoethyl-Sephadex
DH	delayed hypersensitivity
EAA	experimental allergic arthritis of rabbits
EDTA	ethylenediaminetetra-acetic acid
EDTA/ME	hypotonic elution solutions
FCA	Freund's Complete Adjuvant
FIA	Freund's Incomplete Adjuvant
Fig.	Figure
g	all rates of centrifugation are quoted as average *g* values
Hb	haemoglobin
HEPES	4-(2 hydroxyethyl)-1-piperazine ethane sulphuric acids
IFA	incomplete Freund's adjuvant
Ig	immunoglobulin
IM	intramuscular injection
i.u.	international units
IV	intravenous injection or infusion
LIF	leucocyte migration inhibition factor
LMT	leucocyte migration test
LTT	lymphocyte transformation test
M	molar
ME	mercaptoethanol
MEM	Eagles Minimum Essential Medium

MI	migration index
ml.	millelitres
MLC	mixed lymphocyte culture
mM	millemolar
M.Wt.	molecular weight
No.	number
NS	not significant, applied to P values
P	probability of significance for t-test
PBS	phosphate buffered saline
PHA	phytohaemagglutinin
PMN	polymorphonuclear cell
PMSF	phenymethylsulphonyl fluoride
PPO	2, 5 diphenyloxazol
POPOP	14 Di (2-(4-methyl 5 phenyl oxazolyl)) benzene
PPD	purified protein derivative of tuberculin
psi	pounds per square inch
RA	rheumatoid arthritis
RAs	rheumatoid arthritics
rbc	red blood cells
RF	IgM rheumatoid factor
SD	standard deviation
SEM	standard error of the mean
SK	streptokinase
SK-SD	streptokinase-streptodornase
TCA	trichloroacetic acid
TRIS	2-amino-2-hydroxymethylpropane-1, 3-diol
TRITON x 100	p-octyl phenoxy-polyoxyethylene
10/150 buffer	150 mM NaCl in 10 mM phosphate pH 7.4
µm	micrometers

CHAPTER 1

INTRODUCTION

CHAPTER 1

INTRODUCTION

SECTION I - DEFINITION OF RHEUMATOID ARTHRITIS

Rheumatoid Arthritis (RA) is a systemic disease in which a non-suppurative inflammatory polyarthritis in the diathrodial joints is the predominant feature. Characteristically it involves peripheral joints (often symmetrically) and runs a sub-acute to chronic course of exacerbation and remission, depending on the degree of synovial involvement.

That joint involvement is the predominant feature is exemplified by the American Rheumatism Association's Diagnostic Criteria, in which nine of the eleven criteria involve joints (Rodnan, 1971).

The aetiology of RA is unknown. Glynn (1969), Bland and Phillips (1972), Barland (1973) and Denman (1975) have all put forward the hypothesis that RA is a disease of viral origin which manifests itself only in genetically pre-disposed people. The pathologically damaged joint which is characteristic of RA is due to an on-going immune response, either to the persisting virus or to antigens evoked during the initial reaction to the infective agent.

SECTION II - THE PATHOLOGY OF JOINT INFLAMMATION IN RA

The pathology of joint inflammation in the chronic lesion has been extensively renewed (Gardner, 1972; Zvaifler, 1973). The synovitis of RA is a predominantly polymorphonuclear (PMN) exudation of the synovial fluid, and a mono-nuclear cell infiltration of the deep synovium. Bränemark (1971) stated that microvascular disturbances constitute a basic phenomenon predisposing to tissue lesions. Weissman (1971) characterised the lesions in RA joints as follows:

a) Margination of leucocytes and their appearance in synovial fluid.

b) Hypertrophy and hyperplasia of synovial lining cells.

c) Infiltration of the synovium by many lymphocytes.

d) The transformation of synovium to granulation tissue which, as pannus, overgrows and invades cartilage.

e) Erosion of cartilage; initially proteoglycan, followed by collagen breakdown then accompanied by chondrocyte death and attempts at regeneration.

(i) Synovial Fluid

In chronic RA the volume of synovial fluid increases from 0.1 - 0.4 ml. to as much as 200 - 400 mls. When seen at arthroscopy the fluid appears turbid, containing fibrinous clots or strands of fibrin which are most conspicuous on the synovial lining cells. The protein content increases to levels obtained for serum. Many agents capable of influencing the coagulation mechanisms are found, e.g. the Hageman Factor (Kellermeyer and Brekennage, 1966) and fibrin. Complement levels are characteristically low when compared with other joint diseases. Patients seropositive for Rheumatoid Factor (RF) usually display high synovial fluid levels of RF. The fluid also contains immunoglobulin (Ig) of other specificities. Many enzymes are found, including proteases, hydrolases and collagenases.

The predominant cell (75% - 90%) is a mature PMN leucocyte which has a half life in the joint of four hours (Zvaifler, 1973). Its transudation is via the synovial membrane. The remainder of the cells consist of lymphocytes, monocytes, occasional macrophages and synovial lining cells.

(ii) Synovial Cell Changes

There is hyperplasia and hypertrophy of the synovial lining cells when the synovium appears oedematous and inflamed and protrudes into the joint space as slender villous projections. The lining cell layers, normally appearing as 1 - 3 cells deep, are 6 - 10 cells deep. Multinucleate giant cells are common, and located in areas of lining hyperplasia or in the subintimal layer. These are derived from the A cells of the synovial lining and were originally thought to be unique to RA. They have since been observed in other forms of chronic arthritis.

A fibropurulent exudate frequently present in interstitial regions between the lining cells occasionally causes these lining cells to assume a palisading appearance with their cytoplasmic processes extending into the fibrinoid material (Fassbender, 1971). Persistant vascular permeability and a deficient synovial fibrinolytic system are unlikely to be the entire explanation of the persistance of fibrin within the chronically inflamed joint. Repeated small intra-articular haemorrhages may contribute to its persistance (Caughey and Highton, 1967).

Following the onset of inflammation synovial cells are able to speed up their metabolism and Lindner (1971) suggests that this behaviour may contribute to the characteristic self-perpetuating lesion of RA. Persistant differences in rheumatoid and normal synovial lining cells are also propogated through many generations of cell culture in vitro (Gardner, 1972). This suggests a "primary defect" (Castor, 1971) although it could be due to infection (Rodnan, 1971).

(iii) Infiltration of Synovium

The connective tissue stroma is packed with mononuclear cells. They are thought to leave the post-capillary venules (Ziff 1974) by migrating mainly through the endothelial cells; a process known as emperipolesis. Lymphocytes form perivascular collections composed predominantly of small cells. A large fraction of these undergo transition to form plasma cells, indicating that they were initially of the B cell line (Kobayashi and Ziff, 1973; Ishikawa and Ziff, 1976). However a high proportion of lymphocytes with the surface markers characteristic of T-lymphocytes are present (Sheldon and Holborow, 1975; Van Boxel and Paget, 1975; Wangel et al, 1975; Loewi et al, 1975; Abrahamsen et al, 1975, 1976; Bankhurst et al, 1976). In all the reports except those of Sheldon and Holborow, 1975 and Wangel et al, 1975, there was an increase in T-cells when compared with peripheral blood lymphocytes. The reports of the percentages of T- and B-cells in the peripheral blood of rheumatoid patients (Frøland et al, 1973; Sheldon et al, 1974; Brenner et al, 1975) indicate that there is no significant difference from the percentages in control blood. However, Mellbye et al, 1973, reports a decrease in B-cells.

The small lymphocytes migrate into the extravascular region where a mixture of cells is found: small lymphocytes, undifferentiated blast cells, plasma blasts, plasma cells and macrophages, all in significant numbers. The presence of these cell types in close contact suggests the interaction of T- and B-lymphocytes with liberation of lymphokines which attract and immobilize macrophages and stimulate B-cells to produce antibody (Ziff et al, 1974).

It has been calculated that the immunoglobulin synthesising capacity of rheumatoid synovium is similar to that of normal human spleen and lymph node (Smiley et al, 1968). These immunoglobins have different specificities from those synthesized by blood lymphocytes (Herman et al, 1971).

Structures similar in appearance to rheumatoid nodules are found only in the presence of RF. They consist of a central mass of necrotic tissue surrounded by a characteristic palisade of macrophages. The necrosis of the tissues is mediated by the capillaries or substances carried by the capillaries (Fassbender, 1971).

PMNs are occasionally seen but are not a prominent feature of chronic synovitis. Macrophages with iron containing pigment, perhaps from haemorrhages (Muirden, 1968), are found in both superficial and deep layers of the synovium.

(iv) Pannus Formation

Histologically composed of proliferating fibroblasts, numerous small blood vessels, various numbers of inflammatory cells and occasional collagen fibres, a vascular granulation tissue, or pannus, extends over the surface of the cartilage and burrows into the subchondral bone. Pannus and cartilage depletions are almost invariably associated, but cartilage destruction can occur where no pannus exists (Hamerman et al, 1967).

(v) Destruction of Cartilage

Uehlinger (1971) states that the nutrition of joint cartilage is provided by diffusion of the joint fluid to the line of calcified cartilage (a maximum depth of 5 mm.). The fibrinous exudation following primary microvascular changes increases the viscosity of the fluid, decreasing its ability to diffuse. The proteoglycans are dissolved, perhaps by enzymes from the chondrocytes, and the joint cartilage systems and the collagen fibres can then be broken by mechanical stress (Holt, 1971). Any enzymatic activity from the synovial fluid must first attack the surface proteoglycans, since intact cartilage does not allow diffusion of large molecules such as proteases (Muir, 1971). However, if the situation envisaged by Holt (1971) occurs, then this might alter the diffusion capacity of the cartilage. Also,

the regenerated proteoglycans are of smaller molecular weight (Muir, 1971) which might alter its diffusion capacity. Given these alterations in the basic structure and characteristics of cartilage it is possible to envisage a situation where the action of collagenase could lead to a breakdown of collagen and the formation of antigenic material. Herman and Carpenter (1975) discuss fully the antigenicity of cartilage.

SECTION III - INFLAMMATION

Inflammation is a process which begins following sublethal injury to tissue and ends with permanent destruction of tissues or with complete healing (Ebert and Grant, 1974).

The inflammatory process can be summarized as follows:

As a consequence of a change in the microcirculation in the vicinity of tissue insult due to the action of chemical, thermal, mechanical or biological "irritants" capillary permeability increases permitting exudation of protein and leucocytes to remove either the inciting agent or damaged tissue. This is an obvious oversimplification of a process dependent upon a series of interrelated and controlled events. It must be emphasized that in considering the role of any possible participant, mediator or reaction in this process one must be aware of the initiating stimulus, the severity of the injury, the organ or tissue affected and the species investigated. A generalized series of events are shown in Figure 1.

A. OUTCOME OF INFLAMMATION

Once formed, the inflammatory exudate can suffer one of four fates:

a) It can resolve, leaving virtually normal tissue.

b) It can become organised to form a scar.

c) It can suppurate, forming an abcess if massive PMN accumulation and tissue degradation exist.

d) It can become a chronic inflammation.

B. CHRONIC INFLAMMATION

The difference between acute and chronic inflammation is predominantly quantitative, chronic inflammation being an inflammatory response that persists, certainly for months if not years.

Granulomatous chronic inflammation is characterised by

persistant and highly cellular reactions, which are often destructive. The classic examples are tuberculosis, leprosy and syphilis. Fungi may cause similar damage, as do certain chemicals, minerals and foreign bodies. Chronic granulomatous inflammation of unknown aetiology includes sarcoidosis and RA (Spector, 1974).

The macrophage is the major cell involved in chronic inflammation. During this process macrophages will form giant cells, epitheloid cells and histocytes.

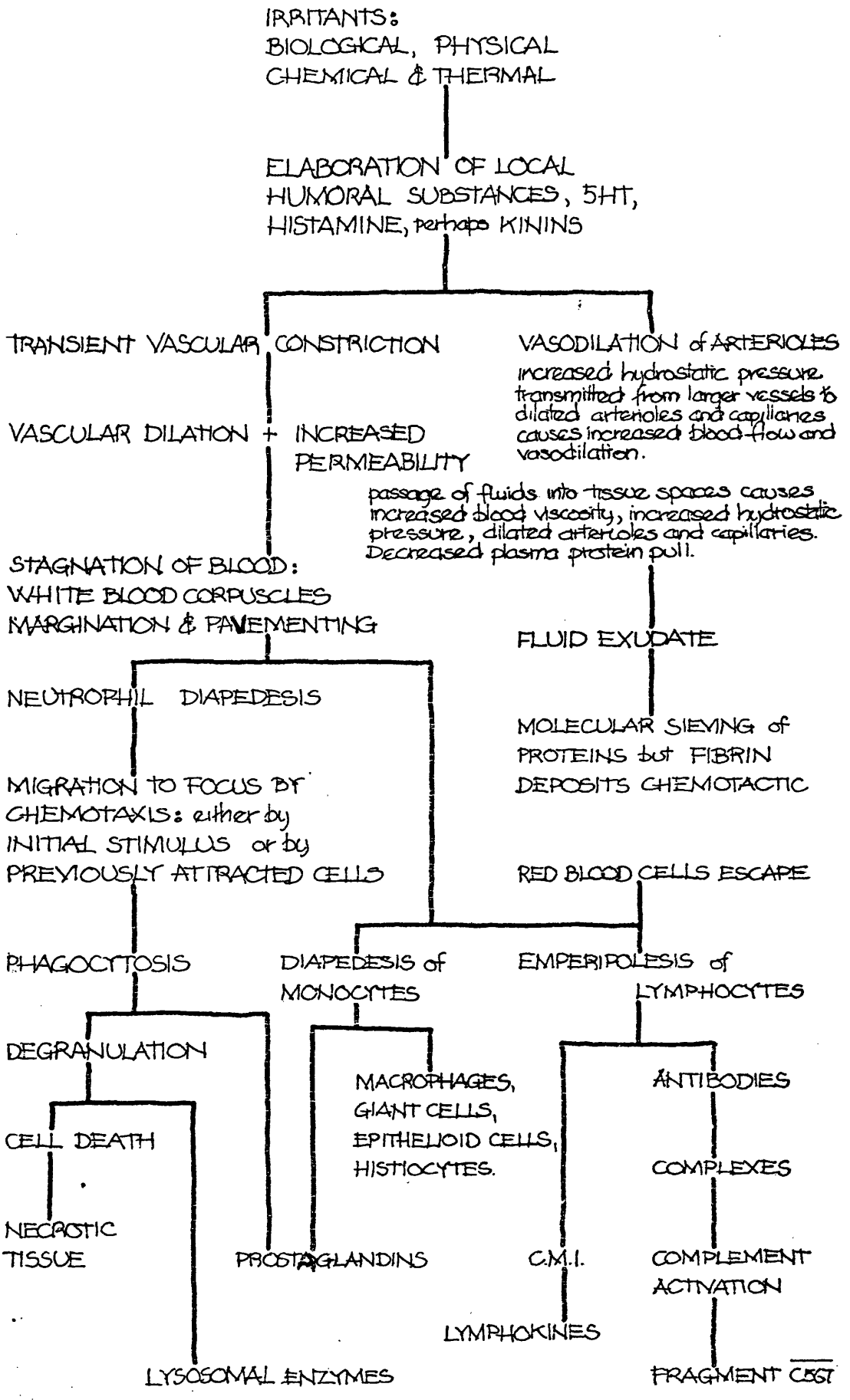
The fibroblast is prominent when organisation is occurring, as in the periphery of a tubercle or when the reaction is fibrinogenic as in silicosis. It most probably originates from local connective tissue cells and it secretes collagen.

Lymphocytes enter the inflammatory area from the early stages of the reaction. These cells are of bone marrow origin (Spector and Willoughby, 1968) but their prevalence depends only on the nature of the inciting stimulus. If the irritant carrageenan is used lymphoid cells are rarely seen. Similarly, only marrow cells enter a lesion induced by Freund's Incomplete Adjuvant (FIA). Freunds Complete Adjuvant (FCA) induces a delayed hypersensitivity response associated with infiltration by lymphocyte cells; possibly as part of a recirculatory mechanism. Spector (1974) concludes that the presence of members of the lymphoid series is, in fact, evidence that a particular inflammatory reaction is associated with an immune reaction response.

A wide variety of radio-labelled irritants have been used in the study of chronic lesions. Spector et al (1968) states that there is no observed instance where a granulomatous reaction is not accompanied by intracellular persistence of irritant or where disappearance of irritant is not accompanied by disappearance of reaction. This does not preclude a situation where the persistent stimulus may arise during the acute inflammatory reaction against

the initiating stimulus. There might be continuous or recurrent production of the stimulus. Alternatively there could be incomplete clearance of the irritant as in the incomplete degradation of irritants by macrophages or inadequacy of lysosomal enzymes or sequestration (Spector 1974).

An excess of antibody over antigen in the formation of immune complexes leads to granuloma formation as opposed to transient inflammation, (Spector and Heesom, 1969). These lesions develop in thymectomised animals (Rothwell and Spector, 1972).



SUMMARY of the INFLAMMATORY PROCESS

FIG 1

SECTION IV - DELAYED HYPERSENSITIVITY

A. T-LYMPHOCYTES

It is generally accepted that there are two different small lymphocyte populations (Raff, 1972):

a) T-lymphocytes, processed by, or in some way dependent on, the thymus and responsible for cell-mediated immunity.

b) B-lymphocytes, bursa dependent and concerned in the synthesis of circulating antibody (Roitt, 1974).

T-lymphocytes are a heterogeneous population of lymphocytes whose members have surface receptors (of unknown chemical constitution) with variable affinity for different antigenic determinants. On exposure to antigen, T-lymphocytes of appropriate specifity are activated. The main manifestations of their action are in transplantation rejection, cytotoxicity, delayed hypersensitivity and T-cell help and suppression of B-cell responses.

During activation T-cells synthesize and secrete mediator substances called lymphokines (Dumonde et al, 1969). These substances are named after the test situation in which their action can be demonstrated in vitro. Their in vivo significance is to a great extent a matter of debate. The lymphokines can act directly on target cells, invading organisms and the vasculature. Others attract, activate and in various ways modify the behaviour of immunologically non-specific cells which may become the principal effectors of cellular hypersensitivity; i.e. B-lymphocytes, monocytes, macrophages, basophilic leucocytes and other granulocytes.

B. DEFINITION OF DELAYED HYPERSENSITIVITY

The classic example is the tubercular reaction, i.e. the inflammatory area which evolves if a person who has previously been exposed to Mycobacterium Tuberculosis is challenged by skin test with a preparation, usually Purified Protein Derivative (PPD) of M. Tuberculosis. This is characterised by a prolonged time course,

several hours elapsing before gross evidence of a developing process.

The fundamental property is the dependence of these reactions on sensitised lymphocytes. Landsteiner and Chase (1942) and Chase (1945) revealed that delayed hypersensitivity (DH) reactions could only be transferred from sensitised to normal animals by means of living lymphoid cells and not by immune sera. Lawrence (1970) has shown transfer with viable lymphocytes, non-viable lymphocytes and extracts from such cells, which he terms Transfer Factor, and which transfers a state of DH not associated with antibody formation.

Cellular hypersensitivity can exist in the absence of detectable antibody and equally need not accompany the production of antibody to a given antigen. The instigating antigen is usually protein or contains a large protein constituent. Most polysaccharides are unable to induce DH unless conjugated with protein when the specificity is for the protein-carbohydrate complex (Turk, 1967).

C. HISTOLOGY OF DELAYED HYPERSENSITIVITY REACTION

The histology is characteristic but by no means specific, thus perivascular accumulations of mono-nuclear cells, a hallmark of these reactions, do not only occur in DH reactions. The appearance of the lesion can also be greatly modified by the presence of specific antibody (Dvorak, 1974).

The initial exudation is mono-nuclear and this differs from ordinary inflammatory lesions (Dvorak, 1974). Neutrophils do participate to a certain extent but contamination of the skin test with irritant, e.g. bacterial endotoxin, results in reactions severe enough to involve tissue necrosis and the co-existence of anaphylactic and Arthus reactions, all of which are characterised by neutrophils, may lead to a falsely high estimate of their frequency.

In guinea pigs, after challenge with PPD, the local site initially contains a sparse infiltrate of mono-nuclear cells and some neutrophils in the deep dermis and sub-cutis, and, more diffusely, through the superficial dermis. Lymphatics are dilated and fixed tissue cells, including fibrocytes and endothelial cells, appear activated. These changes occur in sensitized, non-sensitized and tolerant animals.

After six hours vascular permeability has started increasing. Positive reactions can be recognised by the presence of perivascular infiltration of mono-nuclear cells in the deep dermis, sub-cutis and, more diffusely, in the upper dermis. The vessels around which the cells accumulate are venules and small veins, and within their lumens are a variety of leucocytes, most commonly mono-nuclear, frequently in mitosis, and said to pass through endothelial cells by a process analagous to emperipolesis.

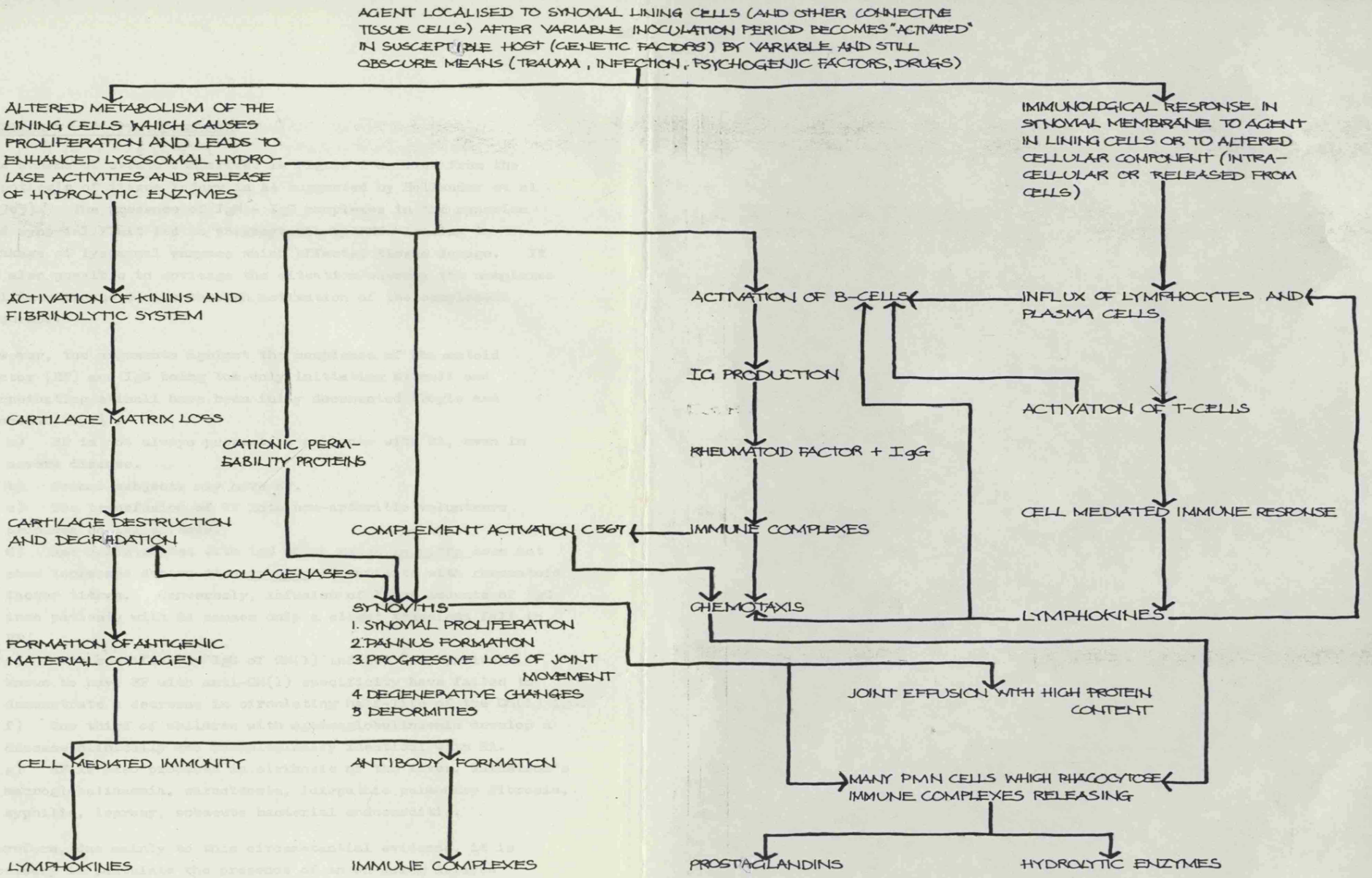
The cellular accumulations occur progressively and by 24 hours infiltrating cells form prominent perivenous cuffs. In some cases dermal lymphatics are packed with cells which may infiltrate the epidermis and other epithelial structures.

The morphological classification is difficult. Kosunen et al (1963) classified them as 5 - 15% monocytes, 25% histiocytes and approximately 20% small lymphocytes. The other 40 - 50% he classified as medium or large lymphocytes shown to belong to a population of cells that was rapidly dividing both before and after arrival at site. Bosman and Feldman (1970) categorised these 40 - 50% as monocytes, macrophages and precursors, while Aström et al (1968) regards them as activated lymphocytes. Dvorak (1974) concludes that the vast majority belong to a rapidly dividing population ultimately derived from the bone marrow and without specificity for antigen. They are attracted and activated by the

few antigen reactive cells (Rocklin, 1976). Bone marrow cells of the type and in the proportions reported here are not unique to DH reactions and have in fact been shown to participate in a variety of non-immunological inflammatory lesions (Volkman and Gowans, 1965).

The round cell accumulation which is a regular feature of DH is a consistent finding in the rheumatoid synovium (Zvaifler, 1973).

In the lymph nodes there is a proliferation of cells in the paracortical (thymus dependent) zone which also contains large cells with pyrininophilic cytoplasm termed immunoblasts thought to derive from small lymphocytes. Following antigen stimulation which leads to DH these immunoblasts appear in large numbers and proliferate rapidly. They and their descendants are responsible for lymph node enlargement. They appear in the draining sinuses and so enter the circulation. They are the only lymphoid cells which 'home in' to inflammatory sites (Asherson and Allwood, 1973). There is an increased level in RA, systemic lupus erythematosis (SLE)(Bacon et al, 1971; Bacon et al, 1975; Delbarré et al, 1975) and polymyalgia rheumatica (PMR) (Eghtedari et al, 1976), but not in psoriatic arthritis, osteoarthritis (OA) or gout (Delbarré, 1975) suggesting that immunoblast levels are not related to inflammation (Delbarré, 1975). Several studies of RA show correlation between inflammatory activity and immunoblast levels suggesting changes in inflammatory activity are due to an immune reaction (Crowther et al, 1969a; Crowther et al, 1969b). Immunoblasts are present in the circulation in both viral and bacterial infections, and their presence suggests an immunological basis for disease. It is difficult to distinguish between B and T immunoblasts (Delbarré, 1975) but in most responses B cells predominate (Hall et al, 1967).



POSSIBLE PATHOGENIC MECHANISMS IN RHEUMATOID ARTHRITIS

FIG 2

SECTION V - PATHOGENESIS

The scheme of events summarized in Figure 2 derives from the hypothesis of tissue injury in RA suggested by Hollander et al (1965). The presence of IgM - IgG complexes in the synovium and synovial fluid led to phagocytosis by PMNs' leading to leakage of lysosomal enzymes which effected tissue damage. It is also possible to envisage the situation whereby the complexes initiate tissue damage through activation of the complement sequence.

However, the arguments agsinst the complexes of Rheumatoid Factor (RF) and IgG being the only initiating stimuli and perpetuating stimuli have been fully documented (Boyle and Buchanan, 1971):

a) RF is not always present in patients with RA, even in severe disease.

b) Normal subjects may have RF.

c) The transfusion of RF into non-arthritic volunteers produces no ill effects.

d) Red cells coated with IgG which react <u>in vitro</u> does not show increased destruction <u>in vivo</u> in patients with rheumatoid factor titres. Conversely, infusion of large amounts of IgG into patients with RA causes only a slight transient fall in RF.

e) More specifically IgG of GM(1) infused into patients known to have RF with anti-GM(1) specificity have failed to demonstrate a decrease in circulating half-life of the GM(1) IgG.

f) One third of children with agammaglobulinaemia develop a disease clinically and radiologically identical with RA.

g) RF is also produced in cirrhosis of the liver, Waldstrom's macroglobulinaemia, sarcoidosis, idiopathic pulmonary fibrosis, syphilis, leprosy, subacute bacterial endocarditis.

Therefore, due mainly to this circumstantial evidence, it is necessary to postulate the presence of an on-going delayed hypersensitivity reaction against some antigen present in the

chronically inflamed rheumatoid joint. In the following paragraphs the evidence will be put forward to support the presence of a cell-mediated immune reaction in RA.

SECTION VI - ANIMAL ARTHRITIS

Much of the evidence for the importance of cell-mediated immune reactions being important in RA derives from studies of various experimental models of chronic immune arthritis produced in laboratory animals. There are two experimental animal models of arthritis which have been widely studied in an attempt to elucidate the mechanisms of joint inflammation. One model is the adjuvant-arthritic rats (Pearson, 1956) and the second is the experimental allergic arthritis of rabbits (Dumonde and Glynn, 1962).

A. ADJUVANT ARTHRITIS OF RATS

Adjuvant arthritis is induced in rats by injecting Freund's Complete Adjuvant (FCA) into one of the hind foot pads. Not all strains of rat are equally susceptible, indicating a genetic influence. The inflammatory condition which subsequently develops after a minimum of ten days involves intraphalangeal, carpal, tarsal and ankle diarthrodial joints and synarthrosis of the tail. Extra-articular manifestations, which may be strain specific, include balanitis and urethritis, uveal tract inflammation and, later, dermatitis with alopecia, which has a histopathology similar to human psoriasis. Nodular lesions develop in the external ear and, less frequently, elsewhere in the skin with underlying vasculitis. It is a syndrome frequently compared with Reiters Syndrome (Hadler, 1976).

Because it follows injection of FCA investigators have focussed on the immunological aspects, and particularly cellular immunological aspects of the syndrome.

The obligate ten day latent period and the mono-nuclear infiltration of the inflamed synovium are reminiscent of the hallmarks of delayed hypersensitivity. By careful attention to the time of harvesting of donor cells, strain of rat and number of cells transferred,

passive transfer has been accomplished (Rawson and Wood, 1964; Whitehouse et al, 1969; Quagliata and Phillips-Quagliata, 1972).

Pearson and Wood (1963) suggested that the disease process is the result, at least in part, of DH to disseminated antigen, probably derived from the injected tubercle bacillis, possibly wax fraction D. Alternatively the animal mounts a response to an altered tissue component linked to the Mycobacterium tuberculosis which is specific to this model. A suggested non-specific mechanism is that the M. tuberculosis causes alteration in the host tissue, revealing new antigenic sites, and thus causing the animal to become hypersensitive to its own tissue, (Dumonde and Glynn, 1962; Glynn, 1968).

The leucocyte migration test has been used to study the onset of delayed hypersensitivity to BCG and an extract of an inflammatory lesion. The hypersensitivity to the inflammatory extract parallelled the disease course. Berry et al (1973) concluded that an endogenous antigen was involved.

B. EXPERIMENTAL ALLERGIC ARTHRITIS OF RABBITS

Experimental allergic arthritis in rabbits is a chronic disease which is produced by immunising rabbits with a soluble antigen in FCA and then injecting the antigen intra-articularly (Dumonde and Glynn, 1962). The arthritis has been shown to last for over two years (Consden et al, 1971). At first fibrin was used as the initiating antigen but later it was realised that any antigen could be used.

Using radiolabelled antigen for the secondary challenge it has been established that most of the antigen disappears rapidly from the joint (Consden et al, 1971). Doble et al (1973), using horse radish-peroxides as antigen, noted that it disappeared completely from the synovium. However, some does remain localised as immune complexes in the surface layers of cartilage, ligaments and menisci (Cook et al, 1972a). Further similarity to RA is the finding of immunoglobulins and C3 trapped in collagenous joint

tissue obtained from patients with the disease (Cook et al, 1972b). Fox and Glynn (1975) showed persistence of antigen, IgG and C3 in animals immunised with both FCA and FIA, but the animals injected with FIA recovered. Therefore although the idea of persisting complexes activating complement and thus initiating tissue damage (Hollander, 1965) is an attractive proposition, this does not seem to be the sole mechanism acting in rabbit arthritis.

As has been suggested for rats (Pearson and Wood, 1963) the disease process could result from delayed hypersensitivity to a disseminated tubercle antigen. However, Hirschowitz et al (1977) has shown that excision of the injection site granuloma one day before intra-articular challenge did not affect the development of the arthritis.

MIF is produced by synovial cultures from rabbits with EAA suggesting a continuing delayed hypersensitivity reaction (Stastny et al, 1973; Steihl et al, 1975). Injection of MIF intra-articularly causes arthritis (Andreas et al, 1972) histologically comparable to a delayed hypersensitivity action (Andreas et al, 1972; Ishikawa, 1973).

Lymphokines have also been found in the synovial fluid of rabbits with EAA (Stastny and Ziff, 1971). Such studies are hampered by the fact that these mediators of CMI are as yet poorly defined chemically and are, thus far, identifiable only by their biological actions.

The synovitis associated with a pronounced local response in which lymphocytes and plasma-cell infiltration is prominent (Glynn, 1968).

The various observations of EAA lead to the suggestion that delayed hypersensitivity to some unknown antigen is necessary for the chronicity of the arthritis.

C. OTHER ANIMAL MODELS

It has been shown that macrophages from mice with arthritis induced by Mycoplasma pulmonis are inhibited by extracts of normal synoval membranes (Harwick et al, 1973). This lends support to the unmasking theory of arthritis induction. (See Page 15).

Induction of granulomatous arthritis lesions in ankles of neonatally bursectomised chickens which persist for many weeks has to be achieved (Oates et al, 1971). The experiments show that the inflammatory mechanisms of cell-mediated immunity alone are able to provide sufficient basis for the development of allergic arthritis.

SECTION VII - DELAYED HYPERSENSITIVITY IN RHEUMATOID ARTHRITIS

As has been outlined in the earlier part of this chapter the histology of the chronically inflamed joint is quite consistent with an ongoing delayed hypersensitivity response. This has led to research into the feasibility of CMI being involved in RA and to the pathogens and antigens capable of eliciting the DH response.

Viral infection of cells causes an alteration in the cell surface membrane of the infected cell. The infected cells are eliminated by T cells which reorganise the cell surface as foreign. Thus, with the emphasis on a viral aetiology for RA, it is necessary to consider the possibility that there is an ongoing CMI reaction. Much of the evidence of a CMI pathogenesis for RA has been reviewed by Yu and Peter (1974).

Arthritis has been induced by a graft-versus-host reaction in joints. An intense synovitis follows the injection of spleen cells of Wistar rats into the knee joints of F_1 hybrid recipients (Bacon et al, 1973). The graft-versus-host reaction is regarded as a pure CMI reaction.

Having established the ability of a joint to support a cell-mediated immune reaction the evidence - admittedly circumstantial - that CMI plays a role in the persistence of inflammation in RA will be presented:

a) There is evidence that thymus derived lymphocytes are found in at least the same proportions in synovial effusions as in peripheral blood in rheumatoid patients (Frøland et al, 1973; Winchester et al, 1973; Vernon Roberts et al, 1974; Sheldon et al, 1974; Utsinger, 1975; Brenner et al, 1975; van de Putte et al, 1976). Most workers have reported an increase in the proportion of T cells (Winchester et al, 1973; Frøland et al, 1973; Vernon Roberts et al, 1974; Sheldon et al, 1974) or a decrease in B cells (Brenner et al, 1975).

As has been stated before (Sect. VII,a) most workers (Van Boxel and Paget, 1975; Abrahamsen et al, 1975; Loewi et al, 1975; Bankhurst et al, 1976) report an increase in the proportion of T cells in synovium when compared with peripheral blood. Some workers (Sheldon and Holborow, 1975; Wangel et al, 1975) report a decrease in T cell proportion. It is possible that the methods of preparing lymphocytes from the synovium, which include enzyme digestion, affect the cell surface. Van Boxel and Paget (1975) found no difference in the proportions of T and B cells in peripheral blood before and after treatment with collagenase and deoxyribonuclease. These enzymes had been used to isolate the lymphocytes from inflammatory tissue.

T cells are present and, presumably, able to mediate a delayed hypersensitivity response.

b) Lymphocyte depletion by prolonged thoracic duct drainage ameliorates the activity of RA (Wegelius et al, 1970; Paulus et al, 1973; Yu et al, 1973). This has also been demonstrated in rats with adjuvant disease (Yu and Peter, 1974)
These are the same thoracic duct cells which were used successfully to transfer AA from one rat to another of the same strain (Quagliata and Phillips-Quagliata, 1972) although lymph node or spleen cells have been used (Pearson, 1963).

c) Seronegative rheumatoid-like diseases occur in patients with agammaglobulinaemia (Good, 1957).

d) Mediators of cellular immunity - lymphokines - are present in the synovial fluid or RA patients (Stastny et al, 1973) and in synovial membrane culture supernatants (Steihl et al, 1975). They are evaluated by their ability to cause inhibition of macrophage migration and blastogenesis of mouse spleen cells. The presence of cytotoxin in synovial fluid has also been reported (Peter et al, 1971).

The difficulties of detecting lymphokines in heterogeneous biological fluids have been highlighted by Maini (1975).

However, it has been reported that MIF may also be produced by B-lymphocytes (Yoshida, 1973) in equal if not greater quantities than T cell production (Rocklin, 1974). These observations cast doubt on the validity of lymphokines as markers of T cell activity. Experiments carried out by Bloom and Shevach (1975) on guinea pigs provided evidence that a T cell helper effect was necessary for B cells to produce lymphokines. Bloom and Shevach (1975) concluded that MIF production is a valid qualitative assay of T cell competence but it cannot be regarded as a quantitive measure of T cell function.

A. PUTATIVE ANTIGENS

Assuming that there is an ongoing delayed hypersensitivity response in rheumatoid arthritic joints it is necessary to consider the possible candidates for the role of antigen. They are infectious agents, immunoglobulins, components of joint fluid, components of cartilage, fibrous tissues and synovial tissue.

a) Infectious agents:

The role of an infectious agent in the aetiology of RA has been well received (Bland and Phillips, 1972; Barland, 1973; Denman, 1975). The hypothesis of an infective agent is attractive for the following reasons:

1. Arthritis may accompany many infectious diseases in man; for example rubella and gonorrhea.
2. Many chronic infective diseases, such as subacute bacterial endocarditis, infectious hepatitis, pulmonary tuberculosis, syphilis, leprosy etc. may stimulate the production of rheumatoid factor (RF). This RF differs from that in rheumatoid arthritis in that it reacts with human IgG (as in latex fixation) rather than mammalian IgG (as in sheep cell agglutination test) and its titres are lower.
   It must be born in mind that RF is also produced in cirrhosis of the liver, Waldenstroms macroglobulinaemia, sarcoidosis and idiopathic pulmonary fibrosis.

3. Arthritis in animals can be caused by a variety of infectious agents with close clinical and pathological similarities to RA, for instance pigs infected with Erysipilothrix Insidusa (Hadler, 1976) and these also exhibit serum RF.
4. The disease process exhibits many clinical features suggestive of an infective process especially in children, who present with high fever, marked enlargement of the lymph nodes and spleen, together with high ESR and PMN leucocytosis.
5. Agammaglobulinaemics have low resistance to infection and suffer from a variety of connective tissue disease (Rotstein and Good, 1961). The hypothesis is that the micro-organism sets up an immune response and the ongoing response is directed against the breakdown products.

   (i) Although many organisms have been suspected, or even isolated, no organism has been isolated with any degree of regularity.

   (ii) Transfer of disease into healthy joints by synovial fluid has failed (Levinsky, 1951).

   (iii) There is no increased prevalence of disease in the spouses of sufferers of RA (Dalakos, 1970). Laine et al (1962) showed no increase of prevalence in nursing staff.

(i) Bacterial Infection

Diphtheroid bacilli were isolated from 21 of 78 synovial membranes and from 12 of 126 specimens of synovial fluid obtained from patients with RA (Stewart et al, 1969), while other joint diseases yielded few organisms. However, more stringent techniques greatly reduced the frequency with which diphtheroids were isolated (Claseur and Biersteken, 1969). Maini et al (1970) tested diphtheroid organisms in the LMT and reported negative results.

There have been both positive and negative reports on the isolation of Mycoplasma from RA joints. However the chances of contamination are high (Sabin, 1967) and evidence that established cell lines carry this kind of organism has tended not to support the pathogenic relationships of these organisms to RA.

Having successfully isolated Mycoplasma from rheumatoid joints (Williams, 1968) Mycoplasma fermentans was used as the test antigen in LMT (Williams et al, 1970) and results were obtained indicating that rheumatoid leucocytes were specifically inhibited. IgG adsorbs to Mycoplasmas and it could have been the IgG which was responsible for the specific inhibition of rheumatoid leucocytes in the LMT (Frøland and Gaarder, 1971).

Denman (1970) stressed that the hypervascular synovial membrane with its rich content of macrophages is an obvious nidus for secondary infection and septic arthritis is a recognised complication of the disease even in patients not receiving corticosteroids (Kellgren et al, 1959). It is know that during systemic infection antigen appears in the joints (Zvaifler, 1973).

(ii) Viral Infection

A variety of means have been used to explore the possibility of a virus as the aetiologic agent. Ultrastructural studies of the rheumatoid synovial membrane have revealed no conclusive evidence of a virus (Györkey et al, 1972; Denman, 1976).

Analyses of sera for antibodies to a variety of viruses have been carried out and although there have been reports of increased anti-measles and anti-rubella antibodies others have reported no difference (Denman, 1975). Nor is there any evidence that the antibody is specifically synthesised in the infected joint Stanford, 1974).

Using conventional virological technique or rheumatoid synovial fibroblasts no virus has been isolated (Smith and Hamerman, 1969). However there was increased resistance to infection with rubella

and Newcastle Disease of fowls Virus (NDV)
in the case of rheumatoid cells. This has been attributed to the increased hyaluronic acid content of the rheumatoid cells compared to the non-rheumatoid (Patterson RL, et al, 1975). It is possible that the virus is non-lytic and would not be detected by normal means. Various other means have been tried, all producing negative results (Norval and Marmion, 1976).

No tests for DH have been attempted using viruses as antigens.

(iii) Immunoglobulins in Rheumatoid Arthritis

Rheumatoid factor (RF) is an antibody with specificity for antigenic sites on the autologous IgG heavy chain determinants (common specificities are GM(a), 'nm-a' and Ga) (Natvig,Munthe and Gaarder, 1971).

Approximately 85% of rheumatoid patients' serum contains RF when tested for by classical methods, i.e. Rose-Waaler (Copeman, 1972).

225 complexes have been found in rheumatoid sera (Franklin, 1957), which dissociate to yield one IgM molecule and five IgG molecules. The IgM displays RF activity. It is this RF which is detected by the Rose-Waaler and other in vitro tests and only 60 - 80% of rheumatoid patients contain this rheumatoid factor (Copeman, 1972).

In its reactions with human IgG, IgM RF activates complement through its own complement attachment sites via the classical pathway (Zvaifler and Schur, 1968). Although evidence is still scanty non-rheumatoid RFs appear to be poor complement activators (Bianco et al, 1974).

Some rheumatoid sera on centrifugation yield complexes of intermediate size (9-17S) which are composed entirely of IgG molecules which on dissociation yield rheumatoid factor activity (kunkel et al, 1961). Free IgG RF is often present in rheumatoid sera and the levels are the same in both seronegative and seropositive individuals (Holborow, 1977).

IgG rheumatoid factor is produced in the affected synovial membrane of seronegative as well as seropositive rheumatoid patients. Complement binding in the synovial membranes is identified more closely with IgG than IgM deposits and in the phagocytic synovial lining cells from seronegative patients' joints, complement may be demonstrated in company with IgG alone (Kinsella et al, 1970).

High molecular weight IgG complexes have been found in rheumatoid synovial fluid as well as serum (Winchester et al, 1970) and have also been eluted from synovial membranes (Munthe and Natvig, 1971). Whether from serum or from joint fluid these display the ability to fix complement via the classical pathway by binding C1q (Lambert et al, 1975).

There is some evidence that alternate pathway complement activation takes place in the rheumatoid joint (Gotze et al, 1972). Allotype analysis of the IgG protein content of complexes derived from joints shows that it is of restricted type and hence likely to have been synthesized in the joint rather than to have reached it from extra-articular sources. Pepsin digestion of rheumatoid synovial membrane sections shows that the Fab fragments of the immunoglobulin product of many of the IgG plasma cells present in both seropositive and seronegative patients possess rheumatoid factor-like anti-IgG activity, but this is blocked <u>in vivo</u> through complex formation with adjacent Fc portions of other intracellular IgG molecules which presumably have identical combining specificities, (Munthe and Natvig, 1971). Thus the solely IgG intermediate complexes in rheumatoid serum and joint fluids appear to take origin as unique self-associating IgG RF molecules (Pope et al, 1974) synthesized in the rheumatoid synovial membrane plasma cells.

There is well documented evidence of an autoimmunity of the humoral type which occurs in RA. It is also possible that the complexes formed can to a great extent be responsible for the damage to the rheumatoid joint. For reasons which have been discussed before (see Sect. V) humoral autoimmunity does not provide a complete explanation for the pathogenesis of rheumatoid arthritis.

There have been attempts to show the presence of a delayed hypersensitivity reaction to immunoglobulins, especially IgG in rheumatoid patients. These have been summarised in the introduction to this thesis (see Section VII).

Chamberlain et al (1970) elicited an Arthus reaction in 6/11 RAs and only 1/15 controls to intradermal injection of heat aggregated IgG. No delayed hypersensitivity reaction was observed. Runge and Mills (1971) observed no reaction to autologous native and aggregated autologous IgG in nine RAs and three controls.

Attempts to induce lymphocyte transformation of RA lymphocytes using native and aggregated IgG has been unsuccessful in some hands (Kăcaki et al, 1969; Runge and Mills, 1971) but successfully carried out by others using autologous IgG and IgM (Dorner et al, 1969; 1974) and using aggregated IgG in autoserum (Kinsella, 1974).

There have been numerous reports of specific inhibition of RA leucocytes in the LMT using native IgG (Massoud et al, 1976), autologous native IgG in agarose test (Weisbart et al, 1975) using native and aggregated IgG (Frøland and Gaarder, 1971; 1973) and aggregated IgG (Brostoff et al, 1973). Eibl and Sitko (1975) induced inhibition of human monocyte migration by aggregated homologous and autologous IgG.

(iv) Joint Fluid

Joint fluid has been used as a source of antigen in tests for delayed hypersensitivity mainly due to the assumption that irrespective of the initial location of the antigen it would eventually be found in the synovial fluid.

The use of synovial fluid in skin tests elicited negative results (Landsbury, 1955). Subsequent investigations were able to detect an *in vitro* response of buffy coat lymphocytes to synovial fluid

from patients with RA (Kinsella, 1970). Autologous synovial fluid induced blast formation in 7 out of 10 rheumatoids and 2 out of 10 osteoarthritics (Okamoto et al, 1973). Rheumatoid lymphocytes underwent blast transformation in response to rheumatoid and control synovial fluids more frequently than control lymphocytes (Lycette, 1965).

Kinsella (1976) showed that autologous rheumatoid synovial fluid was an effective stimulant of rheumatoid lymphocytes, whereas autologous non-rheumatoid fluids were not effective. Allogeneic rheumatoid fluid was as effective as autologous against rheumatoid lymphocytes and more effective against non-rheumatoid lymphocytes than non-rheumatoid fluid. Non-rheumatoid fluid has very little effect.

Synovial fluid lymphocytes and synovial fluid from most patients with RA induced blastogenesis of autologous peripheral blood lymphocytes.

It could be that there are lymphokines in the rheumatoid fluid inducing these transformations (Stastny et al, 1973; 1975).

There are plentiful antibody-antigen complexes in rheumatoid synovial fluid (Zvaifler, 1973) and these can stimulate even non-immunized lymphocytes to transform (Block-Shtader, Hirschhorn and Uhr, 1968).

It is possible for RF-IgG complexes to induce transformation (Dorner, 1969; 1974; Kinsella, 1974).

Ciobanu (1975) reported the ability of fibrin to cause increased ^{3}H thymidine incorporation in rheumatoid cell cultures when compared with controls.

Fibrin has been isolated from rheumatoid synovial fluid and it has been shown to inhibit leucocytes in the LMT (Hall, 1974).

(v) Cartilage Components

Patients with RA have a much higher incidence of antibodies against denatured and native human collagen in their sera than control subjects (Michaeli et al, 1971; Steffen, 1972; Menzel et al, 1976). Anti-collagen antibodies have also been detected in the synovial fluid (Cracchiolo et al, 1972). Collagen and IgG have been detected in identical places in the RA inclusions of synovial fluid using double-staining immunofluorescence (Steffen, Ludwig and Knapp, 1974). Some of the antibody at least is produced in the rheumatoid synovial membrane (Mestecky and Miller, 1975).

Herman et al (1973) set up cultures of RA and normal leucocyte to various cartilage antigenic components, i.e. crude protein polysaccharide complex (PPC) and protein polysaccharide light fraction and reported that the rheumatoids were stimulated more than the controls.

Statistically significant specific inhibition of migration of rheumatoid leucocytes by collagen when compared with the control leucocytes was reported (Bernacka, 1972).

(vi) Synovial Tissue Antigens

It is the synovium which is infiltrated with the inflammatory cells and the synovial cells which appear abnormal in culture (Norval, 1976). Therefore perhaps there is an immune reaction against the synovial cells.

The aetiology of RA could be attributed to a viral agent, which would account for the differences in cultures of rheumatoid synovial fibroblasts (Ishimoto, Temin and Strominger, 1966).

It had been shown that rheumatoid synovial fluid can cause lymphocyte transformation (Lycette, 1969; Okamoto, 1973; Kinsella, 1970, 1976) and it was conceivable that these stimulatory substances had arisen from the synovium.

Landsbury et al (1955) used test material from synovial fluid, synovial tissue and rheumatoid nodule. It was filtered and cultured to test sterility. This was injected subcutaneously in 20 RAs and 20 controls and induced no skin reaction. Multz et al (1968) injected homogenized synovial tissue and standard antigens S/C into 16 RA and 17 non-RA patients. RAs were less reactive to the standard antigens: mumps and PPD, and to homologous synovial tissue. 4 out of 9 RAs gave maximal responses to autologous synovial tissue. All 5 non-RAs injected with autologous tissue gave minimal or weaker responses. Braunsteiner (1961) found greater reactivity to synovial homogenates in rheumatoids.

Rothenberger and Thiele (1970) first reported that using the LMT as an in vitro correlate of DH they were able to induce specific inhibition of migration of rheumatoid leucocytes when compared with control leucocytes using a crude RA synovial homogenate, but not with non-rheumatoid homogenates (Rothenberger and Thiele, 1971a). Using a similar homogenate in the LTT they were unable to elicit positive results. This they attributed to the high incorporation of tritiated thymidine in the control cultures (Rothenberger and Thiele, 1971b).

In 1973 Bacon et al reported specific inhibition of rheumatoid leucocytes when compared with healthy, gouty or osteoarthritic controls in the LMT using a rheumatoid synovial homogenate. The inhibitory material was not obtained from a psoriatic or osteoarthritic synovium.

Since then there have been two conflicting reports of use of synovial homogenates as antigen in the LMT.

One reported no difference in the migration of RA and age and sex matched controls using rheumatoid synovial homogenate as antigen (Panayi, 1975).

A second (Thonar and Sweet, 1976) reported a specific inhibition of rheumatoid leucocytes when compared with control leucocytes using normal synovial homogenate as the antigen.

SECTION VIII - INTRINSIC IMMUNOLOGICAL DEFECT

Many experiments have been carried out in an attempt to determine whether rheumatoid arthritic patients have any defect in their ability to mount delayed hypersensitivity reactions. These experiments have included *in vivo* testing of delayed hypersensitivity by intradermal injection of standard antigens known to elicit a response in a large percentage of a control population. It has been reported that rheumatoids have depressed skin reactivity to common antigens, i.e. PPD, streptokinase-streptodornase, candida albicans, mumps, trychophytin (Waxman et al, 1973; Andrianakos et al, 1977). Waxman et al (1973) correlated this anergy to duration of disease, while Adrianakos et al (1977) reported that the degree of anergy was related to age but not to duration of disease. Normal skin responses (Azoury et al, 1967; Muller, 1971) and increased responses (Hiemeyer, 1971) have also been reported.

The *in vitro* tests have been either mitogenic stimulation or mixed lymphocyte cultures as tests of lymphocyte function.

Most reports of mitogenic stimulation (Giswold and McIntosh, 1973; Waxman et al, 1973; Laine and Knight, 1974; Sturrock, 1975; Lockshin et al, 1975; Silverman et al, 1976; Rawson and Huang, 1974a) have noted a decreased response of RA cells to PHA stimulation. However the responses are not statistically significantly lower than control responses (Waxman et al, 1973; Lockshin et al, 1975; Sturrock, 1975). Silverman et al (1976) used more than one concentration of the mitogen, established a dose response curve and then compared the areas under the curve of RA patients with either normal controls or controls with chronic inflammatory diseases e.g. tuberculosis. They reported two groups of rheumatoid patient: those exhibiting normal responses and those with consistently and statistically significantly low responses who had severe erosive disease. Rawson and Huang (1974b) showed that

RA lymphocytes did not bind PHA as well as control lymphocytes. Crout et al (1976) showed that decreased responses to PHA were obtained after incubation of peripheral blood lymphocytes in synovial fluid.

Responses to Concanavalin A (Con A) - another T cell mitogen - have not been so frequently documented and again there is divergence of results. Sturrock et al (1975) reported no difference in the response of rheumatoid and normal cells while Lockshin et al (1975) and Silverman et al (1976) reported a decrease in the response of rheumatoid peripheral blood cells. Rawson and Huang (1974b) reported no difference in the ability of RA and control cells to bind Con A.

Pokeweed mitogen - a B cell mitogen - induced a reduced response in rheumatoid cells which was not statistically significant (Lockshin et al, 1975) while Silverman et al (1976) and Sturrock et al (1975) found no difference.

On the whole there is a tendency for rheumatoid cells to respond less well to mitogenic stimulation, especially T cell. However it is extremely difficult to show that this decrease in response is statistically significant.

Rheumatoid cells have also been cultured in the one-way mixed lymphocyte culture (MLC) which is used as a means of assessing thymus derived cell function (Roitt, 1977).

Studies have shown normal responses of rheumatoid cells stimulated by normal allogeneic cells (Astorga and Williams, 1969; Hedberg et al, 1971; Rawson and Huang, 1974; Metzger et al, 1974; Caperton et al, 1975). Although rheumatoid cells from unrelated donors mixed together showed decreased responses (Astorga and Williams, 1969) Williams et al (1973) found that pre-incubation of normal cells with certain rheumatoid and SLE γ-globulin fractions decreased their reactivity in the MLC. Using pooled donor

stimulating cells it was found that reactivity of rheumatoid cells was stimulated when the cultures were carried out in pooled human sera and depressed when compared with normals if the cultures were carried out in autologous sera (Keystone et al, 1976). Keystone et al (1976) suggest that experimental details could account for differences in results, e.g. percentage of lymphocytes in stimulatory cells (Astorga and Williams, 1969), biased recovery of different populations of mononuclear cells, limited antigen pool, ratio of stimulatory cells to responding cells.

SECTION IX - TESTS OF CELL MEDIATED IMMUNITY (CMI)

A. IN VIVO

In human patients the only test for CMI which has been employed with any regularity is skin testing. This suffers from the disadvantage that contaminants which are able to induce Arthus and Schwartzmann reactions of any severity are capable of giving false positives. In man, when the DH is tested to PPD the injection site is studied at 48 hours.

B. IN VITRO

The initial contact between antigen and sensitized T cells leading to the production of the various mediators of cellular immunity is tested by the actions of that mediator on indicator cells. The tests are named according to the particular lymphokine action they are demonstrating, i.e. migration inhibition test (to test for MIF), lymphocyte transformation test, macrophage migration inhibition test (to test for MIF) and cytoxicity tests (to test for cytotoxin) (Pick and Turk, 1972). Thus disparity arises between an in vitro test and the physiological skin test which is dependent not only on the initial contact of cell and antigen, releasing lymphokines, but also on the production of the mediators of inflammation leading to a visible response.

Two tests which have been used with most regularity are the leucocyte migration test and the lymphocyte transformation test:

(i) Leucocyte Migration Test (LMT)

The observation that tuberculin in tissue culture media inhibited the migration of cells taken from either the spleen or lymph node explants of tuberculous rabbits or guinea pigs presented the first description of an in vitro model for studying delayed type hypersensitivity in animals (Rich and Lewis, 1932).

By demonstrating the passive transfer of contact hypersensitivity to normal guinea pigs from hypersensitized animals by means of

lymphoid cells and not serum, the cellular basis for delayed-type hypersensitivity was established (Landsteiner and Chase, 1942). This finding intensified the search for an improved *in vitro* correlate of delayed hypersensitivity, which culminated in the introduction of the capillary tube migration assay of George and Vaughan (1962).

Peritoneal macrophages from tuberculin sensitive guinea pigs were transferred into capillary tubes which were then sealed at one end and placed in tissue culture chambers, allowing the cells to actively migrate across the chamber floor in a fan-like fashion. Delayed hypersensitivity was expressed as a migration index; this was the ratio of the areas, as measured with a planimeter, of the cells' migration in the presence and absence of specific antigen (Tuberculin PPD).

Later it became apparent that the peritoneal exudate cells used in these experiments were a heterogeneous cell population consisting predominantly of macrophages plus a small but significant number of lymphocytes. Studies to assess the contribution of the two cell types demonstrated that the inhibition of cellular migration was due to the liberation of a protein, termed Migration Inhibition Factor (MIF), from sensitized lymphocytes on contact with a specific antigen (Bloom and Bennett, 1966; David, 1966).

The original method of George and Vaughan (1962) was later modified into a two-step assay, and demonstrated delayed hypersensitivity of human lymph node cells to microbal antigens (Thor, 1968). The lymphocytes in question were incubated with specific antigen for 72 hours before the MIF containing supernatant was tested on normal guinea pig macrophage migrations.

A direct migration inhibition of human peripheral blood leucocyte that correlated with the presence of delayed hypersensitivity to brucella antigens *in vivo* was introduced by Bendixen and Sϕborg

(1969). An improvement in method sensitivity was presented by Federlin et al (1971) who correlated migration inhibition with positive Mantoux skin reactions in the detection of tuberculin hypersensitivity.

It had been shown that MIF reduced or inhibited the migration of macrophages and monocytes (Rosenberg and David, 1970) and so in the human situation it seemed necessary to remove polymorphs during leucocyte preparation (Falk, 1971). However, Rocklin (1974) showed that human activated lymphocytes produced a lymphokine which he called leucocyte inhibitory factor (LIF) which had no effect on guinea pig macrophages or human monocytes, but utilizes human PMN cells as indicator cells. He reports that the receptor molecules on monocytes and PMNs are different for MIF and LIF (Rocklin, 1976).

David (1971) states that it is likely that the in vitro system is measuring only some of the initial steps that occur in in vivo reactions of cellular hypersensitivity. It does not take into account the possible release of lysosomal enzymes from macrophages and the subsequent reactions, nor does it assess the effects of other mediators, blood clotting factor or local tissue conditions (i.e. conditions of blood vessels).

When attempting to correlate the leucocyte migration test with delayed hypersensitivity in vivo it must be remembered that much of the earlier work, and the majority of the more definitive work, has been carried out on guinea pig macrophage migration inhibition. However since Rocklin (1974) showed the production of LIF by previously sensitized human lymphocytes in the presence of the specific Ag. the macrophage inhibition test and the leucocyte migration test parallel each other, i.e. the production of lymphokines by sensitized lymphocytes, which act on their respective indicator cells.

(ii) Correlation of In Vivo and In Vitro Observations

a) Animals must be sensitized either by protein antigens in Freund's Complete Adjuvant or by living BCG. Human subjects are sensitized by natural infection. Peritoneal cells from sensitized animals are inhibited by antigen from migrating in vitro and lymphocytes from such animals or from sensitive human subjects produce migration inhibitory factor (MIF) when stimulted by antigen in vitro.

b) Guinea pigs sensitized with antigen-antibody complexes in Freund's Complete Adjuvant by the method of Uhr, Salvin and Pappenheimer (1957) demonstrate delayed hypersensitivity without detectable antibody. Peritoneal exudate cells from such animals are also inhibited from migrating by specific antigen.

c) Guinea pigs immunized by the I.V. or I.M. route without Freund's Complete Adjuvant, which produce certain antibodies but do not exhibit delayed hypersensitivity, yield peritoneal exudate cells which are not inhibited by antigens. In addition lymphocytes from such animals produce no MIF.

d) The specificity of inhibition of migration has been studied with hapten-protein conjugates and chemically defined hapten-oligopeptides (David and Schlosman, 1968). The carrier specificity found correlates with that of DH reactions in vivo and does not exhibit the specificity of the reaction between antigen and humoral antibody.

e) Peritoneal exudate cells are not inhibited from migrating by specific antigen when the donor animals have been made partially tolerant, i.e. they have diminished or absent DH but are still producing antibody.

However numerous investigators have failed when using human peripheral leucocyte as the indicator cells to obtain adequate reproducibility of results (Thor, 1968; Lockshin, 1969; Kaltreider et al, 1969; Alzer et al, 1973). It is probable that disparity of results, difficulty in interpretation and experimental failure could largely be due to lack of precision and experimental design (Maini, 1973).

(iii) Lymphocyte Transformation Test

Leucocytes suspended in serum and a simple culture medium having been left at 37°C in an airtight container with the addition of a small quantity of an extract of red kidney bean, Phaseolus Vulgaris, (PHA) underwent marked changes in morphology. Large active cells appeared with large nuclei and prominent nucleoli. On the third or fourth day of culture mitotic cells were seen (Hungerford et al, 1959; Nowell, 1960). These large active cells are variously referred to in the literature as blast cells, transformed cells, stimulated cells or activated cells. Carstairs (1961, 1962) was the first to show clearly that the small lymphocyte was the source of the dividing cell.

Pearman et al (1963) reported that lymphocytes from tuberculin positive individuals cultured in the presence of PPD produced mitoses whereas lymphocytes from tuberculin negative individuals cultured under the same conditions did not.

Schrek et al (1963) noted that antigen cultures needed 5 days to show blast formation compared with only 3 days for PHA cultures.

It is generally accepted that the transformation and proliferation of lymphocytes when cultured with antigen in vitro parallels that situation in vivo in which antigen stimulated sensitive cells (Pearman, 1963; Valentine, 1971).

Lymphocytes have been stimulated by a wide variety of antigens, including viruses, bacteria, fungi, protozoa and pollen, as well as purified animal proteins, hapten protein conjugate, synthetic amino acids, polymers and drugs (Valentine, 1971).

It was reported (Kasukura and Lowenstein, 1965; Dumonde, Howson and Wolstencroft, 1968) that supernatants from antigen stimulated, previously sensitized lymphocytes are able to cause an increased DNA synthesis in a secondary lymphocyte population. In some cases this was found to be antigen dependent (Valentine and Lawrence, 1969). It was then reported that unstimulated lymphocytes also

release a mitogenic factor *in vitro* (Kasukura, 1970).

However, as stated by Pick and Turk (1972) the secretion of mediators by lymphocytes incubated with mitogen is a phenomenon preceding transformation and is independent of DNA synthesis. Work has been carried out using MIF as the lymphokine because its presence can easily be detected. It has been shown that MIF production is dissociated from lymphocyte transformation and the two phenomena may be induced by functionally separate lymphocyte populations (Chaparas et al, 1971; Bloom et al, 1972; Rocklin, 1973). Bennett and Bloom (1967) suggested separate pathways for the induction of MIF and lymphocyte transformation.

(iv) Correlation of In Vitro and In Vivo Observations

Much effort has been expended in correlating this *in vitro* observation with delayed hypersensitivity *in vivo*. The work has been carried out both on humans and experimental animals.

Leucocytes from approximately 60% of BCG-vaccinated or tuberculous donors were found to respond to tuberculoprotein (Rauch, 1967). However the response correlated well with the passive haemagglutinin test using tuberculoprotein-coated cells and with tuberculin skin tests. Several other authors (Gump et al, 1967) have correlated blastogenesis with tuberculin skin test. The disparity between various workers' results may in part be explained by results of work which show that the degree of transformation is dependent upon the nature of the Mycobacterial antigen used and the source of serum (Heilman, 1966). Failure to obtain tuberculin-induced mutosis with the lymphocytes of patients with active tuberculosis (Pearman et al, 1963) was due to the inhibitory effect of tuberculous serum.

Rabbits received a four week course of intravenous injections of aluminium-precipitated proteins, and four weeks later they were killed and their cells exposed to the antigen *in vitro*. Although a slight non-specific stimulation was often found, this did not obscure the strong response of cells from the immunized animal to

the immunising antigens. A straight line relationship between the rate of DNA synthesis of cultured cells and the log of the antigen concentration was observed (Dutton and Eady, 1964; Dutton and Bulman, 1964).

Oppenheim et al (1967) produced DH in guinea pigs in the absence of detectable antibody, to PPD and guinea pig albumin-orthanilic acid conjugates. Ten days later peripheral cells and cells from lymph nodes, spleen and thymus cultured with stimulated antigen. All cultures containing cells from peripheral lymph nodes and about 40% of the spleen and peripheral leucocyte cultures contained increased numbers of cells in DNA synthesis and increased total DNA synthesis in response to PPD or albumin-orthanilic acid conjugates.

In animals the main evidence for in vitro lymphocyte transformation being a correlate of in vivo DH stems from the carrier specificity of the transformation. Lymphoid cells from animals immunized with hapten-protein conjugates I.V. or in complete Freund's Adjuvant will transform in vitro only when cultured with a hapten-protein conjugate used for sensitization (Dutton and Bulman, 1964; Mills, 1966; Oppenheim et al, 1967).

Carrier specificity of this sort is also required for the elucidation of the DH skin test and the initiation of the secondary antibody response. These two phenomena, like the stimulation of lymphocytes in vitro, require the interaction of antigen with antigen-sensitive cells (Bloom, 1971).

Further evidence is the report of experiments with DNP-poly-L-lysine which have demonstrated that a lysine chain length of seven or more is required for immunogenicity, the elicitation of DH skin response and the stimulation of lymphocytes in vitro (Schlossman et al, 1966; Stulberg and Schlossman, 1968).

In human subjects we rely on the occurance of congenital abnormalities of the immune system. Cooperband (1966) reported

that the quantity of DNA and RNA synthesised by the lymphocytes of hypogammaglobulinaemic patients stimulated with PHA was not significantly different from that of lymphocytes of normal individuals, i.e. they have T cells capable of responding to T cell stimulants. This work was repeated and further extended to include response to S.K., streptolysin O and various other antigens (Bradley and Oppenheim, 1967). Fudenberg (1964) reported normal responses to PHA and streptolysines but hypo-responses to typhoid antigen, diphtheria toxoid and tetanus toxoid. Conversely a child with complete failure of the thymus (Lischner et al, 1967) retained primary and secondary skin homographs, did not show DH responses, and although immunoglobulin levels were normal did not always produce immunoglobulin. The lymphocytes responded poorly to PHA, leucocyte antibodies and other stimulants. The tissue origin of the peripheral lymphocytes was not established, but it supports the idea that the responsive cells in the peripheral blood are thymic in origin.

Work on disease states is equivocal. Patients with Hodgkin's disease who were anergic as revealed by impaired skin DH reactions were correlated with diminished levels of transformation after stimulation of the peripheral lymphocytes of these patients (Hirsch and Oppenheim, 1965; Eisenberg, 1965).

Alternatively work on sarcoid patients with positive kviem skin test were found not to transform when exposed to the antigen *in vitro* (Cowling et al, 1964).

The lymphocyte transformation test is widely accepted as an *in vitro* correlate of *in vivo* delayed hypersensitivity (Bloom et al, 1973), although it can only be said with certainty that the transformation and proliferation of lymphocytes when cultured with antigen *in vitro* parallels those situations *in vivo* in which antigen stimulates sensitive cells (Valentine, 1971).

SECTION X - DEFINITION OF PROJECT

The *in vivo* delayed hypersensitivity reaction can be paralleled *in vitro* by the leucocyte migration test and the lymphocyte transformation tests. These tests will be used to test for the presence of a synovial antigen in crude synovial homogenate and any purified solutions that are prepared.

CHAPTER 2

EXPERIMENTAL

CHAPTER 2

EXPERIMENTAL

SECTION I - MATERIALS

All chemicals and reagents were of analytical grade whenever available and, unless otherwise stated, came from normal commercial sources such as British Drug Houses Ltd., Poole, Dorset.

Radioactive ^{3}H thymidine was obtained from the Radiochemical Centre, Amersham, Bucks.
All tissue culture media were obtained from Wellcome Reagents Ltd., Beckenham, Kent, or Flow Laboratories, Irvine, Scotland.
Anti-human IgG, anti-normal human serum, Test Streptokinase were obtained from Hoechst UK Ltd., Hounslow, Middx.
Sephadex G-100, G-200, Sepharose 6B, DEAE-Sephadex A-50, Ultrogel ACA_{22} from Pharmacia, Uppsala, Sweden.
Cholic acid, TRIS (Hydroxymethyl) amino ethane Buffer, p-octyl phenoxy-polyoxyethylene (TRITON x 100) from Sigma Chemical Co., Kingston Upon Thames, Surrey.
Heparin (5000 i.u./ml) from Evans Medical Ltd., Liverpool.
Diaflo PM10 and XM50 membrane filters from Amicon Corporation, Lexington, Mass.
Spherical macroreticular styrene-divinyl-benzene copolymer (Biobeads SM-2) from BioRad Laboratories, Kent.
5, 2-diphenyloxazole (PPO), 1, 4 Di (2-(4-methyl 5 phenyloxazolyl)) benzene (dimethyl POPOP) from Koch Light Laboratories Ltd., Colnbrook, Bucks.
Chlorhexidine gluconate from ICI Ltd., Macclesfield, Cheshire.
Streptokinase-Streptodornase (Varidase) from Lederle Laboratories, Cyanamid, Gosport, Hants.

ILAX 1020 homogenizer from Scientific Instrument Centre Ltd., London W.C.1.

SECTION II - LEUCOCYTE MIGRATION TEST

A. MATERIALS

All tissue culture supplements used in the migration studies were obtained from Wellcome Reagents Ltd., Beckenham, Kent, and Flow Laboratories, Irvine, Scotland.

Capillary tubes of dimension 0.4 mm internal diameter and length 75 mm were supplied by Drummond Scientific Company, U.S.A.

The migration plates (Sterilin), each containing 12 chambers, were obtained from Arnold R. Horwell, Ltd., Kilburn, London.

The tissue culture medium used in the migration studies was prepared in 100 ml batches by asceptically adding the following supplements in the stated volumes:

10 mls Eagles Medium (MEM)
12.5 mls Horse Serum
2 mls Hepes Buffer (one M)
2 mls Penicillen-Streptomycin (5000 units/ml)
73.5 mls Sterile Water

The addition of 20 mM Hepes (4-(2 hydroxyethyl)-1-piperazine ethane sulphuric acids) yielded a physiological pH of 7.35 at 37°C. All media was filtered (0.45 μm millipore) into sterile glass bottles and stored at 4°C until used.

The Hank's Physiological Salt Solution was diluted from a 10x concentrate to single strength by the addition of sterile water, and adjusted to a final pH of 7.35 using 4.4% $NaHCO_3$.

B. METHOD

The method outlined below was used for all studies in this thesis. Basically the method of Søberg and Bendixen (1969), it was modified as a result of experiments by Brown (1976) carried out in our laboratory.

Having drawn 20 mls blood from a cubital vein into a plastic syringe it was expelled into two 10 ml sterile tubes, each containing 250 units of heparin. To ensure complete mixing of the blood and anti-coagulants the tubes were gently shaken for 2 minutes; the two tubes were then spun at 800g for 10 minutes. The plasma and leucocyte layers, which had settled on top of the erythrocytes, were removed using a No.E10 cannula and 20 ml syringe. After removal of cannula the syringe was inverted and stood vertically in a 37°C incubator to allow spontaneous sedimentation of the remaining erythrocytes.

After 1 hour incubation at 37°C the leucocyte-rich plasma was gently aspirated through a hypodermic needle which was bent at an angle of 45° to the upright syringe into a conical plastic tube. This was centrifuged at 200g for 10 minutes at room temperature. The resulting plasma supernatant was discarded and the leucocyte pellet re-suspended in an excess of Hank's Balanced Salt Solution. Re-suspension was effected by repeated withdrawals into a clean syringe through a No.E18 cannula (Horwell). The spinning at 200g and the re-suspension was repeated and the cell suspension spun for a third time. This final cell pellet, suspended in a small volume of Hank's Solution, was adjusted to a concentration of 4×10^7 cells/ml. Thorough mixing was ensured by whirlimixing and repeated passages through a cannula into a syringe.

Each capillary tube was filled to its maximum capacity (8.5 µl) with the cell suspension and one end immediately sealed with "seal ease" (Clay Adams; Horwells). After the preparation of every 10 tubes the cell suspension in the plastic tube was thoroughly mixed using the cannula and syringe, thus maintaining an adequate distribution of the heterogeneous cell population. At least 30 capillary tubes were prepared from every 20 ml sample, the absolute number depending upon the original cell concentration of the donor's blood. All the leucocyte packed capillary tubes

were placed in a centrifuge tube containing a cotton wool plug and centrifuged at 200g for five minutes. Prior to the completion of this final centrifugation the upper edge of every migration chamber was covered with silicone grease and filled with an excess of Eagles tissue culture medium with or without the test antigen. In every migration plate (12 chambers) used, a control series of migrations were set up.

After centrifugation each capillary was cut at the cell-fluid interface and the leucocyte pellet was immediately anchored by a small amount of silicone grease horizontally onto the floor of a chamber with its sealed end tightly adjacent to the inner wall and its open end extending towards the centre of the chamber. This was repeated for every capillary tube. Chambers were rendered air-tight by applying cover slips onto the previously greased edges. Extreme caution was taken to prevent the introduction of any air bubbles. The migration plates were carefully placed on a horizontal (adjusted using a spirit level) incubator shelf and left for 21 hours at $37^{\circ}C$.

At the end of this incubation period the cells were seen to have moved out from the tube and migrated along the floor of the chamber in a fan-like fashion. Each migration was projected onto paper by a photographic enlarger, the image drawn and its area measured using a planimeter.

C. READING OF MIGRATION AREAS

The human leucocytes used for migration analysis in this study consisted of a heterogeneous population. After 21 hours of incubation two migration areas are discernible: a peripheral monolayer, which has previously been demonstrated to consist predominantly of polymorphonuclear cells (PMNs) and an inner area composed of mononuclear and PMN cells in approximately equal proportions (Rosenberg and David, 1970; Egeberg, Bendixen and Nerup, 1972; Nordquist and Rorsman, 1972).

Migration areas in this thesis are defined from the outer zone which was reported by Jokipii and Jokipii (1974) to be more sensitive and less variable in the detection of inhibition. All migration areas were measured by planimetry and expressed in square centimetres.

The control area of migration was the mean of the areas of migration of leucocytes in media containing no added antigen. Any deviation in the size of this area in test migrations was expressed either as a percentage stimulation or inhibition of control. A students' T-test was used to analyse any significant difference between control and test migrations.

D. CELL VIABILITY

Cell viability was measured using the trypan blue dye exclusion test (Boyse, Old and Caroulinta, 1972). 0.16% trypan blue in isotonic saline solution was incubated with an equal volume of the cell suspension for 10 minutes at 37°C. The cells were examined microscopically within 15 minutes of contact with trypan blue solution. Viable cells excluded the dye.

E. PREPARATION OF APPARATUS

Siliconisation of glassware did not modify the quality of migrations in any way (Brown, 1976).

All glass and plastic apparatus was washed in Rapidex (Arnold Horwell), followed by several rinses in boiling tap water and distilled water.

SECTION III - LYMPHOCYTE TRANSFORMATION TEST

A. SEPARATION OF LYMPHOCYTES FROM PERIPHERAL BLOOD

(i) Materials

Ficoll : Pharmacia Fine Chemicals, Uppsala, Sweden.

Triosil : Nyegaard and Co., Oslo, Norway.

2 mls of Triosil were diluted with 2.4 mls of water and mixed well and added to 10 mls of 9% Ficoll. The Ficoll was made up and stored at -20°C in 10 ml aliquots ready for use.

Ficoll-paque™ : Pharmacia Fine Chemicals, Uppsala, Sweden. This was used in some later experiments instead of Ficoll-Triosil.

R.P.M.I. 1640 : Wellcome Reagents, Beckenham, Kent.

(ii) Method

(Based on Boyums method of lymphocyte separation, 1968). 30 mls of blood were withdrawn from a cubital vein and expelled into three 10 ml sterile tubes each containing 250 units of heparin. To ensure complete mixing of the blood and anticoagulant, the tubes were gently shaken for 2 minutes. The tubes were spun at 200g for 10 minutes. The plasma and leucocyte layers, which settled on top of the erythrocytes, were removed using a No.E10 cannula and 20 ml syringe and aspirated into a 50 ml measuring cylinder and diluted 1:1 with R.P.M.I. 1640. 3 mls aliquots of Ficoll-Triosil or Ficoll-paque™ were placed in conical test tubes and 4 mls of the diluted cell suspension were layered onto this gradient. After spinning at 400g, at 18-20°C for 30 minutes a white band of lymphocytes was visible at the plasma-Ficoll interface. Taking great care to avoid contamination with the Ficoll-paque the lymphocyte-rich layer was removed and washed twice with R.P.M.I. 1640 media, spinning at 100g to pellet the cells.

B. PREPARATION OF LYMPHOCYTE CULTURES

(i) Materials

All tissue culture supplements used in the migration studies were obtained from Wellcome Reagents Ltd., Beckenham, Kent, and Flow Laboratories, Irvine, Scotland.

Microtitre plates (Sterilin), each containing 96 chambers of 0.25 ml volume obtained from Arnold Horwell Ltd., Kilburn, London.

Oxford Sampler micropipetting system and plastic tips - Oxford Laboratories International Corporation, Ireland.

Tritiated Thymidine obtained from the Radio-chemical Centre, Amersham.

P.H.A. (Reagent grade dried) from Wellcome Reagents, Beckenham, Kent.

CULTURE MEDIUM

10 x concentrate medium 199	: 10 mls
Newborn Calf Serum	: 10 mls
Penicillin-streptomycin (5000 units/ml)	: 2 mls
Gentamycin	: 20 µl of 50 mg/ml solution
4.4% $NaHCO_3$ solution	: 4 mls
Sterile Water	: to 100 mls

SCINTILLATION MEDIUM

PPO (2.5 diphenyl oxazole)	: 12.5 g
POPOP (14 Di (2-(4-methyl 5 phenyloxazolyl)) Benzene)	: 0.75 g
Toluene	: 2.5 litres
Glass fibre filter papers GF/C from Ferris & Co. Ltd., Bristol	: Whatman 2.5 cms.

(ii) Method

Having isolated and washed the lymphocytes, they were then re-suspended in Medium 199 containing 10% Newborn Calf Serum, and adjusted to a concentration of 1 x 10^6 lymphocytes/ml. 200 µl of the cell suspension were pipetted into each culture well. The necessary concentration of antigen or mitogen was added in a volume of 10 µl. The control cultures received 10 µl of culture medium. The microtitre plate was covered with a lid of sterilized aluminium foil and incubated in a humidified atmosphere (5% CO_2, 95% air) at

37 °C for 72 hours (mitogen stimulations) or 120 hours (antigen stimulations). Six hours before harvesting [methyl ^{3}H] thymidine specific activity 5.0 curies/mmol was added at a concentration of 0.5/μCi per culture and mixed thoroughly.

The cells were harvested into test tubes and macro-molecules precipitated by the addition of 2 mls ice-cold 10% trichloroacetic acid (TCA). After standing for not less than 30 minutes at 4°C the nucleic acid incorporating the radio-labelled thymidine was filtered onto a glass fibre filter which had first been washed with a mixture of 3 parts ether to 1 part ethanol, and then ether alone. The filter papers were dried at 37°C for 60 minutes and placed in 2" x ½" glass vials, covered with 2.5 mls of the scintillant toluene PPO POPOP. The concentration of tritiated thymidine was counted in a Phillips liquid scintillation analyser.

C. CALCULATING RESULTS

(i) Tritiated Thymidine Incorporation

The antigen or mitogen concentrations were tested in triplicate. The readout was expressed as disintegrations per minute (d.p.m.) and used as a measure of DNA synthesis and ultimately used to quantify cell division. The control value was the mean of the d.p.m. of three lymphocyte cultures not subjected to any stimulation by antigen or mitogen. Any deviation in the d.p.m. of the test cultures was expressed as a percentage of the control.

(ii) Microscopic Examination of Cell Cultures

Each person's lymphocytes were tested in triplicate against each antigen or mitogen concentration and the transformation occuring was measured by the amount of tritiated thymidine incorporated. A fourth culture was set up at each mitogen or antigen concentration and this was examined microscopically at the end of the culture period.

The cell suspension was dropped onto the slide in overlapping circles (Waithe and Hirschhorn, 1973)

It was dried and fixed with methanol.

It was stained with Wrights Stain (B.D.H., Poole, England) for 1 minute. Then diluted 1:1 with buffer pH 6.8 (B.D.H., Poole, England) for 8 minutes. Having been washed thoroughly with pH 6.8 buffer, the slide was allowed to dry.

The cells were examined microscopically and divided into groups on the basis of size: 7.5 um or less, 7.5 to 10.0 um, 10 um to 12.5 um, and greater than 12.5 um.

D. CELL VIABILITY

Cell viability was measured using the trypan blue dye exclusion test (SECTION II, D).

E. PREPARATION OF MATERIALS

All laboratory ware was prepared as previously described (SECTION II, E). It was sterilized by autoclaving at 15 psi, 121^{o}C for 15 minutes.

CHAPTER 3

THE EFFECT OF STREPTOKINASE ON THE MIGRATION OF CONTROL AND RHEUMATOID LEUCOCYTES FROM PATIENTS RECEIVING VARIOUS DRUG REGIMES IN THE LEUCOCYTE MIGRATION TEST.

CHAPTER 3

THE EFFECT OF STREPTOKINASE ON THE MIGRATION OF CONTROL AND RHEUMATOID LEUCOCYTES FROM PATIENTS RECEIVING VARIOUS DRUG REGIMES IN THE LEUCOCYTE MIGRATION TEST

SECTION I - INTRODUCTION

A. DRUG EFFECTS

In addition to the possible intrinsic defect of the immune system in rheumatoid arthritis (Chapter 1, Section VII), it is known that the drugs routinely used to treat these patients do have effects on the immune system (Hurd, 1977). Drugs normally used in the management of RA are as follows (Constable, 1975):

(a) Non-steroidal anti-inflammatory drugs, e.g. aspirin, indomethacin, ibuprofen, naproxen, flurbiprofen, phenylbutazone, ketoprofen, fenoprofen.

(b) Anti-malarial, i.e. chloroquine, hydroxychloroquine.

(c) Gold salts, i.e. sodium aurothiomalate.

(d) Chelating agents, i.e. D-penicillamine.

(e) Immunosuppressives, i.e. azathioprine, cyclophosphamide, methotrexate.

(f) Corticosteroids, e.g. adrenocorticotrophic hormone (ACTH), prednisolone, betamethasone, dexamethasone, triamcinolone

(g) Recently imidazole derivative, i.e. levamisole.

(h) Patients may also be given additional analgesics, e.g. distalgesic, paracetamol.

The aetiology of RA is unknown and the use of the above drugs in the treatment of RA has arisen from empirical observations. The efficacy of these drugs in the treatment of RA has not been shown to be due to any known pharmacological action of the drug. Therefore their effect on DH reactions cannot be predicted. When using the in vitro tests of DH it has been considered good policy to exclude patients on high dose steroids (Caperton, 1975), corticosteroids, chloroquine, azathioprine or other immunosuppressive therapy (Keystone et al, 1976). It was therefore essential to test various patient groups for deviation from the response of a control group of subjects in the LMT using a standard antigen.

B. STREPTOKINASE

Streptokinase is a bacterial extracellular protein produced by group C Streptococcus hemolyticus. It is not a proteolytic enzyme, although it activates human plasminogen through reaction with a serum precursor called a proactivator to form the true activator (De Barbieri, 1965). Its use in the treatment of thromboembolic disease had been accompanied by side effects, later attributed to its antigenicity.

It has been shown that 86% of adults exhibited delayed skin test reactivity to intradermal challenge by appropriate concentrations of streptokinase-streptodernase (SK-SD) (Lawrence, 1954). Confirmation of it being a delayed-type hypersensitivity response was obtained by transferring SK-SD reactivity from sensitized donors to unsensitized recipients with peripheral blood leucocytes (Lawrence, 1952). The histology of the reaction to incubated streptokinase was examined and found to be characteristic of delayed-type hypersensitivity (Biergio et al, 1969).

Work has been carried out to define the antigen which elicits these responses, and it has been found that it is not the streptokinase but a β-globulin, molecular weight 21,500 which is consistently found in the heterologous preparation Varidase (Lederle) (Taylor et al, 1971; Tomar, 1976). Its ability to stimulate lymphocytes in vitro shows good correlation with skin reaction in the same subjects (Tomar et al, 1972).

The antigen has been used to induce MIF production (Rocklin et al, 1972) by cultivating SK-SD with lymphocytes from previously sensitized individuals. The MIF was assayed on guinea pig macrophages.

The inhibition of human leucocyte migration by SK-SD in agarose has been correlated to skin reactivity in the same individuals (Astor et al, 1973). It is therefore a suitable antigen to use in the standardisation and investigation of reproducibility of the leucocyte migration inhibition test.

SECTION II - METHODS

A. STREPTOKINASE-STREPTODORNASE EVALUATION

The commercially available Varidase contains Streptokinase 100,000 units, Streptodornase 25,000 units and 2.0 mg of the preservative thiomersal. These preservatives affect the viability of cells.

It was therefore necessary to remove this preservative before the SK-SD as the test antigen in the LMT.

(i) Removal of Thiomersal from SK-SD

The method used was an adaptation of that described by Rocklin et al (1970).

To the commercial preparation of SK-SD (Varidase) was added 10 mls sterile distilled water. The solution was dialysed against 5 litres of Phosphate buffered saline (PBS) at 4°C for 48 hours, and a further 5 litres for 24 hours at 4°C. The preparation was packed into multidose containers and kept at 4°C for a maximum of 2 weeks.

To ensure that there was no residual inhibitory activity, 10 mls of 0.2 mgs/ml thiomersal solution was subject to the same dialysis procedure and used in the LMT.

(ii) Results

See Tables 1 and 2 for effects of thiomersal on leucocyte migration.

See Fig. 3 for results using SK-SD as antigen in LMT.

TABLE 1:-

(A) RESULTS OBTAINED USING THE DIALYSED VARIDASE SOLUTION (Section II, A (i)) AS ANTIGEN IN THE LMT AT 500 i.u. AND 100 i.u./ml CULTURE MEDIUM.

(B) RESULTS OBTAINED USING THE DIALYSED 0.2 mgs/ml THIOMERSAL SOLUTION (Section II, A (i)) AT EQUIVALENT VOLUMES TO THE SK-SD SOLUTION USED IN (A).

As in all experiments the Migration Index (MI) is calculated from the mean of four readings at each antigen concentration.

A

WITHOUT ANTIGEN	1·0ml soln CONTAINING 500 iu/ml SK-SD	0·5ml soln CONTAINING 100 iu/ml SK-SD	
24·5	14·5	19·5	INDIVIDUAL READINGS
23·6	15·5	23·0	
21·3	16·0	23·3	
23·8	14·9	19·9	
23·3	15·2	21·43	MEAN
1·38	0·66	2·00	SD
CONTROL	65·24	91·97	M.I.

B

WITH OUT ANTIGEN	EQUIVALENT TO 1·0ml soln CONTAINING 500iu/ml SK-SD	EQUIVALENT TO 0·5ml soln CONTAINING 100iu/ml SK-SD	
22·3	22·2	23·9	INDIVIDUAL READINGS
23·2	22·9	23·6	
22·2	22·3	22·2	
23·1	22·2	22·9	
22·70	22·40	23·15	MEAN
·52	0·32	0·76	SD
CONTROL	98·68	101·98	M.I.

TABLE 1

TABLE 2:-

(A) RESULTS OBTAINED USING THE DIALYSED VARIDASE SOLUTION (Section II, A (i)) AS ANTIGEN IN THE LMT AT 1000 i.u. AND 500 i.u./ml CULTURE MEDIUM.

(B) RESULTS OBTAINED USING THE DIALYSED 0.2 mgs/ml THIOMERSAL SOLUTION (Section II, A (I)) AT EQUIVALENT VOLUMES TO THE SK-SD SOLUTION USED IN (A).

A.

WITHOUT ANTIGEN	1.0 ml soln CONTAINING 1000 iu/ml SK-SD	0.5 ml soln CONTAINING 500 iu/ml SK-SD	
25.2	12.4	21.3	INDIVIDUAL READINGS
25.1	12.5	18.0	
26.4	11.9	23.6	
25.8	13.6	19.7	
25.6	12.6	20.7	MEAN
0.60	0.72	2.38	SD
CONTROL	49.22	80.86	M.I.

B.

WITHOUT ANTIGEN	EQUIVALENT TO 1.0 ml soln CONTAINING 1000 iu/ml SK-SD	EQUIVALENT TO 0.5 ml soln CONTAINING 500 iu/ml SK-SD	
25.4	25.1	24.2	INDIVIDUAL READINGS
27.2	23.8	25.1	
27.6	24.4	25.7	
26.8	25.4	24.8	
26.25	24.8	24.95	MEAN
1.36	0.91	0.62	SD
CONTROL	94.48	95.05	M.I.

TABLE 2

Figure 3:-

DOSE RESPONSE OF STREPTOKINASE-STREPTODORNASE (SK-SD) ON MIGRATION OF LEUCOCYTES FROM PATIENTS WITH RHEUMATOID ARTHRITIS ●———● AND AGE AND SEX MATCHED HEALTHY CONTROLS ▲- - - - -▲.

Each point is the mean of eight subject responses ± one SD.

There is no significant difference between the rheumatoid and the normal responses.

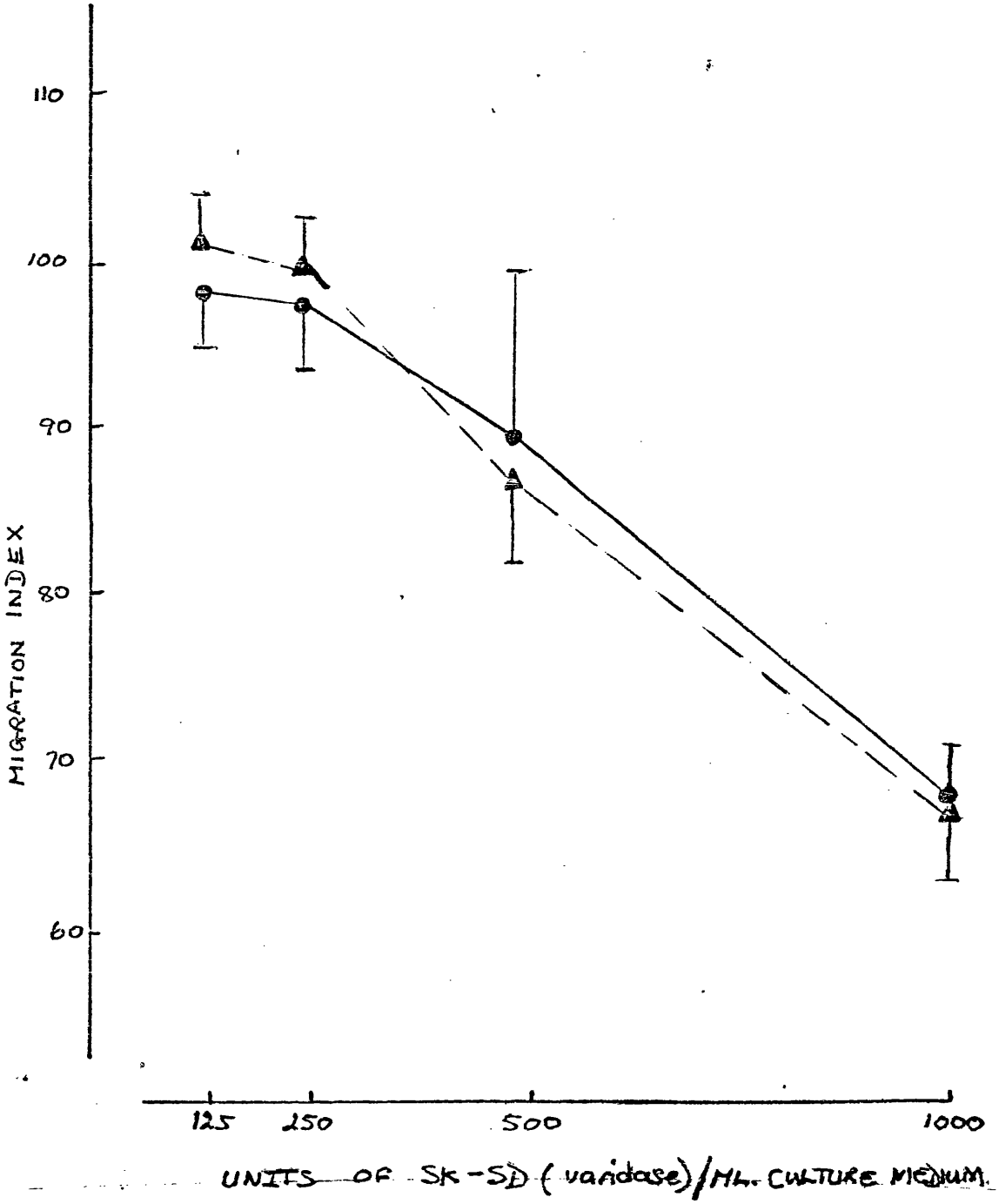

110
100
90
80
70
60
MIGRATION INDEX
125
250
500
1000
UNITS OF SK-SD (vaidase)/ML. CULTURE MEDIUM

Fig: 3

B. TEST STREPTOKINASE EVALUATION

A second commercially available preparation is Test Streptokinase. This contains no preservative, and is therefore easier to handle.

(i) Results

See Figure 4.

Figure 4:-

RESPONSE OF RHEUMATOID ARTHRITIS PATIENTS AND AGE AND SEX MATCHED HEALTHY SUBJECTS TO 500 i.u. TEST STREPTOKINASE (HOECHST) IN THE LMT (Section II, B).

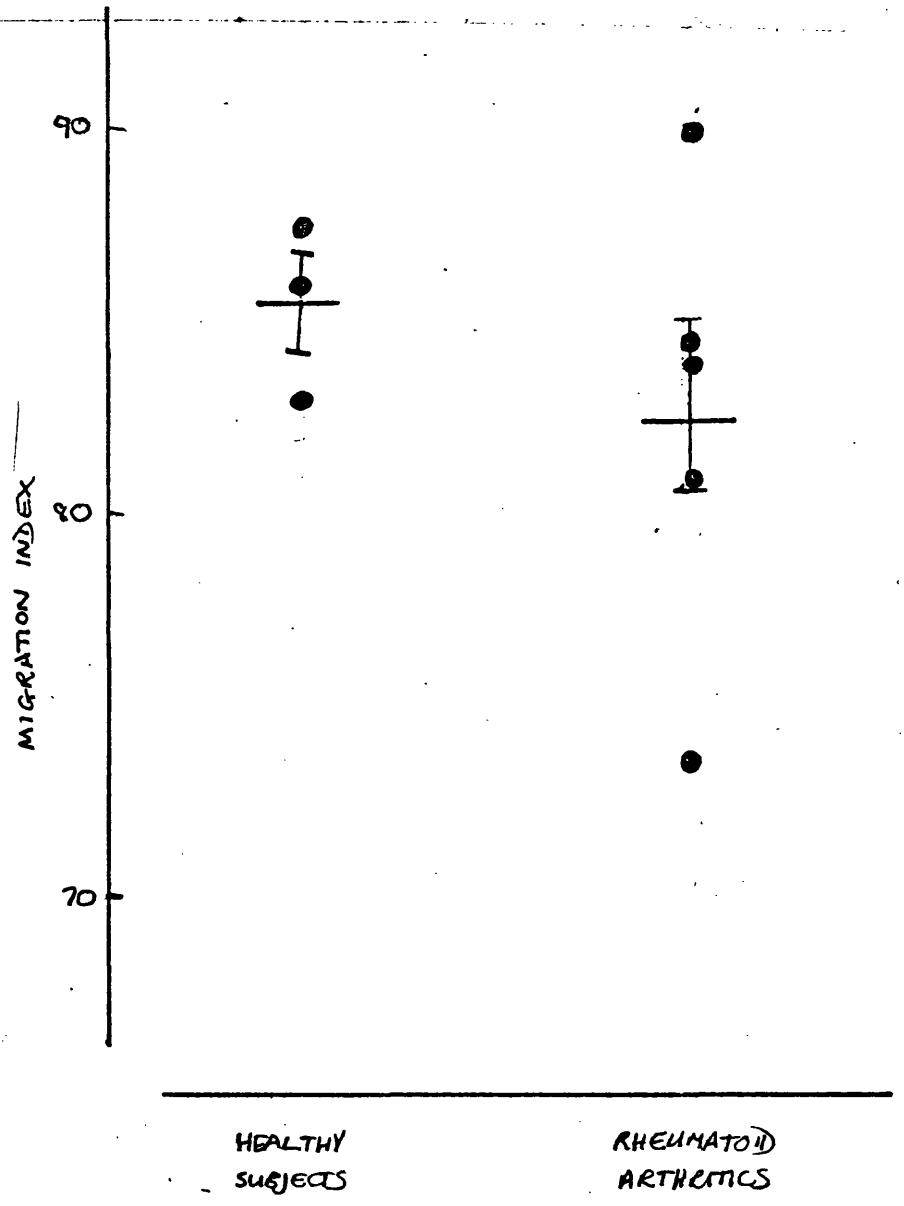

90
80
70
MIGRATION INDEX
HEALTHY SUBJECTS
RHEUMATOID ARTHRITICS

Fig: 4

C. COMPARISON OF PATIENT GROUPS

Various patient groups were tested against 1000 i.u. of streptokinase (Test Streptokinase, Hoechst) in the LMT.

(i) Results

See Figure 5.

Figure 5 :-

RESPONSE OF HEALTHY AND RHEUMATOID LEUCOCYTES TO 1000 i.u. STREPTOKINASE IN THE LEUCOCYTE MIGRATION TEST.

Each patient group, as indicated, was compared by Student's T test with the response of the healthy subjects, testing NULL HYPOTHESIS

HEALTHY	less	than patients	on CYCLOPHOSPHAMIDE	$P<0.001$
HEALTHY	less	than patients	on D-PENICILLAMINE	$P<0.001$
HEALTHY	less	than patients	with LATE INACTIVE DISEASE on NSAID	$P<0.05$
HEALTHY	less	than patients	with EARLY ACTIVE DISEASE on NSAID	$P<0.001$
HEALTHY	greater	than patients	on ANTI-MALARIALS	$P<0.001$
HEALTHY	greater	than patients	on CORTICOSTEROIDS	$P<0.001$
HEALTHY	greater	than patients	on GOLD THERAPY	$P<0.001$
HEALTHY	greater	than patients	with LATE ACTIVE DISEASE on NSAID	$P<0.01$
HEALTHY	greater	than patients	with LATE SERO-NEGATIVE DISEASE on NSAID	$P<0.01$

HEALTHY greater than all patients on NSAID $P = 0.6$
which is NOT SIGNIFICANT.

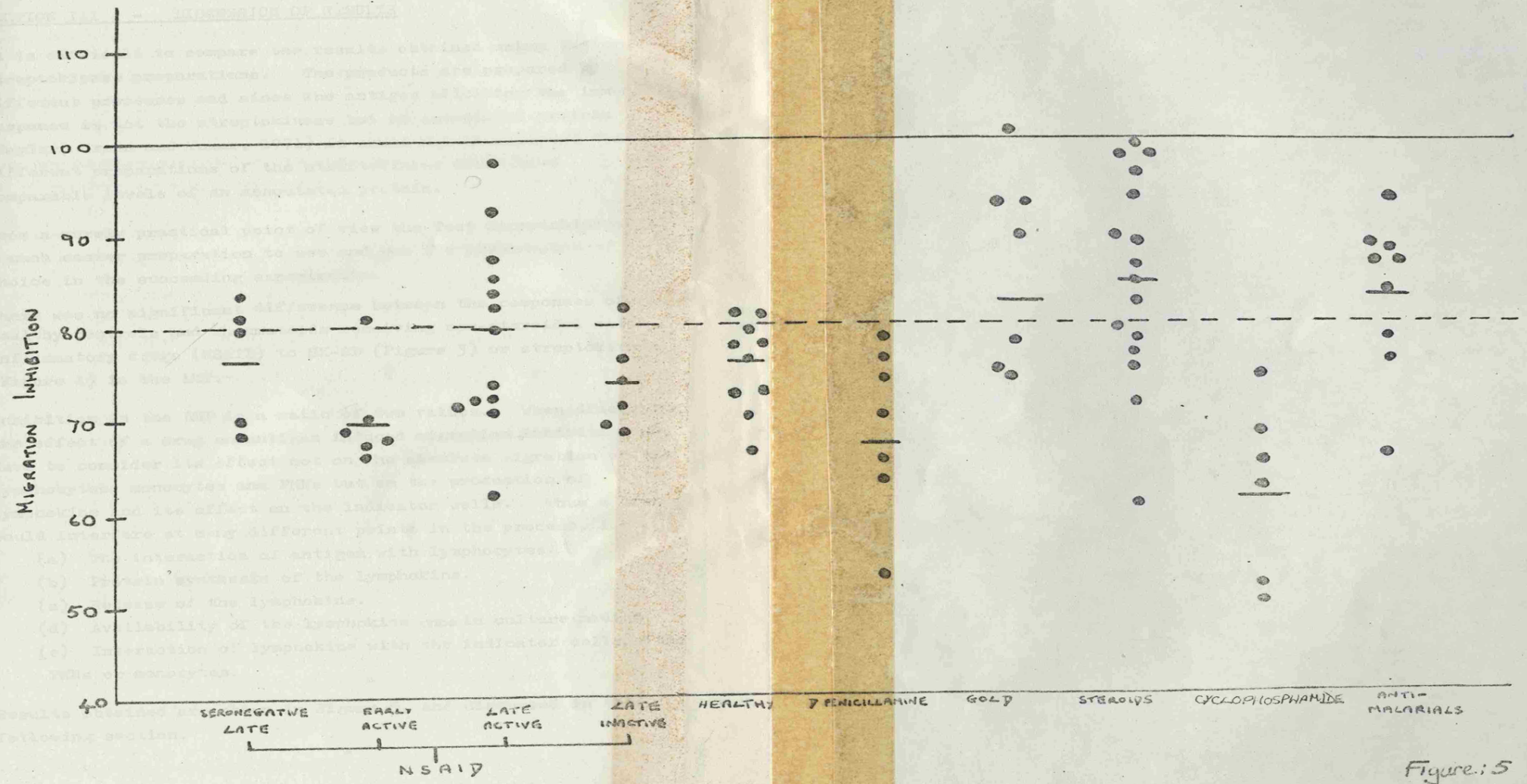

MIGRATION INHIBITION
110
100
90
80
70
60
50
40
SERONEGATIVE LATE
EARLY ACTIVE
LATE ACTIVE
LATE INACTIVE
HEALTHY
PENICILLAMINE
GOLD
STEROIDS
CYCLOPHOSPHAMIDE
ANTI-MALARIALS
NSAID

Figure: 5

SECTION III - DISCUSSION OF RESULTS

It is difficult to compare the results obtained using the two streptokinase preparations. The products are prepared by different processes and since the antigen eliciting the immune response is not the streptokinase but an associated protein (Taylor, Green and Tomar, 1971) it would not be expected that different preparations of the streptokinase would have comparable levels of an associated protein.

From a purely practical point of view the Test Streptokinase is a much easier preparation to use and was the preparation of choice in the succeeding experiments.

There was no significant difference between the responses of healthy controls and rheumatoids receiving non-steroidal anti-inflammatory drugs (NSAID) to SK-SD (Figure 3) or streptokinase (Figure 4) in the LMT.

Inhibition in the LMT is a ratio of two values. When discussing the effect of a drug on antigen induced migration inhibition we have to consider its effect not on the absolute migration of the lymphocytes, monocytes and PMNs but on the production of lymphokine and its effect on the indicator cells. Thus a drug could interfere at many different points in the process, i.e.:

(a) The interaction of antigen with lymphocytes.
(b) Protein synthesis of the lymphokine.
(c) Release of the lymphokine.
(d) Availability of the lymphokine once in culture medium.
(e) Interaction of lymphokine with the indicator cells, either PMNs or monocytes.

Results obtained are shown in Figure 5, and discussed in the following section.

A. NSAID

In vitro indomethacin, phenylbutazone, ibuprofen, aspirin and sodium salicylate were demonstrated to directly impede the random migration of human leucocytes in a dose-related manner. The effect was more pronounced on PMNs than on lymphocytes (Brown, 1976).

Aspirin in physiological dose range will inhibit the incorporation of 3H-thymidine in the lymphocyte transformation reaction in response to PHA. If the lymphocytes were incubated with aspirin and then washed the inhibitory effect was lost (Opelz et al, 1973; Pachman et al, 1973).

When the drugs are given to the patients and the peripheral blood cells tested in vitro it was found that the minimum absolute migration occurred at the maximum salicylate levels (Brown, 1976).

No effects on lymphokine production in vitro have been documented.

In our results we found no significant difference between the various patient groups on NSAID and the healthy control subjects.

B. PENICILLAMINE

There have been reports of effects of penicillamine on inflammation and immune responses in vivo. Acetyl-penicillamine given to rabbits in repeated doses reduced the severity of induced inflammation of the skin. It was concluded that thiols, e.g. penicillamine, had a direct effect on the inflammatory process (Bailey and Sheffner, 1967).

Liyanage and Curry (1972) were unable to show any effect on adjuvant arthritis in rats. It has a bimodal effect on immune responsiveness in rabbits (Tobin and Altman, 1964; Altman and Tobin, 1965; Hubner and Georgiai, 1966).

D-penicillamine has been reported as having no effect on lymphocyte transformation (Zuckner et al, 1970) and thus no significant effect on T cell function (Maini et al, 1975).

Huskisson (1976) reported that penicillamine had been shown to enhance cell-mediated reactions to tuberculin.

Thus results such as those shown in Figure 5 have not been previously reported. However, we conclude that patients receiving penicillamine will show a greater degree of inhibition in the LMT than control or RA receiving NSAID. This could be due to a stimulation of the T cell response.

C. GOLD

Adrianakos et al (1977) reported that gold treatment had no effect on rheumatoid patients' ability to response to standard antigens in a skin testing regime.

When added in vitro to cultures gold inhibits lymphocyte transformation by PHA, PPD or allogeneic cells (Lies et al, 1977). Harth et al (1976) has concluded that gold prevents the amplification of the immune response.

Williams and Bruckner (1971) state that rheumatoids are specifically inhibited when compared with normals in the LMT using Mycoplasma fermentans as antigen. Patients receiving chrysotherapy respond in the same manner as other RAs. However a further report (Brostoff and Roitt, 1974) states that rheumatoids receiving gold and in remission are only inhibited to the same extent as control subjects. They attribute this to the action of gold on M. fermentans.

We found that gold decreased the inhibition of migration of rheumatoid patients to streptokinase in the LMT. Our results were not unlike those of Brostoff and Roitt (1974), but we do not attribute the diminution of the inhibitory response to the same cause. However they were comparing control population, rheumatoid population against aggregated IgG, and the negation of inhibition of the rheumatoid population receiving chrysotherapy caused inhibition to only the same extent as the control population.

We have shown that gold affects the inhibition of migration against standard antigens when the control and rheumatoid population have the same response.

D. ANTI-MALARIALS

Chloroquine will inhibit blastogenesis induced by mitogen (Hurvitz and Hirschhorn, 1965; Panayi et al, 1973) but will not inhibit the release of skin reactive factor from guinea pig lymphocytes (Pick, Krejci and Turk, 1970). It was also found to have a direct action on rabbit PMNs, rendering the cells incapable of responding to a chemotactic factor (Ward, 1966).

Therefore it seems that chloroquine has no effect on the release of lymphokine, but might affect the response of the indicator PMN cells in such a way as to prevent inhibition. This reasoning could explain the results obtained on Figure 5.

E. STEROIDS

There have been many reports of the in vivo and in vitro effects of corticosteroids on leucocytes. It has been stated that in clinical situations in which the glucocorticoids are used to prevent the consequences of CMI reactions, e.g. graft rejection, the steroids do not interfere with the normal processes in the development of DH. Rather they prevent or suppress the inflammatory responses that take place as a consequence (Cohen, 1971).

Following the administration of corticosteroid two observations have been reported, (1) granulocytosis due to increased release from bone marrow and decreased rate of removal (Bishop, 1968) and (2) a lymphocytopenia: following a single dose of cortisol, a 70% decrease in circulating lymphocytes at 4 - 6 hours. T cells are decreased more than B cells due to redistribution (Fauci and Dale, 1974).

In vitro hydrocortisone has a dual action on human leucocyte motility : the movement of total leucocyte population is stimulated while PMNs are inhibited (Stevenson, 1973) at high doses, at low doses PMNs' migration is also stimulated (Stevenson, 1974). Methyl prednisolone increases leucocyte migration under agarose (Bendtzen, 1975). The effect of leucocyte migration inhibitory activity was also abolished by methyl prednisolone added in vitro at therapeutic drug levels (Bendtzen, 1975). Glucocorticoids added to MIF containing supernatants abolishes migration inhibitory activity on guinea pig macrophages (Balow and Rosenthal, 1973) as does dexamethasone (Lockshin, 1972). The production of leucocyte migration inhibitory activity is inhibited by methyl prednisolone in man (Bendtzen, 1975).

In man glucocorticoids also suppress antigen and PHA induced lymphocyte transformation and the mixed lymphocyte reaction (Nowell, 1961; Heilman and Leichner, 1971; Heilman et al, 1973).

Very few experiments have been carried out in which the drug has been given in vivo and the cells (macrophages or leucocytes) tested in vitro. PMNs' migration has been shown to be stimulated (Stevenson, 1976) but there seems to be little information on the production and effect of migration inhibitory activity on cells cultured in vitro without steroid. Hydrocortisone and prednisolone have very little effect on PHA induced transformation, but dexamethasone suppresses it (Fauci, 1976). In guinea pigs hydrocortisone did not suppress functional capabilities on lymphocytes in circulation, while cortisone acetate markedly suppressed antigen-induced lymphocyte blastogenesis and macrophage inhibitory factor production (Balow et al, 1975).

Hydrocortisone and prednisolone have been found to have a direct action on rabbit PMNs, rendering the cells incapable of reacting to a chemotactic factor (Wood, 1966).

In our experiments it seems that the in vivo corticosteroids are either preventing the release of LIF or rendering the PMNs less able to respond to the LIF since the degree of migration inhibition is reduced.

F. CYCLOPHOSPHAMIDE (CY)

In whole guinea pigs CY has been reported to deplete B cells (Turk and Poulter, 1972) and cause an increase in intensity of the DNCB skin reaction (Turk et al, 1972). Mitsuoka et al (1976) reported that low dose CY enhanced both the primary and secondary DH reactions in mice due to an effect on suppressor T cells. In mice it has been reported that the proportion of carrying lymphocytes increases after CY treatment.

In man Alepa et al (1970) and Strong et al (1973) reported that in vitro PHA and PWM stimulation decreased on treatment with cyclophosphamide, but five of the nine patients were also receiving prednisolone. Skin reactivity to standard antigens decreased (Strong et al, 1973).

CY in vitro had no effect on human leucocyte migration under agarose, but it did prevent the production of leucocyte migration inhibitory activity (Bendtzen, 1975).

The observations that CY depletes lymphocytes from the non-thymus dependent areas of the lymphnodes and spleen (Turk and Poulter, 1972) and increases the proportion of Θ carrying cells (Poulter and Turk, 1972) are consistent with our observations that migration inhibition is potentiated by CY treatment. Alternatively the CY could be acting firstly on the short-lived cells which are the suppressor T cells, thus causing a magnification in the CMI response.

In the light of the results presented we felt justified in excluding from our further tests all patients receiving steroids, gold, penicillamine, cyclophosphamide and anti-malarials.

SECTION IV - CONCLUSION

In the light of the results present we felt justified in excluding from further tests all patients receiving steroids, gold, penicillamine, cyclophosphamide and anti-malarials.

It has been shown that the LMT tested against a standard antigen is sensitive enough to detect differences in patient groups on different therapies, an observation which has many applications.

It is important to note that if patients are receiving no anti-rheumatic drugs but only NSAID, then their mean response to the test antigen is not statistically different from the responses of control subjects. Thus any difference in response to further test antigens can be attributed to an effect of the antigen on the cell population, and not an intrinsic difference in the cell populations(**Chapter 1, Section VIII.**)

CHAPTER 4

THE EFFECT OF CRUDE SYNOVIAL HOMOGENATES ON THE MIGRATION OF RHEUMATOID AND CONTROL LEUCOCYTES IN THE LEUCOCYTE MIGRATION TEST

CHAPTER 4

THE EFFECT OF CRUDE SYNOVIAL HOMOGENATES ON THE MIGRATION OF RHEUMATOID AND CONTROL LEUCOCYTES IN THE LEUCOCYTE MIGRATION TEST.

SECTION I - INTRODUCTION

Much work has been carried out in an attempt to define a DH reaction to rheumatoid synovium. The results have been equivocal, but nonetheless encouraging. The hypothesis of aetiology of RA indicates that the synovium is the site of an ongoing immune response, possibly of the CMI type.

The tests of CMI employed, and the antigens which have been used, have been fully discussed in the introduction to this thesis (Chapter 1, Section VII A)

SECTION II - METHODS OF SYNOVIAL HOMOGENISATION

Tissue obtained at synovectomy was kept in a sterile container at 4°C in Hank's solution for a minimum of 1 hour. After removal of fat and cartilage the tissue was minced, hand homogenised and, lastly, sieved through a 60 gauge steel mesh (Bacon et al, 1973). After standing for 16 hours at 4°C the crude suspension was treated in one of two ways to give two different antigen solutions:

(a) "Low Spin" Homogenate
Spin at 1000 g for 20 minutes, which will cause sedimentation of whole cells. Remove supernatant and keep at -20°C.

(b) "High Spin" Homogenate
Spin at 100,000 g for 1 hour. This procedure will cause sedimentation of membrane fragments and is used to establish solubility. Remove supernatant and keep at -20°C.

SECTION III - RESULTS.

Eight synovial specimens were homogenised and subjected to the 1000 g centrifugation and the supernatants tested in the LMT against RA and age and sex matched control buffy cells. The control and rheumatoid cultures were run in parallel.

In the original method the homogenate was settled under gravity for 16 hours. Graph 2 shows results obtained when the homogenate was allowed to settle for 16 hours and then divided into two parts, and the one subjected to centrifugation at 1000 g for 20 minutes.

Although the difference in result was not statistically different the settled homogenate tended to be more inhibitory and when examined microscopically this solution still contained whole cells. Both RA and control cells were equally inhibited by both supernatants.

Figure 7 shows the first results obtained testing RAs and controls against a crude synovial homogenate.

As an initial attempt at purification the supernatant was subjected to a high speed spin, 100,000 g, for 1 hour, such as would be used to establish solubility.

It was found in all five synovial homogenates treated in this way that all the inhibitory material was removed, see Figures 9, 10, 11, 12 and 13. In these cases the protein concentrations of the solutions tested were the same.

In two experiments, see Figures 12 and 13, the low spun homogenate was significantly more inhibitory.

In none of the experiments were rheumatoid leucocytes more inhibited than control leucocytes. In one experiment

(See Figure 7) control leucocytes were inhibited to a greater extent than RA leucocytes and this was statistically significant $P < 0.02$.

At the end of each type of experiment the cells involved in the LMT test were found to be viable as assessed by trypan blue dye exclusion.

The total results obtained when testing rheumatoid and control leucocytes against low spin crude synovial homogenates in the LMT are shown in Figure 16 and against high spin crude synovial homogenates in Figure 17.

Figure 7 :-

DOSE RESPONSE OF FD RHEUMATOID SYNOVIAL HOMOGENATE ON MIGRATION OF LEUCOCYTES FROM PATIENTS WITH RHEUMATOID ARTHRITIS AND HEALTHY AGE AND SEX MATCHED CONTROLS.

At the doses of 320, 160, 40, 20 and 10 μg of protein/ml there was no significant difference between rheumatoid arthritis patients ●———● and control subjects ▲------▲. Each point represents the mean $\pm$ SD of six subjects.

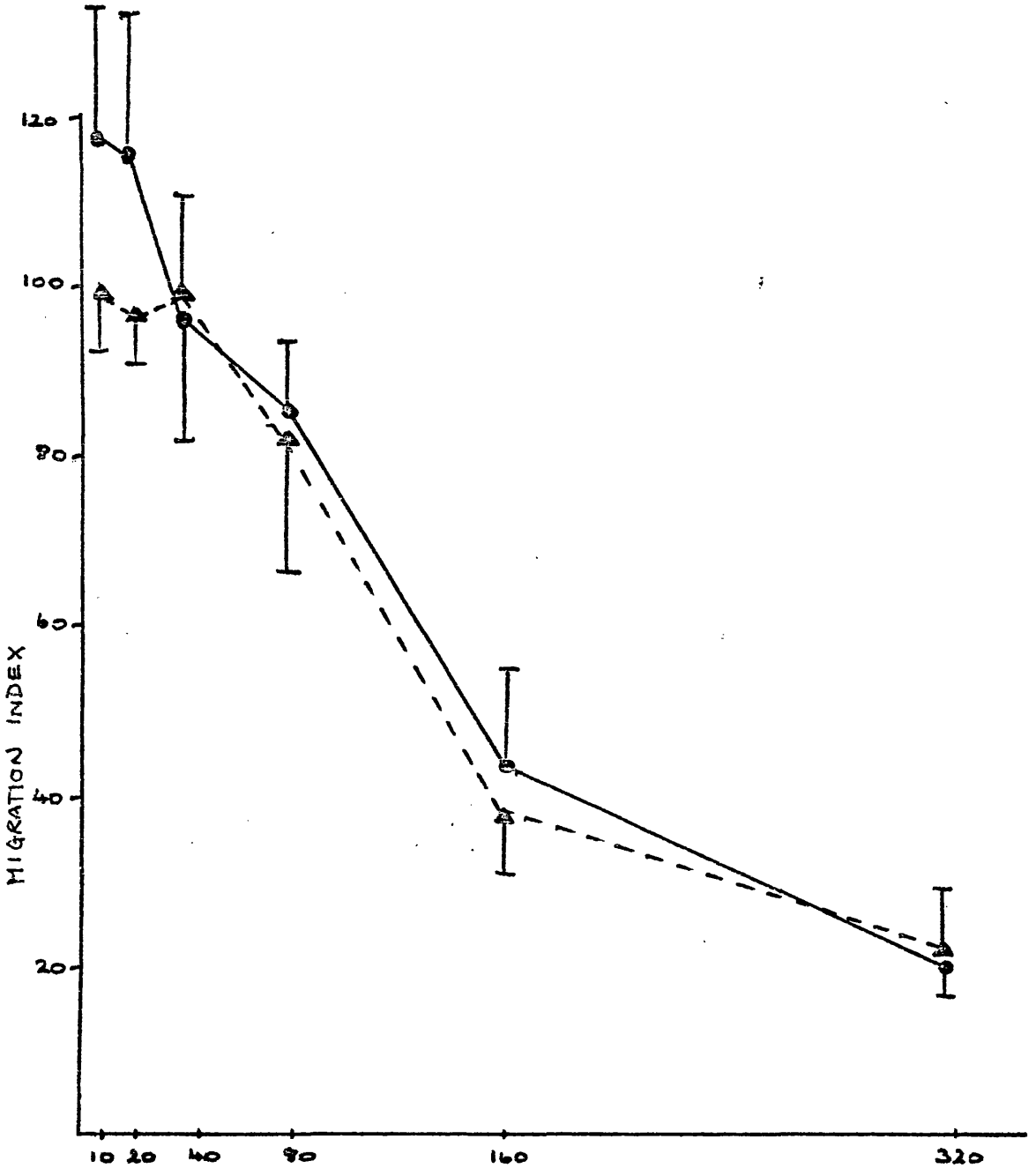

120
100
80
60
40
20
MIGRATION INDEX
10
20
40
80
160
320
µg PROTEIN/ML OF CULTURE MEDIUM

Fig: 7

Figure 8 :-

DOSE RESPONSE OF JC RHEUMATOID SYNOVIAL HOMOGENATES EITHER SEDIMENTED AT 1 *g* (A) OR SPUN AT 1000 *g* FOR 20 MINUTES (B) ON MIGRATION OF LEUCOCYTES FROM PATIENTS WITH RHEUMATOID ARTHRITIS AND HEALTHY AGE AND SEX MATCHED CONTROLS.

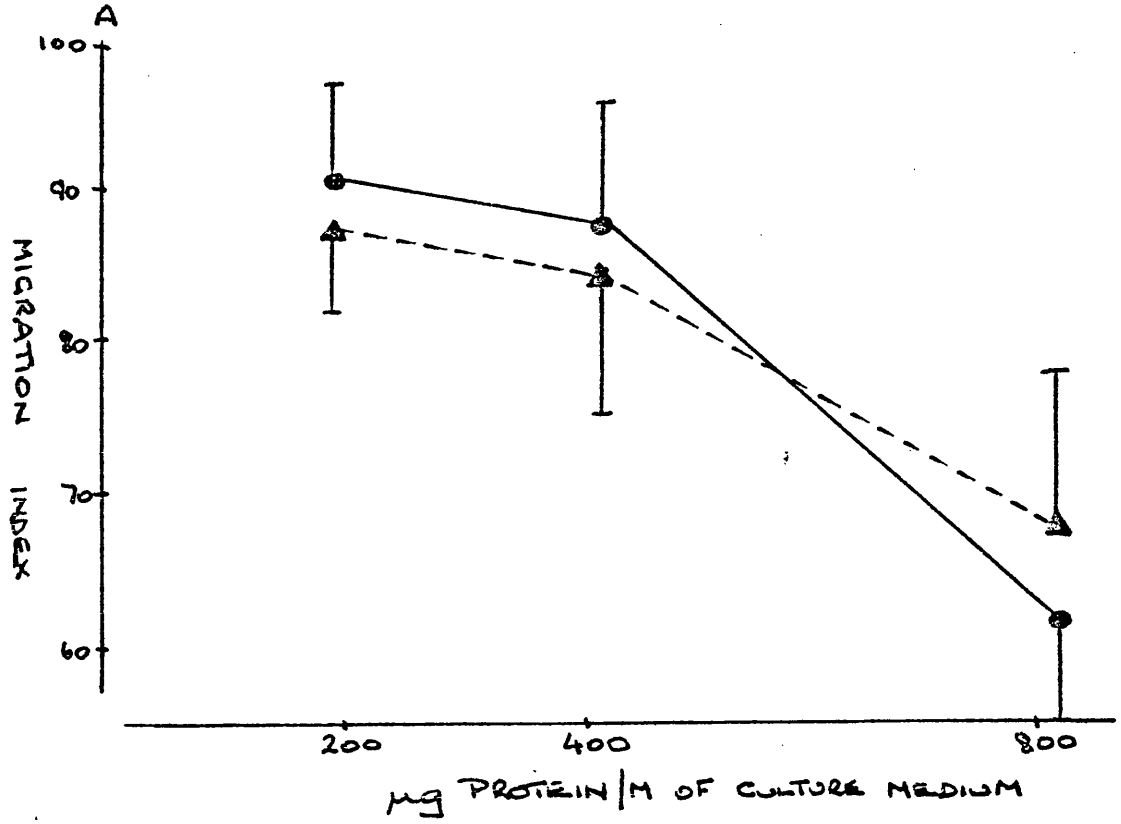
(A) At the doses of 800, 400 and 200 μg of protein/ml of culture medium, there was no significant difference between rheumatoid arthritis patients o——o and control subjects Δ---- Δ. Each point represents the mean $\pm$ standard deviation (SD) of six subjects.

(B) At the doses of 800, 400 and 200 μg of protein/ml of culture medium there was no significant difference, between RAs and controls.

RA settled was more inhibited than RA spun $P < 0.05$.

Control settled was more inhibited than control spun $P < 0.001$.

Combined RA and control settled was more inhibited than combined total spun $P < 0.001$.


A
100
90
80
70
60
MIGRATION INDEX
200
400
800
μg PROTEIN/ML OF CULTURE MEDIUM

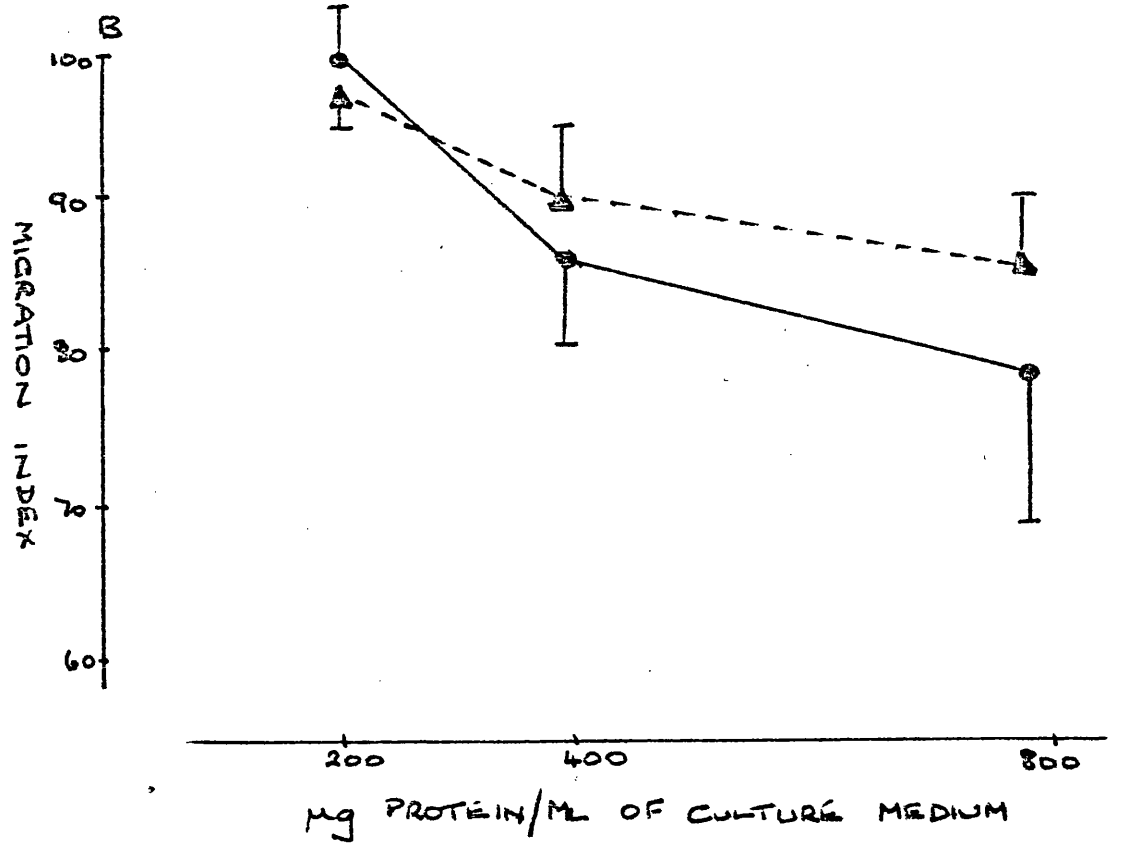

B
100
90
80
70
60
MIGRATION INDEX
200
400
800
μg PROTEIN/ML OF CULTURE MEDIUM

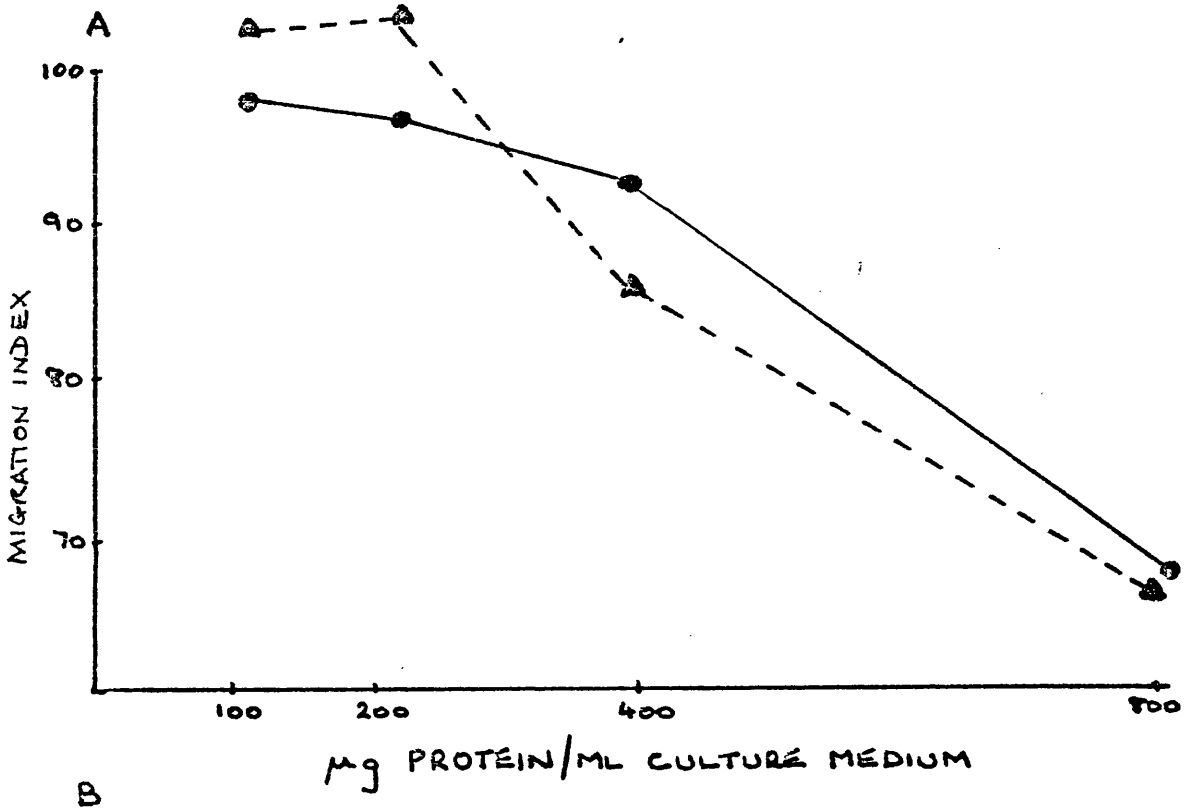
Fig: 8

Figure 9 :-

DOSE RESPONSE OF VM RHEUMATOID SYNOVIAL HOMOGENATES EITHER SUBJECTED TO LOW SPIN (A) OR HIGH SPIN (B) ON MIGRATION OF LEUCOCYTES FROM PATIENTS WITH RHEUMATOID ARTHRITIS AND HEALTHY AGE AND SEX MATCHED CONTROLS.

(A) At 800, 400, 200 and 100 µg/ml there was no significant difference between rheumatoid arthritis patients ●——●, the mean of four observations, and control subjects ▲- - - - -▲, the mean of five subjects.

(B) There were no tests of significance of these readings, since they were means of three or less observations.


A
100
90
80
70
MIGRATION INDEX
100
200
400
800
µg PROTEIN/ML CULTURE MEDIUM

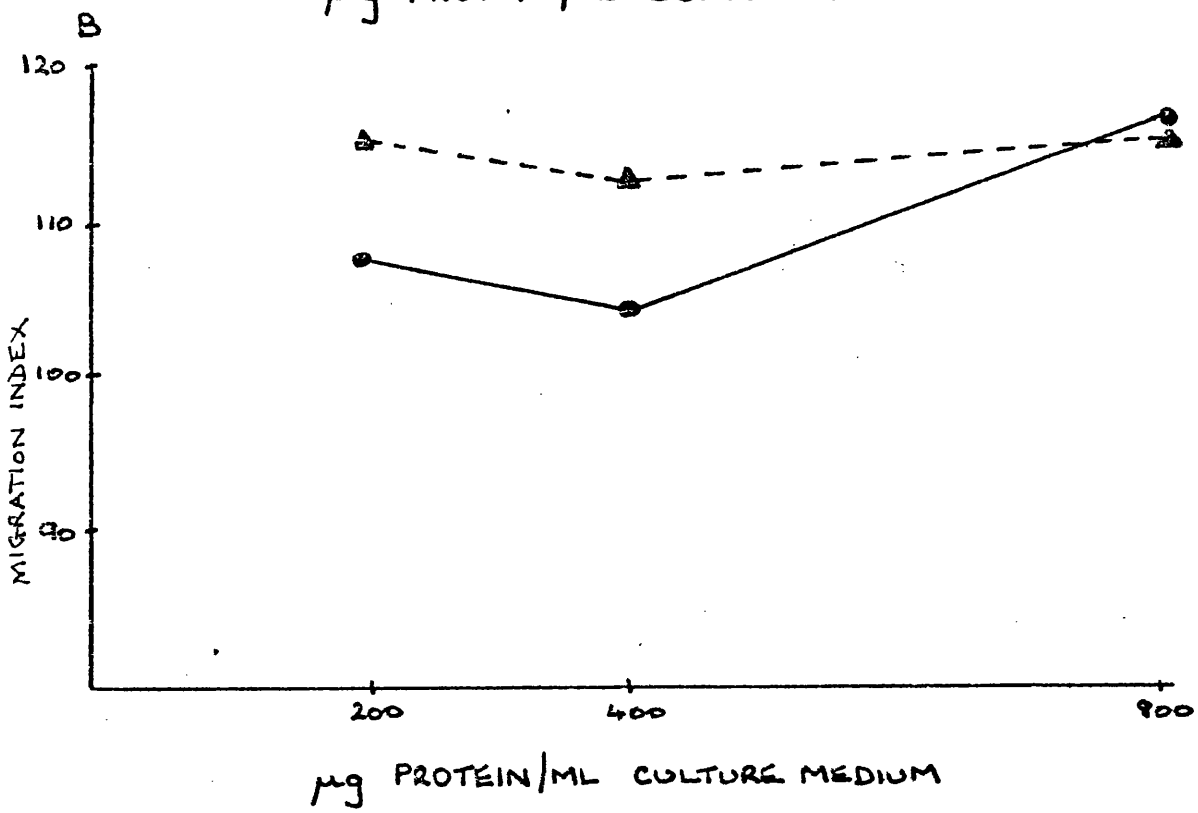

B
120
110
100
90
MIGRATION INDEX
200
400
800
µg PROTEIN/ML CULTURE MEDIUM

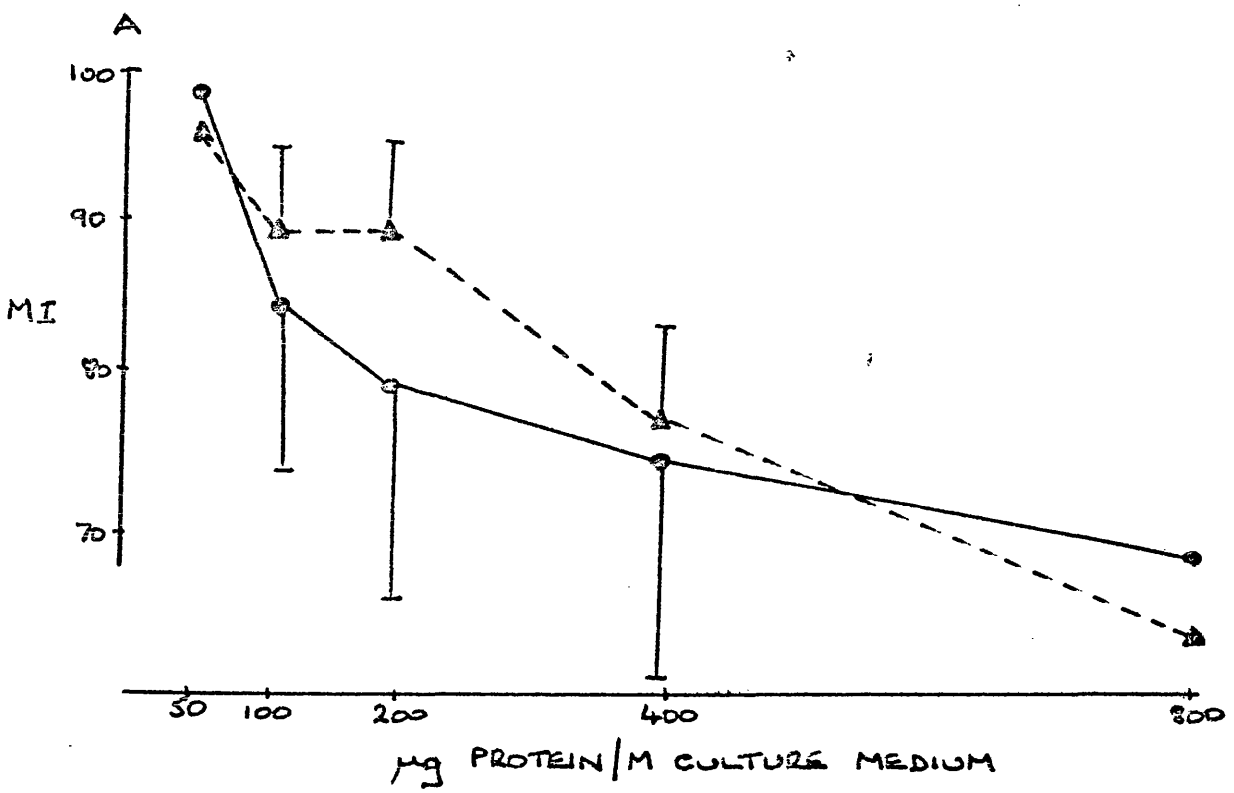
Fig: 9

Figure 10 :-

DOSE RESPONSE OF JB RHEUMATOID SYNOVIAL HOMOGENATE EITHER SUBJECTED TO LOW SPIN (A) OR HIGH SPIN (B) ON MIGRATION OF LEUCOCYTES FROM PATIENTS WITH RHEUMATOID ARTHRITIS AND AGE AND SEX MATCHED CONTROLS.

(A) At 800, 400, 200, 100 and 50 μg/ml, there was no significant difference between rheumatoid arthritis patients ●——● and control subjects ▲---▲. Each point is the mean of five subjects' responses $\pm$ one SD.

(B) At 1600, 800, 400, 200 and 100 μg/ml, there was no significant difference between arthritic and control subjects, each point is the mean of four subjects' response $\pm$ one SD.


A
100
90
MI
70
50
100
200
400
800
µg PROTEIN/M CULTURE MEDIUM

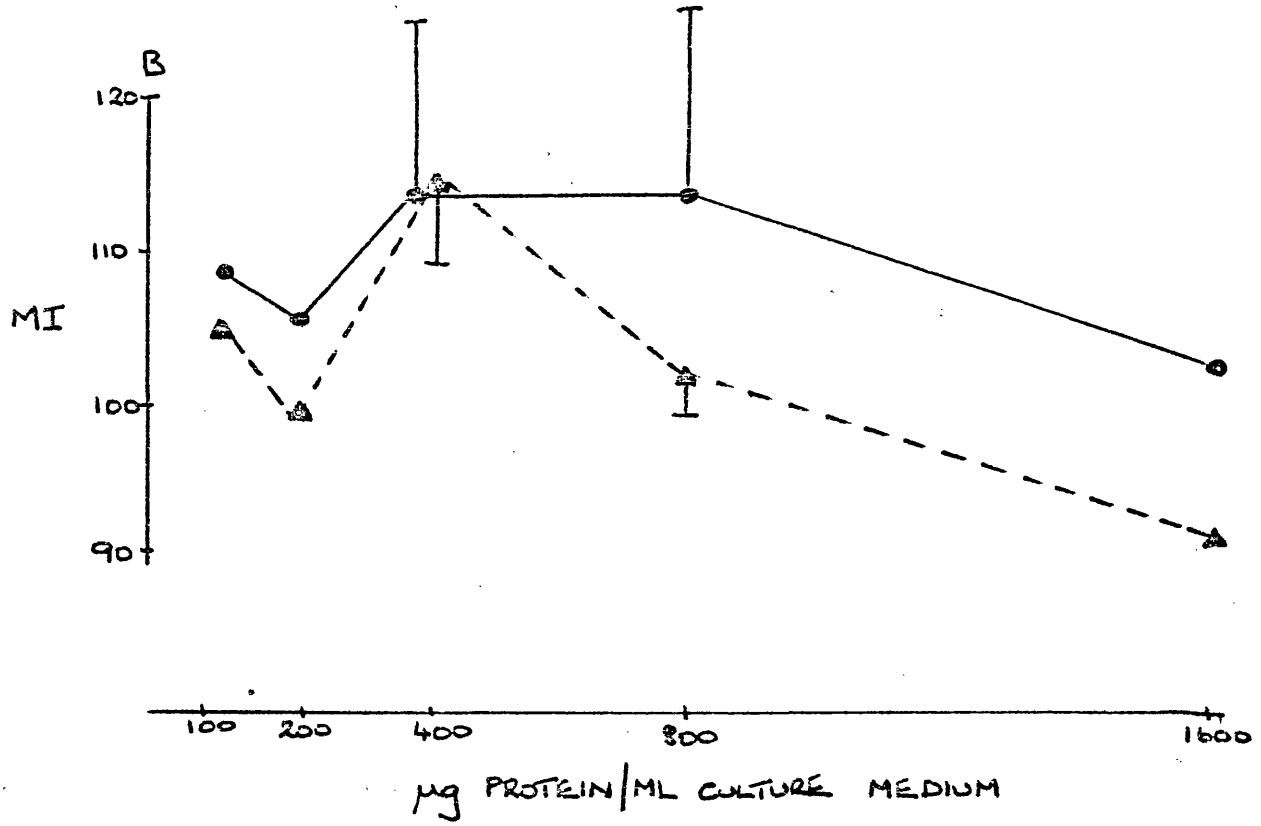

B
120
110
MI
100
90
100
200
400
800
1600
µg PROTEIN/ML CULTURE MEDIUM

Fig: 10

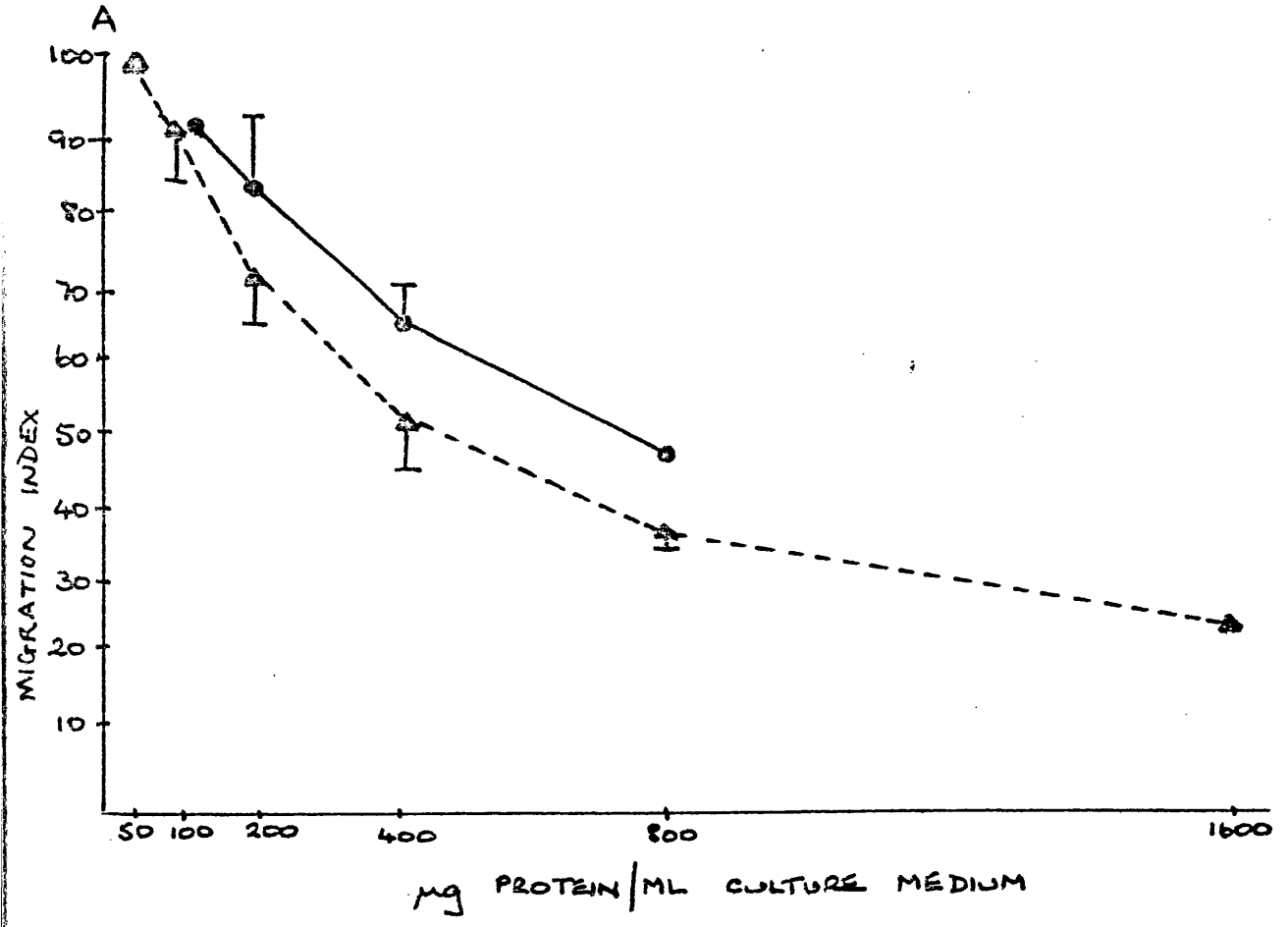
Figure 11 :-

DOSE RESPONSE OF LC RHEUMATOID SYNOVIAL HOMOGENATE EITHER SUBJECTED TO LOW SPIN (A) OR HIGH SPIN (B) ON MIGRATION OF LEUCOCYTES FROM PATIENTS WITH RHEUMATOID ARTHRITIS AND AGE AND SEX MATCHED CONTROLS.

(A) At 400 and 200 μg protein/ml there was no significant difference between rheumatoid arthritis patients ●——● and control subjects ▲- - - -▲ . Each point is the mean of a maximum of six subjects' response $\pm$ one SD.

(B) At 400 and 200 μg protein/ml there was no significant difference between arthritic and control subjects. Each point is the mean of a maximum of six subjects' responses $\pm$ one SD.

The points with no SD are the means of three or less separate observations.


A
MIGRATION INDEX
100
90
80
70
60
50
40
30
20
10
50
100
200
400
800
1600
µg PROTEIN/ML CULTURE MEDIUM

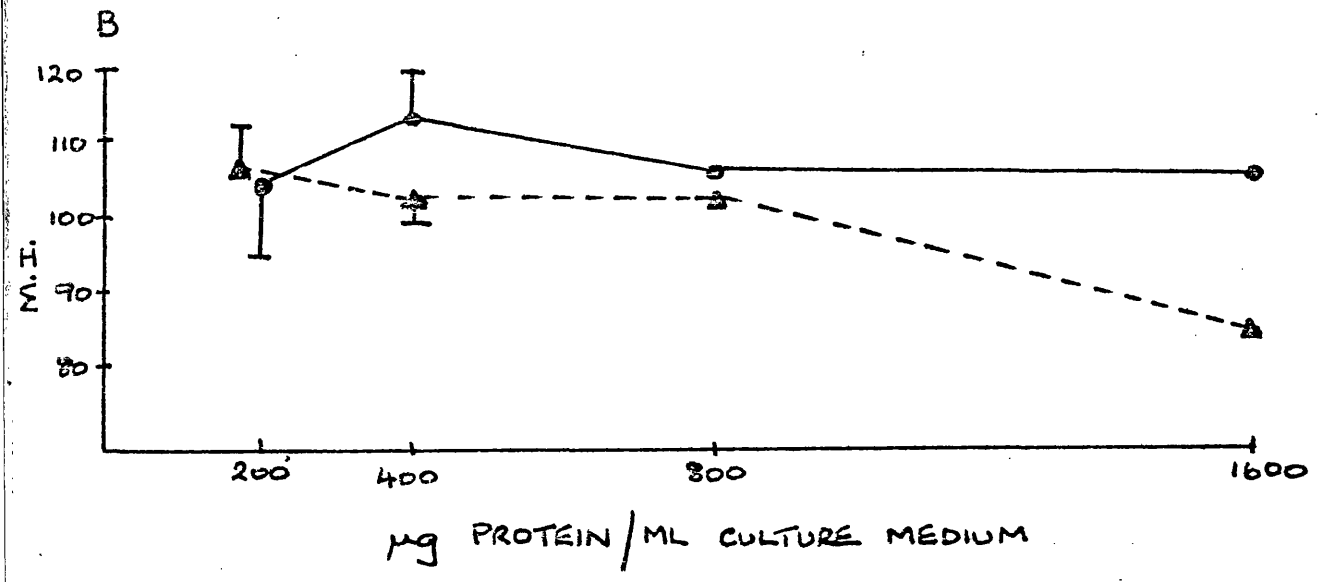

B
M.I.
120
110
100
90
80
200
400
800
1600
µg PROTEIN/ML CULTURE MEDIUM

Fig: 11

Figure 12 :-

DOSE RESPONSE OF JM RHEUMATOID SYNOVIAL HOMOGENATES EITHER SUBJECTED TO LOW SPIN (A) OR HIGH SPIN (B) ON MIGRATION OF LEUCOCYTES FROM PATIENTS WITH RHEUMATOID ARTHRITIS, AND AGE AND SEX MATCHED CONTROLS.

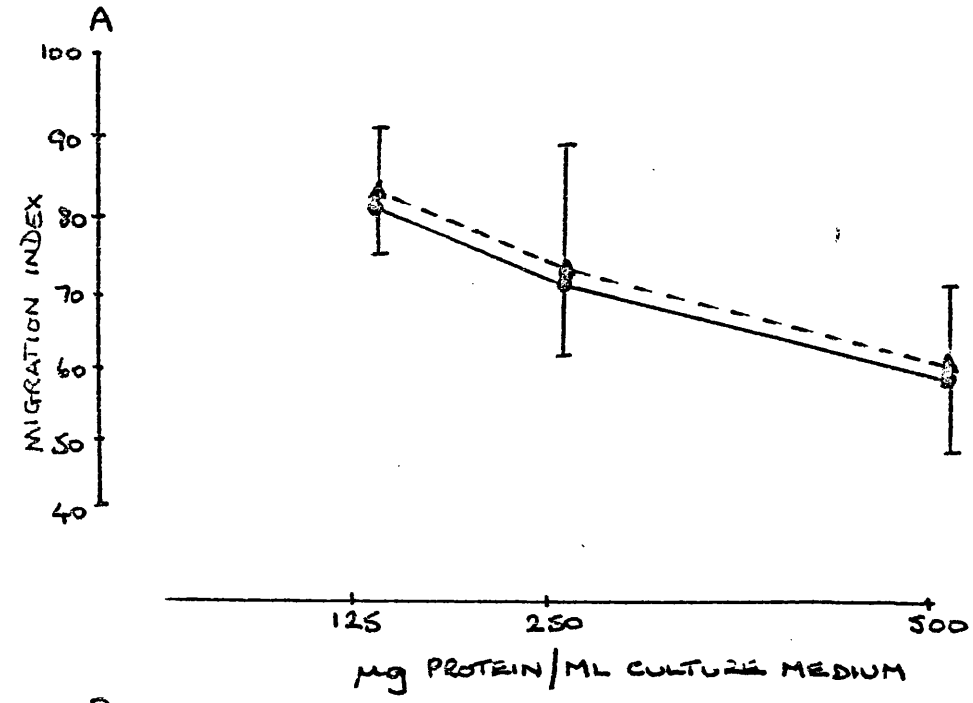
(A) At 500, 250 and 125 µg protein/ml culture medium there is no significant difference between the response of rheumatoid arthritis patients ●———● and control subjects ▲--------▲ . Each point is the mean of four separate observations.

(B) At 500, 250 and 125 µg protein/ml culture medium there is no significant difference between the response of rheumatoid arthritis patients and control subjects. Each point is the mean of four separate observations.

RA response to low spin homogenate is more inhibitory than RA response to high spin homogenate $P < 0.005$.

Control response to low spin homogenate is more inhibitory than control response to high spin homogenate $P < 0.002$.

Total response to low spin homogenate is more inhibitory than total response to high spin homogenate $P < 0.00001$.


A
100
90
80
70
60
50
40
MIGRATION INDEX
125
250
500
µg PROTEIN/ML CULTURE MEDIUM

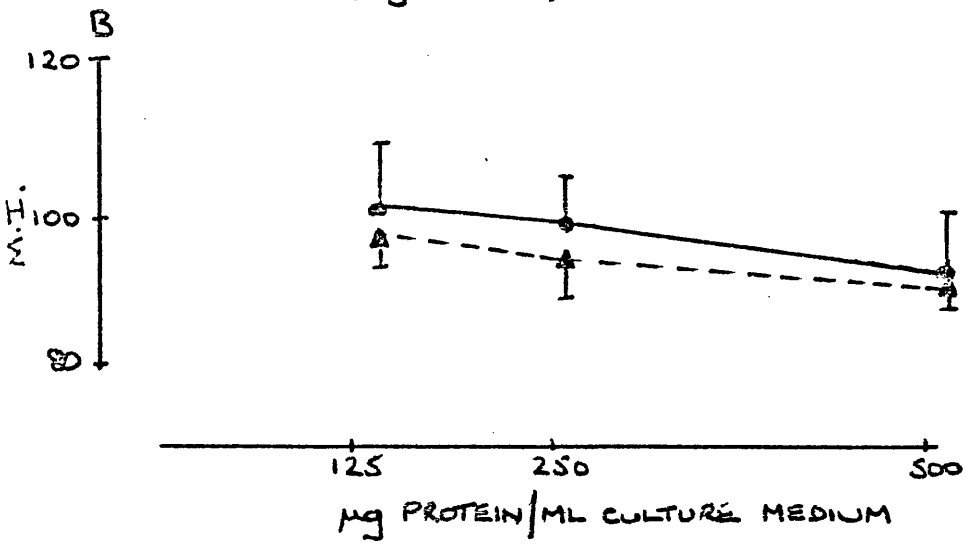

B
120
100
80
M.I.
125
250
500
µg PROTEIN/ML CULTURE MEDIUM

Fig: 12

Figure 13 :-

DOSE RESPONSE OF NS RHEUMATOID SYNOVIAL HOMOGENATES SUBJECTED TO EITHER LOW SPIN (A) OR HIGH SPIN (B) ON MIGRATION OF LEUCOCYTES FROM PATIENTS WITH RA ●——● AND AGE AND SEX MATCHED CONTROLS ▲- - - - -▲.

(A) Each point is the mean of five subjects' response ± one SD. At 500 μg protein/ml culture medium the control leucocytes are more inhibited than the RA leucocytes tested by student t test, P = 0.02.

At 250 μg protein/ml culture medium the control leucocytes are more inhibited than the RA leucocytes, P = 0.025.

(B) At 500, 250 and 125 μg protein/ml there is no significant difference between the response of RA and control subjects. Each point is the mean of four subjects' response ± one SD.

RA response to low spin homogenate is more inhibitory than RA response to high spin homogenate $P < 0.002$.

Control response to low spin homogenate is more inhibitory than control response to high spin homogenate $< P\ 0.0001$.

Total response to low spin homogenate is more inhibitory than total response to high spin homogenate $P < 0.000005$.

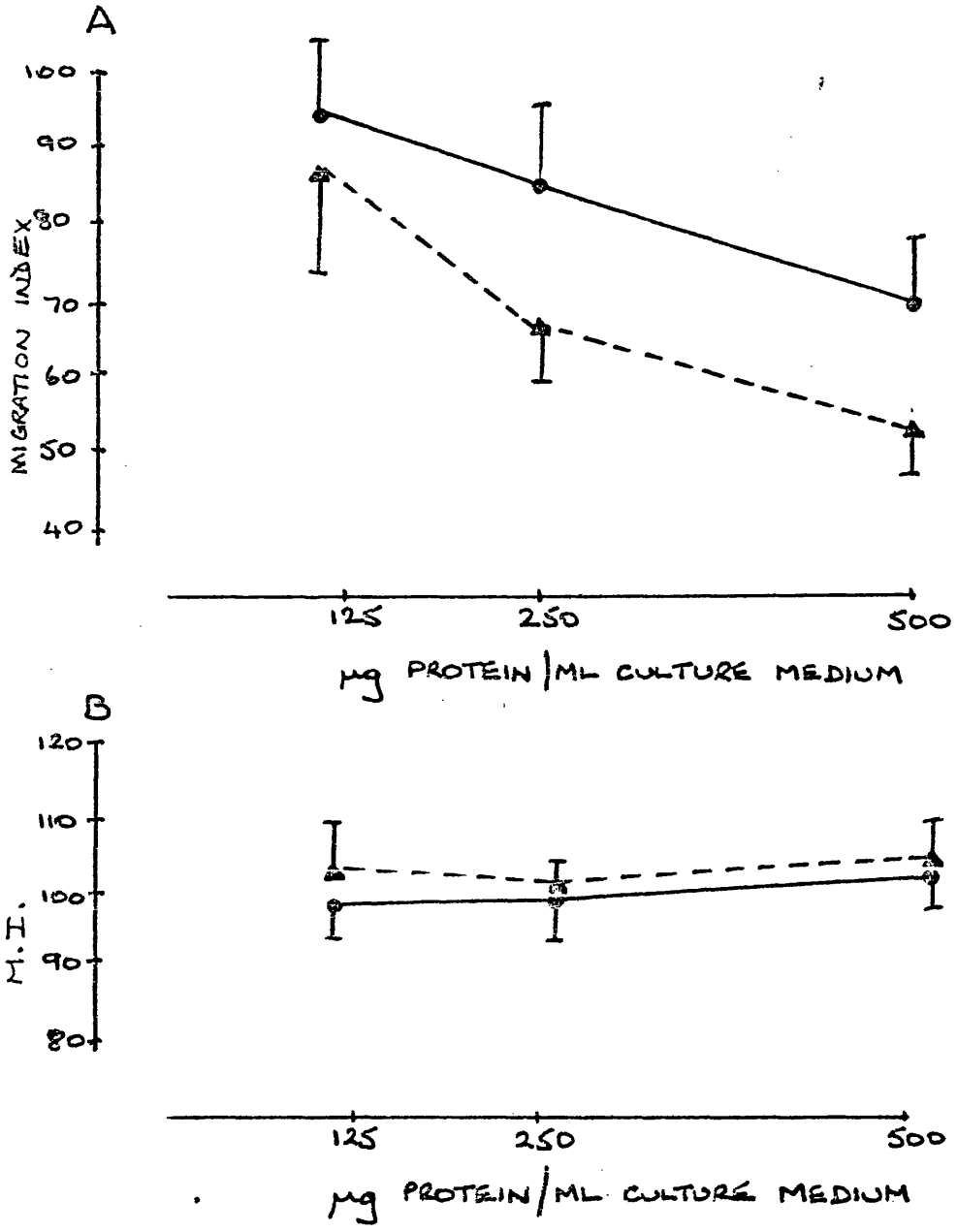

A
100
90
80
70
60
50
40
MIGRATION INDEX
125
250
500
µg PROTEIN/ML CULTURE MEDIUM
B
120
110
100
90
80
M.I.
125
250
500
µg PROTEIN/ML CULTURE MEDIUM

Fig: 13

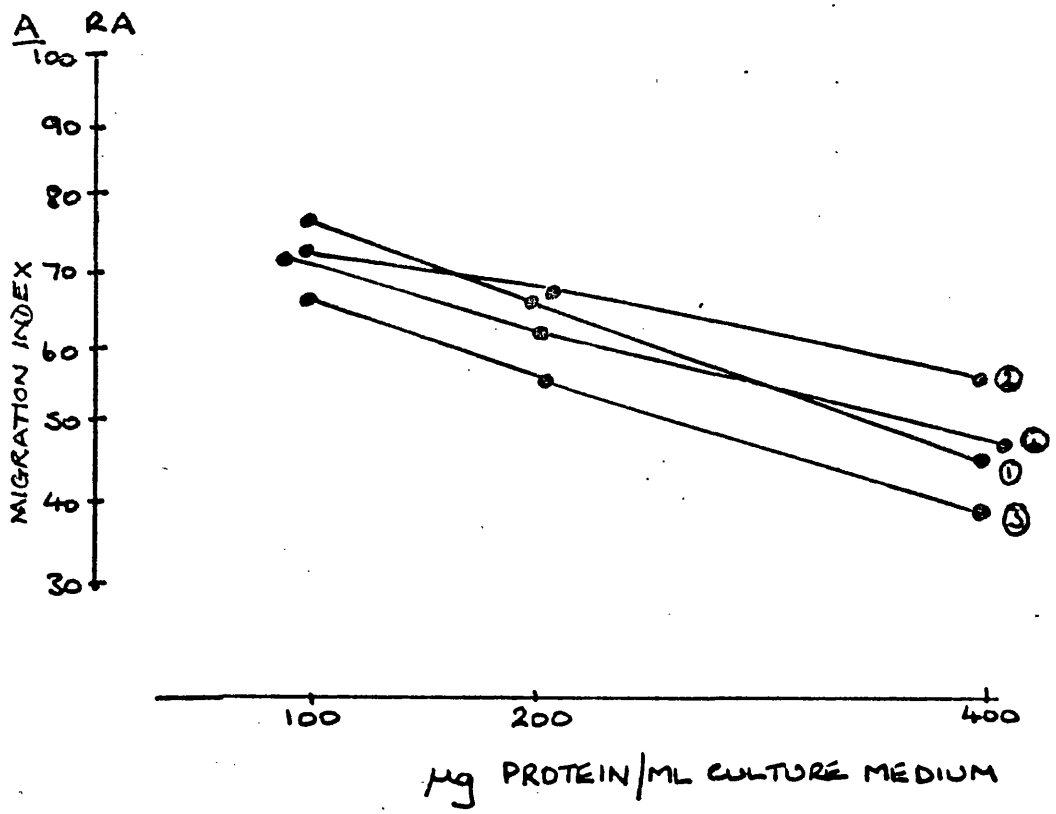
Figure 14 :-

DOSE RESPONSE OF NM RHEUMATOID SYNOVIAL HOMOGENATES SUBJECTED TO LOW SPIN.

(A) The mean response of four RA patients ●——● to 400, 200 and 100 µg/ml on the 1.8.75 (1), 11.8.75 (2), 18.8.75 (3), and the 2.9.75 (4).

(B) The mean response of four age and sex matched controls ▲- - - - - -▲ to 400, 200 and 100 µg/ml on the 1.8.75 (1), 11.8.75 (2), 18.8.75 (3) and the 2.9.75 (4).

No significant difference between RAs and controls run at the same time was found.


A RA
MIGRATION INDEX
100
90
80
70
60
50
40
30
100
200
400
µg PROTEIN/ML CULTURE MEDIUM

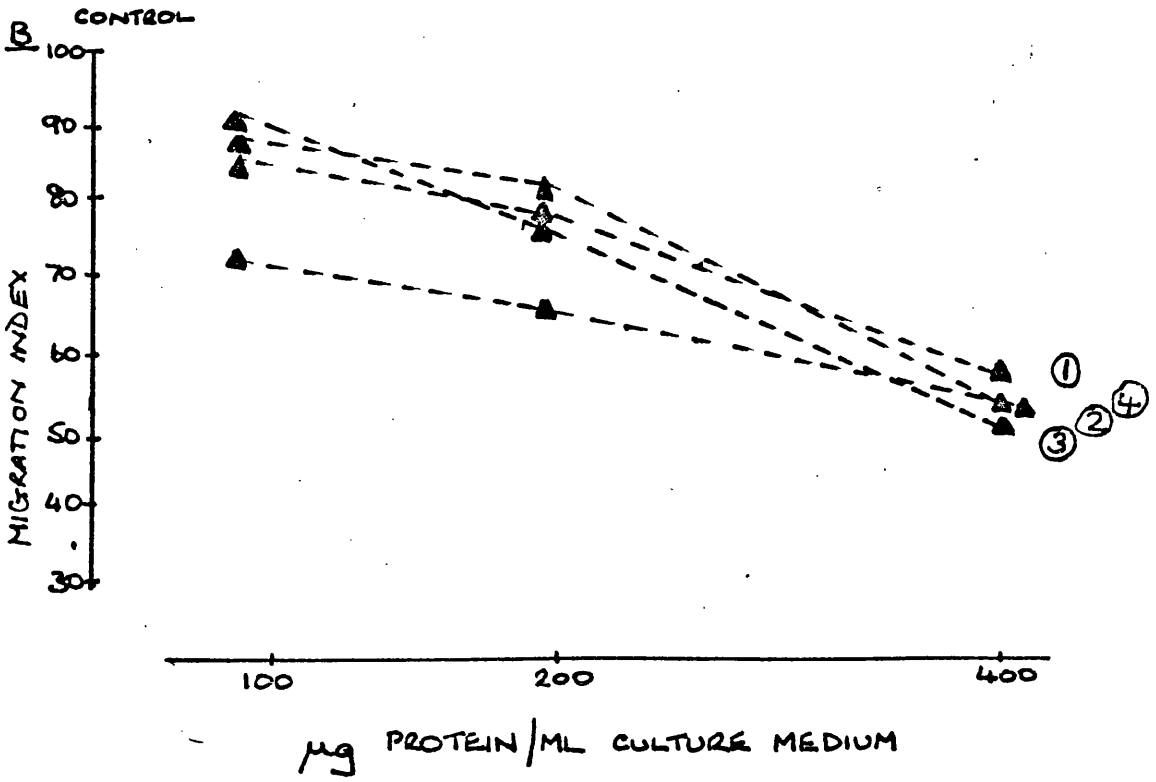

B CONTROL
MIGRATION INDEX
100
90
80
70
60
50
40
30
100
200
400
µg PROTEIN/ML CULTURE MEDIUM

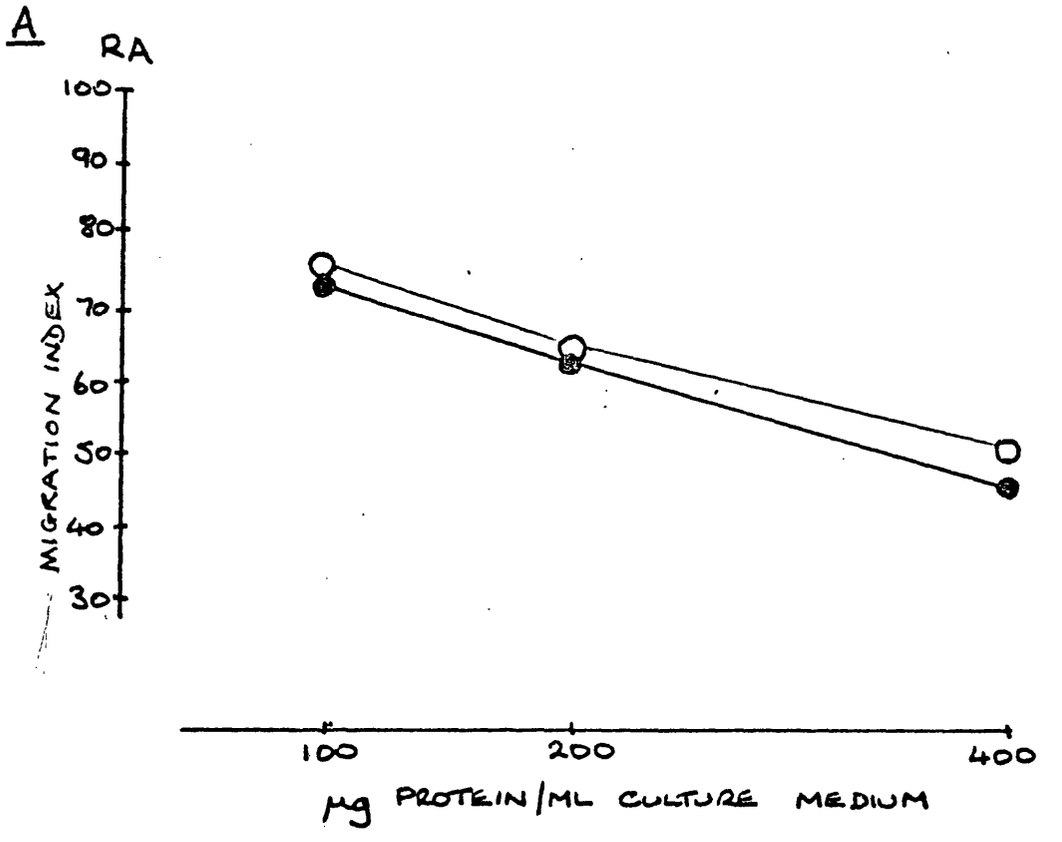
Fig: 14

Figure 15 :-

DOSE RESPONSE OF MV RHEUMATOID SYNOVIAL HOMOGENATES SUBJECTED TO LOW SPIN, THE PROTEIN SOLUTIONS EITHER WITH OR WITHOUT GLYCEROL TO PREVENT DENATURATION OF PROTEIN.

(A) The mean response of eight rheumatoid arthritis patients against crude homogenate with glycerol o——o , or without glycerol ●——●.

(B) The mean response of eight control subjects against crude homogenate with glycerol Δ- - - -Δ, or without glycerol ▲——▲.


A
RA
100
90
80
70
60
50
40
30
MIGRATION INDEX
100
200
400
µg PROTEIN/ML CULTURE MEDIUM

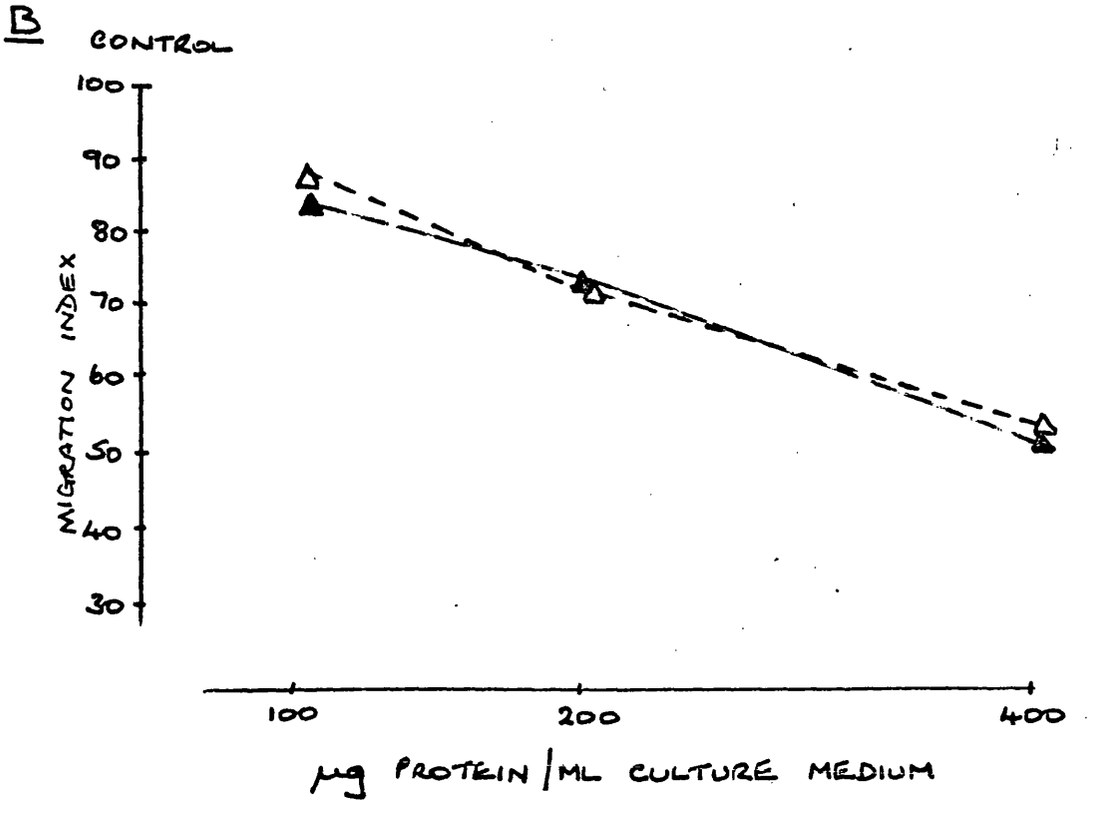

B
CONTROL
100
90
80
70
60
50
40
30
MIGRATION INDEX
100
200
400
µg PROTEIN/ML CULTURE MEDIUM

Fig: 15

Figure 16 :-

DOSE RESPONSE OF ALL THE LOW SPIN CRUDE RHEUMATOID SYNOVIAL HOMOGENATES AGAINST RHEUMATOID ARTHRITIS PATIENTS o———o AND CONTROL SUBJECTS Δ- - - - -Δ.

Each point is the mean of the number of observations indicated at the highest protein concentrations tested for each synovium.

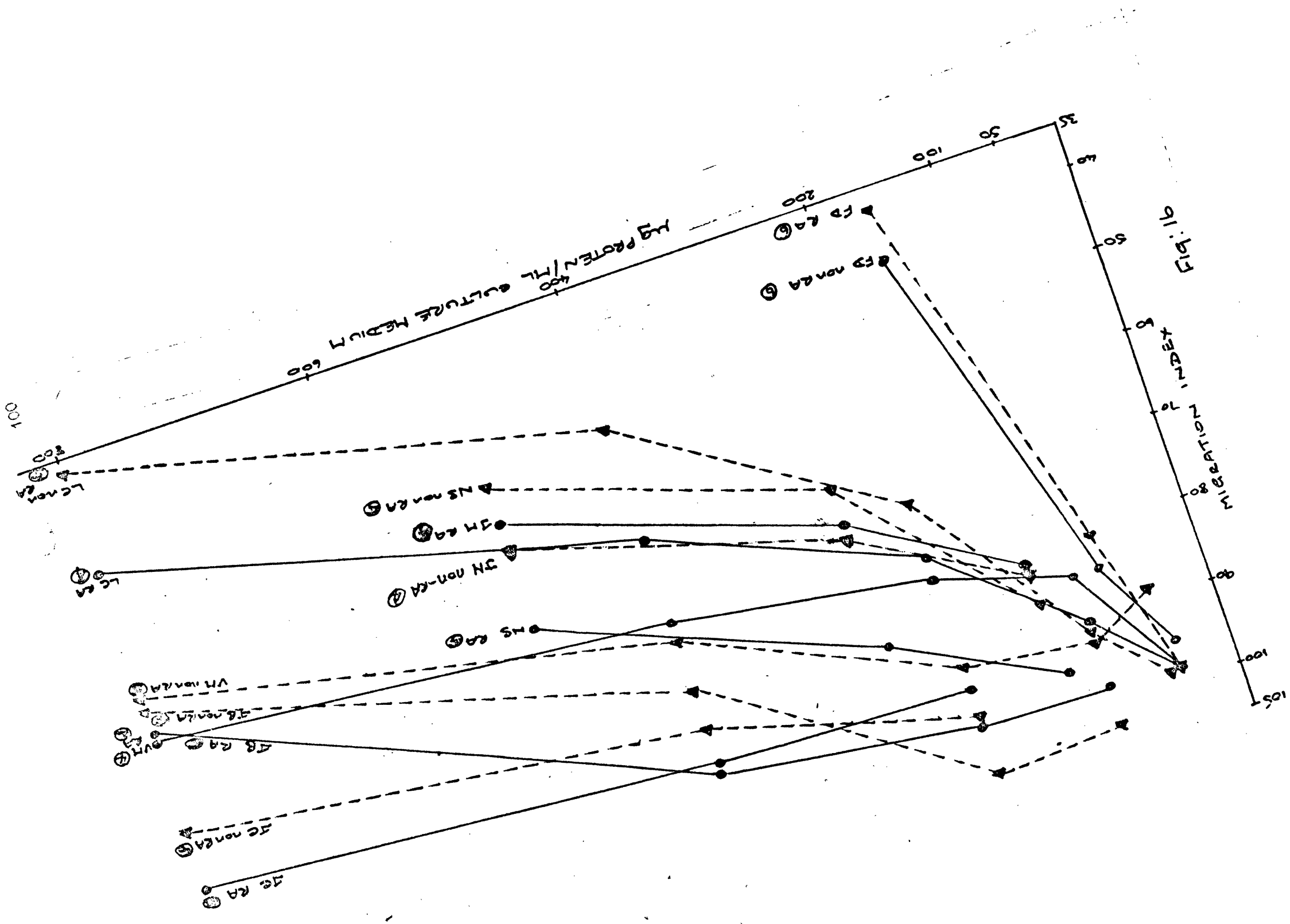

Migration Index
Fig. 1b
mg protein/mL cultured Medium
100
200
300
400
500
100
90
80
70
60
50
40
35

Figure 17 :-

DOSE RESPONSE OF ALL THE HIGH SPIN CRUDE RHEUMATOID SYNOVIAL HOMOGENATES AGAINST RHEUMATOID ARTHRITIS PATIENTS o——————o AND CONTROL SUBJECTS ▲- - - - - ▲.

Each point is the mean of the number of observations indicated at the highest protein concentration tested for each synovium.

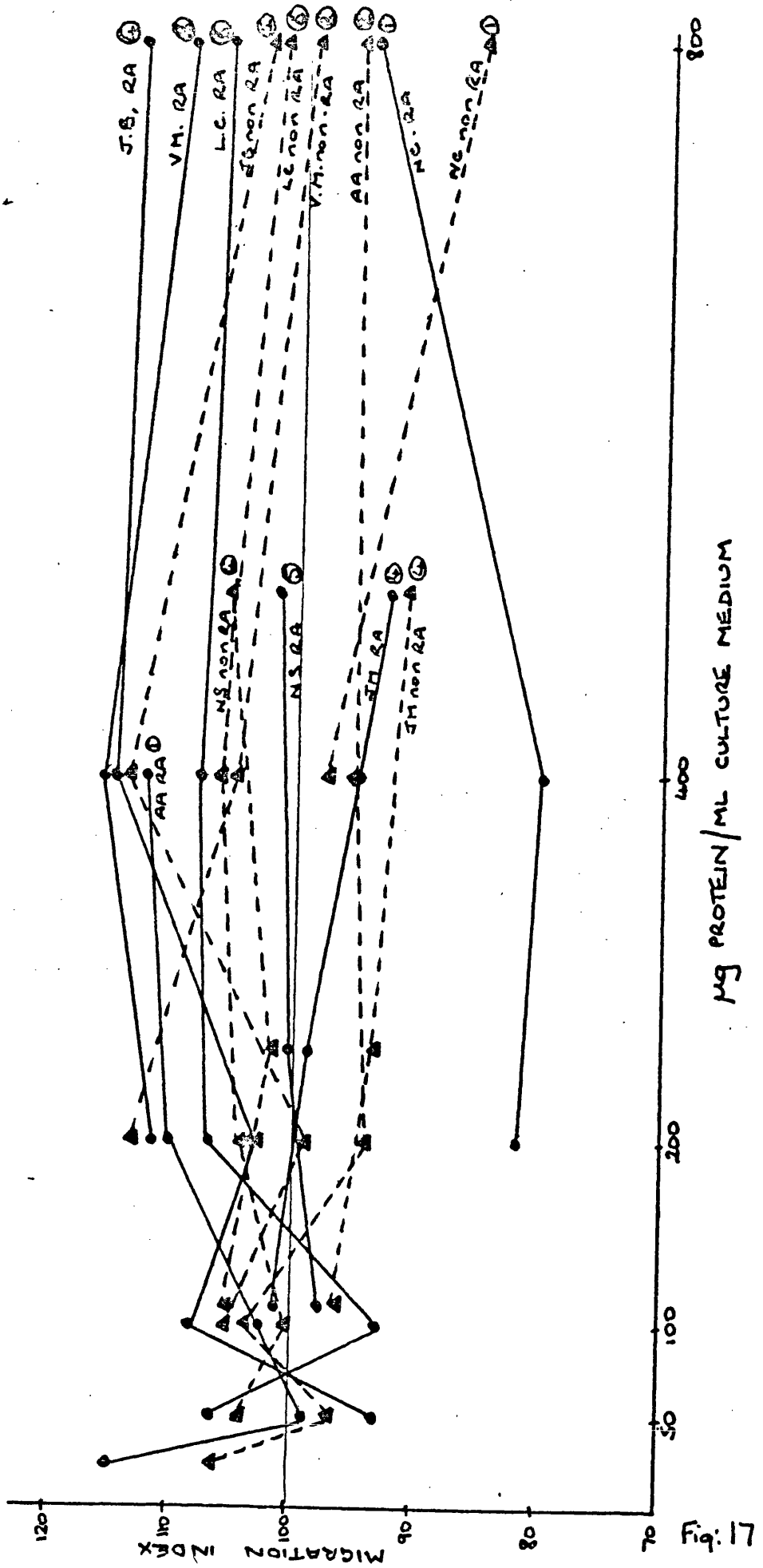

J.B. RA
V.H. RA
L.C. RA
L.C. non RA
V.H. non RA
AA non RA
N.S. non RA
N.S. RA
AA RA
TH RA
TH non RA
MIGRATION INDEX
120
110
100
90
80
70
50
100
200
400
800
µg PROTEIN/ML CULTURE MEDIUM

Fig: 17

SECTION IV - DISCUSSION

The results showed that in our hands rheumatoid synovial homogenates as prepared by Bacon et al (1973) were inhibitory in the LMT. However, in no instances were rheumatoid cells more inhibited than controls. That the inhibitory material was not soluble was shown in five cases (Figures 9 - 11).

It would seem therefore that the inhibitory material would be the membrane fragments or insoluble aggregates of protein. An attempt was made to resuspend the high speed pellet without success.

It has been shown that membrane fragments can still be immunogenic (Viza et al, 1972), and the possibility of absorbed IgG cannot be excluded, perhaps in a manner analogous to the adsorption of IgG by Mycoplasma spp (Williams et al, 1970).

The possibility exists that membranes could physically prevent the buffy cells from migrating by crosslinking. The indicator PMNs, if actively engaged in phagocytosis, might not migrate as far as in control cultures.

One cannot exclude the presence of immune complexes large enough to be spun out. These might cause non-specific lymphokine production (Muller, 1969), though in the light of other work immune complexes seem to cause specific production of MIF in rheumatoid lymphocytes (Eibl and Sitko, 1975).

SECTION V - CONCLUSION

When synovial homogenates are prepared as described in previous papers (Rothenberger and Thiele, 1971; Bacon et al, 1973) inhibition of migration of leucocytes in the LMT is achieved using these homogenates. The inhibitory material is not soluble.

The mechanism of inhibition will be more fully discussed in the light of further experiments.

CHAPTER 5

THE EFFECT OF CELL-MEMBRANE ELUATES ON RHEUMATOID AND CONTROL LEUCOCYTE MIGRATION IN THE LEUCOCYTE MIGRATION TEST

CHAPTER 5

THE EFFECT OF CELL-MEMBRANE ELUATES ON RHEUMATOID AND CONTROL LEUCOCYTE MIGRATION IN THE LEUCOCYTE MIGRATION TEST.

SECTION I - INTRODUCTION

In the preceding chapter work was presented which showed that homogenized rheumatoid synovium could cause inhibition of leucocyte migration in the LMT.

That inhibition of migration is not necessarily an expression of delayed hypersensitivity is known (Brostoff, 1974).

In the light of previously reported results (Bacon et al, 1973; Thonar and Sweet, 1976) it was decided to further investigate the possibilities of DH to synovial membranes in the rheumatoid patient. Attempts were made to extract protein by several methods, and to partially purify the inhibitory fraction using the technique of column chromatography.

It was believed that if a cell-mediated immune reaction was occuring in the rheumatoid joint, then this was directed against altered cell surface proteins expressed on the cells of the synovial membrane. It is known that cells infected with viruses do have altered cell membranes due to the expression of either virus proteins or a virus-induced altered surface protein (Rothman and Lodish, 1977).

SECTION II - EDTA/ME

The initial method chosen was one used by Marchesi et al (1970) to extract spectrin: a protein component of erythrocyte membranes. It had also been used to extract HLA antigens (Verrier-Jones, 1973). The method employed mild non-denaturing conditions in an attempt to obtain the protein in its native conformation.

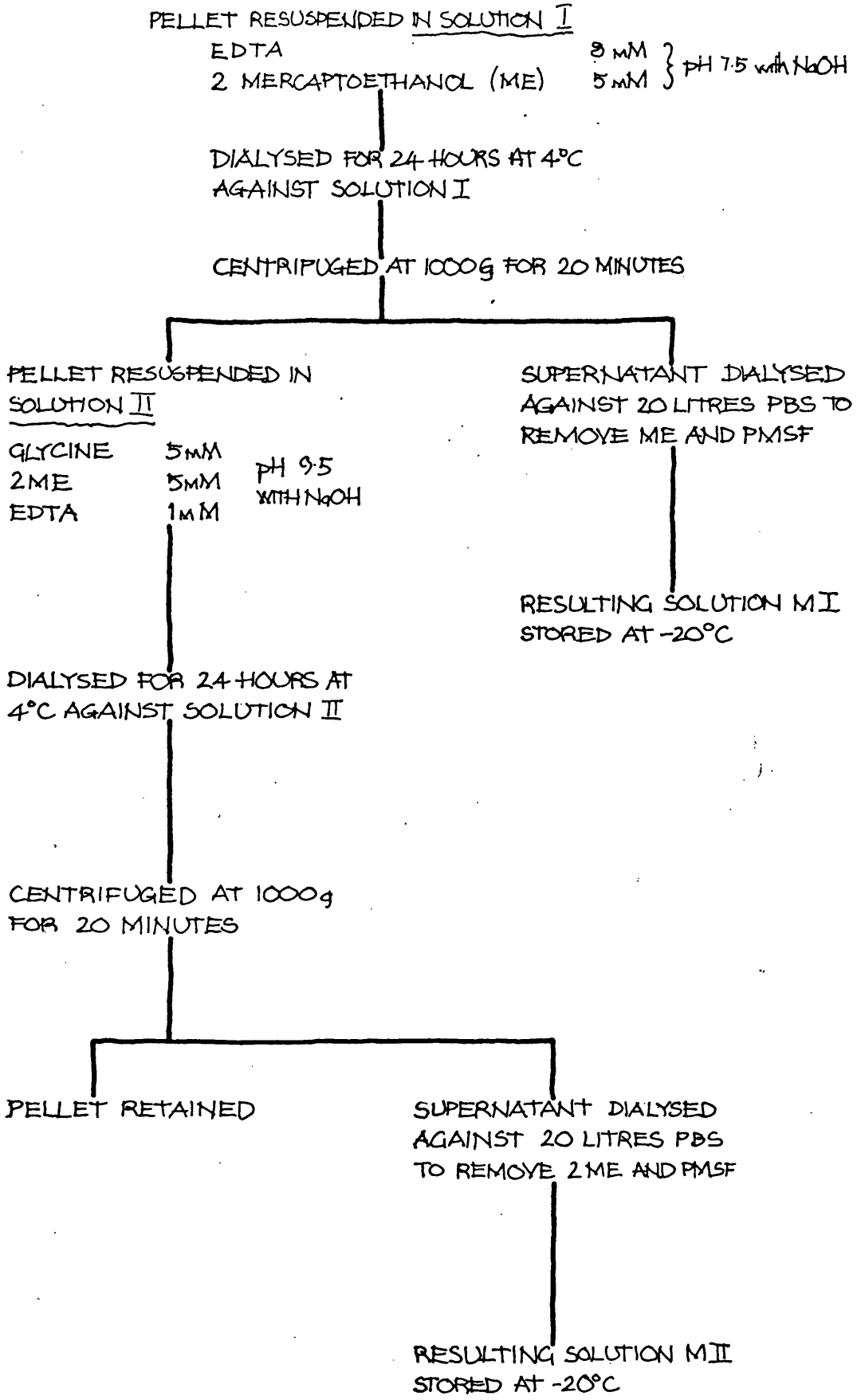
A. METHOD

Tissue obtained at synovectomy was kept in phosphate buffered saline (PBS) containing 2 mM phenylmethyl sulphonyl fluoride (PMSF) as protease inhibitor. After removal of fat and cartilage the tissue was subjected to homogenisation using an ILAX 1020 homogeniser.

The suspension was spun at 1000 g for 20 minutes and the resulting pellet washed three times and subjected to centrifugation. This pellet was then extracted by EDTA/ME (see Fig. 18).

After dialysis against PBS to restore isotonicity the solutions could be used in the LMT. The pH of the solution was 7.4. Marchesi et al (1970) states that the tendency towards aggregation is reduced at alkaline pH and in the presence of salt.

This procedure is comparable with the hypotonic treatment and water extraction used by Bacon et al (1973).



ELUTION PROCEDURE

Fig 18

SECTION III - CHOLATE SOLUBILISATION

About 90-95% of lymphocyte membrane protein is soluble in cholate solution. The resulting solution contained the same relative amounts of all the major polypeptide chains as the original membrane dissolved in sodium dodecyl sulphate (Chauvin and Holliman, 1975).

Neither deoxycholate (Crumpton and Parkhouse, 1972) or cholate (Chauvin and Holliman, 1975) significantly affects the antigenicity of immunoglobulin.

Critical micellar concentration of deoxycholate is approximately 10 mM (0.39%^{W}/v) whereas cholate is 45 mM (1.89%^{W}/v). Therefore a higher concentration of cholate is needed than deoxycholate. However, the molecular weight of cholate (819) is lower than that of deoxycholate (1963). Therefore cholate is more rapidly removed by dialysis. Solutions of cholate do not gel and precipitate as do those of deoxycholate.

A. MATERIALS

phosphate buffered saline

sodium chloride	8.00g/litre
potassium chloride	0.20g/litre
di-sodium hydrogen phosphate	1.15g/litre
potassium dihydrogen phosphate	0.20g/litre

21% ^{W}/v cholate solution: prepare a stock solution of 21% ^{W}/v cholic acid solution and, when ready for use, adjust the pH to 7.4 at 21^{o}C using a 6M-NaOH solution.

B. METHOD

The tissue collected at synovectomy was kept at 4^{o}C in PBS. After homogenisation with ILAX 1020 the resulting cell suspension was spun at 20,000g for 1 hour. The pellet was extracted by the process shown in Fig. 19.

The method was also carried out using the cholate solutions without the pellet. This is referred to as the cholate blank.

PELLET RESUSPENDED: 5 mls 1·5 M TRIS pH 7·4

ADD 5 mls 2% W/V CHOLATE SOLUTION pH 7·4

STIR AT ROOM TEMPERATURE FOR 30 MINUTES

ADD 45 mls DISTILLED WATER

STIR AT 4°C OVERNIGHT

SPIN AT 20,000 g FOR 1 HOUR

DIALYSE AGAINST 4 litres of ISOTONIC SUCROSE

SPIN AT 20,000 g FOR 1 HOUR
RESULTING SUPERNATANT STORED AT −20°C

CHOLATE SOLUBILISATION

Fig 19

SECTION IV - TRITON x 100 SOLUBILISATION

Although bile salts are easier to remove than non-ionics e.g. TRITON x 100, they are not always as mild in their effects on proteins (Helenius and Simons, 1971).

It has been shown that TRITON x 100 (p-octyl phenoxy-poly oxyethylene) can be removed from solutions using BioBeads SM-2 down to a concentration of 0.01% (Holloway, 1973). The method of protein extraction used was that described by Melcher (1975).

A. MATERIALS

Preparation of BioBeads: Methanol (200 ml) was added to 30g BioBeads SM-2 20-50 mesh at 37°C. The mixture was stirred for 15 minutes and the co-polymer beads were collected on a sintered glass funnel and washed with a further 500 mls of methanol. The beads were not allowed to dry, but were immediately washed with a further 1,000 ml glass distilled water. The moist beads were then slowly washed in a chromatography column with 2,000 mls of distilled water. They were stored under water until required.

B. METHOD

The tissue obtained at synovectomy was kept in PBS at 37°C, homogenised with the ILAX 1020 and spun at 20,000 g for 1 hour. This supernatant is comparable to that used in Chapter 3, Section II.

The resulting pellet was extracted as described in Fig. 20 (Holloway, 1973).

PELLET RESUSPENDED IN 10 mls 1% v/v TRITON X-100

INCUBATE AT 37°C FOR 80 MINUTES

ADD 10 mls PBS to give 0.5% TRITON X-100

SPIN AT 20,000g FOR 1 HOUR

TO SUPERNATANT ADD 4 gram BIOBEADS SM-2

STIR OVERNIGHT AT 4°C

FILTER OUT BEADS

CENTRIFUGE AT 20,000g FOR 1 HOUR
RESULTING SUPERNATANT STORED AT -20°C

TRITON X-100 SOLUBILISATION

Fig 20

SECTION V - COLUMN CHROMATOGRAPHY

A. SEPHADEX G-100

Sephadex G-100 was swollen in and equilibrated with 150 mM NaCl in 10 mM phosphate pH 7.4 (buffer 10/150).

90 x 1.5 cm column was poured and equilibrated with 10/150 at 4°C. The void volume was estimated using blue dextran 10/150. The flow rate was 8 ml/hour and 4 ml fractions were collected on an LKB Ultra rac 7000 overnight.

A 280 nm profile was recorded.

B. ULTRAGEL ACA_{22}

Ultragel was obtained pre-swollen and washed extensively with distilled water on a Büchner funnel to remove sodium azide (NaN_2). The washed gel was resuspended in 10/150 (see Section V, A) and a 90 x 1.5 cm column poured. The column was run at 8 mls/hour at 4°C and 4 ml fractions collected.

A 280 nm profile was recorded.

C. SEPHAROSE 6B

Sepharose was obtained pre-swollen and a 90 x 1.5 cm column poured. It was equilibrated in 10/150. The column was run at 8 mls/hour at 4°C and 4 ml samples collected.

A 280 nm profile was recorded.

D. GENERAL

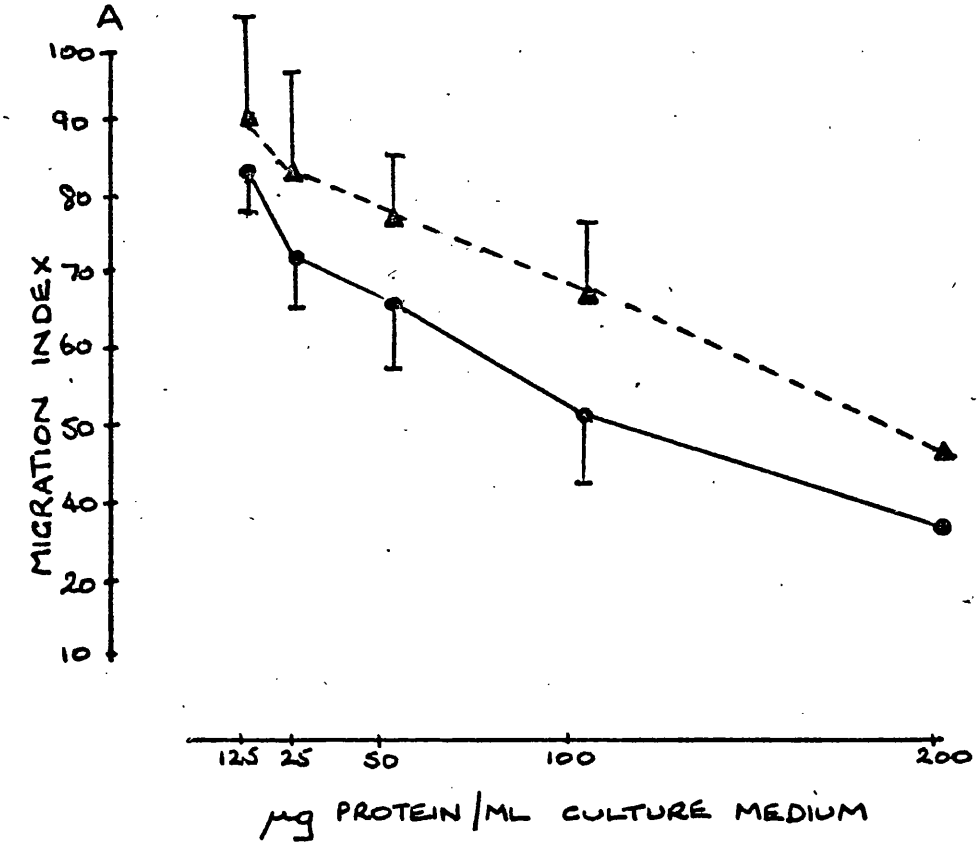
In all cases fractions were pooled as described in the text, and concentrated under nitrogen at 75 psi at 4°C over a Diaflo PM10 membrane (nominal exclusion weight 10,000 daltons.)

Figure 21 :-

DOSE RESPONSE OF EDTA/ME ELUATES OF RHEUMATOID SYNOVIUM (RS) ON MIGRATION OF LEUCOCYTES FROM PATIENTS WITH RHEUMATOID ARTHRITIS o——o AND AGE AND SEX MATCHED CONTROL SUBJECTS▲ - - - - - -▲.
MI (A) OR MII (B) ELUATES.

(A) At the dose of 50 μg protein/ml the mean of eleven rheumatoid responses is significantly different from the mean of eleven control responses $P < 0.01$. At 100 μg and 25 μg/ml the difference is not significant $P > 0.10$; the readings are the mean $\pm$ one SD of five observations.

(B) At the dose of 400, 200, 100, 50 and 25 μg/ml there is no significant difference between rheumatoid and control readings. The readings are the mean of a maximum of four observations.


A
MIGRATION INDEX
100
90
80
70
60
50
40
20
10
12.5
25
50
100
200
µg PROTEIN/ML CULTURE MEDIUM

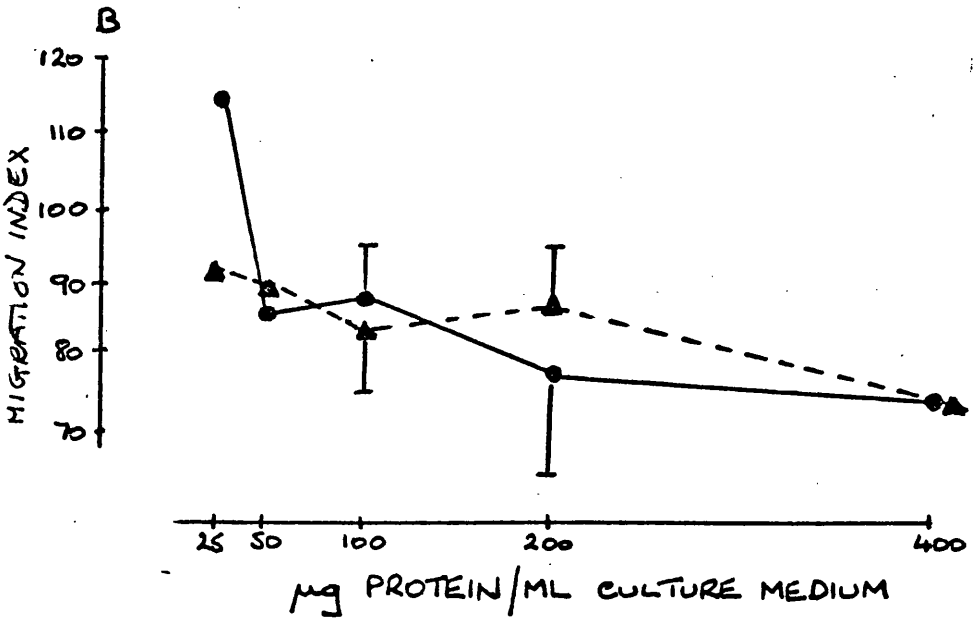

B
MIGRATION INDEX
120
110
100
90
80
70
25
50
100
200
400
µg PROTEIN/ML CULTURE MEDIUM

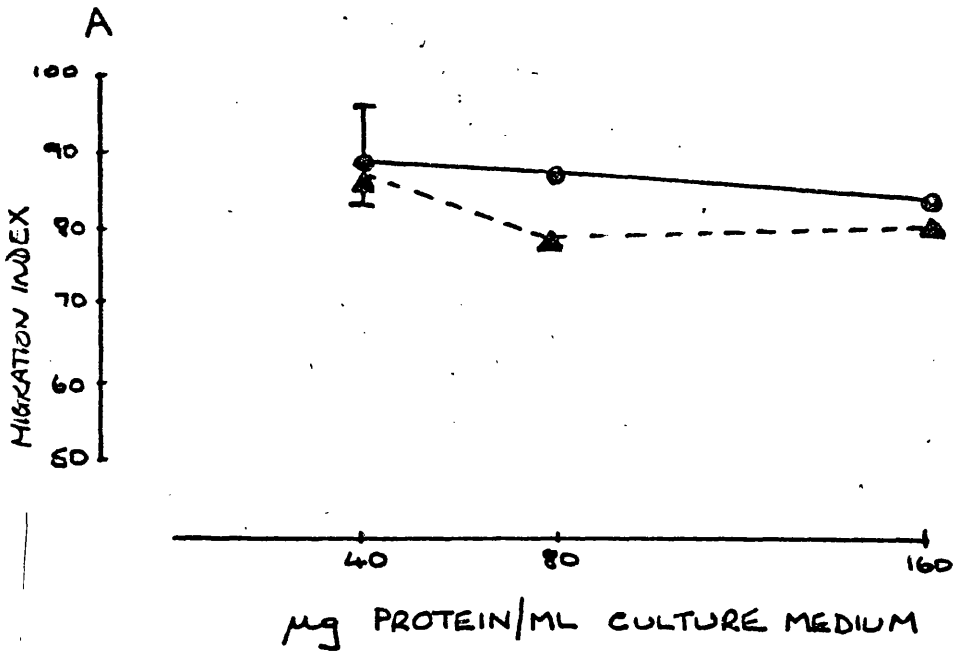
Fig: 21

Figure 22 :-

DOSE RESPONSE OF EDTA/ME ELUATES OF EH RHEUMATOID SYNOVIUM ON MIGRATION OF LEUCOCYTES FROM PATIENTS WITH RA ●——●AND AGE AND SEX MATCHED CONTROLS SUBJECTS ▲- - - - -▲. MI (A) OR MII (B).

(A) At 160, 80 and 40 μg/ml there is no significant difference between rheumatoid or control lymphocytes. The response is the mean of a maximum of four readings ± one SD.

(B) At 200, 100, 50 or 25 μg/ml there is no significant difference between rheumatoid or control lymphocytes. The response is the mean of a maximum of four readings ± one SD.


A
100
90
80
70
60
50
MIGRATION INDEX
40
80
160
µg PROTEIN/ML CULTURE MEDIUM

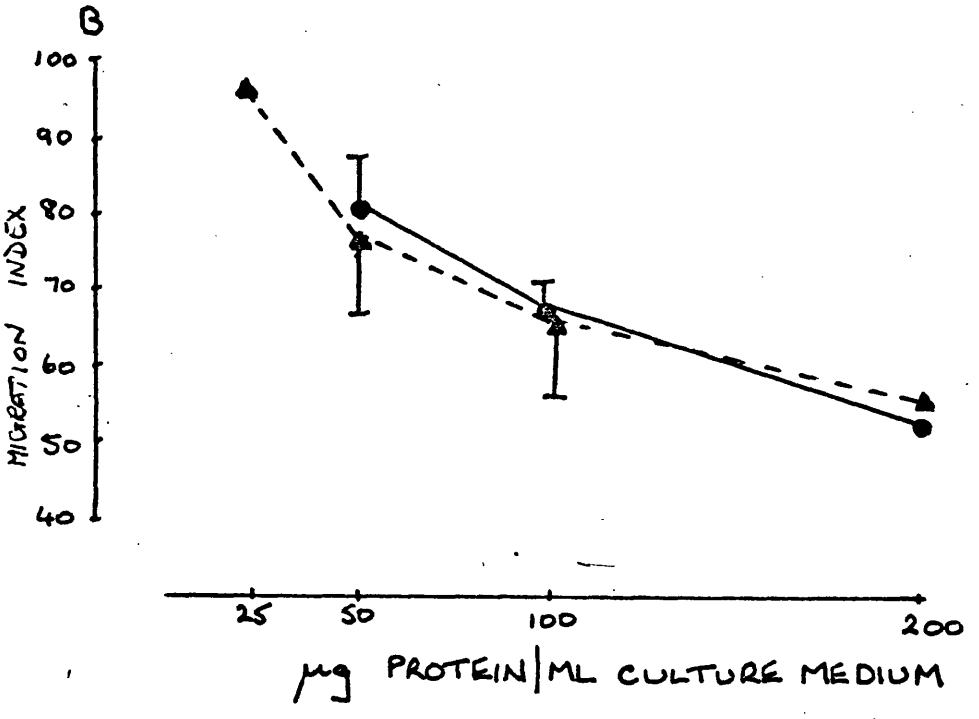

B
100
90
80
70
60
50
40
MIGRATION INDEX
25
50
100
200
µg PROTEIN/ML CULTURE MEDIUM

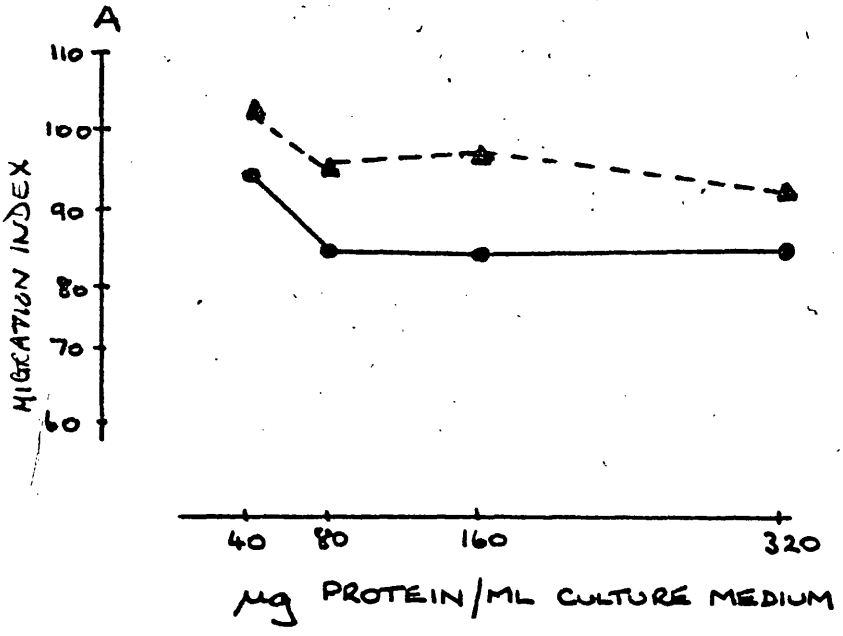
Fig: 22

Figure 23 :-

DOSE RESPONSE OF EDTA/ME ELUATES OF MA RHEUMATOID SYNOVIUM ON MIGRATION OF LEUCOCYTES FROM PATIENTS WITH RHEUMATOID ARTHRITIS o——o AND AGE AND SEX MATCHED CONTROL SUBJECTS Δ---Δ ON MII (B).

(A) At the dose of 320, 160, 80 and 40 μg/ml there is no significant difference between the responses of this mean of a maximum of three subjects' responses to MI eluate.

(B) At the dose of 720, 360, 180 and 90 μg/ml there is no significant difference between the response of the means of a maximum of three subjects' responses to MII eluate.


A
110
100
90
80
70
60
MIGRATION INDEX
40
80
160
320
µg PROTEIN/ML CULTURE MEDIUM

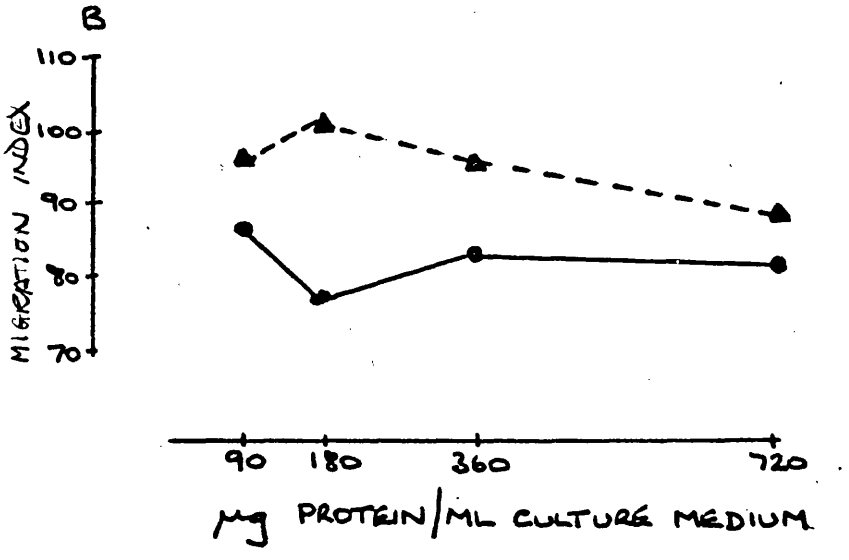

B
110
100
90
80
70
MIGRATION INDEX
90
180
360
720
µg PROTEIN/ML CULTURE MEDIUM

Fig: 23

Figure 24 :-

DOSE RESPONSE OF EDTA/ME ELUATES OF FM RHEUMATOID SYNOVIUM ON MIGRATION OF LEUCOCYTES FROM PATIENTS WITH RA o———o AND AGE AND SEX MATCHED CONTROL SUBJECTS ▲- - - - -▲.

(A) At the dose of 200, 100, 50 and 25 μg/ml there is no significant difference between the means of eleven RA patients and eleven control subjects.

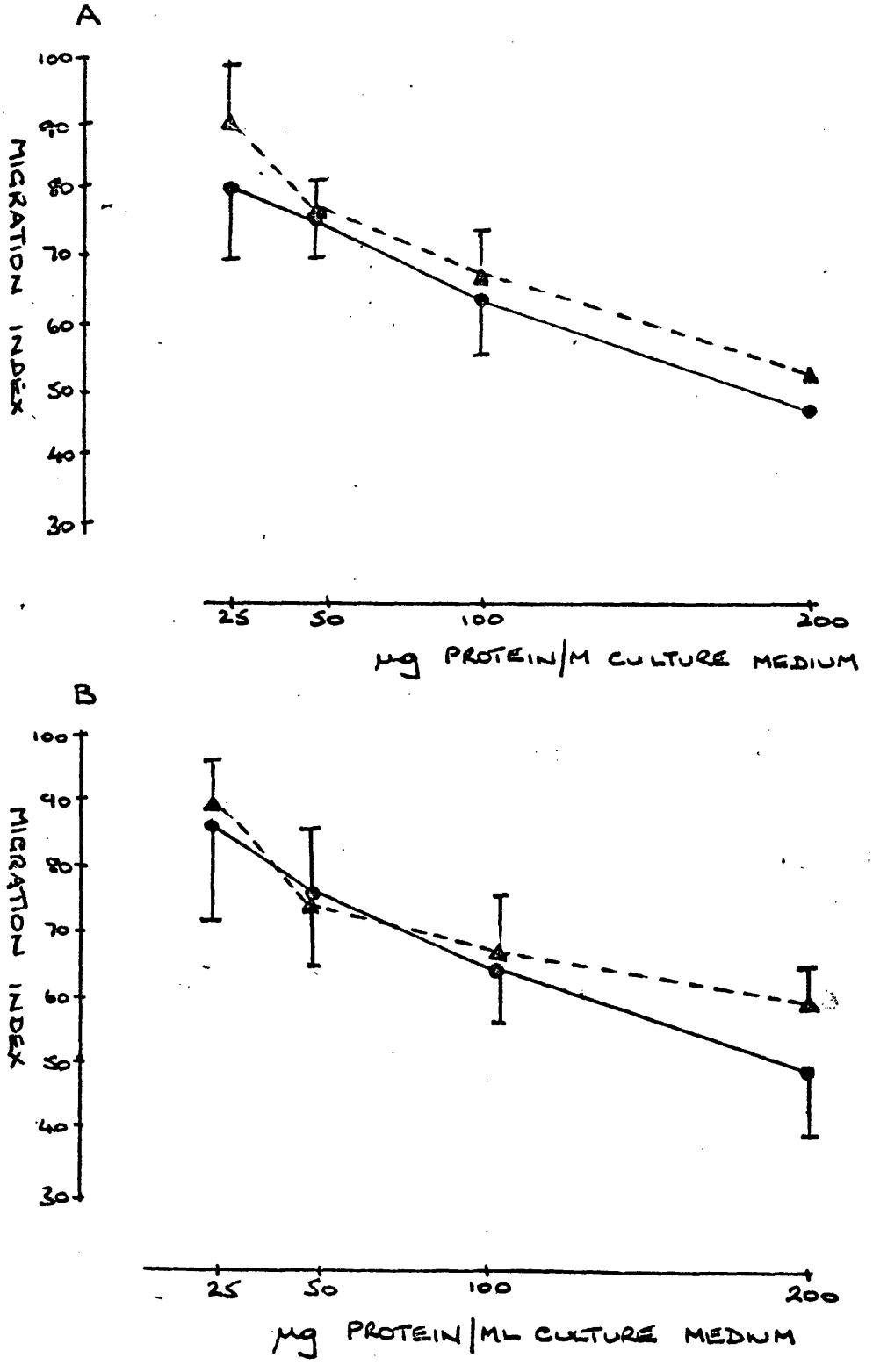
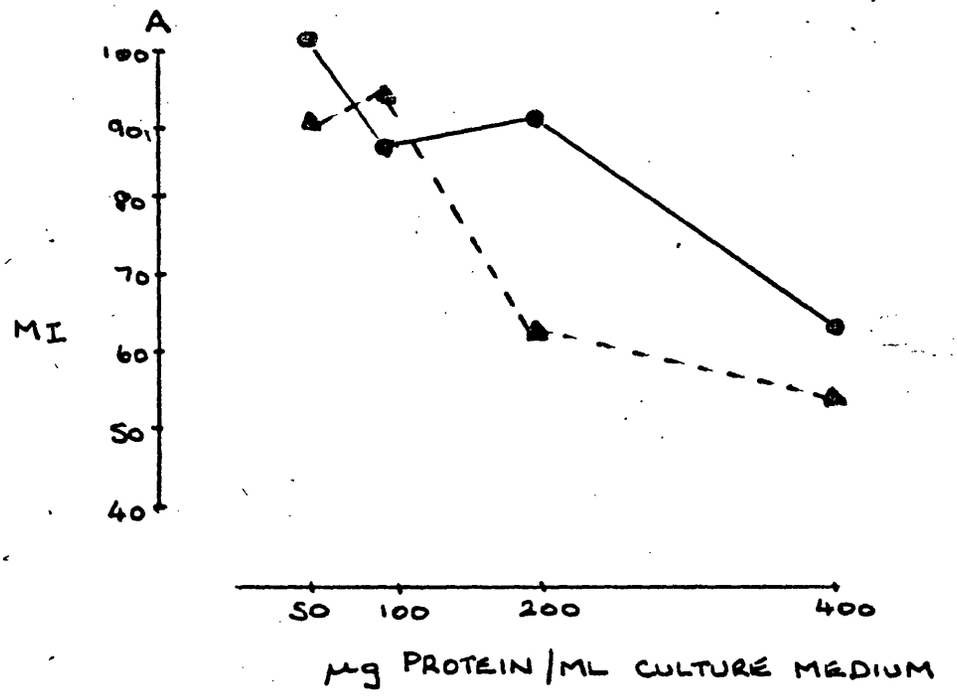

A
MIGRATION INDEX
100
90
80
70
60
50
40
30
25
50
100
200
µg PROTEIN/M CULTURE MEDIUM
B
MIGRATION INDEX
100
90
80
70
60
50
40
30
25
50
100
200
µg PROTEIN/ML CULTURE MEDIUM

Fig: 24

Figure 25 :-

DOSE RESPONSE OF EDTA/ME ELUATES OF RHEUMATOID SYNOVIUM III(A) AND III(B) AGAINST ONE RHEUMATOID o——o AND ONE AGE AND SEX MATCHED HEALTHY CONTROL A- - - - - A AFTER 100,000 g FOR ONE HOUR, INDICATING THAT THE SOLUTION IS STILL INHIBITORY.


A
100
90
80
70
60
50
40
MI
50
100
200
400
µg PROTEIN/ML CULTURE MEDIUM

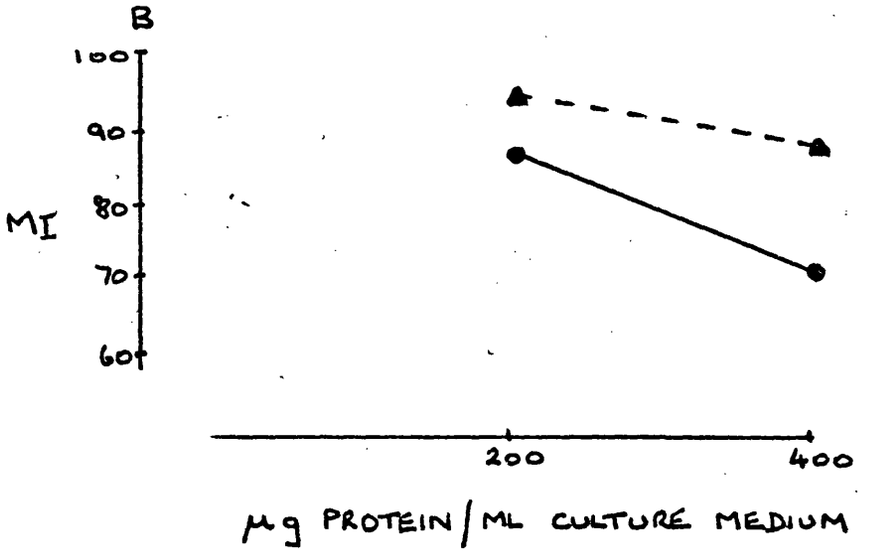

B
100
90
80
70
60
MI
200
400
µg PROTEIN/ML CULTURE MEDIUM

Fig: 25

Figure 26 :-

DOSE RESPONSE OF EDTA/ME ELUATE OF GR RHEUMATOID SYNOVIUM AGAINST RHEUMATOID ●——● AND AGE AND SEX MATCHED CONTROL SUBJECTS' ▲- - - - - ▲ LEUCOCYTES IN THE LMT.

At 100 μg, 50 μg and 25 μg/ml there was no significant difference between the RA and control responses. Each point represents the mean of four subjects' responses.

At 200 μg the control population is more inhibited than the rheumatoid $P < 0.005$.

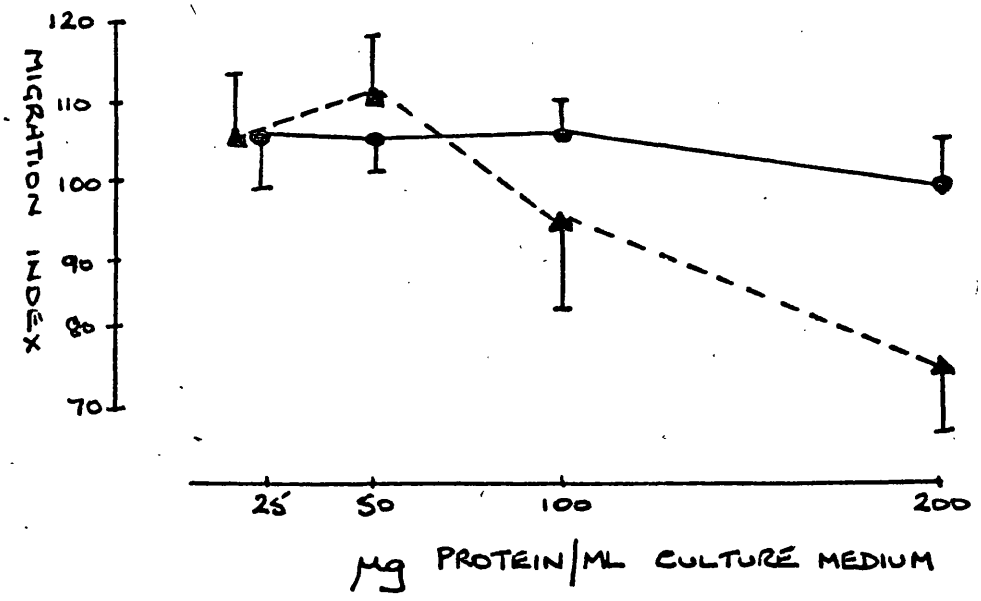

120
110
100
90
80
70
MIGRATION INDEX
25
50
100
200
µg PROTEIN/ML CULTURE MEDIUM

Fig: 26

Figure 27 :-

DOSE RESPONSE OF EDTA/ME EXTRACTS OF LC RHEUMATOID SYNOVIUM AFTER 1,000 *g* FOR 20 MINUTES OR 100,000 *g* FOR 1 HOUR AGAINST RHEUMATOID o——o AND CONTROL A- - - - -Δ LEUCOCYTES.

(A) The low spin solution was inhibitory at 400 µg/ml after low speed spin. After high speed spin over half the protein is removed, and what remains is not inhibitory.

(B) The low spin solution was not inhibitory. Each point represents the mean of three or less readings.

The results in this Figure compare with those in Figure 25, where the protein concentration of the spun homogenate was adjusted to that of the original solution.

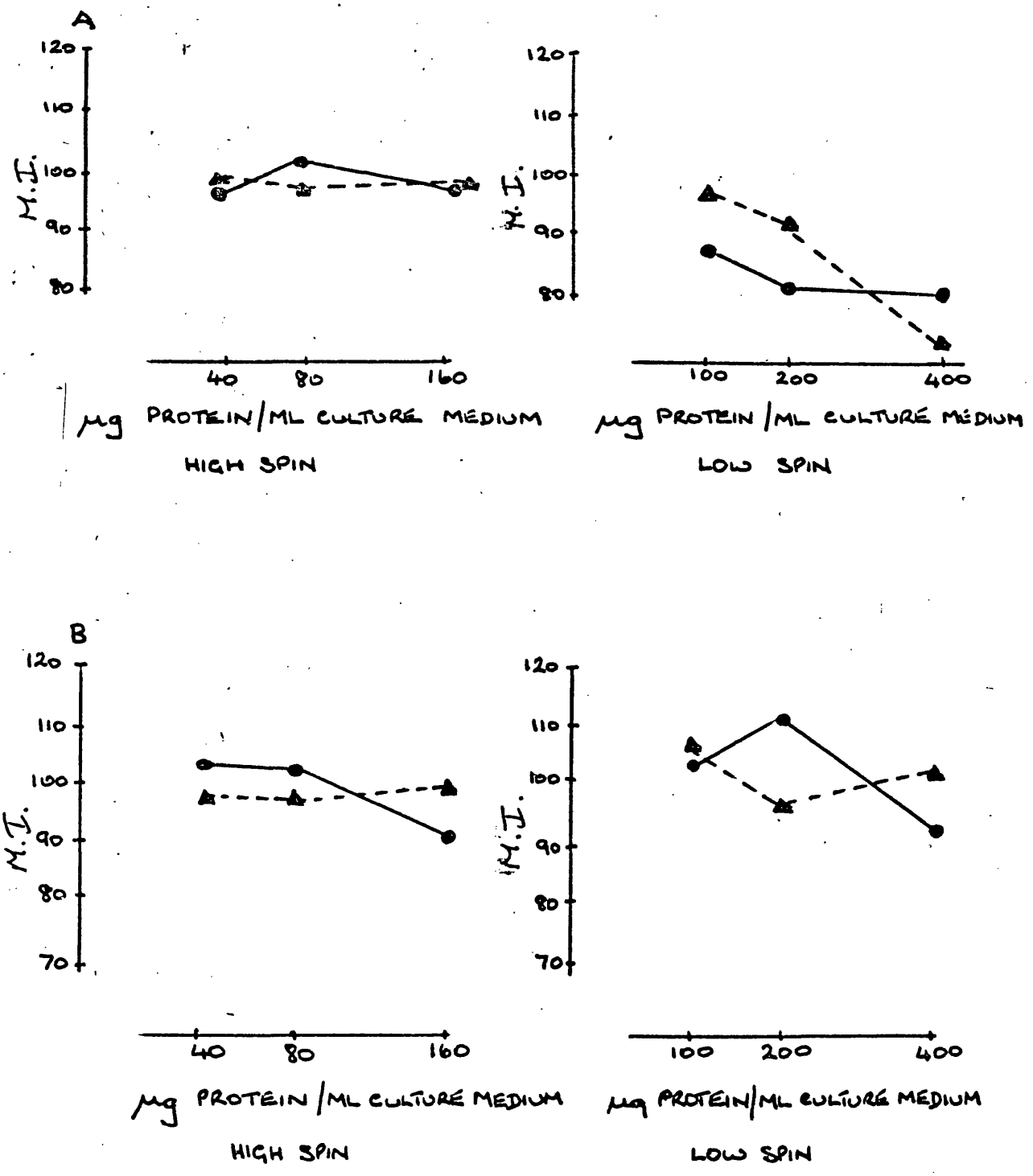

A
120
110
100
90
80
M.I.
40
80
160
μg PROTEIN/ML CULTURE MEDIUM
HIGH SPIN
120
110
100
90
80
M.I.
100
200
400
μg PROTEIN/ML CULTURE MEDIUM
LOW SPIN
B
120
110
100
90
80
70
M.I.
40
80
160
μg PROTEIN/ML CULTURE MEDIUM
HIGH SPIN
120
110
100
90
80
70
M.I.
100
200
400
μg PROTEIN/ML CULTURE MEDIUM
LOW SPIN

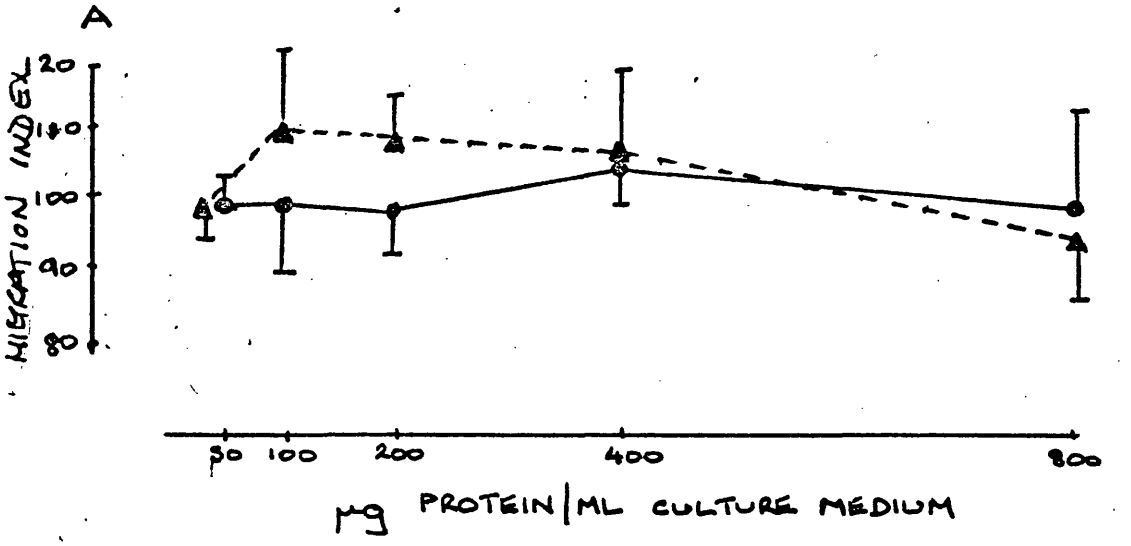
Fig: 2.7

Figure 28 :-

DOSE RESPONSE OF EDTA/ME ELUATES MI(A) AND MII(B) OF LD PIGMENTED VILLONODULAR SYNOVIAL SPECIMEN AGAINST RHEUMATOID ARTHRITIS PATIENTS o——o AND AGE AND SEX MATCHED CONTROL SUBJECTS Δ- - - -Δ IN THE LEUCOCYTE MIGRATION TEST.

No inhibition occurred even at high protein concentration.

Each point represents the mean of five subjects' responses $\pm$ one SD.


A
MIGRATION INDEX
120
110
100
90
80
50
100
200
400
800
µg PROTEIN/ML CULTURE MEDIUM

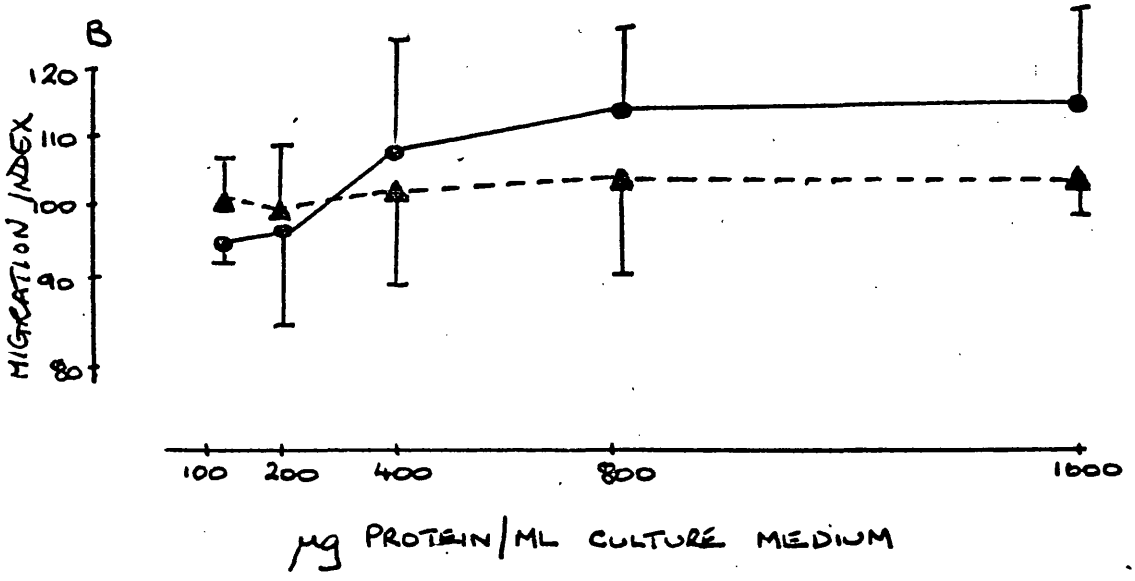
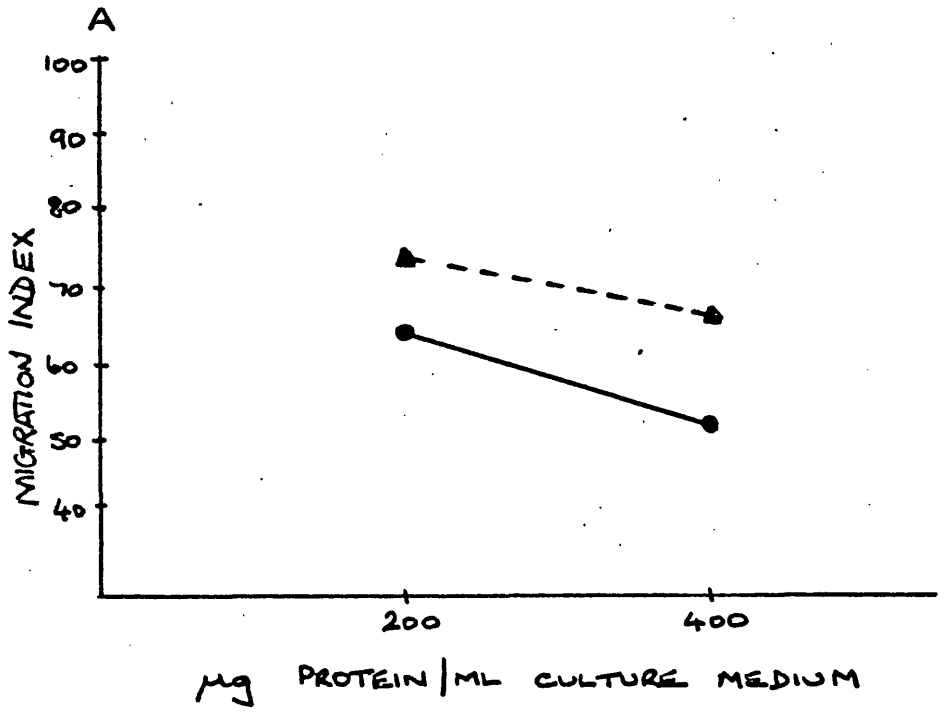

B
MIGRATION INDEX
120
110
100
90
80
100
200
400
800
1600
µg PROTEIN/ML CULTURE MEDIUM

Fig: 28

Figure 29 :-

DOSE RESPONSE OF EDTA/HCl ELUATES MI(A) AND MII(B)
OF DR ANKYLOSING SPONDYLITIC SYNOVIAL SPECIMENS
AGAINST RHEUMATOID ARTHRITIS PATIENTS ●————●
AND AGE AND SEX MATCHED CONTROL SUBJECTS ▲- - - -▲.

Each point is the mean of three subjects' response.


A
100
90
80
70
60
50
40
MIGRATION INDEX
200
400
µg PROTEIN/ML CULTURE MEDIUM

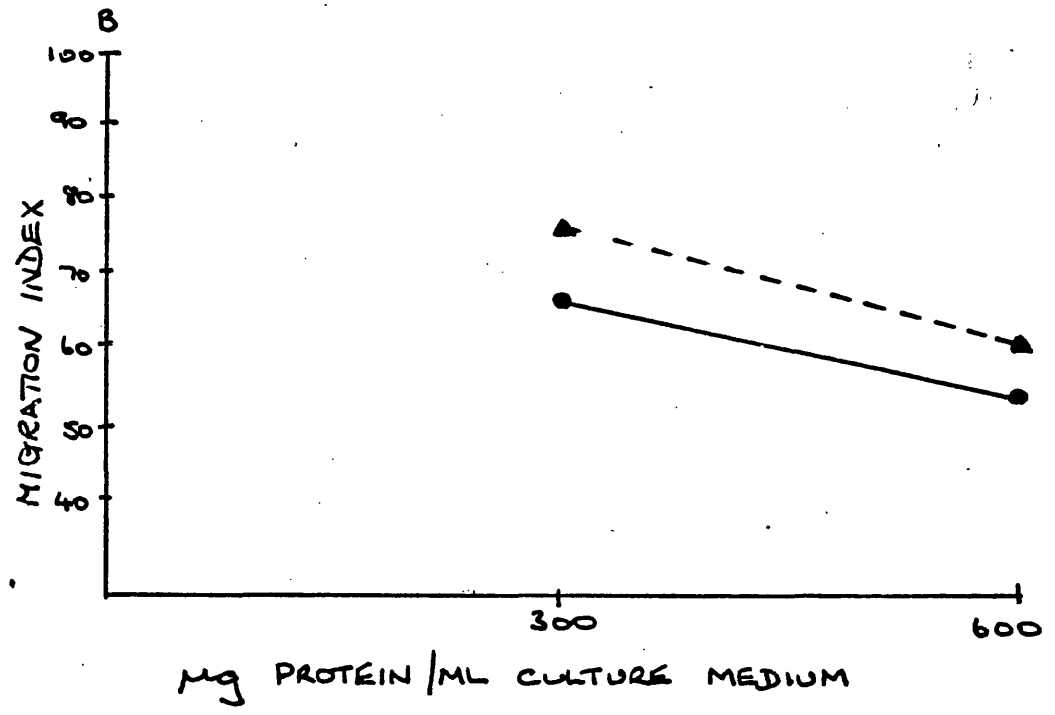

B
100
90
80
70
60
50
40
MIGRATION INDEX
300
600
µg PROTEIN/ML CULTURE MEDIUM

Fig: 29

Figure 30 :-

DOSE RESPONSE OF ALL THE EDTA/MEM MI ELUATES AGAINST RHEUMATOID ARTHRITIC PATIENTS' ●——●
AND AGE AND SEX MATCHED CONTROL SUBJECTS'
▲ - - - - - ▲ LEUCOCYTES IN THE LMT.

LD = pigmented villonodular synovitis
DR = ankylosing spondylitis
All others are rheumatoid arthritis synovial specimens.

The eluates from the RA and AS synovial specimens were inhibitory at 200 µg/ml except LC.

The figures in brackets are the number of observations made for each point.

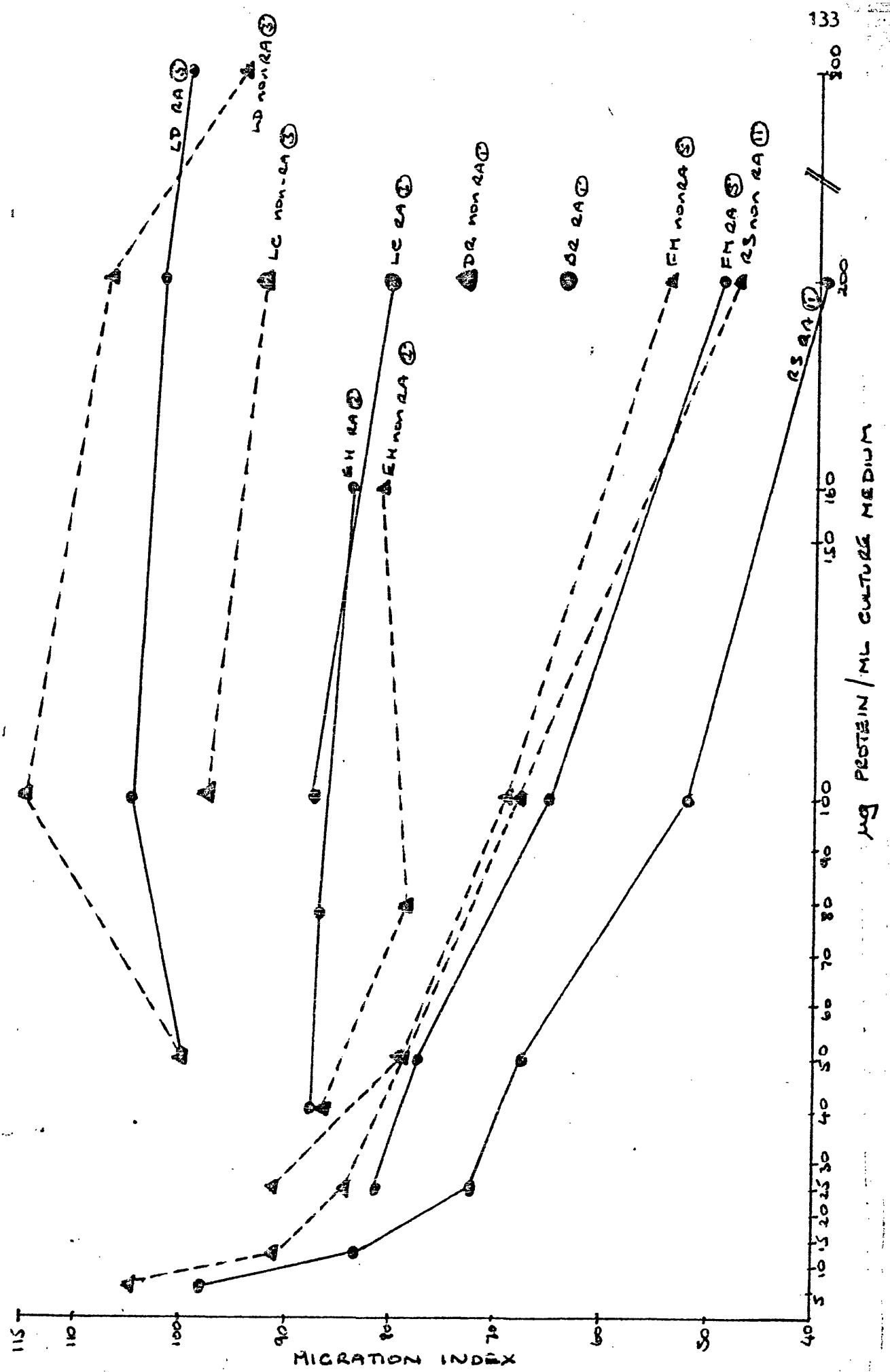

LD RA
LD non RA
LC non-RA
LC RA
EH RA
EH non RA
DR non RA
DR RA
FM non RA
FM RA
RS non RA
RS RA
MIGRATION INDEX
115
110
100
90
80
70
60
40
5 10 15 20 25 30 40 50 60 70 80 90 100 150 160 200 300
µg PROTEIN / ML CULTURE MEDIUM

Fig: 30

Figure 31 :-

DOSE RESPONSE OF ALL THE EDTA/ME MII ELUATES AGAINST RHEUMATOID ARTHRITIS PATIENTS' ●——● AND AGE AND SEX MATCHED CONTROL SUBJECTS' A- - - - - A LEUCOCYTES IN THE LMT.

LD = pigmented villonodular synovitis
All others are rheumatoid arthritis synovial specimens.

See Fig. 30.

LD RA ③
LD non RA ③
GR RA ④
RS non RA ②
RS RA ②
GR non RA ④
FM non RA ⑤
EH non RA ⑤
EH RA ⑤
FM RA ⑤
120
110
100
90
80
70
60
50
40
MIGRATION INDEX
1600
200
100
50
25
µg PROTEIN/ML CULTURE MEDIUM

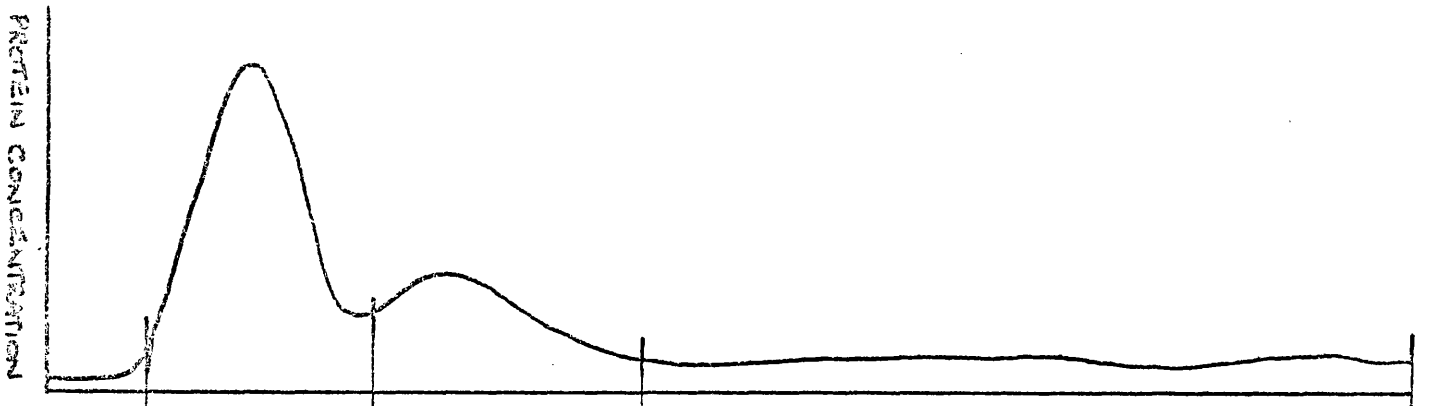
Fig: 31

Figure 32 :-

RESPONSE OF LEUCOCYTES FROM RHEUMATOID ARTHRITIC PATIENTS AND AGE AND SEX MATCHED CONTROLS SUBJECTS TO FRACTIONS OBTAINED FROM A SEPHADEX G-100 ANALYSIS OF RS EDTA/NE MI ELUATE IN THE LMT.

See Fig.21

The trace of protein concentration is obtained by absorbance at 280 nm.



APPROXIMATE MOLECULAR WEIGHT	>120,000	80,000–120,000	< 80,000
PROTEIN CONCENTRATION /ML MEDIA	50 µg	70 µg	
MEAN MIGRATION INDEX TESTING LEUCOCYTES FROM			
a) 6 RHEUMATOIDS ± 1 SD	64·0 ± 11	86·0 ± 10	96·0 ± 4
b) 6 CONTROLS ± 1 SD	82·0 ± 7	92·0 ± 4	92·0 ± 4
STUDENTS T TEST	P < 0·02	NS	NS

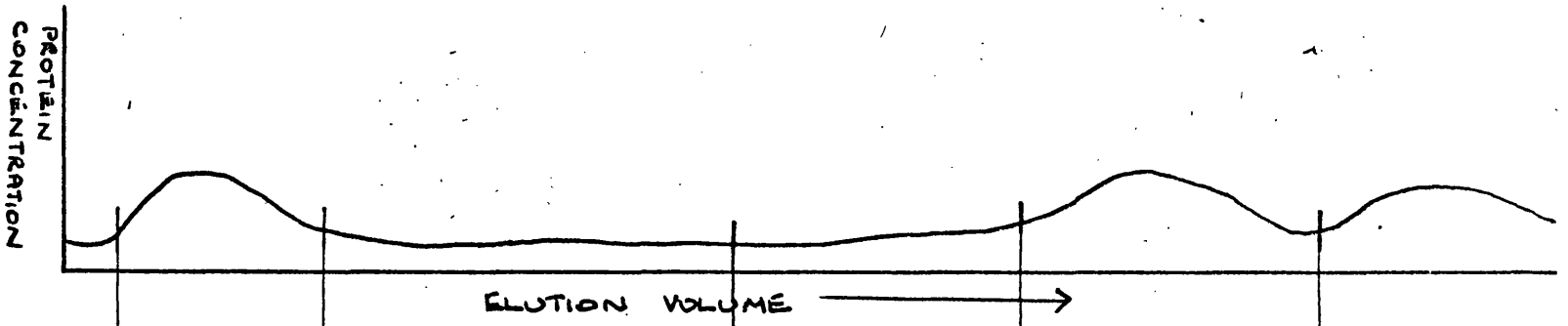
Fig. 22

Figure 33 :-

RESPONSE OF RHEUMATOID ARTHRITIC AND AGE AND SEX MATCHED CONTROL LEUCOCYTES IN THE LMT TO FRACTIONS OBTAINED FROM AN ULTRAGEL ACA22 ANALYSIS OF RS EDTA/ME MI ELUATE.

See Figure 32 and 21.

The trace of protein concentration is obtained by measurement of absorbance at 280 nm.



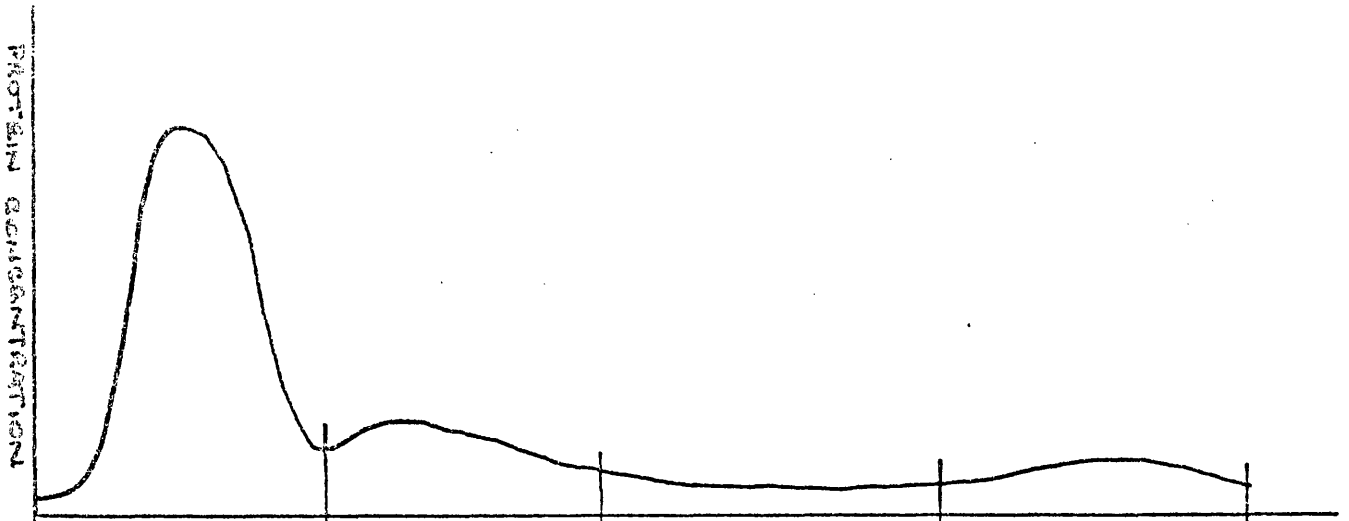
APPROXIMATE MOLECULAR WEIGHT	>800,000	400,000	150,000	100,000	60,000
PROTEIN CONCENTRATION /ML MEDIA	30µg	10µg	30µg	30µg	100µg
MEAN MIGRATION INDEX TESTING LEUCOCYTES FROM					
a) 6 RHEUMATOIDS ± 1SD	69.0±7	89.0 ± 12	88.0 ± 9	96.0 ±7	94.0 ±12
b) 6 CONTROLS ±1SD	65.0±5	89.0±5	80.0 ± 8	86.0±7	89.0 ±6
STUDENTS T TEST	NS	NS	NS	NS	NS

Fig: 33

Figure 34 :-

RESPONSE OF RHEUMATOID ARTHRITIC PATIENTS AND AGE AND SEX MATCHED CONTROL LEUCOCYTES IN THE LMT TO FRACTIONS OBTAINED FROM A SEPHADEX G-100 ANALYSIS OF EDTA/ME MII ELUATE FM RHEUMATOID SYNOVIAL SPECIMEN.

A known volume of the original solution 200 µg protein/ml culture medium was layered on the column and the fractions concentrated back to the original volume, thus the protein concentration is expressed as ≡ 200 µg.

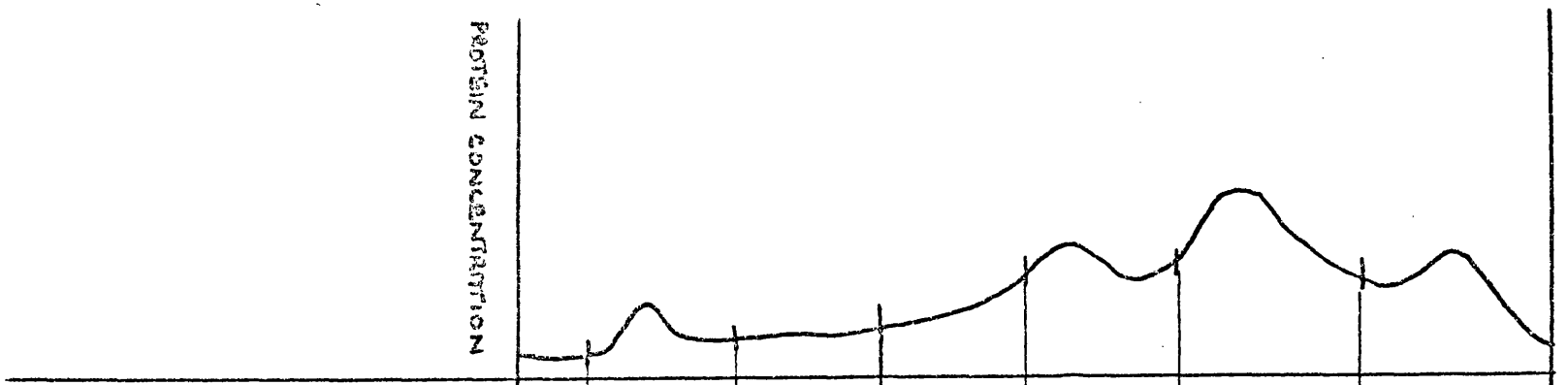


APPROXIMATE MOLECULAR WEIGHT	>120,000	80—120,000	10 - 80,000	<10,000	ORIGINAL SOLUTION 200 µg
PROTEIN CONCENTRATION / ML MEDIA.	= 200 µg	= 200 µg	= 200 µg	= 200 µg	
AGAR MIGRATION INDEX NEUTRAL LEUCOCYTES FROM					
2 RHEUMATOIDS	61.0±10; 79.0±6	80±1 80.0±2	97.0 104.0±4	91.0±7	47.0±10
2 CONTROLS	66.0±10, 77.0±3	82±2 76.0±15	102.0 91.0±8	94.0±4	61.0±4
STUDENTS T TEST	NS	NS	NS	NS	NS

Figure 35 :-

RESPONSE OF RHEUMATOID ARTHRITIC PATIENTS AND AGE AND SEX MATCHED CONTROL LEUCOCYTES IN THE LMT. TO FRACTIONS OBTAINED FROM AN ULTRAGEL ACA22 ANALYSIS OF EDTA/ME MI ELUATE FM RHEUMATOID SYNOVIAL SPECIMEN.

See Figure



APPROXIMATE MOLECULAR WT	800,000	400,000	200,000	150,000	100,000	60,000
PROTEIN CONC./ML MEDIA	28 µg	18 µg	33 µg	53 µg	146 µg	20 µg
MEAN MIGRATION INDEX TESTING LEUCOCYTES FROM						
a) 2 RHEUMATOIDS	83·5 ± 5	93·0 ± 4	94·5 ± 2	91·0 ± 4	95·0 ± 0	99·0 ± 1
b) 2 CONTROLS	82·5 ± 6	88·5 ± 13	93·5 ± 2	92·0 ± 0	99·0 ± 1	97·0 ± 6
STUDENT T TEST	NS	NS	NS	NS	NS	NS

Fig. 35

Figure 36 :-

DOSE RESPONSE OF HIGH SPIN CRUDE HOMOGENATE OF AA RHEUMATOID SYNOVIAL SPECIMENS AGAINST ONE RHEUMATOID SUBJECT.

There was no inhibition at any dose levels.

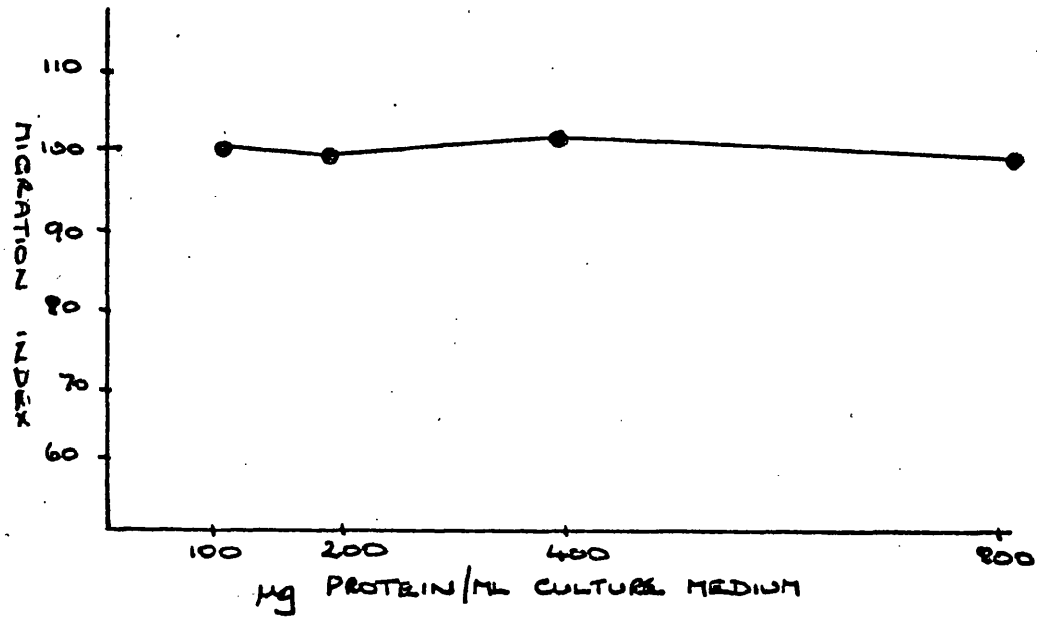
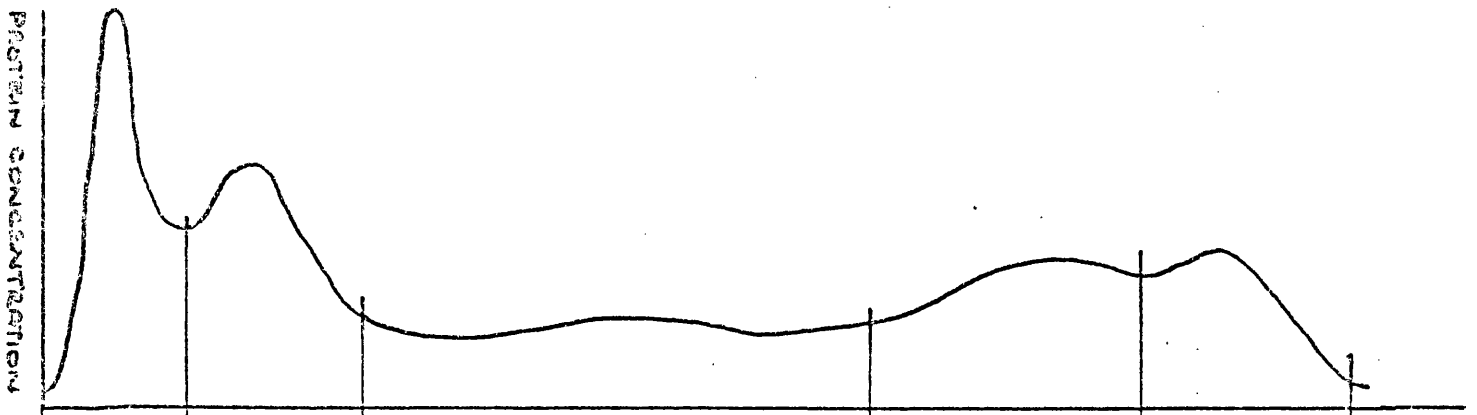

110
100
90
80
70
60
MIGRATION INDEX
100
200
400
800
μg PROTEIN/ML CULTURE MEDIUM

Fig: 36

Figure 37 :-

RESPONSE OF RHEUMATOID ARTHRITIC AND CONTROL LEUCOCYTES TO FRACTIONS OBTAINED FROM A SEPHADEX G-100 ANALYSIS OF HIGH SPIN CRUDE HOMOGENATE OF AA RHEUMATOID SYNOVIUM.

The trace obtained measuring absorbance at 280 nm is broadly similar to RS MI eluate, Fig. 32, but AA is not inhibitory.



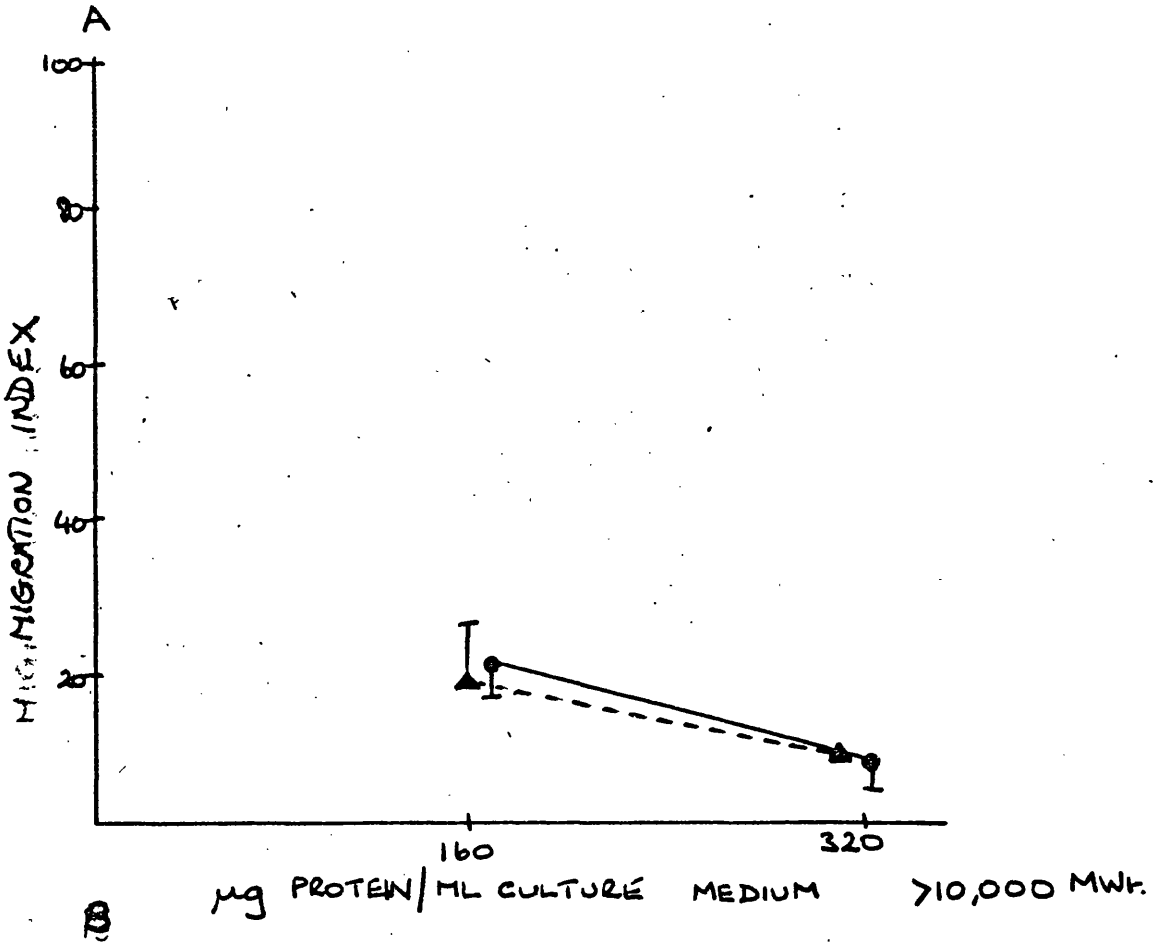
APPROXIMATE MOLECULAR WEIGHT	>120,000	100,000	80,000 – 30,000	30,000 – 10,000	<10,000
PROTEIN CONCENTRATION /ML MEDIA	40 µg	80 µg			
MEAN MIGRATION INDEX MIGRATING LEUCOCYTES FROM					
a) RHEUMATOID	106	103	97	98	
b) CONTROL	93	101	96	102	

Figure 38 :-

DOSE RESPONSE OF CHOLATE EXTRACT FROM AJ RHEUMATOID SYNOVIUM AGAINST RHEUMATOID ARTHRITIS o———o AND CONTROL Δ- - - - - Δ LEUCOCYTES IN THE LMT.

(A) Protein of molecular weight greater than 10,000 daltons (See Chapter 5, Section III).
(B) Protein of molecular weight less than 10,000 daltons (See Chapter 5, Section III).

There is no signficant difference between rheumatoid and control leucocytes. Each point represents the mean of four subjects' responses.


A
100
80
60
40
20
MIGRATION INDEX
160
320
μg PROTEIN/ML CULTURE MEDIUM >10,000 MWt.

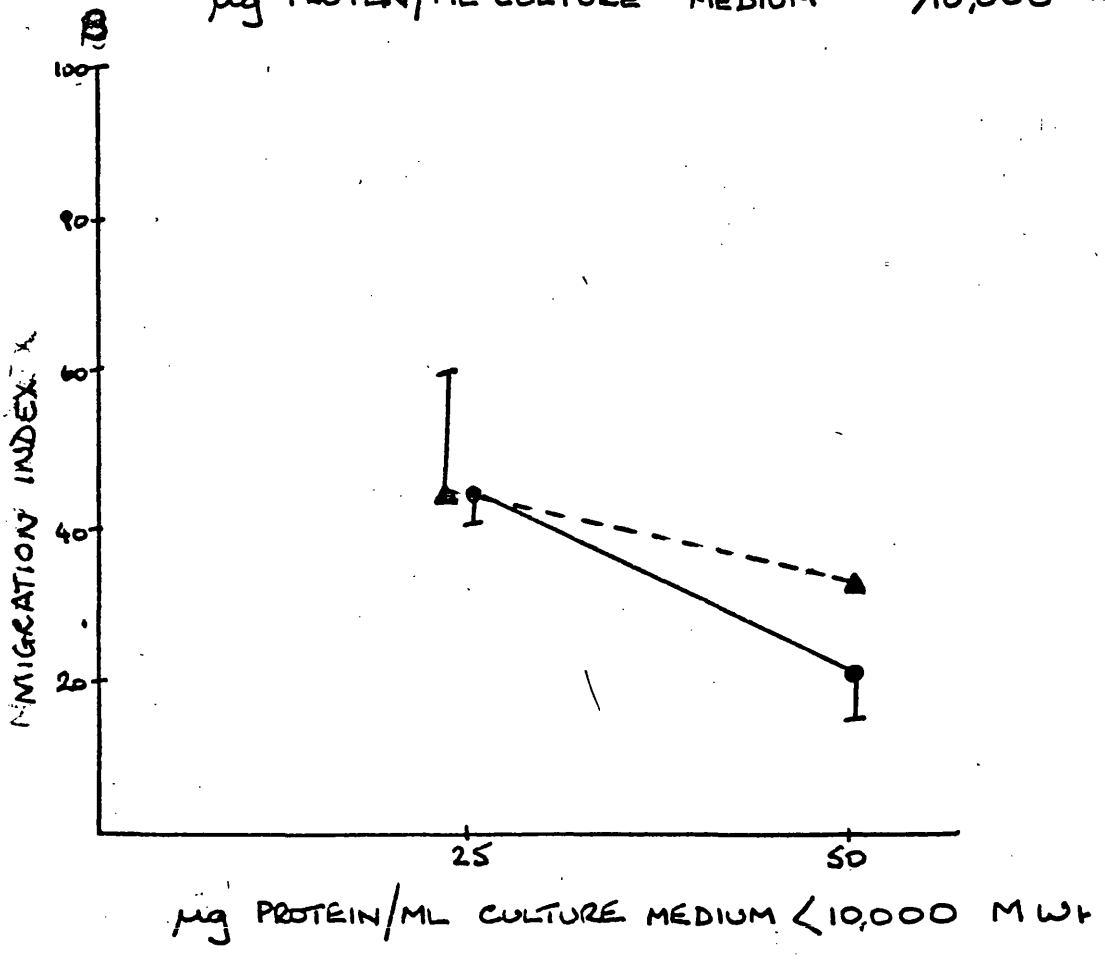
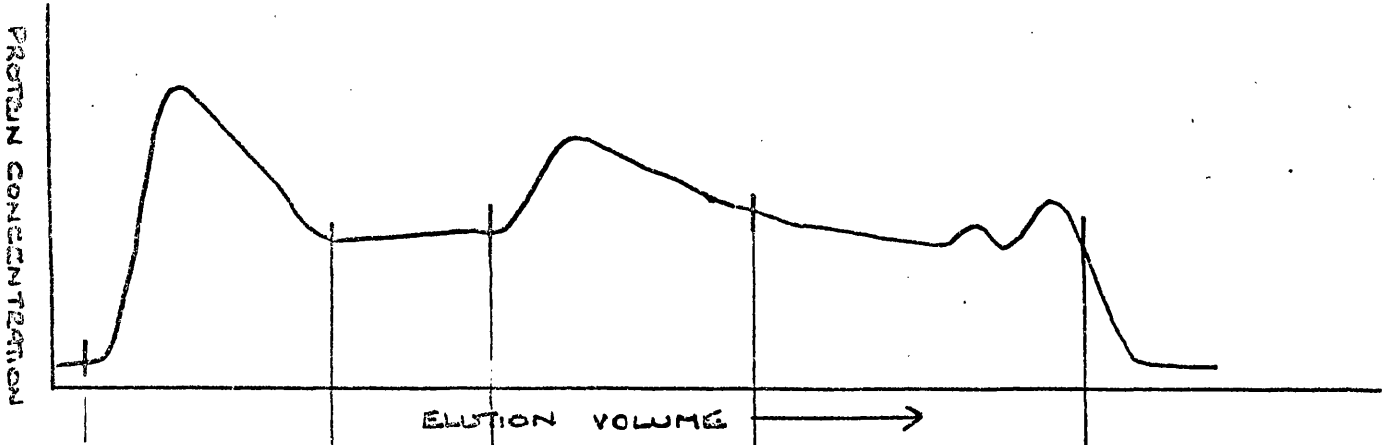

B
100
90
60
40
20
MIGRATION INDEX
25
50
μg PROTEIN/ML CULTURE MEDIUM <10,000 MWt

Fig: 38

Figure 39 :-

RESPONSE OF RHEUMATOID ARTHRITIC AND AGE AND SEX MATCHED CONTROL LEUCOCYTES IN THE LMT TO FRACTIONS OBTAINED FROM A SEPHAROSE 6B ANALYSIS OF A CHOLATE SOLUBILIZATION OF AJ RHEUMATOID SYNOVIUM.

The protein is of molecular weight greater than 10,000 daltons.



APPROXIMATE MOLECULAR WEIGHT	$\geq 2 \times 10^6$	700,000	150,000	20,000
PROTEIN CONCENTRATION / ML MEDIA	4 µg	13 µg	62 µg	25 µg
MEAN MIGRATION INDEX TESTING LEUCOCYTES FROM:				
a) 3 RHEUMATOIDS	83.0 ± 6.5	92.0 ± 6	83.0 ± 15	22.0 ± 8
b) 3 CONTROLS	81.0 ± 8.5	93.0 ± 3	84.0 ± 5	30.0 ± 2
STUDENTS T TEST	NS	NS	NS	NS

Fig. 30

Figure 40 :-

RESPONSE OF RHEUMATOID ARTHRITIC AND AGE AND SEX MATCHED CONTROL LEUCOCYTES TO FRACTIONS OBTAINED FROM A SEPHAROSE 6B ANALYSIS OF CHOLATE SOLUBILIZATION OF AJ RHEUMATOID SYNOVIUM.

The protein is of molecular weight less than 10,000 daltons.

See Fig. 39.

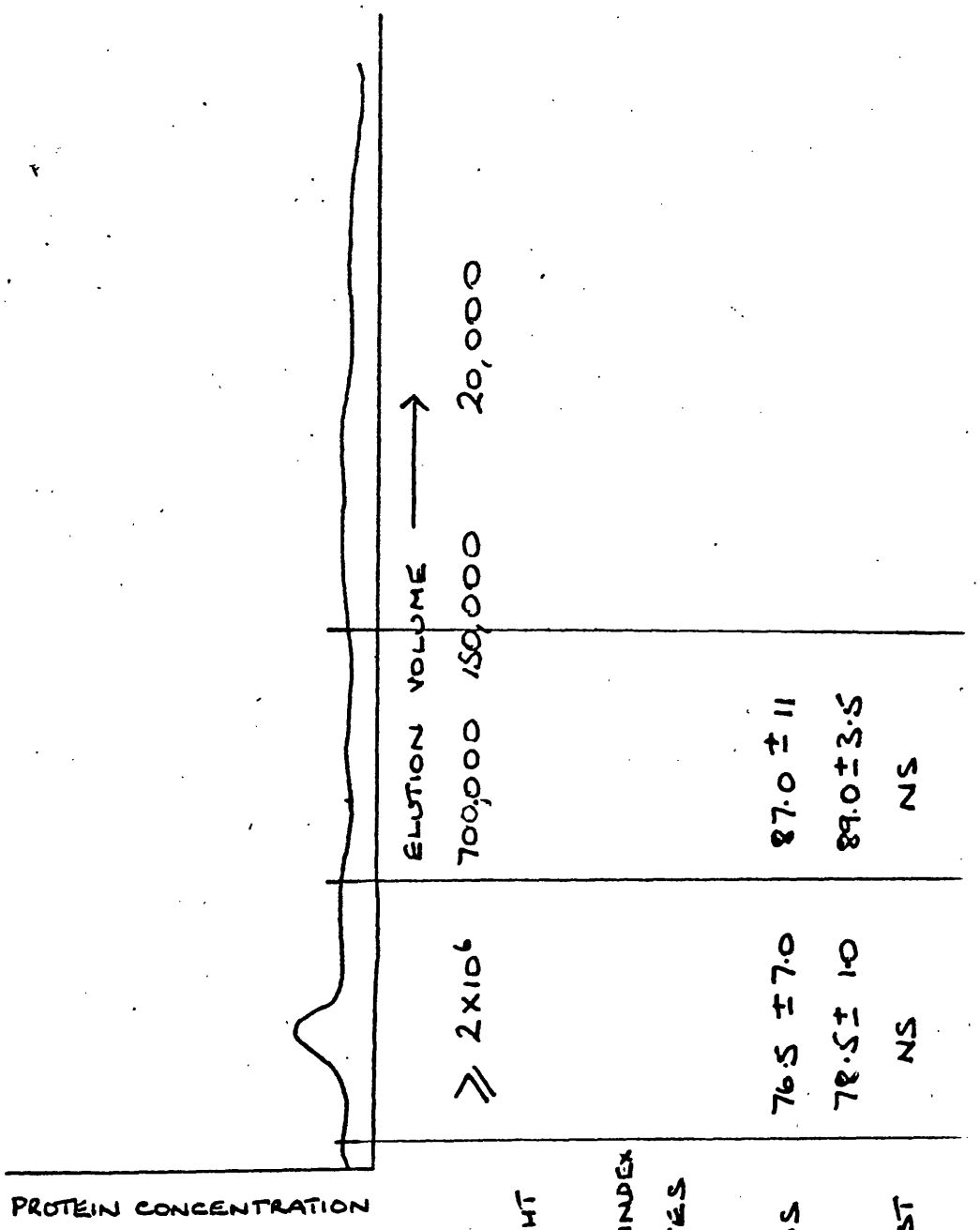
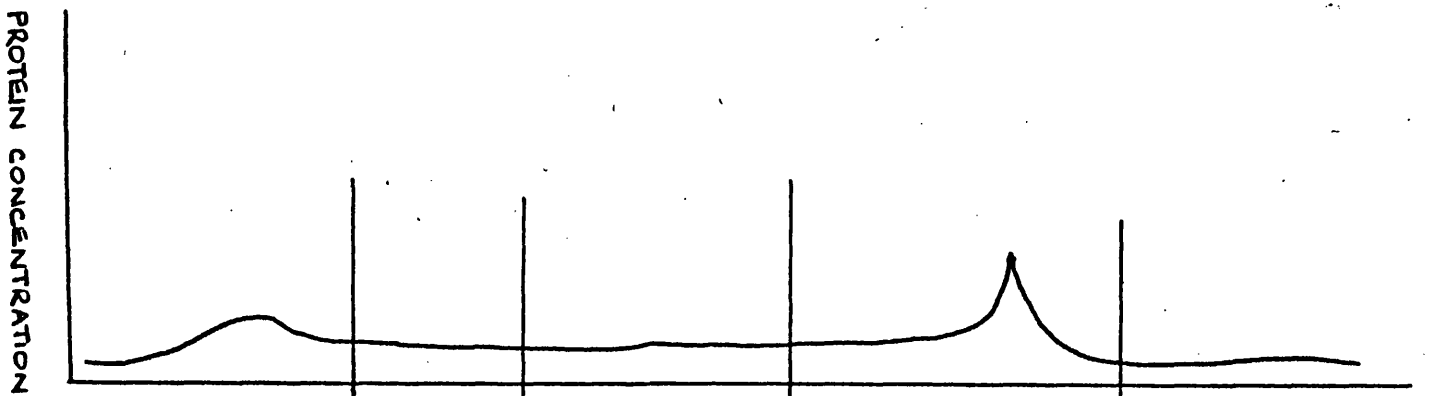

PROTEIN CONCENTRATION
ELUTION VOLUME
700,000
150,000
20,000
APPROXIMATE MOLECULAR WEIGHT
≥ 2X10⁶
MEAN MIGRATION INDEX TESTING LEUCOCYTES FROM
a) 2 RHEUMATOIDS
76.5 ± 7.0
87.0 ± 11
b) 2 CONTROLS
78.5 ± 10
89.0 ± 3.5
STUDENTS T TEST
NS
NS

Fig: 40

Figure 41 :-

RESPONSE OF RHEUMATOID AND AGE AND SEX MATCHED CONTROL LEUCOCYTES TO FRACTIONS OBTAINED FROM A SEPHAROSE 6B ANALYSIS OF A CHOLATE BLANK SOLUTION.

There should be no protein present. See Figs. 39 and 40.



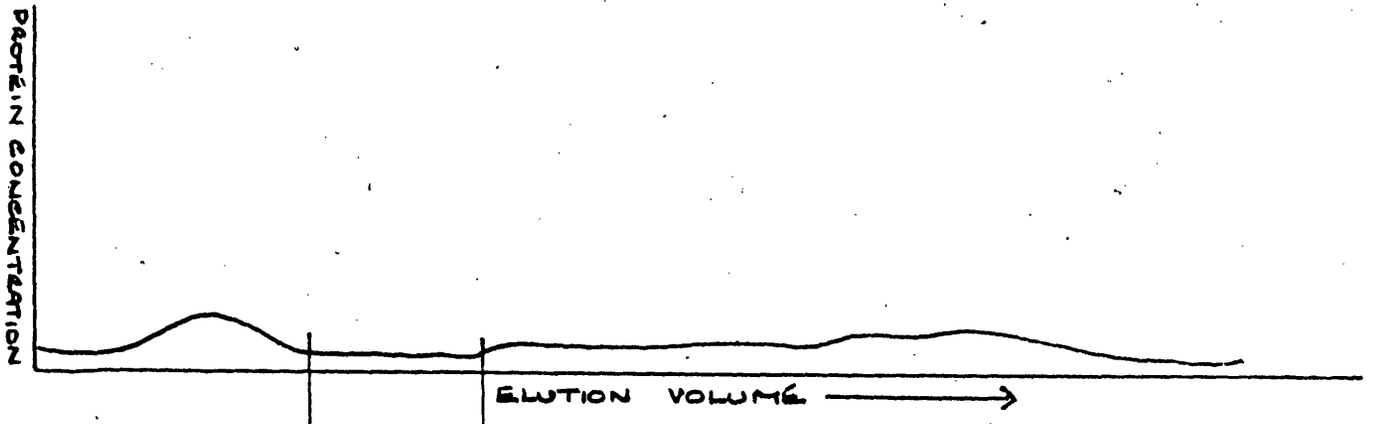
APPROXIMATE MOLECULAR WT.	$\geq 2\times10^6$	709,000	150,000	29,000
FOLIN CIOCALTEAU PROTEIN DETERMINATION	10 µg	0 µg	17 µg	10 µg
MIGRATION INDEX FROM CONTROL	88·0	81·0	77·5	32·2

Fig 41

Figure 42 :-

RESPONSE OF RHEUMATOID ARTHRITIC AND AGE AND SEX MATCHED CONTROL LEUCOCYTES TO FRACTIONS OF GIVEN MOLECULAR WEIGHT OBTAINED FROM A SEPHAROSE 6B ANALYSIS OF CHOLATE SOLUBILIZATION OF DH RHEUMATOID SYNOVIUM; IN THE LMT.

The Student T test is not applied to these results because they are the mean of only 2 readings.



APPROXIMATE MOLECULAR WEIGHT	$\geq 2 \times 10^6$	700,000
FOUNCIOCALTEAU PROTEIN ESTIMATION /ML MEDIA	40 µg	55 µg
MEAN MIGRATION INDEX MEASURING LEUCOCYTES FROM		
a) RHEUMATOIDS	90.0±4	101.0±1
b) CONTROLS	97.0±1.5	100.0±2

Fig. 42

Figure 43:-

DOSE RESPONSE CURVE OF HIGH SPIN PBS SUPERNATANT OBTAINED AFTER HOMOGENIZATION OF MA RHEUMATOID SYNOVIUM AGAINST RHEUMATOID ●——● AND AGE AND SEX-MATCHED CONTROL SUBJECTS ▲----▲; IN THE LMT.

Supernatant is comparable with crude homogenates see Fig. 17

There was no significant difference between rheumatoid and control responses.

Each point represents the mean of a maximum of 4 subjects response.

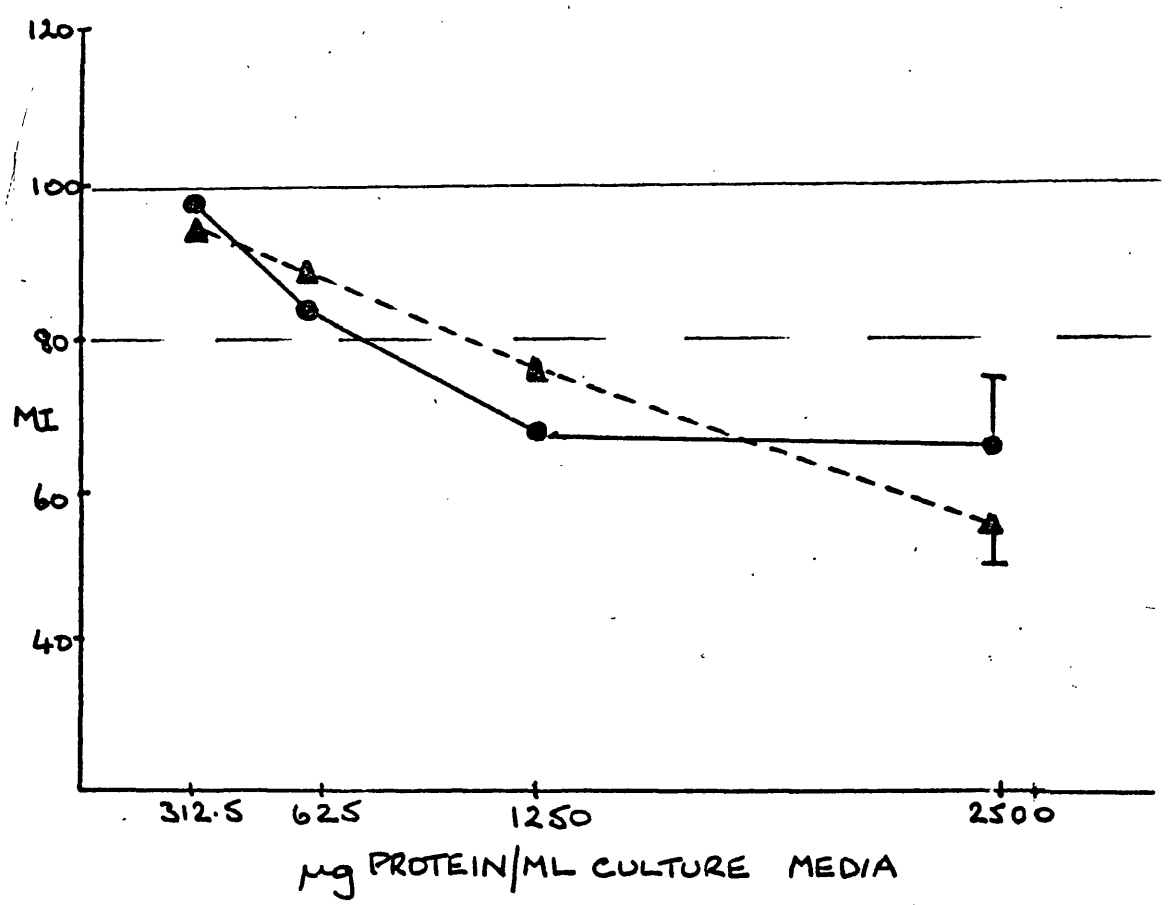
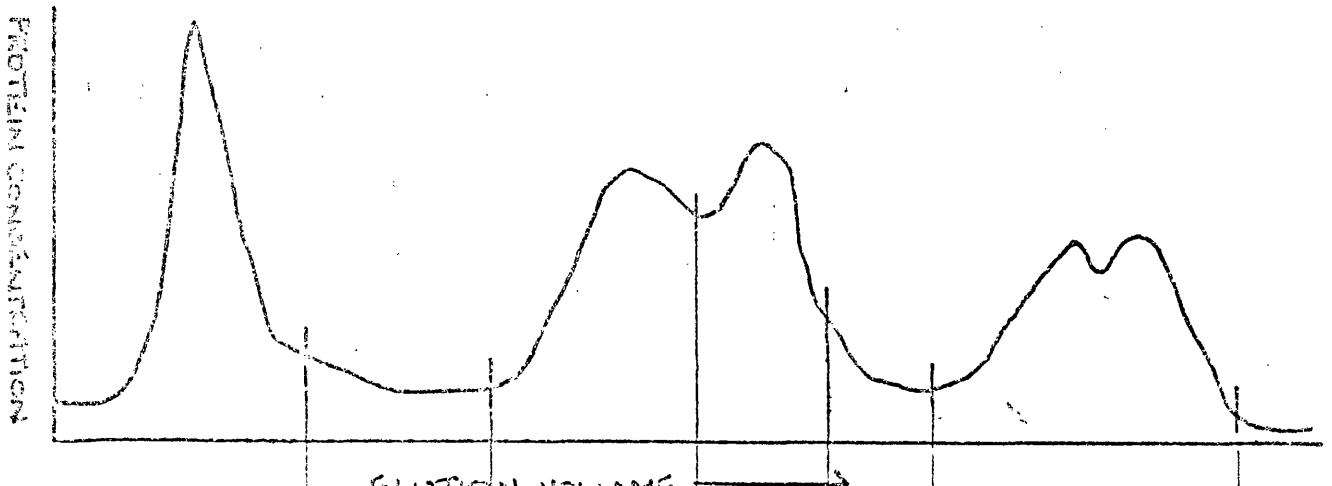

120
100
80
MI
60
40
312·5
625
1250
2500
µg PROTEIN/ML CULTURE MEDIA

Fig: 43

Figure 44:-

RESPONSE OF RHEUMATOID AND AGE AND SEX MATCHED CONTROL LEUCOCYTES TO MA PBS SUPERNATANT (SEE CHAPTER 5, SECTION IV B) FRACTIONATED ON A SEPHAROSE 6B COLUMN, IN THE LMT.



APPROXIMATE MOLECULAR WEIGHT	$\geq 2\times10^6$	700,000	200,000	70,000	30,000	<10,000
PROTEIN CONCENTRATION/ML MEDIA	30mg	100mg	220mg	550mg	190mg	18mg
MEAN MIGRATION INDEX TESTING LEUCOCYTES FROM						
a) 2 RHEUMATOIDS	100.0 ± 5	97.0 ± 7	101.0 ± 0	96.0 ± 1	102.0 ± 6	99.25 ± 4
b) 2 CONTROLS	99.25 ± 9	99.0 ± 3	99.0 ± 6	96.0 ± 4	96.5 ± 5	88.5 ± 10
STUDENTS T TEST	NS	NS	NS	NS	NS	NS

Fig. 4.4

Figure 45 :-

DOSE RESPONSE CURVE OF TRITON X100 EXTRACTED PROTEIN FROM MA RHEUMATOID SYNOVIUM AGAINST RHEUMATOID ●——● AND AGE AND SEX MATCHED CONTROL SUBJECTS ▲---▲ IN THE LMT.

There is very little inhibition and there is no significant difference in response of rheumatoid and control leucocytes.

Each point represents the mean of 4 subjectes responses.

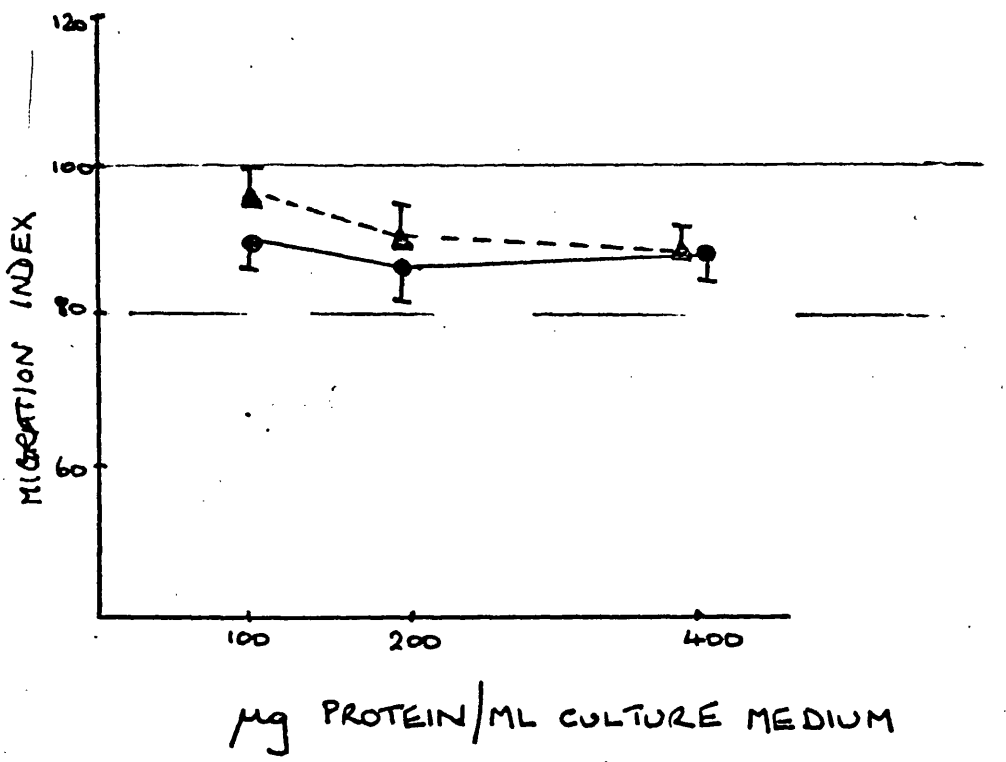

120
100
80
60
MIGRATION INDEX
100
200
400
µg PROTEIN/ML CULTURE MEDIUM

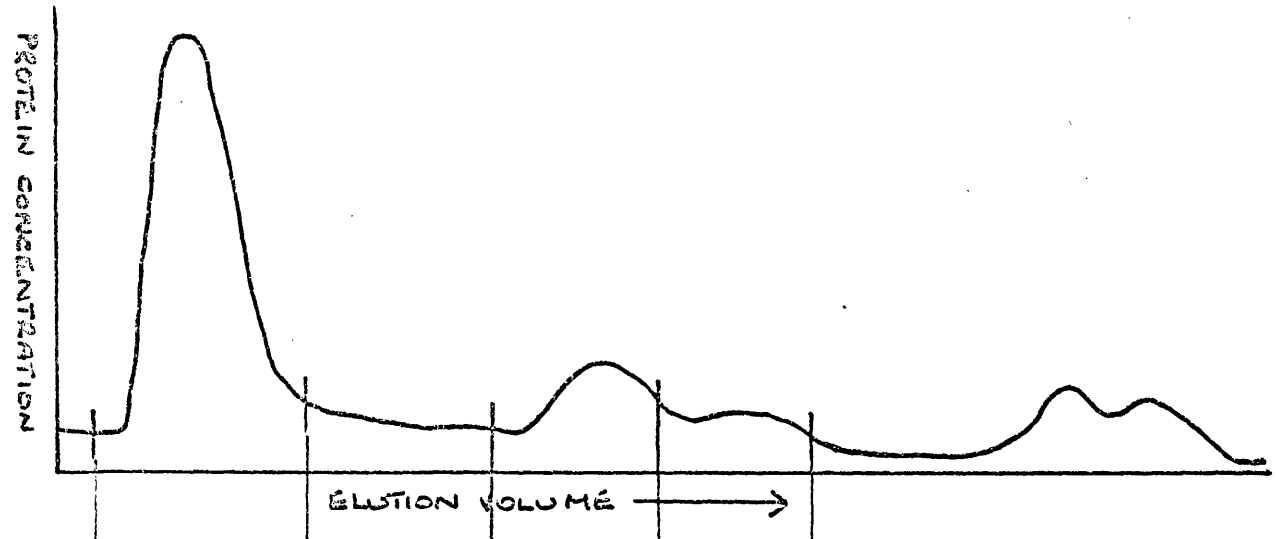
Fig: 45

Figure 46 :-

RESPONSE OF RHEUMATOID ARTHRITIC AND AGE AND SEX MATCHED CONTROL LEUCOCYTES TO FRACTIONS OF GIVEN MOLECULAR WEIGHT OBTAINED BY SEPHAROSE 6B ANALYSIS OF MA RHEUMATOID SYNOVIUM., IN THE LMT.

MA synovium had been subjected to aTriton X100 solubilization

Fraction of molecular weight 200,000 is more inhibitory for rheumatoid than control leucocytes $P < 0.05$.



APPROXIMATE MOLECULAR WEIGHT	$\geq 2\times10^6$	700,000	200,000	70,000
PROTEIN CONCENTRATION/ML MEDIA	30 µg	45 µg	40 µg	60 µg
MEAN MIGRATION INDEX TESTING LEUCOCYTES FROM				
a) 3 RHEUMATOIDS	93.8 ± 8.5	95.2 ± 7.5	84.0 ± 11	94.0 ± 10
b) 3 CONTROLS	95.0 ± 7	97.5 ± 3	100.7 ± 6	95.8 ± 6
STUDENTS T TEST	NS	NS	$P<0.05$	NS

Fig 4b

SECTION VI - RESULTS

A. EDTA/ME EXTRACTION

Nine synovia were subjected to this elution technique. It was found that material inhibitory in the LMT could be extracted by this method. It was to be found in both the MI and MII eluates of the rheumatoid and the ankylosing spondolytic (AS) synovia (Figs. 21 - 27).

Inhibitory material could not be extracted from a pigmented villonodular synovium(Fig. 28). Of the rheumatoid synovia, all except one (Fig. 23) yielded material which was inhibitory at 200 µg or less. The crude homogenates (Chapter 4) except one (Fig. 7) were not inhibitory at this protein concentration. The AS synovium also yielded inhibitory material.

If the inhibition of migration caused by the crude homogenates was an immunological, not a physical, effect, then we are possibly concentrating the inhibitory protein by this method of extraction.

It was found that at a given protein concentration if the solution was subjected to high speed spin the majority of the inhibitory material was removed (Fig. 27). However, if after spinning the supernatant is concentrated to the same protein concentration as the unspun eluate, the soluble protein was as inhibitory as the protein which had not been subject to centrifugation (Fig. 25).

The three synovia on which the most work was carried out were compared. It can be seen that inhibitory material could be present predominantly in MI (RS Fig. 21) or MII (EH Fig. 22) or equally distributed (FM Fig. 23).

In one case there was a significant difference between the responses of rheumatoid and control leucocytes (RS Fig. 21). It was found that the inhibitory material was present in the MI eluate and that rheumatoid leucocytes were inhibited more than control leucocytes. This specificity was significant only at the 50 µg/ml culture medium.

The protein solution was subject to column chromatography. Sephadex G-100 analysis indicated that the majority of the inhibitory material was $\geq$ 120,000 molecular weight. This protein retained its specificity for inhibition of rheumatoid leucocytes and the results were statistically significant. Some inhibitory material was also present in the fraction with M.Wt. $>$ 80,000 but $<$ 120,000 (Fig.32).

The same protein solution fractionated on an Ultragel column gave slightly differing results. There was still protein at approximately 100,000 M.Wt. which was slightly inhibitory but not specific for RAs. The majority of the inhibitory material appeared in the fraction M.Wt. $>$ 800,000. This had no specificity for RA leucocytes in the LMT (Fig. 33).

The eluates from FM (Fig. 23) werealso subject to analysis by column chromatography. The MII eluate was analysed on Sephadex G-100. Again the majority of inhibitory material was in the $\geq$120,000 M.Wt., and also some in the approximately 100,000 M.Wt. fraction (Fig. 34). The MI eluate was fractionated on Ultragel ACA 22 (Fig. 35). The most inhibitory material was $>$ 800,000 M.Wt., although all fractions were to a certain extent inhibitory.

A protein solution derived from a crude synovial homogenate subject to high speed spin (Chapter 3, Section II, B) (Fig. 36) was analysed on Sephadex G-100 (Fig. 37). The proportions of the different M.Wt. proteins were similar to those in the MI and MII eluates (Figs. 32 and 34), but there was an extra peak at 10-80,000 M.Wt. None of these fractions were inhibitory.

B. CHOLATE SOLUBILIZATION

Two rheumatoid synovia were subject to this cholate solubilization.

The cholate extract was concentrated over a Diaflo filter (cut off 10,000 M.Wt.) and the solutions containing protein of greater than 10,000 M.Wt. and less than 10,000 M.Wt. were tested in the LMT.

It was found that despite its very low protein concentration the protein solution of M.Wt. less than 10,000 was extremely inhibitory (Fig. 38b). The solution containing protein of greater than 10,000 M.Wt. was also extremely inhibitory (Fig.38a).

The solution containing high molecular weight protein was fractionated on a Sepharose 6B column (Fig. 39). All fractions were inhibitory, fraction 1 more than fraction 2 or 3. Fraction 4 was the most inhibitory. It should contain very little protein, since all protein of <10,000 M.Wt. has been removed.

The solution containing protein of less than 10,000 M.Wt. was fractionated on a Sepharose 6B column. Although no high molecular weight protein was present due to its previous treatment (see above) all the fractions were again inhibitory (Fig. 40).

A cholate solution which had been subject to the same procedures but using no synovial homogenate,i.e. a control solution, was also fractionated on Sepharose 6B (Fig. 41). This was found to be as inhibitory as the eluate containing the protein.

A second rheumatoid synovium was subject to the same solubilization technique and fractionated on a Sepharose 6B column (Fig. 42). This contained very little protein which was not inhibitory.

C. TRITON x 100 SOLUBILIZATION

A Triton solubilization was carried out on one rheumatoid synovium.

A PBS supernatant which is the same as the high speed crude homogenate (Chapter 3, Section II, B) was tested for inhibitory activity which was present only at very high protein concentration, i.e. 1,250 and 2,500 µg/ml culture medium (Fig. 43).

This protein solution was subject to column chromatography on a Sepharose 6B column (Fig. 44). None of the fractions contained inhibitory material.

The TRITON x 100 solubilized protein was tested in the LMT and found to be only slightly inhibitory and the inhibition did not titrate out (Fig. 45). When fractionated on a Sepharose 6B column all the fractions were very slightly inhibitory but none to any great extent (Fig. 46).

SECTION VII - DISCUSSION

A. EDTA/ME

A protein was eluted which was inhibitory in the LMT. That it did not act by killing the cell was tested using trypan blue dye exclusion. The extracted protein was inducing inhibition by a non-toxic means.

Weight for weight the protein in these eluates was more inhibitory than that in the crude homogenates.

That the inhibitory material cannot be removed by spinning indicates that a true extraction has occurred.

The inhibitory material is soluble. The extraction procedure can be compared with that used by Bacon et al (1973), who showed that using hypotonic solutions an inhibitory material could be removed from rheumatoid synovial specimens.

Synovia from all patients suffering from inflammatory conditions (RA and AS) yielded this inhibitory material.

The non-inflammatory pigmented villonodular synovium did not yield any inhibitory material. Although only one inflammatory non-rheumatoid synovium was tested, it might be that the inhibitory material is not associated specifically with RA, but with chronic inflammation.

When column chromatography was carried out, inhibitory material appeared in the void volumes using Sephadex G-100 and Sepharose 6B or Ultragel. To elute in the void volume of Sepharose 6B or Ultragel indicates that the protein has a M.Wt. of 800,000 or greater.

In view of the nature of the extraction procedure, it seems unlikely that protein possessing M.Wt. 800,000 daltons is a native protein. There is no hard evidence for such a statement. The possibility still exists that there could be antigen-antibody complexes present.

The fraction of M.Wt. approximately 100,000 from column chromatography has also tended to be inhibitory. This is of interest, since the protein cannot be antigen-antibody complexes once the molecular weight is too low.

It must be borne in mind that since this method does not employ solubilising agents only proteins are extracted. The carbohydrate content of the cells is virtually untouched (Tanner and Boxer, 1972) by such a procedure. Thus glycoproteins are not removed by this method. Many of the integral membrane proteins are glycoprotein, and it is accepted that the carbohydrate is responsible for cell-surface specificity (Tanner and Boxer, 1972). Membrane proteins synthesised in response to viral infection are glycoproteins (Rothman and Lodish, 1977).

It is possible that the inhibition caused by these proteins is due to their ability to alter the cell-surface charge in a manner analogous to that proposed for the action of NSAID (Brown, 1976).

B. CHOLATE EXTRACTION

From the traces from column fractionation it would seem that a great quantity of protein has been solubilised by the cholate. What is significant is that much of this protein is of low molecular weight, contrasting with both EDTA/ME and TRITON x 100 extraction, when the vast majority of protein appeared in the void volume.

It is unfortunate that when a cholate blank solution was run down the column the fractions obtained caused a similar degree of inhibition to those fractions containing protein. The majority of the cholate was removed from the solution by dialysis (Chauvin and Holliman, 1975). The very small quantity which remained was obviously not removed by column fractionation.

The method by which cholate causes inhibition of leucocyte migration is debatable. The cell fractions were still viable as indicated by trypan blue dye exclusion.

C. TRITON x 100 EXTRACTION

The amount of protein present was comparable to that obtained by the EDTA/ME procedure. The amount in the void volume after Sepharose 6B analysis was not much less than that obtained from RS MI void volume after Ultragel Analysis (Fig. 33). The RS protein was inhibitory, whereas the TRITON extracted protein was not.

It is possible that the trace of the column fractionation is misleading, since TRITON x 100 absorbs at 275 nm. The focusing instrumentation on the LKB UltraRac 7000 is not accurate and is set to measure the absorbance of proteins at 280 nm. It is unlikely that TRITON would interfere with a Folin-Ciocalteau protein determination.

TRITON does not cause alteration in the antigenicity of proteins (Helenius and Simons, 1971). Since much of the protein is high molecular weight, aggregation would seem to have occurred on removal of the detergent. Why this protein does not cause inhibition of migration, when the EDTA/ME eluted protein does, is unresolved.

This method of extraction was carried out on only one synovium, and it would be wrong to place too much emphasis on these results.

SECTION VIII - CONCLUSION

Soluble inhibitory material was eluted from synovial membranes using EDTA/ME. The inhibitory material was perhaps associated with the ongoing inflammatory reaction as opposed to being specific for RA: the AS synovium yielding inhibitory material while the pigmented villonodular synovium did not. From only one synovium was inhibitory material obtained which was specific for rheumatoids.

In an attempt to extract more of this inhibitory material two detergent extractions were employed. Both of these proved unsatisfactory. There are great difficulties inherent in using detergent solubilizations on material intended for use in systems utilizing viable cells, principally the complete elimination of the detergent. Despite aggregation occuring on the removal of detergent it was to be hoped that different proteins with different antigenic sites would be extracted than using hypotonic salt solutions. This was not borne out by the results presented.

CHAPTER 6

THE EFFECT OF NATIVE AND AGGREGATED IMMUNOGLOBULIN G ON RHEUMATOID AND CONTROL LEUCOCYTE MIGRATION IN THE LEUCOCYTE MIGRATION TEST

CHAPTER 6

THE EFFECT OF NATIVE AND AGGREGATED IMMUNOGLOBULIN G ON RHEUMATOID AND CONTROL LEUCOCYTE MIGRATION IN THE LEUCOCYTE MIGRATION TEST.

SECTION I - INTRODUCTION

Rheumatoid factors have been implicated in the pathogenesis of rheumatoid disease. The effects of these immunoglobulins in the LMT are well documented (Chapter 1, Section VII, A (iii)).

It would be useful to repeat these results to see if the method employed will give results comparable to those published.

SECTION II - PREPARATION OF IMMUNOGLOBULIN G

A. NATIVE IgG

The method is based on that of Ishizaka et al (1965). A quantity of DEAE Sephadex A50 was swelled in a 10mM phosphate buffer containing 0.002% chlorhexidine gluconate, pH 7.4 and stored at 4°C.

Serum was taken and placed in a 100 ml beaker. The DEAE suspension was filtered on a Büchner funnel to remove excess buffer. It was added to the previously prepared serum to form a paste, and stirred for 1 hour at 4°C. During this procedure all proteins bind to the DEAE except IgG. The mixture of DEAE Sephadex and serum was filtered and washed with 10 mM phosphate buffer. The filtrate was concentrated by pressure dialysis at 4°C under N_2 over a Diaflo Ultrafiltration Membrane M50 - molecular weight exclusion 50,000. The concentrate was dialysed for 16 hours against a 10/150 (Chapter 5, Section V,A) chlorhexidine gluconate buffer pH 7.4.

The protein concentration was determined by the Folin-Ciocalteau method (Lowry et al, 1951). The solution was divided into small aliquots and stored at -20°C.

B. AGGREGATED IgG

The previously prepared native IgG solution was incubated at 63°C in a shaking-waterbath for 1 hour. The solution was divided into small aliquots and stored at -20°C.

C. TEST OF IDENTITY

The IgG was shown to be immunochemically pure using immunoelectrophoresis against commercial anti-human IgG and anti-normal human serum.

Figure 47 :-

EFFECT OF NATIVE AND AGGREGATED IgG 250µg/ml on MIGRATION OF LEUCOCYTES FROM PATIENTS WITH RHEUMATOID ARTHRITIS AND HEALTHY AGE AND SEX MATCHED CONTROLS, IN THE LMT.

AGGREGATED IgG

Testing 10 RAs and 10 controls the aggregated IgG causes greater inhibition of the rheumatoid leucocytes $P < 0.001$

NATIVE IgG

Testing 7 RAs and 7 controls the native IgG causes greater inhibition of the rheumatoid leucocytes $P < 0.001$

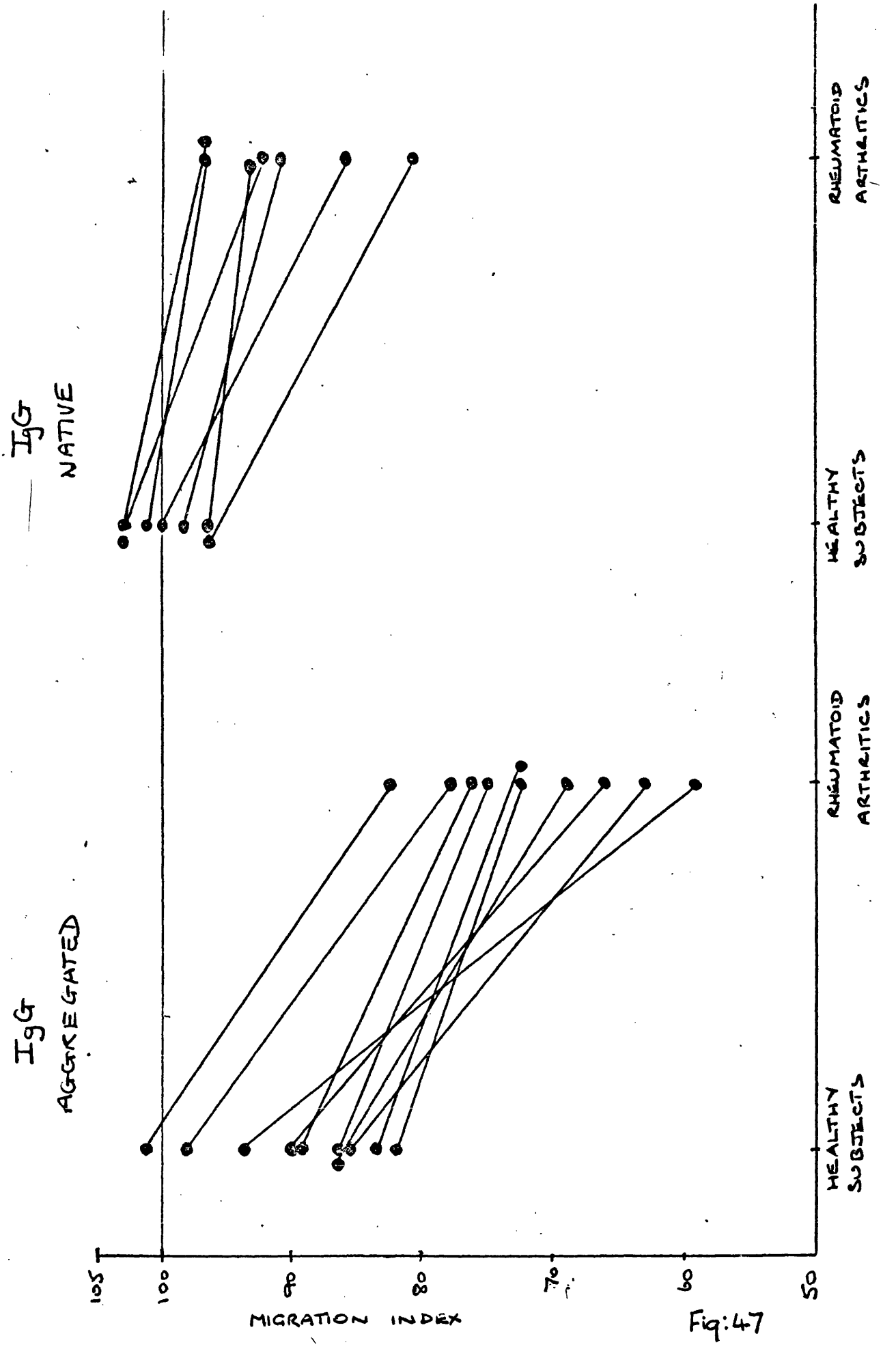

IgG
NATIVE
IgG
AGGREGATED
HEALTHY SUBJECTS
RHEUMATOID ARTHRITICS
HEALTHY SUBJECTS
RHEUMATOID ARTHRITICS
105
100
90
80
70
60
50
MIGRATION INDEX

Fig:47

Figure 48 ;-

DOSE RESPONSE OF NATIVE IgG (A) AND AGGREGATED IgG (B) ON MIGRATION OF LEUCOCYTES FROM PATIENTS WITH RHEUMATOID ARTHRITIS ●—● AND HEALTHY AGE AND SEX MATCHED CONTROLS ▲- -▲, IN THE LMT.

(A) At all dose levels the native IgG causes significantly more inhibition in the RA leucocytes compared with controls.

At 100µg $P < 0.001$
At 200µg $P < 0.001$
At 400µg $P < 0.001$
At 800µg $P < 0.001$

(B) At all dose levels theaggregated IgG causes significantly more inhibition in the RA leucocytes compared with controls.

At 100µg $P < 0.001$
At 200µg $P < 0.001$
At 400µg $P < 0.001$
At 800µg $P < 0.001$

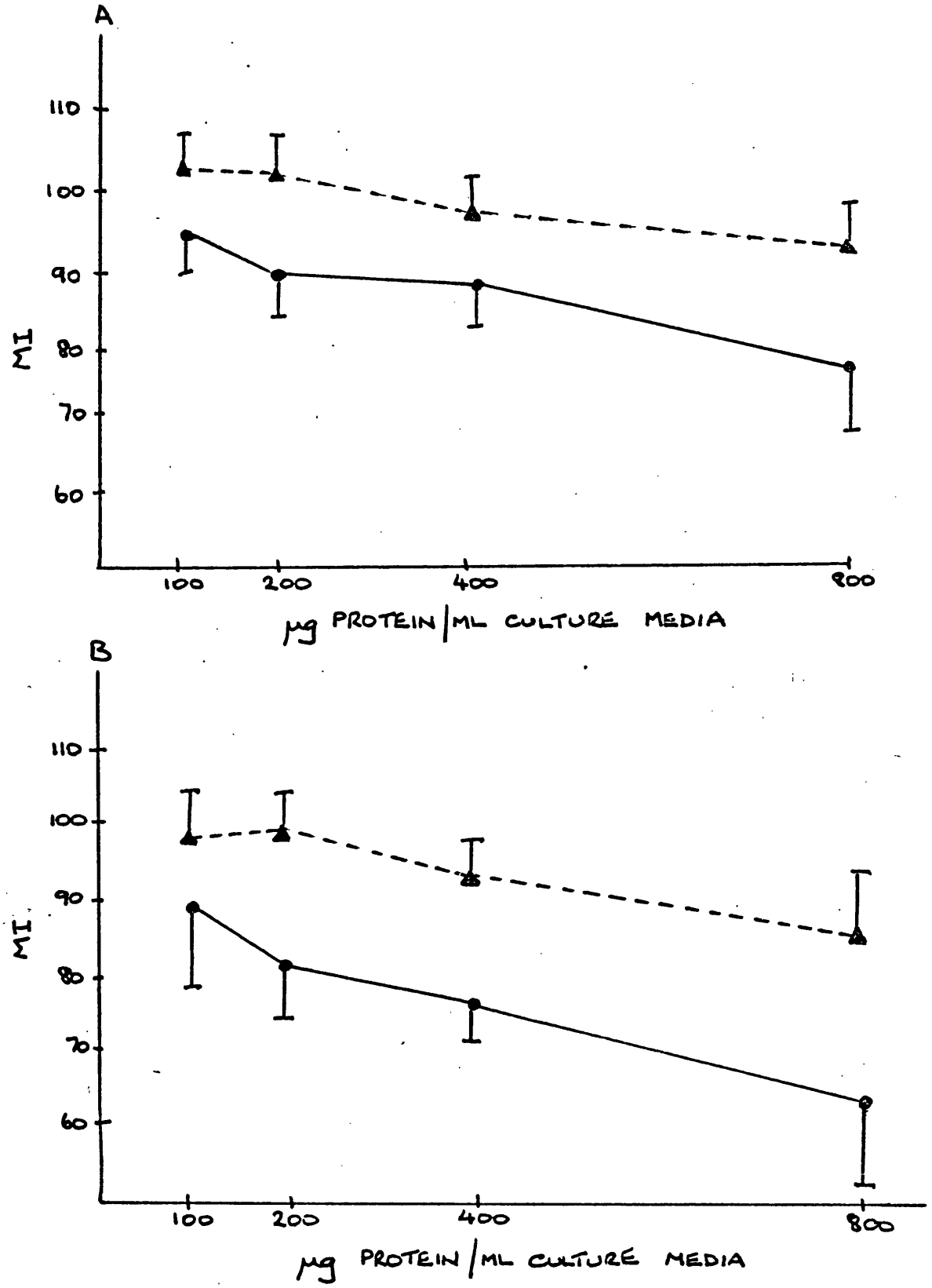

A
110
100
90
80
70
60
MI
100
200
400
800
µg PROTEIN/ML CULTURE MEDIA
B
110
100
90
80
70
60
MI
100
200
400
800
µg PROTEIN/ML CULTURE MEDIA

Fig: 48

SECTION III - RESULTS

Experiments had been carried out to establish that chlorhexidine gluconate in the concentration used in these experiments was not inhibitory in the LMT.

The results presented show that both native and aggregated IgG will cause specific inhibition of rheumatoid leucocytes in the LMT.

Fig. 47 shows that both native and aggregated IgG will inhibit rheumatoid and control lymphocytes in the LMT over a range of concentrations.

Fig. 48: a concentration was chosen which would be comparable with previously published work, i.e. 250 µg protein/ml culture medium. At this concentration rheumatoid lymphocytes were significantly inhibited more than controls in the presence of aggregated IgG $P < 0.001$

In the presence of native IgG rheumatoid lymphocytes were again more inhibited than controls $P < 0.001$

SECTION IV - DISCUSSION

It has been previously been reported that native and aggregated IgG will cause inhibition of migration in the LMT. In particular it causes specific inhibition of rheumatoid leucocytes (Frøland and Gaarder, 1971, 1973; Brostoff, Howell and Roitt, 1973; Massoud et al, 1975; Sany et al, 1975).

It is not known whether the observed effect in the LMT is due to a T-cell effect, i.e. production of lymphokines, or is caused by other means, i.e. cytophilic antibody causing cross-bridging between cells (Brostoff, 1974).

In our test system we also showed specific inhibition of rheumatoid cell migration using native IgG. This would seem to decrease the possibility of inhibition due to a binding effect. The IgG was prepared from a non-rheumatoid subject, which would preclude the presence of immune complexes in the native IgG solution. Thus inhibition could not be caused by the presence of complexes in the native IgG solution.

What exactly is causing inhibition is a mystery since RF is an antibody directed against the Gm locus which is exposed by immune complex formation and probably aggregation of IgG. Therefore if this is an expression of delayed hypersensitivity it must be directed against some components of normal native IgG.

In previous experiments using aggregated IgG there was no correlation between the degree of migration inhibition and the Rose-Waaler titre. However, in those patients in which IgG antiglobulins were detected there was a correlation between the antibody titres and the degree of inhibition. In those patients lacking this antibody the inhibition appeared to be a true expression of delayed hypersensitivity (Brostoff, Howell and Roitt, 1973).

Immune complexes are known to cause lymphocyte transformation in non-sensitised individuals (Moller, 1969; Block-Schtader et al, 1968). For the purposes of this discussion it has been assumed that alteration in IgG conformation on heat aggregation is the same as the alteration on complex formation (Givol et al, 1974).

Bibl and Sitko (1975) showed that antigen-antibody complexes caused specific inhibition of rheumatoid mononuclear peripheral blood cells. There was no correlation with the Rose-Waaler titre. When cultured with immune complexes 16/20 RA patients' lymphocytes produced MIF as assayed on guinea pig peritoneal macrophages, but only 2/20 control lymphocytes produced MIF. Weisbart et al (1975) showed production of MIF by aggregated IgG by autologous rheumatoid leucocytes. The evidence indicates that immune complexes and aggregated IgG could cause immunologically specific inhibition in the LMT.

SECTION V - CONCLUSION

The experiments presented in Chapter 3 indicated that there was no difference in rheumatoid and control leucocytes' response to an ubiquitous antigen : streptokinase.

When rheumatoid and control leucocytes are tested against the various synovial homogenates and eluates it has been difficult to demonstrate a difference in response.

In these experiments it has been shown that there is an antigen which consistently induces differing responses in control and rheumatoid leucocytes in the LMT, i.e. IgG in both native and aggregated forms.

CHAPTER 7

THE EFFECT OF ERYTHROCYTE MEMBRANE HOMOGENATE ON THE MIGRATION OF CONTROL AND RHEUMATOID LEUCOCYTES IN THE LEUCOCYTE MIGRATION TEST

CHAPTER 7

THE EFFECT OF ERYTHROCYTE MEMBRANE HOMOGENATE ON THE MIGRATION OF CONTROL AND RHEUMATOID LEUCOCYTES IN THE LEUCOCYTE MIGRATION TEST

SECTION I - INTRODUCTION

In an attempt to elucidate the mechanisms involved in the inhibition of leucocyte migration by synovial homogenates, red blood cells were subjected to a homogenisation and spinning procedure comparable with that used for the synovial specimens.

The aim of the following experiments was to determine whether leucocyte migration could be inhibited by cell membrane fragments.

SECTION II - METHODS

A. HOMOGENISATION

20 ml blood from a cubital vein drawn into a plastic syringe was expelled into two 10 ml sterile tubes, each containing 250 units heparin. To ensure complete mixing of the blood and anti-coagulant the tubes were gently shaken for 2 minutes. The tubes were than spun at 800g for 10 minutes. The plasma and leucocyte layers were removed leaving the lower erythrocyte layer. The red blood cells (RBC) were washed in distilled water to effect lysis and the ghosts pelleted by spinning at 3000g for 30 minutes; the supernatant was then poured off.

(a) The pellet was subjected to the washing and spinning process six more times. The ghosts were then homogenised using an ILAX 1020 homogeniser. They were allowed to stand overnight at 4°C and then spun either at 1000g for 20 minutes or 20,000g for one hour. These directly parallel low and high spin homogenates prepared from the synovium (Chapter 4, Fig. 6). These preparations were made isotonic using 10x concentrated PBS. The protein determinations were carried out using the Lowry method (1951). The solution was divided into aliquots and frozen down to store at -20°C.

(b) The original supernatant was made isotonic using 10x concentrated PBS. The protein was estimated using Folin-Ciocalteau reagent (Lowry, 1951). The solution was divided into aliquots and stored at -20°C, haemoglobin preparation.

B. PREPARATION OF SEPHADEX G-200

Sephadex G-200 was swollen in and equilibrated with 150 mM NaCl in 10 mM phosphate buffer pH 7.4. A 90 x 1.5 cm column was poured and equilibrated with 10/150 at 4°C. The void volume was estimated using blue dextran in 10/150. The flow rate used was 8 ml/hour and 4 ml fractions were collected on an LKB UltraRac 7000 overnight. A 280 nm profile was recorded.

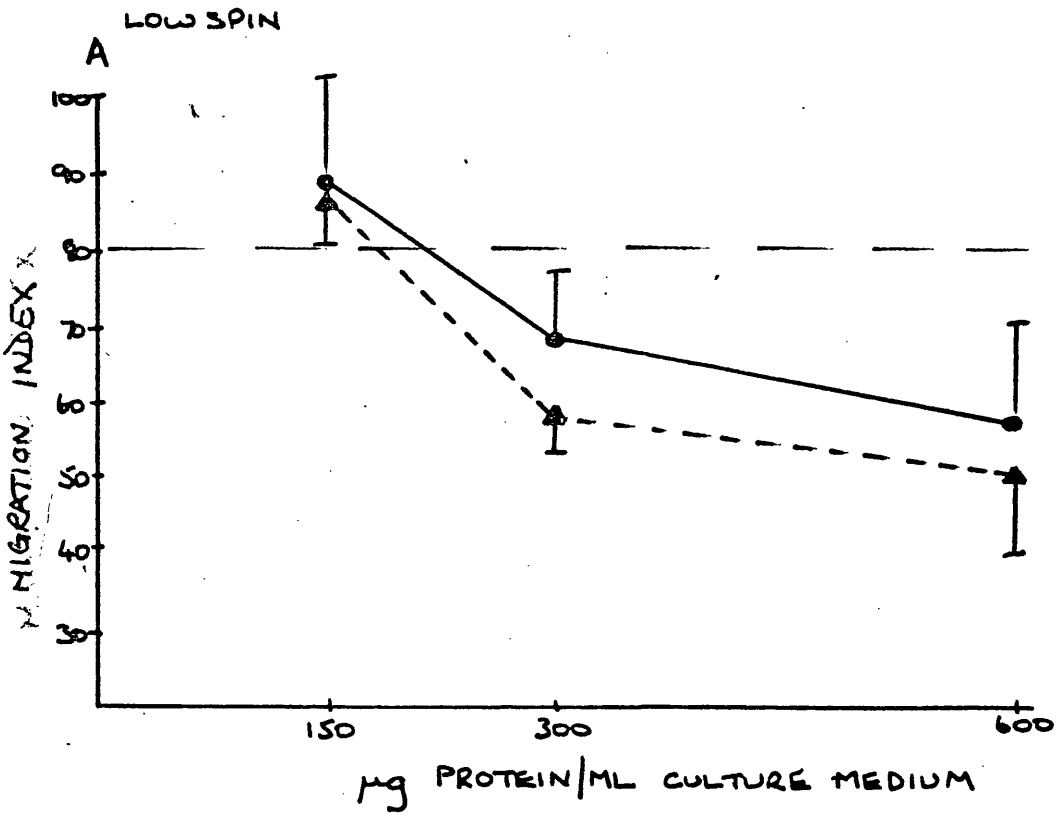
The solution was concentrated over a Diaflo XM50 filter (nominal exclusion, M.Wt. 50,000.)

Figure 49 :-

DOSE RESPONSE OF HOMOGENIZED HUMAN RBC SPUN AT 1,700g FOR 20 MINUTES (A) AND 20,000g FOR 1 HOUR (B) ON MIGRATION OF LEUCOCYTES FROM PATIENTS WITH RHEUMATOID ARTHRITIS AND AGE ●——● AND SEX MATCHED CONTROLS ▲- - -▲, IN THE LMT.

(A) At 600 and 150µg/ml there is no significant difference between RA and control responses.
At 300µg/ml the difference is significant $P < 0.001$

(B) There is no significant difference at any of the "antigen" levels.


LOW SPIN
A
% MIGRATION INDEX
100
90
80
70
60
50
40
30
150
300
600
μg PROTEIN/ML CULTURE MEDIUM

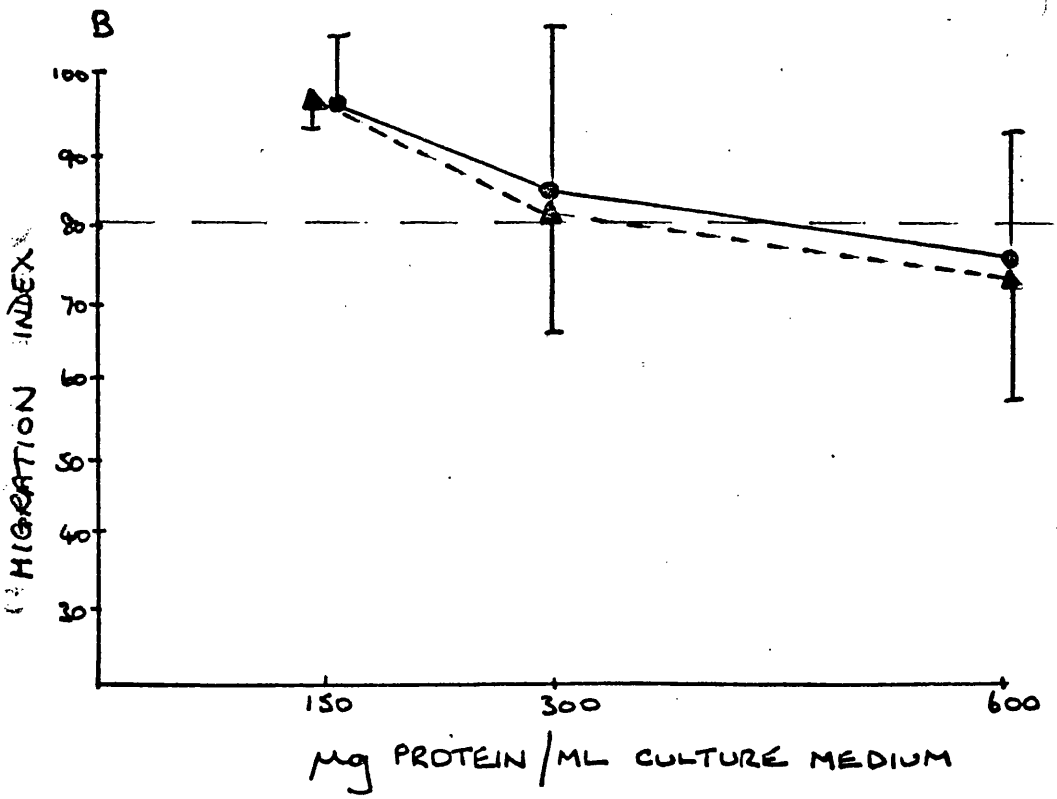

B
% MIGRATION INDEX
100
90
80
70
60
50
40
30
150
300
600
μg PROTEIN/ML CULTURE MEDIUM

Fig: 49

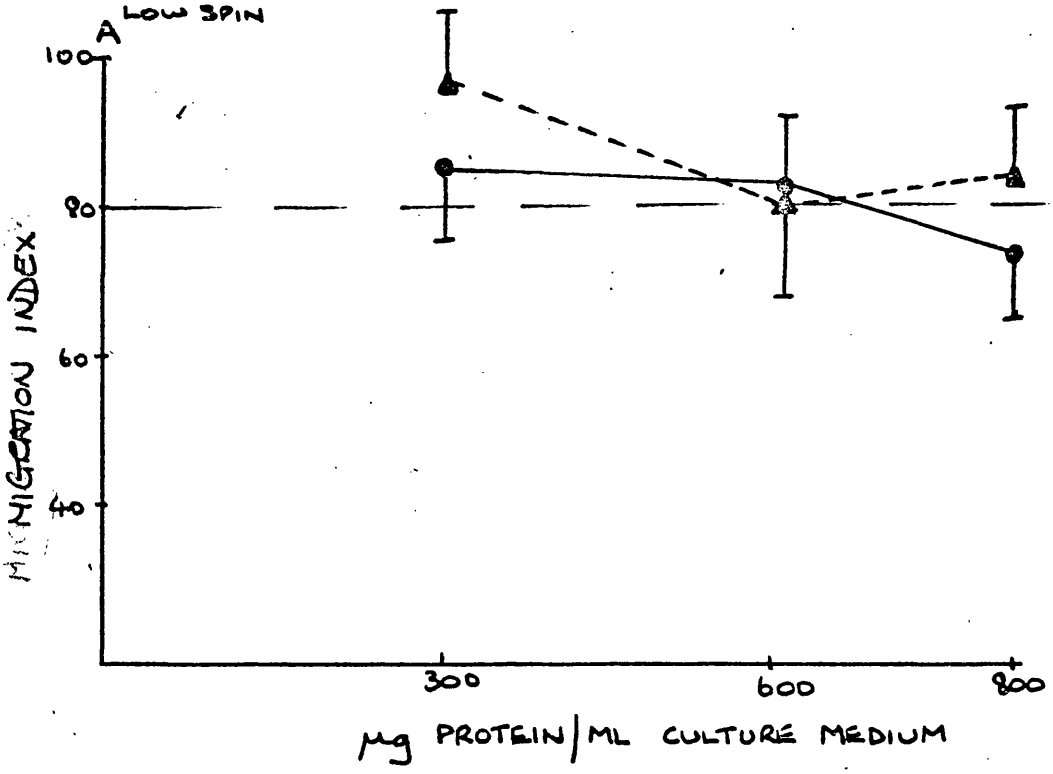
Figure 50:-

DOSE RESPONSE OF HAEMOGLOBIN SOLUTION (preparation p.185) SPUN AT 3,000g FOR 20 MINUTES (A) AND 20,000g FOR 1 HOUR(B) ON MIGRATION OF LEUCOCYTES FROM PATIENTS WITH RHEUMATOID ARTHRITS AND AGE AND SEX MATCHED CONTROLS: IN THE LMT.

(A) At 800ug/ml control more inhibited than RAs $P < 0.01$
At 300ug/ml RA more inhibited than controls $P < 0.01$
At 600ug/ml there is no significant difference

(B) At 1,200, 600, and 300ug/ml there is no significant difference between control and rheumatoid response.

RA response o—o mean of 7 patients responses
Control response Δ- -Δ mean of 7 subjects responses


A LOW SPIN
100
80
60
40
MIGRATION INDEX
300
600
800
µg PROTEIN/ML CULTURE MEDIUM

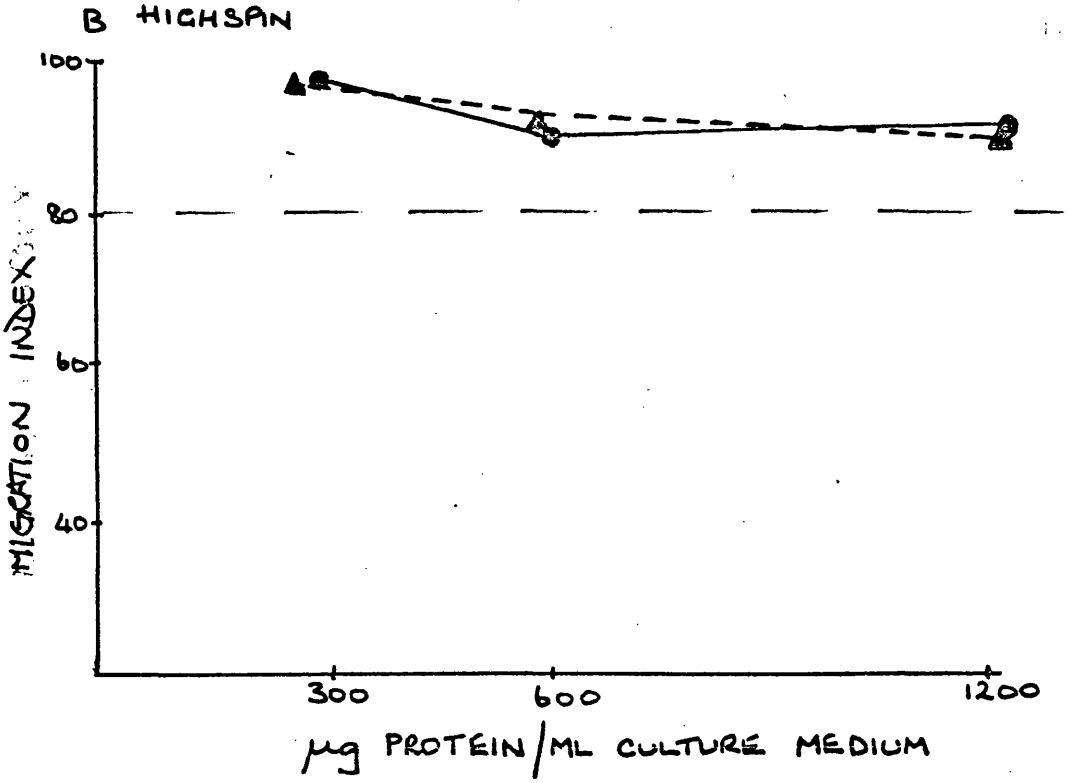

B HIGHSPIN
100
80
60
40
MIGRATION INDEX
300
600
1200
µg PROTEIN/ML CULTURE MEDIUM

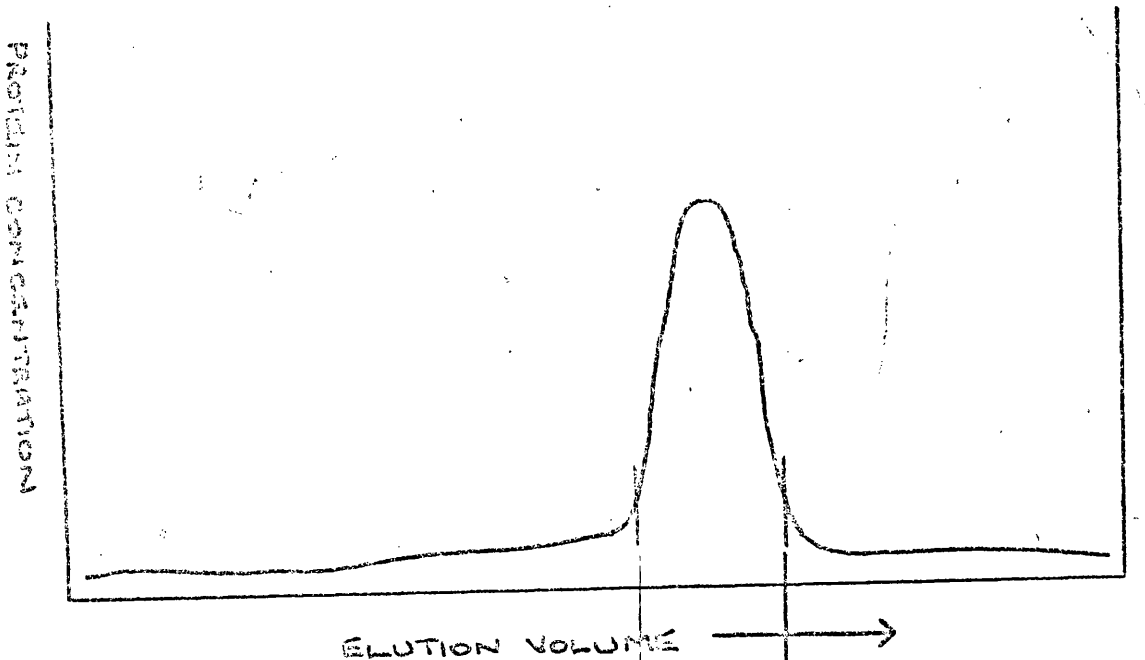
Fig: 50

Figure 51 :-

RESPONSE OF RHEUMATOID AND AGE AND SEX MATCHED CONTROL LEUCOCYTES IN THE LMT TO HAEMOGLOBIN FROM LYSED RBC FRACTIONATED ON A SEPHADEX G200 COLUMN AND TO THE ORIGINAL HAEMOGLOBIN PREPARATION, IN THE LMT

A known volume of the original solution 800ug/ml of protein was layered on the column and the haemoglobin containing fractions were concentrated back to the same volume. thus the protein concentration is expressed as $\equiv$ 800ug/ml.

The original haemoglobin solution was more inhibitory for rheumatoid leucocytes $P < 0.001$



APPROXIMATE MOLECULAR WT.	62,000	NOT APPLICABLE
PROTEIN CONC./ML MEDIA	800 µg	800 µg
MEAN MIGRATION INDEX TESTING LEUCOCYTES FROM ± ONE S.D.		
a) 3 RHEUMATOIDS	81·5 ± 4	7 RHEUMATOIDS 75·0 ± 9
b) 3 CONTROLS	80·5 ± 11	7 CONTROLS 83·5 ± 10
STUDENTS T TEST	NS	NS

Fig 4

Figure 52 :-

DOSE RESPONSE OF SUPERNATANTS OF HOMOGENIZED RBC SPUN AT 1,700g FOR 20 MINUTES (●——●), 20,000g FOR 1 HOUR (○——○) AND HAEMOGLOBIN (▲- - -▲) PREPARED ON SEPHADEX G200 (see Fig. 51) AGAINST RHEUMATOID AND CONTROL LEUCOCYTES -COMBINED RESULTS - IN THE LMT.

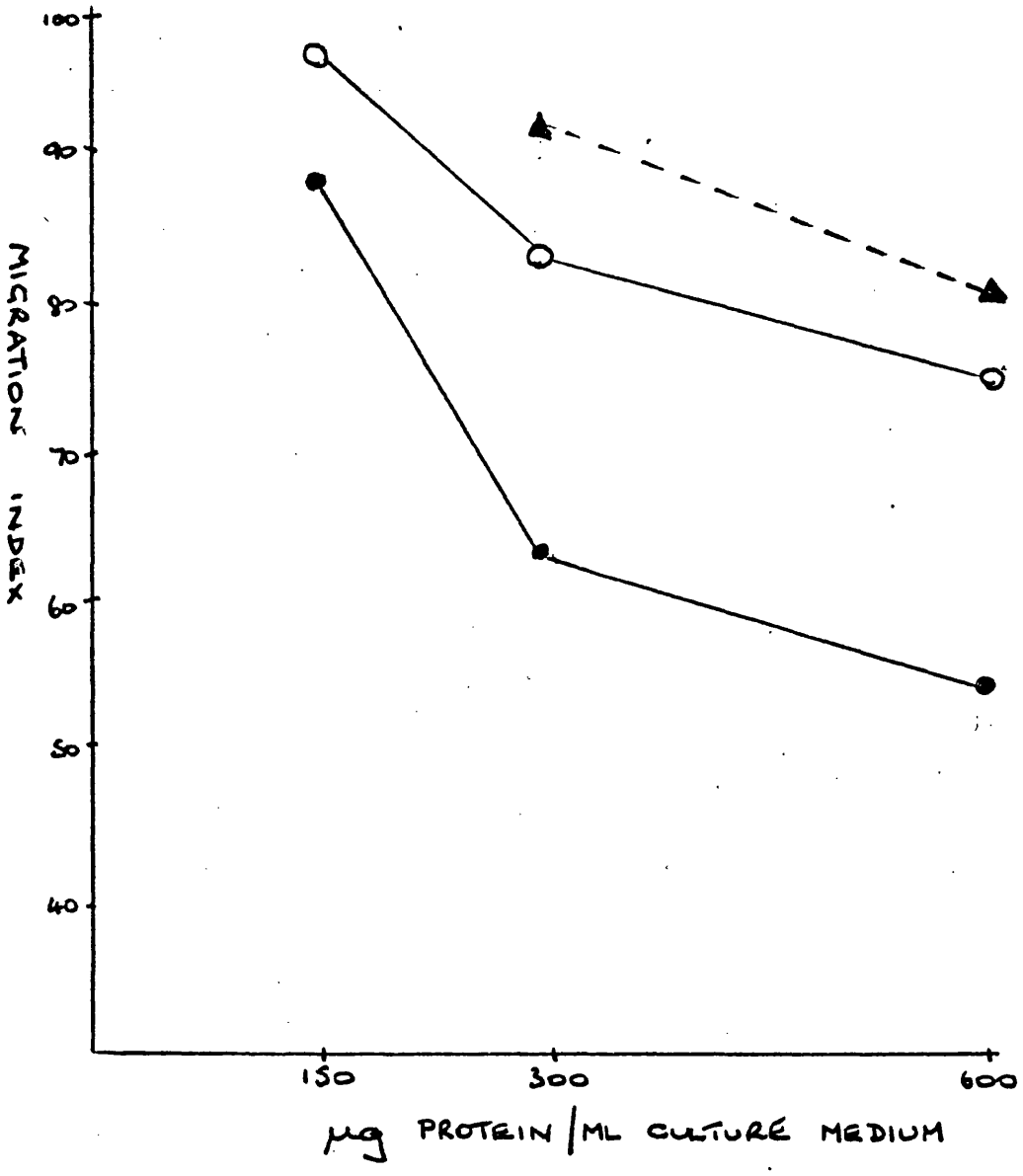

100
90
80
70
60
50
40
MIGRATION INDEX
150
300
600
µg PROTEIN / ML CULTURE MEDIUM

Fig: 52

Figure 52 :-

DOSE RESPONSE OF SUPERNATANTS OF HOMOGENIZED RBC SPUN AT 1,700g FOR 20 MINUTES (●——●), 20,000g FOR 1 HOUR (○——○) AND HAEMOGLOBIN (▲- - -▲) PREPARED ON SEPHADEX G200 (see Fig. 51) AGAINST RHEUMATOID AND CONTROL LEUCOCYTES -COMBINED RESULTS - IN THE LMT.

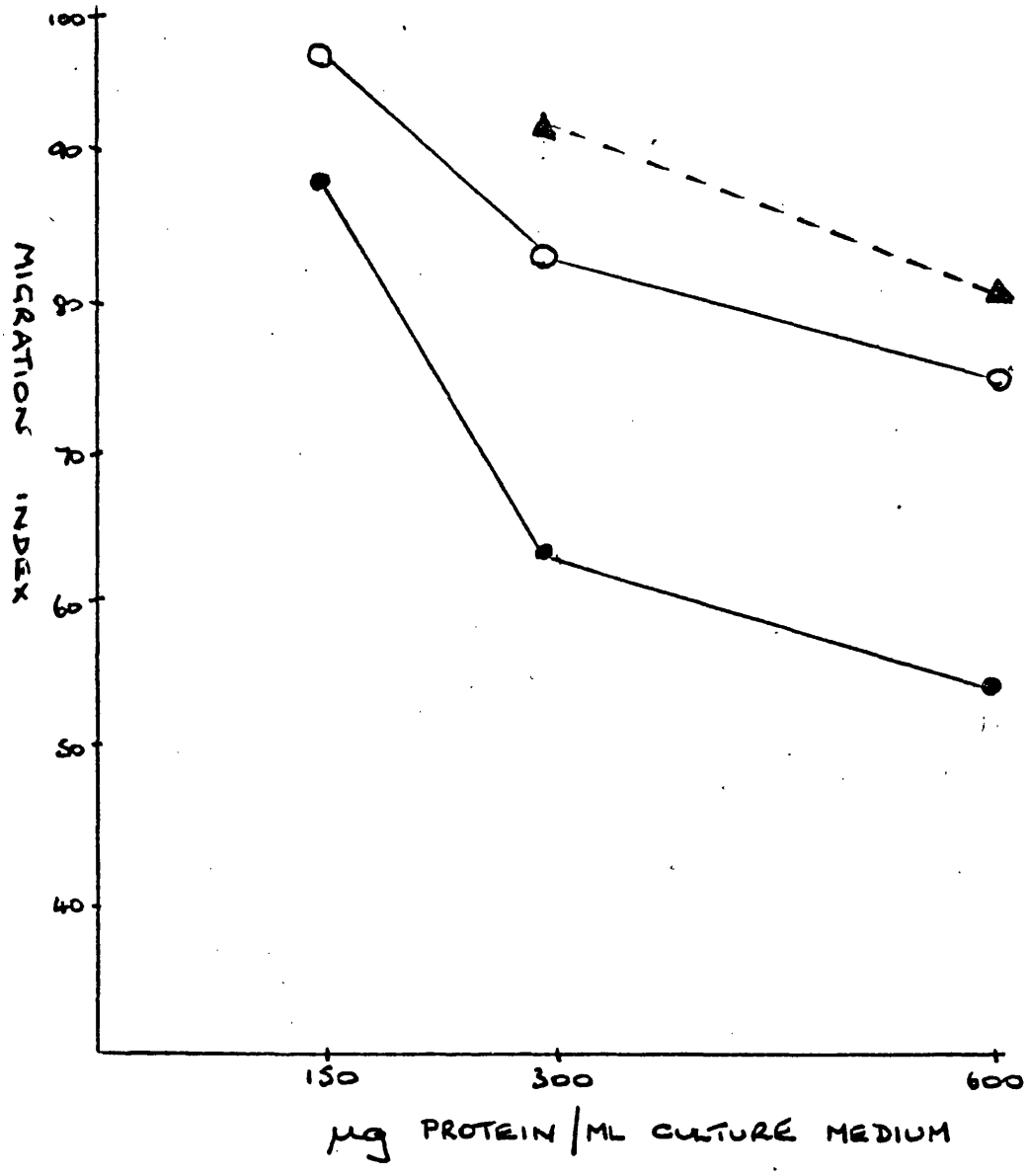

100
90
80
70
60
50
40
MIGRATION INDEX
150
300
600
µg PROTEIN / ML CULTURE MEDIUM

Fig: 52

SECTION III - RESULTS

Results illustrated in Fig. 49a show that homogenised red blood cell (RBC) membranes were inhibitory for both control and RA leucocytes in the LMT. There was no difference in response between RAs and controls.

When the supernatant was subjected to high speed centrifugation it was not possible to spin out all the inhibitory activity (Fig. 49b), although it was considerably reduced.

A haemoglobin preparation was prepared from the same RBC and tested as antigen in the LMT (Fig. 50). It was shown that 600 ug of haemoglobin/ml of culture medium was slightly inhibitory, (Fig. 50a). When subjected to high spin the haemoglobin preparation lost some of its inhibitory activity (Fig.50b). The degree of inhibition obtained using high and low spin haemoglobin preparations are not significantly different from each other. The haemoglobin preparation was subject to analysis on a Sephadex G-200 (Fig. 51) and the fraction tested in the LMT. It was found that the only peak eluted in the 22-25th fractions corresponded to a M.Wt. of 68,000 daltons and was haemoglobin. When this inhibitory activity was compared with the unfractionated solution, the haemoglobin accounted for all the inhibitory activity.

SECTION IV - DISCUSSION

Homogenised RBC membranes are inhibitory in the LMT. Unfortunately not all the inhibitory material can be spun out of the homogenates, in contrast to the results with crude synovial homogenates (Chapter 4, Section III). The situation was further complicated because haemoglobin was inhibitory.

The inhibition produced by these materials was the same in both rheumatoid and control leucocytes.

It is not possible to directly compare the results just presented with those in Chapter 4, Fig. 16,17. However, it is possible to say that the amount of inhibition is of a similar magnitude.

SECTION V - CONCLUSION

It is possible to induce inhibition of migration in the LMT using RBC membrane homogenates.

This inhibition is not specific for rheumatoid cells.

CHAPTER 8

THE EFFECT OF PUTATIVE ANTIGENS ON RHEUMATOID AND CONTROL LYMPHOCYTES IN THE LYMPHOCYTE TRANSFORMATION TEST

CHAPTER 8

THE EFFECT OF PUTATIVE ANTIGENS ON RHEUMATOID AND CONTROL LYMPHOCYTES IN THE LYMPHOCYTE TRANSFORMATION TEST

SECTION I - INTRODUCTION

In the previous chapters results have been presented to show that leucocyte migration can be inhibited by streptokinase (Chapter 3), crude synovial homogenates (Chapter 4), various protein solutions eluted from synovial cells (Chapter 5), immunoglobulin G (Chapter 6) and red blood cell membranes (Chapter 7).

It has been reported that an in vivo delayed hypersensitivity reaction can be detected in vitro by inhibition of leucocyte migration in the LMT. It is also known that this is not the only means by which leucocyte motility is altered so as to produce leucocyte migration inhibition in this test (Brostoff, 1974; Bryant et al, 1966).

The lymphocyte transformation test (LTT) has also been used as an in vitro correlate of in vivo DH. Again the transformation can be caused by a variety of mitogens, and not only a sensitizing antigen (Loeb, 1974).

However, the ability of a substance to induce blast formation would add weight to the argument that the leucocyte migration inhibition is due to the production of the specific lymphokine LIF.

SECTION II - EXPERIMENTAL

A. MATERIALS

The antigens tested were crude synovial homogenate and a combined MI and MII eluate.

In addition standard cultures were set up using Test Streptokinase. SK-SD is known to cause lymphocyte transformation (Spitler et al, 1972; Tomar et al, 1972; Rocklin, 1974). In this case the antigen used was Test Streptokinase (Hoechst), the reactions of which have not been well documented.

Cultures were also set up using PHA, a standard mitogen, as a positive control for the test system.

B. METHODS

(i) Culture Technique

The culture technique used was that described in Chapter 2, Section III.

(ii) Antigen Preparation

a) Test Streptokinase was made up as described in Chapter 3, Section

b) Crude homogenate was that used from JC, see Chapter 4, Fig. 8 Homogenate subjected to low spin was used.

c) A variety of MI and MII eluates which had previously been shown to be inhibitory in the LMT (Chapter 5, Figs. were pooled. They were filtered through an 0.22 μ filter (millepore). A Folin-Ciocalteau protein determination was carried out (Lowry, 1951). This protein solution was then tested in the LTT.

TABLE 3 :-

DOSE RESPONSE OF PHA AT GIVEN CONCENTRATIONS AGAINST HEALTHY (C) AND RHEUMATOID ARTHRITIC (RA) LYMPHOCYTES IN THE LYMPHOCYTE TRANSFORMATION TEST.
THE CELLS WERE HARVESTED ON DAY 3 OF CULTURE.

The stimulation is presented as a TRANSFORMATION INDEX.

SUBJECT	DISEASE STAGE	BACKGROUND COUNTS	μg PHA/ML CULTURE MEDIUM				
			1·0	5·0	20·0	100·0	500·0
JH	RA	5314	1·67	1·53	8·48	4·70	1·11
JM	C	[illegible]	14·70	4·47	12·27	9·03	7·15
NH	C	[illegible]	2·60	6·26	7·28	4·45	1·09
JM	C	[illegible]	–	–	10·63	13·72	3·85
JM	C	[illegible]	–	6·48	9·60	10·60	0·89
JC	C	1948	3·17	3·57	1·82	2·52	1·34
GC	RA	2996	1·26	1·06	2·01	1·45	1·16
MT	RA	752	–	13·60	39·02	20·35	–
MT	RA	561	–	7·41	12·15	8·44	–

Table:3

TABLE 4 :-

DOSE RESPONSE OF PHA AND TEST STREPTOKINASE AT GIVEN CONCENTRATIONS AGAINST RHEUMATOID AND CONTROL LYMPHOCYTES IN THE LYMPHOCYTE TRANSFORMATION TEST.

PHA cultures harvested on day 3
Streptokinase cultures harvested on day 5
The stimulation is presented as a transformation index

Soil Type	Soil Reaction (pH)	Organic Carbon (%)	Available Nitrogen kg/ha				Available Phosphorus kg/ha				Available Potassium kg/ha			
			Low	Medium	High	Very High	Low	Medium	High	Very High	Low	Medium	High	Very High
JH	RA	517	0.9	445	94	3.6	5.0	20.0	100.0	200.0	1.5	2.2	1.5	1.4
JM	C.	516	5.9	10.1	0.6	-	10.1	5.4	3.1	1.7	3.6	1.9	1.5	2.2
JW	C	1013	6.5	5.4	3.1	1.7	1.5	1.9	1.5	1.1	1.5	1.5	1.5	1.4
M.	RA.	878	10.1	21.8	13.7	5.2	7.0	5.4	2.1	1.1	1.5	1.5	1.5	1.4

Table: 4.

TABLE 5:-

DOSE RESPONSE OF PHA AND COMBINED ELUATES AT GIVEN CONCENTRATIONS AGAINST ONE RHEUMATOID'S AND ONE CONTROL'S LYMPHOCYTES IN THE LYMPHOCYTE TRANSFORMATION TEST.

PHA cultures harvested on day 3

Combined eluates cultures harvested on day 5

Two series of cultures were run in parallel

The stimulation is presented as a transformation index.

Table 5

Species	Strain	Serial No.	µg PHA/ml. culture medium			Serial No.	Combined eluates µg protein /ml. culture medium			
			5.0	20.0	100.0		25.0	50.0	100.0	200.0
MT	R1	374	11.1	15.0	12.7	2383	2.2	2.5	2.0	1.1
						2373	2.2	3.3	1.8	1.8
SH	C	1309	6.6	4.7	3.5	1904	2.8	3.3	5.9	4.2
						6761	0.7	0.6	0.8	1.6

TABLE 6:-

DOSE RESPONSE OF PHA AND CRUDE HOMOGENATES AT GIVEN CONCENTRATIONS AGAINST ONE RHEUMATOID'S AND ONE CONTROL'S LYMPHOCYTES IN THE LYMPHOCYTE TRANSFORMATION TEST.

PHA cultures harvested on day 3

Crude homogenate cultures harvested on day 5

The stimulation is presented as a transformation index.

JW and WW cells also respond to a test antigen - streptokinase - see Table 6.

Subject	BACK-Ground Counts	Disease Category	BACK-Ground Counts	Malaria Parasite / 100 WBC									
MC	1013	C	873	100.0	50.0	35.0	18.9	1.3	1.0	0.8	0.7	0.5	0.1
MV	878	A	1191	100.0	50.0	35.0	18.9	1.3	1.0	0.8	0.7	0.5	0.1
MK	1013	C	873	100.0	50.0	35.0	18.9	1.3	1.0	0.8	0.7	0.5	0.1
MV	878	A	1191	100.0	50.0	35.0	18.9	1.3	1.0	0.8	0.7	0.5	0.1

Table: 6

TABLE 7 :-

DOSE RESPONSE OF PHA AT GIVEN CONCENTRATIONS AGAINST NORMAL LYMPHOCYTES IN THE LYMPHOCYTE TRANSFORMATION TEST.

Cells harvested on days 1,2,3,4,5,6,8, and 10.

The stimulation is presented as a TRANSFORMATION INDEX.

Sl. No.	Count/ml	µg PHA/ml culture medium		
		5.0	20.0	100.0
1.	516	0.60	1.46	1.36
2.	938	1.42	1.22	1.70
3.	732	4.70	26.60	9.20
4.	190	3.50	17.90	9.90
5.	738	6.33	11.90	7.52
6.	6361	1.03	1.62	1.05
7.	3613	1.95	2.00	1.86
8.	89242	0.87	0.88	0.71

Table: 7

TABLE 8:-

DOSE RESPONSE OF TEST STREPTOKINASE AT GIVEN CONCENTRATIONS AGAINST NORMAL LYMPHOCYTES IN THE LYMPHOCYTE TRANSFORMATION TEST.

Cells harvested on days 1,2,3,4,5,6,8,10.

The stimulation is presented as a TRANSFORMATION INDEX.

S. No.	Organism	i.u. STREPTOKINASE / ML CULTURE MEDIM		
		50	100	200
1.	516	1.01	0.70	0.93
2.	838	1.06	1.10	0.90
3.	732	0.79	0.72	2.57
4.	190	1.31	1.19	2.67
5.	738	0.69	5.93	4.45
6.	6361	0.95	0.80	0.60
8.	2613	1.90	2.40	2.72
10.	88242	0.35	0.35	0.68

Table: 3

Figure 53 :-

MICROSCOPIC EXAMINATION OF CULTURES OF RHEUMATOID AND CONTROL LYMPHOCYTES WITH PHA.

CULTURE No. 1 = no mitogen
CULTURE No. 2 = 5ug/ml culture medium
CULTURE No. 3 = 20ug/ml culture medium
CULTURE No. 4 = 100ug/ml culture medium

Cells divided into groups by size: $< 7.5\mu$, 7.5 - 10.0μ, 10.0 - 12.5μ, 12.5 - 15.0μ, and $> 15.0\mu$.

Each histogram represents the mean of 16 readings $\pm$ ONE SD.

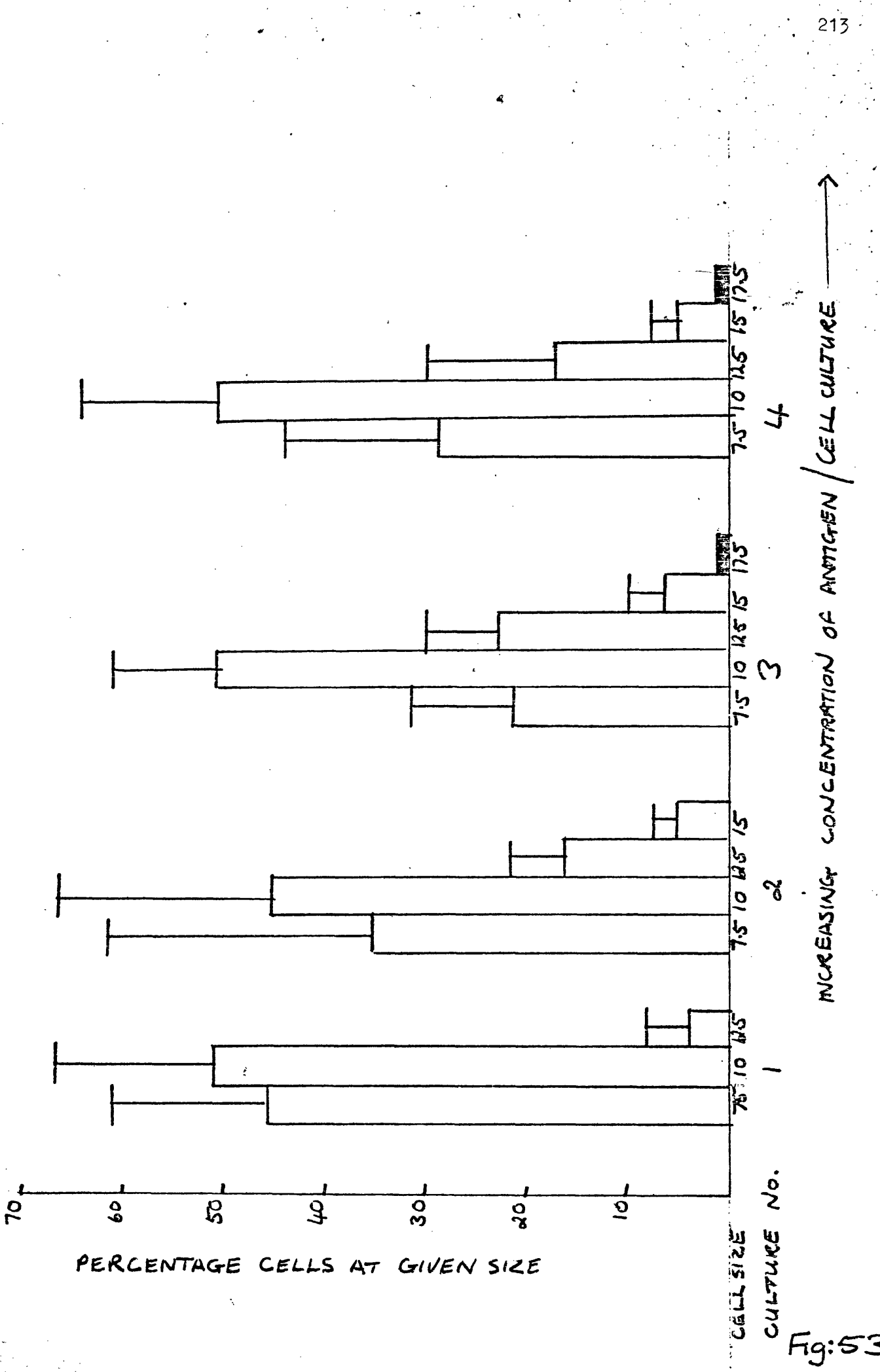

70
60
50
40
30
20
10
PERCENTAGE CELLS AT GIVEN SIZE
CELL SIZE
CULTURE No.
1
2
3
4
INCREASING CONCENTRATION OF ANTIGEN / CELL CULTURE

Fig: 53

Figure 54 :-

MICROSCOPIC EXAMINATION OF CULTURES OF RHEUMATOID AND CONTROL LYMPHOCYTES WITH TEST STREPTOKINASE:

CULTURE No. 1 = no antigen

CULTURE No. 2 = 5 µg/ml culture media

CULTURE No. 3 = 20 µg/ml culture media

CULTURE No. 4 = 100 µg/ml culture media

Cells divided into groups by size: $<$ 7.5 µ, 7.5 - 10.0µ, 10.0 - 12.5 µ, 12.5 - 15.0µ and $>$15.0µ.

Each histogram represents the mean of 4 readings $\pm$ ONE SD

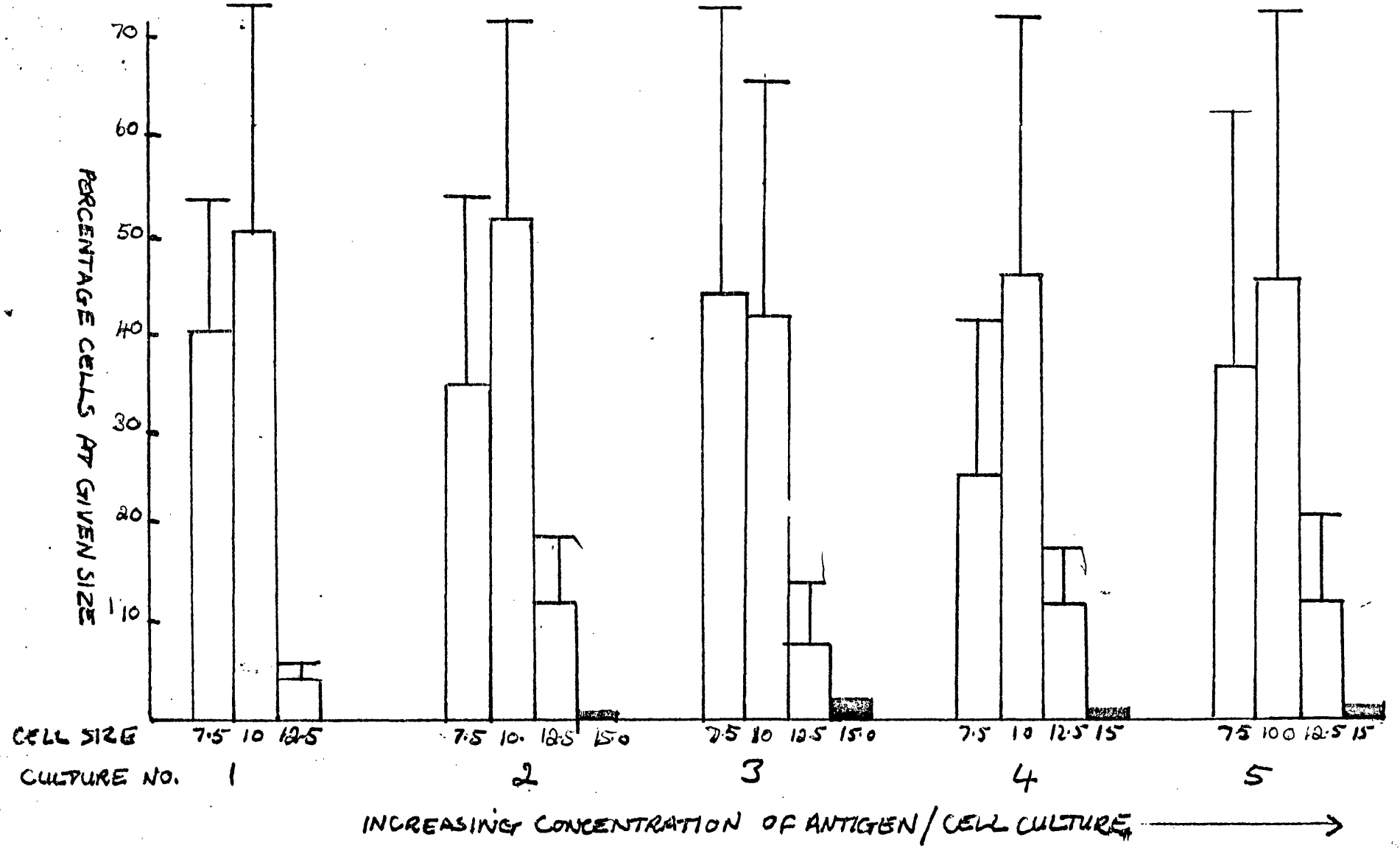

PERCENTAGE CELLS AT GIVEN SIZE
70
60
50
40
30
20
10
CELL SIZE
7·5 10 12·5
7·5 10· 12·5 15·0
7·5 10 12·5 15·0
7·5 10 12·5 15
7·5 10·0 12·5 15
CULTURE NO. 1 2 3 4 5
INCREASING CONCENTRATION OF ANTIGEN / CELL CULTURE

Fig. 54

Figure 55:-

MICROSCOPIC EXAMINATION OF CULTURES OF RHEUMATOID AND CONTROL LYMPHOCYTES WITH COMBINED ELUATES p199.

CULTURE No. 1 = no antigen
CULTURE No. 2 = 25µg/ml culture medium
CULTURE No. 3 = 50µg/ml culture medium
CULTURE No. 4 = 100µg/ml culture medium
CULTURE No. 5 = 200µg/ml culture medium
CULTURE No. 6 = 400µg/ml culture medium

Cells divided into groups by size: $<$ 7.5µ, 7.5µ - 10.0µ, 10.0 - 12.5µ, $>$ 12.5µ.

Each histogram represents the mean of 14 readings $\pm$ ONE SD.

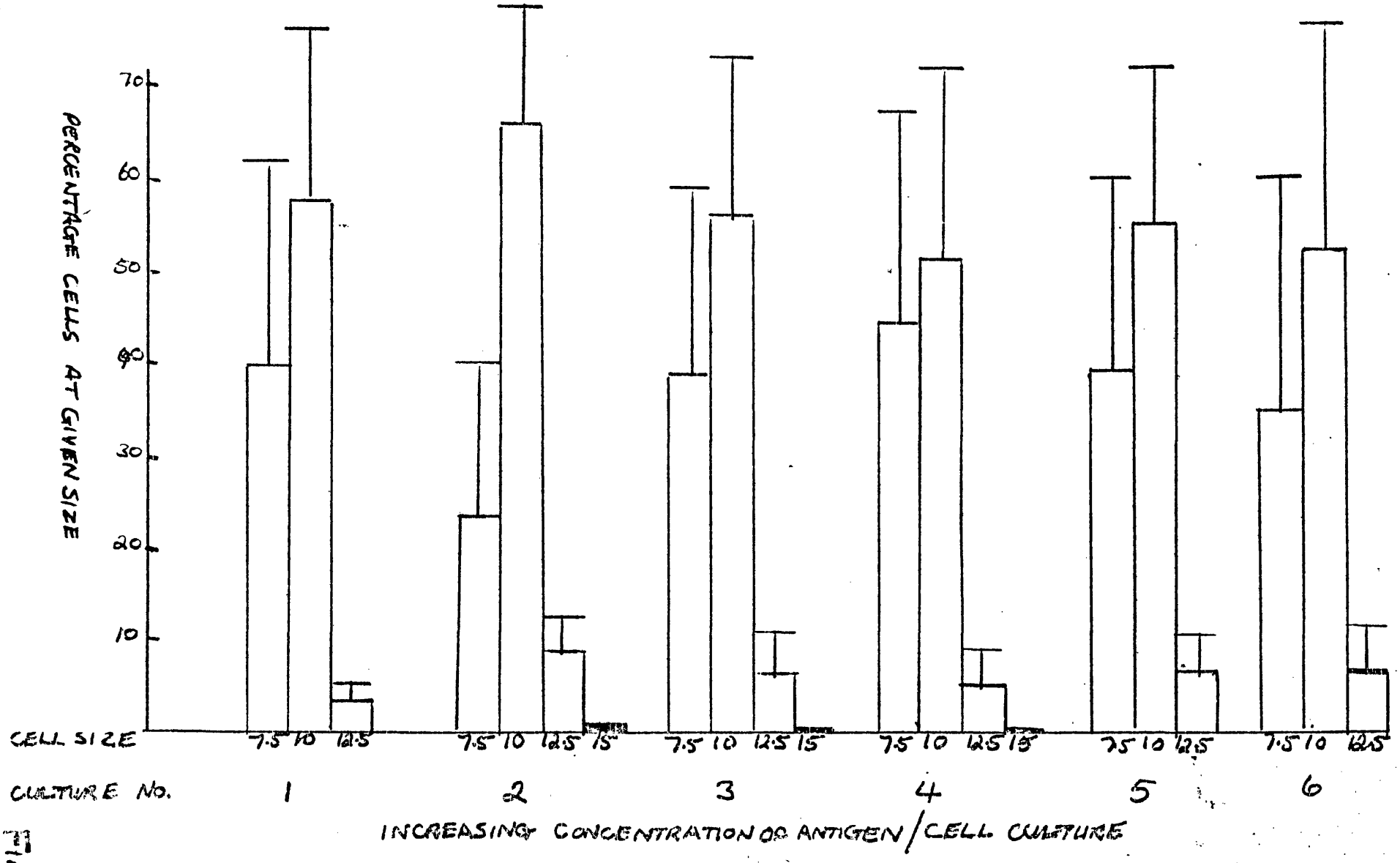

PERCENTAGE CELLS AT GIVEN SIZE
70
60
50
40
30
20
10
CELL SIZE
7.5 10 12.5
7.5 10 12.5 15
7.5 10 12.5 15
7.5 10 12.5 15
7.5 10 12.5
7.5 10 12.5
CULTURE No.
1
2
3
4
5
6
INCREASING CONCENTRATION OF ANTIGEN / CELL CULTURE

Fig 55

Figure 56 :-

MICROSCOPIC EXAMINATION OF CULTURES OF RHEUMATOID AND CONTROL LYMPHOCYTES WITH CRUDE HOMOGENATE(JC).

CULTURE No. 1 = no antigen
CULTURE No. 2 = 25µg/ml culture medium
CULTURE No. 3 = 50µg/ml culture medium
CULTURE No. 4 = 100µg/ml culture medium
CULTURE No. 5 = 200µg/ml culture medium
CULTURE No. 6 = 400µg/ml culture medium

Cells divided into groups by size: $\leq$ 7.5µ, 10.0 - 12.5µ, 12.5 - 15.0µ.

Each histogram represents the mean of 11 responses $\pm$ ONE SD.

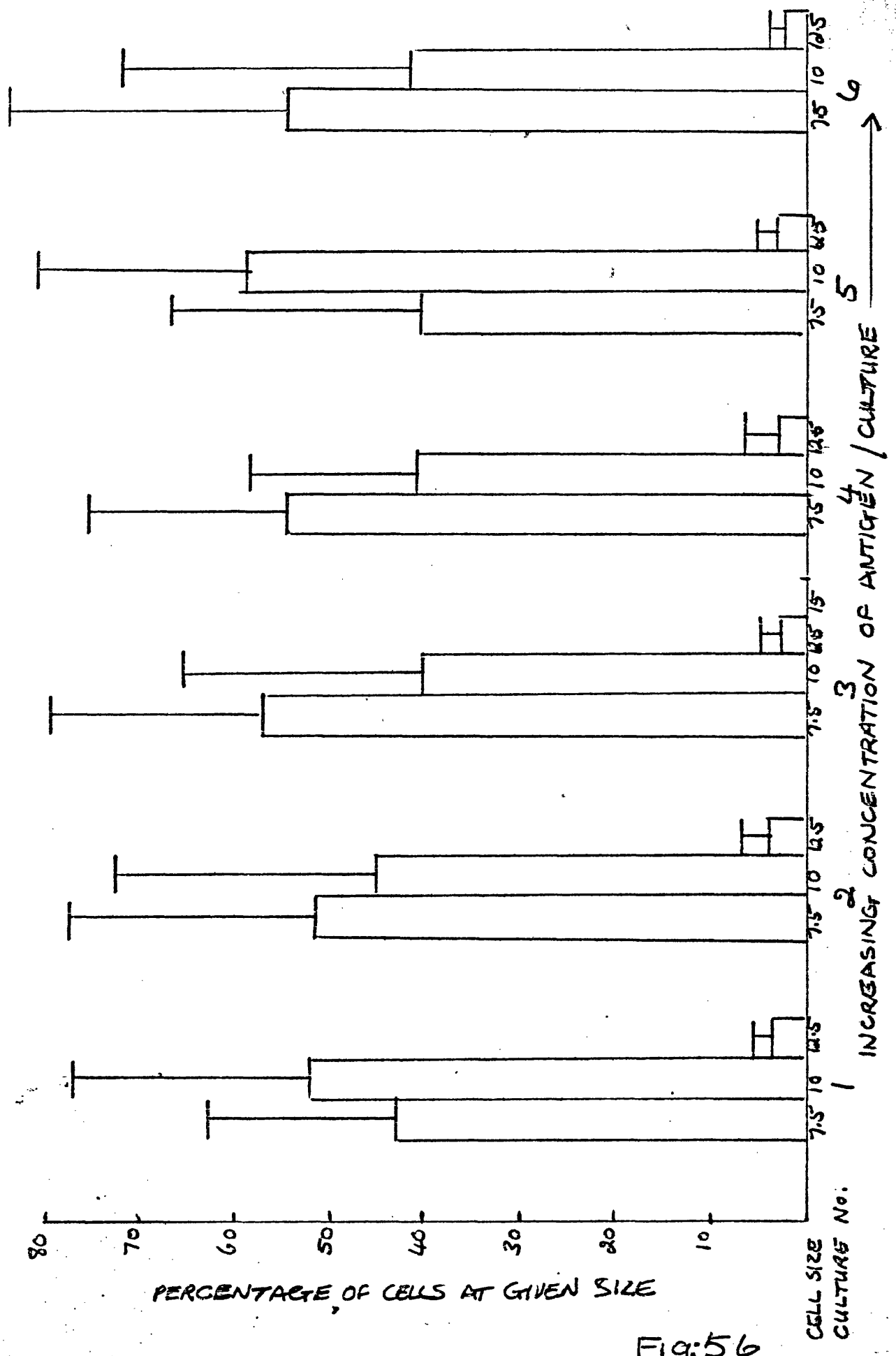

80
70
60
50
40
30
20
10
PERCENTAGE OF CELLS AT GIVEN SIZE
CELL SIZE
CULTURE No.
1
2
3
4
5
6
INCREASING CONCENTRATION OF ANTIGEN / CULTURE
7.5
10
12.5
15

Fig:56

SECTION III - RESULTS

The initial cell population contained not less than 99% lymphocytes. The contaminating cells were usually erythrocytes, occasionally PMNs. The viability of this population of cells was at least 99% as tested by trypan dye exclusion (Boyse et al, 1972).

The cell population after culture for three days both with and without mitogen contained at least 99% viable cells.

The cell population after culture for five days contained not less than 97% viable cells, except when cultured with 500 or greater i.u. streptokinase or 400 µg/ml or greater of the crude homogenate.

Results are expressed as a Transformation Index (TI).

$$TI = \frac{\text{mean dpm for test culture containing antigen or mitogen}}{\text{mean dpm for control culture}}$$

Three cultures were set up at each antigen or mitogen concentration.

a) Phytohaemagglutinin Cultures

Results are presented in Table 3 and Fig. .

In Table 3 the results are presented as TI derived from the incorporation of ^{3}H thymidine in DNA.

Fig. 53 shows the results expressed as size distribution of the cultured cells. In the presence of PHA the number of large cells, i.e. greater than 10.5 µ diameter, increases from less than 5% in control cultures to 25% in the test culture containing 20 µg of PHA.

A series of cultures were set up to determine on which day PHA induced maximum stimulation of lymphocytes. This was day 3.

A similar series of cultures were set up for streptokinase. The day on which maximum stimulation occurred was day 5, although ^{3}H thymidine incorporation was slightly increased on day 4.

In future experiments the PHA cultures were harvested on day 3, and the test antigens on day 5.

Since these results appear to validate our culture system, the test antigens were next used in this system.

b) Streptokinase

Table 4 presents the results obtained using streptokinase as our test antigen. Fig. 54 is the microscopic examination of a further 12 cultures. The control cultures after five days contain less than 5% large cells, whereas the test cultures consistently contain more than 5%.

c) Combined Eluates

The combined eluates (Table 5) show some increased ^{3}H thymidine incorporation. The microscopic examination indicates that again the control cultures contain less than 5% of large cells, while the test cultures contain more than 5% (Fig. 55).

d) Crude Homogenates

The crude homogenates caused no increase in ^{3}H thymidine incorporation (Table 6). The microscopic examination indicates that the number of large cells are the same in test and control cultures. (Fig. 56)

SECTION IV - DISCUSSION

The results presented cannot be taken as convincing evidence of the ability of the test substances to cause blast transformation, but trends can be elucidated.

Both RA and control lymphocytes transformed in response to PHA. The low incorporation of ^{3}H thymidine must be attributed to the culture technique. A micro-culture technique was used, based on that employed by Froebel et al (1975), who successfully used it for both antigenic and mitogenic cultures.

Certain criticisms must be made of the system as developed by myself.

a) PHA was reagent grade used primarily to agglutinate erythrocytes (Li and Osgood, 1949).

b) Risk of contamination is very high since the foil overcaps do not form as good a seal as one would obtain using culture bottles.

c) The cells form a pellet in the bottom of the culture well, which is difficult to redistribute on addition of ^{3}H thymidine. The redistribution is perhaps not helped by the cell agglutinating properties of the crude PHA used.

The streptokinase did cause transformation. This validates the results obtained in Chapter 3 using this particular streptokinase preparation as an antigen in the LMT.

The crude homogenates did not cause blast transformation of the lymphocytes, despite their being inhibitory in the LMT. Crude homogenates have been used to stimulate lymphocytes in the LMT. Hazleman et al (1975) used homogenised artery and muscle in polymyalgia rheumatica, while Esiri (1973) used muscle in myopathy. Many workers have failed to cause stimulation of

blast transformation with crude homogenates, including Rothenberger and Thiele (1970b) using crude synovial homogenates. The possibility exists that this could be due to the crude nature of the antigen presentation, and not to the lack of antigen. The possibility also exists that the fragments are so large that they mask the receptors, or they could induce agglutination of the cells preventing movement.

The eluates did cause transformation in both rheumatoid and control lymphocytes. These protein solutions had, of course, caused inhibition of migration in the LMT.

SECTION V - CONCLUSION

Using the microculture technique it is possible to show stimulation of rheumatoid and normal lymphocytes with combined MI and MII synovial eluates.

The crude homogenate was not stimulatory in this culture system.

The implication of these results will be discussed in the light of results obtained by testing the homogenates and eluates in the LMT.

The streptokinase preparation caused lymphocyte transformation.

CHAPTER 9

DISCUSSION

CHAPTER 9
DISCUSSION

TABLE 9 :- CAUSES OF INHIBITION OF MIGRATION IN THE LEUCOCYTE MIGRATION TEST

Author	Year	Observation
Packalen and Wasserman	1971	Thyroglobulin can cause inhibition of migration of guinea pig peripheral blood leucocytes passively sensitized with $\gamma 2$ serum fraction of an immune animal.
Brostoff, Howell and Roitt	1973	Bridging mechanism for the inhibition seen when rheumatoid arthritic cells are cultured in the presence of aggregated IgG, i.e. cytophilic IgG forms complexes with the aggregated IgG.
Brostoff	1974	PMNs are capable of releasing a factor which will cause migration inhibition of human leucocytes.
Eibl and Sitko	1975	Antigen-antibody complexes can cause production of MIF by rheumatoid lymphocytes. They believe the difference in response of the rheumatoid and control lymphocytes is quantitive and not qualititive.
Bryant et al	1966	Inhibition of white blood cell migration after ingestion of latex particles.
Brown	1976	Leucocyte migration - both lymphocyte and PMN - inhibited by NSAID *in vitro* due to a change in surface charge induced by the small drug molecules.
-	-	A toxic effect of the putative antigen on the cell metabolism which does not necessarily cause cell death.

SECTION I - INTRODUCTION

The aim of this discussion is to examine the results presented in the preceding chapters in the context of of published work. The mechanisms of inhibition of migration in the LMT and stimulation in the LTT of the various putative antigens will be explored. The possibility that the effect might not be that of a trigger antigen, but perhaps that of a perpetuator of chronic inflammation, is discussed.

SECTION II - CAUSES OF INHIBITION OF MIGRATION IN THE LMT

This work was carried out assuming that the LMT and the LTT were *in vitro* correlates of *in vivo* delayed hypersensitivity. Evidence culled from the literature to substantiate this assumption was presented in Chapter 1, Section IX, B (ii) and IX, B (iv). During the course of the experimental work it has become obvious that leucocyte migration can be inhibited by a variety of mechanisms.

The causes of inhibition of leucocyte migration have been summarized in Table 9. Brostoff (1974) has reviewed the mechanisms for inhibition of both peripheral blood leucocytes and peritoneal macrophages.

SECTION III - SIMILAR RESPONSES OF RHEUMATOID AND CONTROL LEUCOCYTES TO A STANDARD ANTIGEN

When RA patients received only NSAID the mean response of their leucocytes in the LMT to the antigen streptokinase was the same as healthy control volunteers. However, the variation within the rheumatoid population was greater than that of the control population - i.e. one SD = 13·7 compared with 6·5 - (Chapter 3, Fig. 5). The rheumatoid population

had larger standard deviations than the control in many of the experiments carried out. The results are reproducable from week to week when the same antigen is used to test the same subjects' leucocytes (Fig. 14). The difference in response between rheumatoid individuals in the LMT is such that when using Student's t-test as a test of significance only gross differences will be significantly different. Streptokinase caused an increased incorporation of ^{3}H thymidine and an increase in the number of large blast-like cells in the LTT.

SECTION IV - DISSIMILAR RESPONSES OF RHEUMATOID AND CONTROL LEUCOCYTES TO A TEST ANTIGEN - IgG

The results obtained using native and aggregated IgG as an antigen in this test showed that there is an antigen to which rheumatoid and control leucocytes give statistically significant different results (see Figs. 47 and 48).

A. COMPARISON OF THE RESULTS REPORTED IN CHAPTER 6 WITH THOSE IN THE LITERATURE

The results reported in Fig. 47 are tested against 250 µg of native or aggregated IgG/ml culture medium. For rheumatoids the MI was 92 and 72 respectively, and for controls the MI was 100 and 90. The results for the aggregated IgG compare well with those obtained by Brostoff et al (1973), which were 92 and 77 using 250 µg/ml of aggregated IgG as antigen. Massoud (1975) reports MIs of 112 and 81 respectively. Frøland and Gaarder (1971) used 270 µg/ml of aggregated IgG and the reported MIs were 92 and 66 for control and RA leucocytes respectively. They obtained migration indeces of 106 and 92 testing control and rheumatoid leucocytes against 400 µg/ml native IgG. In Fig. 48, migration indeces of 97 and 89 are reported. Of interest is that the source of IgG varies from experiment to experiment, some using

commercial preparations (Fr/land and Gaarder, 1971), others preparing their own by the method of Ishizaka (1965) (Brostoff et al, 1973).

Thus the results reported by the various researchers using IgG are consistent from report to report.

SECTION V - SIMILAR RESPONSES OF RHEUMATOID AND CONTROL LEUCOCYTES TO ERYTHROCYTE HOMOGENATES

In Chapter 7 evidence was presented that red blood cell homogenates cause inhibition of leucocyte migration. The mechanism of action has not been elucidated.

A. COMPARISON OF RESULTS REPORTED IN CHAPTER 7 WITH THOSE IN THE LITERATURE

Bendixen (1969) used homogenised kidney, liver and adrenal gland as control antigens for experiments using intestinal muscle tissue homogenate as a test antigen in the LMT. He states that he titrated out "toxic inhibition" of these tissues in control subjects at 100 µg/ml for the kidney, 150 µg/ml for liver, and 200 µg/ml for adrenal gland. The quantity of tissue homogenate was measured as µg protein/ml of culture medium. Therefore tissue homogenates can cause non-specific inhibition of leucocyte migration in the LMT. Using our synovial tissue homogenates (Fig. 16) they are invariably inhibitory at 400 µg/ml, but the inhibitory activity titrates out between 200 and 50 µg/ml. This is the same order of magnitude as that reported by Bendixen (1969).

The preparation of our erythrocyte membrane homogenate did vary from that used for the crude synovial homogenate. Firstly an ULAX 1020 homogeniser was used, which is much more effective than the hand homogeniser and sieve which were used for the rheumatoid synovial homogenates (Chapter 4). The RBC homogenate was also spun at 3000 g, because spinning at 1000 g for 20 minutes still left much membranous material in the supernatant when examined microscopically.

B. NATURE OF INHIBITORY MECHANISM

It is perhaps obvious that it is easier to homogenise a single cell suspension than a tissue such as synovium, complete with its basement membrane and fibrinous deposits. The nature of the inhibition is unknown, but could be due to phagocytosis of membrane fragments by the PMNs. The mature erythrocyte does not express HL-A antigens on its surface, so it is unlikely to be an antigenic inhibition (Kahan et al, 1973). Haemoglobin was also found to be inhibitory, but its mechanism of action is also unknown. It is important to state that the synovial specimens were washed thoroughly before homogenisation, and so contamination with haemoglobin should be minimal.

SECTION VI - SIMILAR RESPONSES OF RHEUMATOID AND CONTROL LEUCOCYTES TO CRUDE SYNOVIAL HOMOGENATES

A. COMPARISON OF RESULTS REPORTED IN CHAPTER 4 WITH THOSE IN LITERATURE

(i) Synovial homogenates

In preceding experiments (Chapter 4) it has been shown that rheumatoid synovial homogenates were inhibitory in the LMT, but were not specific for rheumatoid leucocytes. When previous reports of specific inhibition of rheumatoid leucocytes in this test system are examined in depth certain discrepancies become apparent. Bacon et al (1973) report specific inhibition of rheumatoid leucocytes using a rheumatoid synovial homogenate as antigen at a protein concentration of 45 µg protein/ml culture medium. They report no specific inhibition of rheumatoid leucocytes induced by 90 µg/ml of a psoriatic synovial homogenate. Osteoarthritic synovial homogenate was also ineffective. Rothenberger and Thiele (1971) do not state their protein concentration, but they find specific inhibition of rheumatoid leucocytes only with rheumatoid synovial homogenates, and not with an unspecified non-rheumatoid synovial homogenate. In a previous paper (Rothenberger and Thiele, 1970a) they report a protein concentration of 3.5 µg/ml used in the cultures which caused specific inhibition of rheumatoid leucocytes in the LMT.

Experiments have been carried out using high concentrations of albumin (1600 ugs/ml) in the IMT. These concentrations were not inhibitory, but these migration areas were more difficult to read accurately (Morgan, 1974) as the protein concentration increases.

Thonar and Sweet (1976) quantify their antigen concentration as 2 mg wet weight/ml which makes it impossible to compare these protein concentrations with the other reports. They are the only workers to report a specific inhibition of rheumatoid leucocytes migration using normal synovium as antigen. They also report that rheumatoid cells were inhibited more than control populations by a homogenate of cartilage and uvea-retina. It is thus impossible to compare these results with the others reported. Therefore unlike the reports of various workers of experiments using IgG which were reproducible, these reports bear little relationship to one another.

(ii) Other tissue homogenates

This type of experiment using crude tissue homogenates has not been confined to synovial homogenates against rheumatoid peripheral cells. Work has been carried out using homogenised intestinal mucosa in ulcerative colitis (Bendixen, 1969); liver homogenates in alcoholic hepatitis (Mihas et al, 1975) and liver homogenates in active chronic hepatitis, primary biliary cirrhosis and cryptogenic cirrhosis (Smith et al, 1972). All workers report specific inhibition of the test population of leucocytes. Homogenised muscle in Crohn's disease (Bendixen, 1969) and homogenised muscle and thymus in myasthenia gravis (Behan et al, 1975) did not cause specific inhibition.

(iii) Discrepancy in methodology

In the above experiments the methodolgy is of interest since none of the reports contain dose/response curves. The

inhibitory activity of the tissue homogenate is titrated out against control or normal leucocytes. Then the test population is tested with the first dose that was not inhibitory for the control leucocytes. It is possible that a bias is introduced into the results. (Doniach et al, 1975).

B. NATURE OF INHIBITORY MATERIAL IN THE CRUDE SYNOVIAL HOMOGENATES

It is possible that the inhibitory material is fibrin which has been shown to be inhibitory in very low concentrations for both normal and rheumatoid leucocytes (Hall 1974). However, it has been suggested that rheumatoid fibrinogen is altered when compared with that from normal subjects, and that the rheumatoid fibrinogen will induce specific stimulation of rheumatoid peripheral blood lymphocytes which normal fibrinogen will not (Ciobanu, 1975). Falk (1970) has shown inhibition of migration of human mononuclear cells using lymphocyte cell fragments as a source of HL-A antigen, however the leucocytes had to be taken from subjects previously sensitized to the antigen by other grafts or other means. There was no "non-specific" inhibition which would parallel the non-specific blast transformation induced by HLA in the MLC.

Biberfeld (1974) has shown that after immunization with mycoplasma both inhibition of migration and stimulation of transformation were positive but after months only this lymphocyte transformation test was positive.

Since it is possible to spin out all the inhibitory activity from the crude synovial homogenates it is likely that the inhibition is due to a physical barrier, a non-specific toxic effect or a decrease in migration caused by phagocytosis.

C. OTHER SOURCES OF ANTIGEN IN THE RHEUMATOID JOINT

Other sources of antigen from the rheumatoid joint have been used. Joint fluid has been used as antigen in the LTT and has been shown to cause stimulation of rheumatoid lymphocytes (Kinsella 1970, 1973, 1976; Crout , 1976). It is logical to assume that anything produced in the synovium by the various cells would eventually find its way into the synovial fluid. It is also true that substances could be degraded by the various enzymes known to be present and might not maintain their original conformation.

It is of interest that autologous fluids are more stimulatory than homologous (Kinsella 1976). It is easy to dismiss the stimulatory material as IgG which has been shown to be stimulatory in the LTT

(Dorner, 1969, 1974; Kinsella, 1974). Crout (1976) reports that not all the synovial fluids contained RF and that immune complexes were not present in all fluids and some contained neither.

Macrophage cytoplasmic membranes localize the antigen used to induce immune synovitis (Webb et al, 1972). Runge (1976) has taken autologous synovial fluid macrophages and used them to stimulate peripheral blood cells. Some stimulation occurs but not enough to be consistent with the presence of foreign antigen.

One system which has not been examined in such depth as a source of antigen is cartilage although some work has been done (Chapter 1 Section VII A). Jasin (1975) showed that in experimental allergic arthritis antigen-antibody complexes become sequestered in cartilage. PMN's still attempt to phagocytose these complexes causing release of enzymes with their inflammatory effect (Chapter 1. Figs. 1 and 2).

SECTION VII - EFFECTS OF SYNOVIAL ELUATES IN THE LMT

The synovial specimens collected were subjected to 3 protein elution or solubilization techniques.

(A) (i) EDTA/ME EXTRACTION.

Hypotonic solutions will elute lipoprotein (Bacon et al, 1973). Bacon et al (1973) succeeded in eluting a protein which maintained its specificity for inhibition of rheumatoid leucocytes. No rheumatoid-specific inhibitory material was eluted from the psoriatic membrane. In the results presented in Chapter 5 only one rheumatoid synovium yielded material which was specifically inhibitory for rheumatoid leucocytes (RS Fig. 21) and the difference shown to be statistically valid. The protein retained its specificity in the void volume protein after fractionation on Sephadex G 100 (Fig. 32) and lost it after fractionation on Ultragel ACA 22 (Fig. 33) although inhibitory material still eluted in the void volume. In all other cases there was a tendency for slightly increased inhibition of rheumatoid leucocytes but this was not statistically significant. It must be noted that the ankylosing spondylitic synovium yielded inhibitory material whereas the pigmented villinodular synovium did not. Thus, on rather slim evidence it would seem that the inhibitory

material could be associated only with inflammatory reactions.

(ii) Nature of inhibitory material.

In chapter one the inflammatory reaction was examined in some detail (Fig. 1) and in particular the inflammatory mechanisms contributory to the rheumatoid arthritic synovitis (Fig. 2). There are many possibilities for the origins of the inhibitory material. There are lymphocytes (T and B), plasma cells, synovial cells A and B present in the inflamed synovium. The inhibitory material could have been eluted in the membrane of any of the mentioned cell types and be either a neo-antigen or HL-A antigen. In addition the protein solution could contain fibrin or immune complexes. The protein could be acting in a non-specific manner such as haemoglobin (Chapter 7) or NSAID (Brown, 1976). Cracchiolo et al (1972) washed the synovial specimens six times to be certain of removal of "non-fixed" immune complexes. The homogenised synovial specimens used in these experiments were washed three times before being subjected to the elution procedure so the majority of the complexes should have been removed. To remove fixed complexes the homogenates were subject to elution with high molarity or acidic buffers. Our technique involved slightly alkaline water which should not remove the complexes. Hall (1978) has shown that large complexes i.e. those which run in the void volume of Sepharose 6B - a minimum molecular weight of 2×10^6 daltous - can cause inhibition of leucocyte migration at concentrations as low as 5 mg/ml. The rheumatoids are specifically inhibited when compared with control subjects. Immune complexes appear to cause production of MIF only by rheumatoid lymphocytes (Eibl and Sitko 1975) as does aggregated IgG in autologous rheumatoid subjects (Weisbart, 1975). HLA antigens are known to be eluted by this method (Verrier Jones, 1973). After fractionation the inhibitory material is of molecular weight 80 - 100,000 daltons (Fig. 33) or 800,000 by Ultragel ACA 22 analysis or 2×10^6 by Sepharose 6B analysis. Soluble HLA antigens have a molecular weight of 31,000 (Kahan et al, 1973). Carbohydrates do not contribute to the antigenic determinants of HLA antigens (Prat and Comoglio, 1975). As previously stated lymphocytes cell fragments used as a source of HLA antigens did not cause inhibition of migration unless the subjects had been previously sensitized (Falk, 1970). Tanner and Boxer (1972) report that proteins which

cannot be easily solubilized by gentle aqueous procedures represent about half of the erythrocyte "ghost" protein. They carry a large proportion of the cell surface carbohydrates. Tanner and Owens (1971) report that following the extraction method of Marchesi (1970) which was used in chapter 5 the insoluble residue consists of protein 47%, total phosphate 58%, cholesterol 57%, hexose 75% and salic acid 50% of that present in intact membrane. Therefore the glycoproteins are not removed.

B. DETERGENT SOLUBILIZATION

Viral-coded membrane antigens are generally glycoproteins (Rothman and Lodish 1977) and glycoproteins are generally integral membrane proteins which can be stabilized only by detergent methods (Helenius and Simons, 1971). It was decided to subject the synovium to detergent solubilization. Deoxycholate will solubilize 95% of plasma cell membrane (Chauvin and Holliman, 1975) and this seemed an ideal way of solubilizing the total protein content. However, even the control blank solutions were inhibitory (Figs 40, 41.) so that the cholate effect on the leucocytes could not be separated from any effect of solubilized membranes. It is known that numerous substances, among which are microbial products and other naturally occurring compounds, several surfactants and pharmacological agents which elicit metabolic processes which may be indistinguishable from those that occur during phagocytosis. The common property is an effect on the surface of the cell. It has been shown that the detergent deoxycholate can do this (Elsbach, 1973). There is very little difference between deoxycholate and cholate so presumably the latter can elicit the same type of response. Phagocytosis of particles causes inhibition of leucocyte migration due to the alteration in the respiratory pathways (Bryant et al, 1966). This is perhaps the mechanism of inhibition observed when the cholate extracted protein was used as antigen in the LMT.

The detergent Triton X100 is not known to have the same effects on leucocytes as deoxycholates (Elsbach, 1973). In fact in the LMT the Triton X100 extracted protein had no inhibitory effect at all. After column fractionation when the protein in the void volume was at a similar concentration to that extracted by EDTA/ME from rheumatoid synovium (Fig. 32) there was still no inhibition. It is possible

TABLE 10 :- CAUSES OF STIMULATIO OF LYMPHOCYTE TRANSFORMATION IN THE LYMPHOCYTE TRANSFORMATION TEST.

Author	Year	Category	Stimulatory substance
LOEB	1974	non-specific	PHA Con A PWM
			Divalent cations: Hg^{2+}, Zn^{2+}, Ni^{2+}.
			Bacterial products e,g, streptolysin 0 lipopolysaccharide
			Antibodies against cell surface components Anti-immunoglobulin sera
			Enzymes : trypsin chymotrypsin papain
BLOCH-SCHTADER et al	1968	non-specific	Antigen-antibody complexes.
MOLLER	1969	non-specific	
LOEB	1974	specific	Antigen to which the animal has been sensitized.
		unknown	HLA antigens

that some Triton still remains. It could react either with the cell membrane to block receptors or with the protein preventing its attachment to receptors. Perhaps in its absence i.e. in the EDTA/ME extraction the proteins assume a different conformation than in its presence.

C. CONCLUSION

The protein which is inhibitory in the LMT is not present in the original crude homogenate after high speed spin so it would seem to have arisen during the EDTA/ME elution procedure, presumably from the insoluble fraction spin out of the crude homogenates.
The nature of the protein is a matter of speculation but it seems to be present only in inflammatory exudates. It is unlikely that it is of viral origin since these produce glydoproteins (Rothman and Lodish, 1977) which are not eluted by EDTA/ME technique (Tanner and Boxer, 1972). Therefore it is a cell membrane antigen, it cannot arise by viral coding.

VIII. CAUSES OF TRANSFORMATION IN THE LTT.

Loeb (1974) gives a comprehensive review of the causes of blast transformation in the lymphocyte transformation test. See Table 10.

IX. COMPARISON OF LMT with LTT.

When the contents of this Table are compared with those causing inhibition of migrates in LMT. See Table 9 only 2 types of compound will cause both inhibition of LMT and stimulation of LTT i.e. specific antigen and antibody-antigen complexes. Our protein mixture contains a variety of proteins and it is conceivable that more than one active protein might be present, one having an effect in the LMT and a different one in the LTT.

X. THE EFFECT OF CRUDE HOMOGENATES ON LTT.

The crude homogenates were not found to be stimulatory. There have been reports of specific stimulation of patient groups crude tissue homogenates. Artery antigen has been used against patients with polymyalgia rheumatica (PMR) (Hazleman, 1975), muscle in PMR (Esiri, Maclennan and Hazleman, 1973) and muscle in polymyositis (Saunders et al, 1969) which is the only confirmed observation (Esiri et al, 1973).

In our situation due to the lack of stimulation in the LTT it would be possible to attribute the inhibition of the LMT to a non-specific toxicity or phagocytosis.

XI. THE EFFECT OF EDTA/ME eluates on LTT.

The EDTA/ME eluates were stimulatory in the LTT. Unfortunately both RA and control responded to these proteins. The stimulatory material could be immune complexes but it is unlikely for the reasons discussed previously (Chapter 9, Section VII) i.e. immune complexes would not be equally inhibitory for RA and controls in the LMT. It could be an antigen but one to which both RA's and controls had been sensitized. It is possible that the excised synovium became infected possibly during preparation and an ubiquitous microorganism to which both rheumatoids and control subjects have been exposed and to which they would react in the in vitro test solution. Only molecular weight prevents it from being HLA antigenic material (Kahan, 1973) which is known to be stimulatory in a soluble form (Viza et al, 1968). It is possible that this material which is stimulatory in the LTT accords with non-degradable biological protein. Protein has been eluted which is stimulatory in the LTT for both rheumatoid and control cells. Its mechanism of action could be antigenic or autogenic.

XII. POSSIBLE FUTURE LINES OF ENQUIRY.

It has been established that the inhibitory material possibly has a molecular weight of 80 - 100,000 (Fig. 32) but more likely it is $>$ 800,000(Fig. 32). One of the first lines of enquiry would be to establish the approximate molecular weight of the stimulatory material. Thus the possibility of the presence of one or more active proteins could be established. The nature of the material in the eluates which cause inhibition of migration of leucocytes and stimulation of transformation of lymphocytes should be investigated. One of the major points of debate has been the presence or absence of immune complexes. These can be identified using the following biological techniques: Clq precipitation, inhibition of platelet aggregation, mixed cryoglobulins, ultracentrifugation and electron microscopy of pellet and inhibition of antibody - dependent cytotoxicity (Cream, 1973 , Johnson et al, 1975, Hay et al, 1976, Penttinen, 1977). It would be of value to determine the presence or

absence of fibrinogen and collagen which could be carried out using immunoelectrophoresis. Anti-immunoglobulin sera could be used as a preliminary to the testing for immune complexes or to establish the immunoglobulin class of immune complexes are present. These tests should be carried out on the high spin supernatants from the crude homogenates (Chapter 4 section II) which are not inhibitory and the EDTA/ME eluates which are inhibitory in the LMT. It is of interest that the active components were only eluted from inflammatory arthritides. It would be useful to test this further by extracting some non-inflammatory synovial specimens. The material might be associated with one or more of the cell types present in the inflammatory nidus. The individual cell types are T cells, B cells, macrophages, A and B cells of the synovial membrane, PMN's. The inflammatory arthritides are associated with granuloma formation and this rapidly proliferating tissue may produce this material. By looking at inflammation in another tissue e.g. Crohn's disease it might be possible to establish its presence in association with granuloma formation. The disadvantage of using the gut is the presence of bacteria and the inevitable endotoxins associated with them.

XIII. IMPLICATIONS OF THIS WORK

A. Assessment of test system.

One of the basic shortcomings of this work is the relative lack of specificity of the test procedures. Inhibition of leucocyte migration and stimulation of lymphocyte transformation can be induced by many types of compound (Chapter 9, Figs. 9 and 10). The variation of response in the test populations is very large. This is particularly true of the rheumatoid population. It is perhaps not surprising. We are testing an immunological parameter and there are various immunological abnormalities of this group of patients. The presence of high titres of rheumatoid factor, anti-DNA antibodies and high levels of IgG are serological examples. The proportions of T and B cells in the peripheral blood has been a subject of much debate (Chapter 1, Section VII), as has been the immunological capability of rheumatoid leucocytes. There has been much work carried out in search of a basic immunological defect of rheumatoid lymphocytes (Chapter 1, Section VIII). Work involving antigenic stimulation of cell cultures using PHA has suggested the inability of RA cells to respond as well as healthy controls. This

work culminated in the paper of Silverman et al (1976) They defined two populations of rheumatoid patient: those with the same responses as a normal population and those with significantly lower responses who had severe erosive disease . Thus it would seem that RA's are a heterogeneous population whose intrinsic response would vary and who would be expected to respond less well to a given stimulus.

B. Assessment of Antigen Preparation.

Given this variation in test population the LMT might be too insensitive to pick up the presence of an antigen which would only be present in small quantities. Compounding the insensitivity of the test population is the heterologous nature of the 'antigen' especially in the crude preparation. Even if antigen were present its membranous nature could physically impede the attachment of enough antigen molecules to receptor sites to initiate the necessary changes in membrane to cause an obvious response. Even when the nature of a neoantigen is known and the means to detect it are chemically defined, its actual characterization in a different tissue can be difficult (Unger and Panayi, 1977). In this situation an unknown is being tested for using a variable indicator. Consideration of the theories of the aetiology of RA leads to the assumption that viral infection may be the factor leading to initiation of the immune response. It is known that virally-coded antigens may only be expressed on the cell membrane during defined stages of the cell cycle (Denman, 1975). In our experiments the inhibitory material was protein not glycoprotein which is normally the chemical composition of viral-coded antigens.

C. Conclusion.

In these experiments using many synovial preparations subjected to various biochemical manipulations and tested against many rheumatoid and control subjects there has been no consistent evidence of an antigen to which rheumatoids are specifically sensitized.

D. Implications for aetiology and Pathogenesis of RA.

One point which has arisen from the literature and which is relevant is that many more positive results are obtained on various test systems when autologous material is used as the test 'antigen' (Kinsella, 1976). It might indicate a different initiating stimulus in each individual

leading to the same clinical picture. In the preceding experiments a homologous test system was used, not autologous. A very similar clinical picture is seen in seropositive and seronegative arthritis. Yet it would seem that the immunological defect is more fundamental in the seropositive disease since in seronegative arthritis the switch has been made from the production of IgM to the production of IgG. This concords with the increased severity of disease in patients who present with early RA and a high Rose-Waaler titre.
The perpetuation of the disease becomes the important mechanism in disease pathogenesis. It could be that the patients have inadequately controlled immune responses to the antigen leading to an inability to switch off the immune response. Alternatively the original stimulus induces a different compound which is responsible for the perpetuation of the disease either by its continued presence or by its ability to override the controlling mechanisms of the immune response. In Fig. 2 the perpetuating mechanisms in RA are presented but there seems to be nothing which would be present that would not be present in any other inflammatory response to an immunological stimulus. This leads to the possibil ty that it is the site of the immune inflammatory response which causes the chronicity. If a protein which was too large to be removed through the synovial membrane by diffusion and was incapable of being degraded and removed by the reticulo-endothelial system was present then this could perpetuate the chronicity, especially if it were antigenic and stimulated an ongoing immune response. In our experiments a protein which caused inhibition of migration of both rheumatoid and control leucocytes in the LMT and stimulation of both control and rheumatoid lymphocytes in the LTT was eluted from inflammatory synovium. It could be that it was acting by a mitogenic mechanism or it could be that it was a protein to which both rheumatoids and controls were commonly sensitized and to which both would react in in vitro tests. It persists in the joints of the rheumatoid patient. Its mechanism of action could be likened to that of non-degradable bacterial cell walls (Ginsburg et al, 1976).

XIV. CONCLUDING STATEMENT.

Thus a protein has been eluted which is inhibitory in the LMT for both rheumatoid and age and sex-matched control subjects. It mis stimulatory for both rheumatoid and control lymphocytes in the LTT.

It is perhaps an ubiquitous antigen to which both rheumatoids and control subjects respond in the in vitro tests but which is not cleared by the normal mechanisms in the rheumatoid joint.

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