IMMUNE REACTIVITY TO HEAT SHOCK PROTEINS IN RHEUMATOID ARTHRITIS

by

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to my parents

ABSTRACT

This study was performed to pursue the hypothesis that immune reactivity to heat shock proteins (hsps), in particular to the mycobacterial 65 kDa hsp, was important in the aetio-pathogenesis of rheumatoid arthritis.

Humoral responses to mycobacterial and *Escherichia coli* 65 and 70 kDa hsps, as well as to human 70 kDa hsp, were studied in RA patients, healthy individuals and disease controls. It was found that the IgG antibody levels to the mycobacterial 65 kDa hsp, and not to the *E. coli* homologue, were significantly elevated in rheumatoid arthritis (RA) patients compared with healthy individuals, systemic lupus erythematosus, ankylosing spondylitis and Crohn's disease patients. This was the first indication that antibody responses to the mycobacterial 65 kDa hsp might be important in RA. There was evidence for cross-reactivity of these antibodies with human 65 kDa hsp (i.e. autoreactivity). A 65 kDa protein was found in RA synovial fluid samples and was also present in immune complexes from such fluids. There was no difference in the cellular responses of RA patients and healthy individuals to the mycobacterial 65 kDa hsp.

These findings suggest that the 65 kDa hsp molecule may be relevant to the aetiopathogenesis of RA. The regulatory mechanisms of the immune responses to the 65 kDa hsp remain to be elucidated.

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ABBREVIATIONS

AA	Adjuvant arthritis
AMLR	Autologous mixed lymphocyte reaction
ARC	Antigen responsive cells
AS	Ankylosing spondylitis
ATP	Adenosine triphosphate
BCG	Bacillus Calmette-Guérin
BiP	Immunoglobulin heavy chain binding protein
BSA	Bovine serum albumin
CD	Crohn's disease
CFA	Complete Freund's adjuvant
CPM	Counts per minute
CTR	Healthy controls
DNA	Deoxy-ribonucleic acid
EAE	Experimental autoimmune encephalomyelitis
EBNA	Epstein-Barr nuclear antigen
EBV	Epstein-Barr virus
EDTA	Ethylenediamine-tetraacetic acid
ELISA	Enzyme linked immunosorbent assay
ESR	Erythrocyte sedimentation rate
GRP(S)	Glucose regulated protein(s)
HI-FCS	Heat inactivated foetal calf serum
HI-NBCS	Heat inactivated new born calf serum
HLA	Human leukocyte antigen
HSP(S)	Heat shock protein(s)
IC	Immune complex
IFN-γ	Interferon-gamma
Ig	Immunoglobulin (A, M or G)
IL	Interleukin (-1 or -2)
I-UdR	Iodo-uridine-deoxyribose
kDa	Kilo-Dalton
LDA	Limiting dilution analysis
MHC	Major histocompatibility complex
MT	*Mycobacterium tuberculosis*

NC	Nitrocellulose membrane
NK	Natural killer cells
OA	Osteoarthritis
OD	Optical density
PB	Peripheral blood
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PEG	Polyethylene glycol
PHA	Phytohaemagglutinin
PPD	Purified protein derivative of *Mycobacterium tuberculosis*
RA	Rheumatoid arthritis
RANA	Rheumatoid arthritis nuclear antigen
RF(S)	Rheumatoid factor(s)
RNA	Ribonucleic acid
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SF	Synovial fluid
SFMC	Synovial fluid mononuclear cells
SI	Stimulation index
SLE	Systemic lupus erythematosus
TB	Sonicate of *Mycobacterium tuberculosis*
TCR	T cell receptor
Tcs	Contra-suppressor T cells
TEMED	Tetramethyl-ethylenediamine
Th/i	Helper/inducer T cells
TNF	Tumour necrosis factor
Ts	Suppressor T cells
Ts/i	Suppressor/inducer T cells
TWEEN	Polyoxyethylene sorbitan monolaurate
VAC	Sonicate of *Mycobacterium vaccae*
VBS	Veronal buffered saline
v/v	Volume/volume
WE	Water extract of *Mycobacterium tuberculosis*
w/v	Weight/volume

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CHAPTER 1

GENERAL INTRODUCTION

Rheumatoid arthritis

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1.1 RHEUMATOID ARTHRITIS

a. Definition of rheumatoid arthritis

Rheumatoid arthritis is a chronic, symmetrical, inflammatory polyarthritis of unknown aetiology. It occurs in 1-3% of the world population and has a female preponderance. It is characterised by synovitis, largely of the peripheral joints, erosion of the articular cartilage and subarticular bone and ultimately ankylosis (Lotz and Vaughan, 1988; Harris, 1989).

Since there is no diagnostic test with high specificity and sensitivity, the diagnosis of RA is based on the recognition of certain clinical and laboratory features which have been set out as the American Rheumatism Association's diagnostic criteria (Arnett *et al.*, 1988). These include the following:

1. Morning stiffness
2. Swelling of three or more joints for more than 6 weeks
3. Swelling of wrist, metacarpal or proximal interphalageal joints for more than 6 weeks
4. Symmetrical joint involvement
5. Hand X-ray detectable erosions
6. Subcutaneous nodules
7. Positive rheumatoid factor test

In addition to the features mentioned above there are others present in patients with more severe disease such as cutaneous vasculitis, lung involvement (effusion, nodules, fibrosis), cardiac involvement (effusion, constrictive pericarditis), eye involvement (episcleritis, scleritis, nodules), Felty's syndrome, peripheral neuropathy and amyloidosis (Panayi, 1986; Macfarlane, 1990).

b. Pathology of RA

Although the systemic nature of RA is clear, the disease is primarily a synovitis. The earliest change in the synovium is hyperplasia of the synovial lining layer, which is normally 1-3 layers thick and becomes 6-10 cells deep with fibrin deposition on the joint cavity side of the synovial membrane (Bennett, 1988; Maddison, 1990).

The synovial tissue becomes infiltrated by lymphocytes, plasma cells, macrophages and neutrophils (Abrahamsen *et al.*, 1975; van Boxel and Paget, 1975). Some mononuclear cells are collected into aggregates, particularly around small blood vessels (Ziff, 1974). There are abundant plasma cells producing antibodies which interact with antigen in the joint space, thus activating complement and inducing an acute inflammatory exudate of neutrophils (Kurosaka and Ziff, 1983; Vernon-Roberts, 1986).

The growth of the inflamed synovial tissue over the surfaces of the joint is called pannus (Gardner, 1986). The pannus becomes a fibrous tissue itself and it ultimately replaces the articular cartilage. At that stage, it binds together the articular surfaces and restricts the movement of the joint. In severe cases,

the joint becomes unstable and subluxation and dislocation are then common (Gardner, 1986; Harris, 1989).

c. Pathogenesis of RA

The synovium is rich in cells bearing activation markers (Klareskog *et al.*, 1984; Burmester *et al.*, 1987; Lotz and Vaughan, 1988). Most infiltrating mononuclear cells are T lymphocytes expressing human leukocyte class II antigens (HLA class II antigens) and other differentiation antigens. In the lymphocyte aggregates, helper/inducer ($CD4^+$) cells greatly outnumber the suppressor/cytotoxic ($CD8^+$) cells and are in close apposition to HLA-DR-bearing dendritic cells (Duke *et al.*, 1982). The histological appearance of the rheumatoid synovium suggests that an active immunological process in the deeper layers of the synovium is taking place (Forre *et al.*, 1982b; Duke *et al.*, 1982; Klareskog *et al.*, 1982; Malone *et al.*, 1984; Klareskog *et al.*, 1987).

Consistent with this is the identification of a wide range of mediators involved in immunoregulation, inflammation, cell proliferation, connective tissue breakdown and fibrosis in the rheumatoid joint (Hirano *et al.*, 1988; Saxne *et al.*, 1988; Guerne *et al.*, 1989; Xu *et al.*, 1989). These include interleukin-1, interleukin-2, tumour necrosis factor, interferons and collagenases. During the course of immune processes in the synovium, both activated lymphocytes and macrophages release factors responsible for the activation and proliferation of the synovium, and the general inflammation (Janossy *et al.*, 1981; Talal, 1985; Firestein and Zvaifler, 1990).

The activated T lymphocytes may contribute directly

to tissue destruction via the release of cytokines or may lead to the release of degradative mediators by other cells (Dayer *et al.*, 1986). Activated macrophages release collagenases capable of breaking down cartilage (Lotz and Vaughan, 1988). Collagen and proteoglycan are destroyed in the region immediately adjacent to the leading edge of pannus. Collagenases cleave collagen, thus exposing it to further proteolytic digestion (Maini, 1989).

A significant role for interleukin-1 (IL-1) in mediating the inflammatory and destructive features of arthritis has been suggested since it stimulates release of collagenase from synoviocytes (Mizel *et al.*, 1981; Dayer *et al.*, 1986; Hubbard *et al.*, 1988) and proteoglycanase from chondrocytes, and activates osteoclasts (Gowen *et al.*, 1983).

Tumour necrosis factor α (TNFα) may also be an important mediator of joint destruction, since it induces cartilage breakdown (Saklatvala, 1986), bone resorption (Bertolini *et al.*, 1986) and release of collagenase and prostaglandin E_2 from synoviocytes (Dayer *et al.*, 1985).

Therefore, the destructive invasiveness of cartilage and bone is due to the release of proteolytic enzymes capable of breaking down connective tissue matrix (Gardner, 1986).

The formation of immune complexes in the synovial fluid, their deposition in the synovial tissues and in the superficial layers of articular cartilage may also contribute to inflammation and the breakdown of articular tissue (Jasin and Cooke, 1978). Complexes arise mostly from self-associating IgG produced locally in the joints, or antibodies to constituents of articular tissue or products of inflammation, such as collagen and fibrin (Lotz and Vaughan, 1988). The initiating factor has been

enigmatic, however, since no exogenous antigen has been found in the complexes (Male *et al.*, 1980).

The complexes activate complement, and neutrophils attracted as a consequence ingest the complexes and release large quantities of proteolytic enzymes, free oxygen radicals and arachidonic acid metabolites (Cooke *et al.*, 1985; Chantry *et al.*, 1989). Proteoglycan depletion can be caused by neutral and acid proteases derived from neutrophils in synovial fluid (Okada *et al.*, 1986).

The presence of macrophages and antigen presenting cells expressing large amounts of class II antigen, the production of interleukin-1 and the presence of a large number of lymphocytes of the helper/inducer type in the synovium suggests an ongoing immune response to presented antigen, either persistent exogenous antigen or perhaps a cross-reactive self-antigen (Maddison, 1990).

d. Aetiology of RA

Even though the first clear descriptions of RA were made at the beginning of the 19th century, the cause, course and outcome of the disease are still debatable (Vaughan *et al.*, 1988). The factor(s) responsible for initiating or perpetuating RA has intrigued investigators for decades. Metabolic, endocrine, environmental and genetic factors have all been considered (Kirwan and Silman, 1987; Cooke and Scudamore, 1989).

The areas in which research is most active today are abnormalities in the regulation of the immune system and infection by one or more agents. However, the distinction between the infective and autoimmune causes for RA becomes blurred as

our knowledge of the host-parasite interactions and the regulation of the immune response increases.

i. Infectious arthritis

Although clinically and epidemiologically RA does not behave like an infectious disease (Silman, 1989), a variety of microbial agents have been proposed in the past as aetiological agents. The search for microbial causes of RA was based primarily on:

1. Human arthritides in which the aetiological roles of microbial agents were more explicit e.g. reactive arthritis (Ford, 1986).

2. Similarities between arthritic conditions in animals caused by microorganisms or their products (e.g. adjuvant arthritis) and human RA (Pearson *et al.*, 1961; Cohen *et al.*, 1985).

In reactive arthritis, synovitis follows infection of the gastrointestinal and genitourinary tracks by organisms including *Salmonella*, *Shigella, Yersinia, Chlamydia* and *Campylobacter* (Aho *et al.*, 1985; Ford, 1986; Phillips, 1988). Similarly, Lyme arthritis has been causally associated with the spirochaete *Borrelia burgdorferi* (Goldings and Jericho, 1986) and Group A streptococcal pharyngitis always precedes and initiates acute rheumatic fever (Williams, 1985; Stollerman, 1989). In ankylosing spondylitis, enteric bacteria such as *Klebsiella* had been implicated (Keat, 1986).

Associations of several viral diseases with inflammatory rheumatic syndromes suggested that RA itself could be caused by a virus (Phillips, 1986). The striking synovitis

sometimes seen following rubella infections (Chantler *et al.*, 1985) and the arthritis that might precede the onset of jaundice in infectious hepatitis (Phillips, 1986) were interesting examples of such associations. Rubella virus has been isolated from synovial fluids of patients with a variety of arthritides including adults with RA (Grahame *et al.*, 1983; Chantler *et al.*, 1985; Ford, 1986).

Another example of viral involvement in arthritis is the epidemic syndrome caused by the Ross River virus and which resembles the systemic onset juvenile chronic arthritis (Fraser, 1986). The human parvovirus B19 has been implicated as a cause of an arthropathy in adults that was usually self-limited and did not resemble RA (Reid *et al.*, 1985; White *et al.*, 1985). In addition, a small particle named RA-1 which resembled parvoviruses in morphologic and physico-chemical properties had been isolated from rheumatoid synovial tissue (Simpson *et al.*, 1984).

The Epstein-Barr virus (EBV) was also implicated in RA by several lines of evidence including the presence of elevated antibodies to a nuclear antigen present in EBV-infected B lymphocytes in RA patients (Alspaugh and Tan, 1976; Alspaugh *et al.*, 1981) and the abnormal cellular response to EBV in RA (Tosato *et al.*, 1981; Gaston *et al.*, 1982; Fox *et al.*, 1985; Irving *et al.*, 1985). Also, increased titres to cytomegalovirus in early RA patients provided evidence for a possible viral cause of RA (Male *et al.*, 1982; Venables, 1988).

The possibility of an infectious cause for RA was also stimulated by similarities between animal models of arthritis caused by microorganisms or their products and human

arthritic conditions (Stimpson *et al.*, 1986). In animals, arthritis was induced by bacterial genera such as *Mycobacteria* (Pearson *et al.*, 1961) and *Nocardiae* (Whitehouse, 1982), *Staphylococci* and *Lactobacilli* (Kohashi *et al.*, 1976) and Group A *Streptococci* (Cromartie *et al.*, 1977).

A chronic arthritis closely resembling the human disease was also found to be induced in mice by *Mycoplasmas* (Cole *et al.*, 1975; Johnson *et al.*, 1984). Amongst the other microorganisms implicated over the years in the pathogenesis of RA were *Clostridia* (Olhagen, 1980) and *Diphtheroids* (Duthie *et al.*, 1967).

Although evidence for microbial involvement is available for polyarthritis, no conclusive evidence exists for rheumatoid arthritis. The limited evidence for a microbial cause of human RA consists of a report that shows the isolation of mycoplasma antigens from the synovial fluid of six patients with rheumatoid arthritis (Clark *et al.*, 1988) and the isolation of a parvovirus from rheumatoid synovial tissue (Simpson *et al.*, 1984). Also, elevated IgA antibody levels to *Proteus mirabilis* were found to be present in active RA patients (Ebringer *et al.*, 1989), and *in vitro* studies has shown elevated T cell responses to a *Mycobacterium tuberculosis* antigen in synovial fluid cells of RA patients (Holoshitz *et al.*, 1986; Gaston *et al.*, 1989a; Pope *et al.*, 1989).

There is new evidence, however, for the possible involvement of mycobacteria in the aetiology of RA, which is discussed more extensively later on.

Although intensive investigation has failed to reveal classical microbiological evidence for defined infective agents at sites of RA inflammation, this could be due to the causative factor being only transiently present i.e. the initiating antigen could trigger the aberrant response, which becomes self-perpetuating long after the offending antigen has been cleared (Spector, 1988). Such a theory can also explain the chronicity of the disease. If infection is ultimately responsible for RA, the organism should be one that is not highly contagious, since RA does not have the epidemiological features of classical infectious diseases (Dumonde *et al.*, 1986).

ii. Autoimmune arthritis

A wealth of evidence, however, supports the view that the immune system plays a central role in the pathogenesis of RA. Of particular relevance are the observations that removal of circulating lymphocytes by thoracic duct drainage brings about remission of joint symptoms (Paulus *et al.*, 1977; Karsh *et al.*, 1981b). In addition, other immunosuppressive regimens, including total body irradiation (Kotzin *et al.*, 1981; Trentham *et al.*, 1981; Field *et al.*, 1983; Strober *et al.*, 1985) and the use of cyclosporin (Weinblatt *et al.*, 1987), are also very effective anti-rheumatic therapies.

The autoimmune features of RA include the presence of a variety of autoantibodies, hypergammaglobulineamia, circulating and deposited immune complexes and infiltration of the synovial membrane with mononuclear chronic inflammatory cells (Scott *et al.*, 1981; Panayi, 1986; Bennett, 1988).

1. Autoantibodies

Rheumatoid factors (RFs) are classically autoantibodies which are directed against the Fc region of the IgG. RFs are quantitatively the major autoantibody in RA and they are the basis for much or most of the immune complex formation in the disease, with the potential to activate the complement cascade (Roitt *et al.*, 1982; Fong *et al.*, 1985).

They are produced by B cells in tissues, including synovial tissue (Wernick *et al.*, 1985; Hardy *et al.*, 1987). They occur in the circulating blood in four out of five patients with RA, they can be mainly of the IgG, IgA and IgM class, and the IgM antibodies occur in 70-90% of the RA patients (Dobloug *et al.*, 1980; Aho *et al.*, 1988). High titers of RF have been associated with more severe and active joint disease and with the presence of rheumatoid nodules (Bennett, 1988).

Despite the extremely strong association of RF with RA, they do not appear to cause the disease. They have been shown to occur in other diseases and in healthy individuals (Carson, 1989). Furthermore, transfusion of RF into healthy volunteers does not initiate inflammation.

A number of other autoantibodies have been described in patients with RA (Holborow, 1986). These include anti-collagen antibodies (Klareskog *et al.*, 1986; Charriere *et al.*, 1988; Lotz and Vaughan, 1988; Morgan *et al.*, 1988), autoantibodies to cytoskeletal elements such as keratin (Kataaha *et al.*, 1985, Youinou *et al.*, 1985), and anti-RANA (rheumatoid arthritis nuclear antigen) present only in human B cell lymphocytes transformed by Epstein-Barr virus (Ferrel *et al.*, 1981; Lydyard and Irving, 1988; Venables, 1988). Whether these

antibodies are epiphenomena of the disease or more closely linked to pathogenesis is not known.

2. Immune complexes

The inflamed synovium in rheumatoid arthritis is a site of active antibody synthesis (Maini, 1986). These antibodies contribute to the local formation of immune complexes and are regularly found in the synovial fluids of these patients (Munthe, 1978).

Numerous antibody specificities have been reported in synovial fluid samples including IgG and IgM anti-globulins, complement proteins, nuclear antigens, viral components and collagen (Male *et al.*, 1980). However, analysis of the synovial fluid immune complexes showed that albumin, complement and acute phase reactants, and immunoglobulins were present; with IgG and IgM immunoglobulins being the most abundant components (Male *et al.*, 1980). In addition, a 48 kDa unidentified protein has been reported in circulating immune complexes of RA patients (Melsom *et al.*, 1987).

Immune complexes have been implicated as initiators of tissue damage (Jasin and Cooke, 1978). Autoantibodies forming immune complexes with autoantigens (perhaps RF and IgG) both in joint and in extra-articular sites, would result in activation of the complement cascade and release of proteolytic enzymes, e.g. collagenases (Chantry *et al.*, 1989). It is commonly believed that immune complexes containing RFs constitute a self-perpetuating system amplifying the inflammatory process by complement activation and/or by interaction with monocytes. Thus, immune complexes are not

pathogenic in themselves but they become pathogenic following interaction with cells and complement (Cooke *et al.*, 1985).

The systemic nature of the disease and the presence of non-organ specific autoreactivity has led to the classification of RA as a non-organ specific autoimmune disease.

Accepting that autoimmune processes are responsible for the pathogenesis of RA, the central question concerns the mechanism whereby this autoimmunity (the recognition of self-antigens) is switched on. Self-tolerance must be broken down and this can be achieved by T cell bypass, polyclonal activation or through regulatory defects of the immune system (Cooke *et al.*, 1983; Cooke, 1988). It is quite possible that several different mechanisms for the abrogation of tolerance to self may co-exist in one autoimmune disease (Schwartz, 1989).

1.2 SELF-TOLERANCE AND AUTOIMMUNITY

A central problem for the immune system is distinguishing foreign invaders from the body's self-components. Recognition of the former concentrates the destructive mechanisms of the immune system on the infectious organism; mistaken recognition of the latter results in autoimmunity and in some cases in autoimmune disease (Schwartz, 1989).

a. Induction and maintenance of self-tolerance

Tolerance is a state of specific unresponsiveness to an antigen following initial exposure to that antigen. Both T and B cell tolerance can be induced, and is thought to depend on a range of factors including the time of exposure to antigen during ontogeny, the dose, route of administration and persistence of the antigen (Claman, 1988; Schwartz, 1989). Although the mechanism of tolerance induction remains obscure, the mechanism of the maintenance of tolerance has been the most controversial.

It has been postulated that self-reactive lymphocytes, which might otherwise cause responses, either undergo clonal deletion from the pre-immune repertoire (Kappler *et al.*, 1987; Fowlkes *et al.*, 1988), or remain in a functionally silent form as a result of early or repeated exposure to antigen (clonal anergy), contact with antigen-specific or idiotype-specific suppressor T cells (Jerne, 1984), or antibody-mediated specific immuno-suppression (Houssaint and Flajnik, 1990).

These mechanisms are not mutually exclusive. Clonal deletion within the thymus or bone marrow could be reinforced by antigen-specific suppression in secondary lymphoid tissue (Kappler *et al.*, 1987; DeFranco, 1989; Goodnow *et al.*, 1989;

Schwartz, 1989).

The requirement of both thymic and peripheral tolerance mechanisms is understandable in view of the development of lymphocytes in limited anatomical locations (bone marrow and thymus). Once matured, they circulate throughout the body and might encounter self-components that were not present at the sites of maturation, and therefore could not have induced clonal deletion or anergy (DeFranco, 1989).

b. Breakdown of tolerance and induction of autoimmunity

Autoimmunity represents the loss of self-tolerance and has to be considered in relation to the mechanisms by which an individual maintains tolerance to self-antigens (Cooke, 1988). Different ways in which autoimmunity can be achieved are outlined below.

i. T cell bypass

When self-tolerance is maintained by suppression of the self-reactive T helper/inducer cells (Th/i), the effector T and B cells are also unresponsive as a consequence. However, circumstances leading to the circumvention of these tolerant Th/i cells would lead directly to the triggering of effector T or B cells.

1. Molecular mimicry

The presence of an antigen which shares determinants with the autoantigen (molecular mimicry) could be recognised by Th/i cells. The Th/i lymphocytes are stimulated by

the non-cross-reactive determinants of the antigen, which in turn provide help to the effector T and B cells some of which might also recognise self-determinants and lead to an autoimmune response.

The cross-reactive antigen could be a foreign (microbial) antigen or an altered-self antigen (Oldstone, 1987). Self-antigens can be altered by viral or bacterial infections, as well as drugs (Salama and Mueller-Eckhardt, 1987), thus exposing antigenic determinants that are recognised as foreign. It has been postulated that degraded collagen can act as altered-self in arthritic diseases (Steffen, 1970).

Autoimmunity provoked by molecular mimicry should occur only when the foreign and host determinants are similar enough to cross-react, yet different enough to break immunological tolerance (Oldstone, 1987). Some evidence for the hypothesis that molecular mimicry can cause autoimmune disease in man comes from studies on the pathogenesis of coeliac disease (a digestion defect) and of ankylosing spondylitis (AS). In the first case the cross-reactive epitopes are shared between the wheat protein A-gliadin, which activates the disease, and a protein of a human adenovirus that inhabits the intestinal tract (Kagnoff *et al.*, 1984). In the case of AS the cross-reactivity is between the HLA-B27 variable domain and peptides derived from *Klebsiella pneumoniae nitrogenase* (McGuigan *et al.*, 1985; Keat, 1986).

Another disease in which the molecular mimicry mechanism appears to operate is rheumatic fever (Williams, 1985). Autoantibodies to heart can be detected in a small proportion of individuals several weeks after a

streptococcal throat infection. Several observations on the cross-reactions between group A streptococci and human heart valve glycoproteins have been made (Appleton *et al.*, 1985; Krisher and Cunningham, 1985).

The widely used adjuvant arthritis model (Pearson *et al.*, 1961) also provides evidence for the cross-reactivity between bacteria and the target tissue, the cartilage. This disease is inducible in susceptible rats by immunisation with heat-killed *M. tuberculosis* in complete Freund's adjuvant and can be transferred with lymphocytes (Pearson *et al.*, 1961) and even by a T cell clone reactive to *M. tuberculosis* (Holoshitz *et al.*, 1984) and to proteoglycan components of the joint cartilage (van Eden *et al.*, 1985). In RA, however, the existence of such cross-reactivity may be possible but has not yet been established.

2. Polyclonal B cell activation

Autoimmunity could also develop following stimulation of self-reactive B cells by polyclonal B cell activators. This circumvents the need for T cell help and has been shown to lead to autoantibody production. A wide variety of microbial products (e.g. endotoxin) or even viruses that stimulate B cells (e.g. Epstein-Barr virus) can act as polyclonal B cell activators (Lotz and Vaughan, 1988).

ii. Defective suppression

In the case of self-tolerance being maintained by self-reactive Th/i cell suppression, triggering of an anti-self response can be achieved through defects in the T suppressor

cells (antigen-specific Ts, non-specific Ts or idiotype specific Ts) (Claman, 1988).

Autoimmunity could also arise by activating the contra-suppressor T cells (Tcs) (Gershon *et al.*, 1981; Green and Ptak, 1986; Lehner and Brines, 1988). Tcs make Th/i cells resistant to the actions of Ts cells and so allow Th/i cells to exert their helper activity. Tcs cells, however, are inactive in the absence of Th/i or Ts cells.

iii. Impaired regulation of the idiotype network

The idiotype network could be involved in induction of autoimmunity by triggering a self-reactive T or B cell which carries an idiotype that cross-reacts either with the idiotype on an anti-microbial antibody or with an antigen on the microbe itself (Cooke *et al.*, 1983; Fong *et al.*, 1986). The T cells recognising this idiotype could help all B cells carrying the idiotype to secrete antibody. Some of these antibodies may be autoantibodies (Plotz, 1983; Male, 1986).

These responses should normally be regulated by suppressor T cells directed against the idiotype or the antigen (idiotype-specific Ts or antigen-specific Ts). Defects in either or both of these regulatory T cells could lead to autoimmunity perpetuated by the combination of Th/i anti-idiotype and autoantigen.

Indications for an involvement of anti-idiotypes in autoimmunity have come from reports of the presence of idiotypic cross-reaction between anti-microbial antibodies and autoantibodies in autoimmune disease such as SLE (Lymberi *et al.*, 1985; Horsfall and Isenberg, 1988). In fact, mouse monoclonal

anti-*M. tuberculosis* antibodies carry the cross-reactive 16/6 anti-DNA antibody idiotype. The 16/6 idiotype titre correlates with disease activity in SLE patients (Shoenfeld *et al.*, 1986). The contribution of the idiotype network to human autoimmune disease, however, remains to be established (Zanetti, 1985; Ebling *et al.*, 1988).

iv. Abnormal expression of class II MHC

Another important factor in the maintenance of self-tolerance may be the inability of autoreactive Th/i cells to recognise potentially autoantigenic molecules on cells which do not normally express major histocompatibility complex (MHC) class II antigens. In these circumstances immunological silence prevails because these T cells can only recognise antigen when it is presented in association with class II molecules.

However, inappropriate expression of class II antigens on the target organ has been noted in some autoimmune states (Bottazzo *et al.*, 1983; Hanafusa *et al.*, 1983), including the synovial tissue in RA (Klareskog *et al.*, 1982). This led to the proposition that aberrant expression of MHC class II molecules on a cell carrying an autoantigen could convert that cell into an antigen-presenting cell to the self-reactive Th/i. This could therefore lead to an autoimmune response.

v. Additional factors

In past years it was believed that an anti-self response was harmful and that such responses did not occur normally. It is now appreciated that normal immune responses involve self-self recognition. The presence of B lymphocytes

reactive with a number of autologous antigens (including thyroglobulin, deoxy-ribonucleic acid (DNA) and cardiolipin) (Grabar, 1983; Dighiero *et al.*, 1985), the presence of self-reactive T cells (myelin basic protein reactive T cells) (Maron *et al.*, 1983) in normal adult animals and in man are some examples of such a recognition.

Although autoimmunity can be induced in normal individuals, most people do not necessarily develop autoimmune disease. This suggests that regulation of the autoimmune processes can occur but that in some susceptible individuals these controlling mechanisms are defective. The susceptibility might be due to genetic factors (Stastny *et al.*, 1983; Cooke and Scudamore, 1989), environmental influences (Russel *et al.*, 1987; Salama and Mueller-Eckhardt, 1987) or hormonal factors (Miller and Schwartz, 1982; Lavalle *et al.*, 1987), and immune defects (Shoenfeld and Isenberg, 1989).

There does appear to be a genetic basis for the tendency to develop autoimmunity (Grennan and Dyer, 1988), as evidenced by HLA associations and family studies (Cooke *et al.*, 1983; Aho *et al.*, 1986). For example, the HLA-DR4 haplotype is found in 60-80% RA patients individuals compared to 20% of healthy individuals. However, this association holds for western European Caucasians, while for Israeli Jews and Indians the association seems to be with HLA-DR1 haplotype (Woodrow *et al.*, 1981; Woodrow, 1986; Stastny *et al.*, 1988).

c. Defective immune regulation in RA and autoimmunity

Evidence that defective immune regulation in RA could be responsible for the autoimmune character of the disease (Lydyard and Irving, 1983; Kitas *et al.*, 1988):

i. Decreased number of suppressor cells

Differences in the number of T helper/inducer cells (Th/i) ($CD4^+$) and T suppressor/cytotoxic cells (Ts/c) ($CD8^+$) in healthy individuals and RA patients, as well as between peripheral blood and synovial fluid in the same RA patients have been reported (Young *et al.*, 1984). However, the results from different reports are inconsistent (Cush and Lipsky, 1988).

While some investigators reported no difference in circulating $CD4^+$ and $CD8^+$ T cells (Burmester *et al.,* 1981), others have reported decreased numbers of $CD8^+$ cells in patients with active RA (Goto *et al.*, 1987). Decreased numbers of $CD8^+$ cells were also found in rheumatoid synovium, where the predominant lymphocyte is of the CD4 phenotype (Duke *et al.*, 1982; Klareskog *et al.*, 1982; Poulter *et al.*, 1985). It has been postulated that the relative lack of $CD8^+$ cells could contribute to autoimmunity in RA (Jannosy *et al.*, 1981; Duke *et al.*, 1982; Klareskog *et al.*, 1982).

However, other investigators found fewer $CD4^+$ cells and a greater number of $CD8^+$ cells in the synovium compared to the peripheral blood of RA patients (Fox *et al.*, 1982).

Additional evidence for T cell abnormal regulation has come from the subdivision of the CD4 subset into helper/inducer $CD4^+$ $UCHL1^+$ (CD45RO) and suppressor/inducer

$CD4^+$ $2H4^+$ ($CD45RA^+$) cells (Pitzalis *et al.*, 1987; Akbar *et al.*, 1988; Sanders *et al.*, 1988; Hirohata and Lipsky, 1989; Rothstein *et al.*, 1990; Wallace and Beverley, 1990). It has been shown that in RA patients there was a marked loss of the $CD45RA^+$ subset, particularly in the synovial fluid (Emery *et al.*, 1987; Lasky *et al.*, 1988).

ii. Absence of non-specific suppressor function

The absence of non-specific suppressor function was reported in synovial fluid cells, as determined by *in vitro* activation with the T cell dependent lectin Concanavalin A, despite the presence of CD8 cells (Chattopadhyay *et al.*, 1979; McCain, 1984).

iii. Defective autologous mixed lymphocyte reaction

Autologous mixed lymphocyte reaction (AMLR) is the response of T cells to autologous non-T cells and has been suggested that it may represent a normal non-specific regulatory system for maintaining self-tolerance (Palacios and Fernandez, 1982).

There is a defective AMLR in RA patients (Beck *et al.*, 1981; Forre *et al.*, 1982a; Salmon and Bacon, 1988). When autologous synovial and blood T and non-T cells were separated and then exchanged in standard AMLR experiments, synovial non-T cells strongly stimulated blood T cells even though they responded poorly to blood non-T cells. This may reflect the relatively increased proportion of dendritic cells as potent stimulators in synovial fluid (Waalen *et al.*, 1986). Blood T cells also responded well to autologous synovial T cells, and this may

be due to the large number of class II molecules that appear on the surfaces of activated synovial T cells.

While the synovial T cells served well as stimulators in these AMLR experiments, they responded poorly when challenged with either synovial or blood non- T cells. These observations probably reflect both intrinsic T-cell defects as well as impaired interactions with accessory cells.

Other investigators have found that the proliferative response in the AMLR of RA cells is the same as in controls but the production of interferon-γ (IFN-γ) during the AMLR is greatly diminished (Hasler *et al.*, 1983).

iv. Cytotoxic cells

The cytotoxic activities of RA blood and synovial lymphocytes have been extensively investigated. Natural killer (NK) cells, which are thought to play an important role in *in vivo* tumour surveillance, resistance to acute and chronic viral infections and regulation of the immune system (Herberman and Ortaldo, 1981) are decreased in synovial tissue (Dobloug *et al.*, 1982) and synovial fluid (Silver *et al.*, 1982) compared to peripheral blood.

In view of the probable role of NK cells in immunoregulation this may imply some lack of normal control of the immune response (Karsh *et al.*, 1981a).

1.3 MYCOBACTERIA, AUTOIMMUNITY, RA

a. Mycobacteria and autoimmunity

Among the evidence supporting the notion that mycobacteria may be involved in autoimmunity is the diversity of autoantibodies in sera of both animals and humans infected with mycobacteria (Shoenfeld and Isenberg, 1988). Furthermore, self-antigens and mycobacterial antigens, may cross-react in humoral and cellular responses (van Eden *et al.*, 1985).

Numerous studies have demonstrated the presence of autoantibodies in patients suffering from tuberculosis or leprosy. These include rheumatoid factor (Mathews and Trautman, 1965), antibodies to DNA (Sela *et al.*, 1987), erythrocytes (Murray, 1978), thyroglobulin (Bonomo *et al.*, 1963), spermatozoa (Gupta *et al.*, 1982) and collagen.

A recent study (Sela *et al.*, 1987) has shown that patients with active untreated pulmonary tuberculosis reacted not only with DNA, but also with other synthetic polynucleotides in a similar way to sera of patients with SLE.

Monoclonal anti-DNA antibodies from MRL/lpr mice and humans with SLE have been shown to bind mycobacterial cell wall glycolipids (Shoenfeld *et al.*, 1986). Similarly, monoclonal anti-*M. tuberculosis* antibodies have been shown to bind to DNA and to behave as antinuclear antibodies (Shoenfeld *et al.*, 1986). Moreover, mouse monoclonal anti-*M. tuberculosis* antibodies were recently found to recognise autoantigens such as thyroglobulin, myosin, actin and collagen (Thorns and Morris, 1985).

Recently, the sequence of the variable regions of autoreactive human monoclonals derived from leprosy and SLE

patients has shown a remarkable homology between antibodies from the two sources (Shoenfeld and Isenberg, 1988). This suggests that increased and persistent production of these antibodies, which are probably present in all sera in small quantities (Avrameas *et al.*, 1983), may represent a disorder of regulation, or of maturation of the B cell response. It is not clear whether this is part of a disordered response to cross-reactive epitopes, or a consequence of polyclonal B cell activation, involving a subset of B cells which secrete antibodies of very broad specificity.

Some of the monoclonals to mycobacteria which also bound to DNA carried a common anti-DNA idiotype (16/6) (Shoenfeld *et al.*, 1986). Therefore, regulation of production of these antibodies could be associated with regulation of this idiotype, rather than of the antibody specificity.

Thus, the significance of autoreactive antibodies occurring in mycobacterial diseases is not clear, and similar antibodies probably occur in other chronic infections (Bonomo *et al.*, 1963).

b. Mycobacteria and rheumatoid arthritis

The possibility that arthritis might be related to mycobacteria has been debated for many years. In fact, the rationale for trying gold therapy in RA was an alleged resemblance between the chronic synovitis of RA, and the pathology of tuberculosis (Forestier, 1934; Isaacs and Sturrock, 1974). This sequence of thought seems odd today since gold is ineffective in tuberculosis, and a pathological resemblance between tuberculosis and RA is no longer evident. In the last few

years there has been a revived interest in this topic. There is now some compelling evidence for the role of mycobacteria in the pathogenesis of RA (Rook, 1988).

i. Tuberculin skin tests in RA patients

Linkages between HLA-DR phenotypes and the ability to respond to skin tests with mycobacterial antigens have hinted at an association between rheumatoid arthritis and mycobacterial infections.

RA patients with HLA-DR4 haplotype (a risk factor for RA; Stastny *et al.*, 1988) showed an increased skin-test response to tuberculin (Bahr *et al.*, 1989). In contrast, RA patients with HLA-DR7 haplotype (a protective haplotype) (Stastny *et al.*, 1988) showed low skin-test responsiveness to mycobacteria (Bahr *et al.*, 1989).

Therefore, there is a rather provocative parallel between the effect of DR haplotypes on skin test responses to mycobacteria, and the effect of the same haplotypes on the risk of developing RA.

ii. BCG immunotherapy and RA

Further support for a connection between RA and mycobacteria came from reports that arthritic symptoms occurred in some patients receiving repeated bacillus Calmette-Guérin (BCG) immunotherapy for advanced cancer (Torisu *et al.*, 1978).

The incidence of arthritis was closely correlated with the host's immunological responsiveness to BCG. The arthritic symptoms usually occurred 1-5 months after the first

BCG injection. The symptoms gradually subsided in response to treatment with non-steroidal anti-inflammatory drugs, but were not completely cured while the effectiveness of BCG continued. The symptoms were aggravated by additional BCG injections.

In addition, researchers have described a form of inflammatory polyarthritis in patients with leprosy in Papua New Guinea (Atkin *et al.*, 1987). They have reported an insidious symmetrical arthritis evolving months or years after the onset of leprosy, and quite independent of erythema nodosum leprosum.

iii. Mycobacteria, agalactosyl IgG and RA

A further link between mycobacteria and RA has recently emerged from studies of the N-glycosylation pattern of the Fc of serum IgG. It has been shown that there is an increased number of oligosaccharide moieties whose outer arms lack galactose and terminate in N-acetylglucosamine, in patients with RA compared to age matched controls (Parekh *et al.*, 1985).

This shift in the population of IgG glycoforms towards those with a higher content of agalactosyl oligosaccharides has also been associated with impaired activity of β–galactosyltransferase, the enzyme responsible for addition of galactose residues to terminal N-acetylglucosamine (Axford *et al.*, 1988).

Elevated agalactosylated IgG glycoforms have been demonstrated in juvenile rheumatoid arthritis, tuberculosis, Crohn's disease, and systemic lupus erythematosus with Sjögren's syndrome (Parekh *et al.*, 1988; Rademacher *et al.*, 1988; Tomana *et al.*, 1988).

However, this did not appear to be a characteristic

feature of chronic inflammatory diseases in general, since it has not been found in numerous other conditions such as primary SLE, ankylosing spondylitis, polymyositis, or primary Sjögren's disease (Parekh *et al.*, 1988).

Therefore, similarities in galactose deficiency in the IgG from patients with RA, primary tuberculosis (which is a mycobacterial disease) and Crohn's disease (which has been postulated to be associated with an unusual type of mycobacteria; Burnham *et al.*, 1978; Chiodini *et al.*, 1984; Graham *et al.*, 1987) point rather tantalisingly to some possible connection between an infective agent (i.e. mycobacteria) or cross-reactive autoantigens and RA (Roitt *et al.*, 1988).

iv. Adjuvant arthritis

Adjuvant arthritis an animal model of arthritis which features synovitis, largely of the distal joints, that progresses to pannus formation, erosion of articular cartilage and subarticular bone, and ultimately ankylosis (Pearson, 1964).

It is inducible in genetically susceptible rats by a single intradermal injection of a suspension of killed *Mycobacterium tuberculosis* (MT) in complete Freund's adjuvant (CFA) (Pearson, 1956). Despite its triggering by bacterial immunisation, adjuvant arthritis was felt to be an autoimmune disease because it could be transferred to naive animals via lymphocytes from inoculated rats (Holoshitz *et al.*, 1984).

T cell lines responsive to antigens of *M. tuberculosis* can be derived from the lymph-nodes of rats rendered arthritic by CFA. Among T-cell clones raised from lymph-nodes of affected rats and selected on the basis of their

proliferative response to MT, a clone designated A2b was found to be arthritogenic in irradiated (750 r) Lewis rats, thus confirming the autoimmune hypothesis, while clone A2c could not cause the disease. Both clones could vaccinate against the disease and accelerate remission in rats with established AA (Cohen *et al.*, 1983).

The arthritogenic clone was found to recognise both an antigen of MT and a component of cartilage proteoglycan probably associated with the core protein (van Eden *et al.*, 1985). Thus, the antigen receptor of the arthritogenic clone of T lymphocytes defined a structural similarity between a mycobacterial epitope and an epitope of joint cartilage. This cross-reactivity between MT and cartilage could explain the attack of the joints in AA (Cohen *et al.*, 1985).

It is now clear that both (arthritogenic and protective) clones recognise an epitope formed by the amino acids at positions 180-188 in the sequence of the 65 kilo-Dalton (kDa) antigen of *M. bovis* BCG (van Eden *et al.*, 1988). Unlike immunisation with whole mycobacteria, the administration of the 65 kDa protein emulsified in oil does not induce AA, and the immunised rats show resistance to subsequent attempts to induce the disease by immunisation to whole MT in oil (van Eden *et al.*, 1988). The 65 kDa antigen has recently been identified as a member of a group of conserved proteins, the heat shock proteins (Young *et al.*, 1987).

v. Data linking mycobacteria to RA in man

Several groups have studied the lympho-proliferative responses of T cells from blood or synovial fluid of

RA patients seeking evidence for involvement of an identifiable antigen.

An earlier report in this area was that of Abrahamsen *et al.* (1978), who compared reactivity of synovial fluid and peripheral blood lymphocytes to purified protein derivative of tuberculin (PPD) in patients with RA. They found much greater reactivity in the synovial fluid cells, whereas responses to mitogens and *Candida albicans* were stronger in peripheral blood cells. In contrast, Ivanyi *et al.* (1973) found no increase in responsiveness to PPD in the synovial fluid T lymphocytes.

Holoshitz *et al.* (1986) have assessed the immune response of RA patients to an acetone precipitated fraction of *M. tuberculosis* that cross-reacts with pig and rat cartilage, and which also stimulated the arthritogenic rat T cell clone A2b. They have reported that RA patients have more pronounced T cell responses to the above antigen than patients with degenerative joint disease or healthy controls. In addition, there was a shift in the distribution of mycobacterium responsive T cells into the joints of early RA, suggesting that an epitope cross-reactive with mycobacteria may also be present in human joints.

1.4 HEAT SHOCK PROTEINS

a. Definition and function

Heat shock or stress proteins are an abundant and ubiquitous class of proteins, being produced in organisms as phylogenetically distant as humans and bacteria in response to environmental stress (Lindquist, 1986; Polla, 1988).

The heat shock response was first described in 1962 by Ritossa (1962), who observed the appearance of a new pattern of chromosome "puffs" - areas that are actively transcribing the DNA into messenger RNA (ribonucleic acid), to be translated ultimately into protein- in salivary gland cells of *Drosophila* after exposure to heat. Tissieres *et al.* reported in 1974 that the appearance of this heat induced puffing was associated with the synthesis of specific proteins, thereafter named heat shock proteins (hsps) or stress proteins.

The heat shock response is not induced solely by exposure to elevated temperatures, but by many other forms of cellular injury, including oxidative injury, heavy metals, ethyl alcohol, amino acid analogues, viral infections, inhibitors of energy metabolism, nutrient deprivation or, *in vivo* , ischemia or reperfusion injury (Polla, 1988). Also, increased hsp synthesis has been found following T lymphocyte activation with mitogens and lymphokines (Ferris *et al.*, 1988), and during cellular differentiation (Bensaude and Morange, 1983; Kurtz *et al.*, 1986). Thus, when prokaryotic or eukaryotic cells are exposed to any of the above mentioned agents, there is a post transcriptional inhibition of normal protein synthesis, accumulation of abnormal, denatured or damaged proteins within cells, and induction of transcription and synthesis of hsps (Pelham, 1988).

The term heat shock proteins is used as a general term to describe both heat induced and related, physicochemically induced, proteins. The hsps are referred to by their relative molecular mass. They exist as gene families, including groups at approximately 20, 65, 70 and 90 kDa. The genes of these proteins have been sequenced and a remarkable conservation in the structure of heat shock genes and hsps across species was found (Lindquist and Craig, 1988). Regulation of these genes is complex; some genes are expressed only after cells are stressed, whereas others are expressed constitutively (Neidhardt *et al.*, 1984).

The functions of the hsps have long been a matter of speculation and it is widely assumed that they have a 'nurse-maid' or 'molecular chaperone' functions (Laskey *et al.*, 1978). These terms have been extended to describe proteins whose proposed role is to ensure that the folding of certain polypeptides and their assembly into oligomeric structures occur correctly (Ellis, 1987). The basic argument is that in some cases the transient exposure of hydrophobic or charged surfaces normally involved in domain interactions, either within or between polypeptide chains, can result in 'improper' interactions to produce incorrect structures. The hsps may mediate folding, unfolding, translocating, assembling subunits, or binding and inactivating other proteins, thus ensuring the proper formation of proteins under stress or non-stress conditions (Pelham, 1986). Often, these functions are facilitated by adenosine triphosphate (ATP) hydrolysis (Lindquist and Craig, 1988) and the hsps may be ATP-dependent enzymes. Although the above functions remain obscure, they are clearly fundamental for cell survival from the effects of stress.

Heat shock protein families also include proteins that are related to hsps in amino acid sequence, but whose expression levels are not altered by stress. They are constitutively expressed and behave in essentially the same way as the heat-inducible proteins (Lewis and Pelham, 1985), which raises the question of the function of these proteins in normal unstressed cells. It is possible that the constitutive proteins perform a general repair function as needed. Alternatively, there may be normal cellular events, such as the breakdown of the nuclear envelope at mitosis, which require disruption of specific insoluble structures. Such a function could be consistent with the high concentration of cognate 70 kDa hsp in rapidly growing cells (Lewis and Pelham, 1985).

Another type of proteins related to the hsps is the glucose regulated proteins (grps) which are not heat inducible, but are overproduced when fibroblasts are starved of glucose. Two major groups have been identified: grp78 and grp94 (Pelham, 1986). They are abundant in secretory cells such as hepatocytes and myeloma cells. One possibility is that the grps perform the same function as the hsps but do so within the endoplasmic reticulum, where they are concentrated (Munro and Pelham, 1986).

b. Major families of heat shock proteins

i. The 65 kDa hsp family

Members of this family have been found in bacteria, plant chloroplasts, yeast and humans (Shinnick *et al.*, 1987a, 1987b; Lindquist and Craig, 1988; McMullin and Hallberg, 1988; Jindal *et al.*, 1989). In eukaryotes, they are normal

components of the mitochondrial matrix, although they are encoded in the nucleus and synthesised in the cytosol. The 65 kDa antigen of mycobacteria has been shown to belong to this highly conserved family of heat shock proteins (Young *et al.*, 1987), which includes the *groEL* gene product of *E. coli* (Shinnick *et al.*, 1988). Gillis *et al.* (1985) have described the 65 kDa antigen as cell-wall associated since much of the antigen was found in the insoluble fraction remaining after disruption of the bacterial cells. Others, however, have proposed a periplasmic location for this antigen (Thole *et al.*, 1988a; Karlsson-Parra *et al.*, 1990) and have demonstrated release of the molecule into culture supernatants during growth of mycobacteria under conditions of zinc deficiency (de Bruyn *et al.*, 1987). Computer aided analysis, of the alpha helical content and hydrophobicity of the amino acid sequence, revealed that the 65 kDa antigen is not an integral membrane protein but rather its sequence resembles a soluble protein (Hendrix, 1979).

The amino acid sequences of *M. tuberculosis* and *M. bovis* 65 kDa hsp are identical, while that of *M. tuberculosis* and *M. leprae* 65 kDa are 95% homologous (Neidhardt *et al.*, 1984). The mycobacterial 65 kDa hsp shows a 60% sequence identity to the *E. coli groEL* gene product (Shinnick *et al.*, 1988; Young *et al.*, 1988b), and shows 47% identical residues and an additional 20% conservative changes when compared to the recently cloned human 65 kDa hsp homologue (Jindal *et al.*, 1989; Table 1.1). The high degree of conservation suggest that there has been a strong evolutionary pressure to maintain the primary amino acid sequence of this protein, which in turn suggests that the 65 kDa antigen is of fundamental biological importance.

TABLE 1.1: Amino acid sequencies of *M. leprae*, *E. coli* and human 65 kDa hsps.

Footnotes: The sequence of each peptide is shown using the single letter code for amino acids. Gaps in the sequence alignment are indicated by dashes (-).
The shaded boxes represent T cell epitope containing areas and the clear boxes B cell epitope containing areas.

The function of the mycobacterial 65 kDa hsp has yet to be elucidated. That of GroEL, however, involves the ATP dependent assembly of oligomeric protein structures including the head-tail connector of phage λ (Hemmingsen *et al.*, 1988). This protein accounts for 1-2% of cellular protein synthesis in cells in steady state growth at 37^{o}C, which increases to 10-15% of total synthesis soon after heat shock (Lindquist, 1986; Hemmingsen *et al.*, 1988). The 65 kDa hsp family also includes the plant 65 kDa hsp which interacts with the chloroplast enzyme ribulose bisphosphate carboxylase oxygenase (Rubisco) (Friedman *et al.*, 1984; Lindquist, 1986; Bochkareva *et al.*, 1988; Hemmingsen *et al.*, 1988) and the yeast 65 kDa hsp which is required for the assembly of several mitochondrial proteins in the mitochondrial inner membrane space (Cheng *et al.*, 1989). No functions have yet been described for the mammalian 65 kDa hsp.

The 65 kDa hsp is a highly immunoreactive protein. It is the protein most frequently recognised by monoclonal antibodies raised to mycobacterial extracts (de Bruyn *et al.*, 1987; Thole *et al.*, 1987). Purified 65 kDa hsp can elicit a strong delayed type hypersensitivity reaction in experimental animals (Gillis *et al.*, 1985; Gillis and Job, 1987). In immune mice, Kaufmann *et. al.* (1987) showed that 20% of the mycobacteria reactive T cells recognise it, indicating that it is an immunodominant antigen. There is considerable evidence suggesting that immune response to the 65 kDa hsp may cause development of adjuvant arthritis in rats (Holoshitz *et al.*, 1984; Cohen *et al.*, 1985; Cohen,1986).

In humans, the 65 kDa antigen appears also to be a major immunoreactive protein during the course of an infection

with either *M. leprae* or *M. tuberculosis*, as well as after vaccination with *M. bovis* BCG (Young *et al.*, 1988a). Both antibodies and T cells directed against this antigen can be detected in the sera of patients and vaccinated persons (Emmrich *et al.*, 1986; Oftung *et al.*, 1987; Thole *et al.*, 1987; Young and Elliott, 1989). T cells specific for this antigen were also isolated from healthy individuals (Lamb *et al.*, 1989). The responses appear to be directed against specific, as well as cross reactive, epitopes present on the 65 kDa antigen (Britton *et al.*, 1985; Thole *et al.*, 1985; Emmrich *et al.*, 1986; Mustafa *et al.*, 1986; Kaufmann *et al.*, 1987; Lamb *et al.*, 1987; Oftung *et al.*, 1988; Thole *et al.*, 1988b; van Schooten *et al.*, 1988; Table 1.1). Synovial fluid T lymphocytes from patients with RA showed specific increased lymphoproliferative activity in response to the *M. bovis* recombinant 65 kDa hsp (Res *et al.*, 1988).

ii. The 70 kDa hsp family

The 70 kDa hsp family consists of at least four members: the highly inducible 72 kDa hsp, the constitutively expressed 73 kDa hsp that is virtually indistinguishable from the 72 kDa hsp, the glucose-regulated 78 kDa (grp78) protein which is located in the endoplasmic reticulum, and the grp75 which is located in the mitochondria (Munro and Pelham, 1986; Lindquist and Craig, 1988). Members of this family include the approximately-70 kDa hsp of mycobacteria, the DnaK protein of *E. coli* and the mammalian 70 kDa hsp.

Before heat shock the 70 kDa hsps are found in both cytoplasm and nuclei, but upon heat shock they migrate and subsequently concentrate in the nucleoli (Welch and Feramisco,

1984). The 70 kDa hsp has also been found as an extracellular component of cell cultures (Abou-Zeid *et al.*, 1988), although members of the 70 kDa family are not generally secreted from cells (Lindquist, 1986). In addition, evidence is now emerging that suggests that at least some cells express 70 kDa hsp on their surface when they are stressed (Jarjour *et al.*, 1989).

The *M. tuberculosis* 71 kDa antigen which was found to belong to the 70 kDa hsp family (Young *et al.*, 1988b) exhibits 40% amino acid identity with the comparable part of the *dnaK* gene product of *E. coli* (Bardwell and Craig, 1984). It is also 38% homologous with the human 70 kDa hsp, although in some regions the identity increases to 71% (Young *et al.*, 1987). The *M. tuberculosis* 71 kDa hsp and *M. leprae* 70 kDa hsp sequences are 93% identical (Garsia *et al.*, 1989). The sequence conservation of these proteins attest to their importance.

The 70 kDa hsps bind strongly to ATP and researchers have long speculated that they act as 'chaperones'. Lewis and Pelham (1985) suggested that 70 kDa hsp binds to hydrophobic surfaces of damaged proteins, thereby facilitating disruption of inappropriate hydrophobic interactions. The 70 kDa hsp then uses ATP hydrolysis to release itself from the 70 kDa hsp/protein complex (Chappell *et al.*, 1986).

Members of the 70 kDa hsp family were demonstrated to have an 'unfoldase' action in facilitating translocation of nascent polypeptides across endoplasmic reticulum and mitochondrial membranes (Chirico *et al.*, 1988; Deshaies *et al.*, 1988). One of the best-studied examples of such a

function is that of heavy chain binding protein (BiP), which belongs to the 70 kDa hsp family (Munro and Pelham, 1986). BiP binds to the immunoglobulin heavy chain within the endoplasmic reticulum until association with the immunoglobulin light chain occurs, and thus prevents premature self assembly of heavy chains (Dorner *et al.*, 1987; Hendershot *et al.*, 1987). In the absence of light chains, BiP remains permanently bound to to the heavy chain, retaining it within the lumen of the endoplasmic reticulum.

Another interesting role of the 70 kDa hsps might be in antigen presentation. A 72-74 kDa peptide-binding protein that participates, along with class II MHC molecules, in the presentation of pigeon cytochrome *c* peptides by mouse B cells to responding T cells has been identified as a member of the 70 kDa hsp family (Lakey *et al.*, 1987; Vanbuskirk *et al.*, 1989). Interestingly, the human major histocompatibility complex was found to contain genes encoding the 70 kDa hsp in the class III region between the complement and the tumour necrosis factor genes (Sargent *et al.*, 1989).

From known functions of some members of the 70 kDa hsp family it seems that this family includes members that are functionally diverse. The *E. coli* 70 kDa hsp has a role in initiation of DNA replication (Sakakibara, 1988). The grp78 is an immunoglobulin heavy chain binding protein (BiP) (Munro and Pelham, 1986; Dorner *et al.*, 1987; Hendershot *et al.*, 1987), and the 73 kDa hsp has an uncoating enzyme activity that releases clathrin from coated vesicles in the presence of ATP (Ungewickell, 1985). The 70 kDa hsp of mycobacteria has been identified as a target of humoral and cellular immune responses

during mycobacterial infection in man (Britton *et al.*, 1986).

iii. The 90 kDa hsp family

The 90 kDa hsp family includes the inducible and constitutive 90 kDa, and the glucose-regulated 94 kDa (grp94) proteins (Pelham, 1986). The 90 kDa hsp is highly abundant, constituting up to 1% of the total protein even in unstressed cells and homologues of the mammalian protein have been identified in other vertebrates, *Drosophila*, yeast and bacteria (Riehl *et al.*, 1985; Bardwell and Craig, 1987; Latchman *et al.*, 1987). The mammalian 90 kDa hsp is normally present predominantly within the cytoplasm but tends to move to the nucleus with heat shock (Welch and Suhan, 1986). However, there are reports of surface expression of an hsp-related 90 kDa protein on macrophage-like cells from synovial fluid of rheumatoid arthritis patients (Jarjour *et al.*, 1989).

Despite its conservation and abundance, no definite function has yet been assigned to this protein. However, it has been found to interact with steroid hormone receptors, and with actin and tubulin (Pelham, 1986; Norton *et al.*, 1989). It has therefore been suggested that the 90 kDa hsp masks the DNA-binding site of the steroid hormone receptors until a hormone is positioned within the hormone-binding site. When this happens, the 90 kDa hsp is released enabling the hormone/receptor complex to bind to DNA (Miller, 1989).

The glucose regulated counterpart of the 90 kDa hsp, grp94, which is located in the endoplasmic reticulum, is thought to be involved in the assembly of other proteins.

It has been reported that some patients with SLE

have elevated autoantibodies to the 90 kDa hsp (Minota *et al.*, 1988b). However, further investigation is required for identifying a possible role of this hsp in immune and inflammatory responses.

iv. The low molecular weight hsps

This family includes the 18 kDa antigen of *M. leprae* (Booth *et al.*, 1988) and the 12 kDa hsp of *M. bovis* BCG (Minden *et al.*, 1984) which shows a 45% homology with the *E. coli* GroES protein (Hemmingsen *et al.*, 1988).

It also includes the histone H2B (Sanders, 1981); the ubiquitin, a protein essential for chromosome organisation through its interaction with nucleosome histones (Bond and Schlesinger, 1985); the 28 kDa hsp; the 32 kDa hsp induced in some cells by exposure to ultraviolet light and chemical carcinogens (Caltabiano *et al.*, 1986; Keyse and Tyrell, 1987, 1989) and the 47 kDa hsp, a novel glycoprotein that binds collagen (Winfield, 1989).

During heat shock these proteins migrate to the nucleus and during recovery they return to the cytoplasm. Their functions are still highly speculative (Lindquist and Craig, 1988; Hurst, 1990).

c. Stress proteins as antigens

The observation that hsps are important targets of the immune response to mycobacterial infection and the knowledge that the hsps are conserved and abundant in other organisms suggests that hsps are likely to be immune targets in many other infections (Kaufmann, 1990). Indeed, the involvement of stress

proteins in immune recognition is not restricted to mycobacterial infection and antigens recognised as members of hsp families have been identified in immune responses to a variety of infections. In Q fever, the GroEL homologue of *Coxiella burnetii* has been recognised as a major antigen (Vodkin and Williams, 1988).

Similarly, major antigens from parasites responsible for malaria (Bianco *et al.*, 1986; Ardeshir, 1987), schistosomiasis (Hedstrom *et al.*, 1987), filariasis (Selkirk *et al.*, 1987) and leishmaniasis (Smith *et al.*, 1988) have been found to belong to the 70 kDa hsp gene family.

The presence of stress proteins among immune targets in a variety of human pathogens suggests that the stress response may be a general component of infection (Young *et al.*, 1988a). The major factors which contribute to the hsps being targets of the immune response to a broad spectrum of infections are the following (Lamb *et al.*, 1989; Young and Elliott, 1989):

i. The abundance of these proteins under conditions of stress, such as an infection, might play a role (Polla, 1988). In addition to hsps produced by the host, infectious agents may respond to the host environment by producing hsps and that these proteins can be important immune targets. In fact, the ability of bacterial pathogens to produce heat shock proteins has been implicated in their survival within macrophages (Christman *et al.*, 1985; Morgan *et al.*, 1986).

ii. Some hsps may be intrinsically antigenic. They

may be particularly amenable to processing and presentation to the immune system by antigen presenting cells (Young and Elliott, 1989; Young, 1990).

iii. Moreover, since the hsps from different pathogens possess a high degree of sequence homology, it can be proposed that their apparent immunodominance is a reflection of repeated challenge to the immune system resulting in a high frequency of lymphocytes directed to conserved antigenic determinants (Lamb *et al.*, 1989).

d. Stress proteins as autoantigens

Because of the remarkable molecular mimicry (epitope homology) between microbial and self hsps, the risk of autoimmunity is high (Winfield, 1989). Cross-reactive immune responses could be induced by any bacterial or parasitic infection following presentation of the antigen in a manner which results in circumvention of tolerance mechanisms e.g. by T cell bypass (Cooke, 1988).

Presentation of a self-like determinant may fail to stimulate lymphocytes due to host tolerance (Schwartz, 1989). Chronic presentation of such determinants could result in breaking of tolerance and production of an autoimmune response.

It is possible that the homologous parts of these proteins are associated with a conserved function, and variable regions of the molecules are important for species specific interactions with other protein components in the cell (Young, 1990).

Detailed analysis of the antigenic determinants

involved in recognition of hsps by antibodies and T lymphocytes will be important in evaluating the potential role of these antigens in induction of protective immunity and autoimmune pathology.

e. Stress proteins as vaccines

The stress proteins may also have 'immunoprophylactic' potential for a broad spectrum of human pathogens (Koga *et al.*, 1989; Young and Elliott, 1989). Thus, because of the sequence conservation, it is possible that an antigen from one pathogen could immunise against another pathogen. Exposure to foreign stress proteins early in life might, in fact, induce a degree of immunity to a variety of infectious agents. If so, the presence of this immunity could provide an explanation for the observation that, only a fraction of infected individuals actually acquire clinical disease (Young, 1990). Because the stress proteins stimulate the body to produce antibodies, they could be candidates for subunit vaccines against these pathogens (Young *et al.*, 1988b; Young and Elliott, 1989).

The structural similarity of stress proteins, however, may cause problems for the human host. Repeated injections of a protein vaccine may break down the host's self-tolerance. The antibodies produced may turn around and attack host stress proteins causing an autoimmune reaction. At the moment, it is unclear who benefits from the stress proteins, the pathogen or the host. But either way, manipulating the level of stress proteins during infection may reduce the severity of infection (Young *et al.*, 1988a; Young, 1990).

1.5 CLINICAL FEATURES OF OTHER DISEASED GROUPS USED IN THIS STUDY

In the studies to be described the main test group was the rheumatoid arthritis patients, although patients with systemic lupus erythematosus (SLE), ankylosing spondylitis (AS) and Crohn's disease were used as diseased control groups. In some assays, patients with osteoarthritis (OA) were also included. The features of the above diseases are outlined below.

a. Systemic lupus erythematosus

Systemic lupus erythematosus is a connective tissue disease of unknown aetiology. SLE is characterised by vasculitis, synovitis, anaemia, fever, rashes, pericarditis, pleurisy, nephritis, or central nervous system disease, and the presence of a wide variety of autoantibodies (Steinberg, 1988).

There is no one clinical abnormality that definitely establishes the diagnosis, nor is there a single diagnostic test for the disorder. As a result, criteria have been developed in an attempt to include patients with SLE and to exclude patients with other disorders (Tan *et al.*, 1982).

The disease is more common in women. The clinical course of SLE is often unpredictable and characterised by periods of remission and chronic or acute relapses.

The signs and symptoms are thought to be caused by the autoantibodies that react with self constituents and initiate inflammatory responses. The initiation of this process may be multifactorial and may be different in different individuals. These factors are currently poorly understood. It is clear though that patients with SLE produce autoantibodies reactive with

nuclear (such as DNA, histone) (Tan, 1982; Muller *et al.*, 1988), cytoplasmic (anti-lysosomal, ssRNA, ribosomal ribonucleo-protein) (Pincus *et al.*, 1971) and cell membrane (Jacob *et al.*, 1986) antigens. Also antibodies to erythrocytes, granulocytes, lymphocytes and macrophages and many of these are pathogenic (Winfield, 1985). SLE is often classified as an immune complex type disorder.

b. Ankylosing spondylitis

Ankylosing spondylitis is an inflammatory disease of the sacroiliac joints and spine. It is a chronic but fluctuating seronegative inflammatory disorder with exacerbations and periods of symptomatic and objective quiescence (Calin, 1988). Bony ankylosis and deformity are the end-results in only a small proportion of severely affected individuals. Other features include peripheral joint arthritis, acute anterior uveitis and rarely aortic valve disease (Calin, 1990).

Although AS was formerly considered a predominantly male disease, several studies now suggest that there may be a more uniform sex distribution, although it is frequently more severe in males. It affects up to 0.5 to 1% of the population and the onset of the disease is usually between 20-30 years of age (Moll, 1986). There is a strong association between HLA-B27 antigen and AS (Archer *et al.*, 1988). HLA-B27 is present in more than 90% of patients with AS (Brewerton, 1984).

The cause of AS remains unknown. However, the serological cross-reactivity between a determinant of HLA-B27 positive spondylitic cells and certain gut bacteria, such as *Klebsiella*, is highly considered as being important in the

aetiology of AS (Geczy *et al.*, 1980; McGuigan *et al.*, 1985; Struthers, 1985; Keat, 1986; Benjamin and Parham, 1990).

c. Crohn's disease

Crohn's disease is a subacute and chronic inflammatory process of unknown cause that may involve any part of the intestinal tract, especially the distal ileum, colon, and anorectal region (Donaldson, 1989). Extraintestinal manifestations such as arthritis, ankylosing spondylitis, and erythema nodosum may precede or strongly influence the presenting syndrome (Mayer and Janowitz, 1988).

The incidence of Crohn's disease is approximately equal in males and females, and the most frequent age of onset is from 15 to 30 years. There is no diagnostic laboratory test for the disease (Rosenberg, 1988).

From the time of the first descriptions of this condition, interest in the aetiology has emphasised possible bacterial or other microbial causes (Kirsner and Shorter, 1982). The similarity of the inflammatory response to that with mycobacterial infection, the fever and toxaemia in some patients, and the response of some to antimicrobial therapy have been recurrent clinical observations (Burnham *et al.*, 1978; Mekhjian *et al.*, 1979; Graham *et al.*, 1987). Sporadically, microbial isolates have been made from tissues of patients with Crohn's disease (Chiodini *et al.*, 1984), including cell wall-deficient mycobacteria (Parent and Mitchell, 1978) and RNA viruses (Gitnick *et al.*, 1979). However, evidence for an aetiologic role for any of these agents has been lacking. Nevertheless, the search for microbiologic agents represents one current approach to understanding the

aetiology of the inflammatory bowel diseases.

Whether or not microorganisms are directly implicated in the aetiology of the disease, they may be involved in the pathogenesis in concert with altered immune mechanisms (Elson *et al.*, 1986). Some of the extraintestinal manifestations of inflammatory bowel disease suggest the presence of antigen-antibody complexes in these sites (Elmgreen *et al.*, 1985; Kagnoff, 1989).

d. Osteoarthritis

Osteoarthritis (OA) is a common age-related seronegative joint disease. It is characterised by degeneration of cartilage, bony remodelling and outgrowth of bone. The disease advances with age and peaks at 70 or over 70 years of age. The development of OA appears to be joint malfunction, usually due to changes in articular cartilage (Howell, 1988; Hutton, 1990).

1.6 AIMS OF THIS STUDY

In view of the implication of the mycobacterial 65 kDa heat shock protein in the induction of adjuvant arthritis in rats (van Eden *et al.*, 1988), the possibility was raised as to whether the immune reactivity to heat shock proteins, and in particular to the mycobacterial 65 kDa hsp, might be important in patients with rheumatoid arthritis.

To investigate this possibility, antibody responses to crude mycobacterial antigens (Chapter 3) and to mycobacterial and *E. coli* 65 and 70 kDa hsps (Chapter 4) were studied in RA sera. The mycobacterial 65 kDa hsp antibodies of RA patients were examined for specificity (Chapter 5) and autoreactivity (Chapter 6). A limited study of mononuclear cell responses to mycobacterial antigens in RA patients was also carried out (Chapter 7).

CHAPTER 2

MATERIALS AND METHODS

Antigens and antibodies

- Preparation and source of antigens
- Processing of clinical material
- Estimation of protein in antigen preparations
- Source of antibodies

Detection of antigens

- Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)
- Western blotting
- Visualisation of proteins immobilised onto NC membrane

Detection of antibodies

- Detection of antibody by an enzyme linked immunosorbent assay (ELISA)
- Detection of antibodies by Western blotting
- Estimation of total serum or synovial fluid IgA, IgM and IgG by radial immunodiffusion
- Purification of mycobacterial 65 kDa hsp serum antibody

Separation of cells and cellular assays

- Separation of peripheral blood mononuclear cells
- Separation of synovial fluid cells
- Counting of cells, culture medium, culture conditions

- Irradiated cells
- Cell proliferation assay
- Estimation of frequency of antigen specific lymphocytes in peripheral blood and synovial fluid

Study groups

- Rheumatoid arthritis patients
- Systemic lupus erythematosus patients
- Ankylosing spondylitis patients
- Crohn's disease patients
- Osteoarthritic patients
- Healthy individuals

Statistical analysis

2.1 ANTIGENS AND ANTIBODIES

a. Preparation and source of antigens

i. A water extract of *Mycobacterium tuberculosis* $H_{37}Ra$ (Difco Laboratories, Detroit, U.S.A.) was prepared as follows. A known amount of heat-killed desiccated *M. tuberculosis* was ground with an homogeniser, suspended in double distilled water at 1 mg/ml, stirred for 8 hours at 4°C, centrifuged for 30 mins at 15000 g and the supernatant was lyophilised. The lyophilisate was dissolved in the minimum amount of double distilled water, the protein content was estimated and adjusted to 1 mg/ml with double distilled water. This antigen preparation was stored in aliquots at -20°C until use and is referred to as WE.

ii. Sonicates from *Mycobacterium vaccae* (NCTC 11659) and *Mycobacterium tuberculosis* were kindly provided by Dr. J.L. Stanford (Medical Microbiology, University College and Middlesex School of Medicine, London), and were prepared as described by Lema and Stanford (1984). These antigen preparations are referred to as VAC and TB respectively.

iii. A *Candida albicans* sonicate preparation was kindly provided by Dr. G.A.W. Rook (Medical Microbiology, University College and Middlesex School of Medicine, London).

iv. Three influenza strains, the Mississippi/1/85,

Rumania/290/87 and Bangkok/1/79, were kindly provided by Professor J. Skehel and Dr. R. Gonsalez (National Institute for Medical Research, Mill Hill, London). Equal amounts of each strain were mixed to form an antigen preparation referred to as the 'flu' preparation.

v. The recombinant 65 kDa heat shock protein (hsp) antigen from *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) was kindly provided by Dr. J.D.A. van Embden (National Institute of Public Health and Environmental Hygiene, Bilthoven, Netherlands). It was purified from overproducing *Escherichia coli* K-12 cells using the method of Thole *et al.* (1987).

Briefly, the recombinant cells were grown, disrupted with an ultrasonic probe, centrifuged and the bacterial supernatant was subjected to ammonium sulphate precipitation and then fractionated through an ion exchange chromatography column, at which stage the 65 kDa antigen was obtained. This antigen is referred to as mycobacterial 65 kDa hsp.

vi. The *Escherichia coli* 65 kDa antigen was kindly provided by Drs. D.B. Young and A. Mehlert (Medical Research Council, Tuberculosis and Related Infections Unit, Hammersmith Hospital, London). It was purified from *E. coli* (strain TG1) cells transformed with pND5 vector, which over-expressed a 60 kDa protein known as GroEL, using the method by Chandrasekhar *et al.* (1986).

The purification procedure involved ammonium sulphate precipitation of the cell supernatant after ultrasonic cell disruption, gel filtration of the supernatant and fractionation

by an ion-exchange chromatography. This antigen is referred to as *E. coli* 65 kDa hsp.

vii. A human preparation of the 65 kDa hsp antigen, purified from human placental tissue (Dudani and Gupta, 1989), was kindly provided by Professor R.S. Gupta (McMaster University, Ontario, Canada). This antigen is referred to as human 65 kDa hsp.

viii. The *Mycobacterium tuberculosis* 71 kDa hsp was kindly provided by Drs. D.B. Young and A. Mehlert. The purification of the recombinant 71 kDa antigen of *M. tuberculosis* was based on the high affinity of 71 kDa for adenosine triphosphate (ATP).

Briefly, the 71 kDa antigen was purified from *E. coli* (strain TG1) transformed with a multi-copy plasmid (pUC8) expressing the mycobacterial protein (Maniatis *et al.*, 1982). The recombinant cells were disrupted using an ultrasonic probe and the supernatant was used for the purification of 71 kDa antigen, using the method described by Welch and Feramisco (1985). The 71 kDa antigen was contained in the ATP eluate obtained during ATP-agarose affinity chromatography of the bacterial supernatant. This antigen is referred to as mycobacterial 70 kDa hsp.

ix. The *Escherichia coli* 70 kDa antigen, which is known as DnaK, was purified according to the method by Welch and Feramisco (1985), using *E. coli* (strain TG1) cells (Mehlert and Young, 1989). This antigen is referred to as *E. coli* 70 kDa hsp and was provided by Drs. D.B. Young and A. Mehlert.

x. A human preparation of 70 kDa hsp was purified

from heat-shocked peripheral blood lymphocytes. Cells were subjected to heat-shock for 2 hours at 45^{o}C, disrupted by two freeze-thaw cycles, and the cell supernatant was used for the purification of the 70 kDa antigen as described by Welch and Feramisco (1985). This antigen was kindly provided by Drs. D.B. Young and A. Mehlert and is referred to as human 70 kDa hsp.

xi. Six synthetic peptides of *Mycobacterium leprae* 65 kDa hsp were kindly provided by Dr. J. Lamb (Medical Research Council, Tuberculosis and Related Infections Unit, Hammersmith Hospital, London (Lamb *et al.*, 1987).

xii. Phytohaemagglutinin (PHA) was obtained from Sigma (St. Louis, U.S.A).

b. Processing of clinical material

i. Preparation of serum

Blood samples were collected in 10 ml sterile glass tubes (Becton-Dickinson, Cowley, U.K.). The blood was left to clot at room temperature and the tubes were spun at 800 g for 10 mins at room temperature. The supernatant (serum) was collected and used either immediately or stored in aliquots at -20^{o}C until use.

ii. Preparation of synovial fluid

Synovial fluid (SF) samples were collected in universal containers (Sterilin Ltd, Teddington, U.K.) containing 200 µl of heparin (1000 units/ml, CP Pharmaceuticals, U.K.). They were spun at 500 g for 20 mins at room temperature to remove

cells and particulate material. The supernatants were then either used immediately or stored at -20°C until use.

Synovial fluid samples intended to be resolved by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) were treated further as follows. After the removal of cells, SF samples were spun at 800-1000 g for 30 mins and filtered through a 0.45 µm filter (Schleicher and Schüll, Dassel, F.R.G.) to remove particulate material. The protein content of the samples was measured and adjusted to 4 mg/ml with 12 M urea and 35 µl/ml of 0.5 M 2-merchaptoethanol. The samples were incubated for 6 hours at a 40°C water bath, diluted 1:1 with phosphate buffered saline (PBS, 0.1 M, pH 7.4) /0.02 M iodoacetamide (Sigma), dialysed against PBS/0.02 M iodoacetamide overnight at 4°C and once against PBS. The treated SF samples were stored at -20°C until required.

iii. Separation of immune complexes from synovial fluid

The separation of the immune complexes from synovial fluid was based on their decreased solubility in polyethylene glycol (PEG) solutions (Male *et al.*, 1980). SF samples were spun at 500 g for 20 mins at room temperature to remove cells and particulate material. Immune complexes were precipitated in a 2% PEG 6000 (Sigma) solution.

Briefly, 500 µl of SF were mixed in 10 ml round-bottomed tubes (Sterilin) with 100 ml of a 12% PEG solution in veronal buffered saline (VBS) (pH 7.6). The tubes were incubated overnight at 4°C, centrifuged at 800 g for 20 mins at 4°C to collect the precipitated immune complexes, and the

precipitates were resuspended with 6 ml of 2% PEG in 0.01 M ethylenediamine-tetraacetic acid (EDTA) in VBS. The tubes were centrifuged at 800 g for 20 mins at 4°C and the precipitates were redissolved in 500 µl VBS and incubated at 37°C for 1 hr to ensure complete solubilisation. At this stage the immune complexes were used or stored at -20°C until required.

c. Estimation of protein in antigen preparations

Protein estimation was determined using a Bio-Rad protein assay kit (Bio-Rad, Richmond, U.S.A.), based on the Bradford dye (Coomassie brilliant blue G) binding procedure (Bradford, 1976; Spector, 1978) with bovine serum albumin (BSA) (Sigma) as a standard.

d. Source of antibodies

i. The monoclonal and polyclonal antibodies used for the identification of 65 kDa and 70 kDa proteins are shown below.

For the identification of 65 kDa hsp protein the following antibodies were used:

TB78: mouse IgG1 monoclonal antibody reactive against the 65 kDa protein of *M. tuberculosis* and *M. bovis* BCG, and the *E. coli* 65 kDa hsp (Coates *et al.*, 1981; Ivanyi *et al.*, 1985; Young *et al.*, 1987). This antibody was kindly provided by Professor J. Ivanyi (Medical Research Council, Tuberculosis and Related Infections Unit, Hammersmith Hospital, London).

F67-2 and F67-13: mouse monoclonal antibodies reactive against the 65 kDa protein of *M. tuberculosis*, *M. bovis* and *M. leprae* (Kolk *et al.*, 1984; Buchanan *et al.*, 1987; Thole *et al.*, 1988a). These antibodies were highly cross-reactive with the 65 kDa protein from a wide range of mycobacterial species. They were kindly provided by Dr. A.H.J. Kolk (Royal Tropical Institute, Amsterdam, Netherlands).

SF8: mouse IgM monoclonal antibody raised against the *M. tuberculosis* 65 kDa protein. It cross-reacts with the human synovial fluid 65 kDa protein. The SF8 was kindly provided by Dr. G.A.W. Rook (Medical Microbiology, University College and Middlesex School of Medicine, London).

Anti-P1: rabbit polyclonal antibody against the 63 kDa protein from Chinese hamster ovary cells which cross-reacts specifically with the corresponding 63 kDa protein from human placenta (Gupta *et al.*, 1982; Gupta and Dudani, 1987; Jindal *et al.*, 1989). The anti-P1 was kindly provided by Professor R.S. Gupta (McMaster University, Ontario, Canada).

For the identification of 70 kDa hsp protein the following antibodies were used:

L7: mouse IgG1 monoclonal antibody raised against the 70 kDa protein antigen of *M. leprae*. It cross-reacts with the *M. tuberculosis* and *M. bovis* BCG 71 kDa proteins, and with the *E. coli* 70 kDa hsp (Britton *et al.*, 1985, 1986; Garsia *et al.*, 1989).

51A: mouse IgG2 monoclonal antibody raised against the *M. tuberculosis* 71 kDa protein. It is highly cross-reactive with the 71 kDa protein from other mycobacterial

species (Engers *et al.*, 1986). It was kindly provided by Dr. O. Closs (National Institute of Public Health, Oslo, Norway).

The TB78, F67.2, F67.13, L7 and 51A antibodies were characterised at a World Health Organisation-sponsored workshop (Engers *et al.*, 1985, 1986).

A selection of mouse antisera to *E. coli* 65 kDa hsp and to *M. tuberculosis* 71 kDa hsp were kindly provided by Dr. D.B. Young and were used for pilot experiments.

iv. The $F(ab')_2$ fragments of goat anti-human immunoglobulins (IgA, IgM and IgG) horseradish peroxidase conjugated were obtained from Sigma.

v. The $F(ab')_2$ fragments of rabbit anti-mouse immunoglobulins horseradish peroxidase conjugated were obtained from Dako Ltd (High Wycombe, Bucks, U.K.).

2.2 DETECTION OF ANTIGENS

a. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Proteins in antigen samples were resolved by SDS-PAGE according to the discontinuous buffer system of Laemmli (1970) on slab gels which consisted of 10% (w/v) acrylamide in the separating gel and 4% (w/v) acrylamide in the stacking gel.

The final concentrations in the separating gel were as follows: 0.26% (w/v) N,N'-bis-methylene acrylamide, 0.375 M Tris(hydroxymethyl)aminomethane-HCL (pH 8.7), 0.06% (w/v) ammonium persulphate, 0.1% (w/v) SDS, 0.06% (v/v) tetramethyl-ethylenediamine (TEMED) in double distilled water.

The final concentrations in the stacking gel were: 0.13% (w/v) N,N'-bis-methylene acrylamide, 0.125 M Tris(hydroxymethyl)aminomethane-HCL (pH 6.8), 0.1% (w/v) ammonium persulphate, 0.1% (w/v) SDS, 0.1% (v/v) TEMED, 10 mM EDTA in double distilled water.

The test samples were dissolved in buffer of 0.0625 M Tris(hydroxymethyl)aminomethane-HCL (pH 6.8), 2% (w/v) SDS, 10% (v/v) glycerol, 5% (v/v) 2-mercaptoethanol and 0.001% bromophenol blue. They were then boiled for 2 mins in a water bath.

A mixture of protein markers (MW-SDS-200; Sigma) was always included on each gel and was used for the determination of the molecular mass of the unknown protein bands.

The electrophoresis was carried out using a multi slab gel apparatus (Bio-Rad) with an actual separating-gel surface of

14 cm X 14 cm. The gels had 15 sample tracks in their majority and test samples were only placed in the inner 13 tracks. The amount loaded onto the gel for electrophoresis depended on the type of the test preparation (single protein or mixture of proteins) and on the subsequent form of staining of the electroblotted preparations (direct protein staining or immunostaining), and are shown in section 2.2c.

The electrophoresis was carried out at room temperature either overnight (for approximately 15 hrs) with a constant current of 6 mA/gel or at 20 mA/gel for 90 mins (until migration through the stacking gel was completed) and at 40 mA/gel for approximately 4 hrs (until migration through the separating gel was completed). The electrophoresis was complete when the bromophenol blue marker reached the bottom of the gel.

b. Western blotting

The proteins separated by SDS-PAGE were transferred from the electrophoresis gels to nitrocellulose (NC) membrane (pore size 0.45 μm; Schleicher and Schüll) (Towbin *et al.*, 1979) using a semi-dry electroblotter (Ancos, Denmark) as described by Kyhse-Andersen (1984) with a constant current of 0.8 mA/cm^2 for 90 mins at room temperature.

At the end of the electroblotting procedure, the NC membranes were cut lengthwise using a sharp scalpel in order to separate the different tracks containing the test antigens. To facilitate the correct separation of the tracks, the NC membranes were briefly (30 secs-1 min) emerged in Ponceaus stain (0.2% (w/v) Ponceaus stain (Sigma) and 3% (v/v) tetra-acetic acid in double distilled water) to locate the protein tracks. Destaining

was carried out by washing the NC membranes with excess double distilled water. The divided NC segments were then prepared accordingly, either for direct staining of the proteins or for immunodetection of the proteins, as explained in the following section.

c. Visualisation of proteins immobilised onto NC membrane

i. Direct staining of proteins

After electroblotting, the antigen coated nitrocellulose membranes destined to be stained for proteins were washed with excess PBS, supplemented with 0.05% polyoxyethylene sorbitan monolaurate (Tween 20; Sigma), for 1 hr at 37^{o}C. Further washes, 3-5 times for 20 mins each time at room temperature with gentle agitation, were carried out to ensure that no particulate matter was left on the NC membranes which could interfere with the staining procedure.

The NC membranes were then incubated overnight at room temperature either in 0.2 ml Aurodye (a colloidal gold solution; Janssen Life Sciences Products, Belgium) per cm^2 of NC membrane, or in PBS-Tween 20 containing 1 µl/ml india ink (Pelican fount india drawing ink, Pelican AG, D-3000) (Hancock and Tsang, 1983). Stained blots were rinsed thoroughly in distilled water, air-dried, covered in aluminium foil to be protected from light and stored at room temperature.

It should be noted that the loading amount required on the gel for efficient ink staining of the NC immobilised proteins was approximately 10-15 µg of total protein for test samples that consisted of a mixture of proteins (such as WE, TB

and VAC antigens, synovial fluid and immune complex samples) and 1-4 μg for single protein samples (such as 65 kDa hsp and 70 kDa hsp). For Aurodye staining, the amounts were 10 times less than the above stated amounts because of the high sensitivity of the dye. The Aurodye was tended to be used for samples of limited quantity.

ii. Immunodetection of proteins

The immunodetection of blotted proteins on NC membranes was based on an adaptation of the method by Burnette (1981). The NC membranes were incubated in PBS-0.05% Tween 20-1% BSA, at room temperature for 3 hours or overnight at $4^{o}C$ with gentle agitation, to saturate additional protein binding sites. At this stage the NC membranes could be used immediately or stored until required. The NC membranes for later use were air-dried, covered in filter paper and aluminium foil and stored for several weeks at $-20^{o}C$.

The saturated NC membranes were then incubated with a specific test antibody (monoclonal or polyclonal) appropriately diluted in PBS-0.05% Tween 20-1% BSA buffer (usually 1/400-1/800 dilutions were used unless otherwise stated), overnight at $4^{o}C$ with constant agitation.

The NC membranes were then washed in PBS-0.05% Tween 20-1% BSA buffer (five 5-minute washes with agitation) and incubated with the second (indicator) antibody horseradish peroxidase-conjugated (affinity purified $F(ab')_2$ fraction, 1/800 dilution in PBS-0.05% Tween 20-1% BSA buffer) directed against the immunoglobulins of the first antibody. The incubation was for 2 hours at room temperature and the blots were washed as above

after the end of the incubation period.

The indication (colour) reaction involved the incubation of the blots in 1 volume of 0.4 mg/ml of 4-chloro-1-naphthol (Sigma) in methanol, 5 volumes of 5 mM Tris-HCL pH 7.6, and 0.01% of H_2O_2 final concentration. This gave a deposit of an insoluble product and the reaction was terminated after 20-30 mins by washing the blots with distilled water. The blots were air-dried, covered in aluminium foil to be protected from light and stored at room temperature.

It should be noted that one track of the test preparation to be immunoanalysed was always stained directly for protein. This was called the reference lane and was used for the visualisation of the protein bands available in the test preparation, their corresponding molecular mass determination and the identification of the bands stained after immunodetection.

The loading amounts required for efficient direct protein staining of the reference lane were shown in the previous section. As for the amounts required for immunodetection of the proteins, it was usually found to be 3-5 times greater than that required for ink staining (i.e. 30-60 μg of total protein).

The mixture of known-molecular weight-proteins used to determine the molecular weight of unknown protein bands consisted of: carbonic anhydrase (molecular weight 29 kDa), ovalbumin (45 kDa), bovine plasma albumin (66 kDa). phosphorylase b (97 kDa), β-galactosidase (116 kDa) and myosin (205 kDa).

2.3 DETECTION OF ANTIBODIES

a. Detection of antibody by an enzyme linked immunosorbent assay (ELISA)

The antigens were coated at optimal concentrations which were 10 µg/ml for WE, TB, VAC, *Candida albicans* and 'flu' preparation, and 1-5 µg/ml for the heat shock proteins and synthetic peptides, in carbonate buffer (0.05 M, pH 9.6) onto immunoplates (Nunc, Roskilde, Denmark) and incubated overnight at 4°C. Excess antigen was washed off with PBS (0.1 M, pH 7.4) containing 0.05% Tween 20 (PBS/Tween).

The test sera were plated in doubling dilutions from 1/50 to 1/400 in PBS/Tween in duplicate and incubated for 2 hours at room temperature.

After further washes with PBS/Tween, the affinity purified $F(ab')_2$ fragments of horseradish peroxidase conjugated human antibodies developed in goat (Sigma) were added at 1/1000 dilution in PBS/Tween and incubated overnight at 4°C.

The washing process was repeated and 0.5 mg/ml of 2,2'-azinobis (3-ethyl benzthiazoline sulphonic acid) (Sigma) in citrate phosphate buffer (0.1 M, pH 4.1) with 0.35 µl/ml H_2O_2 vol20 (6% w/v) was added. After approximately 30 mins the reaction was stopped with 96 mg/ml of sodium fluoride (Sigma) in double distilled water and the absorbance was measured at 650 nm using a Titertek multiscan ELISA reader (Flow Laboratories, Irvine, U.K.).

Throughout the assay the volume of reagents added per well at each step was 100 µl and each washing step was carried out at room temperature and was repeated three times with 5-minute intervals.

Analysis and expression of antibody levels measured by ELISA

In order to standardise the reaction in each ELISA plate, a known positive healthy serum was included in each plate and the test sera were compared against this in each plate. A negative control serum was also included.

For the IgM and IgA assays, cord serum was used as a negative control, while for the IgG assays an agammaglobulinaemic serum (kindly provided by Dr. D. Webster, Northwick Park Hospital, London) and a myeloma IgG (kindly provided by Professor F.C. Hay, St. George's Hospital Medical School, London) were used. For each individual assay the same positive and negative controls were used.

The antibody levels were expressed as optical density (OD) ratios and were calculated as follows:

$$\text{OD ratio} = \frac{OD_{650} \text{ of test serum}}{OD_{650} \text{ of positive healthy serum}}$$

The OD_{650} value was expressed relative to the blank (a well with PBS/Tween but without serum) of the ELISA. The negative controls never gave values greater than 0.004 OD units.

The serum dilutions used for the calculations were those within the linear phase of the antibody binding curve and were 1/100 dilution for the IgA ELISA of the mycobacterial 65 kDa antigen and 1/200 for all the other assays.

b. Detection of antibodies by Western blotting

In the detection of serum antibodies directed against synovial fluid antigens, the antigens were immobilised on NC membranes. The synovial fluid samples were electrophoretically separated and Western blots were prepared. The synovial fluid SDS-PAGE gel prepared for this analysis was mostly of the 3-track type.

Normally each gel consisted of 15 separate tracks (6 mm/track). The preparative gels consisted of 3 tracks, one for the molecular weight markers (6 mm), a second one for a reference lane (6 mm) and a third one (~96 mm) for the SF sample. This type of gel was used for the detection of antibodies directed against synovial fluid antigens. Using this form of gel a greater number of SF tracks would be obtained per gel, and therefore a greater number of serum samples could be tested for antibodies. In addition the resolution of the protein bands was more clear since there was no 'smiling' effect on each track apart from the end ones.

The NC membranes containing the immobilised proteins transferred from the polyacrylamide gels were treated as the membranes destined for the immunodetection of proteins (section 2.2c). However, the first antibody applied on the saturated NC membranes was human serum (1/40 dilution in PBS-0.05% Tween-1% BSA buffer).

c. Estimation of total serum or synovial fluid IgA, IgM and IgG by radial immunodiffusion

The estimation of total serum or synovial fluid IgA, IgM, and IgG immunoglobulins was performed by single radial immunodiffusion using NOR-Partigen immunodiffusion plates (Behring Diagnostics, U.S.A.) according to the manufacturers' instructions.

d. Purification of mycobacterial 65 kDa hsp serum antibody

Affinity column chromatography was used for the purification of mycobacterial 65 kDa hsp antibodies from healthy and rheumatoid arthritis sera.

Two milligrams of the recombinant *M. bovis* 65 kDa hsp were coupled to 2 ml of Affigel-10 (Bio-Rad), according to the manufacturers' instructions. 1 ml of a 1/100 dilution of the test serum in PBS was loaded onto the column and the bound antibody was eluted with glycine/HCL (0.1 M, pH 2.8) and neutralised using equal volumes of double strength borate buffer (0.1 M, pH 8.3). This procedure was repeated for a minimum of three times for each serum, in order to deplete the test sera from the 65 kDa hsp antibodies present in them.

2.4 SEPARATION OF CELLS AND CELLULAR ASSAYS

a. Separation of peripheral blood mononuclear cells

The separation of peripheral blood mononuclear cells (PBMC) was based on the method by Böyum (1968). Peripheral blood was collected in universal containers (Sterilin) containing 200 µl of heparin. The blood was then diluted 1:1 with sterile RPMI 1640 (Gibco Ltd, U.K.) at room temperature. Approximately 12 ml of diluted blood was layered onto 9 ml of Lympho-paque (density 1.086 g/ml; Nyegaard, Norway) in universal containers, which were centrifuged at 400 g for 30 mins at room temperature.

The interface cells (PBMC) were removed by aspiration, placed in a universal containing cold RPMI 1640 supplemented with 5% heat inactivated (56^{o}C for 40 mins) new born (HI-NBCS, Gibco) or foetal calf serum (HI-FCS, Gibco) and washed.

The washing procedure involved a 7-8 mins centrifugation at 400 g at 4^{o}C, the supernatant was removed, the cell pellet was resuspended in cold RPMI 1640-5% HI-NBCS or HI-FCS and the washing procedure was repeated. The cells were then kept in RPMI 1640-5% HI-NBCS or HI-FCS and on ice until the set up of the experiment, when the final wash was carried out using cold RPMI 1640 only.

b. Separation of synovial fluid cells

Synovial fluid samples were collected in heparin (200 µl) containing universals. The SF was centrifuged at 500 g for 20 mins at room temperature. The cell pellet was resuspended in RPMI 1640 supplemented with 5% HI-NBCS or HI-FCS and a 'pinch'

of carbonyl iron (GAS Corporation, New York, U.S.A.) was added. The cells were rolled at 37°C for 45-60 mins and the iron-containing phagocytic cells were removed by standing the tubes on a magnet for a few minutes. Cells in the supernatant were collected and the SF mononuclear cells were separated by density gradient centrifugation as described above for PBMC.

c. Counting of cells, culture medium, culture conditions

Cell counts were performed using an improved Neubauer chamber (Weber, England) and an ethidium bromide/acridine orange stain to distinguish viable (green) and non-viable (orange) cells, according to Lee *et al.* (1975).

All cellular work was carried out under sterile conditions. Cells were cultured in RPMI 1640 supplemented with 10% autologous serum, 2 mM glutamine (Flow), penicillin (100 units/ml; Glaxo Laboratories Ltd, U.K.) and streptomycin (100 µg/ml; Glaxo). In some limiting dilution analysis experiments, the 10% autologous serum was substituted by 20% autologous plasma due to the limited availability of serum. All cell cultures were incubated in a 5% CO_2 humidified atmosphere at 37°C.

d. Irradiated cells

Cell preparations were irradiated (2500 rads) by placing them in a ^{60}Cobalt source for the necessary length of time, washed once and resuspended at the required concentration in culture medium.

e. Cell proliferation assay

Mononuclear cell populations were cultured at 10^5 cells/well in flat-bottomed 96-well microtitre plates (Nunc) in culture medium, in the absence and presence of different concentrations of stimulant (antigen). At least 3 replicate wells were set up for a particular set of experimental conditions. The plates were incubated at 37°C in a 5% CO_2 humidified atmosphere for the required length of time (usually 5 days).

Proliferation was assessed during the last 18 hours of the culture period by the incorporation of 50 µl ^{125}Iodo-uridine-deoxyribose (^{125}I-UdR; specific activity 10 µCi/ml; Amersham, U.K.). The cells were harvested onto glass fibre filters using a Titertek multichannel cell harvester (Flow), and the radioactivity of the discs was counted on a gamma-counter (Crystal multi detector gamma system, United Technologies Packard, U.K.).

Background counts, consisting of wells containing medium only that were treated the same way as above, were subtracted from all the readings.

f. Estimation of frequency of antigen specific lymphocytes in peripheral blood and synovial fluid

Limiting dilution analysis (LDA) (Lefkovits and Waldmann, 1984) was used for the estimation of the precursor frequency of WE (water extract of *M. tuberculosis*) specific lymphocytes in peripheral blood and synovial fluid samples (van Oers *et al.,* 1978).

Varying numbers of mononuclear cells (100, 500, 2500, and 12500 cells per well) were cultured together with antigen at

its optimal concentration (10 μg/ml), in the presence of $3x10^4$ autologous-irradiated (2500 rads) mononuclear cells in flat-bottomed 96-well microtitre plates in a final volume of 200 μl/well.

Thirty replicate wells were set up at each cell concentration in the presence of antigen and absence of antigen. The plates were incubated at an angle (45^o) to facilitate cell contact for 3-4 days, and flat for the rest of the incubation period.

The assessment of positive wells was made visually (presence of lymphoblasts, areas of active proliferation) by inverted phase contrast microscopy after the 6th-7th day of incubation. It was found that the discrimination between responding and non-responding cultures was improved by extended the culture period to 9 days. The accuracy of the visual assessment was tested by the use of ^{125}I-UdR incorporation as a parameter for stimulation on the 9th day of the culture period. The precursor frequencies obtained by visual observation and incorporation of ^{125}I-UdR were similar (i.e. they showed a <10% variation).

The estimation of precursor frequency was performed according to Lefkovits and Waldmann (1984). Assuming that antigen responsive cells (ARC) are randomly distributed among the culture wells, the number of ARC per well follows a Poisson distribution expressed in the following function:

$$F(x)=(u^x/x)\ e^{-u}$$

This is the probability of x ARC/well when the mean number of ARC at that cell density is u. The probability of

obtaining a culture containing no ARC is given by $Fo=e^{-u}$, and when u=1, Fo is 0.37. Thus, when N cells per well result in 37% non-responding cultures, the frequency of the ARC is 1/N which can be calculated by plotting the natural logarithm of the fraction of negative wells/cell concentration against the number of cells/well plated (Figure 2.1).

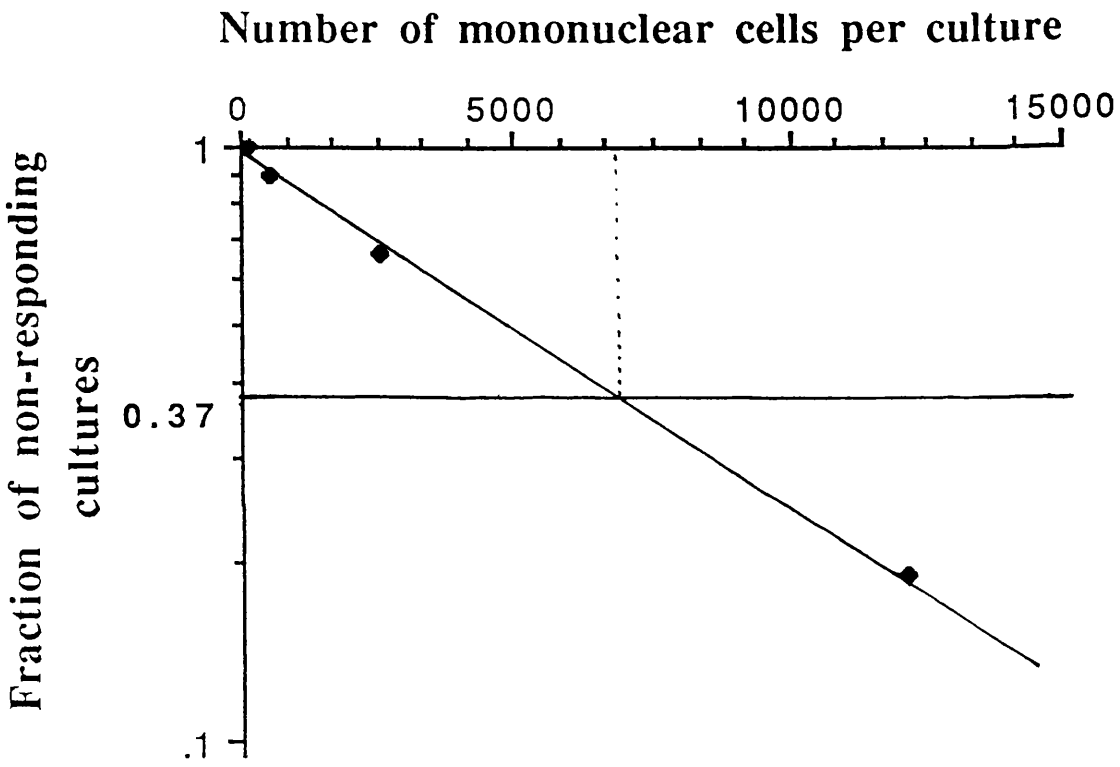


FIGURE 2.1: ESTIMATION OF PRECURSOR FREQUENCY OF WE REACTIVE MONONUCLEAR CELLS BY LIMITING DILUTION ANALYSIS. The WE antigen (10 μg/ml) induced proliferation was measured in cultures with 100, 500, 2500 and 12500 responding cells/well in the presence of $3x10^4$ γ-irradiated autologous mononuclear cells. Thirty wells were set up for each cell concentration.The frequency of the limiting cell type could be read as the inverse of the number of cells at which 37% of the cultures are non-responding.

2.5 STUDY GROUPS

a. Rheumatoid arthritis patients

Peripheral blood, synovial fluid and serum samples were obtained from patients attending the outpatient clinic of the Bloomsbury Rheumatology Unit at University College and Middlesex School of Medicine, London, and were kindly provided by Drs. A. Bhalla, B. Colaco, M. Corbett, J. Edwards, G. Papasavvas, M. Richter, M. Shipley and M. Snaith.

The patients fulfilled the American Rheumatism Association's criteria for classical RA (Arnett *et al.*, 1988). The information available on the majority of patients was their age, sex, disease activity and duration, treatment of disease, status of seropositivity, erythrocyte sedimentation rate, rheumatoid factor levels (IgM, IgA and IgG), total serum immunoglobulin levels (IgM, IgA and IgG) and HLA-DR tissue type.

i. The disease activity of the patients was assessed according to their joint disease activity and divided into highly active, moderately active and inactive (Young *et al.*, 1980).

Highly active disease meant widespread synovitis of joints which were either hot, red, tender or cold, clammy and densely thickened. Early morning stiffness often lasted for several hours.

Moderately active disease meant that synovitis or early morning stiffness was present in a few joints only or in tendon sheaths.

Inactive disease meant that early morning stiffness was less than half an hour in duration and any joint symptoms could be ascribed to mechanical damage or primary or

secondary osteoarthritis.

For the purpose of statistical analysis of the data and because of the small number of moderately active patients, the highly and moderately active patients were treated as one group named active patients.

ii. The erythrocyte sedimentation rates (ESR) were measured by the standard Westergren method (Kushner, 1989) and ESR values greater than 30 mm/hr were taken as increased activity (the presence of inflammation or tissue injury or lymphoproliferative disorders).

iii. The status of seropositivity was assessed by latex agglutination test according to Hudson and Hay (1989).

iv. The rheumatoid factor levels were measured by an ELISA and were provided by the Rheumatology Research Department at University College and Middlesex School of Medicine, London.

v. The total serum immunoglobulin levels were measured as shown in section 2.3c.

vi. The HLA-DR tissue typing of the patients was courtesy of the Immunology Department at the London Hospital, London.

vii. The patients receiving treatment were divided into

those receiving first line, second line and third line drugs (Capell, 1990). The majority of patients who were on second or third line drugs were receiving first-line drugs as well.

The first-line drugs included the non-steroidal anti-inflammatory drugs such as aspirin, indomethacin, ketoprofen, naproxen, piroxicam and salicylate (Platt and Dick 1986).

The second-line drugs included the disease-modifying drugs such as gold, penicillamine and sulphasalazine (Berry, 1986).

The third-line drugs included azathioprine, cyclophosphamide, prednisolone and methotrexate (Corke *et al.*, 1986; Myles, 1986).

The clinical and serological characteristics of the patients used in each assay are shown in the individual chapters.

b. Systemic lupus erythematosus patients

Serum samples were collected from patients attending the outpatient SLE clinic of the Bloomsbury Rheumatology Unit at University College and Middlesex School of Medicine, London, and were kindly provided by Dr. D.A. Isenberg. The patients fulfilled four or more of the American Rheumatism Association's revised criteria for the classification of the disease (Tan *et al.*, 1982).

The information available on the patients was their age and sex, disease activity and treatment, and HLA-DR tissue type.

i. The disease activity was graded according to a scoring index corresponding to predominant clinical manifestations of the disease (Isenberg *et al.*, 1984; Symmons *et al.*, 1988). The scoring system is shown in table 2.1.

The activity was assessed as high or severe (grade 4 activity, 8 points or more), moderate (grade 3 activity, 5-7 points), mild (grade 2 activity, 2-4 points) and inactive (grade 1 activity, 0-1 points) (Table 2.1).

ii. The patients receiving treatment were on azathioprine only (4/18) or on azathioprine and prednisolone (6/18).

iii. The HLA-DR tissue typing of the patients was courtesy of Professor J.R. Bachelor (Department of Immunology, Royal Postgraduate Medical School, London).

TABLE 2.1: Disease activity scoring system for systemic lupus erythematosus patients.

Clinical feature	Score
Horizontal 10 cm visual analogue scale of well being (5 cm or an increase of > 2 cm since previous visit)	1
Pyrexia (>37.5° C, not due to infection)	1
Lymphadenopathy (not due to infection)	1
Arthralgia and/or myalgia	1
Pleuritis and/or pericarditis	2
Vasculitis skin rash (1) grade 1	0
grade 2	0
grade 3	1
Raynaud's active	1
Cerebral involvement (2) grade 1	1
grade 2	1
grade 3	3
Renal proteinuria (+ or more)	
Proteinuria trace + hypertension Proteinuria trace + stable urea/creatinine	1
Increasing proteinuria ± Rising blood preasure Increasing proteinuria ± Rising urea/creatinine	3
Easy bruising / bleeding	1
Steroids	
Prednisolone 0 - 4 mg/day	0
5 - 24 mg/day	1
25 mg +/day	2

Footnotes: (1) Grading of skin involvement:

grade 1: <4/9 body surface involved, no infarction or 2 or 3 in healing phase.

grade 2: >4/9 body surface involved, no infarction, ulceration or tropic change.

grade 3: any distribution with infarction, ulceration or tropic change.

(2) Grading of cerebral involvement:

grade 1: frequent vascular headaches and/or visual phenomena or grade 2 or grade 3 recovering.

grade 2: disturbance of mood or clouding of consciousness with normal functioning.

grade 3: neurological deficit developed within last month or disturbance of mood or clouding of consciousness inconsistent with normal functioning.

c. Ankylosing spondylitis patients

Serum samples were collected from patients attending the AS clinic of the Bloomsbury Rheumatology Unit at University College and Middlesex School of Medicine, London, and were kindly provided by Drs. F. Yüksel and A. Ebringer.

The patients fulfilled the New York criteria for ankylosing spondylitis (Bennett and Burch, 1968; Moll and Wright, 1973). The information available on the patients was their age, sex, activity and treatment of disease.

i. The patients were divided according to their disease activity into active, slightly or moderately active and inactive (Ebringer *et al.*, 1988).

Active patients were those who had both an ESR value greater than 30 mm/hr and a total serum IgA greater than 300 mg/dl.

Slightly active were considered those who had either an ESR value greater than 30 mm/hr or a total serum IgA value of ≥300 mg/dl.

Inactive patients were defined as those having both an ESR value of <30 mm/hr and a total serum IgA value of <300 mg/dl.

ii. The patients on treatment were receiving non-steroidal anti-inflammatory drugs, mainly indomethacin.

d. Crohn's disease patients

Serum samples were kindly provided by Professor J. Lennard-Jones and colleagues (St. Mark's Hospital, London). The

information available on the patients was their age, sex, activity and treatment of disease.

The patients were divided according to their disease activity into slightly active (C-reactive protein <12 μg/ml and Harvey-Bradshaw clinical indices of <5) and moderately active (C-reactive protein >12 μg/ml and Harvey-Bradshaw clinical indices of >5) (Harvey and Bradshaw, 1980). The clinical activity indices were based on:

i. General well-being (very well=0, slightly below par=1, poor=2, very poor=3, terrible=4).
ii. Abdominal pain (none=0, mild=1, moderate=2, severe=3).
iii. Number of liquid stools per day
iv. Abdominal mass (none=0, dubious=1, definite=2, definite and tender=3).
v. Complications: arthralgia, uveitis, erythema nodosum, aphthous ulcers, pyoderma gangrenosum, anal fissure, new fistula, abscess (score 1 per item).

Patients receiving treatment (5/21) were on prednisolone (4/5) and on azathioprine (1/5).

e. Osteoarthritis patients

Serum and synovial fluid samples were kindly provided by Dr. D.A. Isenberg (Bloomsbury Rheumatology Unit, University College and Middlesex School of Medicine, London).

The information available on the osteoarthritic patients was limited. They were males and females with an

average age of 75 years that were referred to the above clinic due to joint pain, stiffness and loss of motion, and were diagnosed as osteoarthritic patients.

f. Healthy individuals

The healthy peripheral blood and serum samples were kindly offered by laboratory personnel of the Immunology, Rheumatology Research and Medical Microbiology Departments of University College and Middlesex School of Medicine, London.

2.6 STATISTICAL ANALYSIS

The Mann-Whitney U-Rank two tailed test was used to compare the antibody levels of two groups.

The Wilcoxon rank test for paired samples was used to compare antibody levels in paired blood and synovial fluid samples.

The Spearman's rank correlation coefficient test was used to analyse correlations between antibody levels and a variety of other parameters of the individuals tested, such as age, duration of disease, ESR values, rheumatoid factor levels and total serum immunoglobulin levels.

It should be noted that the statistical analysis was carried out only for groups with 4 or more entries to be compared, since the statistical values obtained from the comparison of groups with a few (<4) entries were not reliable (Campbell, 1981).

The statistical analysis was carried out using the STSC Statgraphics version 2.0 computer software package.

CHAPTER 3 SERUM ANTIBODY LEVELS TO MYCOBACTERIAL ANTIGENS IN RHEUMATOID ARTHRITIS PATIENTS

Introduction

Characteristics of study groups in chapter 3

Results

- Antibody levels to WE, TB and VAC antigens in rheumatoid arthritis sera
- Correlations between antibody levels to the WE, TB and VAC antigens
- Antibody levels to the mycobacterial antigens with respect to age, sex, duration of the disease, disease activity and treatment, seropositivity and total serum immunoglobulin levels in RA patients
- Anti-mycobacterial antibody responses and HLA-DR haplotype

Discussion

3.1 INTRODUCTION

Although different models for rheumatoid arthritis have been proposed and studied, its cause in humans remains unknown. The favoured aetiological model for rheumatoid arthritis is an immunological reaction to one or more microbes, or autoantigens cross-reactive with them, in a genetically susceptible host. Although the identification of such an agent has been the target of considerable study with only limited success, new hope emerges from reports on the aetiology of adjuvant arthritis in rats.

Adjuvant arthritis can be induced in rats by immunisation with mycobacteria in oil (Pearson, 1956). The disease can be transferred to susceptible rats by T cell clones specific for *Mycobacterium tuberculosis*, which also recognise an acetone precipitable fraction of *M. tuberculosis* and a component of cartilage proteoglycan (Holoshitz *et al.*, 1984; van Eden *et al.*, 1985). This double recognition points to antigenic mimicry between *M. tuberculosis* and this autoantigen. The *M. tuberculosis* protein epitope is contained within a peptide composed of the amino acids at position 180-188 of the mycobacterial 65 kDa heat shock protein (van Eden *et al.*, 1988).

The possibility was raised as to whether RA too might be characterised by reactivity to mycobacterial antigens. As an

initial approach, antibody responses to mycobacterial antigens were studied in sera from RA patients and healthy individuals.

Furthermore, since mycobacterial responses have been associated with certain HLA-DR haplotypes (Ottenhoff *et al.*, 1986; Bahr *et al.*, 1988b; Palacios-Boix *et al.*, 1988), correlations between antibody responses to mycobacterial antigens and HLA-DR haplotypes were examined.

The mycobacterial antigens tested were a water extract of *M. tuberculosis* (WE), sonicates from *M. tuberculosis* (TB) and *M. vaccae* (VAC). The antibody levels were measured by an enzyme linked immunosorbent assay (ELISA).

3.2 CHARACTERISTICS OF STUDY GROUPS IN CHAPTER 3

TABLE 3.1: Characteristics of rheumatoid arthritis patients and healthy individuals used to study antibody levels to crude mycobacterial antigens.

	Rheumatoid Arthritis			Healthy Controls
Ig Class	IgA	IgM	IgG	IgA, IgM, IgG
Number of donors	64	85	75	37
Male	14	19	20	9
Female	50	66	55	28
Average Age (Years)	59	58	58	35
(range)	33-84	30 - 84	30 - 84	24 - 63
Duration of disease	mean 11 years, range 2-20 years			↑ Not applicable ↓
Activity of disease (1)				
Active	15	19	19	
Inactive	39	52	47	
Not known	10	14	9	
Treatment (2)				
First-line drugs	26	35	32	
Second-line drugs	7	10	9	
Third-line drugs	7	7	7	
No treatment	16	22	22	
Not known	8	11	5	
Seropositivity (3)				↑ Not known ↓
Seropositive	23	30	26	
Seronegative	23	25	26	
Not known	18	30	23	

Footnotes: (1) Activity was assessed as shown in section 2.5.

(2) First-line drugs: non-steroidal anti-inflammatory drugs (e.g. indomethacin, naproxen)
Second-line drugs: disease-modifying drugs (e.g. gold, penicillamine, sulphasalazine)
Third-line drugs: prednisolone, azathioprine, methotrexate.

(3) Seropositivity was assessed by latex agglutination test.

3.3 RESULTS

a. Antibody levels to WE, TB and VAC antigens in rheumatoid arthritis sera

The IgG, IgA and IgM antibody levels to the WE, TB and VAC mycobacterial antigens were measured by ELISA in sera from rheumatoid arthritis patients and healthy individuals. The antibody levels were expressed as optical density ratios (OD_{650} of test serum /OD_{650} of a standard healthy serum used as positive control). The characteristics of the RA patients and healthy individuals used in this part of the study are shown in table 3.1.

The RA patients were found to have raised IgA serum antibody levels to the WE ($p<0.01$), TB ($p<0.0001$) and VAC ($p<0.0001$) antigens in comparison to healthy controls (Figure 3.1a). Sera from 19/64, 29/64 and 22/64 RA patients showed IgA levels higher than the mean+2 standard deviations (SD) of the healthy control group levels to WE, TB and VAC antigens respectively.

In contrast, the IgG and IgM antibody levels to the same antigens were lower ($p<0.001$ for the WE and TB assays, $p>0.05$ for the VAC assay) in RA patients than in healthy controls (Figures 3.1b and 3.1c respectively).

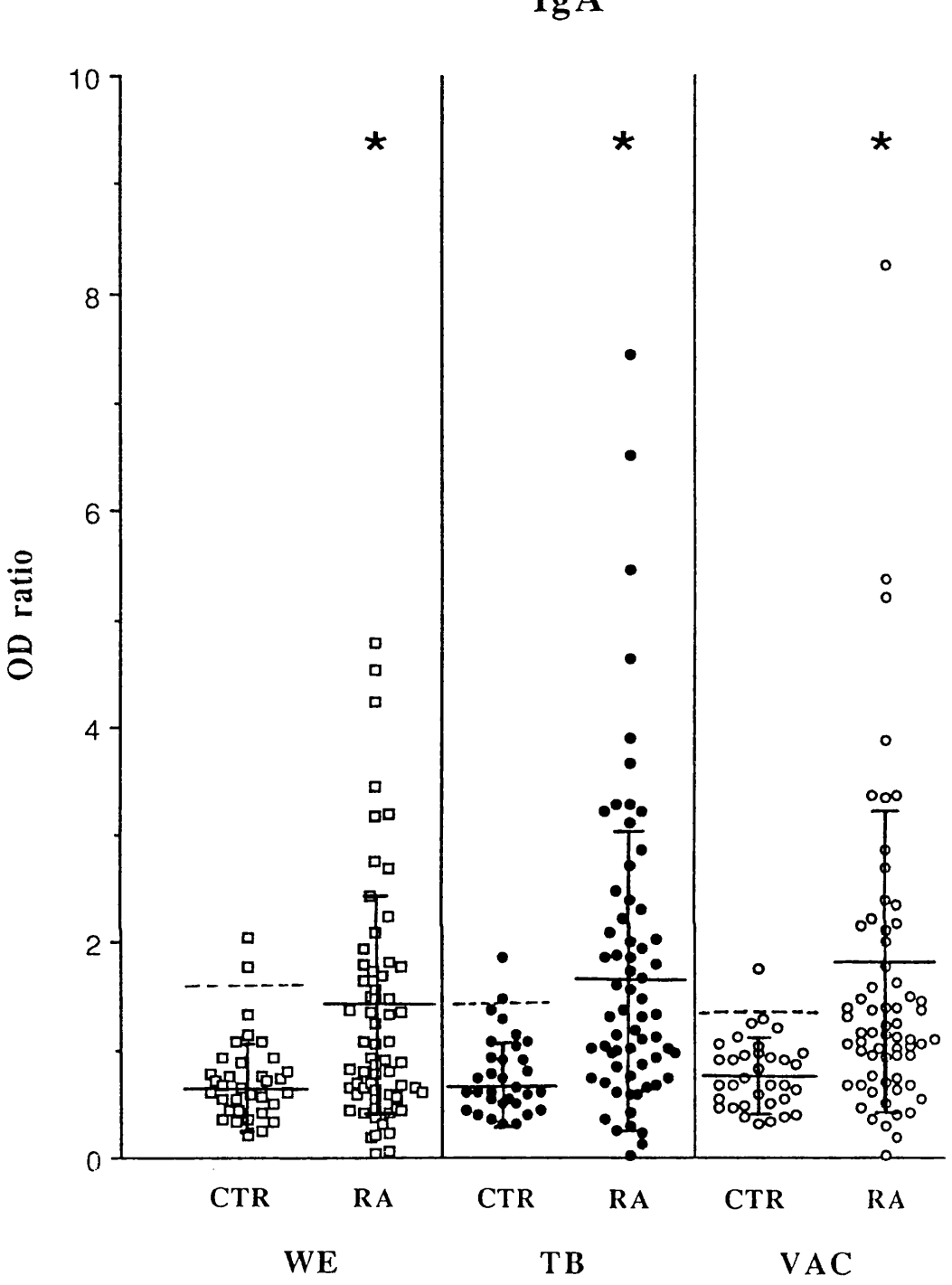


FIGURE 3.1a: IgA SERUM ANTIBODY LEVELS TO WE, TB AND VAC MYCOBACTERIAL ANTIGENS IN HEALTHY INDIVIDUALS (CTR) AND RHEUMATOID ARTHRITIS PATIENTS (RA). WE: water extract of *M. tuberculosis*; TB: sonicate of *M. tuberculosis*; VAC: sonicate of *M. vaccae*. The antibody levels are expressed as optical density ratios (OD_{650} ratio = OD_{650} of test serum /OD_{650} of standard healthy serum used as a positive control). The horizontal lines represent the mean OD ratio for each group and the vertical lines represent the standard deviation (SD) for each group. The dotted lines define 2SD above the mean OD ratio of the healthy control group. * $p<0.01$.

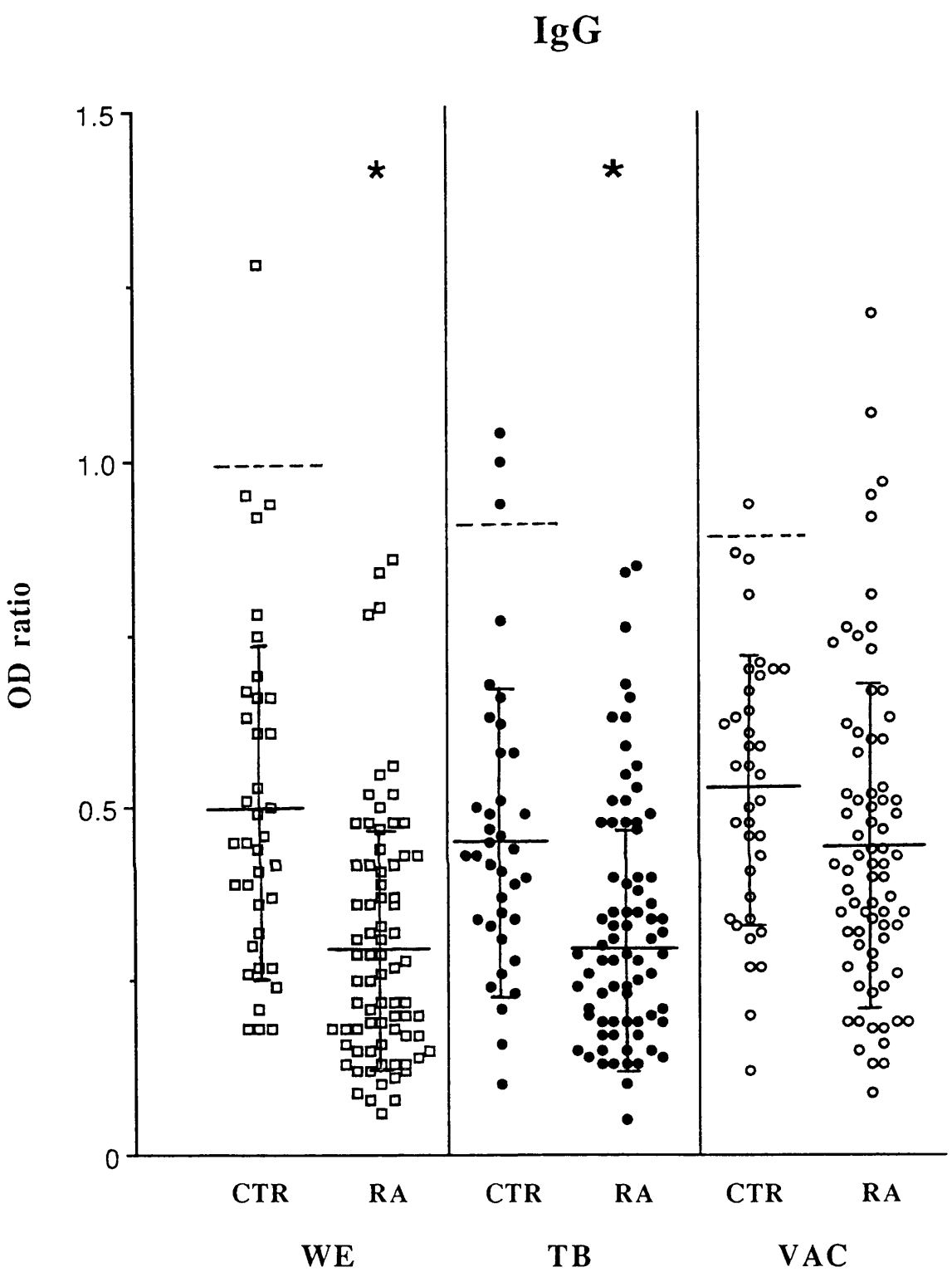


FIGURE 3.1b: IgG SERUM ANTIBODY LEVELS TO WE, TB AND VAC MYCOBACTERIAL ANTIGENS IN HEALTHY INDIVIDUALS (CTR) AND RHEUMATOID ARTHRITIS PATIENTS (RA). The antibody levels are expressed as in figure 3.1a. The horizontal lines represent the mean OD ratio for each group and the vertical lines represent the standard deviation (SD) for each group. The dotted lines define 2SD above the mean OD ratio of the healthy control group. * $p<0.001$.

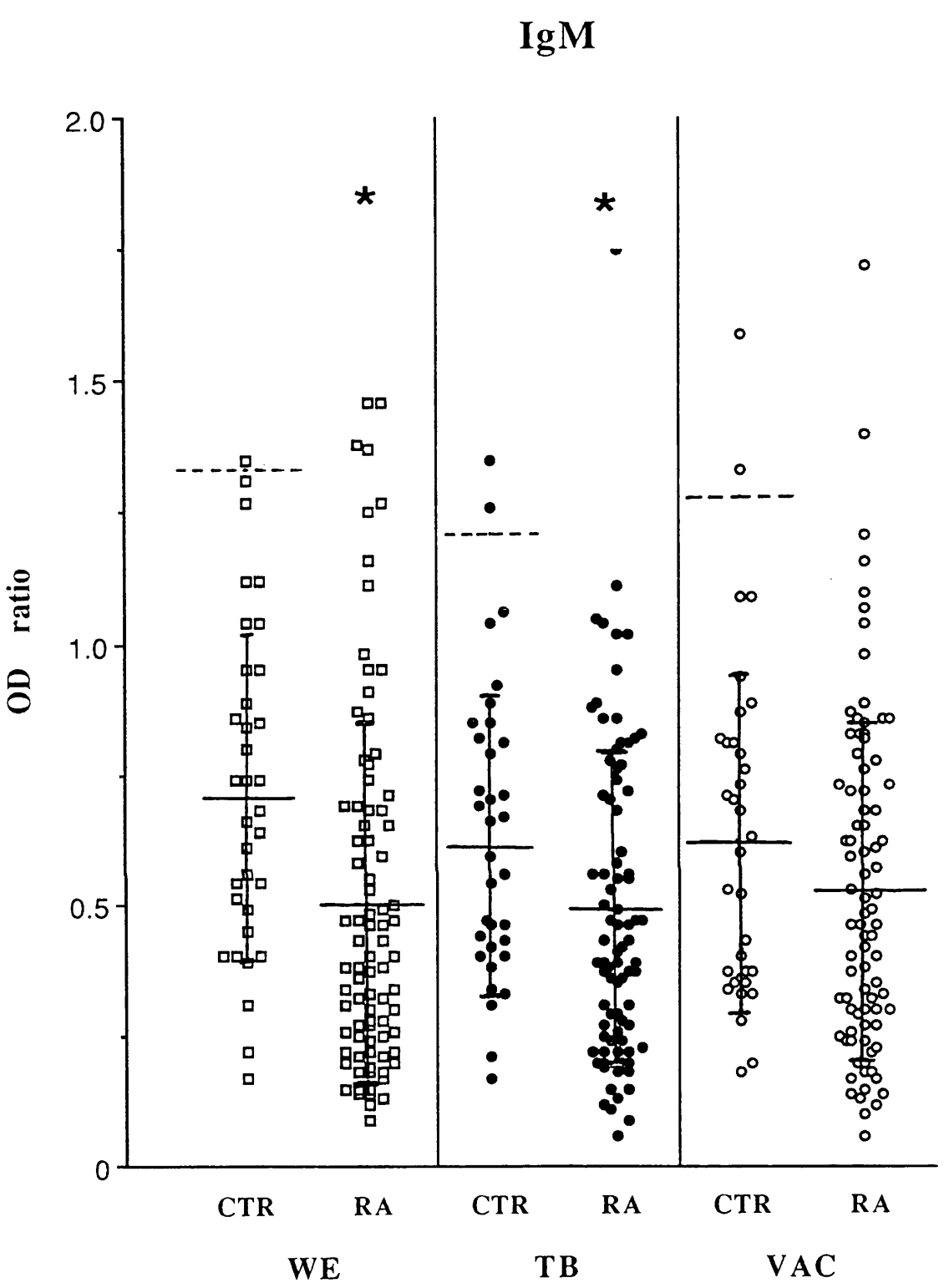


FIGURE 3.1c: IgM SERUM ANTIBODY LEVELS TO WE, TB AND VAC MYCOBACTERIAL ANTIGENS IN HEALTHY INDIVIDUALS (CTR) AND RHEUMATOID ARTHRITIS PATIENTS (RA). The antibody levels are expressed as in figure 3.1a. The horizontal lines represent the mean OD ratio for each group and the vertical lines represent the standard deviation (SD) for each group. The dotted lines define 2SD above the mean OD ratio of the healthy control group. * $p<0.01$.

b. Correlations between antibody levels to the WE, TB and VAC antigens

The WE, TB and VAC antigens, being crude preparations, were likely to consist of several antigenic molecules. In order to examine whether or not the antibodies directed against these preparations were, in fact, directed against common or cross-reactive antigens, the antibody levels to these preparations were tested for correlations.

Strong correlations were found between antibody levels to WE, TB and VAC antigens of the RA sera ($r>0.80$, $p<0.001$). This was true for all three immunoglobulin (Ig) classes studied. In fact, analysis of the RA patients with IgA antibody levels higher than the mean+2SD of the healthy control group level (19/64 in the WE assay, 29/64 in the TB and 22/64 in the VAC assay), has shown that while 13 of them had antibodies to all three antigen preparations, 7 individuals had antibodies primarily against the TB and VAC, 6 against the TB and WE, 2 against the VAC only and 3 against the TB antigen preparation only. This suggested that the antibodies detected to the three antigen preparations were directed not only against common or cross-reactive but also against specific epitopes.

Correlations between antibody levels to the three mycobacterial antigens were also seen in the healthy control group for all three Ig classes studied ($r>0.50$, $p<0.01$).

c. Antibody levels to the mycobacterial antigens with respect to age, sex, duration of the disease, disease activity and treatment, seropositivity and total serum immunoglobulin levels of RA patients

To ensure that the antibody levels to the crude mycobacterial antigens were specific and not influenced by certain characteristics of the patients (such as age, sex, duration and disease activity, treatment, seropositivity and total serum immunoglobulin levels), statistical analysis was carried out.

IgA, IgG and IgM antibody levels were tested for possible associations with the above characteristics and the results are shown below. However, the results concerning the IgA antibody levels are shown in greater detail in table 3.2.

i. Antibody levels with respect to age and sex

Since the average age of the healthy control group (35 years) was much lower than that of the RA group (59 years), the data were analysed for any evidence that the age of the donors influenced the antibody titres. However, no correlation was found between age and antibody levels in either group ($r<0.2$, $p>0.2$).

The antibody levels of male and female donors were not significantly different either ($p>0.05$) and this argued against the antibody response being sex linked.

ii. Antibody levels with respect to disease activity and duration

The IgG and IgM anti-mycobacterial antibody levels of the patients with high disease activity did not differ from

those with low disease activity ($p>0.05$). However, the elevated IgA antibody levels were found to be associated not only with high disease activity (assessed by the joint disease activity, section 2.5), but with erythrocyte sedimentation rate (ESR) values as well (Table 3.2).

In contrast, no association was found between IgA, IgM and IgG antibody levels and the duration of the disease, which ranged from 2-20 years ($r<0.1$, $p>0.4$).

iii. Antibody levels with respect to treatment of disease

The antibody levels of patients receiving treatment were not significantly different from those receiving no treatment ($p>0.05$).

iv. Antibody levels with respect to total serum immunoglobulin levels and seropositivity

Since one of the features of rheumatoid arthritis patients is known to be hypergammaglobulineamia, it was important to check whether the high antibody levels seen in the RA group were due to elevated serum immunoglobulin levels. However, no correlation was found between antibody levels and total serum immunoglobulin levels of the same Ig class ($r<0.5$, $p>0.1$).

In addition, the anti-mycobacterial antibody levels of seropositive patients were not significantly different from those of seronegative patients ($p>0.05$).

TABLE 3.2: Analysis of IgA antibody levels to WE, TB and VAC antigens in rheumatoid arthritis patients with respect to age, sex, duration and activity of disease, treatment, seropositivity and total serum IgA levels.

	WE	TB	VAC
Male (14)	1.73±1.45	2.74±2.33	2.17±1.62
Female (50)	1.11±0.96	1.46±0.99	1.40±1.36
p value (1)	0.19	0.06	0.06
On treatment (40)	1.37±1.27	1.95±1.69	1.82±1.64
No treatment (16)	0.98±0.51	1.17±0.49	0.99±0.31
p value	0.92	0.25	0.17
Seropositive (23)	1.26±1.15	1.95±1.53	1.76±1.34
Seronegative (23)	1.41±1.27	1.75±1.71	1.39±1.03
p value	0.82	0.17	0.09
Active (15)	1.67±1.11	2.65±2.00	2.06±1.28
Inactive (39)	1.06±0.99	1.37±1.13	1.42±1.50
p value	0.015	0.008	0.014
Antibody levels	1.07±0.89	1.49±0.86	1.31±0.72
Age (years)	mean ± SD: 59.12±13.70 (min 33, max 84)		
r,p values (2)	0.029, 0.66	0.017, 0.90	0.038, 0.78
Antibody levels	1.07±0.89	1.49±0.86	1.31±0.72
Duration of disease (years)	mean ± SD: 10.21±5.85 (min 2, max 20)		
r,p values	-0.058, 0.64	-0.074, 0.56	-0.102, 0.42
Antibody levels	1.07±0.89	1.49±0.86	1.31±0.72
ESR (mm/hr)	mean ± SD: 20.26±18.65 (min 1, max 73)		
r,p values	0.29, 0.043	0.34, 0.018	0.32, 0.023
Antibody levels	1.07±0.89	1.49±0.86	1.31±0.72
Total serum IgA (mg/ml)	mean ± SD: 3.27±2.49 (min 1.10, max 7.97)		
r,p values	0.57, 0.10	0.63, 0.065	0.53, 0.15

Footnotes: (1) The p value was obtained from the Mann-Whitney U-Rank two tailed test.
(2) The r, p values were obtained from the Spearman's Rank correlation coefficient test.
The antibody levels are presented as the mean optical density ratio and standard deviation (SD) for each group.

d. Anti-mycobacterial antibody responses and HLA-DR haplotypes

Since responses to mycobacteria have been reported to be associated with certain HLA-DR haplotypes (Ottenhoff *et al.*, 1986; Bahr *et al.*, 1988b; Palacios-Boix *et al.*, 1988), it was examined whether the anti-mycobacterial antibody responses in this study were HLA-DR linked.

Table 3.3 shows the frequencies of HLA-DR haplotypes in the RA group. The healthy donors used in this study were not tissue typed. It should be noted that the frequency of some haplotypes was too low for meaningful statistical analysis of their relationship to antibody levels, so only data for HLA-DR1, 2, 3, 4, 5, 6 and HLA-DR7 haplotypes were considered.

HLA-DR1 positive RA patients were found to have higher IgA antibodies to the WE ($p>0.05$), TB ($p=0.05$) and VAC ($p<0.05$) antigens compared to non-DR1 patients (Table 3.4). In addition, HLA-DR6 positive patients had higher IgG antibody levels to the WE ($p<0.05$) and TB ($p<0.01$) antigens compared to HLA-DR6 negative individuals (Table 3.4).

The antibody levels of HLA-DR2, 3, 4, 5 and HLA-DR7 positive individuals did not differ from those lacking these particular haplotypes ($p>0.05$).

TABLE 3.3: Frequency of HLA-DR haplotypes in the RA group.

	Rheumatoid Arthritis			Healthy Controls
Ig Class	IgA	IgM	IgG	IgA, IgM, IgG
HLA-DR1	14	15	15	Not known
HLA-DR2	11	12	13	
HLA-DR3	16	19	16	
HLA-DR4	26	47	43	
HLA-DR5	6	10	8	
HLA-DR6	10	15	12	
HLA-DR7	8	7	7	
HLA-DR8	4	4	3	
HLA-DR9	0	1	1	
HLA-DR10	3	2	2	
HLA-DR11	3	3	1	
HLA-DR13	1	1	1	
Number of donors studied	64	85	75	37

TABLE 3.4: Comparison of IgA (HLA-DR1 positive versus HLA-DR1 negative individuals) and IgG (HLA-DR6 positive versus HLA-DR6 negative individuals) antibody levels to WE, TB and VAC antigens in RA patients.

		WE	TB	VAC
IgA	HLA - DR1 (14)	1.75±1.30	2.30±1.43	2.07±1.30
	Non - DR1 (50)	1.17±0.97	1.63±1.45	1.43±1.37
	p value	0.060	0.050	0.020
IgG	HLA - DR6 (12)	0.37±0.14	0.43±0.14	0.51±0.21
	Non - DR6 (63)	0.29±0.19	0.31±0.18	0.44±0.23
	p value	0.026	0.010	0.30

Footnotes: The antibody levels are presented as the mean optical density ratio and standard deviation for each group.

The p value was obtained from the Mann-Whitney U-Rank two tailed test.

3.4 DISCUSSION

The study of serum IgA, IgG and IgM antibody levels to WE, TB and VAC mycobacterial antigens has shown that RA patients have significantly raised antibody levels of the IgA class to these antigens compared to healthy individuals. In contrast, the IgG and IgM antibody levels were lower in the RA group. Interestingly, the TB and VAC antigen preparations were also used by Bahr *et al.* (1988b) who showed that there was a tendency to raised serum IgA (but not IgG or IgM) antibody levels in Kuwaiti RA patients compared to healthy individuals.

Although there was an association of raised IgA antibody levels with disease activity and ESR values (but not with duration of the disease), the relevance of the mycobacterial antibodies in RA and the preferential elevation of the IgA antibody levels could not be explained at this stage.

The antibody responses to these mycobacterial antigens seemed to be specific and not to be influenced by characteristics of the test population, such as the total serum immunoglobulin levels and status of seropositivity of the test samples. In addition, the antibody responses were not age or sex linked and the treatment of the disease did not influence these responses either.

The antibody responses were analysed for any relationship with HLA-DR haplotypes that were previously shown to be related to RA, such as HLA-DR4, HLA-DR2 and HLA-DR7 haplotypes (Bahr *et al.*, 1988b; Palacios-Boix *et al.*, 1988; Stastny *et al.*, 1988). The HLA-DR4 haplotype, which has been strongly associated with an increased susceptibility to RA (Stastny *et al.*, 1988; Roudier *et al.*, 1988) and an increased skin test responsiveness to tuberculin

(Bahr *et al.*, 1989), occurred with increased frequency in the RA group tested but failed to show any correlation with antibody levels. The lack of correlation was also true for the HLA-DR7 haplotype, which has been associated with decreased risk of developing RA (Stastny *et al.*, 1988) and was correlated with low skin test responsiveness to mycobacteria in RA (Bahr *et al.*, 1989). There was no correlation between antibody levels and the HLA-DR2 haplotype, although low IgA antibodies to the TB antigen preparation were significantly correlated with this haplotype in the study by Bahr *et al.* (1988b).

The HLA-DR haplotypes tested for correlations with antibody levels also included the HLA-DR1, 3, 5 and HLA-DR6 haplotypes. While HLA-DR1 and HLA-DR6 positive patients were found to have higher IgA and IgG antibody levels respectively, than the patients lacking these haplotypes (Table 3.4), no difference was found in the antibody levels of HLA-DR3 or HLA-DR5 patients and those lacking these haplotypes.

However, there have been very few reports of HLA-associated regulation of antibody levels in man, and these have tended to involve class I antigens of the major histocompatibility complex (MHC). Thus, antibody to flagellin (Whittingham *et al.*, 1980) or to peptides of the influenza virus (Spencer *et al.*, 1976) showed associations with HLA-A and HLA-B respectively. In both of these studies the antigen was a purified peptide, in contrast with the antigen preparations used in this study.

Since the WE, TB and VAC antigens were crude preparations and probably consisted of a variety of antigenic molecules, the specificity of the antibodies directed against them was difficult

to be determined. There were, however, strong correlations between the antibody levels to the three antigen preparations, suggesting that most of the antibodies would probably be directed against common or cross-reactive epitopes. On the other hand, the presence of a few individuals with high antibody levels exclusively against one antigen preparation would suggest that the antibodies were directed not only against common or cross-reactive but also against specific epitopes.

At that stage, because of the limited information that was available about the three antigen preparations studied, it was not obvious which epitopes were responsible for the responses measured here. In addition, it could not be established whether these responses were mycobacteria specific.

The presence of anti-mycobacterial antibodies in the serum could reflect immunisation with mycobacteria per se or with antigens cross-reactive with them. Immunisation could be in the form of bacillus Calmette-Guérin (BCG) vaccination or infection with mycobacteria or cross-reactive microbes.

Unfortunately no information was available on the BCG vaccination status of the groups studied. Thus, it could not be established whether the elevated IgA antibodies were due to prior active immunisation with the mycobacteria, although one might expect a similar frequency of vaccination in both healthy and RA groups.

On the other hand, environmental contact with mycobacteria could not be excluded. Even though mycobacteria do not seem to be part of the normal commensal flora in man (Brock, 1979), they are abundant environmental saprophytes and can be found in large numbers in soil and water samples in some parts of

the world (Taussig, 1984). In view of the fact that susceptibility to adjuvant arthritis in rats could be increased or decreased by prior reconstitution of the bowel flora with various bacterial species (Kohashi *et al.*, 1985), the extent of exposure of an individual to mycobacteria might be important. Thus, extensive exposure to mycobacteria might be sufficient to break self-tolerance (e.g. oral tolerance; Challacombe and Tomasi, 1987; Schwartz, 1989) and lead to an autoimmune process. This might explain the preferential elevation of the IgA antibody levels, since IgA antibodies are the major immunoglobulins found on mucosal surfaces and appear to play a role in preventing initial access of microorganisms to portals of entry (Challacombe, 1987).

Although there is no conclusive evidence for the implication of microorganisms in the aetiopathogenesis of RA, the concept of a microbial trigger in its aetiology is still a subject of intense debate and any of the above suggestions could be possible.

Moreover, it is possible that anti-mycobacterial antibodies have a more fundamental role in the aetiopathogenesis of RA, as has been indicated from experimental animal models. The adjuvant arthritis model in rats provided evidence for the cross-reactivity between mycobacteria and cartilage (van Eden *et al.*, 1985). By analogy with the AA model, cross-reactivity between exogenous antigen and self-antigens may be a pathway through which chronic, persistent arthritis could develop in humans. Cross-reactivity between microbial antigens and self-molecules has been seen previously by means of serology (Oldstone, 1987; Chapter 1). If that was true, it would mean that

the antibodies measured here were partly anti-self.

If cross-reactivity between mycobacterial and self epitopes is one of the mechanisms involved in the resulting autoimmune character of RA, as shown for the adjuvant arthritis model, the interesting question concerns the identity of the cross-reactive self-epitopes in man.

CHAPTER 4

SERUM ANTIBODY LEVELS TO MYCOBACTERIAL AND *E. COLI* 65 AND 70 kDa HSPS IN RHEUMATOID ARTHRITIS PATIENTS

Introduction

Characteristics of study groups in chapter 4

Results

- Evidence for the presence of 65 and 70 kDa proteins in the crude WE, TB and VAC mycobacterial antigens
- Antibody levels to the *M. bovis* and *E. coli* 65 kDa hsps in RA sera
- Antibody levels to the *M. tuberculosis* and *E. coli* 70 kDa hsps in RA sera
- Antibody levels to the hsps in relation to age, sex, activity, duration and treatment of disease, total serum immunoglobulin levels, seropositivity, rheumatoid factor and HLA-DR haplotype
- Anti-mycobacterial antibody responses and HLA-DR haplotypes

Discussion

4.1 INTRODUCTION

In order to investigate further the humoral immune reactivity of rheumatoid arthritis (RA) patients to mycobacteria, antibodies to defined mycobacterial antigens were studied.

The *Mycobacterium bovis* 65 kDa has been reported to be an immunodominant mycobacterial antigen (Young *et al.*, 1987) and it could be involved in the aetiology of adjuvant arthritis (van Eden *et al.*, 1988). A recombinant form of the *M. bovis* 65 kDa was therefore used to analyse antibody levels against it in RA sera.

Being a heat shock protein (hsp), the mycobacterial 65 kDa protein would probably cross-react with the 65 kDa hsp from other bacteria (Shinnick *et al.*, 1988; Young *et al.*, 1988a). It was important, therefore, to determine whether its immunogenicity was due to its mycobacterial origin or not, and for this reason the *Escherichia coli* 65 kDa hsp homologue was included in the study.

The 70 kDa hsp antigen has also been shown to be an immunodominant antigen of mycobacteria (Young *et al.*, 1988a) and the *M. tuberculosis* 70 kDa hsp was therefore also included in the study to control for observations that were specific to the 65 kDa hsp antigen or not. The *E. coli* 70 kDa hsp was used for the same reason as the 65 kDa hsp *E. coli* homologue.

To account for any specificity of the immunogenicity of the above antigens for the RA group, the study groups included sera from systemic lupus erythematosus patients (as an example of another non-organ specific autoimmune disease; Steinberg, 1988), from ankylosing spondylitis patients (as an example of an inflammatory arthritic disease; Calin, 1990), from Crohn's disease patients (as a non-autoimmune disease that has been associated with mycobacteria; Burnham *et al.*, 1978; Rosenberg, 1988) and from healthy individuals.

4.2 CHARACTERISTICS OF STUDY GROUPS IN CHAPTER 4

TABLE 4.1a: Characteristics of rheumatoid arthritis patients used to study antibody levels to heat shock proteins (hsps).

Hsps	*M. bovis* 65 kDa	*E. coli* 65 kDa	*M. tuberc. & E. coli* 70 kDa	
Ig Class	A,M,G	A,M,G	A, M	G
Number of donors	48	22	21	41
Male	15	4	3	9
Female	33	18	18	32
Average Age (Years)	55	58	60	57
(range)	27 - 78	30 - 71	33 - 76	31 - 79
Duration of disease	mean 12 years, range 2 - 20 years			
Activity of disease (1)				
Active	21	9	7	20
Inactive	27	13	14	21
Not known	-	-	-	-
Treatment (2)				
First-line drugs	22	10	9	18
Second-line drugs	4	2	3	6
Third-line drugs	6	5	2	4
No treatment	15	5	7	13
Not known	1	-	-	-
Seropositivity (3)				
Seropositive	17	11	8	12
Seronegative	14	7	7	17
Not known	17	4	6	12

Footnotes: (1) Activity was assessed as shown in section 2.5.

(2) First-line drugs: non-steroidal anti-inflammatory drugs (e.g. indomethacin, naproxen)
Second-line drugs: disease-modifying drugs (e.g. gold, penicillamine, sulphasalazine)
Third-line drugs: prednisolone, azathioprine, methotrexate.

(3) Seropositivity was assessed by latex agglutination test.

TABLE 4.1b: Characteristics of systemic lupus erythematosus (SLE), ankylosing spondylitis (AS) and Crohn's disease (CD) patients used to study antibody levels to heat shock proteins (hsps).

	SLE	AS	CD
	For all hsps and Ig classes tested		
Number of donors	18	15	21
Male	1	10	11
Female	17	5	10
Average Age (Years)	42	50	36
(range)	30 - 68	33 - 73	18 - 74
Activity of disease [1]			
Slightly active	9	-	12
Moderately active	2	9	9
Very active	1	-	-
Inactive	6	6	-
Treatment [2]			
First-line drugs	-	14	-
Second-line drugs	-	-	-
Third-line drugs	10	-	5
No treatment	8	1	16
Not known	-	-	-

Footnotes: (1) Activity was assessed as shown in section 2.5.

(2) First-line drugs: non-steroidal anti-inflammatory drugs (e.g. indomethacin, naproxen)
Second-line drugs: disease-modifying drugs (e.g. gold, penicillamine, sulphasalazine)
Third-line drugs: prednisolone, azathioprine, methotrexate.

TABLE 4.1c: Characteristics of the healthy individuals used as controls for the study of antibody levels to heat shock proteins (hsps).

Hsps	*M. bovis* 65 kDa	*E. coli* 65 kDa	*M. tuberc. & E. coli* 70 kDa	
Ig Class	A,M,G	A,M,G	A, M	G
Number of donors	45	18	19	39
Male	12	4	9	19
Female	33	14	10	20
Average Age (Years)	35	30	31	32
(range)	24 - 63	24 - 50	23 - 50	23 - 50

4.3 RESULTS

a. Evidence for the presence of 65 and 70 kDa proteins in the crude WE, TB and VAC mycobacterial antigens

Before considering whether antibodies to the WE, TB and VAC antigen preparations were also directed against the immunodominant mycobacterial 65 and 70 kDa hsps, it was important to examine whether these hsps were contained in the crude mycobacterial preparations.

Using Western blots of electrophoretically separated WE, TB and VAC preparations and monoclonal antibodies specific for the 65 kDa (TB78) and 70 kDa (L7) hsps, a band of approximately 70 kDa was identified in all three antigen preparations, whilst only the TB and WE preparations were found to contain a 65 kDa protein (Figure 4.1).

Thus, the antibodies against the WE and TB antigens could be directed against the 65 and/or 70 kDa proteins, among the other antigenic molecules present in these preparations.

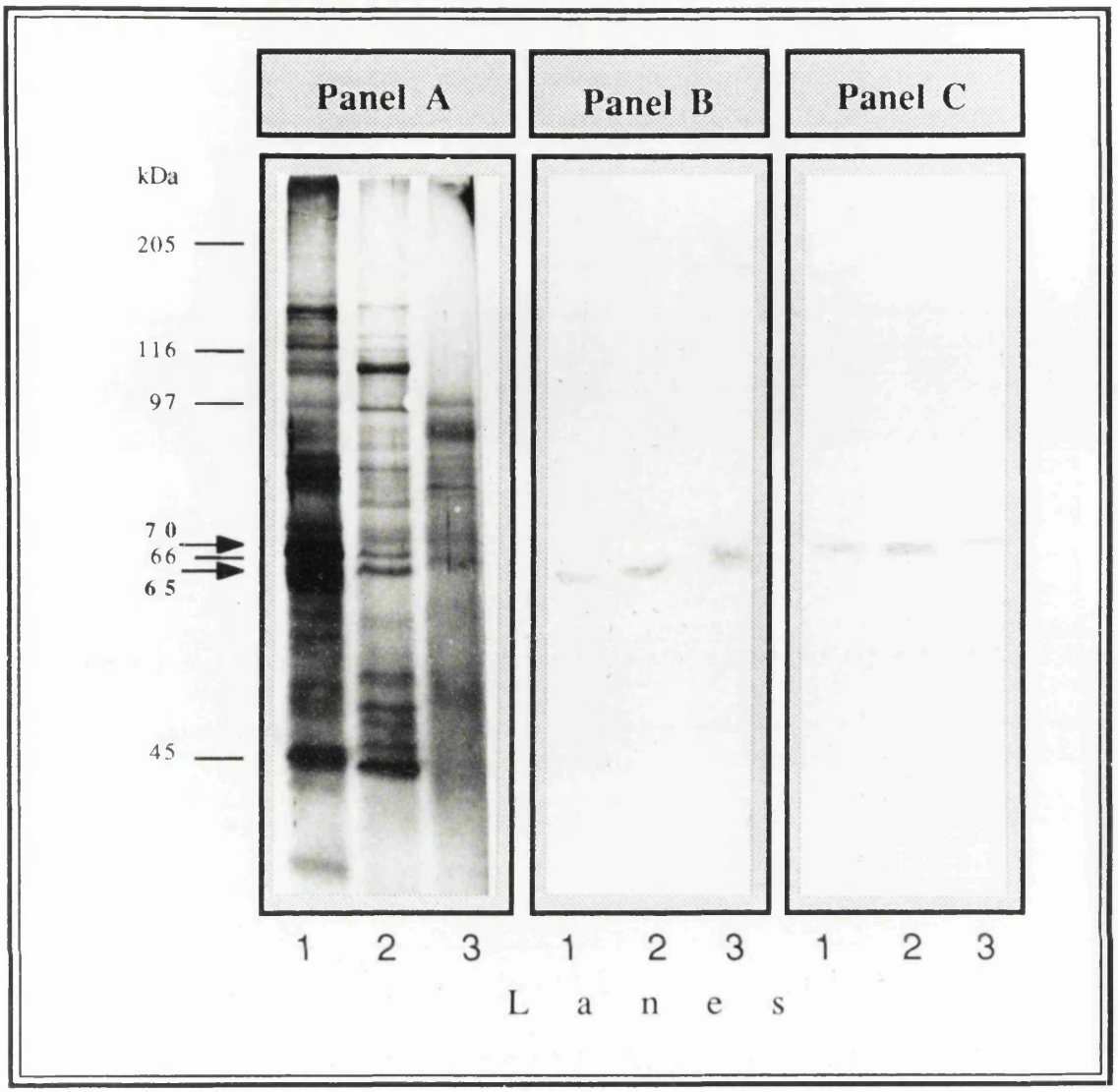


FIGURE 4.1: PRESENCE OF 65 AND 70 kDa PROTEINS IN VAC, TB AND WE MYCOBACTERIAL ANTIGENS.

The VAC, TB and WE preparations were run on a 10% SDS-PAGE and electroblotted.

Western blots of Lane 1: VAC - sonicate of *M. vaccae*
Lane 2: TB - sonicate of *M. tuberculosis*
Lane 3: WE - water extract of *M. tuberculosis*

stained with Panel A: ink
Panel B: TB78 (anti-65 kDa monoclonal antibody)
Panel C: L7 (anti-70 kDa monoclonal antibody)

as described in section 2.2c.

The molecular weight markers are indicated on the left hand side of the figure.

b. Antibody levels to the *M. bovis* and *E. coli* 65 kDa hsps in RA sera

The IgG, IgA and IgM antibody levels to the *M. bovis* and *E. coli* 65 kDa hsp were measured by ELISA in sera from patients with rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), ankylosing spondylitis (AS), Crohn's disease (CD) and in healthy individuals (CTR). The characteristics of these groups are shown in tables 4.1a, 4.1b and 4.1c. The antibody levels were expressed as optical density ratios (OD_{650} of test serum/OD_{650} of a standard healthy serum used as positive control).

i. <u>IgM antibody levels</u>

In all patient groups, the IgM antibody levels to the mycobacterial and *E. coli* 65 kDa hsp antigens were found to be lower than in healthy control sera. However, this decrease of antibody levels was not statistically significantly ($p>0.05$) (Figures 4.2a, 4.2b).

ii. <u>IgA antibody levels</u>

The IgA antibody levels to the mycobacterial 65 kDa hsp were increased in RA ($p<0.00001$), SLE ($p<0.001$), AS ($p<0.001$) and Crohn's ($p<0.001$) patients compared to healthy individuals (Figures 4.3a, 4.3b). However, the greatest increase of antibody levels was that of the RA group. Whereas 11/45 RA sera had antibody levels higher than the mean antibody level+2 standard deviations (SD) of the healthy control group, only 1/18 SLE sera, 3/15 AS sera and 2/21 Crohn's sera were seen to be so.

A further difference between RA and the other diseases was seen in the IgA antibodies to the *E. coli* 65 kDa hsp.

Whereas SLE, AS and Crohn's sera contained significantly raised levels of IgA antibodies to this antigen ($p<0.001$) there was no significant rise in the RA sera ($p>0.05$) (Figures 4.3a, 4.3b). Thus, the increased IgA antibody levels to 65 kDa hsp in RA sera showed some specificity to the mycobacterial homologue.

iii. IgG antibody levels

Another interesting finding was the increase in IgG antibody levels to the mycobacterial 65 kDa hsp in RA patients compared to healthy controls ($p<0.001$) (Figures 4.4a, 4.4b). In contrast, there was no significant increase in IgG antibody levels to this antigen in SLE, AS or Crohn's sera compared to the healthy control group (p values >0.05) (Figures 4.4a, 4.4b). While 10/42 RA patients showed anti-mycobacterial 65 kDa hsp antibody levels higher than the mean antibody level+2SD of the healthy control group, only 3/18 SLE, 2/15 AS and 2/13 Crohn's patients did so.

However, the increase in IgG antibody levels in RA sera was seen only with the mycobacterial 65 kDa hsp homologue (i.e. not with the *E. coli* 65 kDa hsp homologue). The IgG antibody levels to the *E. coli* 65 kDa hsp of the RA, SLE, AS and CD groups were similar to those of the healthy control group (Figures 4.4a, 4.4b).

Thus, both IgA and IgG responses in RA appeared to be increased to determinants that were specific to mycobacterial 65 kDa hsp and not to the homologous determinants shared with the *E. coli* 65 kDa hsp.

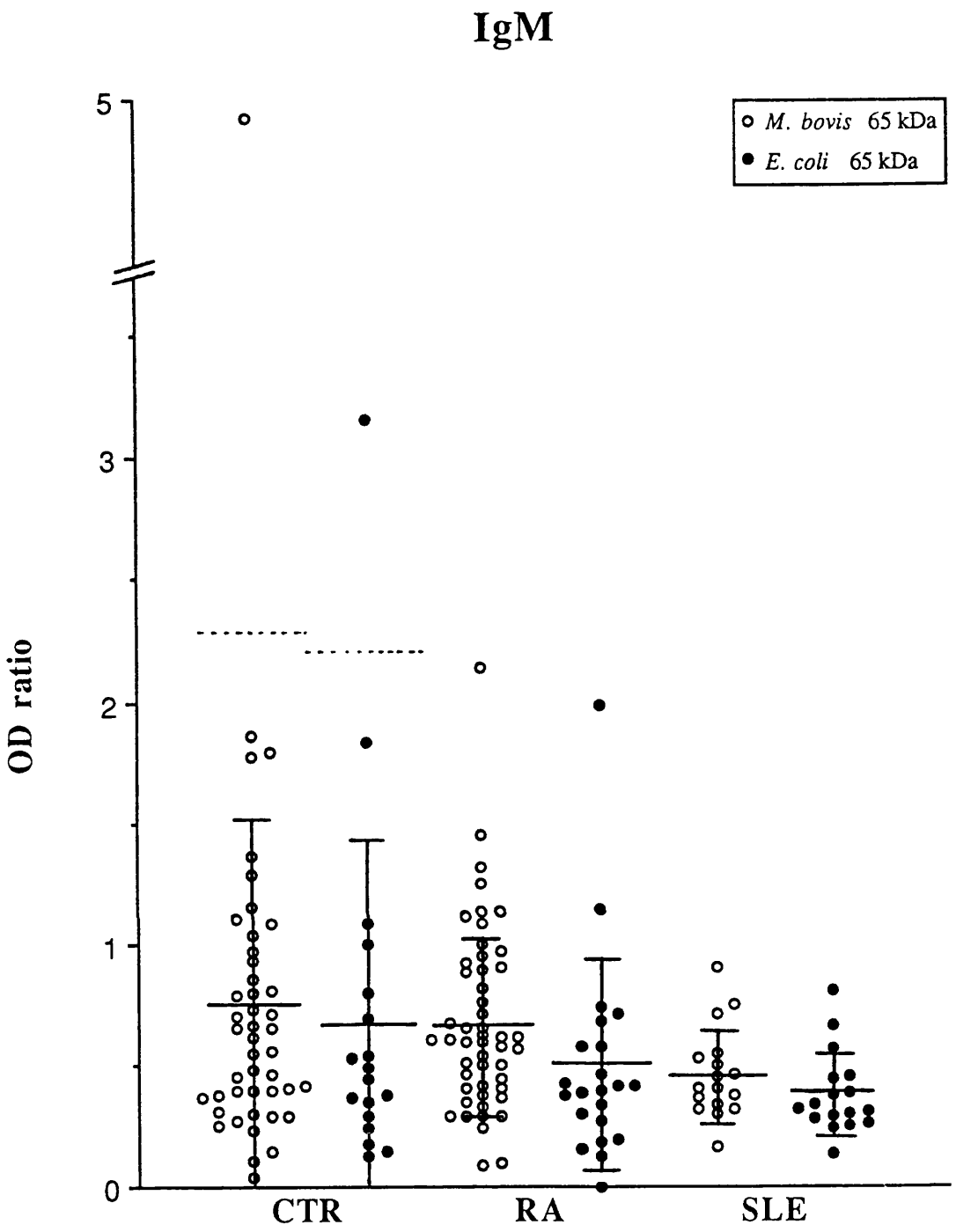


FIGURE 4.2a: IgM SERUM ANTIBODY LEVELS TO *M. BOVIS* AND *E. COLI* 65 kDa HSPS IN HEALTHY INDIVIDUALS (CTR), RHEUMATOID ARTHRITIS (RA) AND SYSTEMIC LUPUS ERYTHEMATOSUS (SLE) PATIENTS. The antibody levels are expressed as optical density ratios for each group (OD_{650} ratio = OD_{650} of test serum /OD_{650} of standard healthy serum used as a positive control). The horizontal lines represent the mean OD ratio for each group and the vertical lines represent the standard deviation (SD) for each group. The dotted lines define 2SD above the mean OD ratio of the healthy control group.

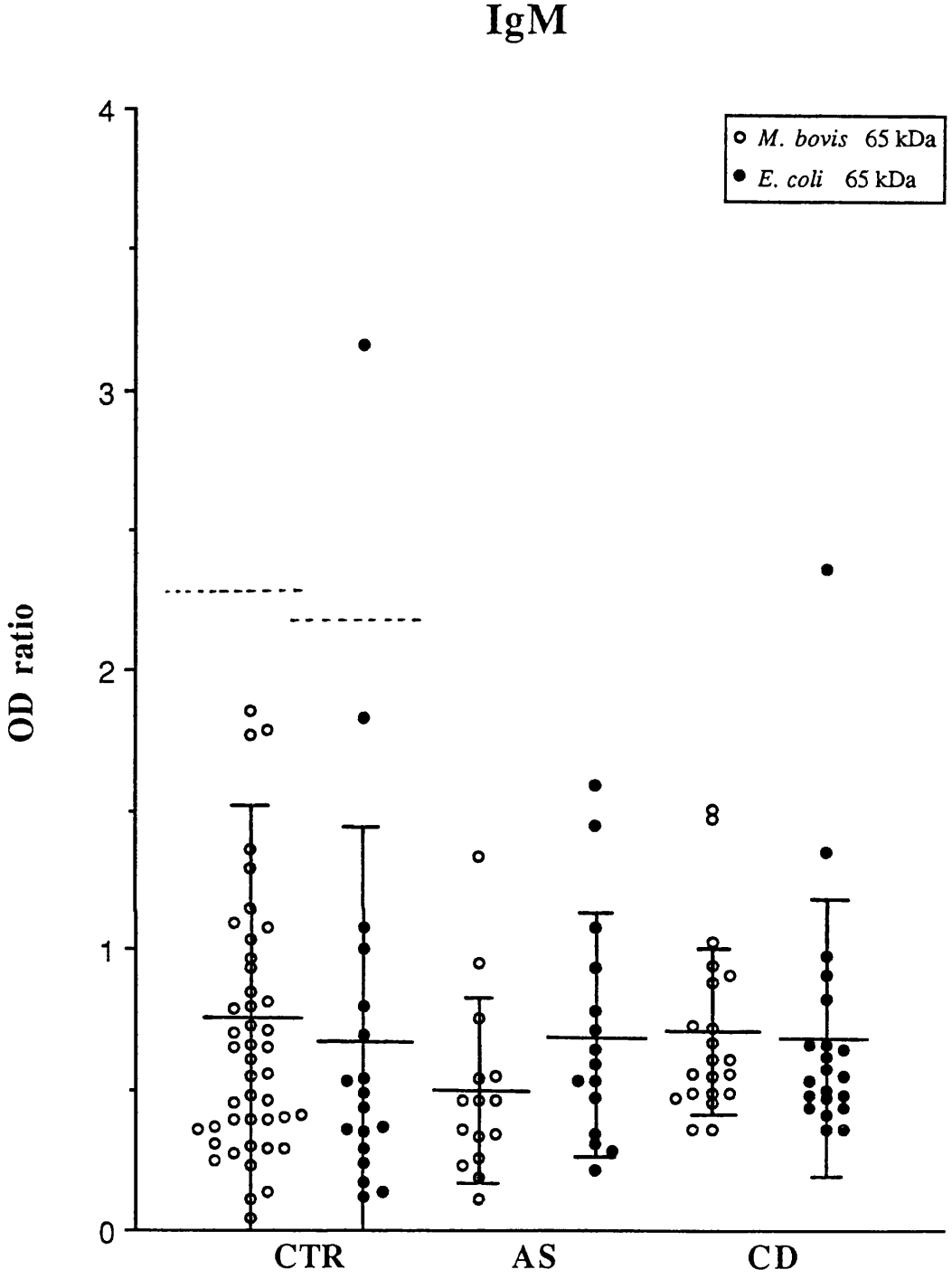


FIGURE 4.2b: IgM SERUM ANTIBODY LEVELS TO *M. BOVIS* AND *E. COLI* 65 kDa HSPS IN HEALTHY INDIVIDUALS (CTR), ANKYLOSING SPONDYLITIS (AS) AND CROHN'S DISEASE (CD) PATIENTS. The antibody levels are expressed as in figure 4.2a. The horizontal lines represent the mean OD ratio for each group and the vertical lines represent the standard deviation (SD) for each group. The dotted lines define 2SD above the mean OD ratio of the healthy control group.

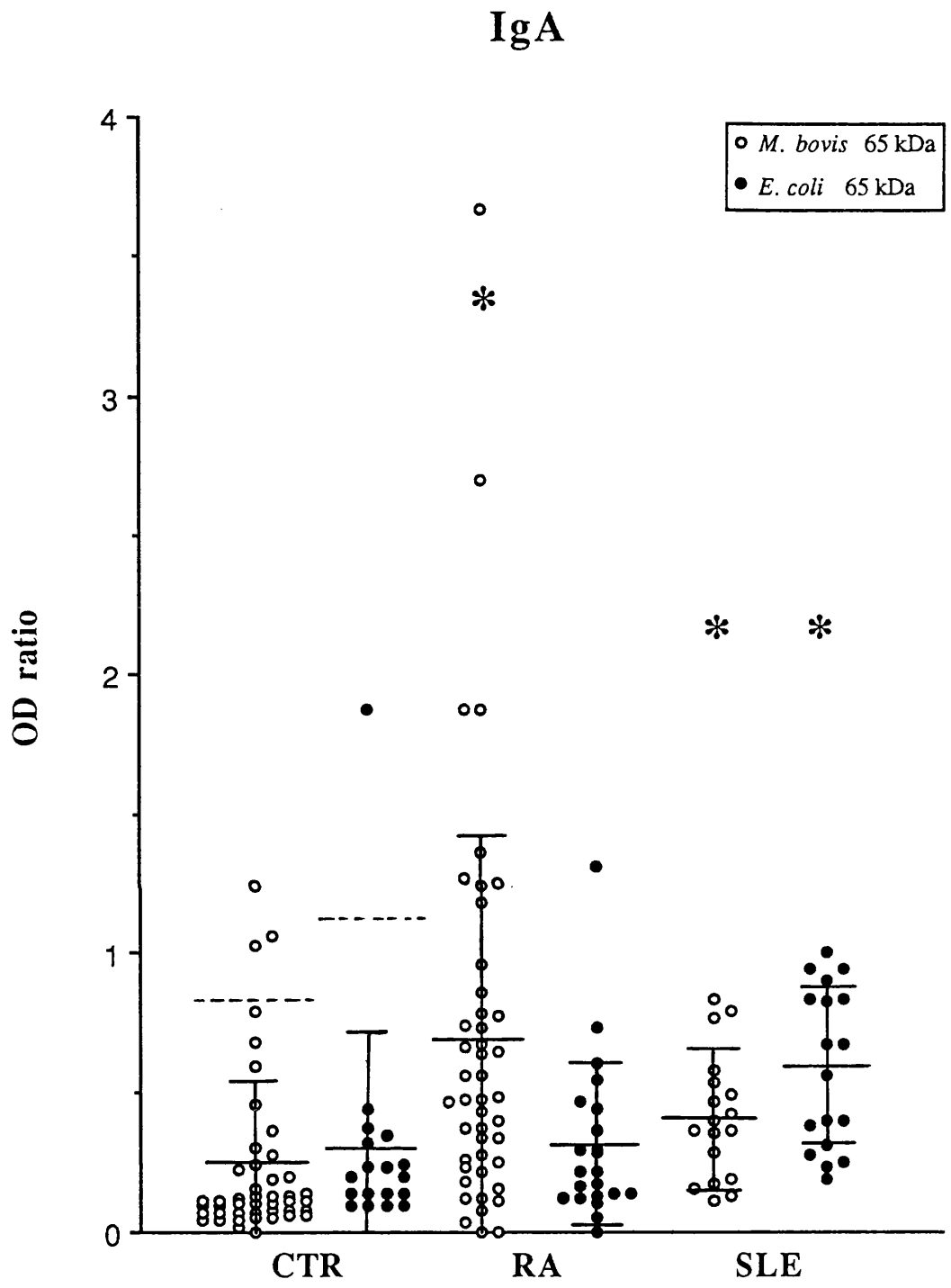


FIGURE 4.3a: IgA SERUM ANTIBODY LEVELS TO *M. BOVIS* AND *E. COLI* 65 kDa HSPS IN HEALTHY INDIVIDUALS (CTR), RHEUMATOID ARTHRITIS (RA) AND SYSTEMIC LUPUS ERYTHEMATOSUS (SLE) PATIENTS. The antibody levels are expressed as in figure 4.2a. The horizontal lines represent the mean OD ratio for each group and the vertical lines represent the standard deviation (SD) for each group. The dotted lines define 2SD above the mean OD ratio of the healthy control group. * $p<0.001$.

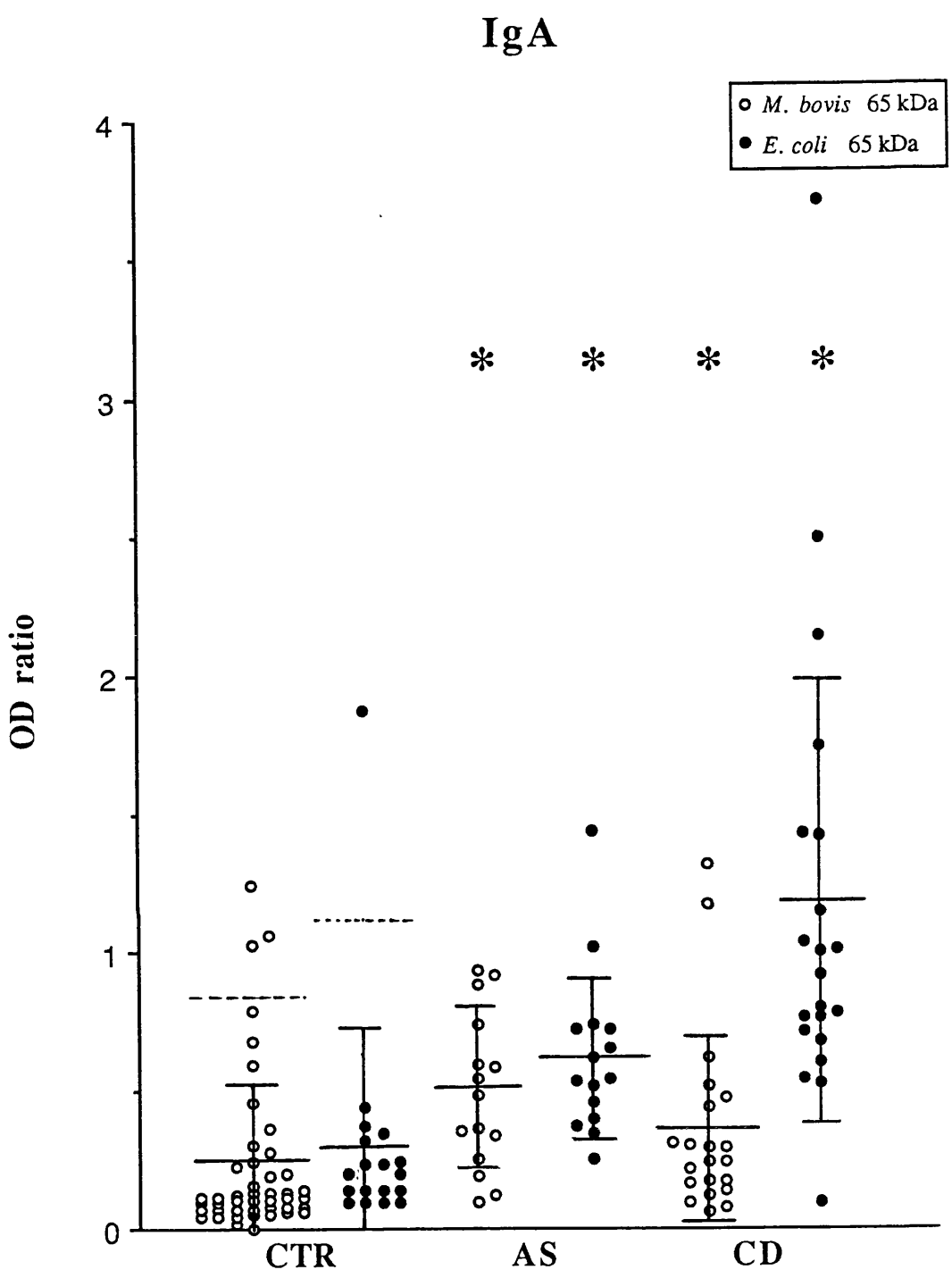


FIGURE 4.3b: IgA SERUM ANTIBODY LEVELS TO *M. BOVIS* AND *E. COLI* 65 kDa HSPS IN HEALTHY INDIVIDUALS (CTR), ANKYLOSING SPONDYLITIS (AS) AND CROHN'S DISEASE (CD) PATIENTS. The antibody levels are expressed as in figure 4.2a. The horizontal lines represent the mean OD ratio for each group and the vertical lines represent the standard deviation (SD) for each group. The dotted lines define 2SD above the mean OD ratio of the healthy control group. * $p<0.001$.

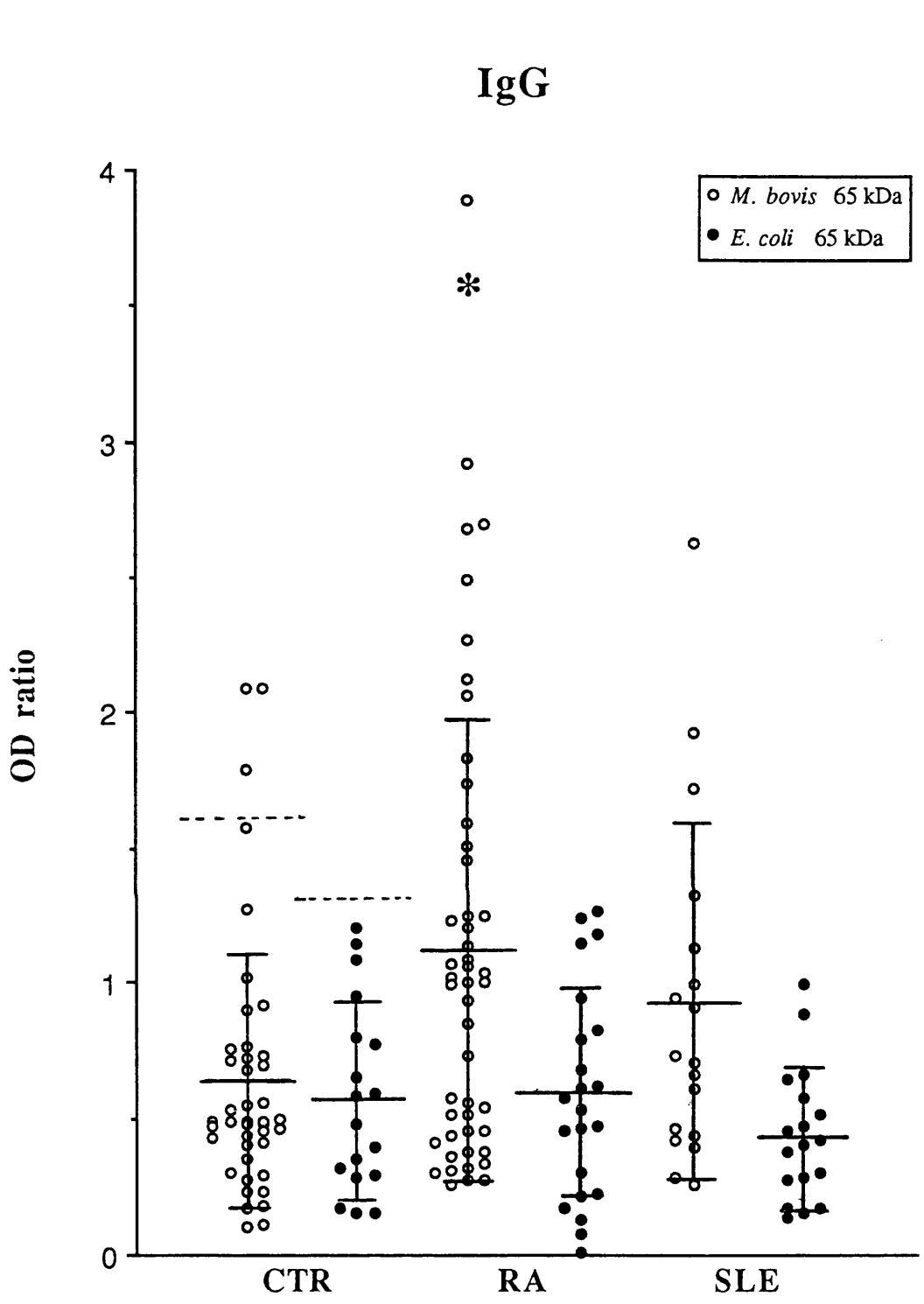


FIGURE 4.4a: IgG SERUM ANTIBODY LEVELS TO *M. BOVIS* AND *E. COLI* 65 kDa HSPS IN HEALTHY INDIVIDUALS (CTR), RHEUMATOID ARTHRITIS (RA) AND SYSTEMIC LUPUS ERYTHEMATOSUS (SLE) PATIENTS. The antibody levels are expressed as in figure 4.2a. The horizontal lines represent the mean OD ratio for each group and the vertical lines represent the standard deviation (SD) for each group. The dotted lines define 2SD above the mean OD ratio of the healthy control group. * $p < 0.01$.

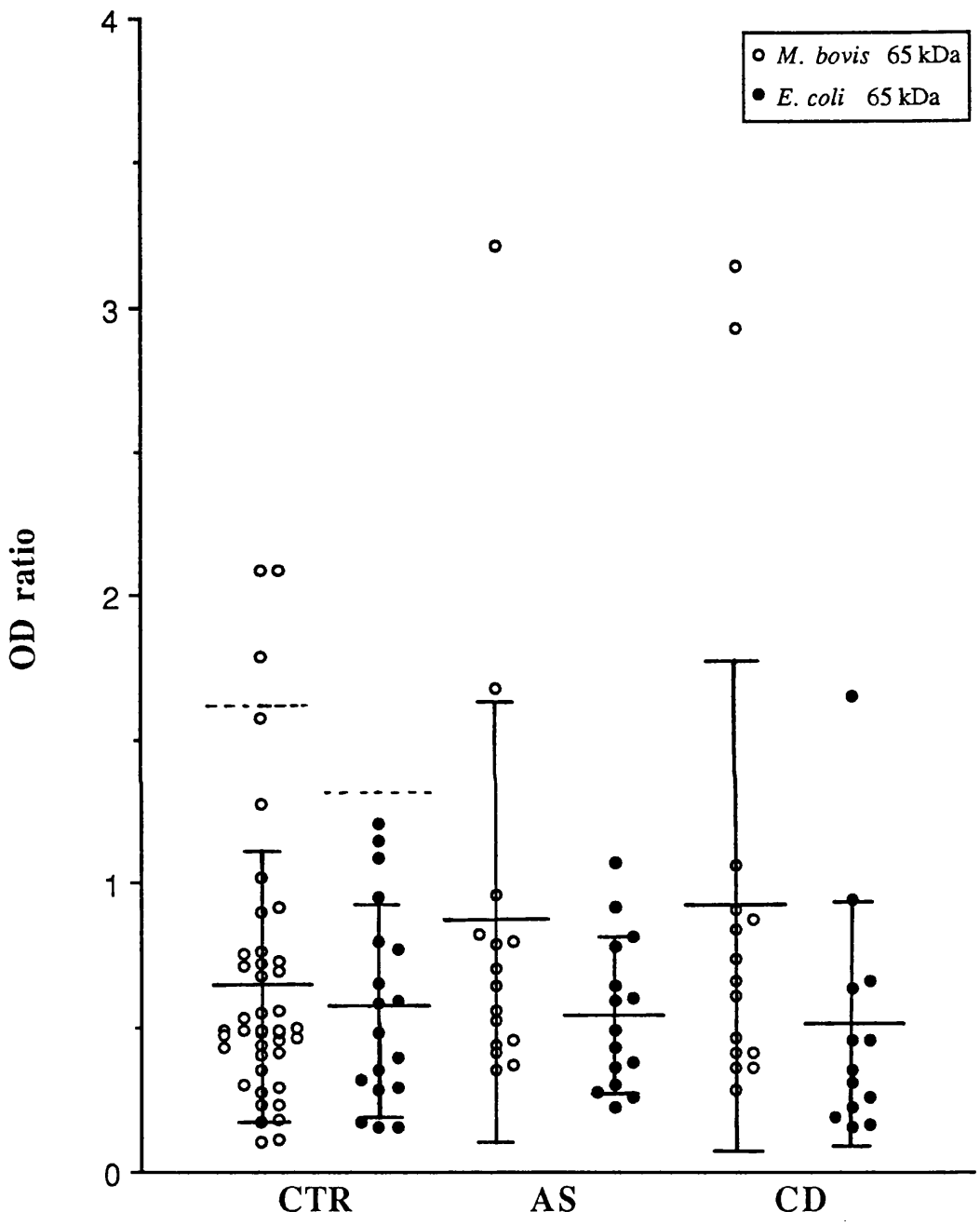


FIGURE 4.4b: IgG SERUM ANTIBODY LEVELS TO *M. BOVIS* AND *E. COLI* 65 kDa HSPS IN HEALTHY INDIVIDUALS (CTR), ANKYLOSING SPONDYLITIS (AS) AND CROHN'S DISEASE (CD) PATIENTS. The antibody levels are expressed as in figure 4.2a. The horizontal lines represent the mean OD ratio for each group and the vertical lines represent the standard deviation (SD) for each group. The dotted lines define 2SD above the mean OD ratio of the healthy control group.

c. Antibody levels to the *M. tuberculosis* and *E. coli* 70 kDa hsps in RA sera

The groups studied for IgG, IgA and IgM antibody levels to the *M. tuberculosis* and *E. coli* 70 kDa hsps were the same as for the 65 kDa hsps. Their characteristics are shown in tables 4.1a, 4.1b and 4.1c.

i. IgM antibody levels

The IgM antibody levels to both mycobacterial and *E. coli* 70 kDa hsps were lower than the healthy control levels in all groups except for the CD group (Figures 4.5a, 4.5b). The decrease in the antibody levels was significant in the RA group for the *E. coli* 70 kDa hsp antibodies ($p<0.05$), in the SLE group for the mycobacterial 70 kDa hsp antibodies ($p<0.05$) and in the AS group for antibodies to both antigens ($p<0.01$). Although the CD group had elevated IgM antibody levels, the increase was not significant ($p>0.05$).

ii. IgA antibody levels

It was found that the IgA antibody levels to the *M. tuberculosis* and *E. coli* 70 kDa hsps were significantly and similarly raised in RA ($p<0.05$) and SLE ($p<0.01$) patients compared to healthy individuals (Figure 4.6a). However, only the IgA antibody levels to the *E. coli* 70 kDa hsp were significantly raised in sera from AS patients ($p<0.001$) (Figure 4.6b). Antibodies to these antigens were not significantly raised in Crohn's patients ($p>0.05$) (Figure 4.6b).

iii. IgG antibody levels

The IgG antibody levels to both of these antigens were also significantly and similarly raised in RA ($p<0.01$) and SLE ($p<0.001$) relative to the healthy controls (Figure 4.7a). Thus, in contrast to the results with the 65 kDa hsps, the IgG antibodies in RA sera did not show greater binding to the mycobacterial than to the *E. coli* gene product.

Moreover, the extent of the increased binding, both in terms of mean antibody level value and proportion of donors with levels higher than the healthy control mean+2SD, was as great in SLE (10/18) as in RA (9/21), whereas using the mycobacterial 65 kDa hsp, the increase was significantly greater in RA.

The IgG antibody levels to both antigens were elevated in the AS and CD groups, although not significantly ($p>0.05$) (Figure 4.7b).

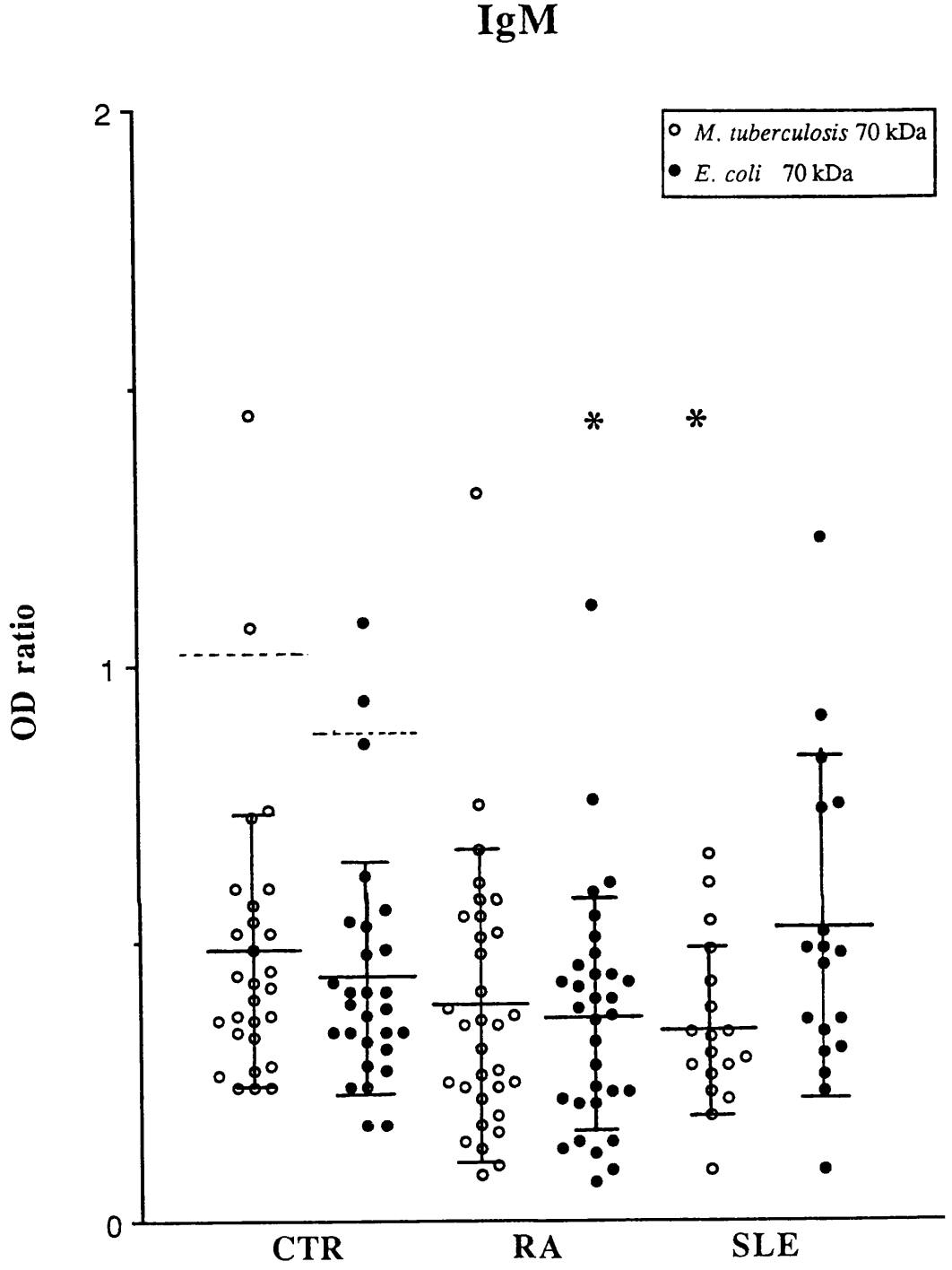


FIGURE 4.5a: IgM SERUM ANTIBODY LEVELS TO *M. TUBERCULOSIS* AND *E. COLI* 70 kDa HSPS IN HEALTHY INDIVIDUALS (CTR), RHEUMATOID ARTHRITIS (RA) AND SYSTEMIC LUPUS ERYTHEMATOSUS (SLE) PATIENTS. The antibody levels are expressed as in figure 4.2a. The horizontal lines represent the mean OD ratio for each group and the vertical lines represent the standard deviation (SD) for each group. The dotted lines define 2SD above the mean OD of the healthy control group.
* $p<0.01$.

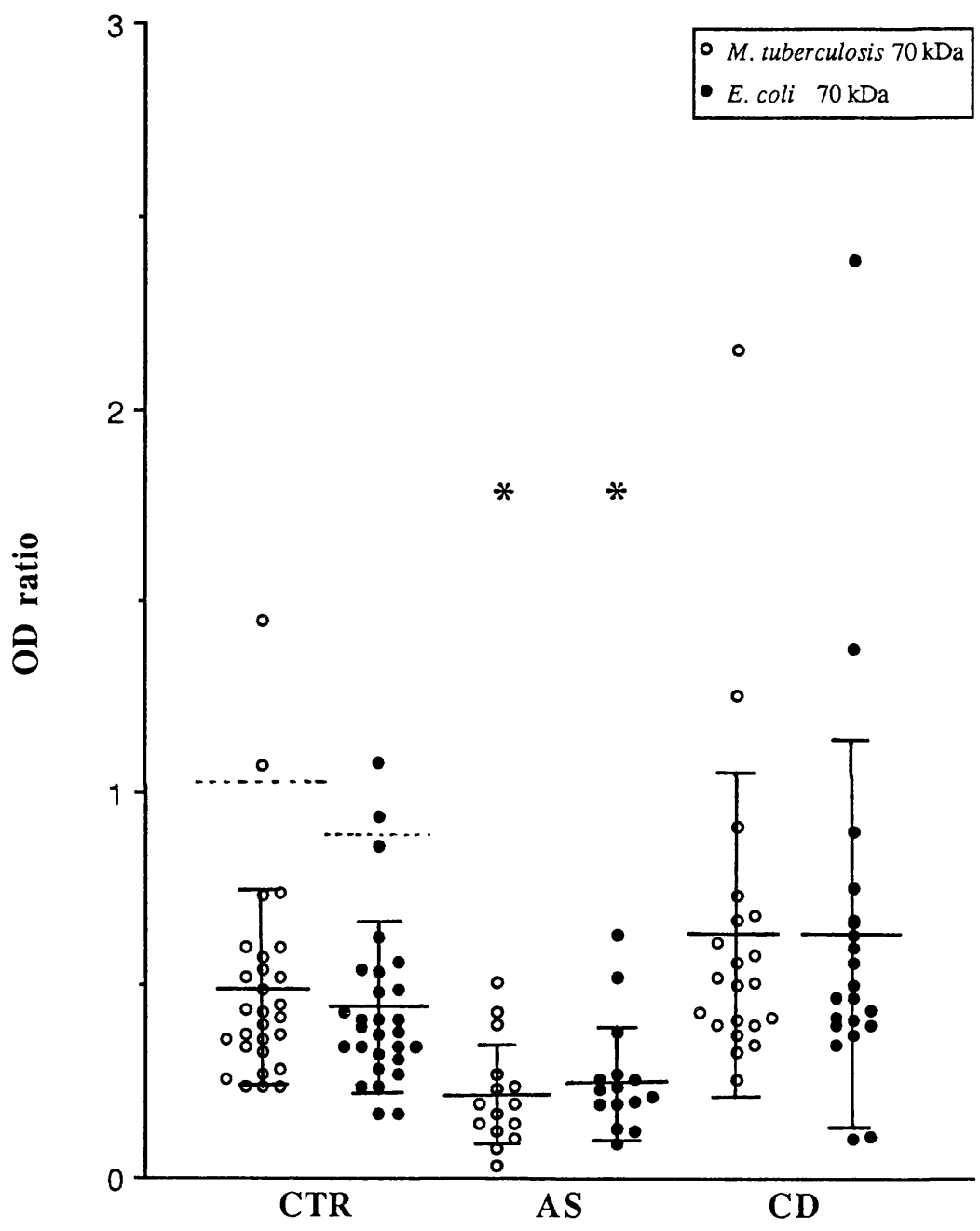


FIGURE 4.5b: IgM SERUM ANTIBODY LEVELS TO *M. TUBERCULOSIS* AND *E. COLI* 70 kDa HSPS IN HEALTHY INDIVIDUALS (CTR), ANKYLOSING SPONDYLITIS (AS) AND CROHN'S DISEASE (CD) PATIENTS. The antibody levels are expressed as in figure 4.2a. The horizontal lines represent the mean OD ratio for each group and the vertical lines represent the standard deviation (SD) for each group. The dotted lines define 2SD above the mean OD of the healthy control group. * $p<0.01$.

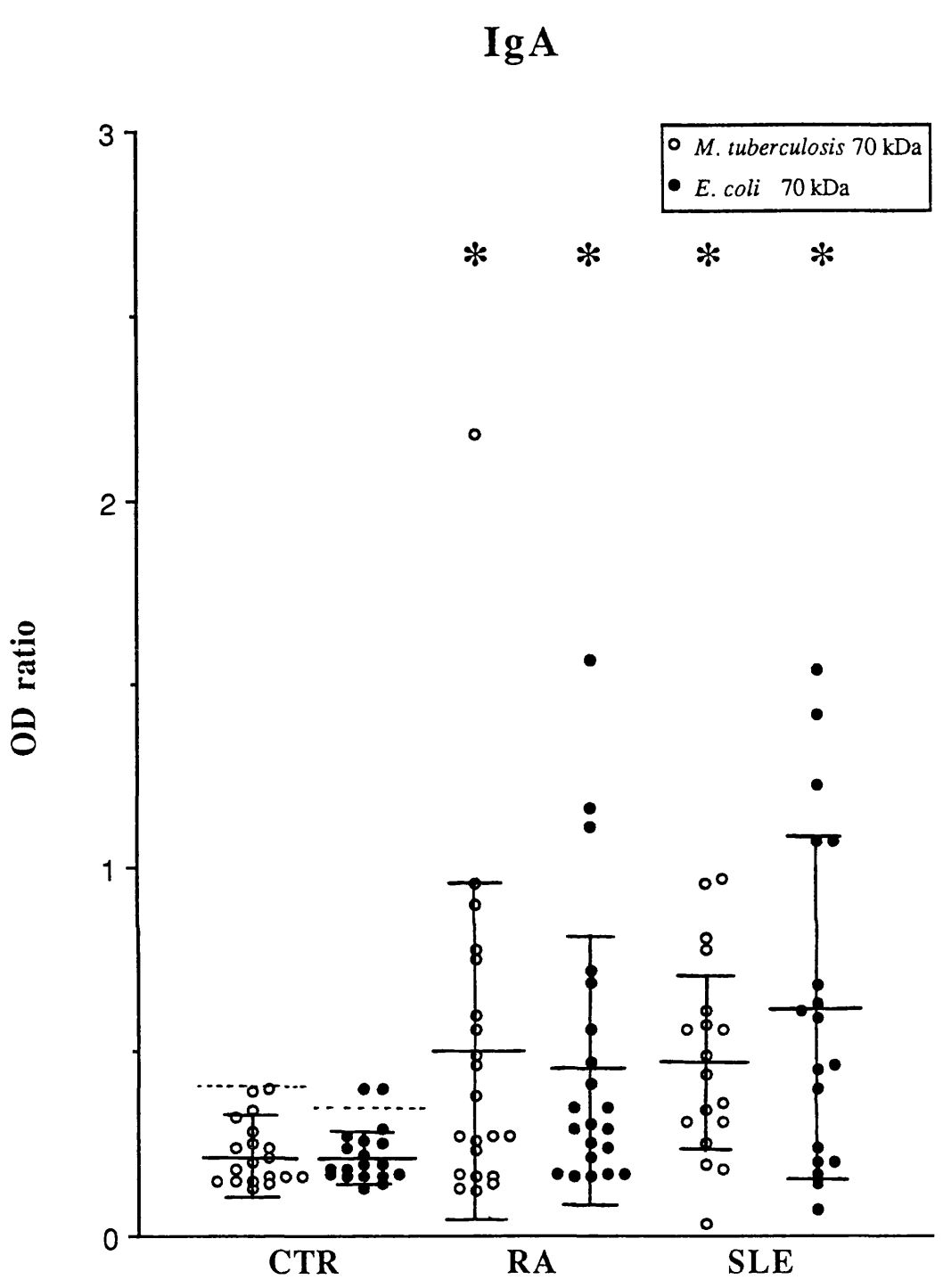


FIGURE 4.6a: IgA SERUM ANTIBODY LEVELS TO *M. TUBERCULOSIS* AND *E. COLI* 70 kDa HSPS IN HEALTHY INDIVIDUALS (CTR), RHEUMATOID ARTHRITIS (RA) AND SYSTEMIC LUPUS ERYTHEMATOSUS (SLE) PATIENTS. The antibody levels are expressed as in figure 4.2a. The horizontal lines represent the mean OD ratio for each group and the vertical lines represent the standard deviation (SD) for each group. The dotted lines define 2SD above the mean OD of the healthy control group. * $p<0.01$.

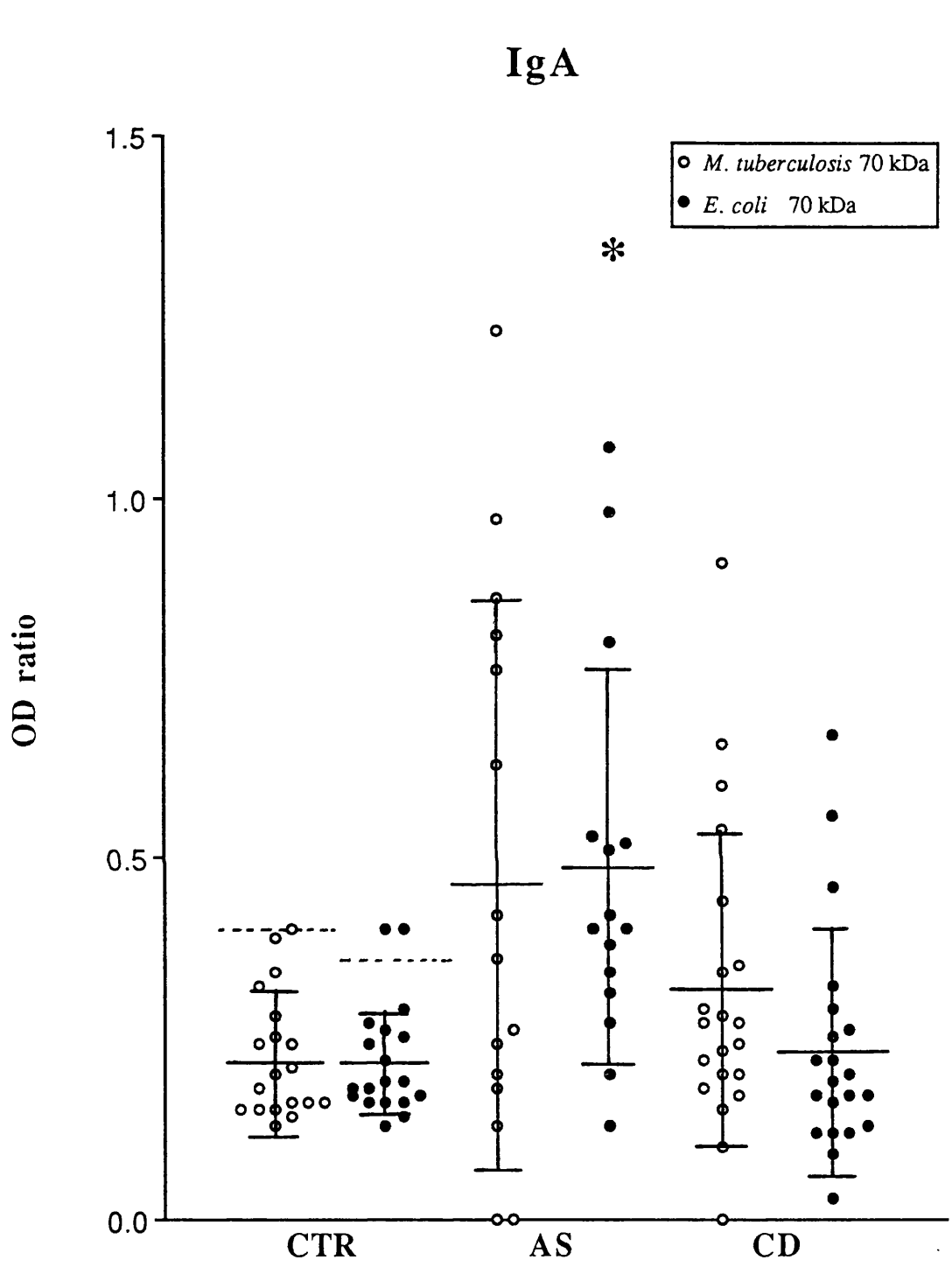


FIGURE 4.6b: IgA SERUM ANTIBODY LEVELS TO *M. TUBERCULOSIS* AND *E. COLI* 70 kDa HSPS IN HEALTHY INDIVIDUALS (CTR), ANKYLOSING SPONDYLITIS (AS) AND CROHN'S DISEASE (CD) PATIENTS. The antibody levels are expressed as in figure 4.2a. The horizontal lines represent the mean OD ratio for each group and the vertical lines represent the standard deviation (SD) for each group. The dotted lines define 2SD above the mean OD of the healthy control group. * $p<0.01$.

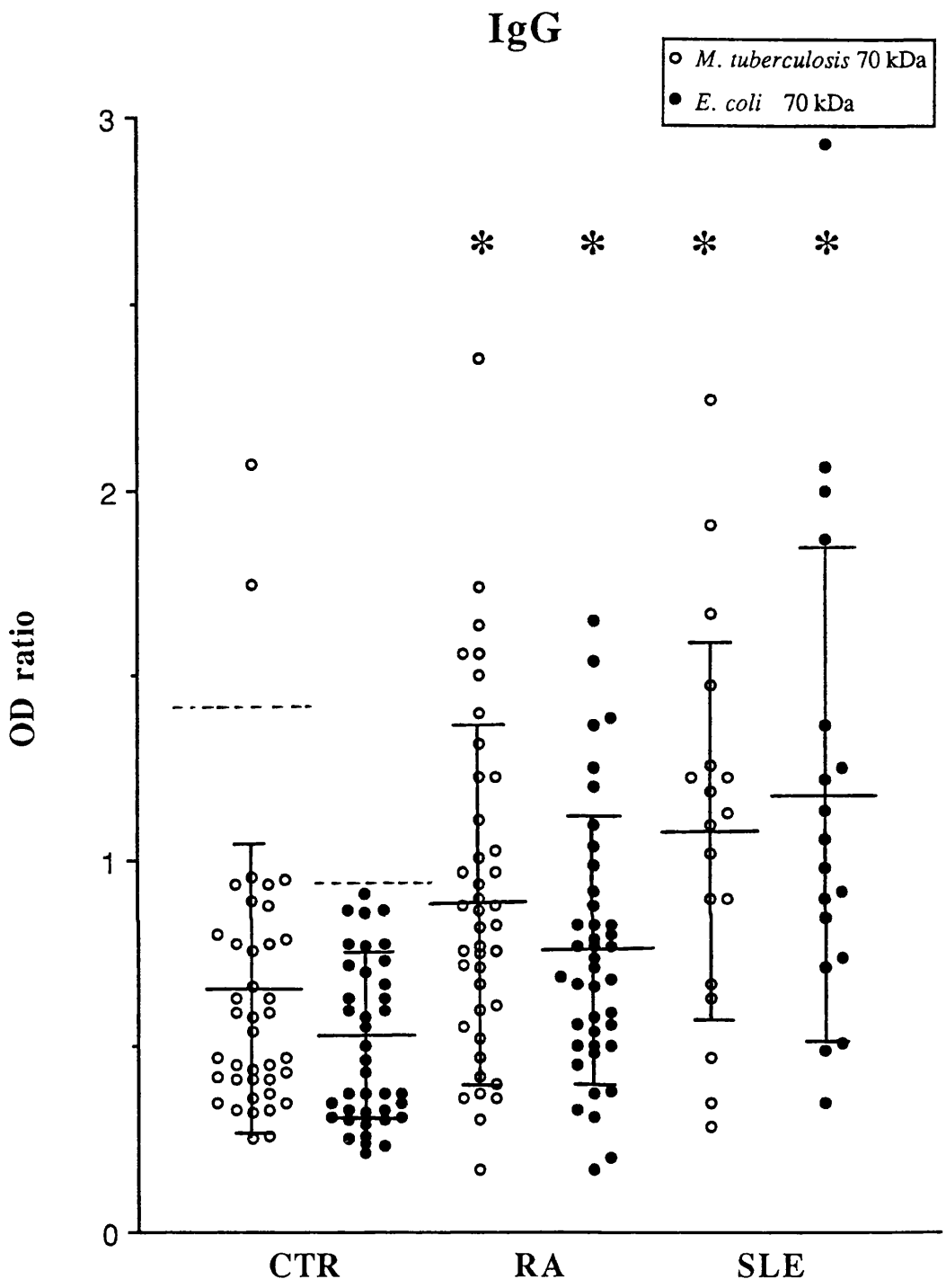


FIGURE 4.7a: IgG SERUM ANTIBODY LEVELS TO *M. TUBERCULOSIS* AND *E. COLI* 70 kDa HSPS IN HEALTHY INDIVIDUALS (CTR), RHEUMATOID ARTHRITIS (RA) AND SYSTEMIC LUPUS ERYTHEMATOSUS (SLE) PATIENTS. The antibody levels are expressed as in figure 4.2a. The horizontal lines represent the mean OD ratio for each group and the vertical lines represent the standard deviation (SD) for each group. The dotted lines define 2SD above the mean OD of the healthy control group. * $p<0.01$.

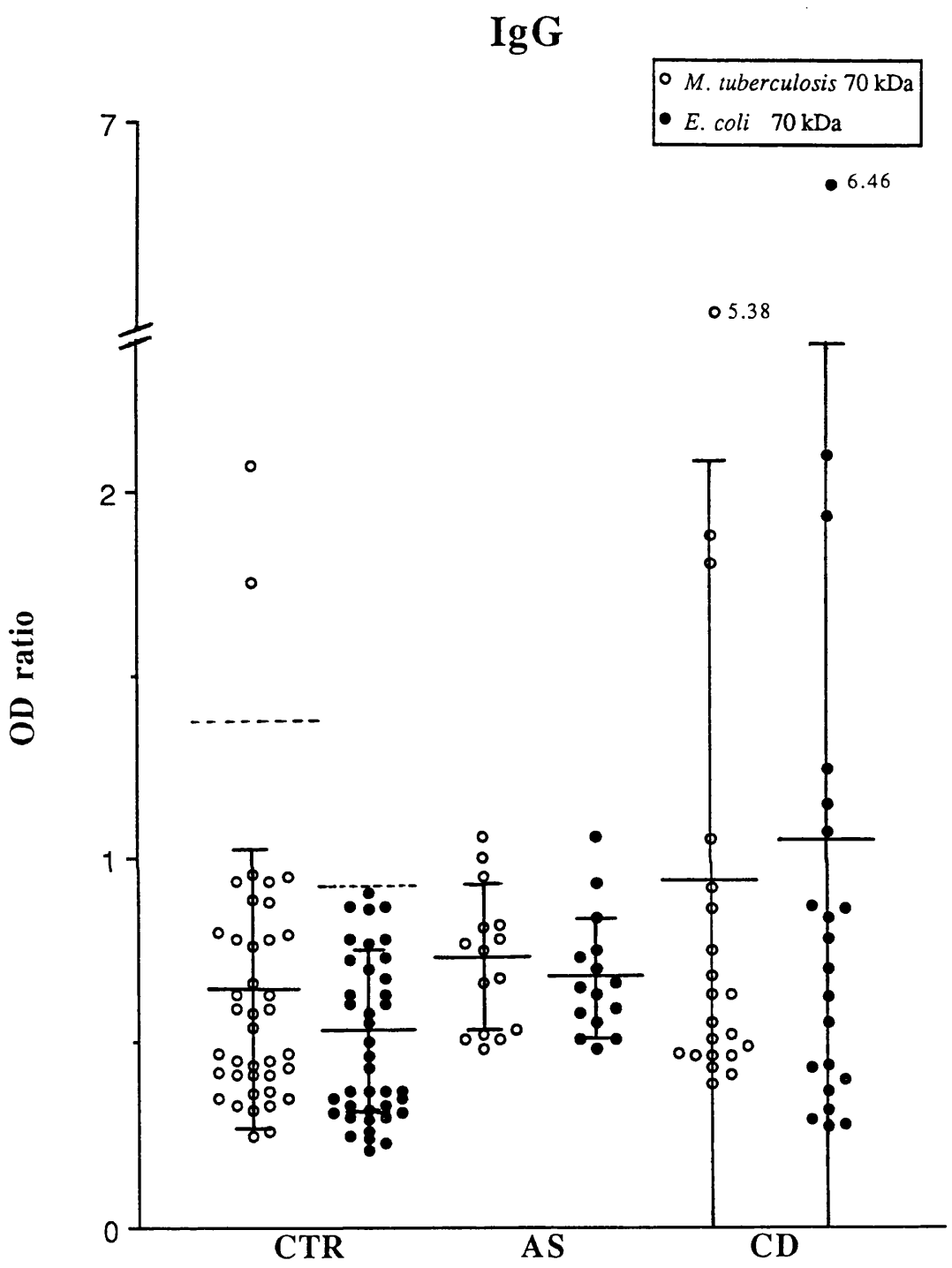


FIGURE 4.7b: IgG SERUM ANTIBODY LEVELS TO *M. TUBERCULOSIS* AND *E. COLI* 70 kDa HSPS IN HEALTHY INDIVIDUALS (CTR), ANKYLOSING SPONDYLITIS (AS) AND CROHN'S DISEASE (CD) PATIENTS. The antibody levels are expressed as in figure 4.2a. The horizontal lines represent the mean OD ratio for each group and the vertical lines represent the standard deviation (SD) for each group. The dotted lines define 2SD above the mean OD of the healthy control group.

d. Antibody levels to the hsps in relation to age, sex, activity, duration and treatment of disease, total serum immunoglobulin levels, seropositivity, rheumatoid factor and HLA-DR haplotype

i. Antibody levels with respect to age

Due to the difference of the average age of the donors in the groups studied (Tables 4.1a, 4.1b and 4.1c), the relationship between age and antibody levels to the hsps was studied. No evidence was found to support a link between antibody levels and age ($r<0.30$, $p>0.1$) in the CTR, RA, SLE and AS groups. However, a correlation was found between IgA antibody levels to the mycobacterial 65 kDa hsp and age in patients with Crohn's disease ($r>0.90$, $p<0.01$).

ii. Antibody levels with respect to sex

Since the distribution of male and female individuals was not uniform in the groups studied, antibody levels of male and female donors were compared to test for any sex linked differences. No statistically significant difference was found between antibody levels of male and female individuals ($p>0.05$).

iii. Antibody levels with respect to disease activity

If antibody levels to hsps were relevant to the disease, they might be found at different levels in relation to the activity of the disease. Antibody levels of donors with active disease were compared to those with inactive disease and no difference was found ($p>0.05$).

iv. Antibody levels with respect to duration of disease

The duration of the disease which ranged from 2-20 years was also not found to be associated with differences in the antibody levels ($r<0.1$, $p>0.1$).

v. Antibody levels with respect to treatment of disease

Drug treatment has been known to alter immune responses (Salama and Mueller-Eckhardt, 1987) and the relationship between treatment and antibody levels was therefore examined. No difference was found between antibody levels of patients on drug treatment and patients receiving no treatment ($p>0.05$). Wherever possible, steroidal and non steroidal treatments were compared in relation to their effect on antibody levels, which also proved to be negative ($p>0.05$).

vi. Antibody levels with respect to total serum immunoglobulin levels

It was also determined whether or not the elevated antibody levels were merely due to the presence of elevated total serum immunoglobulin levels. However, no such correlation was found between these two parameters ($r<0.5$, $p>0.1$).

vii. Antibody levels with respect to rheumatoid factor

Furthermore, since rheumatoid factor (RF) might be present in active RA patients, it was essential for the rise in mycobacterial antibody levels to be distinguished from RF

activity. Comparison of IgG and IgA antibody levels with RF activity did not show any correlation ($p>0.05$).

viii. Antibody levels and HLA-DR haplotypes

Since responses to mycobacteria have been reported to be HLA-DR restricted (Palacios-Boix *et al.*, 1988; Bahr *et al.*, 1989), the antibody levels of RA sera to the antigens tested were examined for associations with HLA-DR haplotypes. Table 4.2 shows the frequencies of the HLA-DR haplotypes in the RA and SLE groups. The AS, CD and CTR groups were not tissue typed.

Comparisons of antibody levels to the mycobacterial 65 kDa hsp of RA patients with and without HLA-DR1, 2, 3, 4, 6 and HLA-DR7 haplotypes were considered, since the frequency of the other haplotypes was too low for meaningful statistical analysis. No correlation was found between antibody levels and the HLA-DR4 haplotype, believed to be associated with increased susceptibility to RA (Stastny *et al.*, 1988).

Similarly, no association was found between antibody levels and the rest of the haplotypes tested, apart from a correlation between HLA-DR6 positive individuals who were found to have higher IgG antibody levels to the mycobacterial 65 kDa hsp compared to the non-DR6 ($p<0.05$). However, this p value did not remain significant when corrected for the number of comparisons made.

The lack of correlation between antibody levels and HLA-DR haplotypes was also true for the SLE group.

TABLE 4.2: Frequency of HLA-DR haplotypes in the rheumatoid arthritis and systemic lupus erythematosus (SLE) patient groups.

	Rheumatoid Arthritis				SLE
	M. bovis 65 kDa	*E. coli* 65 kDa	*M.tuberc. & E.coli* 70 kDa		All hsps
Ig Class	A,M,G	A,M,G	A, M	G	A,M,G
HLA-DR1	6	3	6	6	2
HLA-DR2	12	3	4	7	6
HLA-DR3	11	2	5	9	9
HLA-DR4	27	13	8	16	4
HLA-DR5	4	3	0	3	4
HLA-DR6	10	4	6	4	0
HLA-DR7	6	1	1	2	3
HLA-DR8	2	2	1	0	1
HLA-DR9	0	0	0	0	0
HLA-DR10	1	0	0	0	0
HLA-DR11	0	0	1	1	0
HLA-DR13	1	0	0	0	0
Number of donors	48	22	21	41	18

4.4 DISCUSSION

It was interesting to find that the immunodominant 65 and 70 kDa mycobacterial antigens were present in the crude WE, TB and VAC antigen preparations. This implied that at least some of the anti-mycobacterial antibodies found against these preparations in RA patients and healthy individuals (Chapter 3) could be directed against the 65 and/or 70 kDa proteins.

It has been previously shown (Chapter 3) that there were strong correlations between antibody levels to the three crude mycobacterial preparations, suggesting that the antibodies were directed against common or cross-reactive epitopes. The crude antigen preparations, however, contained a plethora of antigens/epitopes (Figure 4.1) and thus other constituents might also be responsible for the strong correlations.

The use of defined mycobacterial antigens to study antibody responses provided more information about the specificity of the antibodies to mycobacteria.

In fact, the IgA and IgG antibody levels to the mycobacterial 65 and 70 kDa hsp antigens were found to be significantly raised in RA patients. The concomitant elevation of the IgA antibody levels to the crude mycobacterial preparations (Chapter 3) would argue in favour of common or cross-reactive epitopes. Assuming that was true, the absence of a parallel elevation of the IgG antibodies to the defined and crude antigens could not be explained. Since the optimal concentration of both defined and crude antigens were used for the assays, differences in the sensitivity of the assays could not be the answer either. However, since the crude antigens consisted of many different epitopes, the relevant ones might not be easily accessible in

these preparations or might be masked by the effect of others.

In general, the study of antibody responses to the defined mycobacterial antigens has shown that patients with RA, SLE, AS, and Crohn's disease could all have increased serum IgA and/or IgG (but not IgM) antibody levels to the mycobacterial and/or *E. coli* 65 and/or 70 kDa hsp. The significance of the presence of antibodies against hsps was not clear yet. Of particular interest was the preferential reactivity to the mycobacterial 65 kDa hsp in RA, because of the implication of this protein in the aetiology of adjuvant arthritis (van Eden *et al.*, 1988).

In fact, antibodies of the IgG class to the mycobacterial 65 kDa hsp appeared to discriminate between RA and the other diseases, as RA was the only group to show increased IgG antibody levels to this protein. This argued in favour of the mycobacterial 65 kDa protein being important in RA. Further support for this concept came from the fact that the IgG antibodies to the *E. coli* 65 kDa hsp protein were not raised in RA.

The preferential immune reactivity to the mycobacterial 65 kDa hsp in RA, was also seen with the elevated IgA antibody levels to this protein but not to its *E. coli* homologue. The IgA antibody levels to the mycobacterial 65 kDa were also raised in SLE, AS and Crohn's patients, suggesting that the elevation of IgA antibodies per se was not disease specific. However, these antibody levels were not only lower than the levels of RA patients, but they were also similar to the *E. coli* 65 kDa hsp antibody levels.

Bahr *et al.* (1988a) have confirmed the observation of high IgG antibody levels to the mycobacterial 65 kDa hsp in Kuwaiti RA patients. In their study, the raised antibody levels to this protein

were even higher than those found in tuberculosis patients, where patients were infected with mycobacteria. The same study, however, did not confirm the significant elevation of IgA antibody levels to the mycobacterial 65 kDa hsp in RA patients (although there was a tendency to raised IgA antibody levels).

The increase of IgG and IgA antibody levels to the mycobacterial 65 kDa hsp but not to the *E. coli* homologue in RA was very interesting, in view of the fact that these heat shock proteins showed a 60% homology in their sequence (Young *et al.*, 1987; Shinnick *et al.*, 1988). Thus, the preferential reactivity to the mycobacterial 65 kDa hsp implied that the antibodies were probably directed against non-cross-reactive epitopes.

Therefore, specific sequences on the 65 kDa hsp of *M. bovis* that were absent from the *E. coli* sequence seemed to be important as targets of immune reactivity in RA. This was also supported by the finding of 65 kDa specific T cell clones isolated from the synovial fluid of a patient with acute arthritis which responded preferentially to an epitope of the 65 kDa hsp that was mycobacteria specific (Gaston *et al.*, 1990). One explanation for the immunodominance of the mycobacterial 65 kDa hsp, and not of the *E. coli* homologue, might be the varying exposure of an individual to a genus such as the mycobacteria against the virtually constant exposure to commensal genera such as *E. coli*.

The use of mycobacterial and *E. coli* 70 kDa hsp preparations, as another control for bacterial hsp gene products, has shown that RA and SLE patients had similarly elevated IgG and IgA antibody levels to both proteins. This argued against a disease specificity with regard to immune reactivity to the 70 kDa hsps and suggested that antibodies to these hsps were

directed against common or cross-reactive epitopes. The lack of disease specificity was supported by the finding of similarly elevated antibody levels to both proteins in RA and tuberculosis sera (Tsoulfa *et al.*, 1989b).

The raised antibody levels to the 65 kDa hsp protein did not correlate with the antibody levels to the 70 kDa hsp protein. In fact, analysis of 10 RA patients with IgG antibody levels to the 70 kDa protein higher than the mean control+2SD showed only 2 patients with parallel high antibody levels to the 65 kDa hsp. Furthermore, some sera with high IgA antibody levels to the 70 kDa had lower levels to the 65 kDa protein. These observations, together with the findings of lower (but not significant) IgM levels in RA patients to all mycobacterial antigens tested, argued against polyclonal B cell activation as a source of elevated antibodies.

The antibody responses seemed to be specific as they were not influenced by total serum immunoglobulin levels or rheumatoid factor levels of the test samples. Also, the age or sex of the individuals tested did not correlate with the antibody levels measured. In spite of the elevated hsp antibody levels in patients' sera, a relationship with disease could not be established since the antibody responses were not correlated in any way with the duration and activity of the disease or even with the treatment of the disease.

No association was found between antibody levels and HLA-DR haplotypes, including HLA-DR4 (a haplotype known to be associated with increased susceptibility to RA) and HLA-DR2 or HLA-DR7 (haplotypes believed to be associated with a significantly decreased risk of developing RA) (Bahr *et al.*, 1988b;

Stastny *et al.*, 1988). Comparable results, with regard to lack of meaningful HLA-DR correlation with antibody levels to the 65 kDa hsp, were also shown by Bahr *et al.* (1988a).

This appeared to be different from data on T cell responses to mycobacteria which have been correlated with HLA-DR4 expression in RA and healthy individuals (Palacios-Boix *et al.*, 1988). Also, with the increased skin-test response to tuberculin in HLA-DR4 positive RA patients (Bahr *et al.*, 1989).

Although the disease specificity for RA of the mycobacterial 65 kDa hsp antibodies was interesting, the lack of an association of the antibody levels to the 65 and 70 kDa hsps with any clinical or serological feature of the patient groups tested made the relevance of these antibodies to disease less obvious. The issue was complicated further by the fact that the 65 and 70 kDa antigens were heat shock proteins.

Because of the high sequence homology of hsps in both prokaryotic and eukaryotic organisms (Lindquist and Craig, 1988), it was not clear as to whether antibodies detected against the 65 and 70 kDa hsps were really against the mycobacterial or *E. coli* organisms and not against hsps from another microbe. Indeed, it was not known whether the response was directed against hsps of the host (self-hsps) or not. At this stage, therefore, the mycobacterial or *E. coli* hsps could not be reliably implicated as the major immunogens leading to the responses measured here and the involvement of other microbes could not be excluded. In fact, antibodies to *Proteus mirabilis* have been shown to be significantly higher in active RA patients compared to healthy controls (Ebringer *et al.*, 1989).

This molecular mimicry of the hsps might prove to be

responsible for the autoimmune character of the RA. The immune system might not differentiate between self and non-self hsps. Therefore, an immune response mounted by the host against an hsp determinant of an infecting agent might cross-react with the host's hsp sequence, leading to breakdown of self-tolerance and to autoimmune processes (Schwartz, 1989).

It is also possible for the host, to mount an exaggerated and potentially harmful response to cross-reactive hsp antigens from other essentially non-pathogenic bacteria. Chronic presentation of a cross-reactive immunogen might occasionally result in breakdown of tolerance and in the production of an autoimmune response (Cooke, 1988). There is now strong evidence that molecular mimicry causes autoimmune disease in humans (Oldstone, 1987).

If, however, the antibody response is directed against mimicked self and microbial hsps, the question is how a situation like this arises. An infection by a microbe can induce synthesis of hsps in both the host and the microbe itself. The hostile environment that usually the host offers to microbes (e.g. macrophages) is stressful enough for them to produce hsps (Christman *et al.*, 1985). On the other hand, infections have been identified as agents for inducing synthesis of hsps by the affected host (Polla, 1988).

It should be noted that cross-reactivity could occur not only between microbial-hsps and self-hsps, but also between microbial-hsps and self non-hsp antigens. In the adjuvant arthritis model it has been shown that such a cross-reactivity existed between mycobacterial 65 kDa hsp and joint cartilage proteoglycan (van Eden *et al.*, 1988). It would be interesting to

know if such a cross-reactivity existed in man and which was the autoantigen involved.

From the data so far, it was not possible to confirm any of the above possibilities as an explanation for the presence and significance of the antibodies to hsps, and for the involvement of the hsps themselves in aetiopathogenesis of RA. It was clearly important to obtain more information about these antibodies and, because of the autoimmune character of RA, it was also important to establish whether any of the antibodies measured in this study were in fact autoantibodies.

CHAPTER 5

SPECIFICITY OF THE MYCOBACTERIAL 65 kDa HSP SERUM ANTIBODY LEVELS IN RHEUMATOID ARTHRITIS PATIENTS

Introduction

Characteristics of study groups in chapter 5

Results

- Serum antibody levels to six synthetic peptides of *M. leprae* 65 kDa hsp in established RA patients
- Serum antibody levels of RA patients in 'early' and 'late' stages of the disease
- Serum antibody levels to the mycobacterial 65 kDa hsp of 12 established RA patients in 3 yearly time intervals

Discussion

5.1 INTRODUCTION

The study of the antibody levels to the mycobacterial and *Escherichia coli* 65 and 70 kDa heat shock proteins (hsps) has suggested a special link between rheumatoid arthritis (RA) and the mycobacterial 65 kDa hsp (Chapter 4).

However, it is not known to which epitope(s) this protein owes its immunodominance in the RA patients. It would be interesting to define the relevant epitope(s) and to examine whether the immunodominant epitope in the adjuvant arthritis model (i.e. amino acids 180-188 of the mycobacterial 65 kDa hsp) is of any interest to the humoral reactivity in RA.

In an attempt to characterise better the mycobacterial 65 kDa hsp antibodies and their importance in the aetio-pathogenesis of RA, the following studies were carried out.

1. Using a small panel of synthetic peptides covering sections of the *Mycobacterium leprae* 65 kDa hsp sequence, including the relevant epitope in AA, it was examined whether there were differences in antibody responses to these peptides in healthy individuals (CTR) and RA patients.

Systemic lupus erythematosus (SLE) and Crohn's disease (CD) patients were also tested as disease control groups.

2. If antibodies to the 65 kDa hsp were important to the initiation of the disease, they might be expected to be present early in the disease. Thus, antibody levels to the mycobacterial 65 kDa hsp were studied in RA patients at the time of diagnosis of the disease and within the following 5 years after the diagnosis.

Candida albicans and a mixture of three influenza strains were used as control antigens, and humoral responses to these antigens were also studied.

5.2 CHARACTERISTICS OF STUDY GROUPS IN CHAPTER 5

TABLE 5.1: Characteristics of 'early' and 'late' rheumatoid arthritis patients used to study antibody levels to *M. bovis* 65 kDa hsp, *C. albicans* and 'flu' antigens.

	' EARLY '	' LATE '
Ig Class	IgA, IgM, IgG	
Number of donors	20	20
Male	6	6
Female	14	14
Average Age (Years)	50	53
(range)	27 - 71	29 - 73
Duration of disease	mean 5 months, range 1 - 7	mean 3.5 years, range 2 - 5
Activity of disease (1)		
Active	12	7
Inactive	8	13
Treatment (2)		
First-line drugs	15	18
Second-line drugs	-	2
Third-line drugs	-	-
No treatment	5	-
Seropositivity (3)		
Seropositive	12	13
Seronegative	7	5
Not known	1	2

Footnotes: (1) Activity was assessed as shown in section 2.5.

(2) First-line drugs: non-steroidal anti-inflammatory drugs (e.g. indomethacin, naproxen)
Second-line drugs: disease-modifying drugs (e.g. gold, penicillamine, sulphasalazine)
Third-line drugs: prednisolone, azathioprine, methotrexate.

(3) Seropositivity was assessed by latex agglutination test.

5.3 RESULTS

a. Serum antibody levels to six synthetic peptides of *M. leprae* 65 kDa hsp in established RA patients

To determine whether or not the fine specificities of the mycobacterial 65 kDa hsp antibodies in RA patients were different from those of the healthy individuals, systemic lupus erythematosus and Crohn's disease patients, antibodies to six synthetic peptides of *M. leprae* 65 kDa hsp (Table 5.2; Young *et al.*, 1988b; Jindal *et al.*, 1989; Lamb *et al.*, 1990) were studied in sera from the above groups. The characteristics of the groups studied are shown in tables 4.1a, 4.1b and 4.1c.

Antibodies to the intact molecule of the recombinant *M. bovis* 65 kDa hsp were studied at the same time and showed the same trend as in previous assays (Chapter 4). Hence, the IgG and IgA (but not IgM) antibody levels were elevated in the RA group compared to the healthy control group ($p<0.05$ for IgG and IgA; $p>0.05$ for IgM) (Figure 5.1).

Furthermore, the IgA antibody levels were also elevated in the SLE ($p<0.05$) and CD groups compared to the healthy control group, although the elevation in the CD group was not significant ($p>0.05$).

i. IgG antibody levels

Comparing the antibody levels to each individual peptide of the four groups studied, it was found that RA patients had significantly higher IgG antibody levels than healthy controls to all six peptides ($p<0.05$), the SLE patients to all ($p<0.05$) but peptide P5 ($p>0.05$), and the Crohn's patients to peptides P1 and P2 only ($p<0.05$) (Figure 5.1).

ii. IgA antibody levels

As for the IgA class, RA patients had elevated antibodies to peptides P4, P5 and P6 ($p<0.05$), the SLE patients to all peptides ($p<0.01$) and the CD patients to peptide P6 only ($p<0.05$) (Figure 5.1).

iii. IgM antibody levels

In contrast, the IgM antibody levels to all synthetic peptides were lower than those of the healthy control group, although this decrease was significant only for the SLE group ($p<0.05$) (Figure 5.1).

TABLE 5.2: Amino acid sequencies of six synthetic peptides of *M. leprae* 65 kDa hsp and the corresponding sequencies of the *E. coli* and human 65 kDa hsps.

		T cell specificity
P1	**65 ... 85**	
M. leprae	Y E K I G A E L V K E V A K K T D D V A G	← mycobacteria
E. coli	F * N M * * Q M * * * * * S * A N * A * *	specific
human	* K N * * * K * * Q D * * N N * N E E * *	
P2	**116 ... 137**	
M. leprae	K R G I E K A V D K V T E T L L K D A K E V	← mycobacteria
E. coli	* * * * D * * * T A A V * E * K A L S V P C	specific
human	R * * V M L * * * A * I A E * K * Q S * P *	
P3	**153 ... 171**	
M. leprae	D Q S I G D L I A E A M D K V G N E G	← cross-reactive
E. coli	* E T V * K * * * * * * * * * * K * *	with *E. coli*
human	* K E * * N I * S D * * K * * * R K *	
P4	**180 ... 196**	
M. leprae	T F G L Q L E L T E G M R F D K G	← autoreactive
E. coli	G L Q D E * D V V * * * Q * * R *	in adjuvant
human	* L N D E * * I I * * * K * * R *	arthritis
P5	**195 ... 219**	
M. leprae	K G Y I S G Y F V T D A E R Q E A V L E E P Y I L	← cross-reactive
E. coli	R * * L * P * * I N K P * T G A V E * * S * F * *	with *E. coli*
human	R * * * * P * * I N T S K G * K C E F Q D A * V *	and human
P6	**390 ... 412**	
M. leprae	R K H R I E D A V R N A K A A V E E G I V A G	← cross-reactive
E. coli	K * A * V * * * L H A T R * * * * * * V * * *	with *E. coli*
human	K * D * V T * * L N A T R * * * * * * * * L *	and human

Footnotes: The sequence of each peptide is shown using the single letter code for amino acids.

Numbering is according to the mycobacterial sequence.

Residues identical to the mycobacterial sequence are shown with an asterisk.

The conservative amino acid changes are underlined.

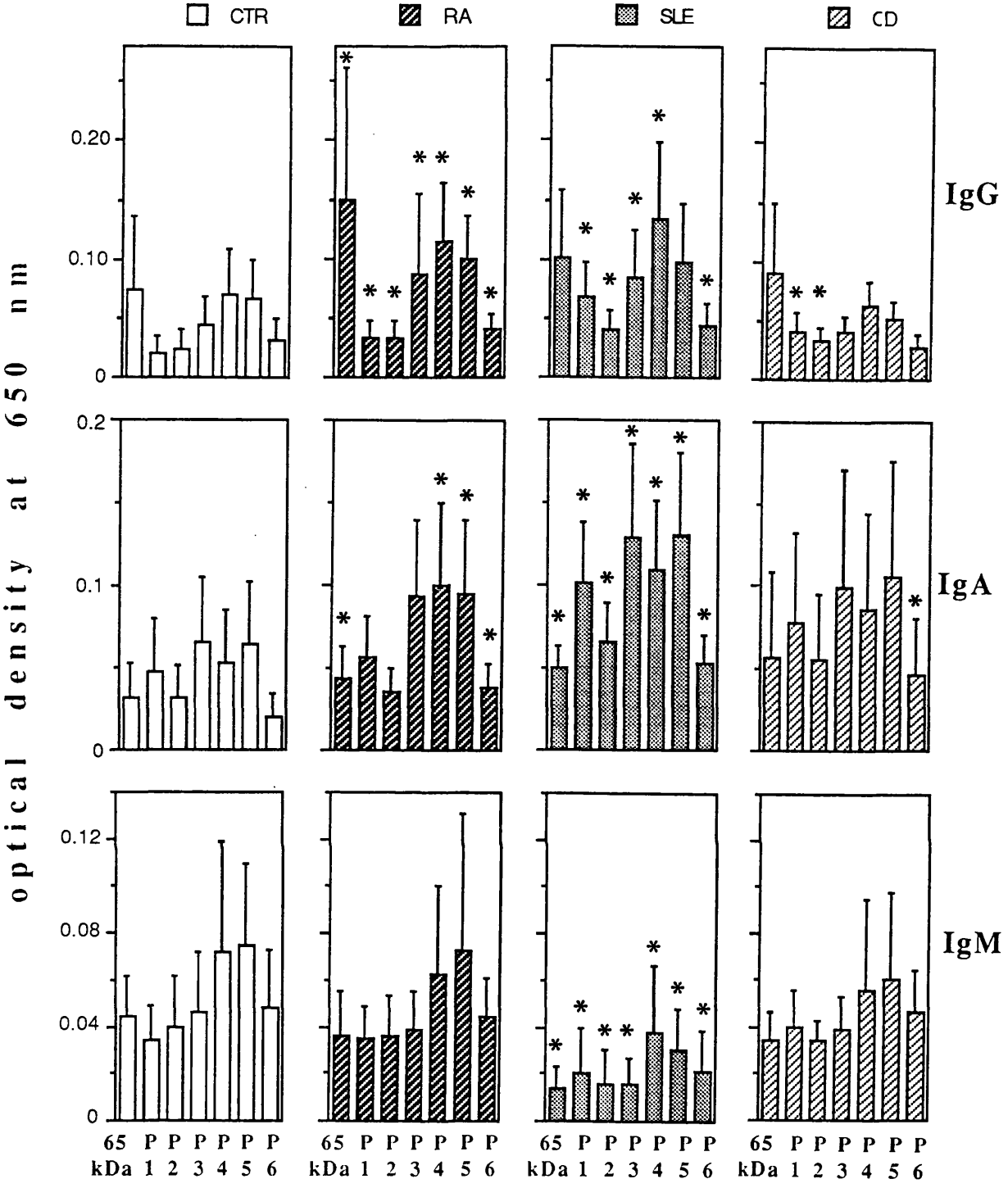


FIGURE 5.1: IgG, IgA AND IgM SERUM ANTIBODY LEVELS TO THE *M. BOVIS* 65 kDa HSP AND TO SIX SYNTHETIC PEPTIDES OF THE *M. LEPRAE* 65 kDa HSP IN HEALTHY INDIVIDUALS (CTR), RHEUMATOID ARTHRITIS (RA), SYSTEMIC LUPUS ERYTHEMATOSUS (SLE) AND CROHN'S DISEASE (CD) PATIENTS. The antibody levels are expressed as optical density values at 650 nm and presented as histograms of the mean optical density for each group. The vertical lines represent the standard deviation for each group. * $p < 0.05$.

b. Serum antibody levels of RA patients in 'early' and 'late' stages of the disease

i. Antibody levels to the mycobacterial 65 kDa hsp

Although the mycobacterial 65 kDa hsp antibodies did not appear to correlate with disease activity or duration in established RA patients (patients with the disease for approximately 10 years) (Chapter 4), such a correlation might exist in the very early stages of the disease.

This could be true especially, if antibodies to the mycobacterial 65 kDa hsp were of some importance to the aetiology of rheumatoid arthritis. In such a case, it would be interesting to see whether the antibody levels were higher at the earlier stages of the disease compared to an established state.

In order to test this hypothesis, antibody levels to the mycobacterial 65 kDa hsp were studied in the sera from 20 RA patients when newly diagnosed ('early' sera) and in sera from the same patients 2-5 years after the diagnosis ('late' sera). The characteristics of these patients are shown in table 5.1. The antibody levels were measured by an enzyme linked immunosorbent assay and were expressed as optical density values at 650 nm.

Although 8/20 and 10/20 patients had higher IgG and IgM antibody levels respectively, at the early stage of the disease compared to the later stage (Figure 5.2), there was no significant difference in the IgG and IgM antibody levels between the 'early' and 'late' sera. However, the IgA antibody levels were found to be significantly elevated at the earlier stages of the disease compared to the later stages, with 16/20 patients showing this elevation (Figure 5.2).

	Mean optical density ± SD		
	IgG	IgM	IgA
Early sera	0.075±0.042	0.053±0.021	0.088±0.038
Late sera	0.088±0.074	0.052±0.022	0.075±0.044
p value	> 0.05	> 0.05	< 0.05

Five serum samples from healthy individuals were also examined for antibodies at the same time, and their antibody levels were found to be lower than those of the 'early' and 'late' sera. The mean antibody level value of these healthy individuals is shown in figure 5.2 with a double-headed arrow.

To determine whether the increase in the IgA antibody levels at earlier rather than at later stages of the disease was specific to the mycobacterial 65 kDa hsp, the same sera were studied for antibody levels to *C. albicans* and to a mixture of three influenza strains.

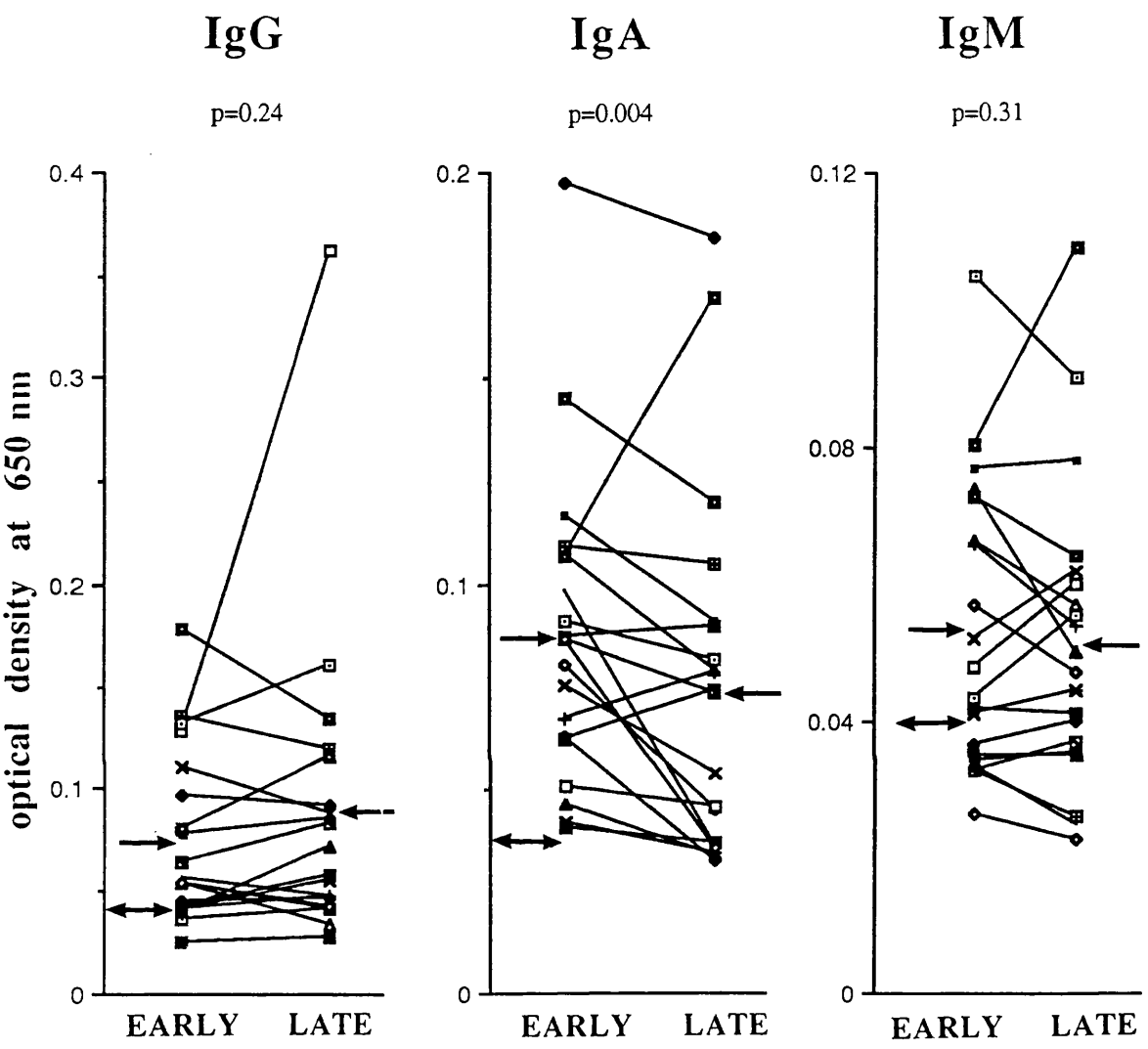


FIGURE 5.2: IgG, IgA AND IgM ANTIBODY LEVELS TO THE MYCOBACTERIAL 65 kDa HSP IN 'EARLY' AND 'LATE' RHEUMATOID ARTHRITIS SERA. The 'early' sera represent the newly diagnosed patients and the 'late' sera represent the same patients 2-5 years after the diagnosis. The antibody levels are expressed as optical density values at 650 nm. The single-headed arrows represent the mean optical density of the 'early' and 'late' sera and the double-headed the mean optical density of the five healthy control sera.

ii. Antibody levels to *C. albicans*

Although 12/20, 15/20 and 10/20 sera had showed elevated IgG, IgA and IgM antibody levels respectively to *C. albicans* at the early stage compared to the late stage of the disease, only for the IgA class of the antibodies this elevation was significant ($p<0.01$) (Figure 5.3).

The antibody levels were expressed as optical density values at 650 nm and the mean value for each group was as follows:

	Mean optical density ± SD		
	IgA	IgG	IgM
Early sera	0.098±0.056	0.109±0.043	0.072±0.045
Late sera	0.087±0.059	0.107±0.040	0.077±0.048
p value	< 0.05	> 0.05	> 0.05

This assay also included 5 healthy sera and their IgA and IgM antibody levels were approximately the same as those found in the 'late' sera, while their IgG antibody levels were lower than those of the 'late' sera (Figure 5.3).

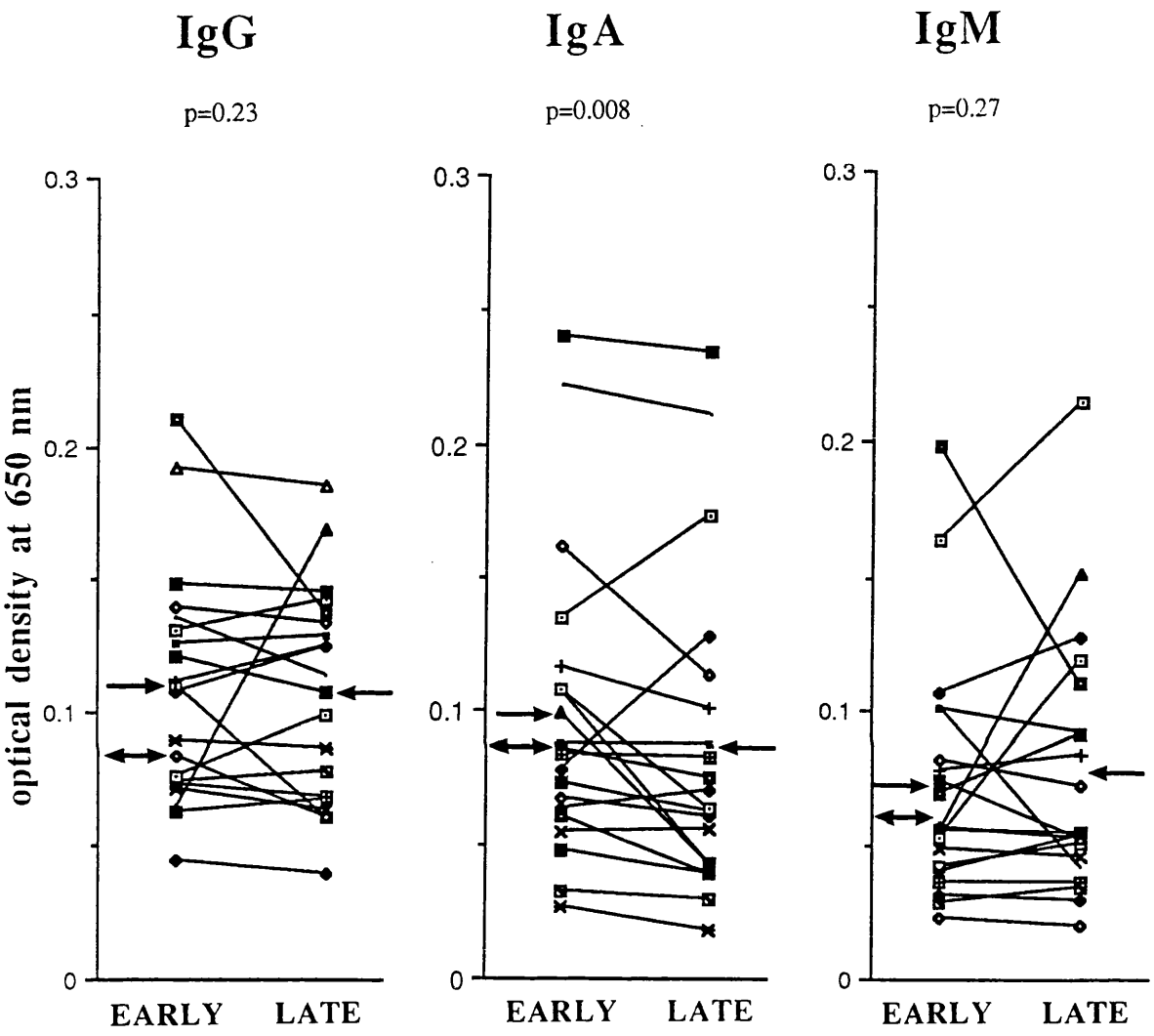


FIGURE 5.3: IgG, IgA AND IgM ANTIBODY LEVELS TO *C. ALBICANS* IN 'EARLY' AND 'LATE' RHEUMATOID ARTHRITIS SERA. The 'early' sera represent the newly diagnosed patients and the 'late' sera represent the same patients 2-5 years after the diagnosis. The antibody levels are expressed as optical density values at 650 nm. The single-headed arrows represent the mean optical density of the 'early' and 'late' sera and the double-headed the mean optical density of the five healthy control sera.

iii. Antibody levels to the 'flu' preparation

As for the IgA and IgM (the IgG class was not tested) antibody levels to the flu preparation (Figure 5.4), there was no significant difference between early and late sera:

	Mean optical density ± SD	
	IgA	IgM
Early sera	0.170±0.089	0.194±0.085
Late sera	0.181±0.133	0.190±0.090
p value	> 0.05	> 0.05

This was true, despite the fact that 13/20 (in the IgA assay) and 11/20 (in the IgM assay) patients had higher antibody levels at the earlier stage of the disease compared to the later stage (Figure 5.4).

The antibody levels of five healthy individuals were also examined and found to be at the same level as those found in the 'late' sera. The mean antibody level of the healthy sera is shown in figure 5.4 as a double-headed arrow.

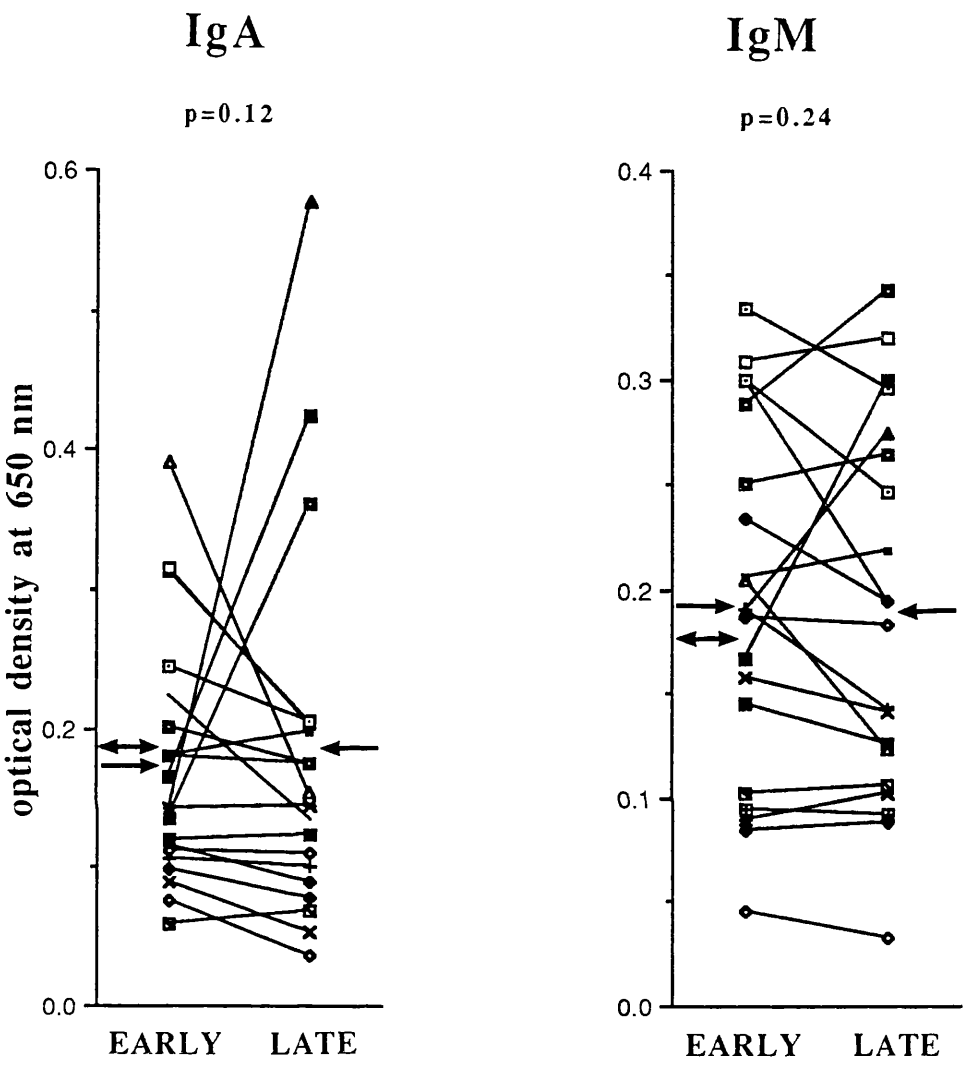


FIGURE 5.4: IgA AND IgM ANTIBODY LEVELS TO THE 'FLU' PREPARATION IN 'EARLY' AND 'LATE' RHEUMATOID ARTHRITIS SERA. The 'early' sera represent the newly diagnosed patients and the 'late' sera represent the same patients 2-5 years after the diagnosis. The antibody levels are expressed as optical density values at 650 nm. The single-headed arrows represent the mean optical density of the 'early' and 'late' sera and the double-headed the mean optical density of the five healthy control sera.

iv. Correlations between antibody levels to the mycobacterial 65 kDa hsp, *C. albicans* and 'flu' preparation

1. IgA class

Although the IgA antibody levels to both mycobacterial 65 kDa hsp and *C. albicans* were higher in the early stages of the disease, no significant correlation was found between these antibodies ($r=0.24$, $p=0.29$). Moreover, this lack of correlation was also true for the IgA antibody levels of the 'late' sera ($r=0.42$, $p=0.065$).

Similarly, there was no correlation between IgA antibodies to the mycobacterial 65 kDa hsp and to the flu preparation in either 'early' ($r=0.22$, $p=0.33$) or 'late' ($r=0.24$, $p=0.29$) sera. This implied that the IgA antibodies were not directed against common epitopes and that there was some degree of specificity in the responses.

2. IgM class

Although the IgM antibody levels to the mycobacterial 65 kDa hsp and to *C. albicans* were not correlated in the 'early' sera ($r=0.40$, $p=0.079$), they did correlate in the 'late' sera ($r=0.55$, $p=0.011$).

Similarly, there was no correlation between IgM antibodies to the mycobacterial 65 kDa hsp and to the flu preparation in the 'early' sera ($r=0.50$, $p=0.23$), whereas there was one in the 'late' sera ($r=0.57$, $p=0.009$).

3. IgG class

As for the IgG antibody levels to the mycobacterial 65 kDa hsp and to *C. albicans,* there was no

correlation in the 'early' sera ($r=-0.430$, $p=0.054$), although there was an inverse correlation in the 'late' sera ($r=-0.56$, $p=0.01$). The lack of direct correlation reinforced the specificity of the antibody responses measured.

There was a direct correlation between antibody levels in 'early' and 'late' sera ($r>0.8$, $p<0.0001$), for all three antigen preparations used and all three immunoglobulin classes studied. In other words, patients' sera that had high antibody levels early in the disease maintained the high antibody levels at the later stages and vice versa.

The total serum immunoglobulin levels of the 'early' and 'late' sera were compared to see whether differences in the antibody levels were due to different amounts of immunoglobulin been present. However, no significant difference was found between the immunoglobulin levels of the 'early' and 'late' RA sera ($p>0.05$).

v. Antibody levels to six synthetic peptides of *M. leprae* 65 kDa hsp in sera from 'early' and 'late' RA patients

Although there was no difference in the fine specificities of the mycobacterial 65 kDa hsp antibodies in established RA patients, SLE and CD patients, and healthy individuals (section 5.3a), it would be interesting to see whether this was also true in 'early' and 'late' sera.

To determine whether antibodies to the mycobacterial 65 kDa hsp might be directed against different epitopes earlier on in the disease in comparison with later stages, antibodies to the same six peptides of *M. leprae* 65 kDa hsp were

studied using 'early' and 'late' RA sera.

Both types of sera showed IgG, IgA and IgM antibodies to all six peptides. However, only the IgA antibodies were significantly elevated in the 'early' sera compared to the 'late' sera ($p<0.05$) (Figure 5.5). This was in agreement with the significant elevation of the IgA antibody levels to the intact *M. bovis* 65 kDa hsp observed in the 'early' sera (section 5.3b).

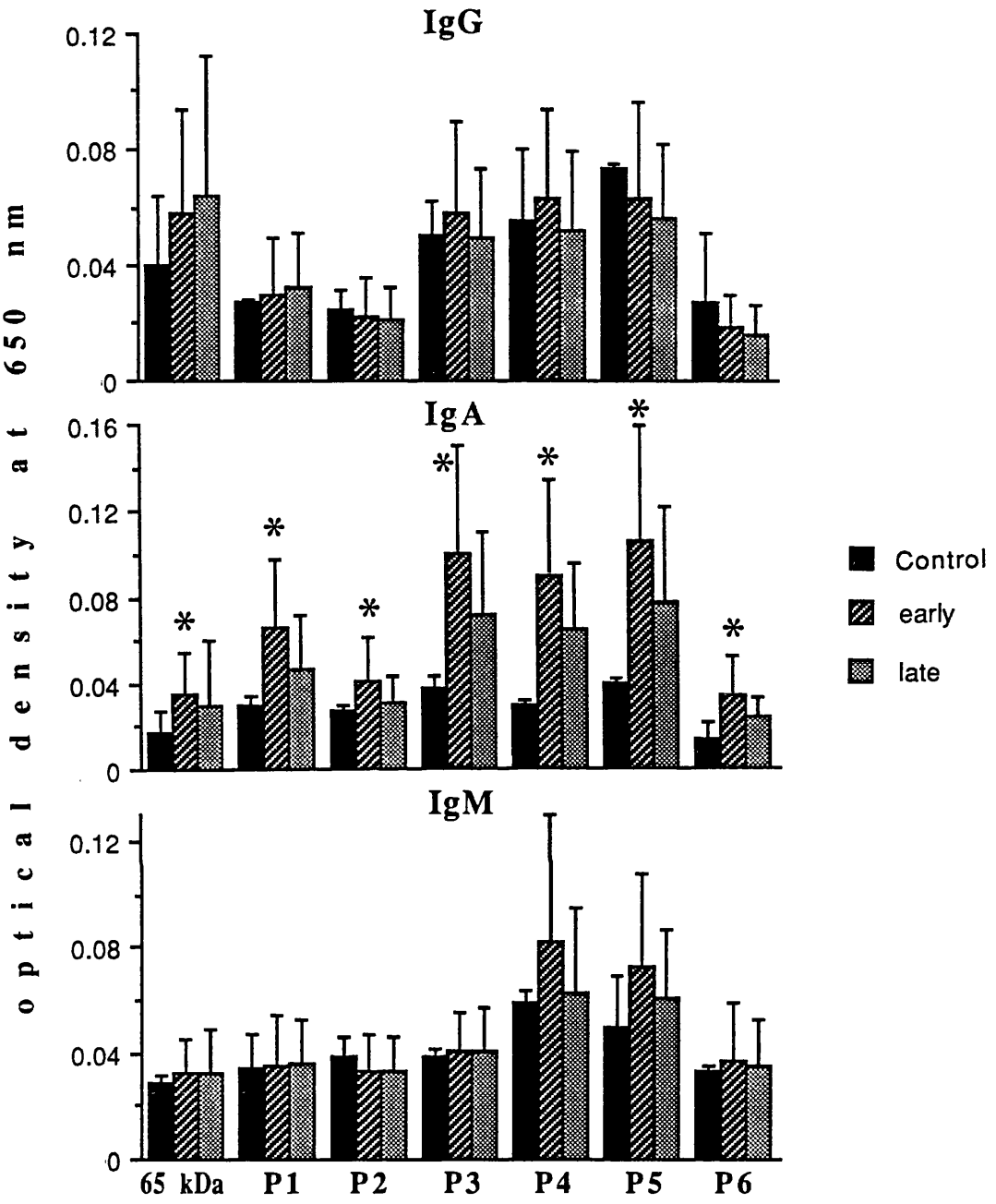


FIGURE 5.5: IgG, IgA AND IgM ANTIBODY LEVELS TO SIX SYNTHETIC PEPTIDES OF *M. LEPRAE* 65 kDA HSP IN 'EARLY' AND 'LATE' RHEUMATOID ARTHRITIS SERA. The 'early' sera represent the newly diagnosed patients and the 'late' sera represent the same patients 2-5 years after the diagnosis. The antibody levels are expressed as optical density values at 650 nm and are represented as histograms of the mean OD for each group. The vertical lines represent the standard deviation (SD) for each group. * $p<0.05$. The p value is the result of the comparison of antibody levels in 'early' and 'late' sera.

c. Serum antibody levels to the mycobacterial 65 kDa hsp of 12 established RA patients in three yearly time intervals

Antibody levels to the mycobacterial 65 kDa hsp in sera from established RA patients (patients with the disease for approximately 10 years) were studied at yearly time intervals, over 3 years, for possible changes associated with the progress of the disease. Twelve patients were selected based on whether they have been previously found to have very high (higher than the healthy control mean+2SD; Chapter 4) or very low IgG or IgA antibody levels to the mycobacterial 65 kDa hsp. Three patients from each category were studied.

The choice of the immunoglobulin class for study was based on the fact that, only the IgG and IgA mycobacterial 65 kDa hsp antibody levels were found to be significantly elevated in the RA patients compared to healthy individuals (Chapter 4).

From the three patients selected as having very high IgG antibody levels, one has shown a drop in antibody levels during the second year of test, but this increased again by the 3rd year (Figure 5.6). This however, did not appear to be associated with a change in the clinical features of the patient. The eleven remaining patients were all found to have almost the same level of antibodies during the three years of study. In other words, if they had high levels of antibodies, IgG or IgA, they stayed high. The reverse was also true. In general, it seemed as though the antibody levels remained somewhat constant.

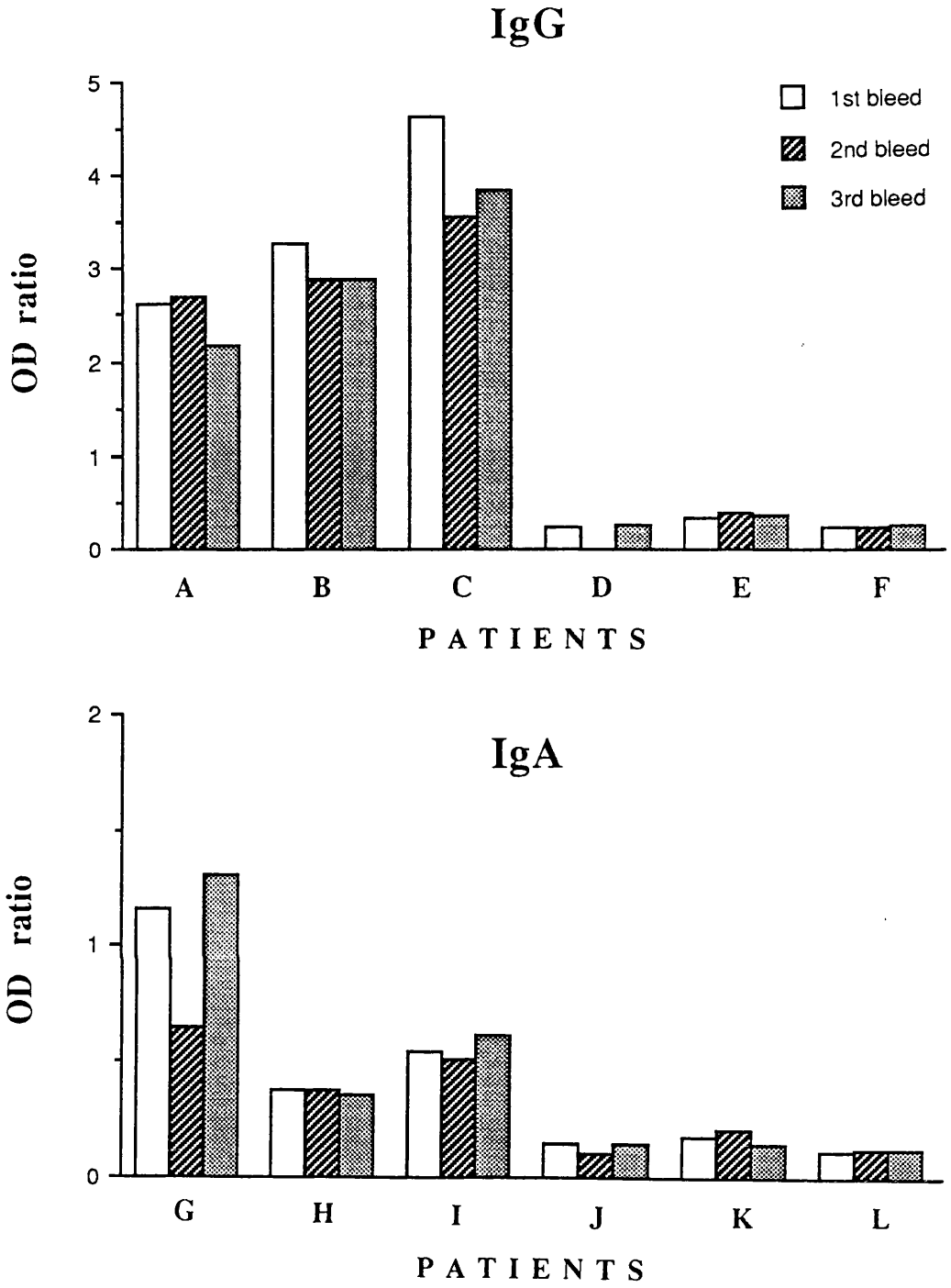


FIGURE 5.6: IgG AND IgA ANTIBODY LEVELS TO THE MYCOBACTERIAL 65 kDa HSP IN 12 RHEUMATOID ARTHRITIS PATIENTS AT THREE YEARLY TIME INTERVALS. The antibody levels are expressed as optical density ratios (OD_{650} ratio = OD_{650} of test serum /OD_{650} of standard healthy serum used as a positive control). Patients A, B, and C were chosen since they had been previously shown to have high IgG mycobacterial 65 kDa hsp antibody levels. Patients D, E, and F were chosen for having low IgG antibody levels; G, H, and I for having high IgA antibody levels; J, K and L for having low IgA antibody levels. The mean OD ratio for the healthy control group was 0.64±0.48 for the IgG assay and 0.23±0.29 for the IgA assay (not shown).

5.4 DISCUSSION

The immunodominance of the mycobacterial 65 kDa hsp antibodies in RA was further investigated by an attempt to characterise the immunogenic component(s) to which the antibody responses were attributed. The use of six synthetic peptides of *M. leprae* 65 kDa hsp to examine antibody specificity in RA, SLE, CD and healthy sera failed to show any difference in the specificities of the mycobacterial 65 kDa hsp antibodies between these groups.

Although all four study groups were found to have antibodies directed against the six peptides, the levels of the antibodies were different. In general, the IgG and IgA (but not IgM) antibody levels to the peptides were elevated in RA, SLE and CD patients compared with those in healthy individuals. This was also in agreement with the antibody levels to the intact *M. bovis* BCG 65 kDa hsp in these study groups (Chapter 4).

It was interesting that antibodies were also directed against peptide P4 which contained the epitope recognised by the arthritogenic clone in the adjuvant arthritis model (van Eden *et al.*, 1988). However, the antibodies directed against P4 were not RA specific and were also found to be elevated in SLE patients.

Since the six peptides tested represented only 1/4 of the 540 amino acid sequence of 65 kDa hsp, the lack of immunodominance of a particular peptide within a group and between the four groups studied might be due to the absence of the immunodominant peptide.

In fact, investigations on B and T cell epitopes of the 65 kDa hsp have identified at least eight different B cell and ten T cell epitope-containing regions (Table 1.1) (Mehra *et al.*, 1986;

Buchanan *et al.*, 1987; Lamb *et al.*, 1987; Thole *et al.*, 1988b; Young *et al.*, 1988b; Lamb *et al.*, 1990). Comparing the stretches of the six synthetic peptides used in this study with the ones of the identified epitopes, it was found that P1, P2, P3, P5 and P6 peptides have been identified as T cell epitopes (Lamb *et al.*, 1987; Young *et al.*, 1988b). In addition, peptides P2, P3, P5 and P6 were partly in B cell epitope-containing regions as well (Mehra *et al.*, 1986; Thole *et al.*, 1988b). However, the B cell epitopes identified by the use of monoclonal antibodies might not necessarily be the immunodominant ones in the human humoral responses.

Occasionally, patients' sera showed stronger binding to one or more of the peptides than to the intact molecule. Since the optimal amounts of each peptide were used in the assays, this could not have been due to the sensitivity of the assays. In addition, the genes encoding the 65 kDa antigen from *M. leprae* and *M. bovis* have been cloned and comparison of the nucleotide sequences of these two genes showed that they differed only in 28 amino acid residues (Mehra *et al.*, 1986; Shinnick *et al.*, 1988). Thus, differences in antibody levels to the intact *M. bovis* 65 kDa hsp as opposed to the *M. leprae* 65 kDa peptides were not attributable to species specific epitopes.

It was interesting though, that the six synthetic peptides derived from the *M. leprae* sequence of 65 kDa hsp differed in only a few amino acids from the recently sequenced human 65 kDa hsp (Jindal *et al.*, 1989) (Table 5.2), indicating that at least some of the antibodies directed against the peptides could be autoantibodies. This could be very important in view of the autoimmune character of RA, but needs to be investigated further.

To provide information about the importance of the

65 kDa hsp in the initiation of the disease, the mycobacterial 65 kDa hsp antibodies were examined in newly diagnosed RA patients ('early' sera) and 2-5 years later ('late' sera).

The IgA antibody levels to the mycobacterial 65 kDa hsp were higher in the 'early' stage of the disease compared to the 'later' stage, which could argue in favour of a primary role for these antibodies in the disease process. Although there was a similar elevation of the IgA antibody levels to the *C. albicans* in 'early' sera, the lack of correlation between elevated IgA anti-mycobacterial 65 kDa hsp and anti-*C. albicans* antibody levels implied a degree of specificity in the responses. Moreover, the absence of a simultaneous increase of the IgA antibody levels to the 'flu' preparation excluded the possibility of a polyclonal activation. In addition, the elevations of the IgA antibody levels did not correlate with levels of the total serum IgA.

Interestingly, the IgA antibody levels to the six synthetic peptides were also elevated in the 'early' sera compared to the 'late' sera, but without any preferential reactivity towards a particular peptide.

The fact that the IgG and IgM mycobacterial 65 kDa hsp antibody levels were similar in the 'early' and 'late' sera did not necessarily exclude a role for these antibodies in the initiation of the disease, but it might not be the same as for the IgA antibodies.

Finally, the observation of virtually constant IgA and IgG antibody levels in 12 established RA patients, during a three year period, was suggestive of a different role of the mycobacterial 65 kDa hsp antibodies in 'early' and 'late' disease.

In summary, the use of the six synthetic peptides covering sections of the *M. leprae* 65 kDa hsp did not show any of the

peptides to be specifically immunodominant in RA. There was an indication that the mycobacterial 65 kDa hsp antibodies could be in part autoantibodies. With regard to studies on 'early' and 'late' sera, it was implied that the IgA antibodies to the mycobacterial 65 kDa hsp might be more relevant to the initiation of the disease, compared to the IgG and IgM antibodies.

However, further studies are required for the identification of the immunodominant epitope(s) of the 65 kDa hsp in humoral responses in RA and the degree of cross-reactivity or autoreactivity of these responses in RA.

CHAPTER 6 AUTOREACTIVITY TO HEAT SHOCK PROTEINS IN RHEUMATOID ARTHRITIS PATIENTS

Introduction

Characteristics of study groups in chapter 6

Results

- Cross-reactivity of *M. bovis* 65 kDa hsp IgG antibodies with human 65 kDa hsp
- Antibody levels to *M. bovis* 65 kDa hsp in synovial fluids of RA patients
- Presence of a 65 kDa protein in synovial fluid and immune complexes from RA patients
- Serum antibody levels to the human 70 kDa hsp in rheumatoid arthritis patients

Discussion

6.1 INTRODUCTION

The remarkable high degree of sequence conservation of the heat shock proteins (hsps), with more than 50% identity between bacterial and mammalian homologues (Bardwell and Craig, 1984; Lindquist, 1986; Jindal, 1989), makes these proteins very strong candidates for potential autoreactivity through mimicry to occur (Oldstone, 1987).

This implies that at least some of the mycobacterial and *Escherichia coli* 65 and 70 kDa hsp antibodies found in human sera (Chapters 4 and 5) might be autoantibodies. In fact, serum antibodies have been found to be directed against synthetic peptides of the *Mycobacterium leprae* 65 kDa hsp that showed high homology with the human 65 kDa hsp sequence (Chapter 5), hence reinforcing such a possibility.

To obtain more information on the possible autoreactivity directed against the hsps, the relevance of the anti-hsp antibodies and the hsps themselves in rheumatoid arthritis, the following studies were carried out.

1. It was tested whether the mycobacterial 65 kDa hsp antibodies found in human sera could bind to the human 65 kDa hsp.

2. Synovial fluid samples were examined for the

presence of mycobacterial 65 kDa hsp antibodies.

3. Since hsps can be produced at sites of inflammation (Polla, 1988), rheumatoid arthritis (RA) synovial fluid samples and immune complexes separated from them were examined for the presence of the 65 kDa hsp itself.

4. Antibodies to RA synovial fluid antigens were studied in sera from healthy individuals, RA and osteoarthritic patients.

5. Serum antibody levels to the human homologue of the 70 kDa hsp were examined in patients with rheumatoid arthritis, systemic lupus erythematosus (SLE), ankylosing spondylitis (AS), Crohn's disease (CD) and healthy individuals (CTR).

6.2 CHARACTERISTICS OF STUDY GROUPS IN CHAPTER 6

TABLE 6.1: Characteristics of rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), ankylosing spondylitis (AS), Crohn's disease (CD) patients and healthy individuals (CTR) used to study antibody levels to the human 70 kDa heat shock protein.

	RA	SLE	AS	CD		CTR
Ig Class	A,M,G	A,M,G	A,M,G	A, M	G	A,M,G
Number of donors	20	18	15	21	13	20
Male	3	1	10	11	6	11
Female	17	17	5	10	7	9
Average Age (Years)	58	42	50	36	33	35
(range)	33 - 76	30 - 68	33 - 73	18 - 74	20 - 51	25 - 63
Activity of disease (1)						Not applicable
Slightly active	-	9	-	12	7	
Moderately active	-	2	-	9	6	
Very active	11	1	9	-	-	
Inactive	9	6	6	-	-	
Treatment (2)						
First-line drugs	9	-	14	-	-	
Second-line drugs	2	-	-	-	-	
Third-line drugs	2	10	-	5	4	
No treatment	7	8	1	16	9	
Not known	-	-	-	-	-	
Seropositivity (3)		Not Known		Not Known		Not Known
Seropositive	7		-			
Seronegative	8		15			
Not known	5		-			

Footnotes: (1) Activity was assessed as shown in section 2.5.

(2) First-line drugs: non-steroidal anti-inflammatory drugs (e.g. indomethacin, naproxen)
Second-line drugs: disease-modifying drugs (e.g. gold, penicillamine, sulphasalazine)
Third-line drugs: prednisolone, azathioprine, methotrexate.

(3) Seropositivity was assessed by latex agglutination test.

6.3 RESULTS

a. Cross-reactivity of *M. bovis* 65 kDa hsp IgG antibodies with human 65 kDa hsp

The high degree of homology between the mycobacterial and human 65 kDa hsps (Jindal *et al.*, 1989) could imply that the same antibodies might be directed against proteins from both origins.

In order to examine this possibility, anti-mycobacterial 65 kDa hsp serum antibodies eluted from a mycobacterial 65 kDa hsp affinity column were tested by ELISA for binding to a preparation of human (placental) 65 kDa hsp. Sera from two patients with rheumatoid arthritis and two healthy individuals that had been previously shown to contain antibodies to the mycobacterial 65 kDa hsp were used.

It was found that the eluted anti-mycobacterial 65 kDa antibodies could bind to the 65 kDa hsp of both mycobacterial and human origin (Table 6.2), suggesting that at least some of the mycobacterial 65 kDa hsp antibodies were autoreactive.

TABLE 6.2: Binding of mycobacterial 65 kDa hsp IgG antibodies (affinity column chromatography purified) to the human 65 kDa hsp.

	Optical density ratio ± SD			
	mycobacterial 65 kDa hsp		human 65 kDa hsp	
	serum	antibodies	serum	antibodies
CTR 1	0.75±0.010	0.68±0.005	0.58±0.005	0.57±0.010
CTR 2	0.43±0.005	0.35±0.010	0.60±0.001	0.35±0.006
RA 1	1.54±0.005	1.50±0.001	1.82±0.005	1.52±0.007
RA 2	2.33±0.006	2.00±0.005	1.78±0.001	1.65±0.005

Footnotes: The binding is expressed as optical density ratio: OD_{650} of the original serum or mycobacterial 65 kDa hsp antibodies (1/200 dilution)/OD_{650} of a healthy serum (1/200). The same healthy serum was used for both the mycobacterial and human ELISA assays. CTR: healthy individual; RA: rheumatoid arthritis patient.

b. Antibody levels to *M. bovis* 65 kDa hsp in synovial fluid samples of RA patients

If antibodies to the mycobacterial 65 kDa hsp were important in the pathogenesis of RA, they might be found in significant amounts at the site of damage, namely the joint. When 34 RA synovial fluid (SF) samples were examined for antibodies to the mycobacterial 65 kDa hsp and compared with the levels found in 50 RA serum samples (non-paired), a significant decrease in IgA, IgG and IgM antibody levels was found in the fluid samples compared to the serum samples ($p<0.001$) (Table 6.3).

Since the serum and synovial fluid samples were not paired, it could not be concluded directly that there were more 65 kDa hsp antibodies in the circulation than at the site of damage in individual patients. It was therefore important to confirm the above observation using paired serum and synovial fluid samples.

Analysis of 23 RA paired serum and synovial fluid samples showed that IgA and IgG, but not IgM, antibody levels were lower in the synovial fluid compared to the serum samples ($p<0.05$ for the IgA and IgG assays, $p>0.05$ for the IgM assay) (Figure 6.1 and Table 6.3).

Interestingly, paired samples from 9 osteoarthritic patients also showed lower IgA, IgG and IgM antibody levels in the synovial fluid samples compared to serum samples (Table 6.3).

Only one ankylosing spondylitis paired sample was available and that showed to have lower antibody levels to the mycobacterial 65 kDa hsp in the synovial fluid compared to the serum. The antibody levels expressed as optical density ratios were as following: IgA (serum=2.89, SF=1.66), IgG (serum=1.44, SF=0.97) and IgM (serum=0.62, SF=0.42).

TABLE 6.3: Comparison of antibody levels to the *M. bovis* 65 kDa hsp in non-paired and paired serum and synovial fluid samples from rheumatoid arthritis patients (RA) and paired samples from osteoarthritic patients (OA).

	IgA	IgG	IgM
Non - paired RA samples			
Serum	0.69±0.71 (45)	1.10±0.83 (48)	0.68±0.38 (50)
SF	0.22±0.16 (34)	0.57±0.86 (34)	0.42±0.26 (30)
p value (1)	0.000048	0.0000065	0.00070
23 paired RA samples			
Serum	3.16±2.92	1.75±1.10	1.75±2.43
SF	2.05±1.81	1.26±0.92	1.10±1.03
p value (2)	0.024	0.042	0.076
9 paired OA samples			
Serum	2.21±1.26	1.46±1.45	1.66±1.27
SF	1.00±0.44	0.62±0.37	0.64±0.36
p value (2)	0.013	0.052	0.027

Footnotes: (1) The p value was obtained from the Mann-Whitney U-Rank two tailed test.

(2) The p value was obtained from the Wilcoxon test.

The antibody levels are presented as the mean optical density ratio and standard deviation (SD) for each group.

If a significant proportion of the antibody which bound to the mycobacterial 65 kDa hsp was autoantibody, the lower antibody levels found in the synovial fluid might be the result of complexing with the human homologue, if this was present in the synovial fluid.

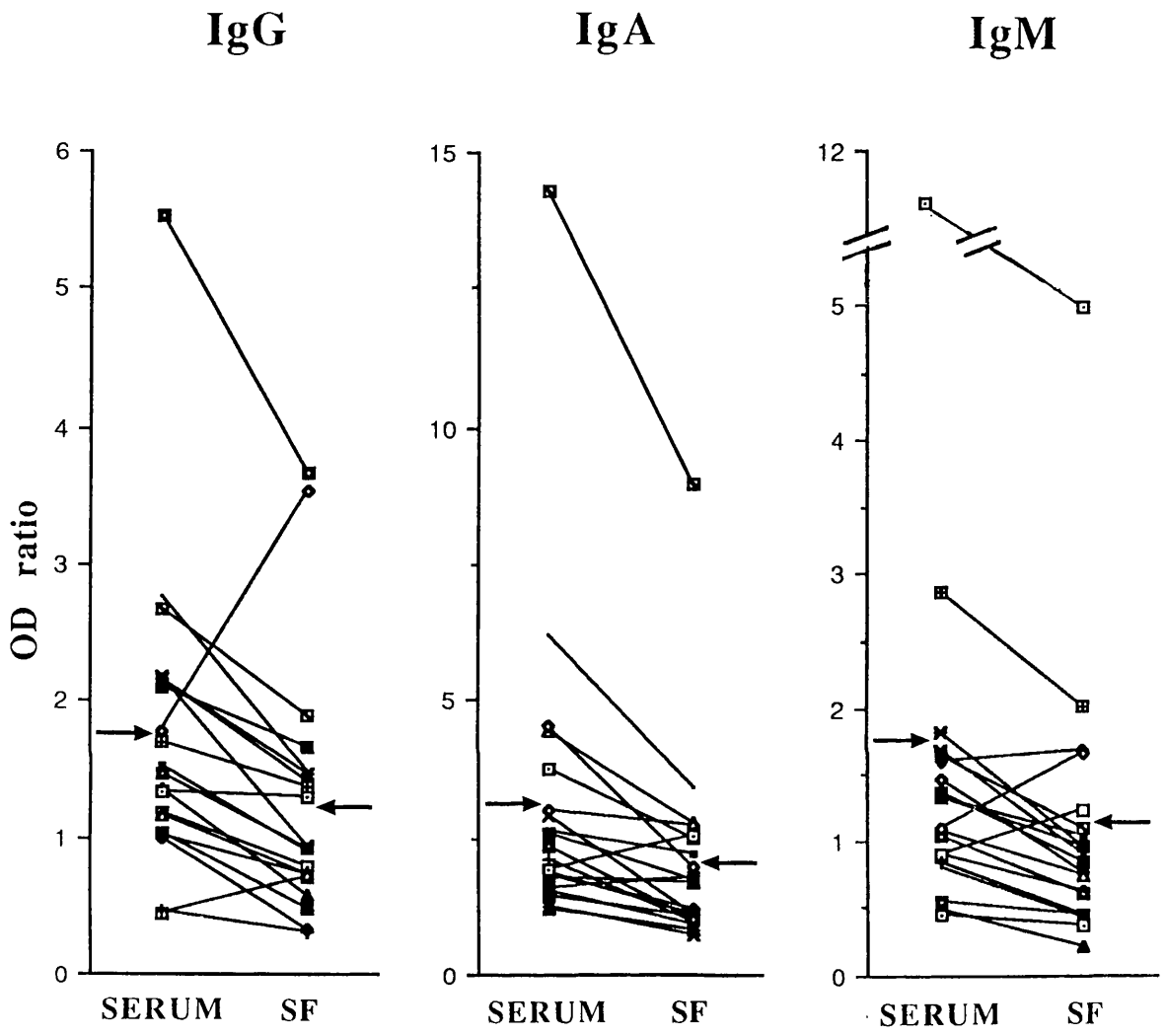


FIGURE 6.1: IgG, IgA AND IgM ANTIBODY LEVELS TO THE *M. BOVIS* 65 kDa HSP IN SERUM AND SYNOVIAL FLUID (SF) SAMPLES FROM RHEUMATOID ARTHRITIS PATIENTS. The antibody levels are expressed as optical density ratios (OD_{650} ratio = OD_{650} of test serum/ OD_{650} of standard healthy serum used as a positive control). The arrows represent the mean OD ratio for each group.

c. Presence of a 65 kDa protein in synovial fluid and immune complexes from RA patients

It seemed possible that, if the 65 kDa hsp was involved in the pathogenesis of RA, it might be found at the site of damage and therefore in the synovial fluid. Hence, the presence of the 65 kDa hsp in RA synovial fluid samples was checked. Furthermore, it was also important to examine whether complexes of anti-65 kDa antibodies with 'human' 65 kDa were present in the synovial fluid samples, as that could partly explain the decreased antibody levels to the mycobacterial 65 kDa hsp in the synovial fluid samples.

Twelve synovial fluid samples and immune complexes from the same SF samples were run in a 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blots were prepared. A track of each sample was stained with ink to reveal all the proteins available for binding. Additional tracks were immunoblotted with monoclonal antibodies specific for the 65 kDa hsp (F67.2 and F67.13) and 70 kDa hsp (L7 and 51A).

It was found that the F67.2 and F67.13 monoclonal antibodies stained a band of approximately 65 kDa molecular weight in both the SF and the immune complexes samples (Figure 6.2). Similarly, the L7 and 51A monoclonal antibodies stained a band of approximately 70 kDa (Figure 6.2).

It was important to determine whether the presence of the 65 kDa was characteristic of RA, or whether it was present in any inflamed joint. Thus, similar Western blots were prepared using SF samples from 2 osteoarthritic patients and from two traumatic knees of otherwise healthy individuals. Using the same

reagents as above, a band of 65 kDa hsp was identified in these samples suggesting that the presence of the 65 kDa hsp in synovial fluid samples was not disease specific.

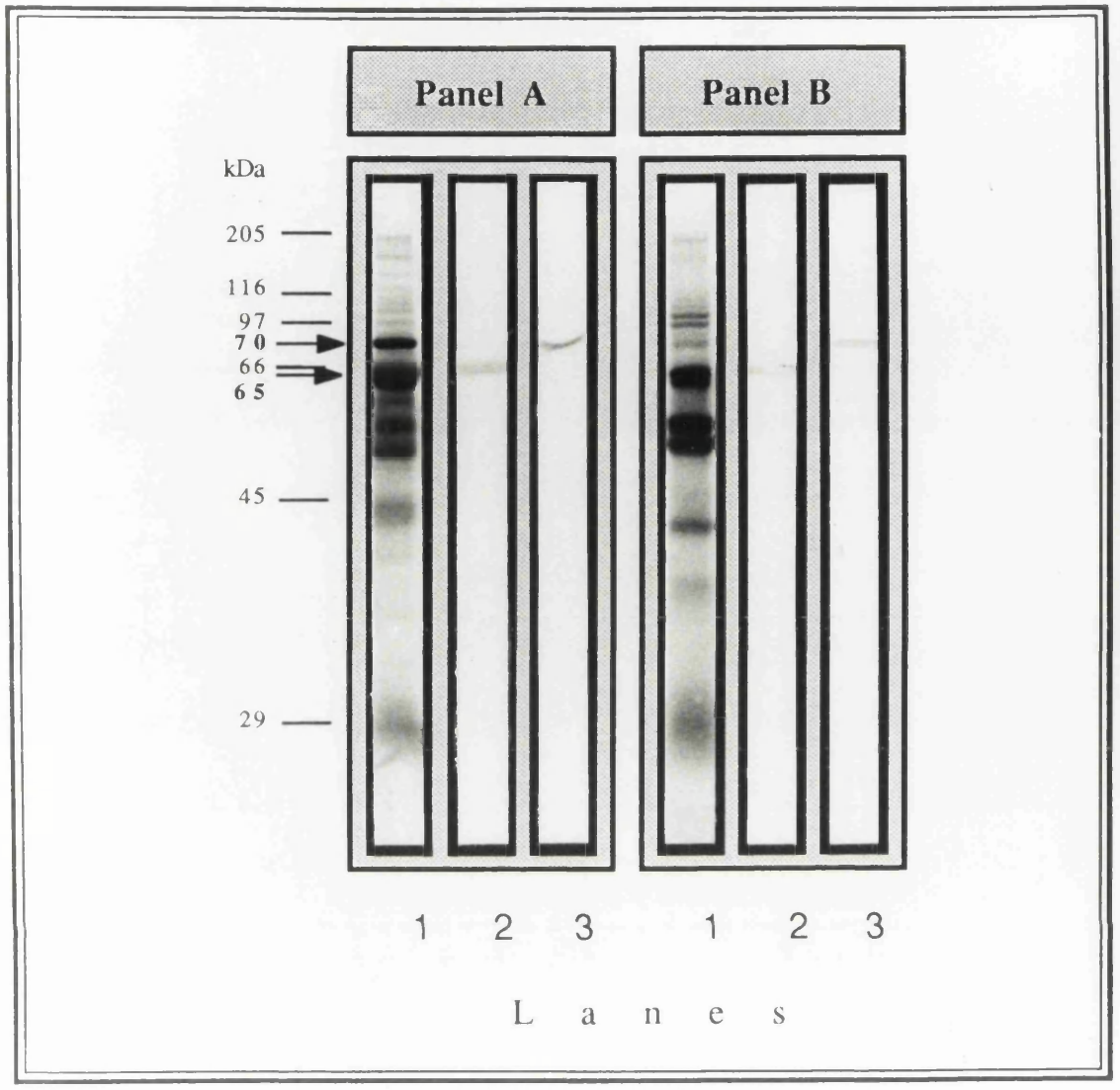


FIGURE 6.2: PRESENCE OF 65 AND 70 kDa PROTEINS IN RHEUMATOID ARTHRITIS SYNOVIAL FLUID (SF) AND IMMUNE COMPLEXES FROM SF.

Western blots of Panel A: synovial fluid
Panel B: immune complexes from synovial fluid

stained with Lane 1: ink
Lane 2: F67.13 (anti-65 kDa monoclonal antibody)
Lane 3: L7 (anti-70 kDa monoclonal antibody)

as described in section 2.2c.

The molecular weight markers are indicated on the left hand side of the figure.

i. Determination of the origin of the 65 kDa protein found in synovial fluid

The synovial fluid 65 kDa protein was further investigated since it could be either of bacterial or of human origin. In order to obtain more information about its origin, SF samples were run in an SDS-PAGE at the same time as a human (placental) and a mycobacterial 65 kDa hsps.

Tracks of each sample were stained with ink, SF8 (monoclonal antibody against the *M. tuberculosis* 65 kDa hsp and cross-reactive with the synovial fluid 65 kDa protein) and anti-P1 (polyclonal antibody against the 63 kDa protein from Chinese hamster ovary cells and cross-reactive with the corresponding 65 kDa protein from human placenta).

The SF8 and anti-P1 antibodies stained a band of approximately 65 kDa in molecular weight in the synovial fluid. In contrast, the same reagents stained a band of approximately 58 kDa in the placental preparation and a band of 62 kDa in the mycobacterial preparation (Figure 6.3).

However, it should be noted that although the above test in its present capacity could not conclusively prove the origin of the synovial 65 kDa hsp, it gave some interesting clues. The differences in the molecular weights of the three proteins and the support for a human origin of the synovial 65 kDa hsp are discussed later on.

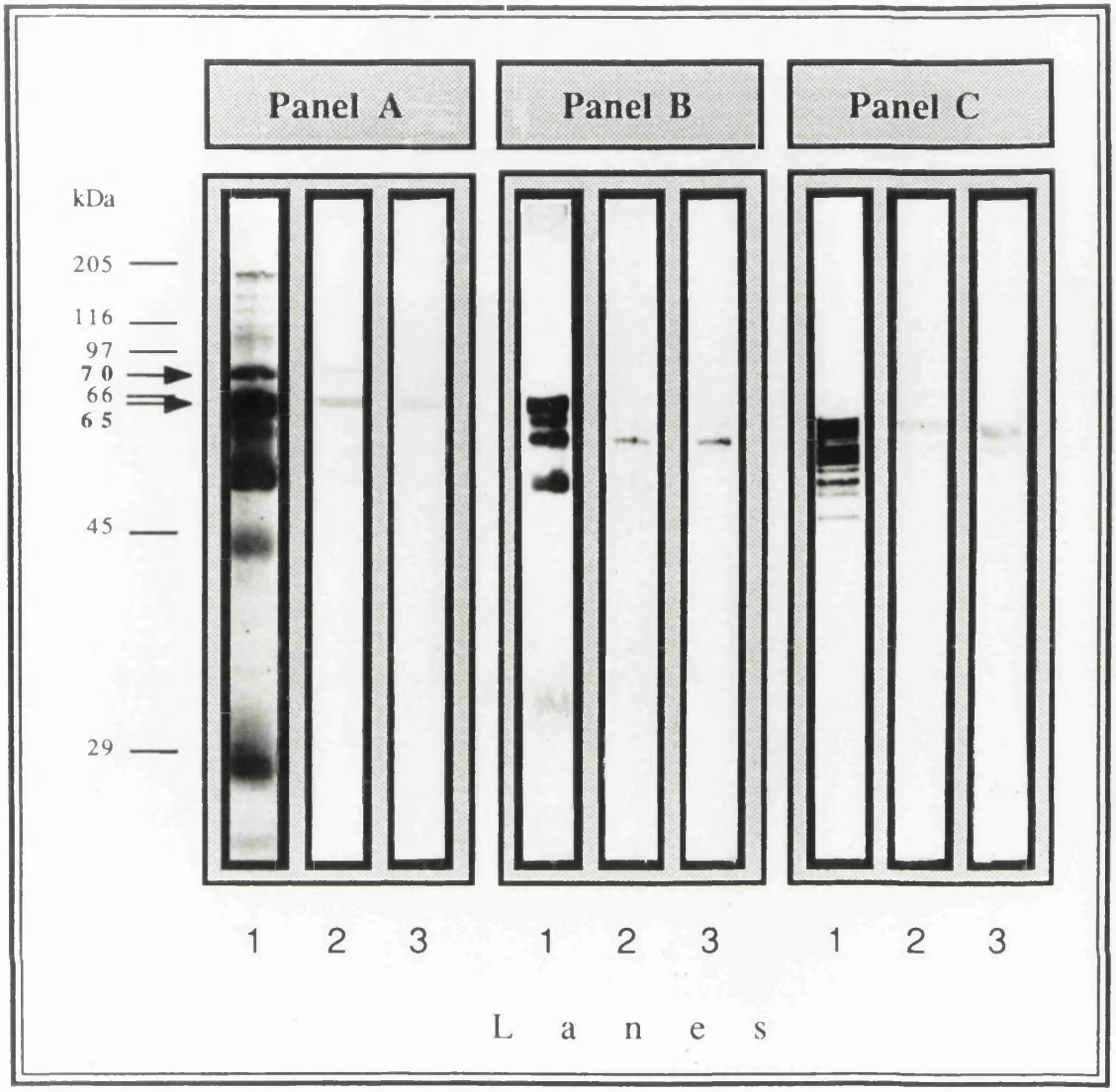


FIGURE 6.3: CLUES AS TO THE ORIGIN OF THE 65 kDa PROTEIN PRESENT IN RHEUMATOID ARTHRITIS SYNOVIAL FLUID.

Western blots of Panel A: synovial fluid
Panel B: human 65 kDa hsp
Panel C: mycobacterial 65 kDa hsp

stained with Lane 1: ink
Lane 2: SF8 (mouse anti-mycobacterial 65 kDa hsp monoclonal antibody cross-reactive with the synovial fluid 65 kDa protein)
Lane 3: anti-P1 (rabbit anti-hamster 63 kDa hsp polyclonal antibody cross-reactive with the human 65 kDa hsp)

as described in section 2.2c.

The molecular weight markers are indicated on the left hand side of the figure.

ii. Serum antibody levels to the synovial fluid 65 kDa protein

The possible autoreactivity towards the 65 kDa protein was further investigated by testing for the presence of antibodies to the synovial fluid 65 kDa by immunoblotting.

Sera from 77 RA patients, 17 patients with osteoarthritis (OA) and 67 healthy individuals were examined. RA synovial fluid samples, previously shown to contain the 65 kDa protein, were subjected to SDS-PAGE. Western blots were prepared and one track from each blot was ink stained to show the presence of approximately 23 protein bands ranging from 18-200 kDa in molecular weight. The remaining tracks were used for immunoblotting and the number of bands recognised by each serum was variable (Figure 6.4).

In order to identify possible specificities between antibody levels to a particular band and a test group, the results were examined by looking at differences in the percentage of donors that recognised a particular band between RA, OA and healthy individuals. There were no major differences between groups as to the number of donors having antibodies directed against a particular antigen. Sera from all three groups studied were found to bind to a 65 kDa as well as to a 70 kDa protein band.

Although the percentage of RA and OA patients having antibodies to the 65 kDa protein was not significantly different from the healthy control group, on visual inspection the intensity of the binding was consistently stronger in the majority of RA patients compared to the OA patients and healthy individuals studied.

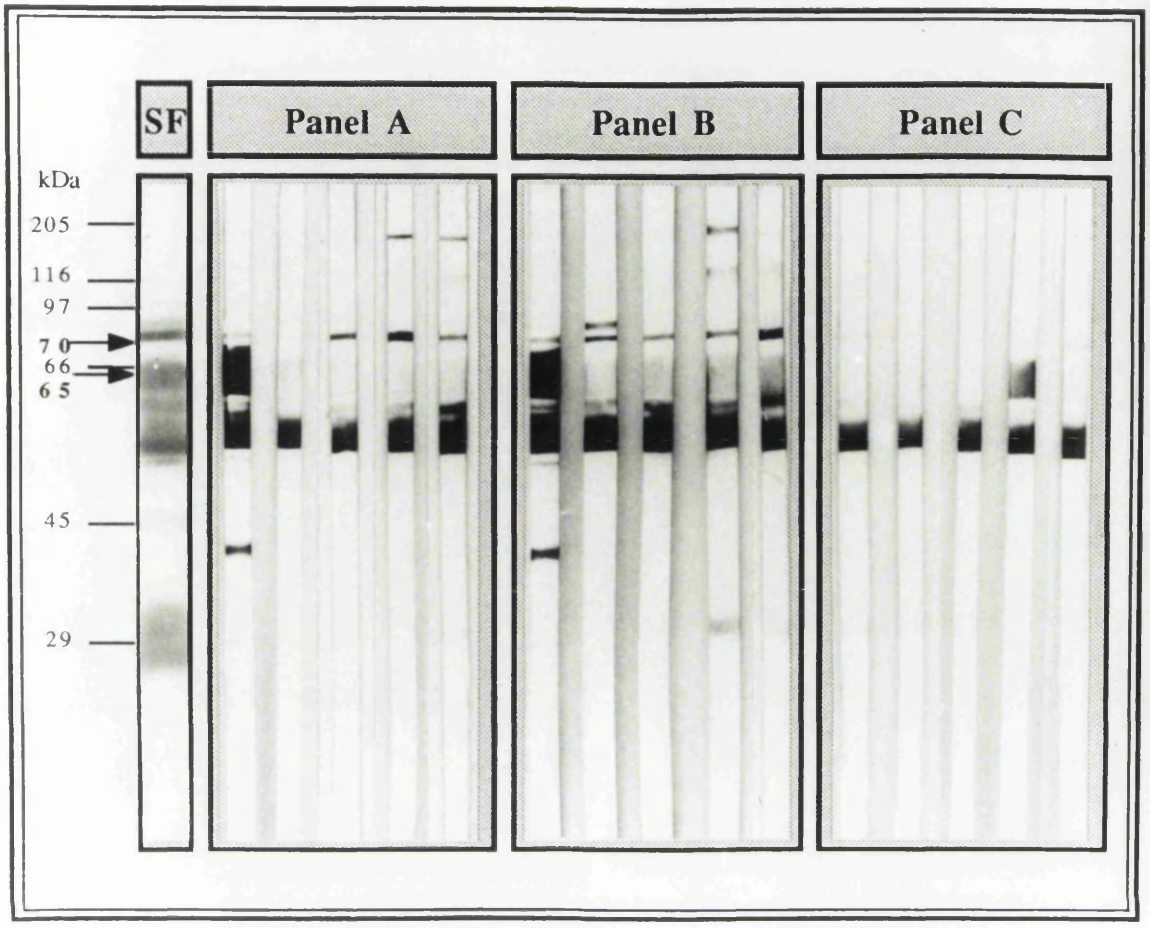


FIGURE 6.4: DETECTION OF ANTIBODIES TO RHEUMATOID ARTHRITIS (RA) SYNOVIAL FLUID ANTIGENS IN SERA FROM HEALTHY INDIVIDUALS (CTR), PATIENTS WITH RA AND OSTEOARTHRITIS (OA).

Western blots of RA synovial fluid stained with:

SF: ink
Panel A: CTR sera
Panel B: RA sera
Panel C: OA sera

as described in section 2.3b.

The sera were used at 1/40 dilution in PBS-0.05% Tween-1% BSA buffer and the second (indicator) antibody was goat anti-human total immunoglobulin $F(ab')_2$ fragment at 1/800 dilution.

The molecular weight markers are indicated in the left hand side of the figure.

d. Serum antibody levels to the human 70 kDa hsp in rheumatoid arthritis patients

Since the immunoblotting studies indicated that antibodies might also be present to the 70 kDa hsp, antibodies to a human preparation of the 70 kDa hsp were examined in 20 healthy individuals, 20 RA, 18 SLE, 15 AS and 13 Crohn's patients. The characteristics of these groups are shown in table 6.1.

i. IgA antibody levels

The RA sera showed increased IgA antibody levels to the human 70 kDa hsp in comparison with the levels of the healthy individuals ($p<0.000001$) (Figure 6.5a). Similarly, increased IgA antibody levels were found in SLE ($p<0.0001$) and AS ($p<0.001$) patients. However, the elevation of the IgA antibody levels in the RA sera was not higher than in SLE ($p>0.05$), although it was higher than in AS sera ($p<0.01$). In contrast, there was no increase of IgA antibody levels in the Crohn's sera (Figure 6.5a).

ii. IgG antibody levels

Interestingly, RA was the only group with significantly increased IgG antibody levels ($p<0.0001$) compared to the healthy control group levels (Figure 6.5b). Although 12/20 RA sera showed IgG levels higher than the mean control +2SD, only 2/18 SLE, none out of fifteen AS and 1/13 Crohn's sera did so.

iii. IgM antibody levels

The IgM antibody levels of the RA and Crohn's sera were similar to those of the healthy control group (Figure 6.5c). However, SLE and AS sera showed significantly lower antibody levels compared to the healthy control sera ($p<0.0001$) (Figure 6.5c).

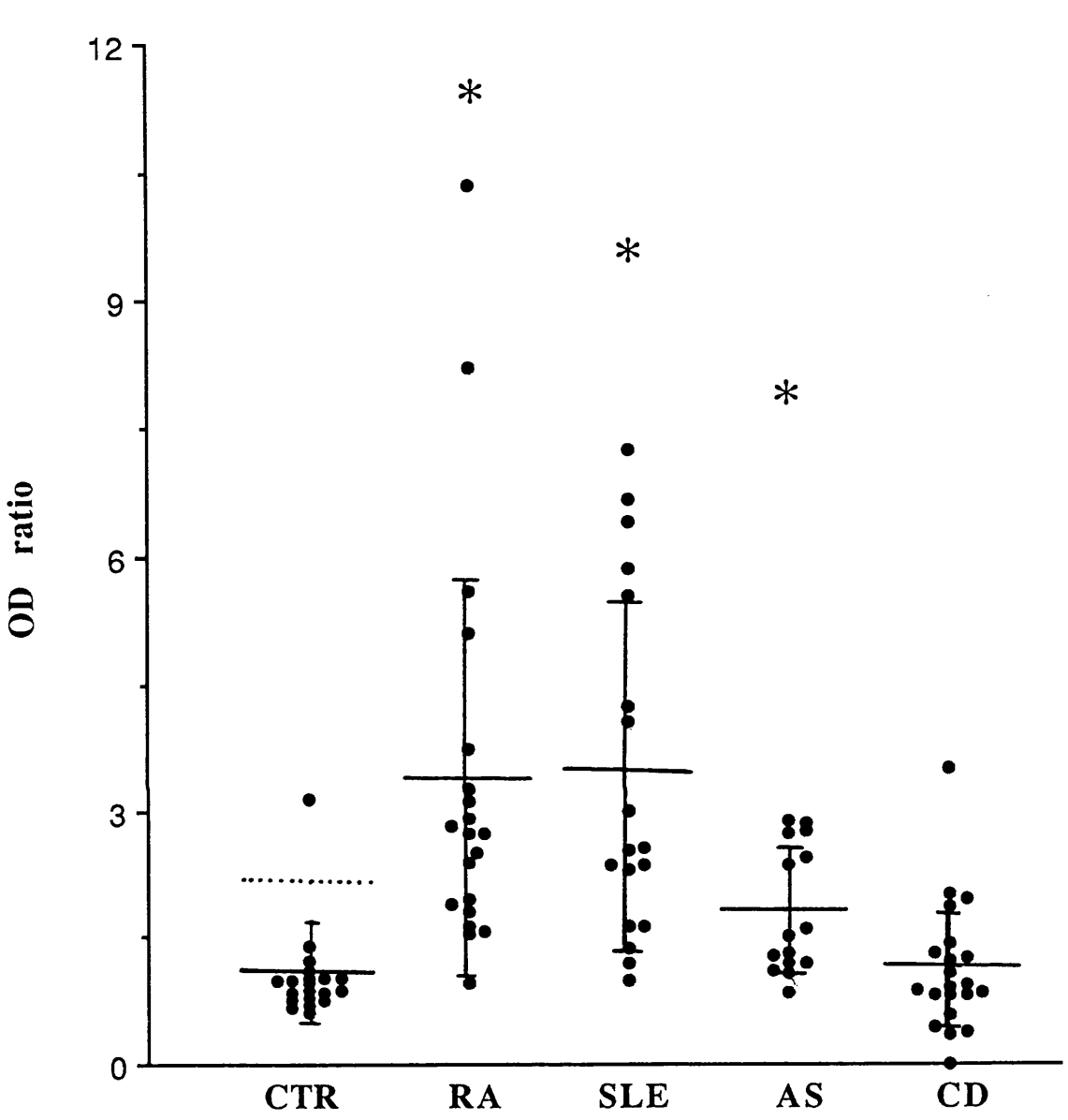


FIGURE 6.5a: IgA ANTIBODY LEVELS TO THE HUMAN 70 kDa HSP IN HEALTHY INDIVIDUALS (CTR), RHEUMATOID ARTHRITIS (RA), SYSTEMIC LUPUS ERYTHEMATOSUS (SLE), ANKYLOSING SPONDYLITIS (AS) AND CROHN'S DISEASE (CD) PATIENTS. The antibody levels are expressed as optical density ratios for each group (OD_{650} ratio = OD_{650} of test serum /OD_{650} of standard healthy serum used as a positive control). The horizontal lines represent the mean OD ratio for each group and the vertical lines represent the standard deviation (SD) for each group. The dotted lines define 2SD above the mean OD ratio of the control group. * $p<0.001$.

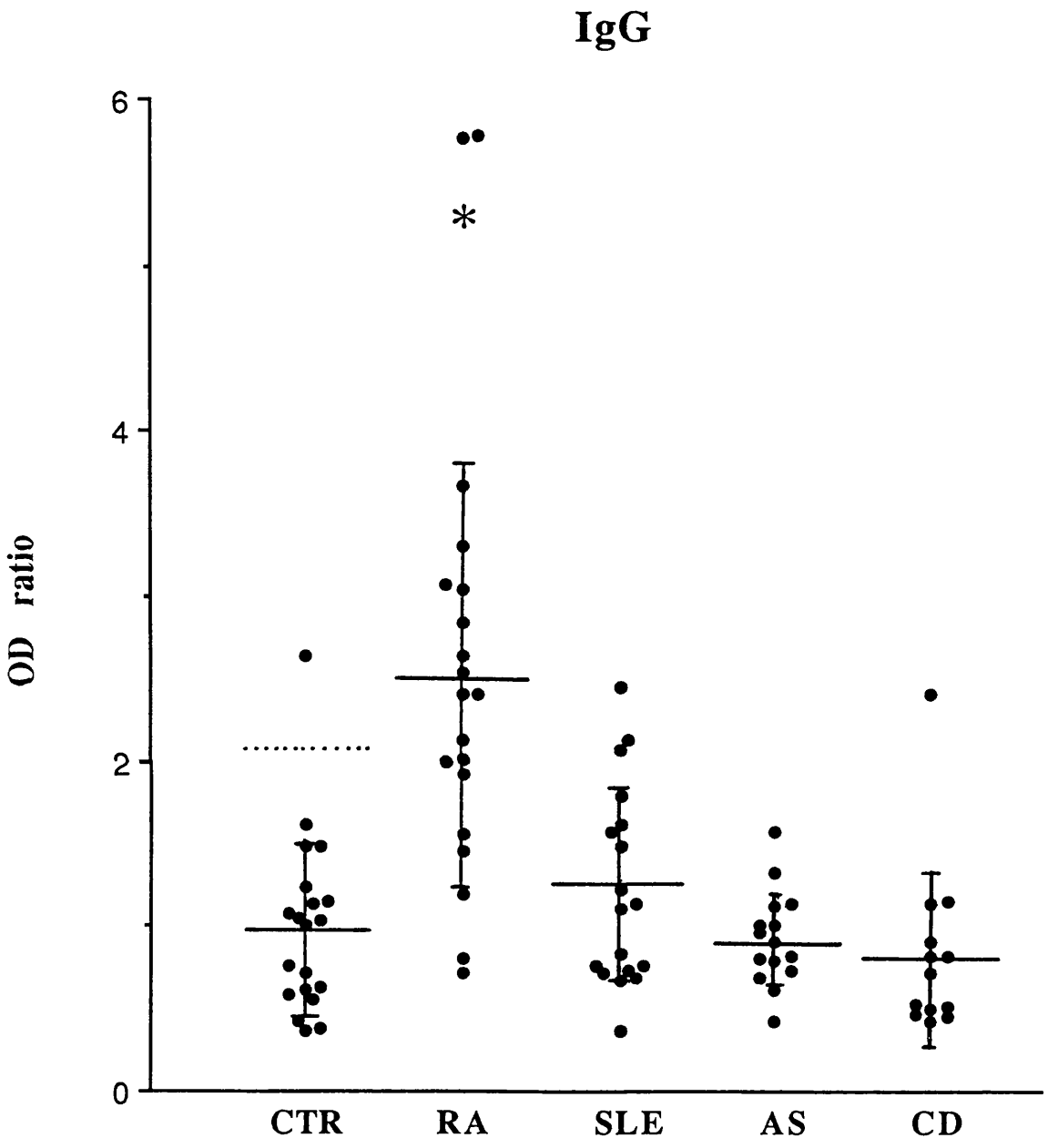


FIGURE 6.5b: IgG ANTIBODY LEVELS TO THE HUMAN 70 kDa HSP IN HEALTHY INDIVIDUALS (CTR), RHEUMATOID ARTHRITIS (RA), SYSTEMIC LUPUS ERYTHEMATOSUS (SLE), ANKYLOSING SPONDYLITIS (AS) AND CROHN'S DISEASE (CD) PATIENTS. The data are presented as in figure 6.5a. * $p < 0.0001$.

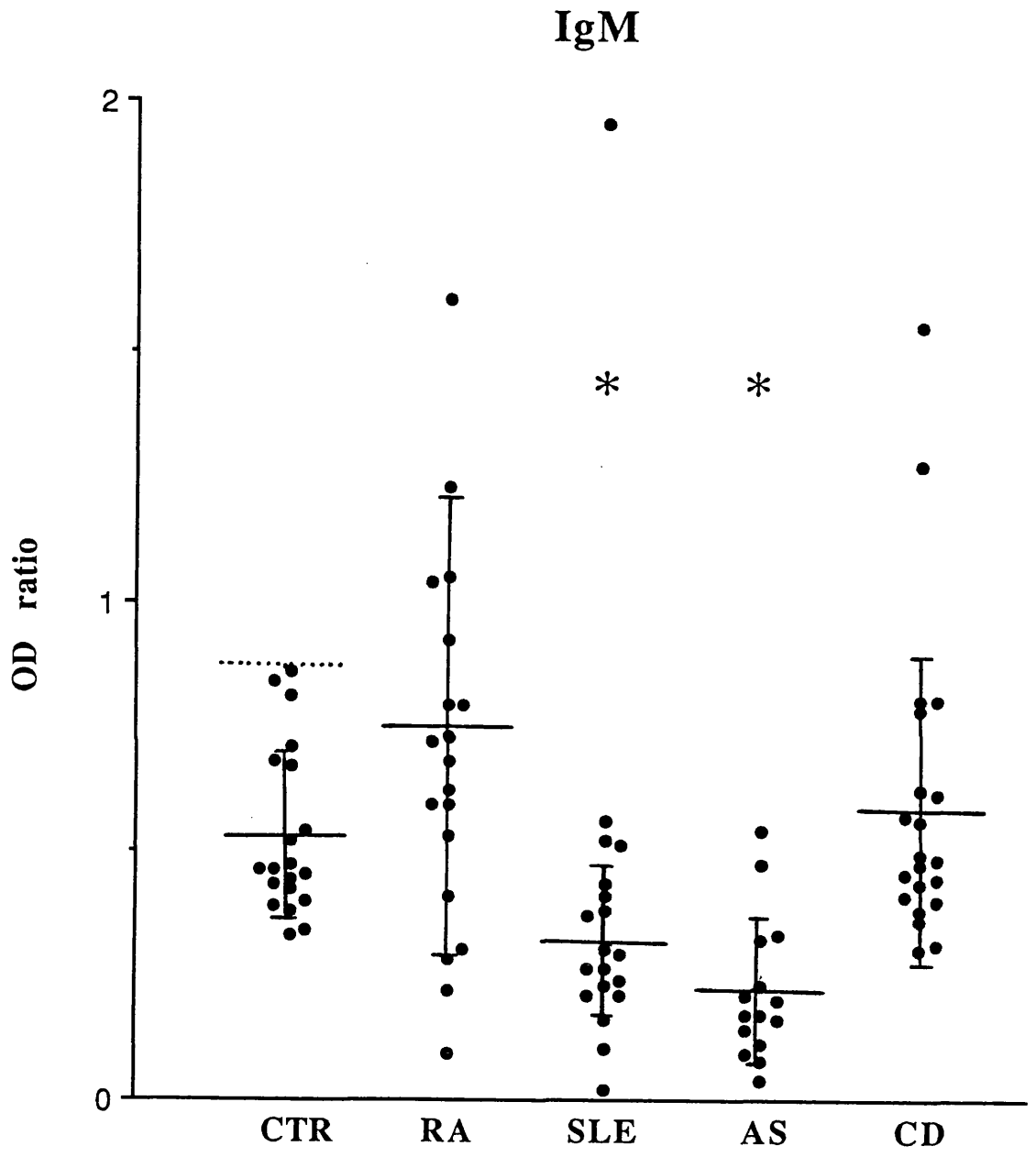


FIGURE 6.5c: IgM ANTIBODY LEVELS TO THE HUMAN 70 kDa HSP IN HEALTHY INDIVIDUALS (CTR), RHEUMATOID ARTHRITIS (RA), SYSTEMIC LUPUS ERYTHEMATOSUS (SLE), ANKYLOSING SPONDYLITIS (AS) AND CROHN'S DISEASE (CD) PATIENTS. The data are presented as in figure 6.5a. * $p<0.0001$.

6.4 DISCUSSION

The presence of a 65 kDa protein has been demonstrated in RA synovial fluid samples. However, it is not known whether this synovial fluid 65 kDa protein is a heat shock protein and the origin of this protein is also unknown.

Assuming that it is a heat shock protein, the synovial fluid 65 kDa protein can be either of microbial (mycobacterial or not) origin or of human origin (self-hsp). In the first case, a microbial infection can place the microbe in the hostile environment of the host and result in the synthesis of microbial hsps (Christman *et al.*, 1985). In the second case, the host may synthesise hsps as a result of an infection (Polla, 1988).

In addition, the inflamed human joint is an environment rich in oxygen free radicals (Blake *et al.*, 1987), which have been identified as inducers of heat shock proteins (Polla, 1988). The synovial joint because of its anatomical location and function is subject to a variety of other stresses as well, including exposure to elevated temperatures either due to joint motion (Tepic *et al.*, 1985) or due to inflammation (Polla, 1988). It seems reasonable, therefore, to assume that the 65 kDa protein is one of the proteins synthesised due to the above hsp inducers.

According to the SDS-PAGE analysis (Figure 6.4), the synovial fluid 65 kDa hsp had a molecular mass slightly higher than that of the corresponding protein purified from the human placenta and of the mycobacterial 65 kDa hsps. Although it was theoretically possible that the 65 kDa protein band identified in the SF was not of human origin, there would have to be a large amount of mycobacterial or other microbial material to account for it in the joint. However, there has been only one report on

parvovirus-like agents been isolated from synovial tissue of patients with severe RA, but not of patients with osteoarthritis (Simpson *et al.*, 1984). This seemed to be an unlikely explanation for the presence of 65 kDa hsp in the synovial fluid samples, in view of the presence of the 65 kDa hsp in both RA and osteoarthritic synovial fluid samples.

There are other possible explanations for the molecular weight discrepancy between the synovial and the placental 65 kDa hsps. For example, the molecule in the joint might be in a different form from that in the placenta. It is not yet known how many forms of this molecule are present in man, although there is evidence for different isoforms and for the existence of two alleles (Waldinger *et al.*, 1988).

Unpublished observations by Sharif, M. and Rook, G. (Medical Microbiology Department, University College and Middlesex School of Medicine; personal communication) have shown, using the same reagents as in this study, that the SF8 monoclonal antibody bound consistently more strongly to the synovial fluid 65 kDa hsp than to the placental preparation. However, exposure of the placental 65 kDa to free radical damage (hypochlorite) enhanced the binding. It was not clear whether those observations merely reflected the fact that the SF8 was selected using RA synovial fluid or whether the cross-reactivity between the synovial and placental 65 kDa hsps was enhanced by the oxidative damage, which might suggest that the 65 kDa hsp in the synovial fluid existed in such a form.

The inflamed human joint is an environment where oxidative damage appears to occur and an oxidative damaged form of the 65 kDa hsp cannot be excluded, as already has been shown

for joint-derived IgG (Blake *et al.*, 1989).

The free radical damage of joint-derived IgG molecule has been shown to result in antigenicity and reactivity with rheumatoid factor (Blake *et al.*, 1989). It is therefore possible that such an alteration could induce the 65 kDa hsp to become a stimulus for formation of immune complexes, thereby promoting and amplifying tissue damage during rheumatoid inflammation. In fact, the presence of this protein has been confirmed in immune complexes from synovial fluids and the notion that some of the IgG and possibly IgA antibodies to mycobacterial 65 kDa hsp cross-react and complex with human 65 kDa hsp is favoured. Thus, complexing of these antibodies with autoantigen in the synovium could result in decreased availability for free 65 kDa hsp antibody. This might be the explanation for the decreased antibody levels to the mycobacterial 65 kDa hsp found in synovial fluid samples as compared to paired serum samples in RA patients.

Immune complexes isolated from synovial fluid of RA patients have been extensively analysed by others (Male and Roitt, 1979; Male *et al.*, 1980) and found to consist predominantly of immunoglobulin and complement components. Although 96% of the constituents in the complexes have been characterised, 4% remained unidentified. It is possible that one of the remaining components is a joint antigen (an hsp or not), a microbial antigen (an hsp or not), or a self-antigen which cross-reacts with a microbial antigen. The 65 kDa protein molecule, being a heat shock protein and showing a high sequence homology between mammalian and microbial homologues (Jindal *et al.*, 1989), would be a suitable candidate. The prospect of such a possibility being

true is very exciting and could explain in part the autoimmune character of RA.

The presence of the 65 kDa hsp in the RA synovial fluid was consistent with its abundant localisation in synovial tissue (De Graeff-Meeder *et al.*, 1989; Karlsson-Parra *et al.*, 1990). Studies with monoclonal antibodies indicated an intracellular distribution of the 65 kDa hsp particularly in the cartilage-pannus junction of human RA synovial joints (Karlsson-Parra *et al.*, 1990) and a prominent staining of synovial lining cells in RA and juvenile RA synovial joints (De Graeff-Meeder *et al.*, 1989). However, in the study by Karlsson-Parra *et al.* (1990) it appeared that at least some of the cells stained positively for 65 kDa hsp were macrophages, which was in contrast with the mitochondrial pattern expected for the 65 kDa hsp (Gupta and Austin, 1987).

In view of the predominantly intracellular distribution of the 65 kDa hsp, the question is how the 65 kDa hsp is found in the synovial fluid. The 65 kDa hsp may readily leak into the synovial fluid from damaged or dead synovial fluid or joint cells (Lydyard *et al.*, 1990) and can become complexed with antibodies, thus contributing to the chronic inflammation of the joint.

It has been shown that 65 kDa hsp reactive T cells can lyse monocytes either pulsed with 65 kDa hsp or non-pulsed but activated (Ottenhoff *et al.*, 1988). Therefore, it is possible that 65 kDa hsp reactive cells exert lytic effects on synovial tissue cells which contain the 65 kDa hsp, hence releasing the protein.

The presence of the 65 kDa hsp in the RA synovial fluid was also consistent with the higher T cell reactivity to the mycobacterial 65 kDa hsp found in the RA synovial fluid cells

compared with the peripheral blood mononuclear cells (Res *et al.*, 1988; Gaston *et al.*, 1989a).

The increased synovial fluid T cell reactivity to this protein, the increased serum antibody levels to it found in RA patients and the presence of it in the synovial fluid mean that this protein might be of pathogenic significance in RA patients. However, the occurrence of 65 kDa hsp in osteoarthritic synovial fluid samples and in fluid from traumatic knees of otherwise healthy individuals argues against an aetiological role for this protein in rheumatoid arthritis.

In spite of the above observations, a pathogenetic role for the 65 kDa hsp in RA cannot be excluded. Furthermore, such a role might also apply to other inflammatory joint diseases. However, other factors could also be involved, which could explain the responses to the 65 kDa hsp in RA.

The antibodies SF8 and anti-P1 used for the identification of 65 kDa hsp in this study were frequently found to bind to bands with multiple molecular weights during Western blotting. The most likely origin of the multiple banding was that the original protein of about 65 kDa underwent progressive proteolytic degradation to multiple fragments each containing a subset of the total antibody binding sites of the full length molecule (Young *et al.*, 1987). A further peculiarity of these antibodies was their ability to bind, although weakly, to IgG heavy chains as well as to the hsp they were directed against. In view of the role of IgG in the autoimmunity of RA (Pope and McDaffy, 1979; Fong *et al.*, 1985), this was quite provocative. Other groups, however, have also shown binding of 65 kDa monoclonal antibodies to SDS-denatured IgG (McLean *et al.*, 1988). Such a cross-reactivity

deserves further analysis, since a highly significant homology was found between a 62 amino acid sequence of the human 65 kDa hsp and a constant domain of the Ig delta chain in the rat (Sire *et al.*, 1982).

Interestingly, RA sera were found to have antibodies directed against the joint 65 kDa hsp. If the 65 kDa hsp was indeed human, these antibodies would be autoantibodies. The production of the autoantibodies arises presumably through breakdown of tolerance to cross-reactive epitopes on the 65 kDa hsp.

Furthermore, in the adjuvant arthritis model, T cells responding to the mycobacterial 65 kDa hsp cross-reacted with the 'unrelated' proteoglycan core protein (van Eden *et al.*, 1985). Hence, it might also be possible for specific anti-65 kDa antibodies to cross-react with other autoantigens in the joint. This implies that mimicked amino acid sequences on other autoantigens can also be the target of the mycobacterial 65 kDa humoral response.

Antigenic proteins similar or closely related to the 65 kDa antigen are also present in a wide variety of other bacteria including *Klebsiella, Shigella*, *Yersinia* and *Campylobacter* species (Thole *et al.*, 1988a), which are associated with human arthritis (Hoiby, 1975; Aho *et al.*, 1985). More interestingly, evidence has been presented by other groups about a cross-reactivity between EBNA-1 (Epstein-Barr nuclear antigen) and a 62 kDa cellular protein present in RA synovium (Fox *et al.*, 1985; Luka *et al.*, 1984). If the 62 kDa in the synovial tissue is in fact an hsp, the possible role of EBV in the aetiology of RA could be studied through a different approach (Lydyard and Irving,

1988).

However, the findings that affinity-purified antibodies to the mycobacterial 65 kDa hsp were able to bind to a human 65 kDa hsp molecule, the presence of the latter free and complexed with antibody in the SF, argued that these antibodies could contribute to pathogenesis in RA and that the human 65 kDa hsp homologue could be one of the antigens involved in the autoimmune responses in RA. However, other functions must clearly be important because antibodies to the synovial fluid 65 kDa hsp were also found in patients with osteoarthritis.

The presence of a 70 kDa band in the synovial fluid and immune complex samples from RA patients and the presence of antibodies to this synovial fluid protein suggested that the autoreactivity could also be directed against the 70 kDa protein. Moreover, IgG serum antibodies to a human preparation of 70 kDa hsp were specifically elevated in RA patients compared to levels of the healthy individuals, SLE, AS and CD patients.

However, the autoreactivity against the 70 kDa hsp was not limited to RA since the IgA antibody levels to this protein were similarly elevated in SLE patients. Minota *et al.* (1988a) have shown the presence of IgG and IgM autoantibodies to a constitutively expressed human 70 kDa hsp in SLE sera. Also, the 70 kDa hsp has been shown by Kubo *et al.* (1985) to be spontaneously synthesised by chondrocytes in severe osteo-arthritic patients compared with healthy chondrocytes. This seems to be a possible explanation for the presence of the 70 kDa protein in osteoarthritic and RA synovial fluid samples. The antibodies to the synovial 70 kDa protein in OA patients, in conjunction with the existence of the 70 kDa protein itself, could

indicate the presence of complexes in the synovial fluid of these patients. However, complexes from OA patients were not tested for the presence of hsps.

Although a number of suggestions have been presented as to the origin and accumulation of the 65 and 70 kDa hsps in synovial fluid, a pathogenetic role of these proteins has not yet been established. It is important to determine whether the presence of these proteins and the antibodies against them are the cause of the disease (primary mechanism) or a consequence of the disease (secondary mechanism). The presence of these proteins in both traumatic (in knees from healthy individuals) and diseased (in RA and OA patients) synovial fluid argued in favour of a role for maintaining and perpetuating rather than causing the disease. However, that responses to the 65 kDa protein may be associated with the primary event was suggested by the finding that T cells responsive to it were present in the joint particularly in early disease (Res *et al.*, 1988).

Since the 70 kDa hsp has been found on the cell surface of B cells and macrophages of joint effusions of RA patients (Jarjour *et al.*, 1989) and has been shown to be involved in antigen presentation (Vanbuskirk *et al.*, 1989), its role in RA might be different to that of the 65 kDa hsp. The cell surface expression would enable viable cells to stimulate an autoreactive response or, conversely, become targets of immunologic attack.

However, further experiments are required to determine the role of these hsps in the aetiopathogenesis of RA.

CHAPTER 7 PERIPHERAL BLOOD MONONUCLEAR CELL PROLIFERATIVE RESPONSES TO MYCOBACTERIAL ANTIGENS IN RHEUMATOID ARTHRITIS PATIENTS

Introduction

Characteristics of study groups in chapter 7

Results

- Proliferative responses of peripheral blood mononuclear cells from healthy individuals and rheumatoid arthritis patients to the WE antigen
- Estimation of precursor frequency of WE reactive PBMC from healthy individuals and rheumatoid arthritis patients
- Proliferative responses of peripheral blood mononuclear cells from healthy individuals and rheumatoid arthritis patients to the recombinant *M. bovis* 65 kDa hsp
- Comparison of peripheral blood mononuclear cell reactivity to the WE and to the mycobacterial 65 kDa hsp antigens

Discussion

7.1 INTRODUCTION

In view of the interesting antibody responses to mycobacterial antigens in rheumatoid arthritis patients (Chapters 3, 4, 5 and 6) and the implication of mycobacteria-specific T cell clones in the pathogenesis of inflammatory arthritis in experimental animals (van Eden *et al.*, 1988), a pilot study was undertaken to examine whether cellular responses to mycobacterial antigens were different in rheumatoid arthritis patients compared to healthy individuals.

The cellular reactivity of peripheral blood mononuclear cells to a water extract of *Mycobacterium tuberculosis* (WE) and to the recombinant 65 kDa heat shock protein of *Mycobacterium bovis* was examined in healthy individuals (CTR) and rheumatoid arthritis patients (RA).

The WE was chosen as it contains a broad range of mycobacterial antigens including the 65 kDa protein (Chapter 4). The *M. bovis* 65 kDa heat shock protein (hsp) was used due to the preferential humoral reactivity to it found in RA patients (Chapter 4; Tsoulfa *et al.*, 1989a) but also since it has previously been implicated in the pathogenesis in the adjuvant arthritis model.

The cellular reactivity was assessed by proliferation of the mononuclear cells as measured by

^{125}Iodo-uridine-deoxyribose incorporation. The study of the cellular reactivity to the WE antigen included the estimation of the precursor frequency of WE reactive cells by limiting dilution analysis.

7.2 CHARACTERISTICS OF STUDY GROUPS IN CHAPTER 7

TABLE 7.1: Characteristics of rheumatoid arthritis patients (RA) and healthy individuals (CTR) studied for:

A: Peripheral blood mononuclear cell responses to the WE antigen.
B: The estimation of WE reactive mononuclear cells.
C: Peripheral blood mononuclear cell responses to the *M.bovis* 65 kDa hsp.

	A		B		C	
	RA	CTR	RA	CTR	RA	CTR
Number of donors	20	20	16	11	26	12
Male	7	10	5	7	10	5
Female	13	10	11	4	16	7
Average Age (Years)	58	35	59	37	65	34
(range)	20 - 74	23 - 50	25 - 76	25 - 50	20 - 76	27 - 44
Activity of disease (1)		Not applicable		Not applicable		Not applicable
Active	7		5		11	
Moderately active	9		9		9	
Inactive	4		2		6	
Treatment (2)						
First-line drugs	8		8		11	
Second-line drugs	5		4		5	
Third-line drugs	2		2		3	
No treatment	5		2		6	
Seropositivity (3)		Not known		Not known		Not known
Seropositive	15		11		18	
Seronegative	5		5		8	

Footnotes: (1) Activity was assessed as shown in section 2.5.

(2) First-line drugs: non-steroidal anti-inflammatory drugs (e.g. indomethacin, naproxen)
Second-line drugs: disease-modifying drugs (e.g. gold, penicillamine, sulphasalazine)
Third-line drugs: prednisolone, azathioprine, methotrexate.

(3) Seropositivity was assessed by latex agglutination test.

7.3 RESULTS

The peripheral blood mononuclear cell (PBMC) responses to the WE antigen, to the mycobacterial 65 kDa hsp and to phytohaemagglutinin (PHA) were based on the proliferation of 10^5 PBMC/well and were measured by ^{125}I-UdR incorporation at the last 18 hours of a five day culture period.

Pilot experiments, where the response of different cell concentrations (from 10^6 to 10^4 PBMC/well) was measured, had shown that responses of 10^5 PBMC/well were optimal.

In addition, the 5 day culture period was based on time-course experiments (from day 2 to day 7) which showed that although for the WE and the mycobacterial 65 kDa hsp antigens the optimal responses were recorded at day 5, for the PHA the peak proliferative response was at day 3 of the culture period. Control experiments showed that the PHA induced proliferation measured at the end of a 5 day culture period represented about 60% of that seen at 3 days. This should be taken into consideration when responses to the mycobacterial antigens are compared to those induced by PHA.

The preliminary experiments were carried out using PBMC from both healthy individuals and rheumatoid arthritis patients.

a. Proliferative responses of peripheral blood mononuclear cells from healthy individuals and rheumatoid arthritis patients to the WE antigen

PBMC proliferative responses to different concentrations of the WE antigen (2 μg/ml-100 μg/ml) were studied in 20 healthy individuals and 20 RA patients. The characteristics of the groups studied are shown in table 7.1. The

proliferative response was measured by ^{125}I-UdR incorporation and expressed as mean counts per minute (cpm) of at least four replicate wells.

Figure 7.1 shows the PBMC proliferative responses of a random selection of CTR and RA individuals to antigen concentrations of 2, 5, 10, 25, 50 and 100 μg/ml. Although the antigen concentration giving the highest proliferative response, in terms of counts per minute, was not the same for all individuals tested, in the majority of them (i.e. in 13/20 CTR and 16/20 RA individuals) the highest response was obtained by 10 μg/ml of the WE antigen. For this reason, the proliferative responses of PBMC to 10 μg/ml of WE antigen were used to compare cellular responses in the CTR and RA groups. These responses are shown in figure 7.2.

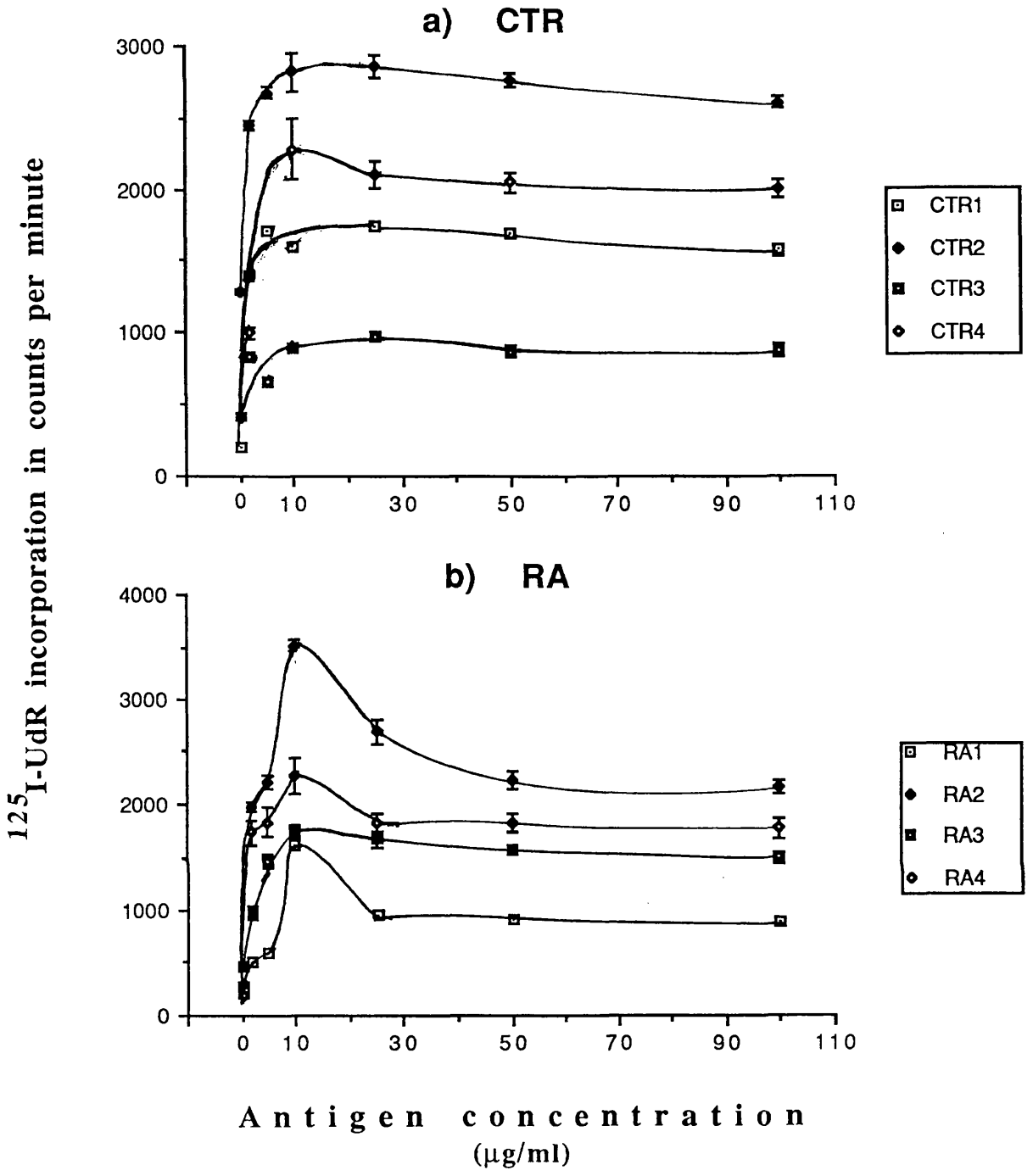


FIGURE 7.1: PERIPHERAL BLOOD MONONUCLEAR CELL PROLIFERATIVE RESPONSES TO DIFFERENT CONCENTRATIONS OF THE WE ANTIGEN IN HEALTHY INDIVIDUALS AND RHEUMATOID ARTHRITIS PATIENTS.

a) PBMC responses of four healthy individuals (CTR).

b) PBMC responses of four rheumatoid arthritis patients (RA).

The proliferative response of 10^5 PBMC/well was measured by ^{125}I-UdR incorporation on day 5 of the culture period and expressed as mean counts per minute of at least four replicate wells.

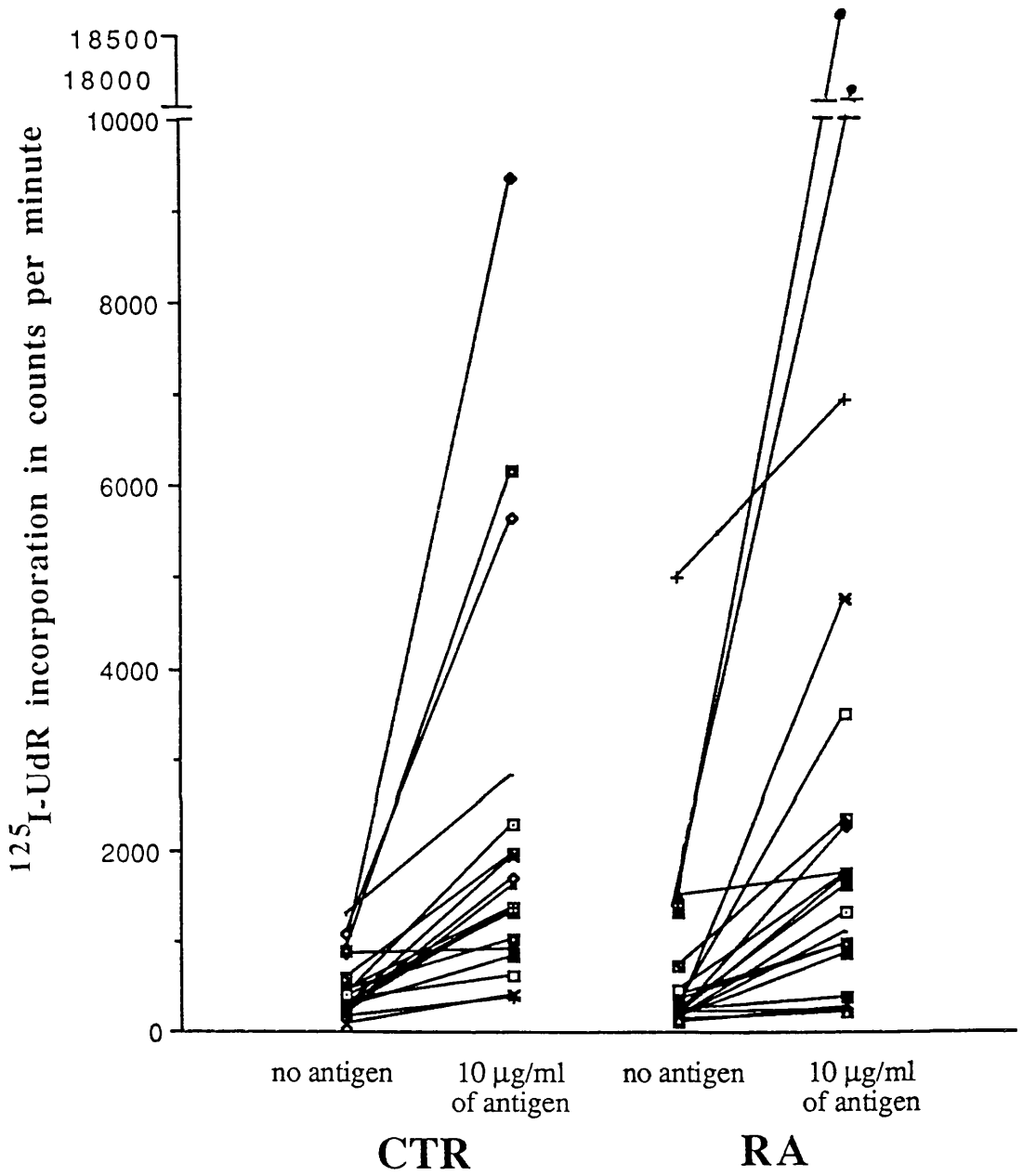


FIGURE 7.2: PROLIFERATIVE RESPONSES OF PERIPHERAL BLOOD MONONUCLEAR CELLS FROM HEALTHY INDIVIDUALS (CTR) AND RHEUMATOID ARTHRITIS PATIENTS (RA) TO THE WE ANTIGEN. The proliferative response of 10^5 peripheral blood mononuclear cells/well from 20 healthy individuals and 20 rheumatoid arthritis patients to 10 µg/ml of the WE antigen was measured by ^{125}I-UdR incorporation on day 5 of the culture period and expressed as mean counts per minute of at least four replicate wells.

The comparison of PBMC proliferative responses to 10 μg/ml of WE antigen in 20 CTR and 20 RA individuals was carried out as following:

a) If responsiveness was arbitrarily defined as one giving a stimulation index of ≥ 5 (SI: the ratio of mean cpm in the presence of antigen divided by the mean cpm without antigen), then 8/20 CTR and 10/20 RA individuals responded to the WE antigen. The mean PBMC proliferative responses of the CTR and RA 'responders' was not significantly different (mean SI of the 8 CTR=7.02±1.58, mean SI of the 10 RA=11.11±5.16, p=0.075).

b) If responsiveness was arbitrarily defined as one where the mean cpm of the test sample (cells plus antigen) was greater or equal to the mean cpm +3SD of the control sample (cells without antigen), then 18/20 CTR and 18/20 RA individuals responded to the WE antigen. The mean PBMC proliferative responses of the CTR and RA 'responders' was not significantly different (mean SI of the 18 CTR=4.90±2.28, mean SI of the 18 RA=7.20±5.89, p=0.57).

Similarly, when the highest proliferative response in terms of counts per minute (irrespective of the antigen concentration to which that response was attributed) was used to compare PBMC responses in CTR and RA groups, the overall responses in the two groups were still no different (mean SI of 20 CTR=4.77±2.69, mean SI of 20 RA=6.90±5.71, p=0.42).

For 4 rheumatoid arthritis patients, synovial fluid samples as well as peripheral blood samples were available. Therefore, the responses of PBMC and SFMC to the WE antigen were examined. It was found that for all antigen concentrations the SFMC responses were higher than those of PBMC in all four

patients. The PBMC and SFMC responses to 10 μg/ml of the WE antigen are shown in table 7.2.

TABLE 7.2: Proliferative responses of peripheral blood mononuclear cells (PBMC) and synovial fluid mononuclear cells (SFMC) to the WE antigen in rheumatoid arthritis patients (RA).

	^{125}I-UdR incorporation in counts per minute			
	PBMC		SFMC	
	no antigen	10 μg/ml of antigen	no antigen	10 μg/ml of antigen
RA 1	502±24	697±39	1103±44	4081±37
RA 2	119±9	260±19	244±15	2293±64
RA 3	161±13	860±37	257±14	1490±19
RA 4	235±16	1613±49	498±23	5577±112

Footnote: The proliferation of 10^5 mononuclear cells was measured by ^{125}I-UdR incorporation and is presented as mean counts per minute of at least four replicate cultures.

Estimation of precursor frequency of WE reactive PBMC from healthy individuals and rheumatoid arthritis patients

To investigate further the cellular immune reactivity to the WE antigen in healthy individuals and RA patients, the number of PBMC responding to the WE antigen was estimated by limiting dilution analysis (LDA).

A pilot LDA study was undertaken with 10 individuals who previously showed responses to 10 μg/ml of the WE antigen in cultures of 10^5 PBMC/well. Ten replicate cultures with cell concentrations of $5x10^4$, 10^4, 10^3 and 10^2 PBMC/well were set up with and without antigen and in the presence of different numbers of γ-irradiated autologous mononuclear cells (from 10^4 to 10^5 cells per well). Responsiveness was arbitrarily defined as one giving counts per minute higher than the mean

cpm+3SD of the same cell concentration without antigen. It was found that while the $5x10^4$ PBMC/well cell concentration gave a positive response in all individuals, the 10^2 PBMC/well cell concentration gave a positive response in only one individual. This indicated that cell concentrations $\geq 10^2$ PBMC/well and $<5x10^4$ PBMC/well could be appropriate for the estimation of the frequency of the WE reactive cells. Therefore, cell concentrations of 10^2, $5x10^2$, $2.5x10^3$ and $1.25x10^4$ were chosen for this study. The 10^2 cell concentration was included in anticipation of obtaining a clone for further analysis. In addition, the above concentrations of the γ-irradiated autologous mononuclear cells did not influence the number of responding cultures and the concentration chosen for the following experiments was $3x10^4$ cells per well.

To ensure that the number of PBMC used was sufficient to produce a measurable response, the response of the different cell concentrations to phytohaemagglutinin (1μg/ml) was measured for each individual tested. The PHA responses of the four PBMC concentrations (in ascending order) were found to range from 980 to 12000 cpm which were equivalent to stimulation indices of 5 and 42 respectively.

The estimation of precursor frequency of WE reactive PBMC was carried out in peripheral blood samples from 11 healthy individuals and 16 RA patients (the characteristics of these groups are shown in table 7.1). The antigen induced mononuclear cell activation was measured visually by phase contrast microscopy and confirmed in some assays by ^{125}I-UdR incorporation as a parameter for stimulation on day 9 of the culture period. The preferential examination of the cultures by

phase contrast microscopy was aimed at using any interesting cultures for further analysis.

Figure 7.3 shows the semi-logarithmic plot used for the estimation of the precursor frequency for three of the individuals tested. A summary of the estimated frequencies for the total number of healthy individuals and RA patients examined is presented in table 7.3. The frequencies observed in the CTR group ranged from 1/16100 to 1/3000, while the frequencies of the RA group ranged from 1/13700 to 1/1140. However, the mean frequencies of the WE reactive PBMC of the CTR (1/8000) and RA (1/7400) groups were not significantly different ($p=0.81$) (Table 7.3).

It should be noted that although individuals with similar precursor frequencies did not necessarily give similar stimulation indices, overall in both CTR and RA groups the frequencies and stimulation indices showed some correlation (Table 7.3).

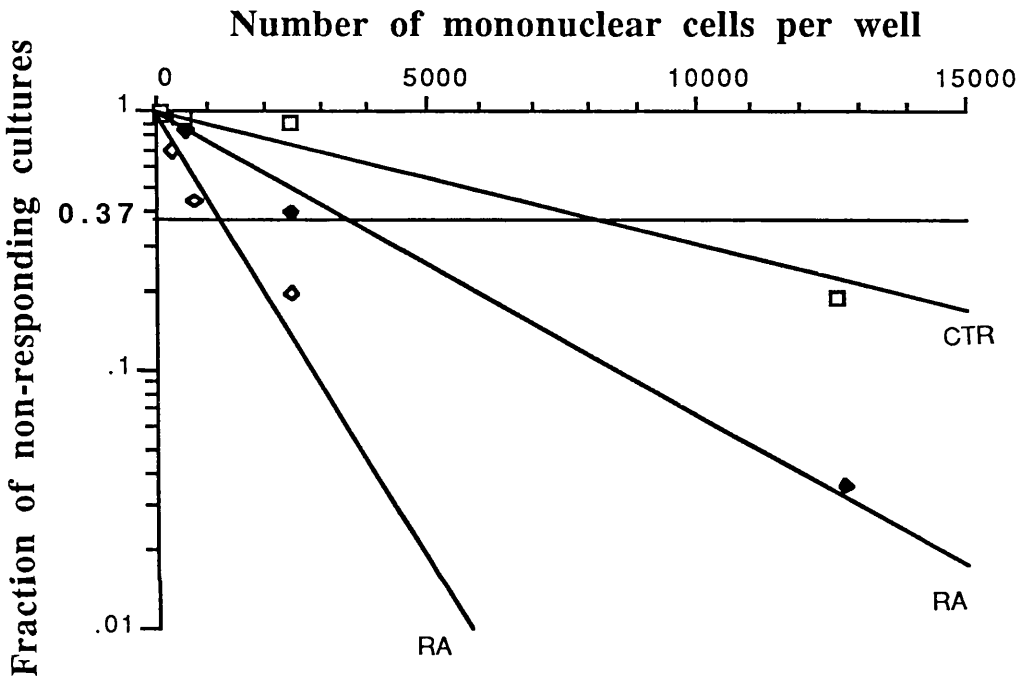


FIGURE 7.3: ESTIMATION OF PRECURSOR FREQUENCY OF WE REACTIVE PERIPHERAL BLOOD MONONUCLEAR CELLS BY LIMITING DILUTION ANALYSIS. The estimation of the precursor frequency is shown for one healthy individual (CTR) and two rheumatoid arthritis patients (RA). The proliferation of 100, 500, 2500 and 12500 mononuclear cells/well in the presence of WE antigen (10 μg/ml) and $3x10^4$ γ-irradiated autologous mononuclear cells was examined. Thirty wells were set up for each cell concentration in the presence and absence of antigen. The frequency of the responding cell could be read as the inverse of the number of cells as which 37% of the cultures were non-responding (section 2.4f).

TABLE 7.3: Precursor frequency of WE reactive peripheral blood mononuclear cells in healthy individuals (CTR) and rheumatoid arthritis patients (RA) estimated by limiting dilution analysis.

CTR	Precursor frequency	Stimulation index (1)	RA	Precursor frequency	Stimulation index
CTR 1	1/16100	1.00	RA 1	1/13700	2.18
CTR 2	1/15330	3.65	RA 2	1/12500	3.74
CTR 3	1/10350	1.70	RA 3	1/12500	1.50
CTR 4	1/9200	3.00	RA 4	1/12500	1.39
CTR 5	1/8780	5.27	RA 5	1/12100	2.76
CTR 6	1/7800	10.14	RA 6	1/9700	9.70
CTR 7	1/5890	6.88	RA 7	1/8700	1.17
CTR 8	1/4300	5.89	RA 8	1/7400	1.49
CTR 9	1/4000	8.10	RA 9	1/7000	3.23
CTR 10	1/3070	2.18	RA 10	1/5700	6.86
CTR 11	1/3000	7.10	RA 11	1/5200	13.55
mean ± SD	8000±4600	4.99±2.92	RA 12	1/5190	2.31
r, p (2)	0.65, 0.038		RA 13	1/5000	7.76
			RA 14	1/3300	23.13
			RA 15	1/3050	13.99
			RA 16	1/1140	11.95
			RA 17	1/1140	5.34
			mean ± SD	7400±4200	6.59±6.11
			r, p	0.65, 0.009	

Footnotes: (1) The stimulation index was obtained from the proliferation of 10^5 peripheral blood mononuclear cells to 10 µg/ml of the WE antigen.

(2) The r, p values were obtained from the Spearman's Rank correlation coefficient test.

The frequency of WE reactive mononuclear cells was also estimated in 4 paired samples of peripheral blood and synovial fluid from RA patients. The estimated frequencies are presented in table 7.4 and as it can be seen for all 4 patients, the frequency of WE reactive cells was higher in synovial fluid mononuclear cells than in PBMC.

TABLE 7.4: Precursor frequency of WE reactive mononuclear cells in peripheral blood (PBMC) and synovial fluid (SFMC) samples from rheumatoid arthritis patients (RA).

	PBMC		SFMC	
	precursor frequency	stimulation index	precursor frequency	stimulation index
RA 1	1/12500	1.40	1/6850	3.70
RA 2	1/13700	2.20	1/1100	9.40
RA 3	1/1140	5.30	1/1100	5.80
RA 4	1/5700	6.90	1/1000	11.20

Footnote: The stimulation index was obtained from the proliferation of 10^5 peripheral blood mononuclear cells to 10 μg/ml of the WE antigen.

It should be noted that the limiting dilution analysis was carried out on an additional 10 individuals (4 CTR and 6 RA) but the frequency of WE reactive PBMC could not be estimated. For 4 CTR and 4 RA individuals the semi-logarithmic titration curves levelled off to an asymptote reflecting the absence of a precursor cell in the cell concentrations examined. This indicated that the frequency of WE reactive cells in those individuals was lower than 1/12500 PBMC. Interestingly, the bulk proliferative responses of those individuals were at background levels, in terms of counts per minute.

For the remaining 2 RA patients, only the 10^2

PBMC/well cell concentration failed to show any proliferation and therefore the titration curves could not be drawn. This suggested that the frequency of WE reactive PBMC in those individuals was higher than 1/100 but lower than 1/500. As far as the bulk proliferative responses were concerned (i.e. the response of 10^5 PBMC/well), these two individuals were 'responders' with stimulation indices of 7 and 12.

b. Proliferative responses of peripheral blood mononuclear cells from healthy individuals and rheumatoid arthritis patients to the recombinant *M. bovis* 65 kDa hsp

Continuing the study of cellular responses to mycobacterial antigens, the mononuclear cell proliferative responses to the recombinant 65 kDa hsp from *M. bovis* were studied in peripheral blood from 12 healthy individuals and 26 RA patients (the characteristics of these groups are shown in table 7.1). The mononuclear cell proliferative response was measured by ^{125}I-UdR incorporation and expressed as mean counts per minute of at least four replicate wells.

The response of PBMC to concentrations of 5, 10, 20 and 40 μg/ml of the 65 kDa hsp antigen were studied. Figure 7.4 shows the PBMC proliferative responses of a random selection of CTR and RA individuals to the above antigen concentrations. Although the antigen concentration giving the highest proliferative response, in terms of counts per minute, was not the same for all individuals tested, in the majority of them (i.e. in 7/12 CTR and 18/26 RA individuals) the highest response was obtained by 10 μg/ml of the 65 kDa hsp antigen. The response to this antigen concentration was used for the comparison of the PBMC responses in the CTR and RA groups. Figure 7.5 shows the responses obtained from the stimulation of CTR and RA PBMC by 10 μg/ml of the 65 kDa hsp antigen.

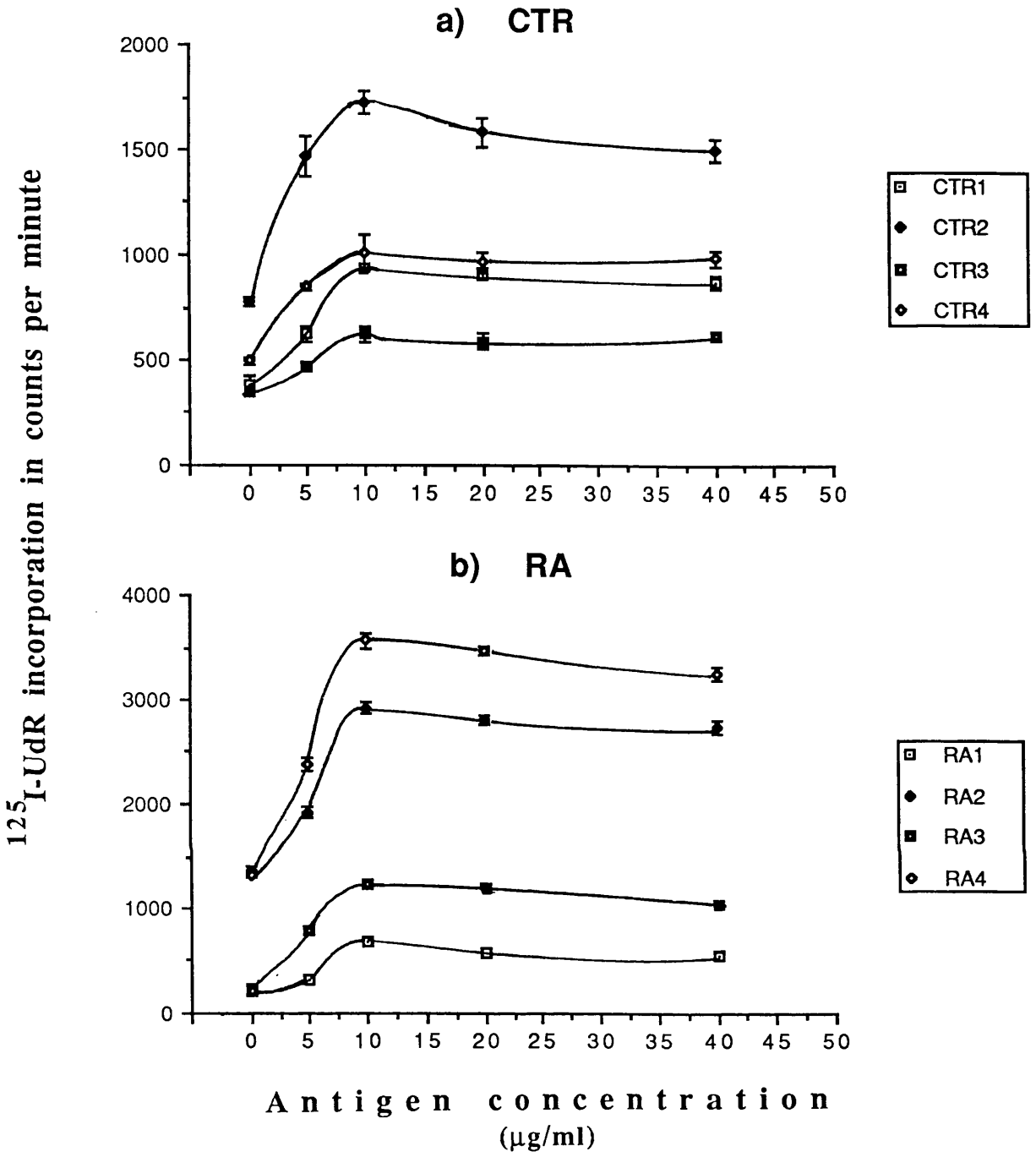


FIGURE 7.4: PERIPHERAL BLOOD MONONUCLEAR CELL PROLIFERATIVE RESPONSES TO DIFFERENT CONCENTRATIONS OF THE MYCOBACTERIAL 65 kDa HSP ANTIGEN IN HEALTHY INDIVIDUALS AND RHEUMATOID ARTHRITIS PATIENTS.

a) PBMC responses of four healthy individuals (CTR).

b) PBMC responses of four rheumatoid arthritis patients (RA).

The proliferative response of 10^5 PBMC/well was measured by ^{125}I-UdR incorporation on day 5 of the culture period and expressed as mean counts per minute of at least four replicate wells.

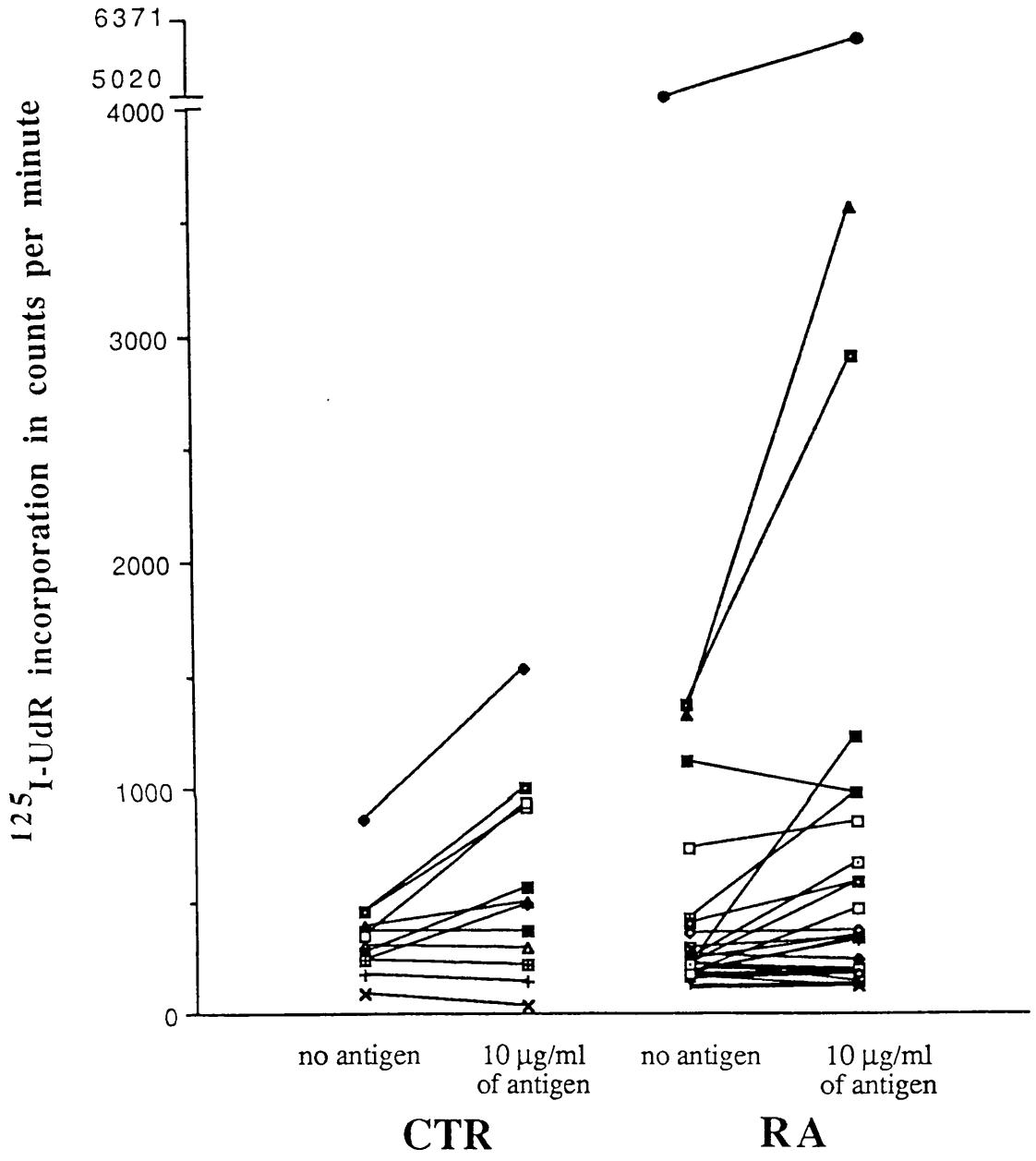


FIGURE 7.5: PROLIFERATIVE RESPONSES OF PERIPHERAL BLOOD MONONUCLEAR CELLS FROM HEALTHY INDIVIDUALS (CTR) AND RHEUMATOID ARTHRITIS PATIENTS (RA) TO THE MYCOBACTERIAL 65 kDa HSP. The proliferative response of 10^5 peripheral blood mononuclear cells/well from 12 healthy individuals and 26 rheumatoid arthritis patients to 10 µg/ml of mycobacterial 65 kDa hsp was measured by ^{125}I-UdR incorporation on day 5 of the culture period and expressed as mean counts per minute of at least four replicate wells.

The comparison of the CTR and RA PBMC proliferative responses followed the same criteria applied for the PBMC proliferative responses to the WE antigen (section 7.3a).

a) If responsiveness was arbitrarily defined as one giving a stimulation index of ≥ 5 (SI: the ratio of mean cpm in the presence of antigen divided by the mean cpm without antigen), then none out of 12 CTR and 1/26 RA individuals responded to the 65 kDa hsp antigen.

b) If responsiveness was arbitrarily defined as one where the mean cpm of the test sample (cells plus antigen) was greater or equal to the mean cpm +3SD of the control sample (cells without antigen), then 7/12 CTR and 13/26 RA individuals responded to the WE antigen. The mean PBMC proliferative responses of the 'responders' was not significantly different (mean SI of the 7 CTR=1.98±0.36, mean SI of the 13 RA=2.33±1.24, p=0.69).

Similarly, when the highest proliferative response in terms of counts per minute (irrespective of the antigen concentration to which that response was attributed) was used to compare PBMC responses in CTR and RA groups, the overall responses in the two groups were still no different (mean SI of CTR PBMC=1.82±0.64, mean SI of RA PBMC=1.73±1.10, p=0.32).

Synovial fluid samples were available for 5 RA patients and their SFMC responses were compared to those of their PBMC responses. It was found that the proliferative response of SFMC was higher than that of PBMC in only one of those 5 individuals. The PBMC and SFMC responses to 10 μg/ml of the 65 kDa hsp antigen are shown in table 7.5.

TABLE 7.5: **Proliferative responses of peripheral blood mononuclear cells (PBMC) and synovial fluid mononuclear cells (SFMC) to the mycobacterial 65 kDa hsp antigen in rheumatoid arthritis patients (RA).**

	^{125}I-UdR incorporation in counts per minute			
	PBMC		SFMC	
	no antigen	10 µg/ml of antigen	no antigen	10 µg/ml of antigen
RA 1	123±20	161±18	182±24	257±34
RA 2	239±26	258±16	169±10	244±16
RA 3	399±33	585±56	1182±99	3263±82
RA 4	153±19	177±21	1387±89	2252±99
RA 5	981±51	1117±53	1103±58	1113±99

Footnote: The proliferation of 10^5 mononuclear cells was measured by ^{125}I-UdR incorporation and is presented as mean counts per minute of at least four replicate cultures.

c. Comparison of peripheral blood mononuclear cell reactivity to the WE and to the mycobacterial 65 kDa hsp antigens

Table 7.6 presents the PBMC responses of CTR and RA individuals to the WE antigen (10 µg/ml; a preparation containing the 65 kDa protein), to the mycobacterial 65 kDa hsp (10 µg/ml), and to phytohaemagglutinin (PHA; 1 µg/ml; a non-specific mitogen).

Since the WE antigen contained the 65 kDa protein, it was initially thought that at least some of the cellular reactivity against the WE antigen might be directed against the 65 kDa protein. However, the comparison of the PBMC reactivities to these two antigens did not show any correlation in both CTR and RA groups. In addition, the proliferative response in terms of counts per minute seemed to be much lower to the mycobacterial 65 kDa hsp compared to the WE antigen. This,

however, can be explained by the fact that the WE preparation consisted of a variety of proteins and that the observed high proliferative response was due to the cumulative responses directed against the different constituents of this preparation. Also, possible differences in the precursor frequencies of the WE and 65 kDa hsp reactive PBMC might explain the difference in the proliferative responses to these two antigens.

The cellular reactivities to the mycobacterial 65 kDa hsp and to the WE antigen seemed to be specific since no correlations were found when they were compared with the cellular reactivity to PHA (Table 7.6).

TABLE 7.6: Proliferative responses of peripheral blood mononuclear cells to WE, mycobacterial 65 kDa hsp and phytohaemagglutinin (PHA) in healthy individuals (CTR) and rheumatoid arthritis patients (RA).

CTR	no antigen	WE	65 kDa	PHA	RA	no antigen	WE	65 kDa	PHA
1	450	1377	918	9800	1	235	1613	343	19290
2	858	903	1535	11200	2	294	3512	329	18247
3	454	1018	998	12262	3	177	1717	184	6709
4	233	1710	489	8490	4	119	260	132	7245
5	366	1337	366	5542	5	242	364	671	11855
6	350	608	935	1640	6	5020	6966	6371	16469
7	383	1314	494	3620	7	1369	18547	2916	11452
8	305	1354	295	14528	8	354	977	371	8760
9	268	835	564	11257	9	206	4765	1230	10940
10	171	383	137	18499	10	730	2355	848	4670
11	233	1373	213	8566	11	1322	18500	3569	12824
12	90	399	37	12600	12	159	1094	178	1045
13	273	1939	-	7600	13	169	1312	331	984
14	588	1963	-	3288	14	186	2277	322	9743
15	197	1608	-	16724	15	416	961	979	1201
16	1293	2824	-	6480	16	215	233	581	14832
17	414	2285	-	4370	17	161	860	123	5342
18	925	9378	-	14640	18	469	1755	-	5846
19	898	6183	-	8764	19	1507	1764	-	7752
20	1071	5648	-	17370	20	148	221	-	3498
					21	226	-	143	18552
					22	194	-	199	12041
					23	108	-	121	6089
					24	217	-	198	8857
					25	258	-	239	12743
					26	399	-	585	3917
					27	153	-	177	8490
					28	1117	-	981	12207
					29	170	-	459	15032

Footnotes: The proliferation of 10^5 peripheral blood mononuclear cells to 10 µg/ml of WE, 10 µg/ml of 65 kDa hsp and 1 µg/ml of PHA was measured by ^{125}I-UdR incorporation and is presented as mean counts per minute of at least four replicate cultures.
The standard deviations are not shown but on average there was <7% variation in the replicate cultures.
[-] Not done

7.4 DISCUSSION

The proliferative responses of peripheral blood mononuclear cells to mycobacterial antigens were studied in rheumatoid arthritis patients and compared to those of healthy individuals. The interest in the cellular responses to mycobacterial antigens in rheumatoid arthritis patients stems in part from the implication of mycobacterial 65 kDa hsp-specific T cell clones in the pathogenesis of experimental inflammatory arthritis in rats (van Eden *et al.*, 1988).

Although it has been suggested that most of the proliferative response to antigens *in vitro* is T cell mediated, in this study the mononuclear cell population was not separated into T and B cells and therefore it cannot be excluded that B cells contributed to the responses measured here. However, since the B cells represent only about 5-15% of the circulating lymphoid pool, it is likely that the bulk cellular reactivity was due to T cell responses.

The proliferative responses of PBMC to either 65 kDa protein-containing WE antigen or to the recombinant *M. bovis* 65 kDa hsp were found to be similar in healthy individuals and RA patients. These data were in agreement with those of Holoshitz *et al.* (1986) but in contrast with the humoral responses to the same antigens, which were found to be significantly higher in the RA patients compared with the healthy controls (Chapters 3 and 4). The lack of difference between CTR and RA PBMC responses to mycobacterial antigens did not necessarily exclude an association between the cellular arm of the immune response to mycobacterial antigens and RA. This was due to rheumatoid arthritis being characterised by an extensive mononuclear cell

infiltration of the synovium and synovial fluid, and it was therefore possible for the SFMC responses to the mycobacterial antigens to be the relevant ones in RA. Hence, the response of SFMC to the above antigens was compared to that of PBMC.

At that stage only five paired samples of peripheral blood and SF were available for studying so the results from these assays could only be treated as preliminary data. Interestingly, the reactivity of the SFMC to both antigens was found to be higher than that of PBMC in the patients studied. The 'increased' reactivity observed in the synovial fluid samples of this study might be due to:

a. The presence of higher precursor frequency of WE and 65 kDa hsp reactive mononuclear cells in the synovial fluid than in the peripheral blood. In fact, the precursor frequency of WE reactive mononuclear cells was higher in synovial fluid samples compared to peripheral blood samples in 4 RA patients tested. Unfortunately, the precursor frequency of 65 kDa hsp reactive cells was not known in those samples.

Overall, the frequencies of the WE reactive cells in peripheral blood samples were not significantly different between the healthy individuals (1/8000) and RA patients (1/7400), and that was also true for the proliferative responses to the WE antigen in these groups.

When interpreting the results of the frequency of WE reactive cells, it should be stressed that this antigen preparation contains a variety of antigens (Chapter 4). The reactive population will therefore consist of multiple clones with different specificities. It is likely that at least some of the reactivity against the WE is directed against the 65 kDa hsp

since this hsp is present in the WE antigen preparation (Chapter 4).

b. Some of the cells in the synovial fluid have been shown to be activated as defined by having interleukin-2 receptors or class II antigens of the major histocompatibility complex (Burmester *et al.*, 1981; Duke *et al.*, 1987; Firestein and Zvaifler, 1987; Jahn *et al.*, 1987; Waalen *et al.*, 1987). However, the controls used in the proliferation assays included cells without antigen and the spontaneous activation was subtracted from the calculations.

c. More efficient antigen presentation in the synovial fluid compared to peripheral blood. In this regard, synovial fluid antigen presenting cells have been shown to have an enhanced ability to support T cell proliferative responses compared to the corresponding cells in the peripheral blood in inflammatory arthritis (Life *et al.*, 1990).

Since these studies were carried out, there have been other reports on cellular reactivity to the 65 kDa hsp. Hence, Res *et al.* (1988), Gaston *et al.* (1989a) and Pope *et al.* (1989) reported greater reactivity to the 65 kDa hsp in the synovial fluid cells of RA patients compared to peripheral blood mononuclear cells from the same patients. In some reports the increased reactivity of SFMC was associated with a joint inflammation of less than three years (Res *et al.*, 1988), while in other reports this was not so (Gaston *et al.*, 1989a).

In this study however, because of the small number of paired samples tested, the mononuclear cell reactivity was not examined for possible correlations with the

onset of inflammation.

With regard to the magnitude of the proliferative cell activation by the WE and the mycobacterial 65 kDa hsp antigens, the response to the WE antigen was higher and the number of 'responders' to the WE antigen was greater than to the mycobacterial 65 kDa hsp. However, this was probably due to the complexity of the WE antigen and consequently the cumulative responses directed against its constituents. Also, differences in the precursor frequencies of the WE and the mycobacterial 65 kDa hsp reactive mononuclear cells could account for such observations. The magnitude of the proliferative responses to PHA was found to be higher than both WE and mycobacterial 65 kDa hsp antigen-induced proliferations. This is not surprising, since PHA is a polyclonal mitogen. The lack of correlation between proliferative responses to the WE, mycobacterial 65 kDa hsp and PHA antigens indicated that there was a specificity in the above responses.

As far as the responses to the mycobacterial 65 kDa hsp were concerned, since the 65 kDa protein has been identified as a highly conserved heat shock protein (Young *et al.*, 1987), it was not possible to assign the antigen recognised by the mononuclear cells to the mycobacterial 65 kDa hsp. Antigens cross-reactive with the *M. bovis* 65 kDa hsp have been found in bacterial species including *Klebsiella*, *Shigella*, *Salmonella*, *Yersinia* and *Campylobacter* (Thole *et al.*, 1988a), all suspected of being involved in human arthritis (Aho *et al.*, 1985). In fact, Gaston *et al.* (1989a) have shown that there was a correlation between the 65 kDa hsp and *Salmonella* specific responses in synovial fluid cells from patients with different arthropathies including

reactive arthritis and RA. Thus, the responses to the 65 kDa hsp might be directed against bacteria other than mycobacteria.

There has been some speculation on the relationship between arthritis and bowel flora in RA, with antibodies to *Proteus mirabilis* being elevated in RA patients (Ebringer *et al.*, 1989). Moreover, it has been shown that susceptibility of germ-free rats to adjuvant arthritis could be increased or decreased by appropriate reconstitution of the bowel flora (Kohashi *et al.*, 1985). Similarly, the bowel flora can induce resistance to the streptococcal cell wall-induced arthritis in rats (van den Broek *et al.*, 1989b). In view of these observations, a similar role for the microbial flora of the human gut cannot not be excluded in human RA.

It should also be noted that the 65 kDa hsp reactive cells, especially those in the synovial fluid, might also be directed against self-epitopes since the 65 kDa hsp has been found in synovial fluid and tissue (De Graeff-Meeder *et al.*, 1989; Karlsson-Parra *et al.*, 1990; Chapter 6). The recognition of the 65 kDa hsp within the joint could be relevant to the pathogenesis of rheumatoid arthritis. It could occur through antigenic mimicry between the immunodominant epitope of the 65 kDa hsp (of mycobacterial or other microbial origin) and a joint self-antigen (a self-hsp or a non-hsp self antigen) (Oldstone, 1987; Polla, 1988; van Eden *et al.*, 1988). Therefore, it would be important to test the ability of the mycobacterial 65 kDa hsp reactive T cells to recognise the human homologue of the 65 kDa hsp or other antigens in the joint. Also, the study of synovial T cell responses to 65 kDa hsps of other bacteria would be required.

Finally, to clarify the 'increased' synovial mononuclear

cell reactivity to the mycobacterial 65 kDa hsp, it is necessary to establish whether or not the relative frequency of the mycobacterial 65 kDa hsp reactive T cells in peripheral blood is the same as that in synovial fluid. Furthermore, it is important to extent this study to the human 65 kDa hsp.

CHAPTER 8

GENERAL DISCUSSION

The significance of immune responses to hsps

- Responses to hsps (self or cross-reactive) as part of the normal immune response
- Responses to hsps as part of autoimmune reactivity

The role of hsp responses in rheumatoid arthritis

- Possible mechanisms by which hsps could be involved in autoimmune pathology of RA
- Can the 65 kDa hsp play a protective role in RA?

Regulation of the immune response to hsps

- The role of the gut
- The role of $\gamma\delta$ T cells

Future experiments

This study was concerned with immune reactivity to heat shock proteins (hsps) in rheumatoid arthritis patients and its possible relevance to the aetiopathogenesis of the disease.

Investigation of the humoral immune responses to hsp antigens, and in particular to mycobacterial hsps, in rheumatoid arthritis patients occupied the larger part of this study. Preliminary data on T cell responses to some of these antigens were also presented.

In brief, it was found that patients with rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), ankylosing spondylitis (AS) and Crohn's disease could all have elevated antibodies to the 65 kDa and/or 70 kDa hsps of mycobacterial, *E. coli* and/or human origin compared to healthy individuals.

However, the IgG antibody levels to the mycobacterial 65 kDa hsp were only elevated in the RA group and that distinguished it from the other disease groups. In addition, this elevation was not observed with the *E. coli* homologue of the 65 kDa hsp, questioning a special relationship between the mycobacterial 65 kDa hsp response and RA (Chapters 3 and 4).

Some of the mycobacterial 65 kDa hsp antibodies were autoantibodies or cross-reactive antibodies, since they also bound to a human homologue of the 65 kDa hsp and were also directed against epitopes with high sequence homology between the mycobacterial and human 65 kDa hsp (Chapter 5).

A 65 kDa protein was found in both synovial fluid samples

and immune complexes from these samples in RA and osteoarthritic patients (Chapter 6). Interestingly, these patients were also found to have antibodies directed against the synovial fluid 65 kDa protein (Chapter 6).

In a limited study, mononuclear cell responses to the mycobacterial 65 kDa hsp were comparable in the peripheral blood of RA patients and healthy individuals, although they were found to be higher in the synovial fluid compared to peripheral blood samples in five established RA patients (Chapter 7).

In view of the above observations, the significance of the humoral and cellular responses to hsps (and in particular to the mycobacterial 65 kDa hsp) in rheumatoid arthritis needs to be evaluated further.

Could immune responses to heat shock proteins or the hsps themselves have a role (causative or protective) in the aetiology or pathogenesis of the disease?

However, before discussing possible aetiopathogenetic roles of the hsps in RA, it should be noted that there is evidence that humoral and cellular responses to hsps are part of the normal immune response. Therefore, it would be most appropriate to examine how reactivity to hsps can coexist in healthy and diseased (RA) individuals. Also, whether this reactivity is autoreactivity, under which circumstances it progresses to autoimmune pathology and what regulates this reactivity.

8.1 THE SIGNIFICANCE OF IMMUNE RESPONSES TO HSPS

a. Responses to hsps (self or cross-reactive) as part of the normal immune response

In assessing the significance of the immune responses to highly conserved antigens such as the heat shock proteins, it is important to determine whether the antigenic determinants involved in the immune responses consist of conserved or variable amino acid residues.

Although species specific epitopes can be recognised as foreign antigens and lead to immunity, epitopes from conserved regions of the protein should be seen as 'self' and should not elicit an immune response due to self-tolerance (Chapter 1). However, under certain circumstances, which will be discussed later, an immune response to 'self-like' determinants may also be induced. Such an autoimmune response need not necessarily result in autoimmune pathology.

In fact, antibodies (partly autoantibodies) to hsps have been found in healthy individuals (this study) and T lymphocytes reactive to self-hsps have been isolated from healthy individuals (Munk *et al.*, 1988; Lamb *et al.*, 1989; Munk *et al.*, 1989). Therefore, antibody and T cell responses to self (or cross-reactive) heat shock proteins seem to be part of a normal immune response.

The presence of autoantibodies and potentially autoreactive T cells in healthy individuals, however, is not unusual. In fact, myelin basic protein specific T cell clones were isolated from normal or experimental autoimmune encephalomyelitis (EAE) resistant rats, and have been shown to produce the disease on injection into susceptible animals (Holoshitz *et al.*,

1983). Similarly, thyroiditogenic T cell lines have been isolated from healthy mice (Maron *et al.*, 1983).

With regard to autoantibodies, high concentrations of acetylcholine receptor antibodies, which have been identified as the cause of myasthenia gravis, have been found in individuals without the disease (Cohen and Cooke, 1986).

If immune responses to hsps are part of a normal immune response, the question is what purpose they serve. It has been suggested that T cells recognising self-hsps in healthy individuals were involved in immune surveillance of autologous cells that were infected, transformed or otherwise stressed, possibly providing a first line of defence against infection (bacterial or viral) and transformation (Koga *et al.*, 1989; Young, 1990). Hence, the healthy individuals might use their capacity to respond to self-hsp determinants to help eliminate autologous stressed cells (Young, 1990). This seems possible since T cells in mice directed against the mycobacterial 65 kDa hsp recognised and lysed MHC class I compatible macrophages which have been activated (and stressed) by interferon-γ or by cytomegalovirus infection (Koga *et al.*, 1989; Kaufmann, 1990).

The lymphocytes that recognise hsp determinants must be capable of discriminating between normal (unstressed) and stressed cells. This is important since many heat shock proteins are constitutively expressed in normal cells, although at lower levels than in stressed cells (Young, 1990).

Normal cells may escape destruction by expressing only substimulatory levels of heat shock protein determinants. In addition, hsps may only be processed and presented efficiently during stress, possibly due to their altered intracellular location

during stress (Lindquist and Craig, 1988). Finally, immune regulatory networks, perhaps involving suppressor or cytotoxic cells, may prevent activation of autoreactive T or B cells under normal conditions.

b. Responses to hsps as part of autoimmune reactivity

The high sequence conservation among eukaryotic and prokaryotic heat shock proteins make these proteins ideal candidates for autoimmune reactivity. The regulatory constraints of the immune response to self/cross-reactive hsps under physiologic conditions might occasionally break down. This could result from defective immune regulation, including defects in the regulatory T cells such as the lack of antigen specific suppression and defective regulation of the idiotypic network (Cooke *et al.*, 1983). Defects in T cell regulation have been shown in RA patients (Chapter 1).

In addition, stimuli which increase the synthesis of heat shock proteins may lead to antigen concentrations sufficient to stimulate autoreactive cells. A variety of physiological events, such as infection (Lamb *et al.*, 1989) or possibly stresses occurring within inflamed joints such as the presence of oxygen free radicals, cytokines and elevated temperatures due to inflammation or joint motion (Kubo *et al.*, 1985; Tepic *et al.*, 1985; Blake *et al.*, 1987; Polla, 1988), could increase the local concentrations of the hsps in this way.

Alternatively, localised changes in the level of expression of MHC molecules could result in increased presentation of self-antigens (Bottazzo *et al.*, 1983).

Inappropriate expression of class II antigens has been noted in the synovial joints of RA patients (Klareskog *et al.*, 1982), and this could be involved in the recognition of self heat shock proteins (Lamb *et al.*, 1989).

The antigen recognition by T cells requires interaction with an MHC molecule on the surface of an antigen-presenting cell, and specificity associated with the antigen-binding site on the MHC molecule will result in preferential recognition of different epitopes on stress proteins by individuals with different haplotypes. The relative frequency of presentation of variable and conserved epitopes will therefore be regulated in part by MHC polymorphism (Lamb *et al.*, 1989).

Since immune reactivity to hsps is not necessarily associated with disease, the question is whether the immune responses to hsps, and in particular to the 65 kDa hsp, could be involved in the autoimmune pathology of RA and how this can be achieved.

8.2 THE ROLE OF HSP RESPONSES IN RHEUMATOID ARTHRITIS

a. Possible mechanisms by which hsps could be involved in autoimmune pathology of RA

i. Breakdown of tolerance through cross-reactivity

Structural mimicry between microbial or other antigens in the environment and self-antigens in an individual can initiate autoimmune disease (Oldstone, 1987). The high sequence homology between the bacterial and human homologues of the hsps, make these proteins potential inducers of cross-reactive autoimmunity (Lamb *et al.*, 1989; Cohen and Young, 1991). Cross-reactivity could occur between:

1. Mycobacterial/microbial hsp and self-hsp antigens.
2. Mycobacterial/microbial hsp and non-hsp self antigens.

A determinant from the variable region of the microbial molecule, say 65 kDa hsp, can be recognised as a foreign antigen and could induce a healthy immune response, which possibly contributes to immunity. Presentation of a determinant from the conserved (cross-reactive) region of the protein should, however, appear as a self-antigen and should not induce an immune response. Because the host's immune system has self-tolerance to its own stress proteins (Schwartz, 1989), it might fail to recognise the pathogen's proteins as foreign. Repeated presentation of a pathogen's stress proteins might break through the established tolerance and a potential autoimmune situation might occur.

Because of the remarkable sequence conservation of the hsps, a response induced by the 65 kDa hsp of one bacterial species could be reactivated by another non-related species. This can have important consequences for the maintenance of the autoimmune condition. Once an anti-microbial hsp response- and thus an anti-self hsp response- is induced by hsps from one microbe, any other invading species might be able to reactivate not only the anti-microbial hsp but also the anti-self hsp response.

Attention has been focused on the possibility that the autoimmune pathology in RA is partly due to cross-reactivity between mycobacterial/microbial 65 kDa hsp and human 65 kDa hsp. This is supported by the following observations:

1. Self-hsps may be produced in the joint as a result of:
    A. A microbial infection, in which case microbial-hsps are also produced (Christman *et al.*, 1985; Polla, 1988).
    B. The presence of cytokines, oxygen free radicals or the elevated temperature in an inflamed joint (Polla, 1988).
2. A 65 kDa protein (possibly a heat shock protein of human origin) has been found in synovial fluid samples free and complexed (Chapter 6).
3. A human 65 kDa hsp has been found in inflamed joints and subcutaneous nodules of RA patients (de Graeff-Meeder *et al.*, 1989; Karlsson-Parra *et al.*, 1990).

4. Anti-mycobacterial 65 kDa hsp antibodies were found to bind to a human 65 kDa hsp preparation (Chapter 6).

5. It was shown that the mycobacterial 65 kDa hsp shared a 47% sequence identity and an additional 20% conservative change with the human 65 kDa hsp homologue (Jindal *et al.*, 1989).

The human autoantigen, however, might not be the self-65 kDa hsp. One possibility that was initially considered, but as yet unsubstantiated, was a cross-reactivity between mycobacterial 65 kDa hsp and joint constituents such as proteoglycan and collagen.

This possibility arose from the fact that in the adjuvant arthritis model in rats, the stimulatory epitope of the mycobacterial 65 kDa hsp molecule (van Eden *et al.*, 1988) was partly homologous to the link protein of rat cartilage proteoglycan (van der Zee *et al.*, 1989). Such a cross-reactivity, between the 65 kDa hsp and a yet-to-be-sequenced human cartilage protein, could not be excluded in human RA (van der Zee *et al.*, 1989). However, there is some evidence that the relevant stimulatory epitope of the 65 kDa hsp in the human RA may not be the same with that in the AA model (van Schooten *et al.*, 1988; Gaston *et al.*, 1990).

In addition, the non-self hsps (or the non-self 65 kDa hsp in particular) could be of mycobacterial origin or of other microbial origin. If mycobacterial antigens are involved in triggering autoreactivity to cross-reactive self-antigens in the joints, immunisation probably arises from environmental contact.

It may also be questioned whether priming of

responses to the 65 kDa antigen during mycobacterial infection or BCG vaccination has an effect on the subsequent immune response to challenge with other environmental bacteria carrying cross-reactive antigens.

ii. Immune complex formation

There is evidence that hsp antigens localise in the RA joint (Chapter 6; McLean *et al.*, 1988; de Graeff-Meeder, *et al.*, 1989; Jarjour *et al.*, 1989; Karlsson-Parra *et al.*, 1990).

RA synovial fluid was found to contain a 65 kDa protein and antibodies against this protein as well as against the mycobacterial 65 kDa hsp. In addition, a 65 kDa protein was also present in immune complexes from synovial fluids (Chapter 6).

It is therefore possible that the formation of the 65 kDa-immune complexes could result in pathology by activating complement, release of cytokines and ultimate cartilage destruction (Cooke *et al.*, 1975; Chantry *et al.*, 1989).

Although it is commonly assumed that hsps are located within the intracellular compartment, evidence is now emerging which suggests that at least some host cells express hsps on their surface when they are stressed (Jarjour *et al.*, 1989; Karlsson-Parra *et al.*, 1990). Such stressed cells might become targets of anti-hsp antibodies and could trigger other potentially pathogenetic autoimmune reactions as well (Lakey *et al.*, 1987; Koga *et al.*, 1989; Vanbuskirk *et al.*, 1989).

It should be noted that if the self-hsp production in RA is due to an infection, the immune reactivity to hsps could have a primary role in the aetiopathogenesis of the disease. In contrast,

if the self-hsp production is a consequence of the existing inflammation in the RA joint, the immune reactivity to hsps could be responsible for the perpetuation of the inflammation rather than the initiation of it. Figures 8.1 and 8.2 show possible routes of a response to hsps which were induced either by an infection (Figure 8.1) or by an existing joint inflammation in RA (Figure 8.2).

That responses to the 65 kDa protein may be associated with the initiation of the disease was suggested by the finding of elevated RA synovial fluid T cell responses to this protein in early disease (Res *et al.*, 1988) and by the elevated IgA antibody levels to this protein in 'early' RA sera (Chapter 5).

On the other hand, the 65 kDa hsp might, in addition, play a pathogenetic role in RA. The 65 kDa hsp has been found in synovial fluid of RA patients and the presence of T cells reactive to self 65 kDa hsp could result in breakdown of tolerance and tissue damage. Such a role, however, could not necessarily be exclusive to RA since elevated synovial T cell responses to 65 kDa hsp have been shown in patients with other arthropathies (Gaston *et al.*, 1989a) and a 65 kDa hsp has been also found in trauma induced and OA synovial fluid (Chapter 6).

It should be noted that if the immune reactivity to heat shock proteins is important in the aetiopathogenesis of RA, the applicable mechanism probably involves more than a cross-reaction between the mycobacterial 65 kDa hsp and the human homologue or a joint antigen.

FIGURE 8.1: Diagram of possible routes of an immune response to a heat shock protein induced by an infection.

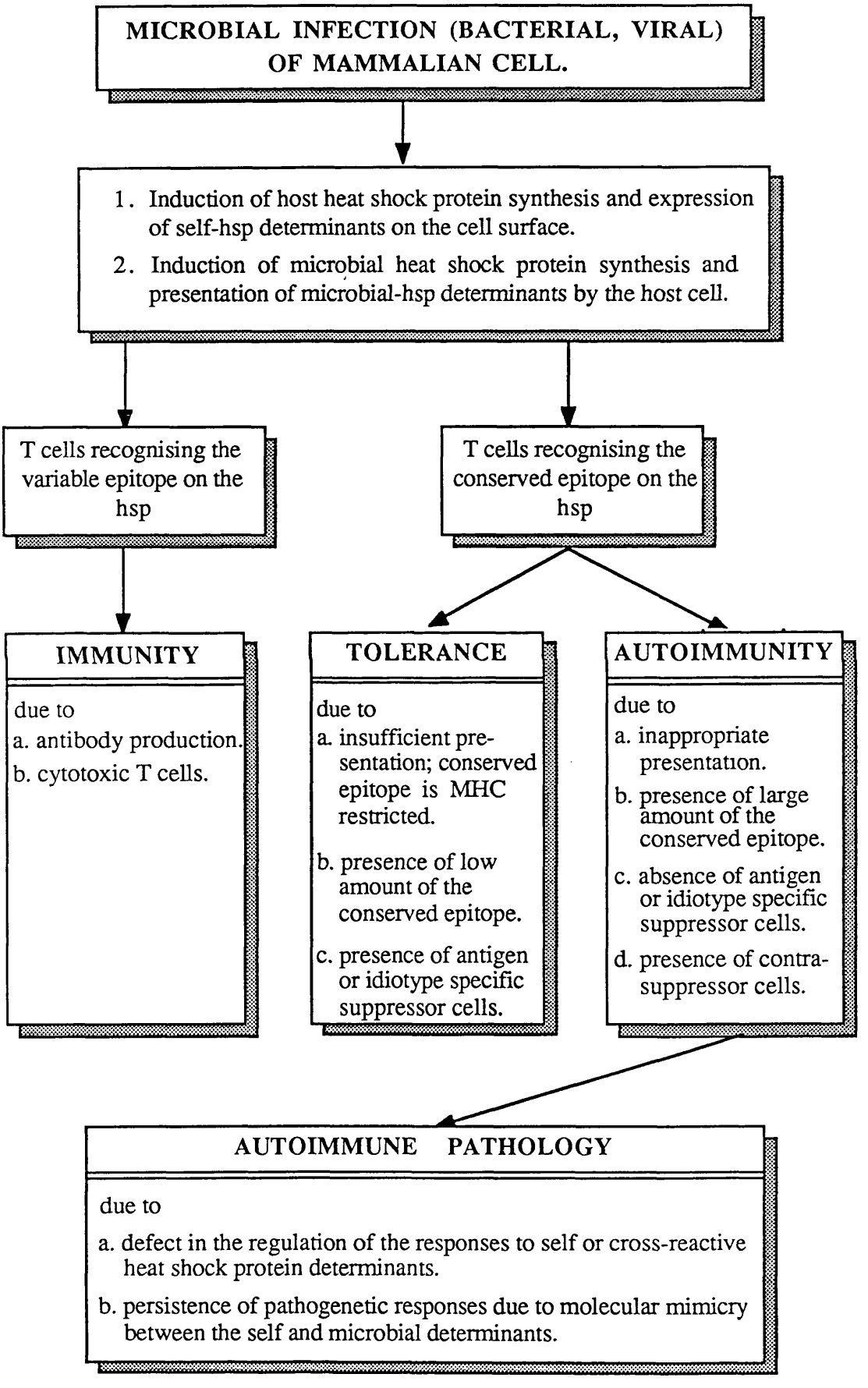
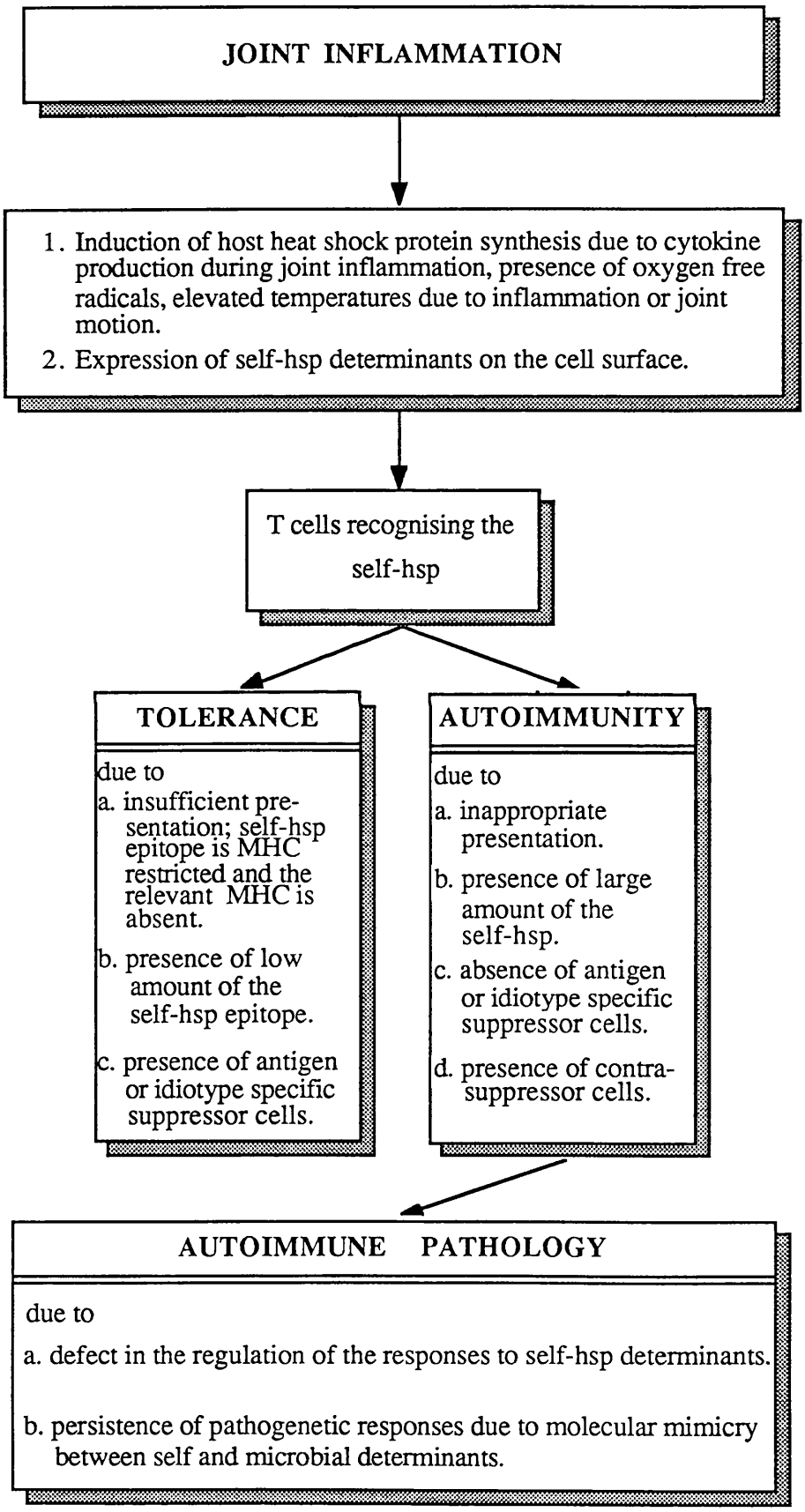


FIGURE 8.2: Diagram of possible routes of an immune response to a self-heat shock protein induced by joint inflammation.



b. Can the 65 kDa hsp play a protective role in RA?

Although pathogenetic roles for the 65 kDa hsp have been suggested above, reports on the ability of the mycobacterial 65 kDa hsp to block arthritis in animal models suggested an aetiologic role of this hsp in arthritis. However, the fact that both 65 kDa hsp-induced and non-65 kDa hsp-induced arthritides could be blocked by this protein argued against such a role.

In the adjuvant arthritis model in rats, the administration of the mycobacterial 65 kDa hsp in oil before the complete Freund's adjuvant blocked the subsequent induction of adjuvant arthritis (van Eden *et al.*, 1988; Yang *et al.*, 1990). Similarly, pretreatment with the mycobacterial 65 kDa hsp blocked the induction of arthritis in the streptococcal cell wall induced arthritis in rats (van den Broek *et al.*, 1989a) and in the pristane induced arthritis in mice (Thompson *et al.*, 1990). Also, the collagen II induced arthritis could be partially blocked by pretreatment with the mycobacterial 65 kDa hsp (Klareskog L., Department of Medical and Physiological Chemistry, Uppsala: personal communication).

In contrast, the *M. leprae* homologue of the 65 kDa hsp blocked the arthritis induced by a synthetic adjuvant (a lipoidal amine CP20961) in the rat, only when combined with the lipoidal amine adjuvant (Billingham *et al.*, 1990). Pretreatment with the 65 kDa hsp had no effect on the non-immunological zymosan-induced arthritis (van den Broek *et al.*, 1989a), which argued against a non-specific anti-inflammatory effect of the 65 kDa hsp.

From the above observations, the 65 kDa hsp appeared

to produce an antigen-specific resistance to subsequent induction of arthritis. The regulatory mechanisms responsible for these actions of the 65 kDa hsp were not clear.

However, there is no information on a protective role of this heat shock protein in man. It is conceivable that immune responses to conserved heat shock protein determinants, probably generated during establishment of natural microbial flora on the skin and in the gut, could provide a general level of protection against infection. If that was true, pathogen derived hsp-like antigens might prove new and important tools for vaccine development (Emmrich and Kaufmann, 1986; Young *et al.*, 1988a; Young *et al.*, 1990).

Immunisation with conserved protein antigens however, whether during natural infection or during vaccination, might also have negative consequences due to the close structural similarity between the stress proteins produced by the pathogens and those produced by the host. Repeated presentation of a pathogen's stress proteins might break through the established self-tolerance, thus precipitating an immune reaction.

In a recent review (Hurst, 1990), it has been suggested that the induction of hsps might be relevant to the therapeutic action of the drug treatment. Therefore, induction of hsps in peripheral blood lymphocytes by gold, prior to their arrival at sites of inflammation, may serve to protect their protein synthesising function against the damaging effects of oxygen free radicals and other mediators, after their arrival in the inflammatory site. This could be consistent with the observation that gold therapy has a restorative or stimulating effect on various aspects of immune function in RA (Smith *et al.*, 1989;

Hurst, 1990). However, no correlation was found in this study between responses to hsps and drug therapy of the RA patients.

The recent implication of the mycobacterial 65 kDa hsp in the induction or prevention of diabetes in non-obese-diabetic mice and the immune reactivity of insulin-dependent diabetes mellitus patients to this protein (Atkinson *et al.*, 1990; Baekkeskov *et al.*, 1990; Elias *et al.*, 1990; Jones *et al.*, 1990) could argue for an immunoregulatory role for the 65 kDa hsp in autoimmune diseases. However, the characterisation of the key 65 kDa hsp epitopes in each case might help to resolve the paradox of different autoimmune diseases seemingly associated with a single 65 kDa hsp antigen.

Although the focus of the immune reactivity to hsps in RA was on the 65 kDa hsp, other heat shock proteins (i.e. 70 kDa hsp) may also play a role in processes that could be important in RA. Their role might not necessarily be the same as that of the 65 kDa hsp.

It should also be noted that if the role of the hsps in RA is in the amplification or perpetuation of inflammation, it is unlikely for this role to be limited to rheumatoid arthritis.

8.3 REGULATION OF THE IMMUNE RESPONSE TO HSPS

a. The role of the gut

The immune response to the 65 kDa hsp has to be under some form of regulation. In the adjuvant arthritis and the streptococcal cell wall induced arthritis models, where the 65 kDa hsp has been shown to be relevant, the incidence of arthritis was found to be strongly influenced by the gut flora (Kohashi *et al.*, 1985; van den Broek, 1989b). This was suggestive of the 65 kDa hsp response being regulated by the nature and quantity of exposure to cross-reactive 65 kDa hsp homologues.

Thus, susceptibility of germ free rats to adjuvant arthritis has been shown to be increased or decreased by appropriate reconstitution of the bowel flora (Kohashi *et al.*, 1985). Gram-positive bacteria were shown to have an enhancing effect and gram-negative a suppressing effect on disease development (Kohashi *et al.*, 1985). Similarly, a state of tolerance to the target antigen, possibly the 65 kDa hsp antigen, was maintained by the bowel flora in the streptococcal cell wall induced arthritis (van den Broek *et al.*, 1989b).

In man, involvement of gut organisms in autoimmune disease has been shown in patients with ankylosing spondylitis, who have a high incidence of intestinal *Klebsiella* (Keat, 1986), and in the Reiter's syndrome which frequently follows episodes of infection with *Salmonella*, *Shigella*, *Yersinia* or *Klebsiella* (Calin, 1989).

The presence of immune reactivity to the mycobacterial 65 kDa hsp in reactive arthritis (Gaston *et al.*, 1989b), which can follow gastrointestinal infections, could not exclude an influential role for the intestinal microbial flora.

Also, the beneficial effects of dietary changes in some RA patients could be partly due to consequent changes in the bowel flora (Panush *et al.*, 1983; Kremer *et al.*, 1985; Kremer *et al.*, 1987).

The precise mechanism whereby the microbial flora can modulate the disease remains uncertain. If the microbial flora exerts a form of regulation of the hsp responses in RA (i.e. through regulation of the oral tolerance), the preferential increased reactivity to the mycobacterial 65 kDa hsp, as opposed to the *E. coli* 65 kDa hsp, could be partly explained by the variable exposure to the mycobacterial hsp in contrast with the virtually constant exposure to hsps of commensal genera (Brock, 1979; Taussig, 1984; Rook *et al.*, 1990).

It is tempting to speculate that the gut plays a regulatory role in the maintenance of oral tolerance to hsp antigens, since there is preliminary evidence for the preferential recognition of hsps by γδ T cells which are localised in epithelial layers (Bonneville *et al.*, 1988; Goodman and Lefrancois, 1988; Lefrancois and Goodman, 1989; Raulet, 1989b; Born *et al.*, 1990).

b. The role of γδ T cells

The regulation of the immune response to hsps might also be related to the T cell subpopulation bearing a γδ-T cell receptor (TCR), which has been suggested of having a biased repertoire for mycobacterial and in particular heat shock proteins (Asarnow *et al.*, 1988; Janeway *et al.*, 1988; Haregewoin *et al.*, 1989; Janis *et al.*, 1989; Kabelitz *et al.*, 1990; Modlin *et al.*, 1989; O'Brien *et al.*, 1989; Raulet, 1989a, 1989b).

γδ-TCR bearing $CD3^+$, $CD4^-$, $CD8^-$ T cells have been

recently shown to reverse oral tolerance when adoptively transferred to orally tolerant mice (Kitamura *et al.*, 1987; Fujihashi *et al.*, 1989). Since these γδ T cells are found in the gut epithelium (Bonneville *et al.*, 1988; Bucy *et al.*, 1988; Lefrancois and Goodman, 1989), they seem to be relevant candidates for the regulation of oral tolerance. Whether this might be their role in RA, however, is not known.

There have been contrasting reports concerning the distribution and number of γδ T cells in RA (Lydyard and van Eden, 1990). Brennan *et al.* (1988) reported increased numbers of γδ T cells in the synovial fluid of RA patients as compared to peripheral blood samples of the same patients, while Smith *et al.* (1990) did not find such a difference. However, γδ T cells have been shown in the synovial membrane of some RA patients (Smith *et al.*, 1990) and a mycobacteria-reactive γδ T cell clone has been isolated from RA synovial fluid (Holoshitz *et al.*, 1989).

Since γδ T cells produce a variety of cytokines (Ferrini *et al.*, 1989; Modlin *et al.*, 1989; Moretta *et al.*, 1989) one might speculate that their prime function in RA could be to maintain immunological tolerance by eliminating autoreactive or stressed cells, which would certainly be expected in abundance in the arthritic joint (Asarnow *et al.*, 1988; Janeway *et al.* 1988; Bernstein, 1989; Rajasekar *et al.*, 1990).

The function of the γδ T cells in RA will be better established when the specificities of these cells in the synovial joint are better defined (Born *et al.*, 1990, Haas *et al.*, 1990).

Interestingly, T cells termed effector contra-suppressor cells (Tcs) have been shown to be involved in the abrogation of oral tolerance (Gershon *et al.*, 1981; Green and Ptak,

1986; Lehner, 1986; Lehner and Brines, 1988). Although the precise phenotype and growth characteristics of these cells is currently a controversial issue, they share common characteristics with the γδ-TCR bearing $CD3^+$, $CD4^-$, $CD8^-$ T cell subset (Gershon *et al.*, 1981; Suzuki *et al.*, 1986; Kitamura *et al.*, 1987; Fujihashi *et al.*, 1989). Therefore, the regulation of the responses to hsps might also be influenced by a network involving Tcs.

In order to define the regulatory mechanisms responsible for the immune reactivity to heat shock proteins, more information is required with regard to the immunological components (i.e. cells, antibodies, mediators) involved in the hsp responses and which are likely to influence the outcome of such a response.

8.4 FUTURE EXPERIMENTS

In this study several points regarding the immune reactivity to hsps in RA have been addressed. However, there are many questions still to be answered regarding the role of the anti-hsp antibodies and the anti-hsp T cell responses in RA, their degree of cross-reactivity or autoreactivity, and their regulation. Thus, a potentially promising area for future investigation would be:

i. To define the relative contribution of conserved and variable antigenic determinants to the overall immune responses (humoral and cellular) to hsps and to identify the immuno-dominant epitope(s). Also, to compare the amino acid sequence(s) of the immunodominant hsp epitope(s) to that of self antigens (such as joint constituents) and other microbial (bacterial or viral) proteins. This could be important in evaluating the potential role of the hsp antigens in induction of protective immunity or autoimmune pathology.

ii. To clarify the elevated response of synovial T cells to the 65 kDa hsp in comparison with that of peripheral blood T cells. It would be interesting to define the proportion of synovial fluid cells which are committed to the 65 kDa hsp and to test 65 kDa hsp-specific synovial T cell clones for cross-reactive responses to joint constituents, including proteoglycan and collagen.

iii. To examine the pathogenetic potential of the anti-hsp antibodies, to define the expression of hsps by cells in RA and to identify the cells involved in the humoral and cellular responses

to hsps.

Equally important is to determine the origin of the 65 kDa protein present in RA synovial fluid samples and in synovial tissue, and to examine the purpose of its presence there.

iv. Finally, to identify the mechanism of regulation of the immune responses to hsps. Part of this would be to define the relative contribution of $\alpha\beta$ and $\gamma\delta$ T cell receptors to T cell reactivity to hsps and the specificities of the $\gamma\delta$ T cells in the RA synovial tissue.

Since recent work in experimental encephalomyelitis has shown that recognition of myelin basic protein by encephalitis-inducing T cell clones is associated with the use of particular V-region genes (Heber-Katz *et al.*, 1989), it will be of interest to determine the TCR V-region genes used to recognise 65 kDa hsp specific epitopes. The identification of the relevant TCR V-region would also be important, in view of a report demonstrating the use of a synthetic TCR V-region peptide to induce specific regulatory immunity against experimental autoimmune encephalomyelitis (Vandenbark *et al.*, 1989).

The immune reactivity to heat shock proteins is currently under intense scrutiny. Whether this will be highly relevant to our understanding of the aetiopathogenesis of rheumatoid arthritis, remains to be seen. However, the observation of elevated mycobacterial 65 kDa hsp antibodies in RA patients, the presence of a 65 kDa protein in synovial fluid samples and the increased T cell reactivity to this molecule in synovial fluid samples have provided a new approach to the study of this disease.

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Elevated IgG Antibody Levels to the Mycobacterial 65-kDa Heat Shock Protein Are Characteristic of Patients with Rheumatoid Arthritis

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We have previously demonstrated raised levels of IgG and IgA antibody to the mycobacterial 65-kDa heat shock protein (hsp) in the sera of patients with rheumatoid arthritis (RA). We have now attempted to determine whether this phenomenon is specific for RA, and whether it is seen only with the mycobacterial homologue of this particular hsp gene family. We therefore screened antibody levels to the mycobacterial and *Escherichia coli* hsp 65, and the mycobacterial, *E. coli*, and human hsp70, in sera from RA, systemic lupus erythematosus (SLE), tuberculosis (TB), ankylosing spondylitis (AS), Crohn's disease, and control donors. RA sera show the greatest increase in IgA binding to the mycobacterial hsp65, but no increase in IgA binding to the *E. coli* homologue. Similarly, only RA and TB sera show increased IgG binding to the mycobacterial hsp65, and we have shown previously that the titre is greater in RA. In contrast, the use of mycobacterial and *E. coli* hsp70 preparations as control bacterial hsp gene products has shown that RA patients do not differ from TB or SLE patients in their antibody binding to these proteins. Moreover, neither IgA nor IgG antibody to the human hsp70 in RA sera were higher than in TB, and the IgA binding was not higher than in SLE. These findings suggest that elevated IgG antibody levels to the mycobacterial hsp65 shows some disease specificity, and further studies with the human homologue and at the T-cell level are required.

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Rheumatoid arthritis (RA) is an immunological disorder of unknown aetiology. It is characterized by synovitis, largely of the distal joints, erosion of articular cartilage and subarticular bone, and ultimately ankylosis.

Recent data have suggested a role for mycobacteria, or auto-antigens cross-reactive with mycobacteria, in the aetiology of this disease.

1. An increase in glycoforms of IgG lacking terminal galactose from the oligosaccharides on the Fc, originally shown to occur in RA [13], has now been shown to occur in diseases of known or possible mycobacterial aetiology, but not in a number of other conditions studied. Thus, agalactosyl IgG increases in tuberculosis, Crohn's disease [15], and leprosy during episodes of erythema nodosum leprosum [7], but not sarcoidosis, systemic lupus erythematosus (SLE), or virus infections [15].

2. The skin-test response to tuberculin has been shown to be increased in DR4$^+$ leprosy [12] and RA [3], but not in DR4$^+$ normal individuals. (DR4 is strongly associated with RA [17].)

3. Adjuvant arthritis in rats has been adoptively transferred by means of T-cell clones recognizing a 65-kDa mycobacterial antigen, and

induction of the disease can be blocked by pretreatment with this antigen in soluble form [21, 22].

This 65-kDa antigen has recently been shown to be a heat shock/stress protein (hsp) [24]. Thus, when prokaryote or eukaryote cells, including those of plants, are exposed to stresses such as heat, virus infections, certain toxins, or oxygen reduction products, there is inhibition of normal protein synthesis and induction of synthesis of hsp [10]. These exist as gene families, including groups at approximately 20, 65, 70, and 110 kDa, some members of which may be expressed even under normal circumstances, while others are absent from cells until they are exposed to stress [14]. Most of these proteins have 'nursemaid' or 'molecular chaperone' functions. Thus, they may be ATP-dependent enzymes which fold, unfold, translocate, assemble subunits, or bind and inactivate other proteins [5, 6]. These functions, which remain poorly understood, are clearly fundamental to cell survival, and appear to have imposed a remarkable degree of sequence conservation. The human-65 kDa protein, the gene for which has recently been cloned, shows 65% homology with the mycobacterial product (R. S. Gupta, personal communication). The high degree of homology between human and mycobacterial 65-kDa hsp makes this protein a very strong candidate for potential autoreactivity through 'mimicry'. Furthermore, in the rat model, T cells responding to the 65-kDa mycobacterial hsp cross-reacted with the 'unrelated' proteoglycan core protein [8]. This implies that 'mimicked' amino acid sequences on other auto-antigens can also be the target of the mycobacterial 65-kDa cellular response, at least in rats.

Our previous studies have shown that serum IgG and sometimes IgA antibody levels to the 65-kDa and also 70-kDa mycobacterial hsp are raised in RA compared with control sera [1, 20]. In order to ascertain the disease specificity of this finding, and to discover whether the mycobacterial 65-kDa hsp is of particular relevance, we now compare levels of antibody binding to the mycobacterial 65- and 70-kDa hsp with binding to the homologous proteins from *E. coli*, and to a preparation of human 70-kDa hsp. We included sera from tuberculosis (TB) as a representative mycobacterial disease, SLE as another non-organ-specific autoimmune disease, ankylosing spondylitis (AS) as another inflammatory disease, and Crohn's disease because it is accompanied by raised agalactosyl IgG, and is conceivably associated with mycobacteria. Our results, considered in conjunction with our previous studies, suggest that there is a special relationship between RA and antibody to the mycobacterial homologue of the 65-kDa hsp.

MATERIALS AND METHODS

Serum collections. RA patients. Sera were collected from patients attending the rheumatology clinics at University College and Middlesex School of Medicine (UCMSM) ($n=50$), and the Mubarak teaching hospital in Kuwait ($n=41$). All these donors fulfilled the American Rheumatism Association criteria for classical RA. The UK donors had a female to male ratio of 3:1, a mean age of 55 years (range 27–78), and a duration of disease from 2–20 years. Twelve were receiving no treatment, 21 were on non-steroidal treatment, and 11 on steroids. Almost 50% of the patients were inactive, and the rest moderately to highly active as assessed according to Young [23]. The erythrocyte sedimentation rate (ESR) was also used as an index of disease activity. The Kuwaiti donors (13 men, 28 women) had a mean age of 30 years (range 18–52) and the duration of the disease varied from 2 to 14 years.

SLE patients. Seventeen female and one male patient, mean age 42 years (range 30–68), were studied as a disease control as SLE, like RA is a non-organ-specific autoimmune disease. Ten of the patients were on steroids while eight were not receiving treatment. Six patients had inactive disease, nine mildly active, two moderately active, and one very active. Disease activity was graded according to a previously published index [9, 18].

AS patients. Fifteen patients with AS were also included in the study as examples of another inflammatory disease. Ten men and five women with a mean age of 50 years (range 33–73) were studied. Fourteen patients were receiving non-steroidal treatment and one was not receiving any treatment. Nine patients had active disease and six inactive as defined by whether they fulfilled both or neither of the following criteria respectively: serum IgA > 300 mg/dl and ESR > 15 mm/h. These sera were kindly provided by Drs F. Yuksel and A. Ebringer (UCMSM, London, UK).

Crohn's disease (CD] patients. Twenty-one CD patients were also studied as a non-autoimmune disease group that has been associated with mycobacteria. Ten women and 11 men with a mean age of 36 years (range 18–74) were studied. Five patients were on steroids while 16 were receiving no treatment. Twelve patients had slightly active disease and nine moderately active. These sera were kindly provided by Professor J. Lennard-Jones and colleagues (St Mark's Hospital, London).

Tuberculosis sera were collected in Kuwait from 90 patients with radiologically and bacteriologically confirmed pulmonary disease. Thirty-one had advanced disease. There were 73 men and 17 women with a mean age of 33 years (range 18–55).

Control sera were obtained from 45 healthy laboratory staff in London (27 women, 18 men, mean age 35 years, range 24–63). Control donors in Kuwait were 79 healthy adult hospital and medical school staff, and blood donors (38 women, 41 men, mean age 31 years, range 19–50).

Antigens used. The recombinant forms of the 65-kDa hsp of *M. bovis* BCG [19] and *E. coli* [4] were used. Antigens belonging to a different hsp gene family, the 70-kDa hsp of *M. tuberculosis*, *E. coli*, and human origin were also included as controls [11]. Homologies within the hsp families are around 65%, whereas there is no homology between the 65- and 70-kDa hsp.

Measurement of antibodies to the stress proteins. Antibodies of the IgM, IgG, and IgA classes were measured in an enzyme-linked immunosorbent assay (ELISA) as previously described [20]. Briefly, antigens were coated at 1–2 μg/ml in carbonate buffer (0.05 M, pH 9.6) onto immunoplates (Nunc, Roskilde, Denmark) and incubated overnight at 4°C. Excess antigen was washed off with phosphate-buffered saline (0.1 M, pH 7.4) containing 0.05% Tween 20 (PBS/T), test sera were added in doubling dilutions from 1:50 to 1:400 in PBS/T in duplicate and the plates were incubated for 2 h at room temperature. After further washes with PBS/T, affinity purified $F(ab')_2$ fragments of horseradish peroxidase-conjugated human antibodies (Sigma, St Louis, Mo., USA) were added at 1:1000 dilution in PBS/T and incubated overnight at 4°C. After further washing, 0.5 mg/ml of 2,2′-azinobis (3-ethyl benzthiazoline sulphonic acid) (Sigma) in citrate phosphate buffer (0.1 M, pH 4.1) with 0.35 μl/ml H_2O_2 (6% wt/vol) was added. The reaction was stopped with 96 mg/ml of sodium fluoride (Sigma) in double distilled water and the absorbance was measured at 650 nm using a Titertek multiscan ELISA reader (Flow, Irvine, UK). The volumes were 100 μl per well, each washing step was repeated three times with 3 min incubation between washes at room temperature, and the same positive and negative control sera were used for each individual assay. For the IgM and IgA assays cord serum was used as a negative control, while for the IgG assays an agammaglobulinaemic serum was used. The Kuwaiti sera were analysed by a slightly modified procedure as previously described [2]. Different standards were used in the two laboratories, so values from the two sets of data are not comparable.

Data analysis. In order to standardize the reaction in each plate, the optical density (OD) ratios were calculated as OD_{650} of test to the OD_{650} of a standard normal serum (positive control) used in each plate. The OD_{650} value is expressed relative to the blank (without serum) of the ELISA; the negative controls (cord serum and agammaglobulinaemic sera) never gave values greater than 0.004 OD units. The serum dilutions used for the calculations were those within the linear phase of the antibody binding curve and were 1:100 dilution for the IgA ELISA of 65-kDa antigen and 1:200 for all the other assays. Since the mean age and sex of the individuals for the groups studied were not the same, the data were analysed for evidence that age or sex influenced the antibody levels. The data were also analysed with respect to drug treatment and activity of the disease.

Statistics. The Mann–Whitney *U*-rank two tailed test was used to compare the antibody levels in control and experimental groups. Comparisons between groups for different drug treatment, sex, and disease activity were also made using the above test. Correlations between antibody levels to hsp, age, and ESR values (for the RA group) were determined using the Spearman rank correlation coefficient. However, statistical analysis was done only for groups with higher than five entries and for this reason ESR values were used as an index of disease activity of the RA group. Statistical analysis was carried out with the STSC STATGRAPHICS software package in London, and the SPSS-X package in Kuwait.

RESULTS

Antibody levels to the mycobacterial and E. coli 65-kDa hsp

In all patient groups, levels of IgM antibody binding to these antigens were found to be lower (though not significantly) than in control sera (Fig. 1).

IgA antibody to the mycobacterial hsp65 was increased in RA ($P < 0.00001$), SLE ($P < 0.001$), CD ($P < 0.001$), and AS ($P < 0.001$) patients (Fig. 2). However, the increase was greatest in RA, and in 11 of the 45 sera the levels were greater than the mean control value + 2SD, whereas this was true for only 1 of the 18 SLE sera, 2 of the 21 CD sera, and 3 of the 15 AS sera.

A further difference between RA and the other diseases was seen in the IgA antibody to the *E. coli* hsp65. Whereas SLE, CD, and AS sera contained significantly raised levels of IgA binding to this antigen, there was no significant rise in the RA sera (Fig. 2). Thus, the increased IgA to hsp65 in RA sera shows some specificity to the mycobacterial homologue. The rise in IgA to the *E. coli* hsp65 in CD patients is likely to be due to their increased exposure to gut flora.

However, the most striking unique feature of RA in this study was the increase in IgG binding to the mycobacterial hsp65. First, no significant increase in IgG binding to this antigen was seen in SLE, CD, or AS sera. Secondly, the increase in IgG binding in RA sera was seen only with the mycobacterial hsp65 homologue (i.e. not with the *E. coli* 65-kDa hsp homologue). Thus, both IgA and IgG responses in RA appear to be increased to the mycobacterial-specific and not the homologous determinants of the 65-kDa hsp of *E. coli*.

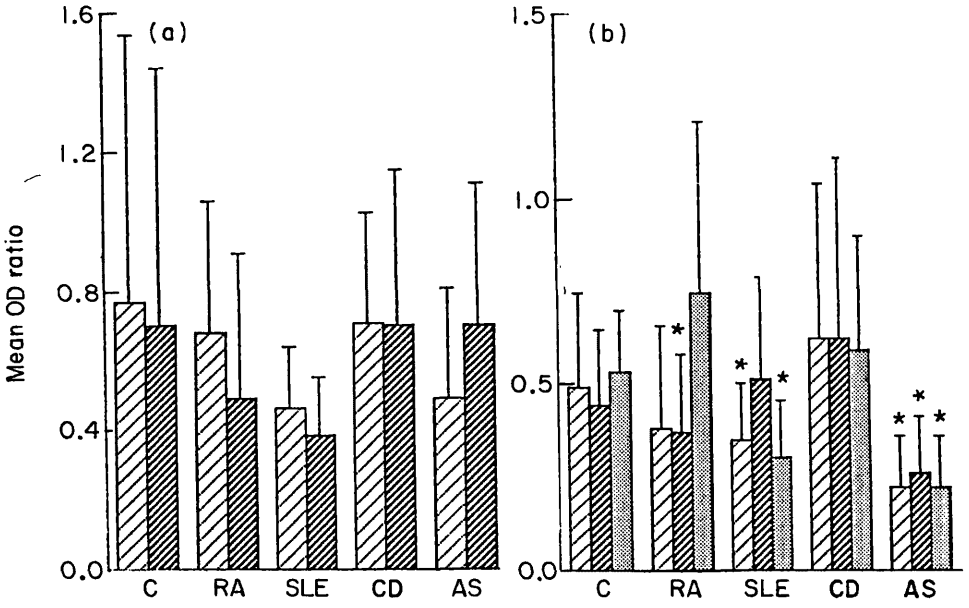

FIG. 1. IgM antibodies to the mycobacterial (▨) and *E. coli* (▩) 65-kDa (a) and 70-kDa heat shock proteins (b), and to the human (▦) 70 kDa were detected in an ELISA (see Materials and Methods) in sera from healthy individuals (C), rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), Crohn's disease (CD), and ankylosing spondylitis (AS) patients. Data are represented as mean optical density (OD) ratios + SD. $*P < 0.05$.

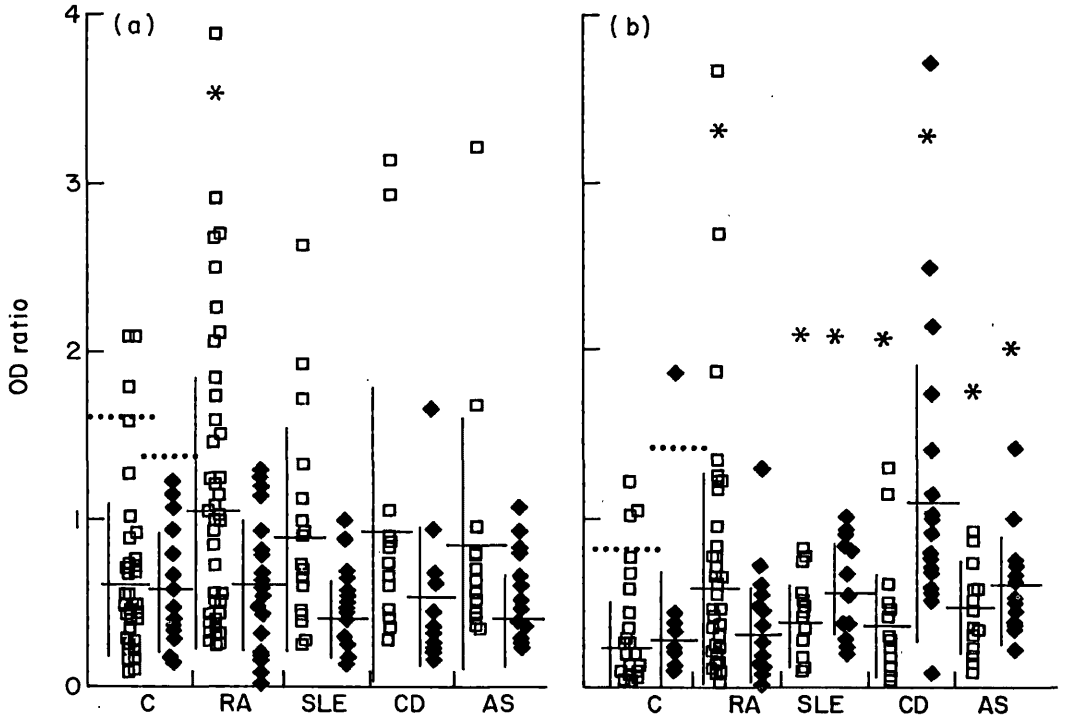

FIG. 2. IgG (a) and IgA (b) antibody levels to the mycobacterial (□) and *E. coli* (◆) 65-kDa hsp were measured in an ELISA (see Materials and Methods) in sera from healthy individuals (C), rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), Crohn's disease (CD), and ankylosing spondylitis (AS) patients. Horizontal lines represent the mean OD ratio for each group and the vertical lines represent the standard deviation (SD) for each group. The dotted lines define 2SD above the control means. $*P < 0.01$.

Antibody levels to the mycobacterial and E. coli 70-kDa hsp

IgA and IgG antibody levels to both of these antigens were significantly and similarly raised in RA and SLE relative to the healthy controls. In addition, only the IgA binding to the *E. coli* 70-kDa hsp was significantly raised in sera from AS patients (Fig. 3). Thus, in contrast to the results with the hsp65 proteins, the IgG antibody in RA sera did not show greater binding to the mycobacterial than to the *E. coli* gene product. Moreover, the extent of the increased binding, both in terms of mean value and proportion of donors with levels more than the control mean + 2SD, was as great in SLE as in RA, whereas with the mycobac-

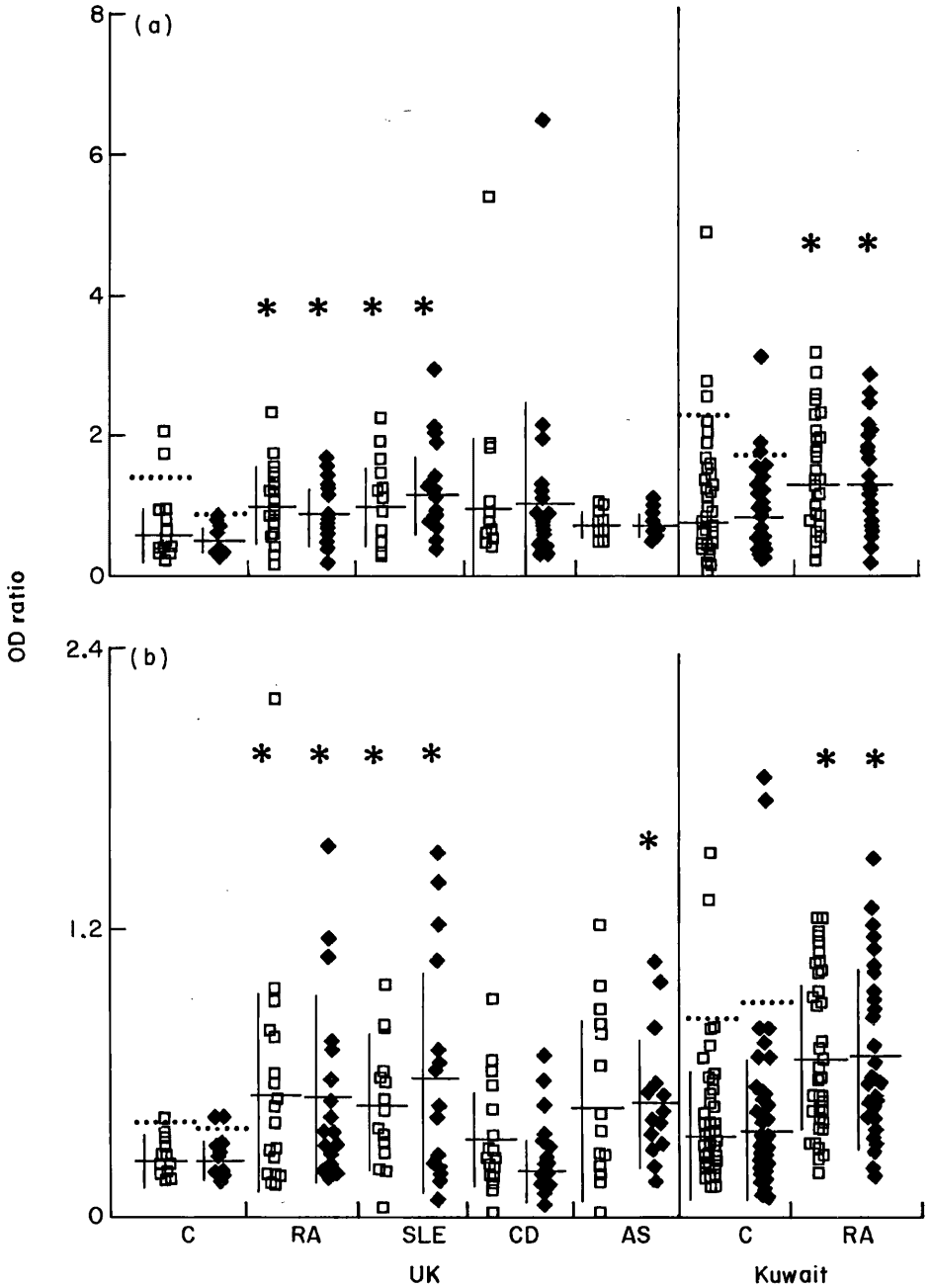

FIG. 3. IgG (a) and IgA (b) antibody levels to the mycobacterial (□) and *E. coli* (◆) 70-kDa hsp were measured by ELISA in serum from healthy individuals (C), rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), Crohn's disease (CD), and ankylosing spondylitis (AS) patients. Sera from healthy individuals and RA patients in Kuwait are also shown. The horizontal lines represent the mean OD ratio for each group and the vertical lines represent the standard deviation (SD) for each group. The dotted lines define 2SD above the control mean. * $P < 0.01$.

TABLE I. IgG and IgA antibody levels to the mycobacterial, *E. coli*, and human 70-kDa hsp measured by ELISA

	Mycobacterial	*E. coli*	Human
IgG			
Healthy adults ($n=79$)	0.52±0.43	0.70±0.39	0.10±0.40
TB patients ($n=90$)	0.83±0.63*	1.00±0.69*	0.26±0.13*
RA patients ($n=41$)	0.81±0.46*	1.01±0.55*	0.24±0.44*
IgA			
Healthy adults	0.20±0.13	0.20±0.18	0.09±0.03
TB patients	0.46±0.36*	0.46±0.28*	0.23±0.15*
RA patients	0.37±0.20*	0.41±0.24*	0.22±0.23*

TB = tuberculosis; RA = rheumatoid arthritis.
*$P<0.001$; results shown are mean absorbance values ± SD.

terial hsp65, the increase was significantly greater in RA.

The results were similar with the Kuwaiti sera (Fig. 3). In this study TB rather than SLE was used as a disease control. As shown in Table I, the rise in antibody binding to the hsp70 antigens was significant, but similar to that seen in TB patients [3].

Antibodies to these antigens were not significantly raised in CD patients.

Antibody levels to the human 70-kDa hsp

RA sera, whether from the UK or Kuwait, showed increased IgG and IgA (but not IgM) binding to the human hsp70 (P values from <0.0001 to <0.000001) (Fig. 4). However, there was no evidence for disease specificity since in all classes the changes were essentially identical in TB (Table I). Similarly, the increase in IgA binding to the human hsp70 in SLE and AS was

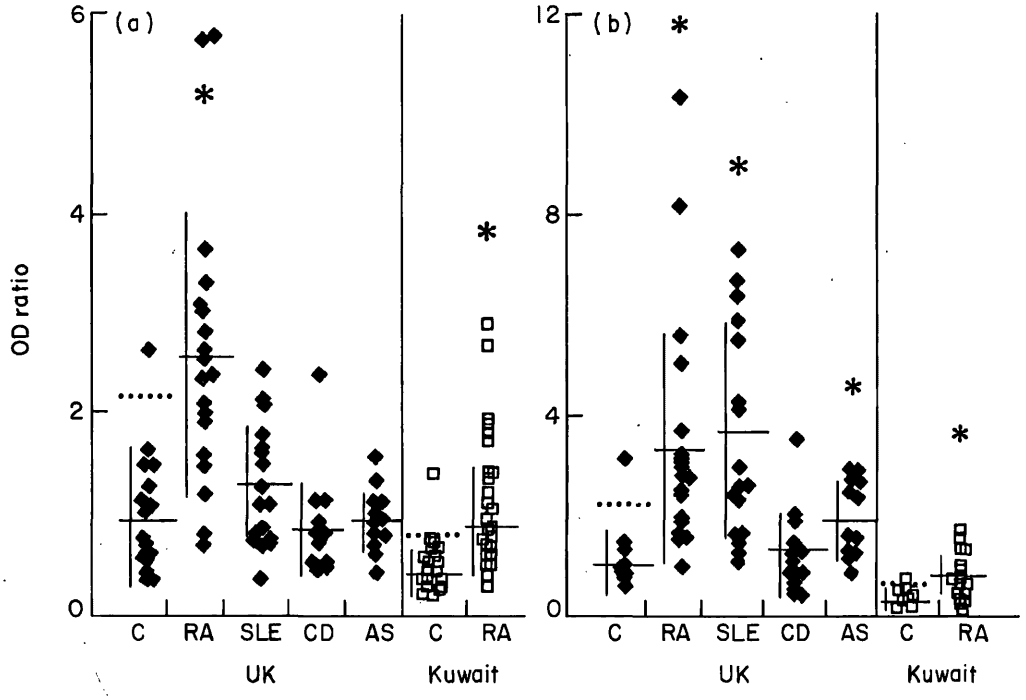

FIG. 4. IgG (a) and IgA (b) antibody levels to the human 70-kDa hsp measured by ELISA in serum from healthy individuals (C), rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), Crohn's disease (CD), and ankylosing spondylitis (AS) patients in the UK (◆) and from healthy individuals and RA patients in Kuwait (□). Horizontal lines represent the mean OD ratio for each group and the vertical lines represent the standard deviation (SD) for each group. The dotted lines define 2SD above the control mean. *$P<0.01$.

similar to that seen in RA. However, SLE and AS differed from RA in that there was no increase in IgG binding to this antigen, and the IgM antibody levels were significantly below those seen in normal sera (Fig. 1).

As seen with the bacterial hsp70 preparations, antibody levels to the human hsp70 were not raised in CD sera.

Antibody levels to hsp in relation to age, sex, drug treatment, and activity of disease

There was no evidence to support a relationship between age and antibody levels in RA and SLE groups ($r < 0.27$). No statistically significant difference was found between the antibody levels in relation to sex, disease activity, or drug treatment ($P > 0.04$). The only correlation that was observed was interestingly between IgA antibody levels to the mycobacterial 65-kDa hsp and age in patients with CD ($r = 0.96$, $P = 0.01$).

DISCUSSION

In this study we show that patients with RA, SLE, CD, AS, and TB can all have increased serum antibody levels to some hsp, but that antibodies, particularly IgG, to the mycobacterial hsp65 discriminate between RA and the other diseases.

The raised antibody levels are unlikely to be due to polyclonal B-cell activation since IgM levels were unchanged, or lower than normal. Furthermore, it is unlikely that our ELISA was influenced by levels of IgA or IgG rheumatoid factor (RF), since there was no correlation between IgA or IgG antibody levels to the mycobacterial hsp65 with levels of RF of these classes (data not shown; $P > 0.588$; $P > 0.302$ respectively).

The data from this and our previous studies [20] point to a degree of disease specificity with regard to the mycobacterial 65-kDa hsp but not to the other hsp tested.

1. RA sera show the greatest increase in IgA binding to the mycobacterial hsp65, but no increase in IgA binding to the *E. coli* gene product.

2. Only RA and TB sera show increased IgG binding to the mycobacterial hsp65, and the titre is greater in RA.

3. In contrast, the use of mycobacterial and *E. coli* hsp70 preparations as control bacterial hsp gene products has shown that RA patients do not differ from TB or SLE patients in their antibody binding to these proteins.

4. Levels of antibody to the human hsp70 in RA sera were not higher than in TB, and the IgA binding was not higher than in SLE, although it was higher than AS. Raised IgG antibody levels to the human hsp70 did distinguish RA from SLE and AS.

5. In contrast, IgA or IgG antibody levels to the antigens tested were not raised in acute rheumatic fever, indicating that elevated antibodies are not due to joint inflammation *per se* (G. M. Bahr *et al.*, submitted). With regard to the latter point, our data did show significantly increased levels of IgG and IgA antibodies to the 70-kDa hsp in both RA and SLE.

The hsp show very high sequence homology not only between bacterial genera, but also throughout all life forms. The fact that IgG antibodies from RA patients affinity purified with mycobacterial 65-kDa hsp bound to a purified human 65-kDa hsp (data not shown) indicates that the 'mycobacterial' antibodies are indeed auto-antibodies. Thus, complexes of these auto-antibodies with autoantigen in the synovium could result in maintenance of chronic inflammation. Using a polyclonal antibody provided by R. S. Gupta and a monoclonal one raised by ourselves we have been able to show a 65-kDa hsp protein in rheumatoid synovial fluid (M. Sharif *et al.*, manuscript in preparation). These same antibodies have demonstrated the presence of the 65-kDa hsp in immune complexes separated by polyethylene glycol precipitation from RA synovial fluids (our unpublished observations). Further analysis of the human 65-kDa hsp response will make use of the recently purified and cloned human 65-kDa hsp (R. S. Gupta, McMaster University, Ontario, Canada, personal communication) and peptides representing parts of the human sequence.

The antibody studies we have undertaken represent a preliminary screening of the possibility that autoimmunity is directed towards the human 65-kDa hsp. Our data on raised levels of antibody in the T-cell-dependent antibody classes of IgG and IgA are consistent with recent data where T-cell proliferation responses in synovial fluid to the mycobacterial form of this antigen are elevated relative to peripheral blood T cells (Ref. 16 and our unpublished observations). The mean-

ing of elevated levels of IgG antibodies to the mycobaterial 65-kDa hsp in RA may be a reflection of a T-cell-mediated response. It is hoped that comparative analysis of recombinant human and mycobacterial 65-kDa hsp will shed light on the extent of humoral and T-cell autoreactivity in RA patients.

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Raised serum IgG and IgA antibodies to mycobacterial antigens in rheumatoid arthritis

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SUMMARY Autoantigens cross reactive with mycobacteria are implicated in the pathogenesis of adjuvant arthritis in the rat, and there are reports of changes in the immune response to mycobacteria in human rheumatoid arthritis (RA). We have therefore examined the IgM, IgG, and IgA antibody levels to crude mycobacterial antigens and to two recombinant mycobacterial heat shock/stress proteins (65 kD and 71 kD) in sera from patients with RA, systemic lupus erythematosus (SLE), and Crohn's disease, and from healthy controls. IgA binding to the crude mycobacterial antigens was significantly raised in RA sera, though IgG and IgM binding tended to be lower than in controls. Both IgA and IgG binding to the heat shock proteins were significantly raised in the RA sera. Smaller significant rises in both classes were seen in sera from patients with SLE, and in the IgA class only to the 65 kD protein in Crohn's disease. The rises in IgG and IgA antibodies to the 65 kD protein in RA were significantly higher than in the other diseases, however. It is interesting that this protein is the one responsible for adjuvant arthritis in the rat.

Key words: antibodies to heat shock proteins, antibodies to stress proteins.

Rheumatoid arthritis (RA) is believed to be an immunological disease, possibly autoimmune, of unknown aetiology. Adjuvant arthritis, which can be induced in rats by immunisation with mycobacteria in oil, is considered by some investigators to be a model of RA. This disease can be transferred to susceptible rats by T cell clones specific for *Mycobacterium tuberculosis*.[1–4] These arthritogenic clones were found to recognise an acetone precipitable fraction of *M tuberculosis*. Interestingly, patients with RA were also reported to have raised T cell responses to an acetone precipitable fraction.[5] This, however, has recently been challenged by the observations that the T cell responses to an *M tuberculosis* antigen in patients with RA are DR4 linked and not associated with RA.[6] More recently van Eden and colleagues have shown that the arthritogenic rat T cell clones recognise a 65 kD protein, which is a component of the acetone precipitable fraction preparation.[7] Evidence that the rat model may indeed be relevant to the human condition is accumulating. Firstly, an association was detected between skin test responsiveness to tuberculin and DR4 in patients with leprosy,[8] and more recently in RA.[9] Secondly, antibody levels to crude mycobacterial sonicates in sera from patients with RA living in Kuwait (a mycobacterium rich environment) showed significant correlations with HLA-DR haplotypes known to be relevant to susceptibility to RA.[10]

The present study examines the possible associations of antibody levels to mycobacteria, and in particular to the 65 kD antigen, implicated in the adjuvant athritis model, in patients with RA living in the UK. It has been found that although patients with RA only showed raised IgA antibodies to crude mycobacterial antigens, IgG as well as IgA antibody levels were raised to the 65 kD and 71 kD antigens.

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Patients and methods

SOURCE OF SERUM SAMPLES

This study was carried out on 85 patients with RA,

as defined by the American Rheumatism Association criteria, attending the outpatient clinic of the Bloomsbury rheumatology unit. Sixty three were female and 22 male, mean age 55 years (range 27–78), duration of disease ranged from two to 20 years, disease activity was based on C reactive protein levels (Abbot Laboratories kit). They were HLA-DR tissue typed.

Eighteen patients with systemic lupus erythematosus (SLE) attending the above clinic were studied as a control disease as SLE is also a non-organ specific autoimmune disease. Seventeen were female and one male, mean age 42 years (range 30–68).

Twenty one patients with Crohn's disease were also included in the study as a non-autoimmune disease group. Ten were female and 11 male, mean age 36 years (range 18–74).

Forty five healthy laboratory staff were studied as a control group. Twenty seven were female and 18 male, mean age 35 years (range 24–63).

SOURCE OF ANTIGENS

A water extract of *M tuberculosis* $H_{37}Ra$ (Difco) was prepared as follows. Heat killed, desiccated *M tuberculosis* were ground with a homogeniser, suspended in double distilled water at 1 mg/ml, stirred for eight hours at 4°C, centrifuged for 30 minutes at 15 000 *g*, and the supernatant was lyophilised. This antigen is referred to as WE. Sonicates of fresh bacilli from *M tuberculosis* (TB) and *M vaccae* (VAC) were also used.[11] The recombinant forms of the 65 kD protein of *M bovis* BCG[12] and the 71 kD protein of *M tuberculosis* (Mehlert and Young, in preparation) were also used. These antigens are referred to as 65 kD and 71 kD respectively.

ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA)

The antigens were coated at 10 μg/ml for WE, TB, and VAC and at 1 μg/ml for 65 kD and 71 kD in carbonate buffer (0·05 M, pH 9·6) onto immunoplates (Nunc) and incubated overnight at 4°C. Excess antigen was washed off with phosphate buffered saline (0·1 M, pH 7·4) containing 0·05% Tween 20 (PBS/T). The test sera were plated in doubling dilutions from 1/50 to 1/400 in PBS/T in duplicate and incubated for two hours at room temperature. After further washes with PBS/T the affinity purified $F(ab')_2$ fragments of horseradish peroxidase conjugated human antibodies (Sigma) were added at 1/1000 dilution in PBS/T and incubated overnight at 4°C. The washing process was repeated, and 0·5 mg/ml of 2,2′-azinobis-(3-ethylbenzthiazoline sulphonic acid) (Sigma) in citrate phosphate buffer (0·1 M, pH 4·1) with 0·35 μl/ml H_2O_2 vol 20 (6% w/v) was added. After approximately 30 minutes the reaction was stopped with 96 mg/ml of sodium fluoride (Sigma) in double distilled water, and the absorbance was measured at 650 nm with a Titertek multiscan ELISA reader (Flow). Throughout the assay the volume of reagents added per well at each step was 100 μl, each wash step was repeated three times with three minutes' incubation between washes at room temperature, and for each individual assay the same positive and negative controls were used.

DATA ANALYSIS

The values of optical density (OD) ratio were calculated as OD_{650} of test/OD_{650} of positive serum control in each plate. The serum dilutions used for the calculations were those within the linear phase of the antibody binding curve and were 1/100 dilution for the IgA ELISA of 65 kD antigen and 1/200 for all the other assays. As the mean age and sex of the individuals for the four groups studied were not the same the data were analysed for evidence that the age or sex influenced the antibody levels. The data were also analysed with respect to activity and duration of the disease.

HLA-DR TYPING

The HLA typing was carried out by The London Hospital, and the frequencies of DR haplotypes in the 85 patients with RA studied were DR1:15, DR2:13, DR3:19, DR4:47, DR5:10, DR6:15, DR7:8, DR8:1, DR9:1, DR10:1, and DR11:1. The data were examined for association between HLA-DR haplotypes (for which not less than five individuals were available) and antibody levels.

STATISTICAL ANALYSIS

The Mann-Whitney U two tailed test was used to compare the antibody levels in the control and experimental groups. The same test was also used to compare antibody levels of patients with RA in association with HLA-DR haplotypes. The Spearman rank correlation coefficient was used to compare antibody levels of the same group of individuals to different antigens as well as to look for correlation between raised antibody levels and sex, age, disease activity, and duration of the disease. Statistical analysis was carried out using the STSC STATGRAPHICS software package.

Results

INCREASED IgA ANTIBODY LEVELS TO CRUDE MYCOBACTERIAL ANTIGENS IN RA

Patients with RA were found to have raised IgA serum antibody levels to the WE ($p<0·01$), TB

($p<0{\cdot}0001$), and VAC ($p<0{\cdot}0001$) antigens in comparison with healthy controls. Serum samples from 19/64, 19/64, and 29/64 patients with RA showed IgA levels more than 2SD above those of the healthy control group levels to WE, TB, and VAC respectively. In addition, the IgG and IgM antibody levels to the same antigens were lower in RA than in controls (Fig. 1). Serum samples from patients with SLE and Crohn's disease were not tested against the crude antigens.

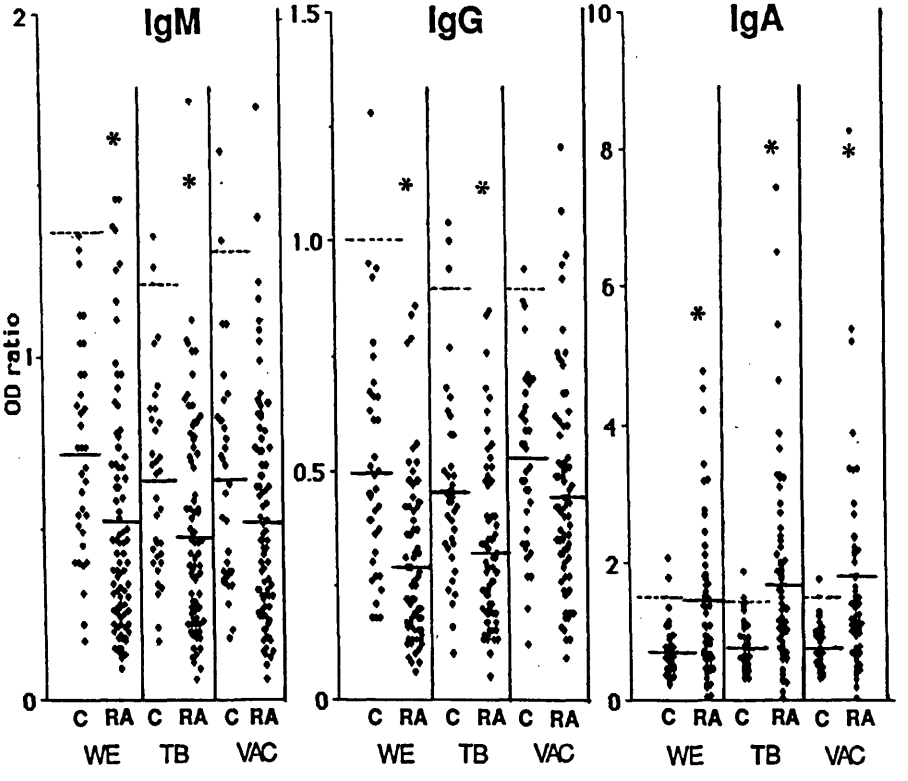

Fig. 1 *Antibody levels to WE, TB, and VAC antigens in healthy controls and patients with RA. The antibody levels are expressed as OD ratios for each group (OD ratio=OD of test/OD of control positive serum in each plate). Horizontal lines represent the mean OD ratio for each group and the dotted lines define 2SD of the control mean.* $p<0{\cdot}01$. *OD=optical density; C=control; RA=rheumatoid arthritis; WE=a water extract of M tuberculosis; TB=sonicates of M tuberculosis; VAC=sonicates of M vaccae.*

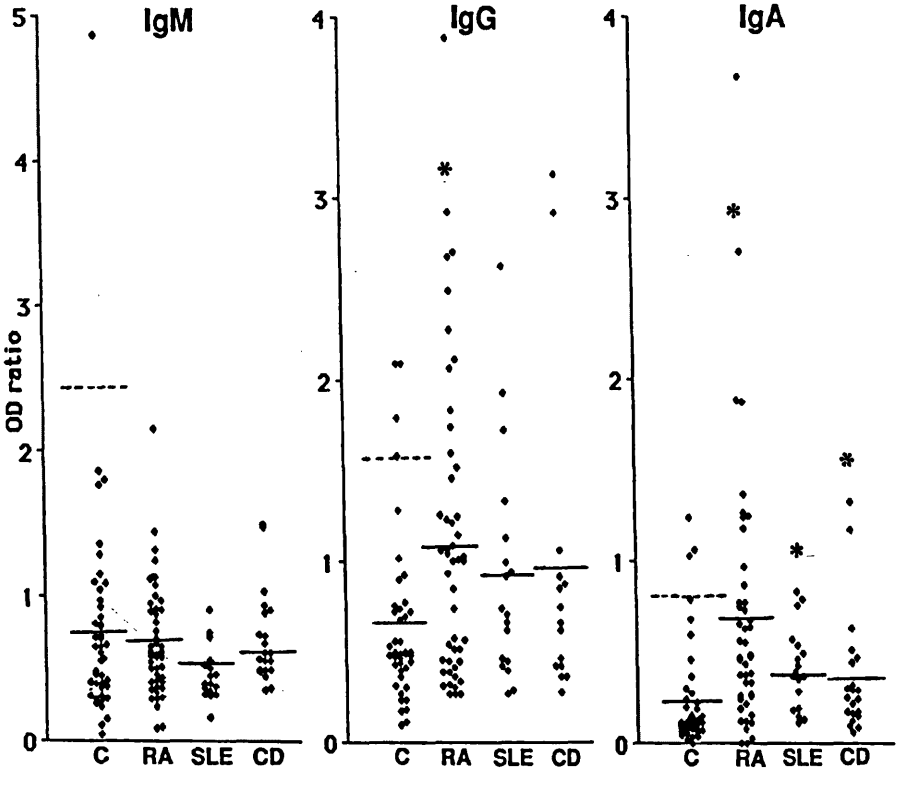

Fig. 2 *Comparison of the antibody levels to the 65 kD protein in healthy controls (C), and in patients with rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), and Crohn's disease (CD). Data presented as in Fig. 1.* $p<0{\cdot}01$.

INCREASED IgA AND IgG ANTIBODY LEVELS TO THE 65 kD AND 71 kD MYCOBACTERIAL ANTIGENS IN RA

When defined mycobacterial antigens were used the differences in antibody levels in RA and controls were not confined to the IgA class only ($p<0{\cdot}0001$). Patients with RA also showed raised IgG antibody levels to both antigens when compared with the controls ($p<0{\cdot}01$), while their IgM levels were lower (but not significantly) to both 65 kD and 71 kD antigens (Figs 2 and 3). Serum samples from 11/45 and 9/19 patients with RA showed IgA levels to the 65 kD and 71 kD antigens respectively which were more than 2SD above those of the healthy control mean. Also, 10/42 (to the 65 kD) and 7/39 (to the 71 kD) RA sera showed raised IgG levels more than 2SD above those of the control values.

ANTIBODY LEVELS TO THE 65 kD AND 71 kD MYCOBACTERIAL ANTIGENS IN PATIENTS WITH SLE AND CROHN'S DISEASE

Patients with SLE were also found to have raised IgG ($p<0{\cdot}001$) and IgA ($p<0{\cdot}001$) antibody levels to both antigens, but their IgM levels were not significantly different from the control levels. Although serum IgG levels were raised in SLE to the 65 kD protein, the quantity of antibodies was lower than that seen in sera from patients with RA (Figs 2 and 3). Only 3/18 sera had IgG levels more than 2SD above those of the controls to the 65 kD and 4/18 to the 71 kD antigens. With regard to IgA levels, only 1/18 sera was more than 2SD above those of the control to the 65 kD, while 10/18 sera were higher to the 71 kD protein.

Patients with Crohn's disease, on the other hand, were also found to have significantly raised IgA antibody levels to the 65 kD antigen ($p<0{\cdot}001$) but again not to the same degree as the RA group (Fig. 2). Only 2/21 sera for the 65 kD antigen had a significantly higher IgA level than the control group.

LACK OF RELATION BETWEEN RAISED ANTIBODY LEVELS AND AGE, SEX, DURATION, AND DISEASE ACTIVITY

Serum samples of patients with RA with significantly higher IgG and IgA antibody levels were compared with RA sera with antibody levels equal to control levels. No correlation was found between antibody levels and age, sex, disease activity, and disease duration. Similarly, the few patients with SLE and Crohn's disease whose levels were more than 2SD above the control mean did not differ clinically from those with normal values.

ANTIMYCOBACTERIAL RESPONSES AND DR HAPLOTYPE

The data on mycobacterial responses were examined for associations between antibody levels and HLA-DR haplotypes as other investigators have suggested such associations. Our data showed no

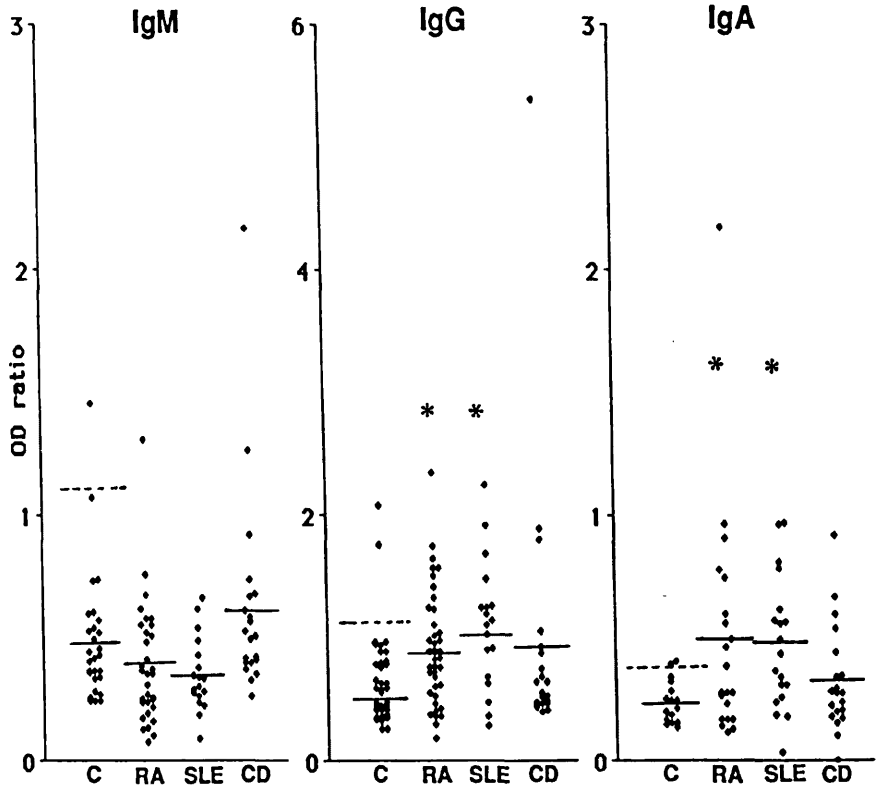

Fig. 3 *Comparison of antibody levels to the 71 kD protein in healthy controls (C), and in patients with rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), and Crohn's disease (CD). Data presented as in Fig. 1. *p<0·01.*

association with the DR haplotypes shown to be relevant to RA, such as DR4, DR2, and DR7. Although DR1 patients with RA showed higher IgA antibodies to the WE ($p<0{\cdot}06$), TB ($p<0{\cdot}05$), and VAC ($p<0{\cdot}02$) antigens than non-DR1 individuals, these p values do not remain significant when corrected for the number of comparisons made.

Discussion

This study has shown that patients with RA have significantly raised antibody levels of the IgA class to the crude mycobacterial antigens WE, TB, and VAC. Our data are consistent with a recent study in Kuwait using TB, VAC, and five similar preparations from other species, where there was a tendency towards raised IgA antibody levels in RA sera, though significance was only reached with *M nonchromogenicum*. All antibody and skin test responses to mycobacteria are high in Kuwait, however, as the environment is rich in mycobacteria.[10]

To obtain more information about the specificity of antibodies to the mycobacteria in the RA sera we examined the levels of the different classes of antibodies to the recombinant 65 kD and 71 kD proteins. Both IgG and IgA levels were increased to these 'clean' antigens. The fact that these proteins show no homology with each other indicates that antibodies to at least two major mycobacterial antigens are raised in these patients with RA.

Raised levels to one recombinant protein did not correlate with the antibody levels to the other protein. In fact, analysis of 10 individual patients' sera with higher levels of IgG antibodies to the 71 kD protein than control sera (OD ratio >mean OD ratio +2SD) showed that two sera were also high for the 65 kD protein, the other eight being low for this antigen. Furthermore, some sera with high IgA levels to the 71 kD protein were lower for the 65 kD protein. These observations, together with the findings of lower (but not significant) IgM levels in patients with RA to all mycobacterial antigens tested, argue against polyclonal activation as a source of raised antibodies. Moreover, it is interesting that in Kuwait, where responsiveness to mycobacteria is very high, this tendency for IgM binding to mycobacteria to be low in RA was significantly correlated with DR7, a haplotype known to be protective in RA.[10]

No significant differences were seen in duration and activity of the disease, sex, or age of the patients with respect to antibody levels. This indicates that although raised levels of antibodies are found in these patients with RA, a relation with the disease itself is not yet clear. In this regard we analysed the relation with DR to determine whether the raised levels could be correlated with known DR haplotypes previously shown to be related to RA—for example, DR4.[13–15] No association was found between antibody levels and DR4. This result appears to be different from data on T cell responses to mycobacteria, which have been correlated with DR4 expression in RA and healthy individuals,[6] and may be important in relation to the pathogenesis in DR4 individuals. A parallel study in Kuwait with the same antigen preparations has shown comparable results with regard to raised antibody levels to the 65 kD protein in RA and lack of meaningful DR correlation (Bahr *et al*, unpublished data).

It is interesting that IgG and IgA antibody levels were raised to both the 65 kD and 71 kD proteins in patients with SLE, suggesting that the increase of antibodies in itself is not disease specific. IgG and IgA antibodies to the 65 kD protein in SLE and IgA in Crohn's disease were lower than in patients with RA, whereas antibodies in SLE sera to the 71 kD protein were raised but not significantly different from those in patients with RA ($p>0{\cdot}1$). This argues in favour of the importance of the 65 kD mycobacterial protein in this disease as previously suggested from cellular studies in the rat model of RA.[7] Further recent data support this concept as IgG antibodies to the 65 kD protein from *Escherichia coli* are not raised in sera from patients with RA (paper in preparation). It is clear, however, that the mycobacterial proteins used here show high degrees of homology with similar proteins in both prokaryotic and eukaryotic organisms. Thus we cannot at this stage reliably implicate the mycobacteria as the major immunogens leading to the responses measured here.

The 65 kD and 71 kD mycobacterial proteins have recently been shown to be heat shock/stress proteins.[16] They show a great degree of homology with human stress proteins, and recent evidence has supported their role in inflammation.[17] Antibodies to a 90 kD heat shock protein have been shown to be raised in SLE sera.[18] The human 70 kD protein has been shown to be spontaneously synthesised by chondrocytes in patients with severe osteoarthritis compared with chondrocytes in healthy controls.[19] In this regard we have shown that IgG and IgA antibodies to the human 70 kD protein are also raised in the sera of these same patients with RA (paper in preparation).

In further studies we hope to evaluate the relevance of the specific antibodies to the various stress proteins (especially the 65 kD) in RA.

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