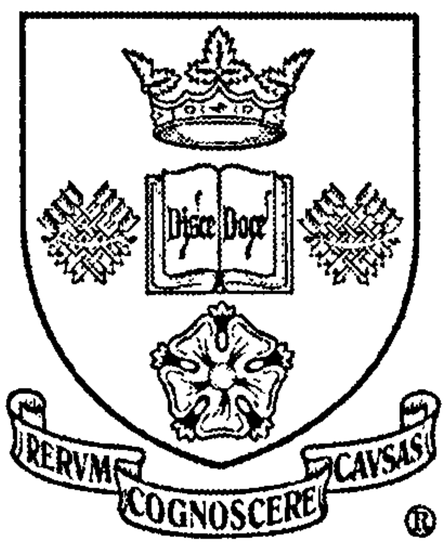
Studies on autoantigens in rheumatoid arthritis

A thesis submitted for the degree of Doctor of Philosophy

University of Sheffield

February 2003



By Devesh Mewar

Summary

This thesis describes the use of phage display for the isolation of autoantigens in rheumatoid arthritis. The potential of the technology is demonstrated by the isolation of an autoantigen, eukaryotic translation elongation factor 1α 1 (eEF1α 1) from a fibroblast cDNA library using rounds of selective enrichment with IgG from RA patients.

Subsequently in order to isolate joint-specific antigens a phage-displayed cDNA library from rheumatoid pannus was generated and screened with analogous procedures. From the clones isolated, putative candidate autoantigens were identified. The presence of anti-eEF1α 1 autoantibodies in approximately 20% of patients with RA was confirmed and extended in larger panels of sera, and the finding of anti-eEF1α 1 shown to be relatively specific for RA. In contrast autoantibodies to the activation-induced negative regulator of T cells, CTLA-4 were not found in contrast to a previous report. The relevance of these findings for the use of antibodies in the diagnosis and prediction of disease characteristics in RA are discussed.

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Abbreviations

amp	ampicillin
APC	Antigen-presenting cell
CD	Cluster designation
CDR	Complementarity-determining region
CII	Type II collagen
CTLA4	Cytotoxic T lymphocyte associated molecule 4
GD	Graves' disease
HLA	Human leukocyte antigen
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
LB	Luria-Bertani
MCTD	Mixed connective tissue disease
MHC	Major histocompatibility complex
NHD	Normal human donors
NK	Natural killer cells
NOD	Non-obese diabetic
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PM	Polymyositis
RA	Rheumatoid arthritis
SLE	Systemic lupus erythematosus
TcR	T cell receptor
tet	tetracycline
TNF	Tumor necrosis factor

Acknowledgements

I would like to thank Dr Gerry Wilson and Dr Phil Watson for their support and enthusiasm for this project as well as practical help. Also all the members of the Molecular Medicine laboratory at the Royal Hallamshire Hospital as well as the members of the Medicine laboratory, Clinical Sciences Centre, Northern General hospital for their help during various parts of the project, particularly Janine Timms and Hazel Holden for oligonucleotides and sequencing in addition to general know-how, Jenny Barton, Helen Kemp, Liz Waterman, Janine Phipps and Laura Herd. I would like to thank Russell Metcalfe for help with FACS. I would like to acknowledge numerous helpful and stimulating discussions as well as practical help from Martin Nicklin, Jon Sayers and Colin Bingle.

The Arthritis Research Campaign funded this project through a Clinical Research Fellowship.

1 Introduction

1.1 Rheumatoid arthritis

Rheumatoid arthritis (RA) is a systemic disease whose characteristic clinical features include a symmetrical polyarthropathy affecting large and small joints often leading to progressive joint destruction. The prevalence in Europe and North America is approximately 1% (Sangha, 2000) with a female to male predominance of around 3:1 and it is therefore a leading cause of disability in these populations. Considerable heterogeneity exists at the clinical level in terms of mode of onset, pattern of joint involvement, severity, extra-articular features and response to treatment suggesting that the term rheumatoid arthritis includes individuals with a spectrum of pathological states all leading to persistent synovial inflammation (Weyand et al., 1998b). Etiopathogenesis is believed to be multifactorial with genetic and environmental factors playing a part in susceptibility and severity.

1.2 The influence of genetic factors in susceptibility to RA

Twin and family studies suggest that genetic factors account for a substantial proportion, perhaps 30-50% of the susceptibility to RA (Barton and Ollier, 2002), (Deighton et al., 1992). This risk can be conveniently expressed as λs, the coefficient of familial clustering and is calculated as the ratio of disease prevalence of siblings of probands to the population prevalence. Values for λs of 5-10 have been estimated for RA (Ollier and Worthington, 1997). Estimating λs for each locus can assess the contribution of individual loci to the overall genetic susceptibility. The association of RA with the serologically defined HLA DR4 was first described more than twenty years ago (Stastny,

1978) and this association has been confirmed in a number of studies although is variable in different ethnic groups. The λs value for this association has been estimated as 1.8 (Marlow et al., 1997) suggesting that HLA DR4 accounts for around one-third of the genetic component of susceptibility. Although this is the major genetic association with susceptibility to RA two-thirds remains unaccounted for and therefore other loci are likely to play a role. A number of approaches including genome wide screening, case-control association studies and family based transmission-disequilibrium testing of candidate genes are currently being employed to search for other disease-associated loci (Barton and Ollier, 2002).

1.3 Pathological changes in RA synovial membrane

Early histological changes in RA synovial membrane include hyperplasia of the intimal lining layer fibroblast-like synoviocytes and macrophages, neoangiogenesis, and the accumulation of a cellular infiltrate in the sublining layer comprising T and B cells, plasma cells, macrophages, dendritic cells, NK cells, mast cells and neutrophils (Tak and Breedveld, 1999). The advent of needle arthroscopy has allowed the description of macroscopic features of synovial inflammation that include macroscopic neoangiogenesis and the formation of villi (Reece et al., 1999). Microscopically three distinct patterns of cellular organization recognised as diffuse, follicular and granulomatous synovitis have been described and appear to correlate with particular cytokine profiles suggesting that each may represent a subgroup of patients with distinct pathogenetic mechanisms (Klimiuk et al., 1997). Later changes include the development of fibrosis and the

destruction of bone and cartilage. This invasive and destructive infiltrate is often referred to as 'pannus'.

1.4 Pathogenic mechanisms in RA

The majority of cells in the intimal and sublining layers are macrophages and activated fibroblasts (Tak and Breedveld, 1999) and are believed to play a major effector role. These cell types show a highly activated phenotype and secrete a number of soluble mediators such as cytokines and matrix metalloproteinases resulting in persistent inflammation and joint damage (Choy and Panayi, 2001). The importance of macrophage-derived mediators such as TNFα is illustrated by the efficacy of anti-TNFα therapy in patients with RA (Lipsky et al., 2000; Maini et al., 1999). Much less clear is the nature of the upstream events that drive the recruitment, proliferation and maintenance of these cells in the synovium. Close spatial arrangements of CD4+ T cells, B cells and dendritic cells amounting to germinal centre-like structures in some cases (Klimiuk et al., 1997) suggests that chronic activation may be occurring, however synovial T cells do not express classical markers of activation such as the IL-2 receptor, nor secrete IL-2 and IFNγ indicating they are in an unusual state of differentiation (*see below*).

1.5 HLA association with RA – the shared epitope hypothesis

Understanding of the molecular basis of the association of particular HLA DR types with RA was significantly advanced in the late 1980s when sequence analysis of disease associated HLA DR molecules from different populations revealed a common amino acid

Table 1. RA associated DRβ alleles – the shared epitope.

	DRβ chain residue					
	57	**67**	**70**	**71**	**74**	**86**
RA associated alleles						
DRB1*0101	D	L	**Q**	**R**	A	G
DRB1*0102	D	L	**Q**	**R**	A	V
DRB1*0104	D	L	**Q**	**R**	A	V
DRB1*0401	D	L	**Q**	**K**	A	G
DRB1*0404	D	L	**Q**	**R**	A	V
DRB1*0405	S	L	**Q**	**R**	A	V
DRB1*0408	D	L	**Q**	**R**	A	G
DRB1*0409	S	L	**Q**	**K**	A	G
DRB1*0410	S	L	**Q**	**R**	A	V
DRB1*0413	D	L	**Q**	**K**	A	V
DRB1*0416	D	L	**Q**	**K**	A	G
DRB1*0419	D	L	**Q**	**R**	A	G
DRB1*0421	D	L	**Q**	**K**	A	G
DRB1*1402	D	L	**Q**	**R**	A	G
DRB1*1406	D	L	**Q**	**R**	A	V
DRB1*1001	D	L	**Q**	**R**	A	G
RA non-associated alleles						
DRB1*0103	D	I	**D**	**E**	A	G
DRB1*0402	D	I	**D**	**E**	A	V
DRB1*0403	D	L	**Q**	**E**	A	V
DRB1*0406	D	L	**Q**	**E**	A	V
DRB1*0407	D	L	**Q**	**E**	A	G
DRB1*0411	S	L	**Q**	**E**	A	V
DRB1*0412	S	D	**Q**	**L**	A	V
DRB1*0414	D	D	**Q**	**R**	A	G
DRB1*0415	D	D	**Q**	**R**	A	V
DRB1*0417	S	L	**Q**	**E**	A	G
DRB1*0418	D	D	**Q**	**L**	A	V
DRB1*0420	D	L	**Q**	**E**	A	G
DRB1*0422	D	L	**Q**	**G**	A	V
DRB1*1401	A	R	**Q**	**E**	A	V
DRB1*0901	V	R	**Q**	**E**	A	V

Table 1. Sequence comparison of RA-associated and non-associated DRB1 alleles in the third hypervariable region. Residues in bold represent those believed to be critical for peptide binding.

motif in the DRβ chain referred to as the "shared epitope" (table 1) (Gregersen et al., 1987). Importantly different DRB1 alleles appear to influence the severity of disease as well as susceptibility with homozygosity for DRB*0401 associated with the most severe phenotype and particularly extra-articular disease (Weyand et al., 1995). A number of structural and functional studies have demonstrated that residues 70, 71 and 74 form part of an alpha helix of the DRβ chain that is critical in determining the peptide binding specificity of each DR molecule (Wucherpfennig and Strominger, 1995). Disease-associated alleles encode a positively charged residue at position 71 (K or R) and hence accommodate peptides with a negatively charged residue at position 4 (P4) of a putative 9 amino-acid peptide bound in the groove of the encoded β chain. The pemphigus vulgaris-associated and RA non-associated DRB1*0402 allele differs from DRB1*0401 and 0404 only by carrying a negatively charged residue at position β71, therefore accomodating peptides with a positive charge at position P4 (Hammer et al., 1995) (table 1). Such studies have provided strong though indirect evidence that the HLA association with RA is due to a functional effect of the associated alleles and not related to a linked susceptibility locus.

The only known function of MHC class II molecules is the presentation of peptide antigen to T cells, supporting the view that specific T cell/MHC class II/peptide interactions are important in the pathogenesis of RA. A number of mechanisms have been proposed to explain how such interactions could directly influence susceptibility (Buckner and Nepom, 2002), all fall into one of two broad categories – the presentation of unique peptide antigens to T cells, or in shaping of the T cell repertoire during thymic

selection. Studies of animal models as well as analysis of T and B cell responses of patients with RA have contributed to attempts to understand the influence of the shared epitope on disease susceptibility.

1.6 Evaluating the shared epitope hypothesis *in vivo*.

That the shared epitope hypothesis is a biological plausibility is supported by considerable evidence, the majority from studies of experimental models. Three such systems are discussed below.

1.6.1 Collagen-Induced Arthritis (CIA) – a disease dependent on specific T cells and antibody directed against a component of articular cartilage.

Type II collagen is specific to diarthrodial joints. Immunisation of susceptible strains of rodents with heterologous type II collagen results in a severe polyarticular arthritis (Trentham et al., 1977). Onset usually occurs around day 28 following the initial immunisation and the disease follows a monophasic course with synovitis, erosions of bone and cartilage and eventual healing by fibrosis and ankylosis (Stuart et al., 1985). Complement fixation by CII-specific antibody has been clearly shown to be the mechanism of pathology (Watson et al., 1987). Using a large panel of congenic B10 mouse strains susceptibility to CIA induced with chick CII was shown to be associated with the MHC class II isotype H-2q (Wooley et al., 1981) and accompanied by strong antigen-specific T cell and autoantibody responses. Other strains also developed CII-specific T cell responses and high titre antibodies but did not develop disease, suggesting that the presentation of specific peptides by disease-relevant MHC molecules may induce

a qualitatively different response in T cells resulting in disease (Wooley et al., 1981). H-2r strains were later also shown to be susceptible to disease induced with bovine or porcine CII but not chick (Wooley et al., 1985). The T cell determinants associated with susceptibility to disease have been characterised in detail. In H-2q mice, strong T cell responses to the peptide 260-267 exist (Brand et al., 1994) and are associated with disease susceptibility. However in H-2r mice, 2 immunodominant determinants have been identified (Myers et al., 1995). Interestingly although T cell responses to both have been shown, disease susceptibility is clearly associated with responses to the less potent determinant in terms of T cell proliferation (Myers et al., 1995), again suggesting that only certain T cell responses can result in disease. Adoptive transfer of disease by T cells to SCID mice has been demonstrated although a booster immunisation was required (Hesse and Mitchison, 2000; Kadowaki et al., 1994), and T cell depleting treatments alleviate disease (Zhang et al., 2002) underpinning the important pathogenic role of T cells in this model.

1.6.2 Human HLA transgenic mice

The MHC encodes large numbers of immunologically active molecules in addition to HLA molecules, and loci in close proximity to each other exhibit linkage disequilibrium resulting in the formation of extended haplotypes (Wilson et al., 1993). Hence a major problem in the analysis of disease associations with HLA has been isolating the contribution of an individual HLA type to disease susceptibility (Hall et al., 1999). The development of transgenic mice expressing human CD4 and the disease-associated DRB1*0401 or disease-neutral DRB1*0402 in the absence of murine class II molecules

has provided a unique *in vivo* model wherein the effects of individual DR molecules on immune responses can be investigated in isolation (Fugger et al., 1994). The use of this approach is illustrated by studies of transgenic mice expressing DRB1*0401 or DRB1*0402 immunised with the candidate antigen human cartilage glycoprotein 39 (*see below*) (Cope et al., 1999). After immunisation a panel of T cell hybridomas was raised from draining lymph nodes and their specificities analysed by measurement of proliferative responses to whole protein and pools of overlapping synthetic peptides spanning the whole molecule. Three immunodominant epitopes in the context of DRB1*0401 and two completely different epitopes in the context of DRB1*0402 were defined (Cope et al., 1999). These three epitopes were the same peptides previously shown to activate T cells from patients with RA (Verheijden et al., 1997). These results clearly demonstrate that disease associated DR alleles present different sets of peptides derived from the same antigen compared to disease non-associated alleles, and that the theoretical predictions of peptide binding to DR molecules are applicable *in vivo*.

Another important observation made in the above system, corroborating studies of CIA is that DR molecules can have antigen-specific effects on the qualitative nature of immune responses. Hence following immunisation of DRB1*0401 mice, when T cells were restimulated with the relevant immunodominant peptides prominent production of IFNγ and TNF was seen, however when T cells from DRB1*0402 mice were stimulated with the appropriate immunodominant peptides little cytokine production was seen, despite equivalent proliferative responses as measured by thymidine incorporation (Cope et al., 1999). Spontaneous arthritis has not been observed in DRB1*0401/murine class II

deficient transgenic mice however they are susceptible to collagen-induced arthritis (Andersson et al., 1998) suggesting that DRB1*0401 is capable of promoting the development of arthritogenic anti-CII specific T cells.

1.6.3 The K/BxN mouse

The K/BxN mouse represents a MHC restricted spontaneous model of arthritis (Kouskoff et al., 1996). The original KRN transgenic strain expresses a single TcR derived from a T cell hybridoma with specificity for a single peptide of bovine ribonuclease restricted by IAk. Transgene-expressing T cells overwhelm central tolerance induction mechanisms and make up a significant proportion of α/β T cells in the periphery. Crossing this strain onto the NOD background resulted in a strain designated K/BxN (KRN/B10 x NOD) that developed an inflammatory arthritis with complete penetrance (Kouskoff et al., 1996). Susceptibility to arthritis was demonstrated by backcrossing and transgenic experiments to be dependent on the NOD-encoded IAg7 MHC class II specificity (Kouskoff et al., 1996). Subsequently the KRN T cells were shown to cross react with a peptide of the ubiquitous enzyme glucose-6-phosphate isomerase (G6PI) presented by IAg7, and the disease shown to be transferable purely with specific IgG against G6PI (Matsumoto et al., 1999). Hence the relevance of this system for the paradigm discussed above is in demonstrating that a joint-specific disease can be dependent on the presentation of a specific peptide by a disease-linked MHC molecule.

The structural and functional studies described above provide clear evidence that the presentation of unique peptides to T cells could be a possible basis for the susceptibility

to RA associated with the shared epitope and strongly implicate T cells as being important in RA. However they do not provide any direct evidence for the pathogenic involvement of T cell responses in human disease. In the following sections the properties of T cells in human disease shall be considered in relation to a possible pathogenic role.

1.7 T cells in RA

1.7.1 Properties of T cells in RA

Rheumatoid synovial membrane is infiltrated with T cells, and topographical arrangements have been noted bearing marked resemblance to those seen in primary lymphoid tissue (Iguchi and Ziff, 1986; Kurosaka and Ziff, 1983; Young et al., 1984). Structures indistinguishable from germinal centres are seen in some patients (Klimiuk et al., 1997). However the composition and structure of the infiltrate is variable and stromal components can dominate the picture (Kurosaka and Ziff, 1983). It is possible that these variations may represent different stages of disease as prospective studies are difficult to perform. Many studies have attempted the characterisation of synovial T cells in terms of cell surface marker expression, cytokine production and clonality based on T cell receptor gene segment usage. Synovial T cells are predominantly CD4 positive, and express cell surface markers such as LFA1, CD69 and CD45RO typical of memory cells (Iannone et al., 1994). This is to be expected as it is only after activation that T cells acquire cell surface molecules such as the integrin VLA4 necessary for interaction with non-lymph node endothelium and therefore the ability to traffic through non-lymphoid

tissues. However contrary to primed T cells in peripheral blood or T cells activated *in vitro*, they express very low levels of CD25, the IL-2 receptor (Iannone et al., 1994). Furthermore the typical T cell cytokines IL-2 and IFNγ have been very difficult to detect in the synovium, although more recently IFNγ protein and mRNA have been demonstrated in synovial tissue and peripheral blood.. This unusual phenotype is likely to reflect continuous recruitment of T cells into the joint (Iannone et al., 1994) and specific survival signals in the highly specialised microenvironment of the synovium (Akbar and Salmon, 1997). Additionally it has been shown that *ex vivo* synovial T cells are hyporesponsive to stimulation via the CD3/TcR complex. A substantial body of work now exists to support the view that chronic exposure of synovial T cells to TNF, as well as other aspects of the synovial cytokine and cellular milieu may explain these observations (Cope, 2002).

There is some evidence to support the view that differentiated memory T cells in RA corresponding to the Th1 subtype may be pathologically relevant (Skapenko et al., 1999) and type 2 cytokines such as IL-4 could be used to regulate their function. Clonal expansions of T cells in synovial tissue and peripheral blood have been reported in RA by many authors (Weyand and Goronzy, 1999). These expansions appear stable over time and are common to multiple involved joints in an individual. Some expanded clonotypes seem to correspond to a subset of CD4+CD28- T cells that are more prone to autoreactivity (Schmidt et al., 1996). Such clonal expansions have been reported in normal individuals and occur more frequently with age (Weyand et al., 1998a). No common antigen specificity or T cell receptor gene segment usage has been identified in RA suggesting that the observed clonal expansions may not be antigen-driven but rather

reflect defects in T cell homeostasis and the stringent requirements for survival and differentiation in the joint (Weyand and Goronzy, 1999).

1.7.2 Anti-T cell therapies

Although much is known about the properties of synovial and peripheral T cells in RA their involvement in disease initiation or persistence remains implied. The ultimate test of the importance of T cells in pathogenesis is the efficacy of therapy directed at T cells. Several early anti-lymphocyte modalities including lymphocytapheresis (Emery et al., 1986), total lymphoid irradiation (Helfgott, 1989), thoracic duct drainage (Ueo et al., 1979) and cyclosporin A (Weinblatt et al., 1987) have been shown to be effective. More recently a number of studies have investigated the use of specific monoclonal antibody therapy directed at T cells (Choy et al., 1998; Isaacs, 2001). Many small-scale open-label studies have suggested that these agents are efficacious, however a number of problems have been encountered, particularly the production of human anti-mouse antibodies and profound lymphopaenia. These effects have been unpredictable and reflect specific properties of the individual antibodies employed; ongoing work in this field is focussed on defining the properties responsible for such effects and engineering variants for clinical testing. T-cell receptor peptide vaccination offers a method of targeting clones of T cells by directing a therapeutic immune response towards clonal expansions of T cells bearing particular α/β receptors. An initial study reported no adverse effects and clinical improvement and further studies are planned (Moreland et al., 1998).

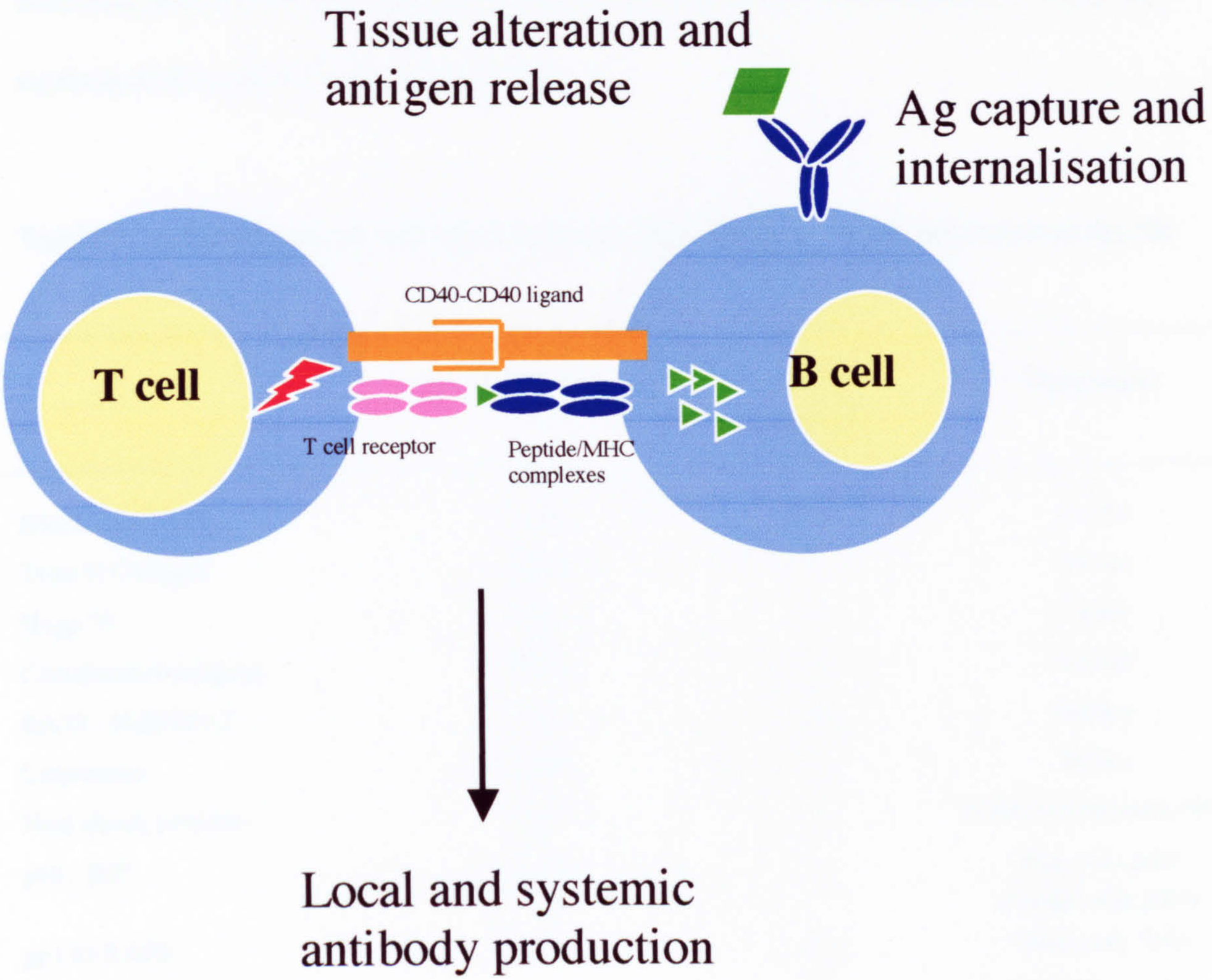
Taking all the available evidence into account, there is much to support the view that T cells are important in the pathogenesis of rheumatoid arthritis, whether due to antigen-specific mechanisms or antigen non-specific recruitment to a unique site of inflammation.

1.8 T – B cell interactions in RA

The production of high-affinity antibodies by B cells is a CD4+ T cell-dependent process. T cell help provided by direct cell-cell interaction involving for instance the CD40-CD40L pair and also secreted mediators is required for class switching and affinity maturation due to somatic hypermutation (Clark and Ledbetter, 1994). In addition B cells can act as highly efficient antigen-presenting cells by internalising soluble antigens via high affinity surface immunoglobulin and presentation to T cells in the context of class II MHC molecules (Clark and Ledbetter, 1994). Rheumatoid factor producing B cells characteristic of RA may have such a function (see below and figure 1), and indeed B cells have been shown to play such a role in animal models of autoimmune disease such as NOD (Falcone et al., 1998) and MRL/lpr (Chan et al., 1999) mice. There is considerable evidence for the interaction of T and B cells in RA. Germinal centre-like structures occur in RA synovium implying close functional relationships between B and T cells and APCs such as follicular dendritic cells (Randen et al., 1995). B cells from RA synovium show evidence of class switching (Rudolphi et al., 1997) as well as somatic hypermutation (Gause et al., 1995) known to be T cell dependent processes. Hence the study of autoantibodies in RA represents a potential approach to identify T cell epitopes. The relative ease of analysis of autoantibodies as compared to T cells makes them potentially very useful as diagnostic or prognostic markers, or tools to attempt to define

subgroups of patients with related clinical or pathogenetic features. These considerations have driven a large body of work attempting to characterise autoantibody responses in RA.

Figure 1. The T – B cell paradigm in rheumatoid arthritis



1.9 Autoantibody systems in RA

A number of autoantigen systems have been described in RA and their clinical associations and role in pathogenicity or as markers of disease examined. These are summarised in table 2 and discussed below.

Table 2. A summary of well characterised autoantigens in rheumatoid arthritis

	Antibody prevalence %	T cell responses[1]	References
Rheumatoid factor	60-80	ND	See text
Type II Collagen	10-30	Y	See text
Hcgp 39	ND	Y	See text
Citrullinated antigens	30-50	ND	See text
RA33 / HnRNPA2	30	ND	See text
Calpastatin	10-50	ND	See text
Heat shock proteins	5-10	Y	(Tishler and Shoenfeld, 1996)
p68 / BiP	30-64	Y	(Blass et al., 2001) (Corrigall et al., 2001)
gp130-RAPS	73	ND	(Tanaka et al., 2000)
Follistatin-related protein	30	ND	(Tanaka et al., 1998)
CTLA-4	18	ND	(Matsui et al., 1999)
eEF1-alpha1	21	ND	(Ditzel et al., 2000)
Lactoferrin	10	ND	(Locht et al., 2000)
Denatured aldolase a	10	ND	(Ukaji et al., 1999)
Osteopontin	15	ND	(Sakata et al., 2001)

Table 2. ND not determined, Y yes, N No

[1]Measurement of T cell responses to antigens varies considerably in terms of the site from which cells were isolated, whether or not ex vivo priming was carried out and the method used for determining response, hence frequencies are not directly comparable and Y or N therefore denotes presence or absence of responses.

1.9.1 Rheumatoid factors

Rheumatoid factors (RFs), the first described human autoantibodies (Waaler, 1940) are directed at the Fc region of IgG and are usually of IgM isotype. They are detectable in up to 10% of normal individuals, 70-80% of patients with RA and in other systemic diseases such as Sjogren's syndrome and many systemic infections (Tighe and Carson, 2001). Rheumatoid factors and the B cells that synthesise and secrete them are believed to have a physiological role as part of the immune system. However RFs from healthy individuals and patients with RA differ considerably. Rheumatoid factors in healthy individuals are synthesised by CD5+ B cells, exhibit low affinity for IgG, polyreactivity and a low ratio of replacement to silent mutations in their CDRs (Borretzen et al., 1997; Mantovani et al., 1993) suggesting the presence of a mechanism to suppress the affinity maturation of RFs. This is supported by the observation that high affinity RFs are deleted in mice (Tighe et al., 1995). In rheumatoid synovium, B cells produce high affinity RFs with multiple replacement mutations in their CDRs (Borretzen et al., 1997) indicating a process dependent on T cell help. A crucial role for CD40 signalling on the production of RF in a transgenic mouse model has been demonstrated (Kyburz et al., 1999) suggesting that bystander T cell help could drive RF synthesis. In addition synoviocytes themselves can support the survival and differentiation of B cells (Edwards et al., 1997). Hence in RA synovium RF-producing B cells could encounter a milieu which would support their survival and provide the T cell help necessary for the production of high affinity RFs (Edwards and Cambridge, 1995) (see figure 1). There is evidence that they could contribute to disease by the formation of immune complexes (Edwards and Cambridge, 1998) and complement fixation (Brown et al., 1982), and also by functioning

as highly efficient antigen presenting cells (Tighe et al., 1993). The correlation of RF status and progression of joint damage in RA (Masi et al., 1976) support a role in pathogenesis although they are by no means an absolute requirement for severe disease and joint destruction.

1.9.2 Type II Collagen

The possible importance of immune responses to type II collagen (CII) was first suggested nearly thirty years ago. The specific expression of this molecule in articular cartilage as well as its ability to induce a destructive arthritis in experimental animals made it an attractive candidate autoantigen in human RA (see above). Antibodies to native and denatured CII have been demonstrated in serum of patients with RA (10-30%) (Clague et al., 1994; Morgan et al., 1987) as well as synovial fluids, and collagen-anticollagen immune complexes described in synovial tissue (Clague and Moore, 1984). A dominant epitope of anti-CII antibodies has been identified (Kraetsch et al., 2001). T cell responses have been found in approximately 50% of antibody positive patients (Snowden et al., 1997). Antibodies to CII are present early in the disease course and appear to decline with time, and may be predictive of a more severe outcome (Cook et al., 1996). However T cell responses to CII are also found in 37% of normal individuals (Snowden et al., 1997), many patients do not demonstrate any form of immune response to CII and no major associations with clinical parameters have been described. Attempts to induce tolerance in patients with RA by the oral administration of collagen have had limited success (Barnett et al., 1998). Hence the relevance of anti-CII T cell responses and autoantibody remains unclear.

1.9.3 Human cartilage glycoprotein 39 (HCgp39)

HCgp39 (also known as YKL-40) is a major secreted product of articular chondrocytes and synovial fibroblasts (Hakala et al., 1993), and based on structural homology is a member of the glycohydrolase family 18, which comprises a large number of pro- and eukaryotic chitinases (Renkema et al., 1998). Its physiological role is unknown, however it does not possess the enzymatic activity of the other chitinases (Hakala et al., 1993) and a recent report has demonstrated that it can promote the growth of synoviocytes and fibroblasts (Recklies et al., 2002). Initial interest in this molecule as a putative autoantigen stemmed from the observation that HCgp39 mRNA was expressed in RA synovium and cartilage but not in normal joints (Hakala et al., 1993). Algorithms for predicting peptide binding to disease-associated HLA DR alleles were used to search for potential T cell epitopes within this molecule and proliferative T cell responses to four peptides measured. Responses were demonstrated in 9/18 RA patients and 2/11 controls. In addition immunization of BALB/c mice with HCgp39 induced a polyarthritis that was preventable by prior nasal administration of the antigen suggesting a possible pathogenic role for autoimmunity directed against HCgp39 (Verheijden et al., 1997). T cell responses against HCgp39 have also been described in patients with osteoarthritis and inflammatory bowel disease (Vos et al., 2000). Recent studies have suggested a wider role for HCgp39 in inflammatory processes and tissue remodelling. Serum concentrations are elevated in patients with RA and correlate with disease activity (Johansen et al., 1999). Synovial fluid levels are 10-fold higher suggesting local production and direct involvement in joint inflammation and tissue breakdown (Johansen

et al., 1993). HCgp39 is synthesised at other sites of inflammation or involution including cirrhotic liver (Johansen et al., 2000), post-lactational breast tissue (Rejman and Hurley, 1988) and by cells of the monocyte/macrophage lineage (Kirkpatrick et al., 1997). A recent study demonstrated that intranasal administration of HCgp39 resulted in the amelioration of arthritis induced in DBA1/j mice with an unrelated antigen, type II collagen, suggesting the possibility of a more generalized effect on immune regulation (Joosten et al., 2000). The involvement of HCgp39 therefore in pathogenic processes in RA is clear, although to what extent this is related to autoimmunity is not established and remains a subject of considerable interest.

1.9.4 Citrullinated antigens – anti-keratin antibody (AKA), anti-perinuclear factor (APF), anti-fillagrin antibody (AFA), citrullinated peptides and Sa

Anti-perinuclear factor (APF) was first described more than thirty years ago by the demonstration of a perinuclear staining pattern with RA sera using indirect immunofluorescence on buccal mucosal cells (Nienhuis and Mandema, 1964), and anti-keratin antibody (AKA) by a similar technique using rat oesophageal sections as substrate (Young et al., 1979). Both factors were found to be highly specific for RA and hence a role in pathogenicity suggested. A large body of work over the last ten years has shed considerable light on the targets of these antibodies. Both factors were shown to share specificity for the cytokeratin-filament aggregating protein fillaggrin (Sebbag et al., 1995; Simon et al., 1993). Moreover this specificity was shown to be dependent on an enzymatic post-translational modification, the conversion of arginine residues to citrulline by the enzyme peptidylarginine deiminase (Girbal-Neuhauser et al., 1999). A

great diversity of synthetic cyclical citrullinated peptides (CCP) based on citrulline-containing motifs of fillagrin have been shown to bind to APF/AKA positive sera (Schellekens et al., 1998) emphasising the importance of citrullinated residues as key antigenic determinants recognised by these antibodies. Recent work has suggested the targets of these antibodies are deiminated forms of α and β fibrin (Masson-Bessiere et al., 2001), which is interesting because fibrin is found abundantly in rheumatoid synovium and has prompted speculation of direct pathological involvement. This is supported by the observation of local production of AKA in RA synovium (Masson-Bessiere et al., 2000). There is also one report of intracellular citrullinated proteins in RA synovium and a suggestion that the presence of citrullinated proteins may be specific for RA (Baeten et al., 2001).

Anti-Sa was first described by immunoblotting of RA sera using spleen and placental extracts as substrate (Despres et al., 1994) and was found to have a similar high specificity for RA (Hueber et al., 1999). The Sa antigen has been identified as the cytoskeletal protein vimentin, and once again antigenicity found to be dependent on citrullination (Menard et al., 2000). A strong correlation has been found between the presence of anti-Sa and anti-CCP (Goldbach-Mansky et al., 2000). These data suggest that APF, AKA, AFA, anti-Sa and anti-CCP are an overlapping group of antibodies directed against citrullinated antigens. Although earlier studies suggested a predictive value for more severe disease, a recent large study of patients with early synovitis suggested that testing was probably of little value over and above traditional assessments (Goldbach-Mansky et al., 2000). Anti-Sa however did seem to define a subgroup of male

patients with a more severe phenotype (Goldbach-Mansky et al., 2000). Considered as a whole, there is much evidence to support the view that antibodies to citrullinated antigens are important in some aspect of the pathogenesis of RA, although their role has not as yet been fully elucidated.

1.9.5 Anti-RA33/hnRNP A2

Antibodies to the 33kDa A2 protein of the heterogeneous nuclear ribonucleoprotein complex (anti-RA33 or anti-hnRNP A2), a component of the spliceosome were first demonstrated in approximately 35% of patients with RA by Western blotting (Hassfeld et al., 1989). They are not specific however occurring in 20% of patients with SLE and 40-60% with MCTD, usually concomitantly with antibodies to U1-snRNP or Sm unlike in RA where these antibodies do not occur (Hassfeld et al., 1995). The major autoantibody binding sites have been shown to be conformation-dependent and found to be different in MCTD patients from RA and SLE (Skriner et al., 1997). Interestingly anti-RA33 has been shown to occur in two experimental models of arthritis, in TNF-transgenic mice and in the arthritis prone MRL/lpr mice (Steiner et al., 1997). The presence of anti-RA33 in SLE appears to define a subset of patients with destructive arthritis emphasising the link with joint pathology (Richter Cohen et al., 1998). Although shown to be present early in the disease, anti-RA33 has not been shown to have any predictive value for disease severity or progression (Goldbach-Mansky et al., 2000).

1.9.6 Antibodies to calpastatin

Calpastatin is a natural inhibitor of the calpains, a family of intracellular calcium-dependent cysteine proteases unique because of their cytosolic rather than lysosomal location. Calpains are over-expressed in diseased synovial tissue (Szomor et al., 1995; Yamamoto et al., 1992) and secreted calpains can degrade components of articular cartilage (Suzuki et al., 1992). Two groups independently described antibodies to calpastatin in approximately 50% of RA sera and although not specific for RA they occur at a higher frequency than in connective tissue diseases (Despres et al., 1995; Mimori et al., 1995). It has therefore been postulated that autoantibodies to calpastatin may potentiate joint destruction by interference with the calpain-calpastatin interaction (Menard and el-Amine, 1996). Some support for this comes from the *in vitro* observation that calpains are released from calpain-calpastatin complexes in the presence of RA sera (Mimori et al., 1995). Antibodies to calpastatin however are not associated with disease severity, and more recent studies have suggested they may be less frequent than previously believed (Lackner et al., 1998). The importance therefore of anti-calpastatin antibodies in RA remains unclear.

1.9.7 Other autoantigens in RA

There are reports of autoantibody and less frequently T cell responses to many other antigens although characterised in less detail than those discussed above. These are summarised in table 2. At the current time the importance of many of these remains a subject requiring further study.

1.10 Autoantibodies as markers of disease

It is clear that the presence of autoantibodies is a feature of RA and despite some overlap the serological profile of RA is distinct from other diseases such as SLE (Blass et al., 1999). The production of autoantibodies is a prominent feature of many diseases including certain malignancies, and in the vast majority they are not directly pathogenic. Many highly specific disease associations with autoantibodies exist and are likely to reflect unique aspects of the pathology in each case (Czaja and Homburger, 2001; Targoff, 2000). Such autoantibodies have proven to be very important diagnostic markers. Hence it is possible that autoantibodies may be useful diagnostic or prognostic markers in RA especially in early disease when it is difficult to differentiate self-limiting synovitis from potentially persistent and destructive disease (Kim and Weisman, 2000) and the importance of early treatment is well established (Fries et al., 1996; Stenger et al., 1998). Data on sensitivity and specificity for the diagnosis of RA is now available for most of the better-characterised antibodies (table 3). A study of a well-characterised cohort of 238 patients with arthritis of recent onset concluded that although anti-Sa, AFA and anti-CCP all had a high diagnostic specificity there was little value over measurement of rheumatoid factor alone (Goldbach-Mansky et al., 2000). A more recent study of 179 patients with established disease confirmed their high diagnostic specificity but demonstrated again that RF status was a better predictor of disease severity (Bas et al., 2002).

Table 3. Diagnostic value of autoantibodies in early arthritis.

	Sensitivity	Specificity	PPV
Rheumatoid factor	0.66-0.75	0.74-0.87	0.80
AFA	0.33	0.93	0.79
Anti-Sa	0.22	0.98	0.88
Anti-CCP	0.41-0.68	0.91-0.96	0.78
AKA	0.26-0.46	0.84-0.94	0.56
RF+ and one or more of above	0.50	0.96	0.90
RF- and one or more of above	0.31	0.73	0.26

Table 3. Diagnostic value of autoantibodies for rheumatoid arthritis in 238 patients with early arthritis.

PPV, positive predictive value. (Modified from Goldbach-Mansky et al 2000 and Bas et al 2002)

Any analysis of RA must take into account the observed heterogeneity of disease (Weyand et al., 1998b), hence it may be possible to define subsets of patients on the basis of serotype and investigate the hypothesis that they represent subgroups of patients sharing common pathological mechanisms or clinical phenotype. For instance in the study described above, anti-Sa defined a group of male patients with more severe disease (Goldbach-Mansky et al., 2000). Antibodies to eukaryotic translation elongation factor 1 alpha are much more frequent in patients with Felty's syndrome (66%) than non-Felty's RA (21%) (Ditzel et al., 2000). Anti-aldolase A has been associated with the presence of bone erosions (Ukaji et al., 1999). It may therefore be possible to gain insights into relevant pathophysiological processes from the analysis of autoantibodies as well as develop markers with a predictive value for particular aspects of disease.

For these reasons, much interest continues in the isolation and characterisation of autoantigens in rheumatoid arthritis. From table 2 it can be seen that antibody responses to a number of self-proteins have been characterised and the significance of a number of these remains unclear. The majority of these studies have used patients with established disease and it is well known that immune responses including those to self-antigens diversify over time to involve multiple epitopes within single molecules and other proteins (intra- and inter-molecular determinant spreading) (Yu et al., 1996). It is easy to envisage this occurring at a site of inflammation such as rheumatoid synovium as a result of antigen release from damaged tissue and subsequent uptake by antigen-presenting cells and presentation by class II molecules. Indeed such a cascade of events has been postulated to be an important process in the development of autoimmunity (Lanzavecchia, 1995). Hence if patients with early RA were studied a different profile of antigens might be encountered. These may be more relevant to disease initiation or better predictors of severity or outcome.

1.11 Aims of this thesis

The aims of this thesis were :

1. To investigate the use of a novel phage display system for the isolation and characterisation of novel autoantibodies from the sera of patients with established and early rheumatoid arthritis.
2. To characterise antibody responses to other recently described putative autoantigens in rheumatoid arthritis.

2 Cloning autoantigens from a fibroblast-derived cDNA library expressed on the surface of filamentous bacteriophage

2.1 Introduction

2.1.1 cDNA cloning is an effective method for characterising novel antigens in rheumatoid arthritis

Methods for the characterisation of novel antigens all rely on the detection of an interaction between antibody from sera or synovial fluid of interest and a complex mixture of potential ligands. Earliest methods include indirect immunoflourescence using fixed cells or tissue sections as antigenic substrate (Nienhuis and Mandema, 1964; Young et al., 1979) and more recently Western blotting against a variety of target tissues (Blass et al., 1995; Lafyatis et al., 1992). The obvious drawback with such techniques is that the targets of these antibodies subsequently require identification. Higher resolution separation of proteins in a complex mixture such as a tissue homogenate can be achieved using 2-dimensional gel electrophoresis and individual proteins identified by MALDI-TOF mass spectrometry (Corrigall et al., 2001), however all these techniques rely upon the target antigen being expressed in the tissue analysed at a high enough level for detection. The isolation of antigens by screening of cDNA libraries offers the advantage that the identity of a gene product interacting with the serum can be established by sequencing, and for this reason screening of bacteriophage λ cDNA expression libraries with RA sera has been attempted by several investigators with some success (Despres et al., 1995; Mimori et al., 1995; Tanaka et al., 2000; Tanaka et al., 1998). This approach also has limitations; proteins may lose antigenicity as a result of denaturation and binding

to solid supports inherent in the procedure, the number of clones that can be screened with reasonable effort and resources is limited to approximately 1 x 10^6, and relatively large quantities of serum are required.

2.1.2 Phage display technology

Phage display was first described in the 1980s (Smith, 1985) and offers a powerful alternative cDNA cloning methodology with a number of theoretical advantages over conventional cloning strategies. In essence foreign DNA fragments are expressed as fusion proteins with filamentous phage gene III or gene VIII, which encode coat proteins (see figure 2). The fusion proteins are then incorporated into mature phage particles, which retain infectivity and display the fusion protein on their surface in a form accessible to antibody. Phage expressing fusion proteins that bind the antibody of interest can be enriched from a complex mixture of 10^{10} to 10^{11} clones by rounds of affinity selection on ligand-coated surfaces and propagation in host bacteria, a procedure now often referred to as "panning". The physical linkage of the fusion protein to its encoding DNA in the phage particle allows identification by sequencing. Random peptide libraries (Parmley and Smith, 1988), libraries of DNA-binding proteins (Rebar and Pabo, 1994) and both single chain antibody (McCafferty et al., 1990) and F(Ab) fragments (Barbas et al., 1991) have been expressed on the surface of phage and used to characterise a number of receptor-ligand interactions in this way.

Figure 2. The structure of filamentous phage

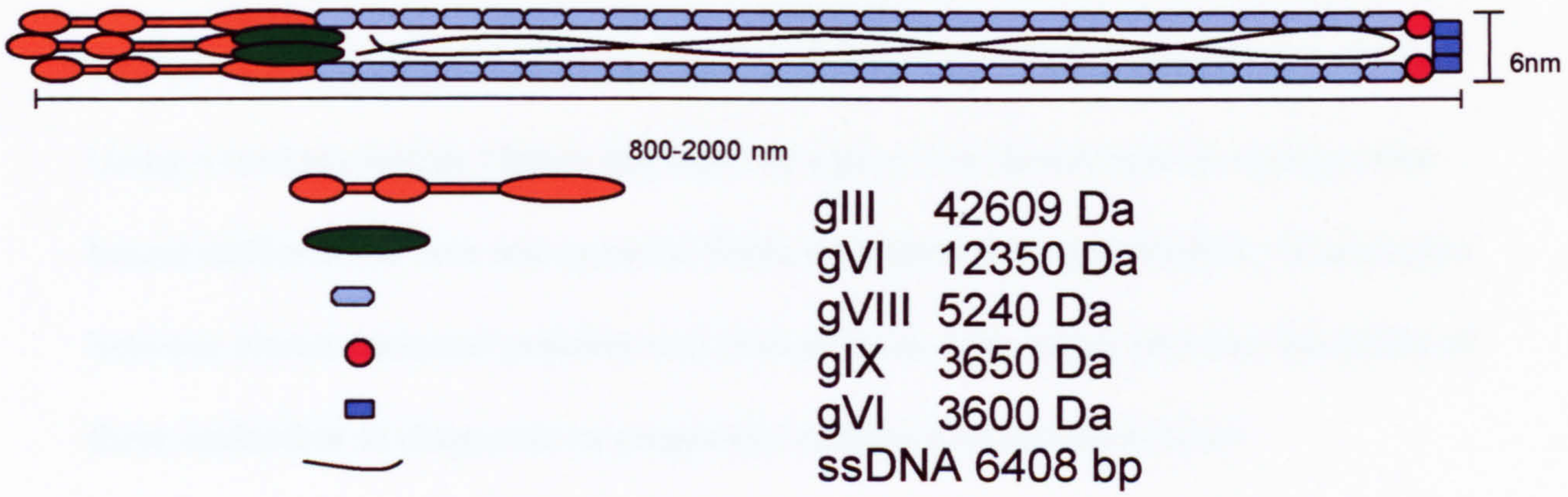


Figure 2. Each particle carries between 3 and 5 copies of the gene III protein, the n-terminal domain of which mediates adsorption to the pilus, and c-terminal domain which is important for assembly. The number of copies of gene VIII is determined by the length of DNA contained by the particle.

One group has previously attempted screening of phage display libraries with RA sera and synovial fluids (Dybwad et al., 1993; Dybwad et al., 1995; Dybwad et al., 1996). Using a random peptide library displayed as a gene VIII fusion peptide epitopes that bound antibodies in sera and synovial fluids were successfully identified. Homologies between immunoselected peptides and viral proteins were noted, however the utility of these antibodies as diagnostic or prognostic markers was not established.

2.1.3 The pJuFo system: a methodology for the display of products of cDNA libraries on phage surfaces.

The phage display systems used for the expression of functional immunoglobulin fragments such as pCBAK8 (Kang et al., 1991) and pComb 3 (Barbas et al., 1991) rely on the use of two independent promoters simultaneously controlling the expression of the Ig heavy chain as an N-terminal coat fusion protein, and the light chain as a secreted molecule. *PelB* leader sequences target the preproteins to the bacterial periplasm where they are able to form functional heterodimers. Such systems however were not suitable for the expression of the products of whole cDNA libraries, as the presence of in-frame stop codons in the 3' untranslated regions of eukaryotic genes would prevent the expression of the phage coat protein. The pJuFo system (Crameri and Suter, 1993) employs the ability of the leucine zippers of *Jun* and *Fos* to form a heterodimer (Kouzarides and Ziff, 1988) to covalently link the products of cDNA libraries to the phage gene III protein (figure 3), thereby establishing a system theoretically capable of allowing the enrichment of ligands from phage-displayed cDNA libraries. The efficacy of this vector has been demonstrated by the selective enrichment of allergens of

Aspergillus fumigatus from a cDNA library using IgE from allergic patients (Crameri and Blaser, 1996).

The work described below represents attempts made to isolate novel antigens in by panning of a fibroblast cDNA library expressed using the pJuFo system with IgG from sera of patients with RA.

Figure 3. Assembly of the pJuFo phage particle

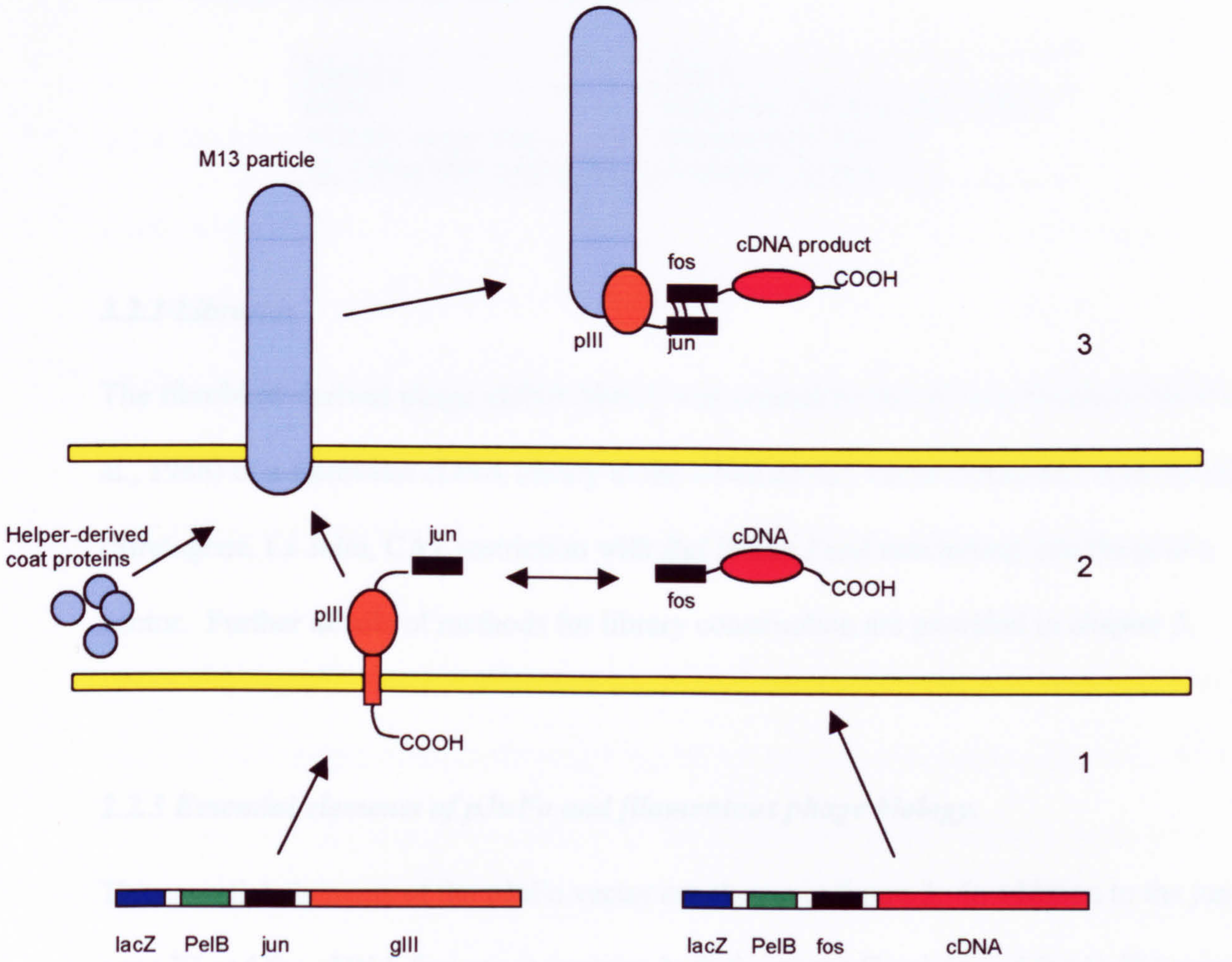


Figure 3. (1) In the cytoplasm of host bacteria *lacZ* promoters drive expression of both *jun*-gIII and *fos*-cDNA product fusion proteins and *pelB* leader sequences direct transport across the inner membrane. (2) The *jun-fos* fusions heterodimerise in the bacterial periplasm and the non-covalent association is further stabilised by disulphide bonds, which form as a result of engineered cysteine residues. The fusion proteins are then incorporated into mature phage particles as a result of the supply of other coat proteins by helper phage and secreted into the extracellular medium (3).

2.2 Materials and methods

2.2.1 Vectors, helper phage and host strains

Resource	Source
pJuFo	Kindly provided by Dr Reto Crameri
VCS M13 helper phage	Stratagene, La Jolla, CA
XL-1 Blue MRF' strain *E Coli*	Stratagene, La Jolla, CA

2.2.2 Libraries

The fibroblast-derived phage cDNA library was created by the in-vivo excision (Short et al., 1988) of a fibroblast cDNA library in the UNIZAP-II l vector obtained commercially (Stratagene, La Jolla, CA), restriction with *Bgl II/Xba I* and subcloning into the pJuFo vector. Further details of methods for library construction are provided in chapter 3.

2.2.3 Essential elements of pJuFo and filamentous phage biology.

The essential elements of the pJuFo vector are shown in figure 3. In addition to the jun-gene III and fos-cDNA fusions, it contains both the phage f1 origin and the ColE1 origin allowing it to be propagated as a plasmid in bacteria as well as packaged into phage particles (phagemid). It does not encode other coat protein genes however; hence the production of mature phage particles requires superinfection with a helper strain that provides the necessary coat proteins. Host cells co-infected with a recombinant pJuFo molecule and helper produce phage particles composed of a mixture of wild-type and recombinant gIII product and wild-type coat proteins. A single phage particle will only contain a single DNA molecule hence a mixture of particles containing wild-type and phagemid genomes will be produced. The presence of the ampicillin resistance marker

allows selection of host bacteria infected with recombinant phage rather than helper alone.

2.2.4 Titration of phage

Filamentous phage infection of host cells occurs by adsorption of the phage particle to the F pilus of the bacterium, an interaction mediated by the phage gene III protein. The genome is then passed through the pilus into the host cell. Pilus genes are located on the F' episome which also encodes tetracycline resistance, hence host cells must be grown in tetracycline-containing media in order to retain the F' episome and infectability.

In order to titre the phage, a single colony of XL-1 blue MRF' streaked from a glycerol stock onto a LB-agar plate containing 12μg/ml tetracycline was used to inoculate 10ml of LB-tet and the culture grown overnight to late logarithmic phase. Dilutions of phage stock in LB were then combined with 1-2ml of host cells and incubated together at room temperature for 15 minutes to allow phage to adsorb onto host cells. Between 2 and 20μl of infected host cells from each dilution were spread onto LB-agar plates containing 12μg/ml tetracycline and 100μg/ml ampicillin. After incubation for 16 hours at 37°C the number of colonies on each plate were counted. The titre of the original phage stock was then calculated according to the formula: (number of colonies) x (total volume of infected cells/volume plated) x (dilution factor) x (1ml/volume of phage dilution used for infection) and expressed as colony-forming units (cfu) per ml. Uninfected host and cells infected with defined amounts of a library of known titre were also plated simultaneously

as controls for contamination of the host with an ampicillin-resistant strain and host infectability respectively.

2.2.5 Growth and propagation of phage

A high titre phage stock was produced by the infection of 1-2ml of host cells with pJuFo as described above. After adsorption of phage 10ml of fresh LB-medium without antibiotic was added to the infected host cells and the culture shaken at 37°C for 1 hour to allow expression of resistance genes. The 10ml culture was superinfected with VCS M13 helper phage at a multiplicity of infection of 20:1 and incubated at room temperature for 15 minutes. The culture was then added to 100ml of fresh LB-medium containing 12µg/ml tetracycline, 100µg/ml ampicillin and 10µg/ml kanamycin and grown overnight with shaking at 37°C. During this time host cells infected with pJuFo secrete mature phage particles into the culture medium. The culture was freed of viable bacterial cells by heating to 65°C for 15 minutes followed by centrifugation. The supernatant was retained and phage particles precipitated by the addition of 0.15 volumes each of 40% PEG 8000 and 5M NaCl. After incubation on ice for one hour phage particles were collected by centrifugation. The pellet thus obtained was resuspended in 1ml of sterile PBS, and clarified of bacterial debris by centrifugation in a microfuge. The phage stock was stored at –20°C. Stocks with titres of 10^9 to 10^{10}/ml of culture supernatant were obtained in this way.

2.2.6 Patients and sera

Two pools of sera were used for panning experiments described. The first pool consisted of serum from ten patients with established RA as defined by the ACR criteria (Arnett et al., 1988) of at least 3 years duration, 6 female and 4 male, and an age range of 58-83. The second comprised 20 patients with early RA, all within 12 months of onset of symptoms. Ethical approval of the South Sheffield Research Ethics Committee for collection and use of all sera was sought and granted.

2.2.7 IgG purification

IgG was purified from pools of sera by affinity chromatography on a protein A-sepharose cartridge (Protein A Antibody Purification Kit, Sigma-Aldrich, Gillingham, UK) according to the manufacturer's instructions. Immunoglobulin G eluted from the cartridge was dialysed twice against 2 litres of PBS for 16 hours each and quantified using a modified version of the Bradford dye-binding assay (Bio-Rad Protein Assay, Bio-Rad, Herts, UK). Purity was judged to be at least 95% by SDS-PAGE and Coomassie blue staining. An example demonstrating typical heavy and light chain bands under reducing conditions is shown in figure 4.

Figure 4. SDS-PAGE analysis of purified IgG

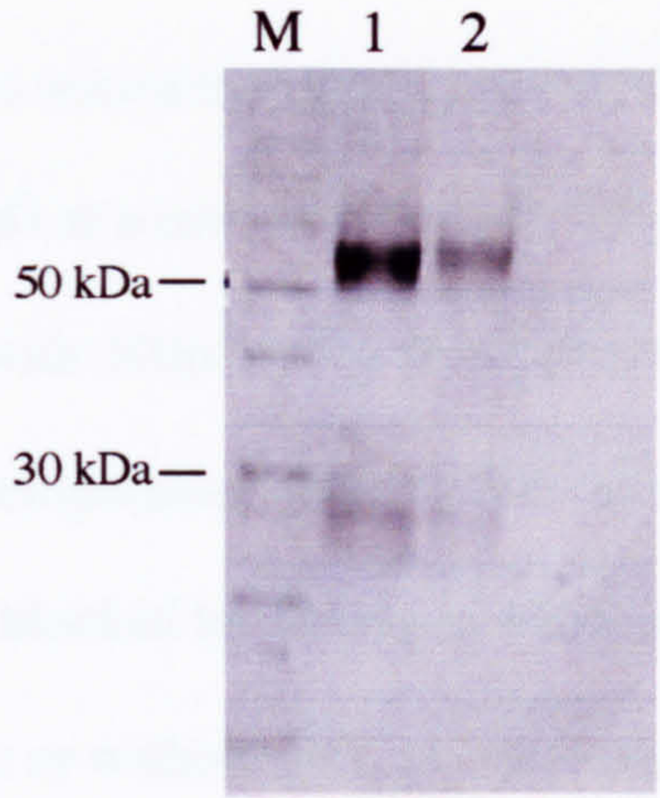


Figure 4. M denotes molecular weight markers, lanes 1 and 2 separate IgG samples.

2.2.8 Panning procedure

Individual wells of a microtitre plate (Costar 3590, Corning, NY) were coated with patient or normal IgG at a concentration of 100µg/ml in PBS overnight at 4°C. Wells were then blocked with 300µl of 5% skimmed milk powder in PBS/tween 0.5% (PBS/tm) for 1 hour at room temperature. During this time approximately 10^{10} cfu of the phage library (50µl) were blocked by adding an equal volume of blocking buffer (10% skimmed milk/1% tween with or without 20% glycerol) and rotating for 1 hour at 4°C. Glycerol has been shown to reduce non-specific adsorption and alter the repertoire of phage-ligand interactions (Kjaer et al., 1998) and it was for this reason that selection was performed both in the presence and absence of glycerol. Blocking buffer was removed from the wells; the blocked phage library applied and binding allowed to proceed overnight at 4°C. On the next day the phage were removed and the plate washed 15 times by filling each well with 200µl of wash buffer (PBS/0.5% tween with or without 10% glycerol) and aspirating. Finally the well was rinsed once in PBS, and bound phage eluted by adding 150µl of 0.1M HCl adjusted to pH 2.2 with glycine. After incubation on ice for 10 minutes the solution was neutralised by the addition of 9µl of 2M Tris. The neutralised solution containing eluted phage was (159µl) was used to infect 2ml of XL1 Blue MRF' as described above. One to 10µl of infected cells were plated on LB-agar tet-amp plates in order to monitor the number of phage eluted. The remainder were amplified as described above in order to produce a high titre phage stock that was used for the next round of panning. The process was then repeated for each round. Three to 4 rounds of panning were generally performed.

2.2.9 PCR analysis of panning clones

PCR employing primers spanning the pJuFo cloning region was used to screen clones for the presence of an insert and assess the approximate size of the insert. In order to prepare a PCR template a single bacterial colony was picked using a sterile micropipette tip and placed in 100µl of sterile LB medium. The colony was disrupted by vortexing and 50µl removed and boiled for 5 minutes using a thermoblock. Bacterial debris was removed by centrifugation in a microfuge at 13000rpm for 5 minutes and 1-2µl of the supernatant used as PCR template. The remaining 50µl was stored at 4°C until the presence of an insert had been confirmed. The following reaction conditions were used:

	Final volume/concentration
Reaction volume	50µl
Magnesium concentration	1.5mM
dNTPs	200mM each
Primers	200mM each

The following cycling parameters were used:

	Temperature	Time
Initial denaturation	95°C	3'
Denaturation	95°C	30"
Anneal	55°C	30"
Extension	72°C	90"
No of cycles	36	

PCR products were analysed by agarose gel electrophoresis and ethidium bromide staining.

2.2.10 Preparation of plasmid DNA and sequencing

Plasmid DNA was prepared from 5ml of overnight culture of each clone grown in LB-tet/amp using the Wizard™ miniprep kit (Promega, Madison, WI) according to the manufacturer's instructions. The DNA was precipitated using ethanol and the pellet washed once with 70% ethanol before resuspending in 10µl of sterile water. Quality and approximate quantity of plasmid DNA was assessed by agarose gel electrophoresis and approximately 500ng used per sequencing reaction. Sequencing was carried out by the core genomics facility of the Division of Genomic Medicine. Sequencing reactions were performed using the ABI PRISM® BigDye™ Terminators version 3.0 cycle sequencing kit (Applied Biosystems, Foster City, CA) and reaction products analysed using the ABI PRISM® 377 DNA sequencer.

2.3 Results

2.3.1 Numbers of phage eluted during rounds of panning

Selective enrichment in panning experiments can be monitored by establishing numbers of phage eluted at each round of panning (Crameri and Blaser, 1996). If successful selection were taking place it might be expected that numbers of phage eluted at each round would increase substantially. Increases of 1000-100,000 fold over 3 to 4 rounds are frequently observed in successful panning using peptide- and antibody-fragment libraries. Therefore numbers of phage eluted at each round were established (table 4).

Table 4. Elution of phage after rounds of enrichment from a fibroblast cDNA library

	Panning round		
	1	2	3
Established RA IgG			
+glycerol	17790	10034	138040
-glycerol	3129	1450	3364
Early RA IgG			
+glycerol	16994	6612	20126
-glycerol	8642	6380	11600

Table 4. Numbers represent the mean based on 2 plates per well panned.

2.3.2 PCR screening of clones from final rounds of panning

In addition to an increase in numbers eluted from each round, another method for monitoring a panning experiment for evidence of selection is to examine the clonality of the eluted phage; an oligoclonal or monoclonal population may arise in response to selection by binding to the selecting antibody. Results of screening of 10 randomly picked clones from round 3 of each panning series described above by PCR across the cloning region are shown in figure 5.

It can be seen from the figure that in both pannings with established RA IgG a relatively diverse mixture of clones appears to exist. In the panning using early RA IgG in the presence of glycerol, clones 2, 5, 8 and 10 appear to have the same size insert. Also of note some clones do not generate a PCR product at all, indeed the majority of clones (9/10) from the panning using early RA IgG. Attempts to sequence the inserts carried by these clones using plasmid DNA and the same sense primer as used for PCR were unsuccessful, suggesting these clones possessed a deletion of the *fos* gene. It appeared possible therefore that despite the absence of significant enrichment in terms of phage numbers selection may nevertheless have been taking place. It was for this reason that the identities of all the inserts carried by the clones shown above was established by sequencing.

Figure 5. PCR amplification of round 3 clones from fibroblast panning.

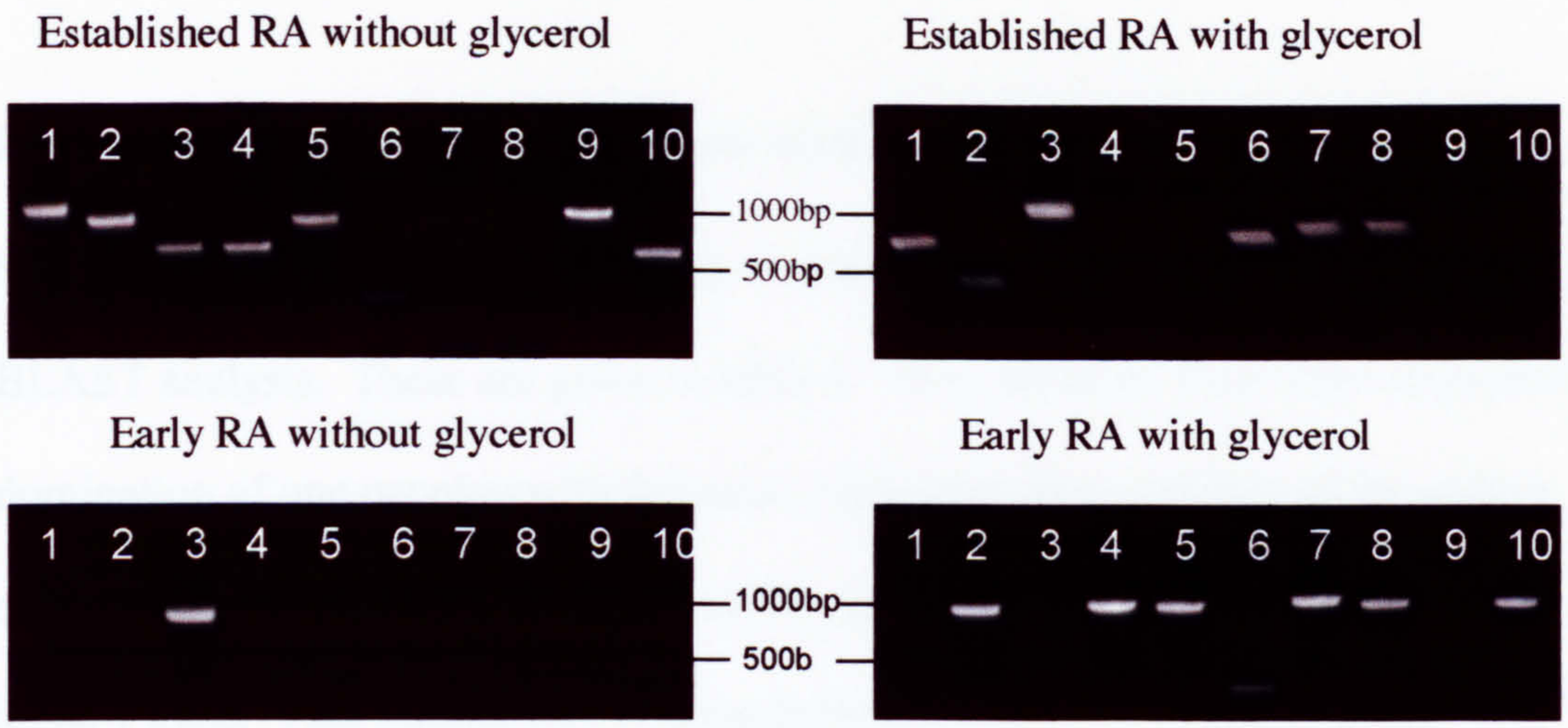


Figure 5. Agarose gel electrophoresis of PCR products from 10 randomly selected clones of round 3 of each panning series. The positions of 500bp and 1000bp molecular weight markers are indicated.

2.3.3 Identities of round 3 clones from each panning series

Sequencing reactions were performed on plasmid DNA and identities established by BLAST analysis. These are given in table 5. Most apparent from these sequences is the domination of one panning with the same clone encoding a portion of the coding sequence and 3' untranslated region of the eukaryotic translation-elongation factor 1-alpha 1 gene. This finding was of considerable interest, for as mentioned above, autoantibodies to this molecule have been demonstrated in patients with Felty's syndrome but also non-Felty's RA. One of these clones, designated FP12 ("fibroblast panning 12") was characterised in detail.

Table 5. Identities of round 3 clones.

Clone	Identity	Accession	Coding
Established RA			
Without glycerol			
1	Ribosomal protein L4	BC005817	CDS
2	KIAA0158	D63878	NON CODING
3	HLA A2	HSHLAA2	NON CODING
4	mitochondrial	M76291.	NON CODING
5	hSIAH2	U76248	NON CODING
9	beta actin	NM001101	NON CODING
10	myoferlin	AF182316	NON CODING
With glycerol			
1	mitochondrial	AF382013	NON CODING
2	EMPTY		
3	ARF 1	XM096195	NON CODING
6	Ferritin heavy chain polypeptide 1	BC015156	CDS
7	Secreted protein of unknown function	BC008823	CDS
8	mitochondrial	AF382011	NON CODING
Early RA			
Without glycerol			
3	Mitochondrial	AF382013	NON CODING
With glycerol			
2	EEF1 alpha-1	NM001402	CDS
5	EEF1 alpha-1	NM001402	CDS
6	EMPTY		
7	RHO GAP	XM085082	NON CODING
8	EEF1 alpha-1	NM001402	CDS
9	EMPTY		
10	EEF1 alpha-1	NM001402	CDS

EEF, eukaryotic translation elongation factor.

2.3.4 Characterisation of EEF1alpha1 clone.

Sequence alignment of FP12 and the full-length mRNA for EEF1a1 is shown in figure 6 and the reading frame of FP12 in figure 7. It can be seen that FP12 encodes the 60 carboxy-terminal amino acids of EEF1a1 and an additional 234 base pairs of the 3' untranslated region up to the poly (A) tail.

Figure 6. Sequence alignment of EEF1a1 and FP12.

```
141    gatatggttcctggcaagcccatgtgtgttgagagcttctcagactatccacctttgggt 200
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
1265   gatatggttcctggcaagcccatgtgtgttgagagcttctcagactatccacctttgggt 1324
403    D  M  V  P  G  K  P  M  C  V  E  S  F  S  D  Y  P  P  L  G

201    cgctttgctgttcgtgatatgagacagacagttgcggtgggtgtcatcaaagcagtggac 260
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
1325   cgctttgctgttcgtgatatgagacagacagttgcggtgggtgtcatcaaagcagtggac 1384
423    R  F  A  V  R  D  M  R  Q  T  V  A  V  G  V  I  K  A  V  D

261    aagaaggctgctggagctggcaaggtcaccaagtctgcccagaaagctcagaaggctaaa 320
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
1385   aagaaggctgctggagctggcaaggtcaccaagtctgcccagaaagctcagaaggctaaa 1444
443    K  K  A  A  G  A  G  K  V  T  K  S  A  Q  K  A  Q  K  A  K

321    tgaatattatccctaatacctgccaccccactcttaatcagtggtggaagaacggtctca 380
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
1445   tgaatattatccctaatacctgccaccccactcttaatcagtggtggaagaacggtctca 1504
463    *

381    gaactgtttgtttcaattggccatttaagtttagtagtaaaagactggttaatgataaca 440
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
1505   gaactgtttgtttcaattggccatttaagtttagtagtaaaagactggttaatgataaca 1564

441    atgcatcgtaaaaccttcagaaggaaaggagaatgttttgtggaccactttggttttctt 500
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
1565   atgcatcgtaaaaccttcagaaggaaaggagaatgttttgtggaccactttggttttctt 1624

501    ttttgcgtgtggcagttttaagttattagtttttaaaatcagtacttttttaatggaaaca 560
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
1625   ttttgcgtgtggcagttttaagttattagtttttaaaatcagtacttttttaatggaaaca 1684

561    acttgaccaaaaatttgtcacagaattttgagacccattaaaaaaagttaaatgag 615
       |||||||||||||||||||||||||||||||||||||||||||||||||||||||
1685   acttgaccaaaaatttgtcacagaattttgagacccattaaaaaaagttaaatgag 1739
```

Figure 6. FP12 sequence is shown above ETEF1a1, numbers refer to the nucleotide positions of mRNA sequence NM 001402 (genbank) or number of nucleotides from the sequencing primer, i.e. 141 represents the end of vector sequence.

It is of interest that this clone is not in frame with the *fos* fusion partner.

Figure 7. Reading frame of clone FP12.

```
DNA: gacgaaaaatccgcgctgcaaaccgaaatcgcgaacctgctgaaagaaaaa
+3:   R  K  I  R  A  A  N  R  N  R  E  P  A  E  R  K  R
+2:  T  K  N  P  R  C  K  P  K  S  R  T  C  *  K  K  K
+1: D  E  K  S  A  L  Q  T  E  I  A  N  L  L  K  E  K

DNA: gaaaagctggagttcatcctggcggcacacggtggttgcagatcttctaga
+3:   K  A  G  V  H  P  G  G  T  R  W  L  Q  I  F  *  N
+2:  K  S  W  S  S  S  W  R  H  T  V  V  A  D  L  L  E
+1: E  K  L  E  F  I  L  A  A  H  G  G  C  R  S  S  R

DNA: actagtggatcccccgggctgcaggaattcggcacgaggatatggttcctg
+3:   *  W  I  P  R  A  A  G  I  R  H  E  D  M  V  P  G
+2:  L  V  D  P  P  G  C  R  N  S  A  R  G  Y  G  S  W
+1: T  S  G  S  P  G  L  Q  E  F  G  T  R  I  W  F  L

DNA: gcaagcccatgtgtgttgagagcttctcagactatccacctttgggtcgct
+3:   K  P  M  C  V  E  S  F  S  D  Y  P  P  L  G  R  F
+2:  Q  A  H  V  C  *  E  L  L  R  L  S  T  F  G  S  L
+1: A  S  P  C  V  L  R  A  S  Q  T  I  H  L  W  V  A

DNA: ttgctgttcgtgatatgagacagacagttgcggtgggtgtcatcaaagcag
+3:   A  V  R  D  M  R  Q  T  V  A  V  G  V  I  K  A  V
+2:  C  C  S  *  Y  E  T  D  S  C  G  G  C  H  Q  S  S
+1: L  L  F  V  I  *  D  R  Q  L  R  W  V  S  S  K  Q

DNA: tggacaagaaggctgctggagctggcaaggtcaccaagtctgcccagaaag
+3:   D  K  K  A  A  G  A  G  K  V  T  K  S  A  Q  K  A
+2:  G  Q  E  G  C  W  S  W  Q  G  H  Q  V  C  P  E  S
+1: W  T  R  R  L  L  E  L  A  R  S  P  S  L  P  R  K

DNA: ctcagaaggctaaatgaatattatccctaatacctgccaccccactcttaa
+3:   Q  K  A  K  *  I  L  S  L  I  P  A  T  P  L  L  I
+2:  S  E  G  *  M  N  I  I  P  N  T  C  H  P  T  L  N
+1: L  R  R  L  N  E  Y  Y  P  *  Y  L  P  P  H  S  *

DNA: tcagtggtggaagaacggtctcagaactgtttgtttcaattggccatttaa
+3:   S  G  G  R  T  V  S  E  L  F  V  S  I  G  H  L  S
+2:  Q  W  W  K  N  G  L  R  T  V  C  F  N  W  P  F  K
+1: S  V  V  E  E  R  S  Q  N  C  L  F  Q  L  A  I  *
```

Figure 7. The frame of the *fos*-fusion protein is indicated in red (+1) and that of EEF1a1 in blue (+3). Stop codons are indicated by asterisks. The highlighted sequence represents the cloning area composed of *Bgl II* and *Xba I* sites.

2.4 Discussion

The objective of work described in this chapter was to selectively enrich clones from a phage display fibroblast cDNA library that bound to autoantibodies from RA sera. Although no large increases of phage eluted at each round were seen, domination of one panning series by a clone encoding a known autoantigen in RA did occur. The discussion that follows focuses on the drawbacks of the methodology employed, ways these may be overcome and whether or not the findings described above can be considered a successful application of this phage display system.

2.4.1 Clonal competition

The great diversity of encoded inserts and displayed polypeptides in terms of size and sequence is the major difference between gene fragment libraries such as used in this work and the majority of phage display libraries successfully used for other applications. Random peptide libraries typically display a great diversity of peptides of small size, while in antibody fragment libraries variation is restricted to complimentarity-determining regions within a conserved structural framework. Such variation in encoded inserts may therefore be expected to have heterogeneous effects on phage particle assembly, infectivity and growth rates and rounds of amplification in liquid media may favour the growth and propagation of non-recombinant or other clones with a growth advantage. In one study using a gene fragment library phage particle yields were found to vary by 100-fold between clones and in some cases by 10^7-fold (Wilson et al., 1997). Other studies have resulted in successful enrichment despite such clonal competition

(Wilson et al., 1998) suggesting that these effects are often unpredictable and do not necessarily preclude a successful panning experiment. Some applications have attempted to limit this problem by performing only 1 or 2 rounds of affinity-enrichment and then attempting to identify binders by plaque lifts (Wilson et al., 1998). This strategy while reducing the number of clones needing to be screened to a few thousand, still suffers from some of the limitations mentioned earlier. We chose to perform no more than three rounds of affinity selection and analyse the population of phage obtained directly by sequencing. The domination of one panning series by a clone carrying a deletion of the *fos* gene is likely to represent an example of such clonal competition. In two series however a relatively diverse population was observed which may be enriched in antigen-encoding clones.

2.4.2 Effects of a complex selecting ligand

In contrast to much previous work using phage display libraries where selection was performed using a pure moiety, the selecting ligand used in this work was a complex mixture, representing the repertoire of IgG molecules found in patient sera. Again these will form a heterogeneous population in terms of binding specificity, affinity and titre all of which will influence the degree of selection applied to individual phage clones and also the degree of enrichment seen. The effects of these various factors on the dynamics of the selection process may mean that enrichment in terms of phage numbers during rounds of panning will not be seen but without precluding a successful selection.

2.4.3 The problem of reading frame

In a study exploring the feasibility of expressing prokaryotic genomic libraries using a phagemid system, a library composed of 100-700bp fragments of *Staphylococcus aureus* genomic DNA fragments was panned using human IgG and fibronectin (Jacobsson and Frykberg, 1995). Panned libraries were screened using oligonucleotide probes to known binding regions of Staphylococcal protein A and fibronectin-binding proteins in order to demonstrate enrichment which was confirmed, however interestingly all five fibronectin-binding clones isolated contained a +1 or –1 frameshift. In a related study, 47 out of 50 binding clones possessed similar frameshifts. The implication is therefore that despite being out-of-frame, a small amount of in-frame polypeptide is produced in the host bacteria because of ribosomal slippage and phage bearing these polypeptides are selected. Out-of-frame clones are likely to possess a survival advantage because they produce much lower levels of foreign protein. None of the coding sequences isolated from this library were in-frame including that of ETEF1alpha1, suggesting that these considerations were likely to be important in this system. The implication for the work described is that an out-of-frame clone must not be discarded as a potential antigen.

2.4.4 How may successful enrichment be monitored?

The considerations discussed above and the differences from the best studied phage display applications impact upon an important practical problem encountered in the work described, that of how to judge the success of a panning experiment. Clearly monitoring eluted numbers of phage, which were initially relied upon may be unreliable. Direct binding assays of clones from panned libraries such as phage ELISA (Crameri and

Blaser, 1996), plaque lifts (Wilson et al., 1998) or dot blots (Barbas et al., 1991) would not overcome the problem of reading frame. This is especially important, as many studies have demonstrated that binders may comprise a relatively small proportion even of a panned library. In the study of Ditzel and colleagues, 1 F(Ab) out of 25 screened bound the selecting substrate (Ditzel et al., 2000). In a recent publication from our laboratory employing the pJuFo system, one clone out of 30 sequenced encoded a fragment of a cell-surface receptor, which was subsequently demonstrated to be an autoantigen (Kemp et al., 2002). The only way to overcome these problems therefore may be to undertake relatively large-scale sequencing and follow up clones that appear to be interesting because of their sequence content.

2.4.5 Successful cloning of an autoantigen or a further example of clonal competition?

A single clone encoding a fragment of EEF1alpha1, a molecule recently demonstrated to be an autoantigen in Felty's syndrome as well as non-Felty's RA dominated one panning series. This did not occur in a parallel experiment performed in the absence of glycerol. It is of much interest that the objective of the study of Kjaer and colleagues (Kjaer et al., 1998) was to isolate antibody fragments from a phage displayed antibody fragment library to this very same molecule, EEF1alpha1 and it was shown that binders could only be isolated in the presence of glycerol. This suggests that EEF1alpha1-antibody interactions may be glycerol-dependent in other contexts also and lends some support to the view that a selective enrichment was seen. No attempts to demonstrate direct binding of the clone to sera were made because the reading frame would prevent expression of significant levels of protein, however 5 of the pool of 20 RA sera used for panning were

confirmed to contain antibodies to ETEF1alpha1 by using a radiobinding assay, described in chapter 4. Although therefore this clone could have been enriched for non-specific reasons, we felt that this was likely to represent a genuine selective enrichment.

Of note, the panning series performed with a pool of 10 established RA sera did not result in selection of this clone, although in fact a small but consistent differential in terms of numbers of phage eluted at each round was seen (table 4). Two coding sequences were represented amongst the relatively small number of clones sequenced. The differences observed between panning with different panels of sera may be due in part to differences in antibody titre although stochastic effects clearly may also occur. It is possible therefore that potentially antigenic clones are over-represented in this series.

2.4.6 Conclusions

The work described in this chapter illustrates the differences between biopanning of gene-fragment libraries with IgG from human sera and previous well-studied applications of phage display. Evidence of clonal competition was observed, however the isolation of a clone encoding a known autoantigen in RA suggests that this can be overcome depending on the complexity of the starting libraries. No simple method for monitoring the success of a panning experiment was found because of the problem of even selectively enriched clones often being out-of-frame. On the basis of these observations a strategy for antigen discovery incorporating limited numbers of rounds of affinity enrichment followed by large-scale sequencing and detailed analysis of candidate

antigens was proposed. These considerations are applied to the screening of a phage-displayed library generated from human rheumatoid pannus described in chapter 3.

3 Construction and screening of a phage-displayed rheumatoid pannus cDNA library

3.1 Introduction

Disease-associated immune responses may be directed at either ubiquitous or organ-specific antigens. Many autoimmune diseases are characterised by both disease-specific and non-specific antibody production. The majority of even highly disease-specific autoantibodies, such as the myositis-specific anti-tRNA synthetase antibodies (Targoff, 2000), are directed against ubiquitously expressed molecules and such antibodies are therefore believed to reflect unique aspects of the pathology in each case. In other situations such as Sjogren's syndrome disease-specific immune responses are directed at both ubiquitously expressed molecules such as SS-A/Ro and SS-B/La, and tissue specific antigens such as the M3 muscarinic receptor (Bacman et al., 1998) and ICA69 (Winer et al., 2002). Evidence from disease models suggests that there may be a hierarchy of antigens with responses to certain molecules being more relevant for disease progression (Winer et al., 2002). A number of antigens characterised in rheumatoid arthritis such as collagen II, hcgp 39, BiP and possibly citrullinated antigens (Baeten et al., 2001) are preferentially expressed in inflamed joints. It is possible therefore that by using libraries derived from the site of inflammation antigens more relevant to disease pathogenesis or progression may be isolated.

Successful screening of a library derived from rheumatoid synovial membrane has been undertaken by only one group yielding the antigens follistatin-related protein (FRP) and gp-130 RAPS (Tanaka et al., 2000; Tanaka et al., 1998), however a relatively small

number of primary recombinants (150 000) were obtained. We sought therefore to generate a more diverse library from primary human RA tissue and express it on the surface of filamentous phage in order to allow screening using the selective enrichment procedures described previously in chapter 2.

3.2 Materials and methods

3.2.1 Construction of a cDNA library in λ TriplEx 2 (BD Clontech) from rheumatoid pannus

Initial attempts to synthesise and clone cDNA using the UNIZAP™ series of lambda vectors (Stratagene, La Jolla, CA) were unsuccessful, so the library described below was generated using the SMART™ cDNA Library Construction Kit (Clontech, Palo Alto, CA).

3.2.1.1 Extraction and purification of messenger RNA

Tissue was obtained from a 64-year-old subject with longstanding rheumatoid arthritis undergoing a total hip replacement. Tissue obtained at surgery was immediately snap-frozen and stored in liquid nitrogen. Light microscopy and haematoxylin and eosin staining confirmed the presence of a typical inflammatory infiltrate. Total RNA was extracted using TRIZOL™ (Invitrogen, Paisley, UK) and mRNA purified using oligo(dT) cellulose (FastTrack® 2.0, Invitrogen). Briefly approximately 1g frozen tissue was homogenised in a pestle and mortar cooled with liquid nitrogen until a fine powder was obtained. The homogenate was added to 20ml of TRIZOL™ reagent and mixed thoroughly to permit complete dissociation of nucleoprotein complexes. Two millilitres of chloroform was added to precipitate protein and DNA, and after separation of aqueous and organic phases the aqueous phase was precipitated with 0.8 volume 100% isopropanol. The pellet of total RNA was resuspended in 100µl of RNAse free water and added to 10ml FastTrack® 2.0 lysis buffer (200mM NaCl, 200mM Tris pH 7.5, 1.5 mM

$MgCl_2$, 2% SDS and proprietary RNAse-protein degrader), incubated at 65°C for 5 minutes and then chilled on ice for 1 minute. The tube was then placed at room temperature and 5M NaCl added to a final concentration of 500mM. At this point the lysate was incubated with 75mg of oligo(dT) cellulose for 60 minutes at room temperature. The beads were then washed first with binding buffer (500mM NaCl, 10mM Tris-Cl pH 7.5) followed by low salt wash buffer (250mM NaCl, 10mM Tris-Cl pH 7.5). The bound mRNA was eluted with 10mM Tris-Cl pH 7.5, precipitated with ethanol and finally resuspended in 10mM Tris-Cl pH 7.5. Quality and quantity of the mRNA obtained was assessed by agarose gel electrophoresis. A yield of approximately 30µg of poly (A) mRNA was obtained from 1g of tissue.

3.2.1.2 cDNA synthesis and cloning

A PCR-based protocol as supplied by the manufacturer was followed. The principle of the SMART method is outlined in figure 8. Approximately 0.5µg of poly(A) mRNA was used as template for first strand synthesis. The following protocol was followed (from manufacturer's instructions):

Component	Volume (µl)
RNA	1
SMART III oligo (10µM)	1
Modified oligo d(T) primer (10µM)	1
H_20	2
Total	5

Heat to 70°C, chill on ice
Then add :

5x 1st strand buffer	2
DTT (20mM)	1
dNTP mix (10mM each)	1
SuperScript™ II RT	1
Total	10

Figure 8. The SMART™ method for cDNA synthesis

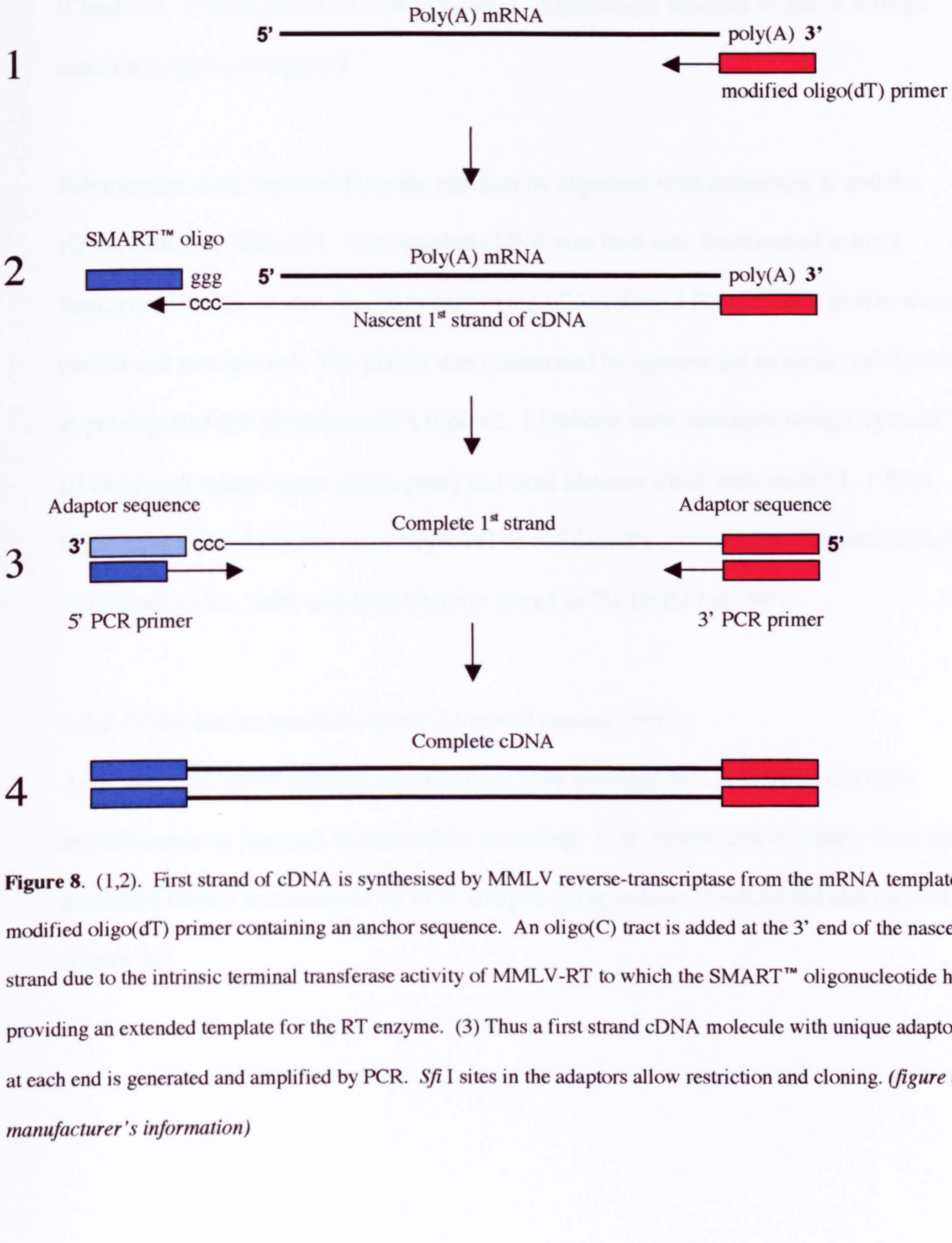


Figure 8. (1,2). First strand of cDNA is synthesised by MMLV reverse-transcriptase from the mRNA template and a modified oligo(dT) primer containing an anchor sequence. An oligo(C) tract is added at the 3' end of the nascent first strand due to the intrinsic terminal transferase activity of MMLV-RT to which the SMART™ oligonucleotide hybridises providing an extended template for the RT enzyme. (3) Thus a first strand cDNA molecule with unique adaptor sequences at each end is generated and amplified by PCR. *Sfi* I sites in the adaptors allow restriction and cloning. *(figure adapted from manufacturer's information)*

The reaction was incubated at 42°C for one hour. Two microlitres of the first-strand reaction were subsequently used as template for PCR using the Advantage 2 PCR kit (Clontech). Fifteen cycles of PCR were used. Agarose gel analysis of 5µl of a 100µl reaction is shown in figure 9.

Polymerases were removed from the reaction by digestion with proteinase K and the cDNA restricted with *Sfi* I. The digested cDNA was then size-fractionated using a Sepharose CL-2B column, fractions containing cDNA from 500 to 2000bp in size were pooled and precipitated. The cDNA was quantitated by agarose gel analysis and ligated to pre-digested and phosphatased λ triplex2. Ligations were packaged using Gigapack™ III Gold packaging extract (Stratagene) and final libraries titred with fresh XL-1 Blue MRF' host cells. Libraries were amplified on solid media according to standard methods (Sambrook et al., 1989) and final libraries stored in 7% DMSO at –80°C.

3.2.1.3 Titre and composition of the λ triplex2 pannus library

Approximately $3x10^6$ primary recombinants were obtained with less than 10% non-recombinance as assessed by blue-white screening. Size distribution of inserts from the amplified library was assessed by PCR analysis using primers flanking the cloning site (figure 10).

Figure 9. SMART PCR product prior to cloning.

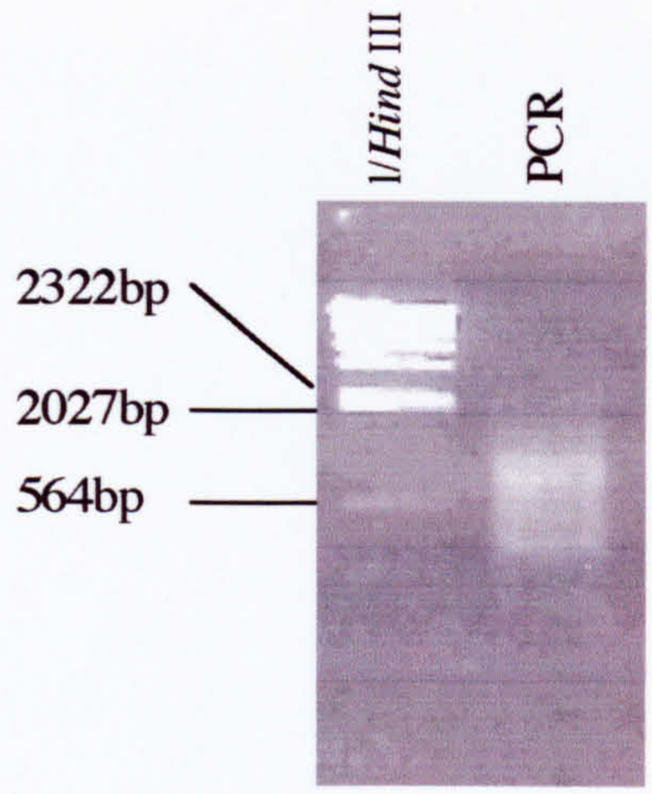


Figure 9. A smear of cDNA can be seen extending from below 500bp to around 2000bp in size. Some prominent bands corresponding to particularly frequent mRNAs can also be seen.

Figure 10. PCR analysis of inserts from l TriplEx2 pannus library

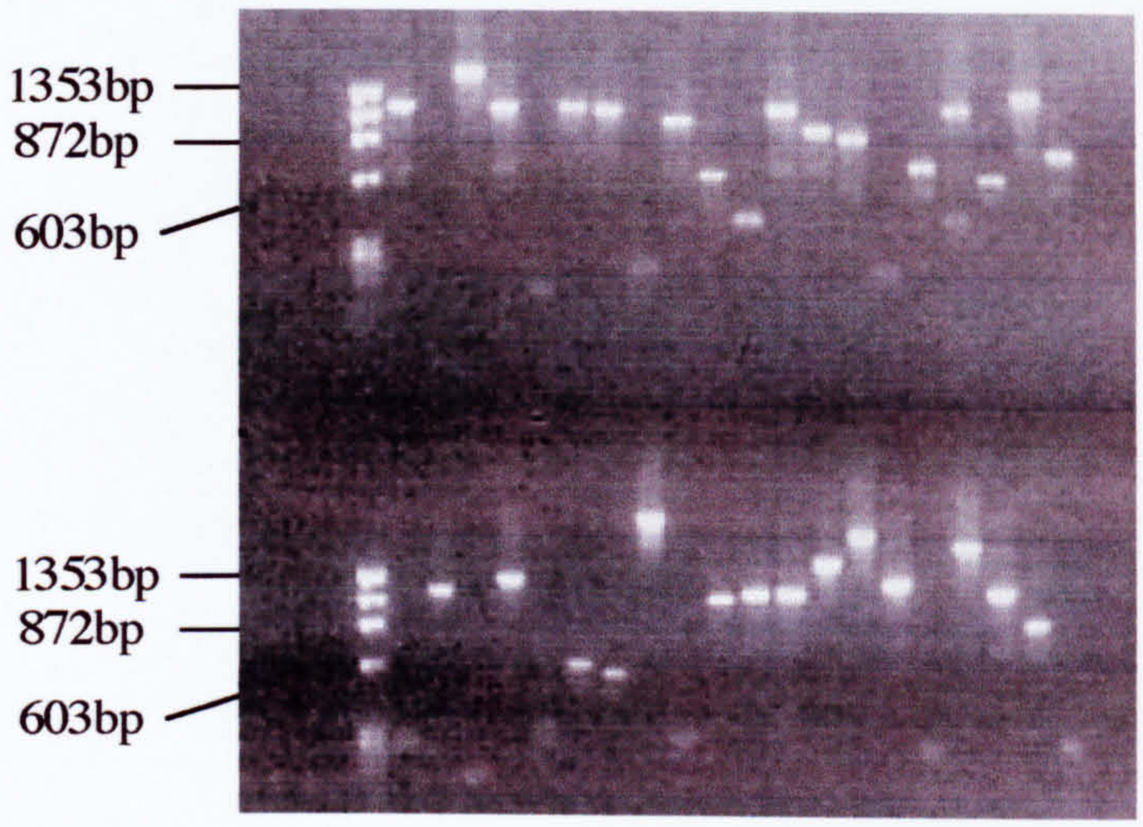


Figure 10. The majority of inserts were sized between approximately 600 and 1100bp (allowing for approximately 200bp of vector sequence in each PCR product). Five of the 40 inserts (12.5%) were above 1353 bp in size.

Composition of the library was also assessed by hybridization with a 1207bp β actin cDNA probe beginning 276bp from the 5' end of the mRNA transcript (total length 1761bp). Fifty-two positive plaques were identified out of approximately 50,000 pfu screened giving a frequency of 0.1%, therefore the library was considered to be representative.

3.2.2 Screening of the λ TriplEx2 pannus library

Approximately 50,000 pfu of the pannus library were plated per 210mm dish (Nunc) on NZY agar using standard methodology . Plaque lifts were performed onto supported nitrocellulose (Hybond™ C Extra, Amersham Pharmacia Biotech, Bucks., UK) and washed in PBS/tween 0.5% for 30 minutes to remove all traces of top agar. Membranes were then blocked for 1 hour at room temperature with 5% skimmed milk in PBS/tween 0.5% and rinsed briefly. A 1/50 dilution of a pool of 10 early RA sera, which had been preadsorbed with E.Coli lysate, was then allowed to react with the filters for 2 hours at room temperature. After four washes in PBS/tween 0.5% bound IgG was detected with alkaline phosphatase-conjugated goat anti-human IgG (Sigma).

3.2.3 Generation of the phage-displayed pannus cDNA library

The λ TriplEx2 multiple cloning site and any insert lies within a plasmid incorporated in the lambda vector. The junction of plasmid and lambda arms is flanked by *loxP* sites, hence transduction of phage into a *Cre*-recombinase positive strain of *E.Coli* such as

BM25.8 and growth at 30°C results in release and circularisation of the pTriplEx2 plasmid, which can then be isolated and propagated in any convenient host (figure 11). Such *in-vivo* excision of the pannus λ TriplEx2 library *en masse* was performed by infection of a culture of BM25.8 with 10^{12} pfu of library followed by subsequent isolation of "maxi-prep" plasmid DNA. Restriction with *Sfi* I released inserts and those in the size range above approximately 600bp were gel-purified and ligated to *Sfi* I-digested and phosphatased pJuFo. Ligations were used to transform high-efficiency electrocompetent XL-1 blue MRF' cells and approximately $2x10^6$ transformants obtained. Recombinance and approximate average insert size was assessed by PCR analysis using flanking primers revealing a similar distribution of insert sizes as the original library (figure 12). The transformed host cells were converted into the final phage particle library by infection with VCS M13 helper phage at a multiplicity of infection of approximately 20:1 and overnight growth in a 100ml culture containing tetracycline, ampicillin and kanamycin as described previously. The culture was clarified of bacterial debris and phage particles collected by PEG/salt precipitation. Five millilitres of final library was obtained with a titre of 10^{12} cfu/ml.

Figure 11. In-vivo excision of the plasmid pTriplEx2

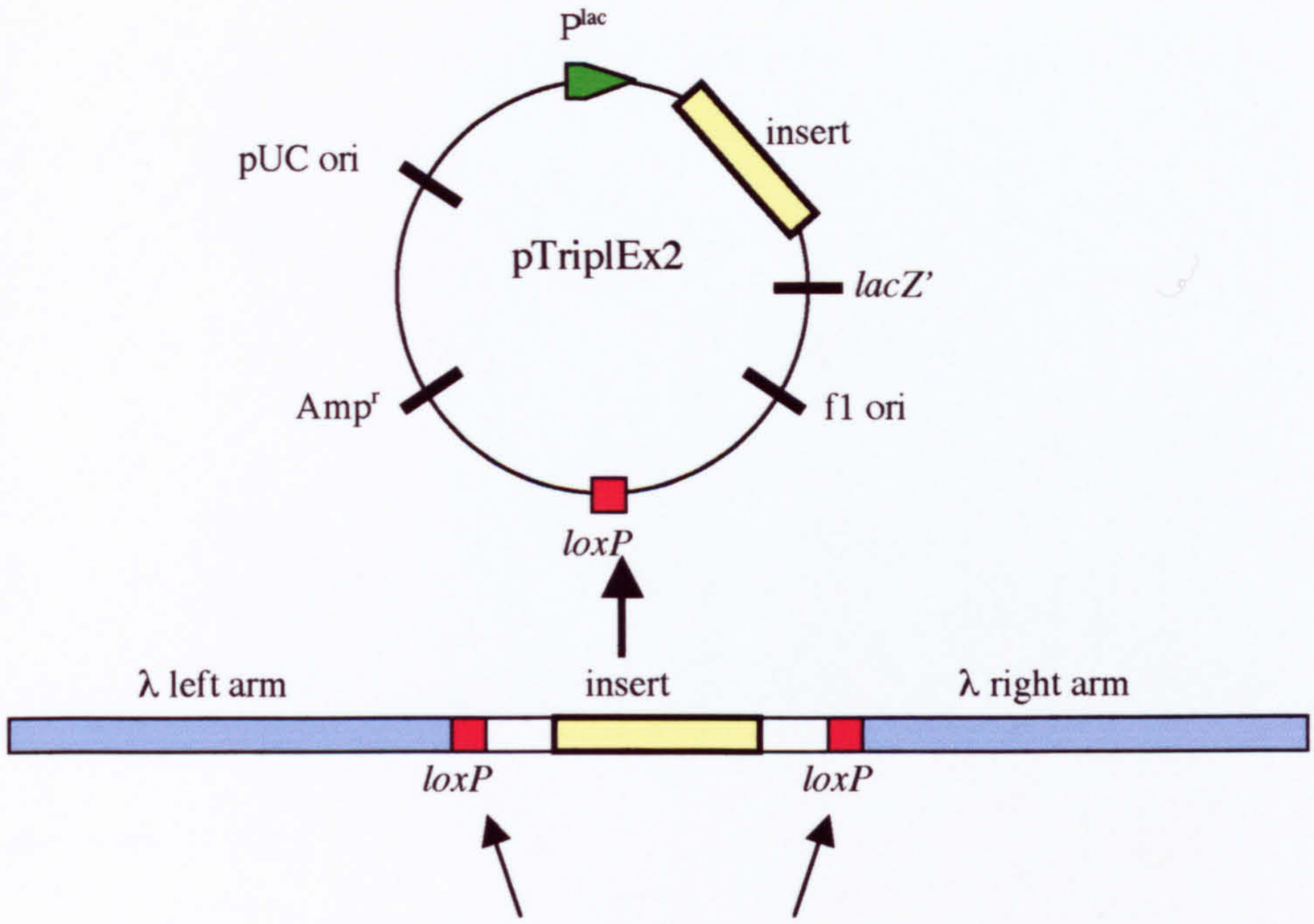


Figure 12. PCR analysis of clones from the pJuFo pannus library

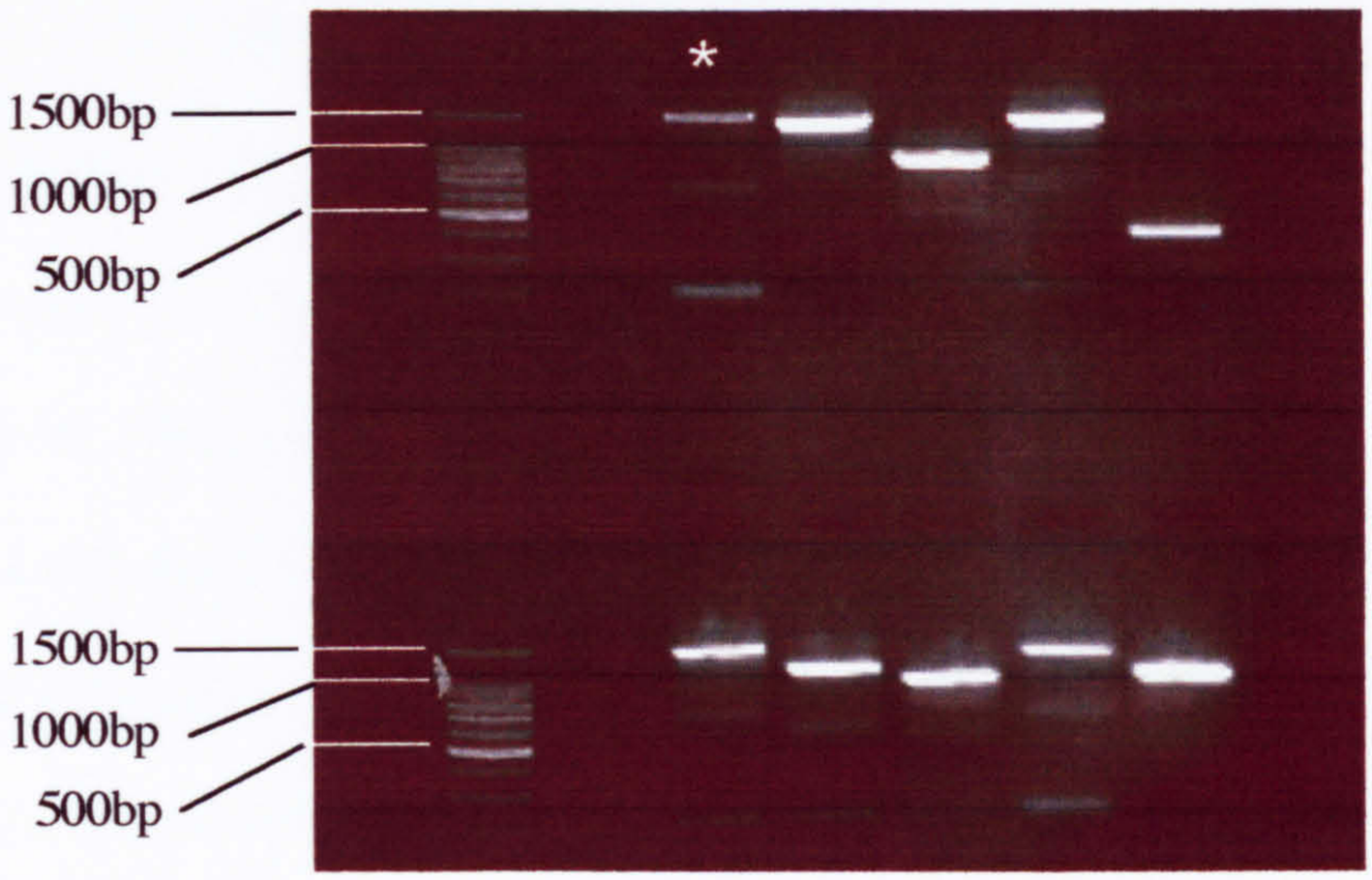


Figure 12. The majority of clones contain an insert of >1000bp. One insert below 500bp is seen and one probable nonrecombinant is seen (*)

3.2.4 Screening of the phage-displayed pannus library by selective enrichment

Dynabeads® Protein A (Dynal® Biotech, Oslo, Norway) were used as the solid substrate for the selective enrichment procedures described below. One hundred microlitres of Dynabeads Protein A has a theoretical binding capacity for human IgG of 25μg (manufacturer's information) as compared to approximately 1μg for passive adsorption to a single well of a microtitre plate (based on manufacturer's information for Costar 3590 high-binding polystyrene 96 well EIA/RIA plates). Hence a much larger number of IgG molecules would be available for binding to phage-displayed ligands. In addition capture of IgG molecules via their Fc regions may prevent loss of binding specificity as a result of the denaturation inherent in passive adsorption to solid surfaces (Butler et al., 1993). Dynabeads Protein A (100μl) were washed in PBS-tween(PBSt) and blocked by incubation with 5% skimmed milk (SM) solution in PBSt for 1 hour at 4°C. The blocking solution was removed and the Dynabeads reacted with a 1/10 dilution of a serum pool composed of 20 patients with early RA (within 6 months of onset of symptoms) containing approximately 100μg of IgG in total. The IgG-coated Dynabeads were then allowed to bind to10^{10} pfu of the phage-displayed library (pre-incubated with 10% normal human serum in 5%SM/PBSt) overnight at 4°C. After 6 washes with PBSt, bound phage were eluted, neutralised and amplified as previously described. After three rounds of panning the diversity of the phage population was established by PCR analysis and sequencing of 40 clones.

3.3 Results

Polymerase chain reaction analysis of 40 clones from the third round of selective enrichment revealed a diversity of inserts ranging from approximately 700 to 1500bp in size. Five clones generated a very small PCR product corresponding to amplification from vector alone and 3 clones did not amplify at all suggesting a deletion of the *fos* gene as encountered previously. The remaining 30 clones were sequenced using a sense primer located in the *fos* gene 134 bp upstream of the cloning site. Identities were established by comparison with DNA sequences deposited in the GENBANK, EMBL and DDBJ databases by BLAST analysis (Altschul et al., 1997). Nineteen clones contained sequences from known genes (summarised in table 6) and 11 clones represented uncharacterised sequences including one for which no database match was found (summarised in table 7). A more detailed analysis of these sequences is presented in the following sections. Sequence alignments were generated using the EMBOSS program (European Bioinformatics Institute, http://www.ebi.ac.uk/emboss/align/) and translations performed using the Baylor College of Medicine Sequence Utilities (http://searchlauncher.bcm.tmc.edu/seq-util/seq-util.html).

3.3.1 Clones encoding known genes.

Table 6. Clones isolated from panning of a phage-displayed pannus cDNA library encoding known genes.

Identity	Approximate size (BP)	Number of clones	Accession of primary match	Sequence content (coding or UTR)
Ig fragments	700-1000	13	Various	CDS
NOLA3 (snoRNP)	700	3	NM018648	Full length including 5' UTR
Mitochondrial NADH subunit 2	700	1	AF382012	CDS
NPL4 (ribosomal protein)	900	1	NM017921	3' UTR
WNT10B	800	1	HSU81787	3' UTR

3.3.1.1 Immunoglobulin gene fragments

Thirteen of the 30 clones sequenced encoded a variety of immunoglobulin fragments. All were light chains and all clones contained coding sequence in addition to 3' untranslated regions. Several explanations for this finding may be proposed. The most likely is that these clones were selected because of direct binding of expressed Ig fragments to free Protein A on the selecting substrate. Although the beads were incubated with an excess of human serum during the coating step, some binding sites will inevitably be empty and the great affinity of protein A for Ig may have led to selective enrichment. An alternative explanation is that these clones were selected by IgG rheumatoid factors in the patient sera. It is also possible that the original library contained such a high frequency of Ig

fragments. However as these clones were not felt to be relevant to the overall aims of the procedure they were not characterised in any more detail.

3.3.1.2 The small nucleolar ribonuclear protein NOLA3

Three of the 30 clones sequenced encoded an identical insert comprising almost the entire *NOLA3* gene including part of the 5' untranslated region. Sequence alignment of clone pJuFo/*NOLA3* and mRNA sequence NM_018648 is shown in figure 13 and the reading frame of this clone in figure 14. As can be seen from the figure 9, 70bp of the 3' untranslated region is included in this clone, which contains 2 stop codons (figure 10). Is it possible therefore that this protein will be displayed on the surface of the phage and be available potentially to bind to antibodies? The reading frame attached to the *fos* frame (shown in blue in figure 14) is in fact different from that of the NOLA3, however as discussed previously in chapter 2 such frameshifts are not uncommon in phage display systems and do not preclude the generation of small numbers of phage particles which express the cognate polypeptide. In addition antibodies against ribonuclear proteins have been characterised in a number of disease states. For these reasons attempts were made to demonstrate antibodies to NOLA3. The *NOLA3* coding sequence was amplified by PCR and cloned into the plasmid pcDNA3 in order generate ^{35}S labelled protein for immunoprecipitation studies, however expression was unsuccessful and therefore it was not possible to establish whether or not this molecule represents a target of antibodies in RA.

Figure 13. Sequence alignment of pJuFo NOLA3 clone.

```
pJuFo/NOLA3        101 GCAGATCTTCTAGAGGCCATTATGGCCGGGGGCGGTCGGGCGGACCACTG  150
                                                   ||||||||||||||||||||
NOLA3(NM_018648)     1 ggccacgaggggaaattgacgaacacgtgacgcggtcgggcggaccactg   50

                   151 CAGACTGAGCGGTGGACCGAATTGGGACCGCTGGCTTATAAGCGATCATG  200
                       ||||||||||||||||||||||||||||||||||||||||||||||||||
                    51 cagactgagcggtggaccgaattgggaccgctggcttataagcgatcatg  100
                                                                     M

                   201 TTTCTCCAGTATTACCTCAACGAGCAGGGAGATCGAGTCTATACGCTGAA  250
                       ||||||||||||||||||||||||||||||||||||||||||||||||||
                   101 tttctccagtattacctcaacgagcagggagatcgagtctatacgctgaa  150
                        F  L  Q  Y  Y  L  N  E  Q  G  D  R  V  Y  T  L  K

                   251 GAAATTTGACCCGATGGGACAACAGACCTGCTCAGCCCATCCTGCTCGGT  300
                       ||||||||||||||||||||||||||||||||||||||||||||||||||
                   151 gaaatttgacccgatgggacaacagacctgctcagcccatcctgctcggt  200
                        K  F  D  P  M  G  Q  Q  T  C  S  A  H  P  A  R  F

                   301 TCTCCCCAGATGACAAATACTCTCGACACCGAATCACCATCAAGAAACGC  350
                       ||||||||||||||||||||||||||||||||||||||||||||||||||
                   201 tctccccagatgacaaatactctcgacaccgaatcaccatcaagaaacgc  250
                         S  P  D  D  K  Y  S  R  H  R  I  T  I  K  K  R

                   351 TTCAAGGTGCTCATGACCCAGCAACCGCGCCCTGTCCTCTGAGGGTCCCT  400
                       ||||||||||||||||||||||||||||||||||||||||||||||||||
                   251 ttcaaggtgctcatgacccagcaaccgcgccctgtcctctgagggtccct  300
                        F  K  V  L  M  T  Q  Q  P  R  P  V  L  *

                   401 TAAACTGATGTCTTTTCTGCCACCTGTTACCCCTCGGAGACTCCGTAACC  450
                       ||||||||||||||||||||||||||||||||||||||||||||||||||
                   301 taaactgatgtcttttctgccacctgttaccccctcggagactccgtaacc  350

                   451 AAACTCTTTGGACTGTGAGCCCTGATGCCTTTTTGCCAGCCATACTCTTT  500
                       ||||||||.||||||||||||||||||||||||||||||||||||||||||
                   351 aaactcttcggactgtgagccctgatgccttttgccagccatactcttt  400

                   501 GGCATCCAGTCTCTCGTGGCGATTGATTATGCTTGTGTGANGCAATCATG  550
                       ||||||||||||||||||||||||||||||||||||||.|||||||||
                   401 ggcatccagtctctcgtggcgattgattatgcttgtgtgaggcaatcatg  450
```

Figure 13. Nucleotide sequence alignment is shown in black and the translated protein sequence in red. The gold shaded sequence represents the *Sfi* I cloning site of the vector.

Figure 14. Reading frame of pJuFo NOLA3 clone

```
DNA: AGATCTTCTAGAGGCCATTATGGCCGGGGGCGGTCGGGCGGACCACTGCAG
 +3:   I  F  *  R  P  L  W  P  G  A  V  G  R  T  T  A  D
 +2:  D  L  L  E  A  I  M  A  G  G  G  R  A  D  H  C  R
 +1: R  S  S  R  G  H  Y  G  R  G  R  S  G  G  P  L  Q

DNA: ACTGAGCGGTGGACCGAATTGGGACCGCTGGCTTATAAGCGATCATGTTTC
 +3:   *  A  V  D  R  I  G  T  A  G  L  *  A  I  M  F  L
 +2:  L  S  G  G  P  N  W  D  R  W  L  I  S  D  H  V  S
 +1: T  E  R  W  T  E  L  G  P  L  A  Y  K  R  S  C  F

DNA: TCCAGTATTACCTCAACGAGCAGGGAGATCGAGTCTATACGCTGAAGAAAT
 +3:   Q  Y  Y  L  N  E  Q  G  D  R  V  Y  T  L  K  K  F
 +2:  P  V  L  P  Q  R  A  G  R  S  S  L  Y  A  E  E  I
 +1: S  S  I  T  S  T  S  R  E  I  E  S  I  R  *  R  N

DNA: TTGACCCGATGGGACAACAGACCTGCTCAGCCCATCCTGCTCGGTTCTCCC
 +3:   D  P  M  G  Q  Q  T  C  S  A  H  P  A  R  F  S  P
 +2:  *  P  D  G  T  T  D  L  L  S  P  S  C  S  V  L  P
 +1: L  T  R  W  D  N  R  P  A  Q  P  I  L  L  G  S  P

DNA: CAGATGACAAATACTCTCGACACCGAATCACCATCAAGAAACGCTTCAAGG
 +3:   D  D  K  Y  S  R  H  R  I  T  I  K  K  R  F  K  V
 +2:  R  *  Q  I  L  S  T  P  N  H  H  Q  E  T  L  Q  G
 +1: Q  M  T  N  T  L  D  T  E  S  P  S  R  N  A  S  R

DNA: TGCTCATGACCCAGCAACCGCGCCCTGTCCTCTGAGGGTCCCTTAAACTGA
 +3:   L  M  T  Q  Q  P  R  P  V  L  *  G  S  L  K  L  M
 +2:  A  H  D  P  A  T  A  P  C  P  L  R  V  P  *  T  D
 +1: C  S  *  P  S  N  R  A  L  S  S  E  G  P  L  N  *

DNA: TGTCTTTTCTGCCACCTGTTACCCCTCGGAGACTCCGTAACCAAACTCTTT
 +3:   S  F  L  P  P  V  T  P  R  R  L  R  N  Q  T  L  W
 +2:  V  F  S  A  T  C  Y  P  S  E  T  P  *  P  N  S  L
 +1: C  L  F  C  H  L  L  P  L  G  D  S  V  T  K  L  F

DNA: GGACTGTGAGCCCTGATGCCTTTTTGCCAGCCATACTCTTTGGCATCCAGT
 +3:   T  V  S  P  D  A  F  L  P  A  I  L  F  G  I  Q  S
 +2:  D  C  E  P  *  C  L  F  A  S  H  T  L  W  H  P  V
 +1: G  L  *  A  L  M  P  F  C  Q  P  Y  S  L  A  S  S

DNA: CTCTCGTGGCGATTGATTATGCTTGTGTGANGCAATCATGGTGGCATCCCC
 +3:   L  V  A  I  D  Y  A  C  V  X  Q  S  W  W  H  P  H
 +2:  S  R  G  D  *  L  C  L  C  X  A  I  M  V  A  S  P
 +1: L  S  W  R  L  I  M  L  V  *  X  N  H  G  G  I  P
```

Figure 14. The reading frame attached to the *fos* protein is shown in blue and the correct NOLA3 frame in red. The TAA stop codon highlighted in green will be ignored as the XL1 Blue MRF' cells used as host bacteria are *supE* and therefore suppress amber stop codons. The cloning site of pJuFo is highlighted in gold.

3.3.1.3 Mitochondrial NADH subunit 2

One clone of the 30 sequenced encoded the c-terminal 209 amino acids and 3' untranslated region of subunit 2 of the mitochondrial enzyme NAD dehydrogenase (*ND2*). Sequence alignment is shown in figure 15. Although not truly enriched the presence of this clone was nevertheless of interest. Occasional reports of anti-mitochondrial reactivity in patients with rheumatoid arthritis do exist (refs). However more specifically a monoclonal antibody produced by a B cell hybridoma generated from rheumatoid synovium has been shown to possess strong anti-mitochondrial activity and stain 38- and 50-kDa bands in mitochondrial extracts (Krenn et al., 1998). NADH2 is a 38kDa protein and it is therefore possible that NADH2 is the target of this antibody. One problem however concerns the expression of this protein in bacteria. NADH2 is encoded by the mitochondrial genome and therefore TGA codons are read as W and not a stop as in eukaryotic or bacterial cells. The clone shown in figure 15 contains 4 such in-frame TGA codons (highlighted in green) and therefore even allowing for ribosomal slippage it seems unlikely that this polypeptide would be expressed in its entirety in the bacterial host cells or on the surface of phage particles used in the screening procedures. It is possible that partial expression is sufficient to display some determinants critical for binding however.

Figure 15. Sequence alignment of pJuFo/*ND2*

```
                 33 CGCGCTGCAAACCGAAATCGCGAACCTGCTGAAAGAAAAAGAAAAGCTGG     82

                399 aaaactaggaatagccccctttcacttctgagtcccagaggttacccaag    448


                 83 AGTTCATCCTGGCGGCACACGGTGGTTGCAGATCTTCTAGAGGCCATTAT    138

                449 gcacccctctgacatccggcctgcttcttctcacatgacaaaaactagcc    498


pJuFo/ND2       139 GGCCGGGCAATCATATACCAAATCTCTCCCTCACTAAACGNAAGCCTTCT    182
                    ||||||||||||||||||||||||||||||||||||.|||||||||
ND2(AF382012)   499 cccatctcaatcatataccaaatctctccctcactaaacgtaagccttct    548
                      I  S  I  M  Y  Q  I  S  P  S  L  N  V  S  L  L

                183 CCTCACTCTCTCAATCTTATCCATCATAGCAGGCAGTTGAGGTGGATTAA    232
                    ||||||||||||||||||||||||||||||||||||||||||||||||
                549 cctcactctctcaatcttatccatcatagcaggcagttgaggtggattaa    598
                     L  T  L  S  I  L  S  I  M  A  G  S  W  G  G  L  N

                233 ACCAAACCCAGCTACGCAAAACCTTAGCATACTCCTCAATTACCCACATA    282
                    ||||||||||||||||||||.||||||||||||||||||||||||||||
                599 accaaacccagctacgcaaaatcttagcatactcctcaattacccacata    648
                      Q  T  Q  L  R  K  I  L  A  Y  S  S  I  T  H  M

                283 GGATGAATAATAGCAGTTCTACCGTACAACCCTAACATAACCATTCTTAA    332
                    ||||||||||||||||||||||||||||||||||||||||||||||||
                649 ggatgaataatagcagttctaccgtacaaccctaacataaccattcttaa    698
                     G  W  M  M  A  V  L  P  Y  N  P  N  M  T  I  L  N

                333 TTTAACTATTTATATTATCCTAACTACTACCGCATTCCTACTACTCAACT    382
                    ||||||||||||||||||||||||||||||||||||||||||||||||
                699 tttaactatttatattatcctaactactaccgcattcctactactcaact    748
                     L  T  I  Y  I  I  L  T  T  T  A  F  L  L  L  N  L

                383 TAAACTCCAGCACCACGACCCTACTACTATCTCGCACCTGAAACAAGCTA    432
                    ||||||||||||||||||||||||||||||||||||||||||||||||
                749 taaactccagcaccacgaccctactactatctcgcacctgaaacaagcta    797
                      N  S  S  T  T  T  L  L  L  S  R  T  W  N  K  L

                433 ACATGACTAACACCCTTAATTCCATCCACCCTCCTCTCCCTAGGAGGCCT    482
                    ||||||||||||||||||||||||||||||||||||||||||||||||
                798 acatgactaacacccttaattccatccaccctcctctccctaggaggcct    847
                     T  W  L  T  P  L  I  P  S  T  L  L  S  L  G  G  L

                483 GCCCCCGCTAACCGGCTTTTTGCCCAAATGGGNCATTATCGAAGAATTCA    532
                    ||||||||||||||||||||||||||||||||||||||||||||||||
                848 gcccccgctaaccggcttttgcccaaatgggccattatcgaagaattca    897
                      P  P  L  T  G  F  L  P  K  W  A  I  I  E  E  F  T

                533 CAAAAAACAATAACCTCATCATCCCCACCATCATAGCCACCATCACCCTC    582
                    ||||||||||||||||||||||||||||||||||||||||||||||||
                898 caaaaaacaatagcctcatcatccccaccatcatagccaccatcaccctc    945
                      K  N  N  S  L  I  I  P  T  I  M  A  T  I  T  L

                583 CTTAACCTCTACTTCTACCTACGGCTAATCTACTCCACCTCAATTACACT    632
                    ||||||||||||||||||||||||||||||||||||||||||||||||
                946 cttaacctctacttctacctacgcctaatctactccacctcaatcacact    995
                     L  N  L  Y  F  Y  L  R  L  I  Y  S  T  S  I  T  L

                633 ACTCCCCATATCT-ACAACGTAA----AAAANAANANNTTNNNANNN        677
                    |||||||||||||| |||||||||    ||||..|.|..||.......
                996 actccccatatctaacaacgtaaaaataaaatgacagtttgaacata      1045
```

3.3.2 Clones encoding uncharacterised sequences

Eleven clones carried uncharacterised sequences (table 7). Databases matches were found for all but one sequence, and EST matches for all except 2 sequences suggesting that the majority represent genuine mRNA transcripts and not PCR artefacts from contaminating DNA for instance. All sequences were screened for the presence of potential open reading frames using the BCM sequence utilities program. In four of the sequences shown, multiple stop codons were found in all three reading frames. Possible reading frames comprising at least 100 amino acids were found in the remaining four sequences however whether these sequences represent polypeptide fragment of uncharacterised genes or non-translated sequence is unclear as none demonstrated any homology to known genes.

Table 7. Clones isolated from a pannus library encoding uncharacterised sequences

Identity	Approximate size (BP)	Number of clones	Accession of primary match	EST matches	Open reading frame	Putative conserved domains or homologies to known proteins
Chromosome 12 unidentified sequence	1500	2	AC026786.5	D44811 BE315280 BU189827 BU625379	Possible	No (Alu repeats)
Chromosome 12 BAC clone	900	2	AC012150	Numerous e.g. BG341239	No	No
Chromosome 19 unidentified sequence	900	1	AC010328	Numerous e.g. BQ938400	No	No (Alu repeats)
Chromosome 1 unidentified sequence	900	1	AL354721	None	Possible	No (Alu repeats)
Chromosome 19 unidentified sequence	1000	1	AC007201	Numerous e.g. BG397564	Possible	No
Chromosome 2 unidentifed sequence from BAC clone RP11-514A9	1000	1	AC012670	Partial e.g. AA582690	No	No
Chromosome 9 unidentified sequence from clone RP11-71A24	1000	1	AL359997	None	No	No
Chromosome 5 unidentified sequence from clone CTD-3075M2	1100	1	AC113358	N57826	Possible	No

3.4 Discussion

The work described above represents attempts made to isolate novel antigens by the construction and screening of a phage-displayed pannus cDNA library with IgG from patients with early rheumatoid arthritis. A library was generated using a PCR-based method and screened with IgG immobilised on Dynabeads Protein A. The majority of the clones recovered were Ig fragments probably because of selection applied by free Protein A on the selecting substrate. Of the remainder, 2 candidate antigens were identified and also a number of uncharacterised sequences isolated most of which are likely to represent background. Factors that are likely to impact on the success of this approach are discussed below.

3.4.1 Library construction

The SMART™ method was chosen for the library construction as availability of poly(A) mRNA was limiting. However there are several reasons why such libraries may not be ideally suited to immunoscreening by any method. The method relies on the addition of an oligo(dC) tract to the end of first strand cDNAs to which the SMART oligonucleotide then hydridises. This oligo(dC) tract is not added if the reverse transcriptase enzyme terminates prematurely and therefore the system is designed to preferentially amplify full-length mRNAs including 5' UTRs which often include stop codons (as seen in the NOLA3 clone, figure 13). Therefore some cDNAs will not be expressed as polypeptides on the surface of phage particles. Clearly this assumes that all mRNAs are intact as otherwise premature termination will take place, this is likely to be responsible for the

generation of truncated mRNAs as in the pJuFu/ND2 clone. In addition first strand synthesis occurs from a modified oligo(dT) primer and hence the library will be biased towards 3' ends of genes. This is manifest in the isolated sequences as the presence of 3'UTRs of genes and C-termini. A random primed library would include a greater diversity of sequences including N-termini and would be a better "antigen library" to screen. However particularly when poly(A) mRNA is limiting this is technically much more difficult and therefore was not attempted in this case.

3.4.2 Methods used for screening

Dynabeads™ Protein A were used to capture IgG directly from serum samples as this method is theoretically least likely to lead to loss of binding specificity as a result of denaturation. This method also has the advantage that IgG purification is not required. However it is possible that serum proteins may adsorb onto the magnetic particles leading to the enrichment of serum-binding factors, and also the domination by immunoglobulin fragment-expressing clones is apparent and has been encountered by others (P.F.Watson, personal communication). An alternative methodology is the biotinylation of purified IgG from patients and immobilisation on Dynabeads™ Streptavidin M-280; however biotinylation itself may also lead to some denaturation of binding sites.

Autoantibodies as yet undiscovered in rheumatoid arthritis may be of low affinity, low titre and be directed at molecules expressed at low levels in cells and tissues. To maximise the chance of detecting a lower affinity interaction all incubations were performed at 4°C in order to slow down the dissociation of low affinity interactions.

However screening was performed by reacting the phage library with beads, which had been pre-coated with IgG. It is possible that reacting the phage with IgG directly in solution might allow multivalent immune complexes to form and capture of these on an affinity matrix may be a more efficient method of detecting rare phage-antibody interactions. Recent work describing the cloning of tumour antigens from a phage-displayed cDNA library from colon cancers provides some evidence that such approaches may be more successful (Somers et al., 2002).

3.4.3 Methods for judging successful enrichment of antigens

As discussed in the previous chapter, great difficulties are encountered when attempting to judge the success of an enrichment experiment using this system. Titration of phage numbers eluted at each round was not performed but a larger number of clones were sequenced than in the experiments described in chapter 2, and it is clear that some clones were enriched. Whether this was because of binding to selecting antibody or for other reasons is unclear. Of the 2 coding sequences isolated, specific problems prevented the use of immunoprecipitation assays and therefore attempts to search for antibodies to either of these molecules will require further work. Subcloning, expression and purification of the *NOLA3* product would allow Western blotting and ELISA studies. The mitochondrial gene NADH2 presents a more difficult problem. Because of mitochondrial codon usage it was not possible to express the cDNA in bacteria or eukaryotic cells directly, and protein purified from cells or tissues would need to be obtained for specific binding studies. Western blotting using microsomal preparations

may give some information on whether sera bound a protein of the relevant size and combined with MALDITOF mass spectrometry may allow identification.

3.4.4 Conclusions

Although many methodological problems remain unsolved, the work described above demonstrates the feasibility of constructing both lambda and phage-displayed cDNA libraries from human rheumatoid synovium. A valuable resource has been established which may be useful not only for the study of antigens but also for the isolation of orphan receptors and ligands important in other aspects of the biology of inflammatory diseases. Further work is required in order to establish whether or not the molecules highlighted above are the targets of autoantibodies in rheumatoid arthritis.

4 Autoantibodies to eukaryotic translation elongation factor 1-α 1 (eEF1α) in rheumatoid arthritis and other systemic diseases.

4.1 Introduction

4.1.1 Eukaryotic translation elongation factor 1-alpha 1(eEF1α)

Eukaryotic translation elongation factor 1-alpha 1 is known to form a complex with both GDP or GTP and aminoacyl-tRNA promoting its binding to 80s ribosomes (Negrutskii and El'skaya, 1998). Although its physiological function is not known it is believed therefore to play a role in tRNA and aminoacyl-tRNA channelling in eukaryotic cells. Antibodies to eEF1α (anti-eEF1α) have been reported only twice previously - in 66% of patients with Felty's syndrome (Ditzel et al., 2000) and 20% of Japanese patients with atopic dermatitis (Ohkouchi et al., 1999). Although a role in the pathogenesis of the neutropaenia of Felty's syndrome was suggested, antibodies were also found in 4/21 non-Felty's RA patients and 1/16 SLE patients tested (Ditzel et al., 2000). Felty's syndrome is known to be strongly associated with HLA DRB1*0401 (Weyand et al., 1995) and it is therefore possible that the primary association of this antibody is with HLA genotype and not neutropaenia, although no analysis of HLA types was performed. In addition Felty's syndrome is associated with severe, erosive disease and other extra-articular features such as vasculitis and it is possible therefore that the primary association of anti- eEF1α is with any one of these phenotypes. Anti- eEF1α could therefore be an important diagnostic or prognostic marker in rheumatoid arthritis and other diseases.

4.1.2 Anti-eEF1α and anti-nuclear and anti-neutrophil cytoplasmic antibodies.

The presence of antibodies to eEF1α in Felty's syndrome correlates with anti-nuclear antibody (ANA) positivity, both nuclear and cytoplasmic staining is seen (Ditzel et al., 2000); this is somewhat unexpected as eEF1α is believed to have a role in correct codon-anticodon selection in the ribosome and therefore predominantly cytoplasmic staining would be expected. In addition staining of neutrophil nuclei was seen but not of lymphocyte nuclei implying a cell specificity. That the nuclear staining is due to anti-eEF1α and not another specificity is supported by the binding pattern of a cloned anti-eEF1α F(ab) fragment (Ditzel et al., 2000). The basis of these observations has not been well established. Anti-nuclear antibodies (Nishimura et al., 1996) (ANA) and anti-neutrophil cytoplasmic antibodies (Mustila et al., 2000) (ANCA) of unknown specificity have been reported in up to 35% and 50% of patients with rheumatoid arthritis and therefore it is possible that anti-eEF1α accounts for a proportion of these. As these tests are in routine clinical use an important aspect of the investigation of anti-eEF1α would be to establish to what extent anti-eEF1α accounts for anti-nuclear and anti-cytoplasmic staining patterns. Additionally it would be of importance to establish the frequency of anti-eEF1α in other disease states characterised by positive ANA such as SLE and other connective tissue diseases.

4.1.3 Anti-eEF1α and neutropaenia

The association of anti-eEF1α with neutropaenia is also of interest. During neutrophil apoptosis induced *in-vitro*, apoptotic bodies containing high levels of eEF1α have been observed at the cell surface (Ditzel et al., 2000) suggesting that at this time the antigen may be accessible to extracellular antibody and the possibility of antibody-dependent pathogenicity. Alternatively destruction of neutrophils by splenic macrophages may result in the presentation of previously sequestered eEF1α and the generation of antibodies. Whatever the mechanism, anti-eEF1α could potentially represent a novel marker for autoimmune neutropaenia and allow differentiation from other causes of neutropaenia such as drug-induced bone marrow suppression, a common clinical problem. Up to 50% of patients with SLE have mild neutropaenia and an association with anti-Ro antibodies has been demonstrated (Kurien et al., 2000; Nossent and Swaak, 1991), however only 20% of patients are anti-Ro positive (Kurien et al., 2000) hence it is possible that other autoantibodies may play a role in pathogenesis. It is therefore interesting that in the study of Ohkouchi et al (Ohkouchi et al., 1999), anti-eEF1α positive patients were found to have lower white cell counts and a higher incidence of facial dermatitis; the authors have suggested that this subgroup of patients may have a lupus-like disease.

Interest in anti-eEF1α initially stemmed from its isolation during the selective enrichment experiments described in chapter 2, however on the basis of the work discussed above we suggest that these antibodies may define a subgroup of patients with RA, have other important disease associations or shed light on pathophysiology. The

presence of anti-eEF1α has only to date been evaluated in 21 patients with non-Felty's RA and 15 patients with SLE. In order to begin addressing these questions we have established a radioimmunoprecipitation assay for anti-eEF1α and used this assay to compare the frequency of anti-eEF1α in panels of sera from patients with established and early RA, SLE, systemic sclerosis, polymyositis and Graves' disease.

4.2 Methods

4.2.1 Construction of the plasmid pcDNA3/efα

The eEF1α open reading frame was obtained by PCR from IMAGE clone cDNA (IMAGE id 4854519) using the following primers

5' cgc gaa ttc atg atg atg atg gga aag gaa aag act cat atc 3'

5' cgc tct aga tca ttt agc ctt ctg agc ttt ctg 3'

to introduce *EcoRI* and *XbaI* restriction sites as well as 3 extra methionine residues (to ensure optimal radiolabelling) into the product. After digestion the PCR product was cloned into the plasmid pcDNA3 (Invitrogen) downstream of the T7 promoter, and the presence of the insert and absence of any PCR-introduced mutations confirmed by restriction digest and sequencing. Plasmid DNA for subsequent use was prepared by use of the Wizard™ midi-prep kit (Promega) according to the manufacturer's instructions.

4.2.2 Production of ^{35}S-methionine labelled protein using an in vitro transcription/translation system.

Linked *in vitro* transcription/translation was performed using the Promega TnT™ rabbit reticulocyte lysate system. Approximately 4μg of plasmid DNA was used as template in a 200μl reaction. A typical reaction is shown below.

Component	Volume(µl)
TnT™ RRL	100
TnT™ reaction buffer	8
TnT™ T7 RNA polymerase	4
Amino acid mixture (minus methionine)	4
(^{35}S) methionine	16
RNAsin® ribonuclease inhibitor 40u/µl	4
Plasmid	4
ddH_20	To 200

The production of an appropriately sized 52kDa polypeptide was confirmed by SDS-PAGE in a 12% gel and autoradiography. A typical example is shown in figure 16.

4.2.3 Immunoprecipitation (IP) assay for anti-eEF1α

All serum samples were clarified by microcentrifugation at 13000rpm for 15 minutes prior to use. Five microlitres of serum and 1µl of IVTT reaction were combined with 50µl of IP buffer (20mM Tris HCl pH8, 150mM NaCl, 1% triton X100, 700u/ml aprotinin) in 1.5ml microfuge tubes and immune complexes allowed to form by incubating the samples on a rotary wheel for 16 hours at 4°C. Immune complexes were then captured by adding 50µl of a slurry of Protein G Sepharose Fast-Flow (Amersham Biosciences, Bucks., UK) equilibrated in IP buffer representing 25µl of dry resin with a predicted binding capacity for human IgG of 425µg (according to manufacturer's information). Following incubation for 1h at 4°C the Sepharose was washed 4 times with 500µl of IP buffer for 30 minutes each time, rotating at 4°C. Following the final wash the amount of ^{35}S-labelled protein bound to the Sepharose was estimated by radioscintillation counting using a Wallac Rackbeta beta counter.

Figure 16. ***Invitro*** **transcription-translation of eEF1a**

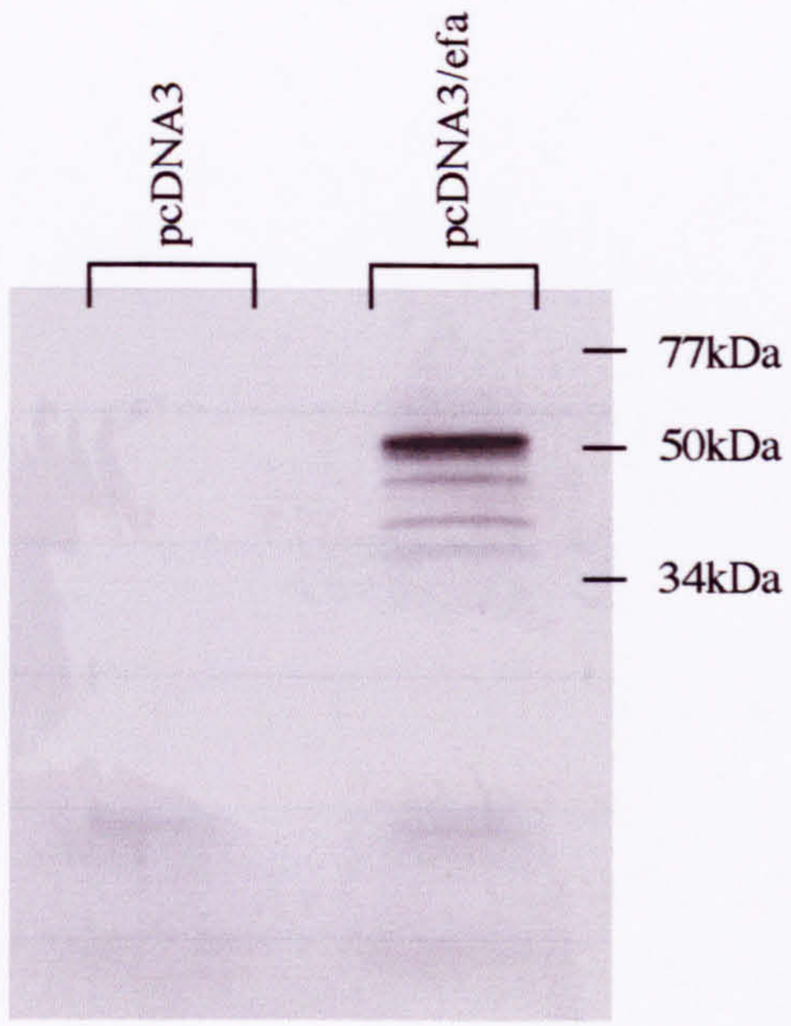


Figure 16. SDS-PAGE analysis of ^{35}S-methionine labelled eEF1a synthesised by *invitro* transcription-translation. One ml of a 200ml reaction was analysed. The positions of molecular weight markers are shown to the right and a negative control reaction using empty pcDNA3 as template is also shown.

4.2.4 Expression and analysis of binding data

Because of the variability between batches of Sepharose and labelling efficiency of different IVTT reactions, each time the assay was performed the same 10 normal sera were tested concurrently with patient samples. Results for each sample were then expressed as binding units calculated according to the following formula:

$$\text{Binding units (sample)} = \frac{\text{cpm(sample) - mean cpm(10 controls)}}{\text{sd (10 controls)}}$$

It can be seen that this value corresponds to the standard normal variate (SNV), and a value of 4 representing 4 sd above the mean of the controls was selected as the cutoff for positivity. In this way results of assays performed on different occasions could be directly compared.

4.2.5 Patients and sera

Local ethics committee approval was sought and obtained. Patients with early RA have been described in section 2.6.6. Patients with established RA formed part of a cohort collected by A S Low, and Dr P Hughes provided sera from patients with SLE and systemic sclerosis. Polymyositis sera were kindly provided by Dr P Plotz, NIH, Bethesda. Graves' sera were provided by Prof A P Weetman. Sera from healthy blood donors were obtained from the Sheffield Blood Transfusion Service with the kind co-operation of Dr Virge James.

4.2.6 HLA-DR typing

HLA-DR typing was performed by PCR amplification using sequence-specific oligonucleotide primers. Typing was performed by A S Low.

4.3 Results

4.3.1 Confirmation of anti-eEF1α reactivity in 20 early RA sera used for selective enrichment

The radioimmunoprecipitation assay described above was applied individually to the 20 RA sera used as a pool for the selective enrichment experiments detailed in chapter 2 and to 20 randomly selected sera from healthy blood donors. Results are shown in figure 17.

Four of the 20 early RA patients were considered positive and 1 borderline on the basis of this assay. In order to ensure that the binding shown above was not due to polyreactivity, all sera were tested in an identical assay for binding to the T cell costimulatory ligand B7.2 (described in chapter 5) and no binding was demonstrated.

Figure 17. Anti-eEF1a reactivity of 20 early RA sera

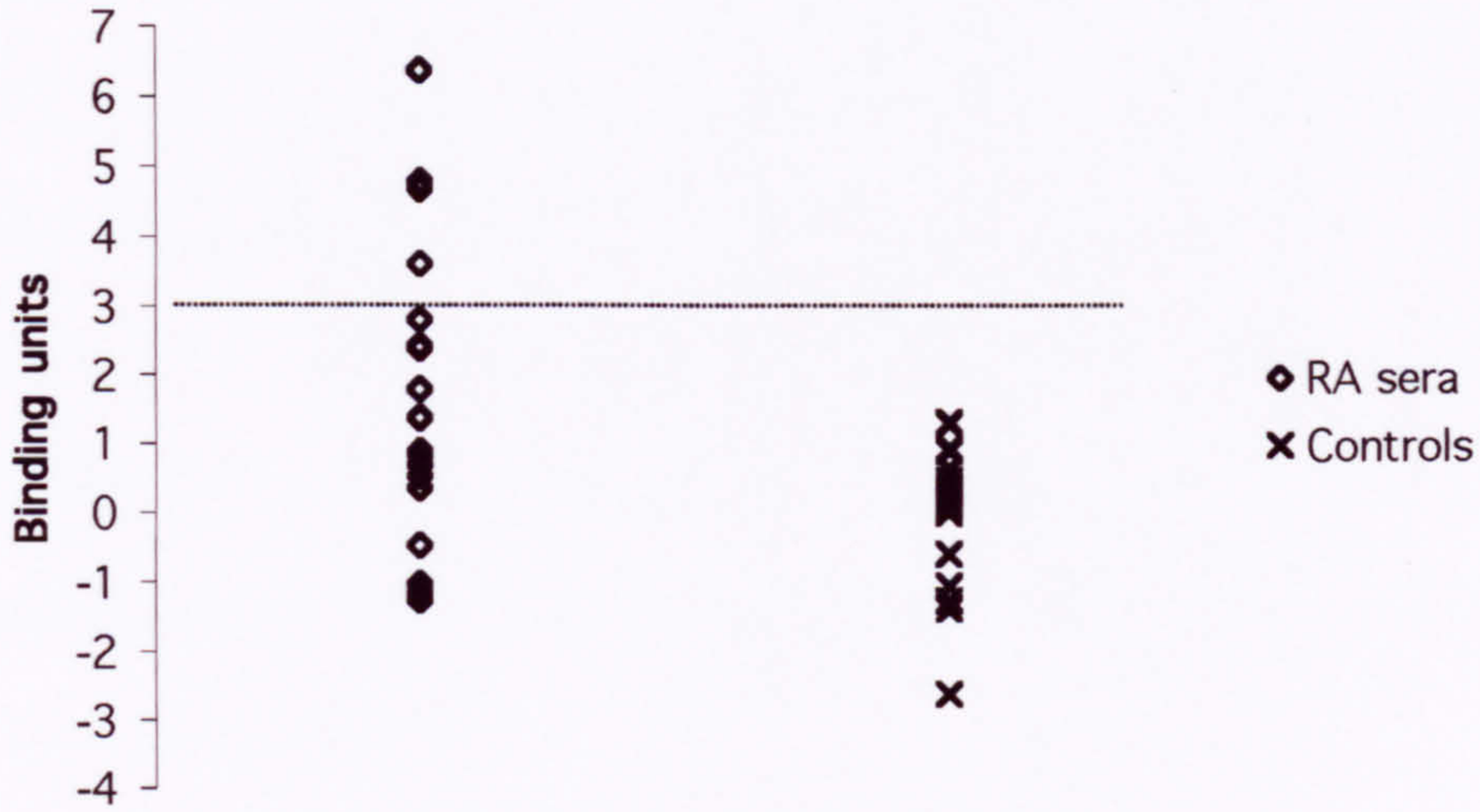


Figure 17. Each symbol represents the mean of duplicates for an individual serum sample. The horizontal line represents an arbitrary cutoff for positivity set at 3 sd above the mean of the control sera – assuming the samples are normally distributed less than 0.5% of samples would be expected to be outside this range by chance.

4.3.2 Anti-eEF1α reactvity in established RA and other systemic autoimmune diseases

The radioimmunoprecipitation assay was then applied to panels of sera from patients with established RA, SLE, systemic sclerosis (SS), polymyositis (PM) and Graves' disease (GD). Binding data for individual sera and frequencies of positive sera are shown in figure 18. In total 50 RA sera were tested and 75 sera from patients with the other autoimmune diseases listed above. Frequencies of positive sera in each group are shown below as a 2 x 2 contingency table :

	RA sera	Non-RA sera	Total
Anti-eEF1α+	11	1	12
Anti-eEF1α−	39	74	113
Total	50	75	**125**

Chi-squared = 12.5, $p<0.001$ confirming that these frequencies are unlikely to have arisen by chance.

4.3.3 Titration curves of positive RA sera

As an additional measure to ensure that binding seen with RA patients was accurate and reproducible, 3 randomly selected sera designated as positive on the first screening were titred for their anti-eEF1α reactivity. Binding in cpm for all 3 and one normal serum tested concurrently for comparison is shown in figure 19.

Figure 18. Anti-eEF1a 1 reactivity in patients with autoimmune diseases

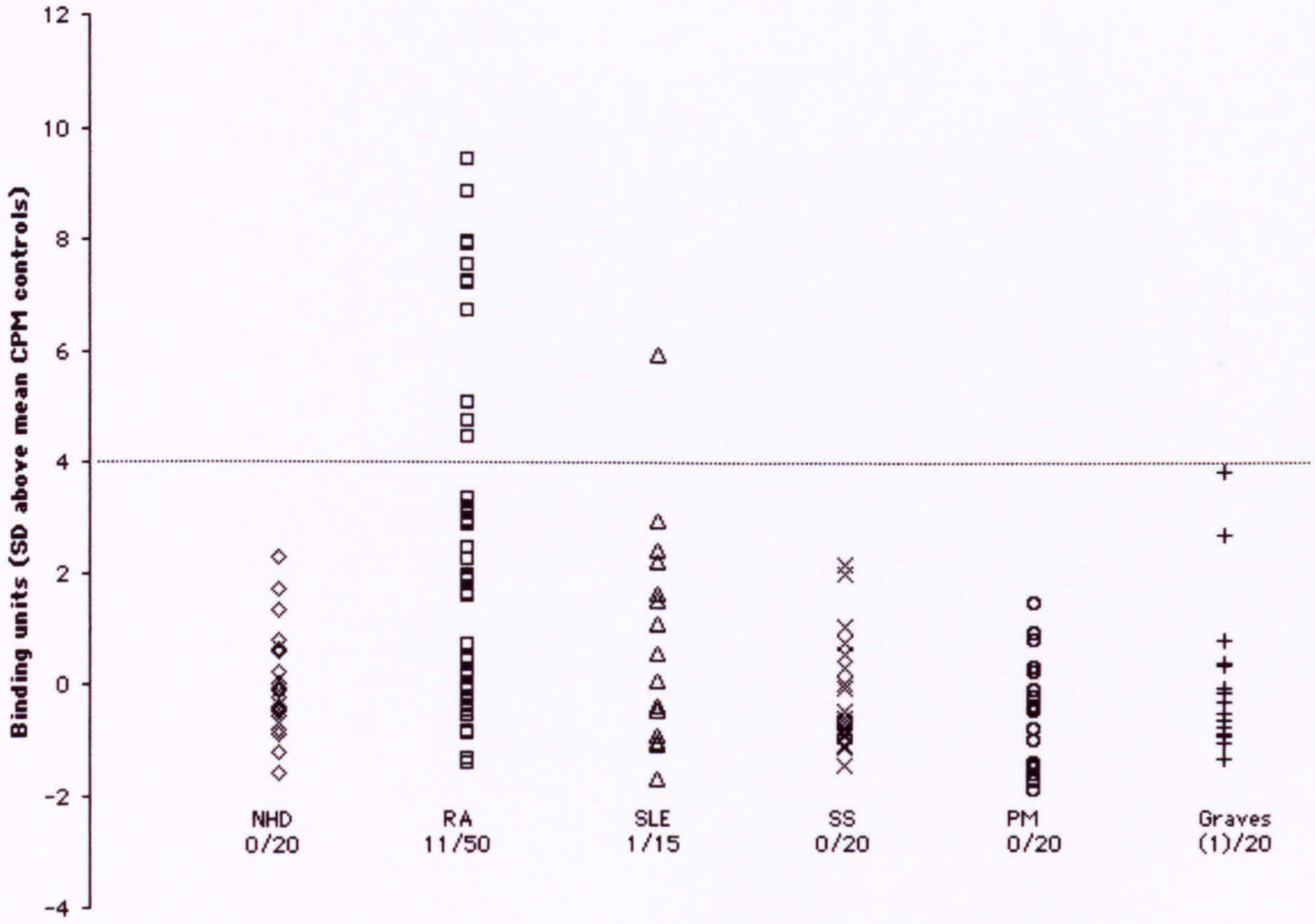


Figure 18. Each symbol represents the mean of duplicate measurements performed for an individual patient serum. Frequency of positive sera are shown below each column.

Figure 19. Titration of three anti-eEF1a positive sera

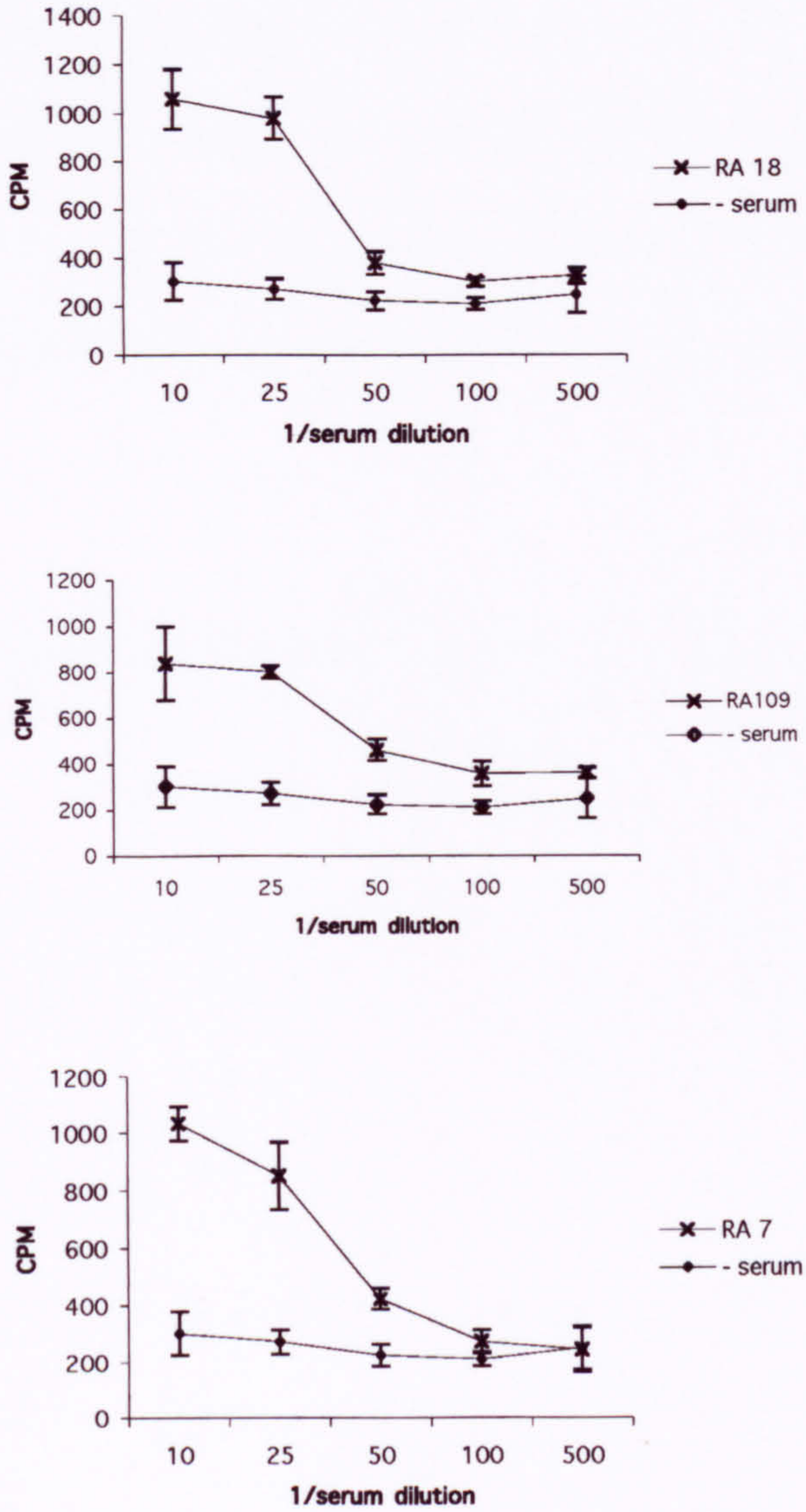


Figure 19. Error bars represent 95% confidence intervals based on triplicates.

4.3.4 Relationship of anti-eEF1α antibody to age, sex, disease duration and HLA alleles

In order to address the question of whether anti-eEF1α reactivity defines a subgroup of patients with RA antibody-positive and negative patients with RA were compared with regard to age, disease duration, gender and carriage of HLA DR*0401, *0101 and *0301 alleles (frequencies of other alleles were too small for any meaningful comparison). Data are shown in table 8.

Table 8. Characteristics of anti-eEF1α positive and negative RA patients

	Anti-eEF1α +	Anti-eEF1α -	*p*
***n*=**	**11**	**38**	
Mean age (yrs)	63	62.8	0.96
Mean disease duration (yrs)	12.3	13.1	0.79
Sex			
Males	2/11	12/38	0.38
Females	9/11	26/38	
HLA DR allele frequencies			
DR*0401	5/22	21/76	0.65
DR*0101	3/22	11/76	0.91
DR*0301	4/22	4/76	0.05

Table 8. Student's *t* test was used to estimate the significance of differences between mean ages and disease duration, and the chi-squared test used to compare the proportions of males and females in each group and carriage rates of DR alleles.

4.4 Discussion

4.4.1 Specificity of the assay for anti-eEF1α antibody

In any immunological assay it is important to ensure that specific binding is being measured, because of the frequent occurrence of polyclonal activation of B and T cells in systemic autoimmune diseases. Specific antibody in a serum can be demonstrated by binding of the cognate antigen but not an irrelevant ligand, and by competition of binding with pure antigen from another source but not by irrelevant ligand. Titration experiments illustrate the relationship of binding with concentration and thereby demonstrate that the binding is due to a serum factor. In addition they provide a means of comparison between sera. We were able to demonstrate that a sample of anti-eEF1α positive sera did not bind a control protein, and that binding was titratable and saturable. However no source of purified antigen or specific antibody was available, hence competition experiments were not performed. These data in addition to the good concordance between the frequency of reactivity detected and that shown by Ditzel *et al* support the view that specific binding was observed in our sera.

4.4.2 Anti-eEF1α as a specific marker for rheumatoid arthritis

Panels of sera from patients with a number of diseases characterised by systemic autoimmunity and varying degrees of joint involvement were tested, however anti-eEF1α reactivity was observed in only 1 of 75 patients without a diagnosis of RA (SLE) compared to 11 of 49 patients with RA. These data give a diagnostic sensitivity of anti-

eEF1α for RA of 22%, a specificity of 98% and positive predictive value of 92%. Although this is an artificial patient population and these figures cannot be directly applied to an outpatient population for instance, they do indicate that anti-eEF1α reactivity is relatively specific for RA. Similar diagnostic sensitivity is seen for the group of patients with early RA, suggesting that anti-eEF1α could be used as a diagnostic marker in early disease - clearly a large prospective study would be required to address this issue.

The single positive non-RA serum was from a patient with SLE. It is of interest that eEF1α was isolated from screening of an endothelial cell cDNA library with lupus sera (Frampton et al., 2000), although the frequency of anti-eEF1α in SLE has not been established. It is also noteworthy that the study of Ozouki et al found an association between anti-eEF1α and an anti-nuclear antibody positive subgroup of patients with facial atopic dermatitis. Anti-eEF1α may therefore be detected in subgroups of patients with SLE.

4.4.3 Anti-eEF1α reactivity may define a subset of patients with rheumatoid arthritis

Anti-eEF1α positive and negative patients were well matched with regard to age, sex and disease duration and carriage of DR B1*0401 and DR B1*0101 but carriage of DR B1*0301 was significantly more frequent in anti-eEF1α positive patients. This is somewhat surprising given the previously described association of this antibody with Felty's syndrome, a phenotype strongly associated with DR B1*0401. This finding raises the possibility that this antibody defines a subset of patients whose disease is associated

with the autoimmune A1 B8 DR3 TNF2 haplotype rather than the shared epitope more characteristic for RA. This might reflect the peptide binding requirements of different MHC molecules or disease processes mechanistically related to DR3- associated autoimmune diseases such as SLE. It would be important to replicate this result however in a larger population.

4.4.4 Why is eEF1α1 an autoantigen in rheumatoid arthritis?

Much accumulating evidence suggests that loss of tolerance to self proteins may occur as a result of post-translational modifications occurring during apoptosis or cellular stress (Utz and Anderson, 1998), such as cleavage by caspases or granzyme B (Casciola-Rosen et al., 1999), and reversible phosphorylation or dephosphorylation (Utz et al., 1997). Apoptotic blebs on the surface of neutrophils contain eEF1α1 as mentioned previously, and it is therefore possible that eEF1α1 is rendered immunogenic by such mechanisms. Antibodies however are much more frequent in patients with rheumatoid arthritis than the connective tissue disease sera tested, suggesting that some aspect of the tissue perturbation involved in joint pathology may result in loss of tolerance to eEF1α1.

4.4.5 Conclusions

Anti-eEF1α reactivity is relatively specific for rheumatoid arthritis although not very sensitive, and antibodies can be detected early in the disease course. Anti-eEF1α could potentially be a useful adjunctive diagnostic marker in patients with joint symptoms. In addition anti-eEF1α may define a subset of patients with a different mechanism of

disease. The frequency of anti-eEF1α1 in other disease states particularly SLE with autoimmune neutropaenia and skin disease needs to be established as it may be a valuable marker in these situations. Production of purified recombinant protein and establishment of an ELISA is currently being pursued in order to attempt to study large numbers of sera.

5 Investigation of autoantibody responses against T cell costimulatory molecules in rheumatoid arthritis

5.1 Introduction

Many transgenic experiments in mice have convincingly demonstrated that the binding of membrane-associated antigens to surface IgM receptors on immature B cells in the bone marrow leads to clonal deletion (Chen et al., 1995; Nemazee and Burki, 1989). Such a mechanism should ensure that effective tolerance exists to those cell-surface molecules, particularly on haemopoietic cells and lymphocytes, which are likely to come into close contact with B cells during their physiological functioning (Cornall et al., 1995). The presence in the periphery of antibodies and by implication B cells with specificity for such molecules might indicate a generalised defect in B cell tolerance induction programmes. Such antibodies may be useful as specific markers of autoimmunity. It is of interest therefore that patients with SLE possess anti-lymphocyte antibodies (Osman and Swaak, 1994) although the specificities of these are incompletely characterised. T cells require a second signal for activation in addition to ligation of the TcR-CD3 complex by MHC/peptide on antigen presenting cells. The best characterised of such costimulatory molecules is the CD28/B7 system. Binding of B7.1 or B7.2 on antigen presenting cells to CD28, which is constitutively expressed on T cells results in the necessary costimulatory signal. Activation of T cells results in expression of the alternative B7 ligand CTLA4. CTLA4 binds to the B7 ligands with 20-50 times higher affinity than CD28 and transduces a negative signal to T cells. Mice deficient in CTLA4

have a profound lymphoproliferative and autoimmune phenotype and therefore this molecule is believed to have an important role in the control of T cell activation (Tivol et al., 1995; Waterhouse et al., 1995). One group has investigated the frequency of antibodies to these molecules in patients with autoimmune diseases and detected antibodies to CTLA4 in 4/49 patients with SLE, 9/48 patients with rheumatoid arthritis, 1/32 patients with systemic sclerosis, 7/22 patients with Behçet's syndrome and 2/15 patients with Sjogren's syndrome (Matsui et al., 1999). Antibodies to B7.2 were found in a much smaller number of patients and antibodies to CD28 and B7.1 in none. Of interest also was the finding that uveitis occurred much less frequently amongst the antibody-positive patients than the antibody-negative patients with Behçet's syndrome, implying an association between antibody status and disease phenotype. It is of interest therefore to corroborate these findings and establish whether or not similar associations can be found in other diseases.

These studies were performed using bacterially produced recombinant antigen in the form of β galactosidase fusions that were adsorbed onto solid surfaces for ELISA. However autoantibodies in general arise in response to native protein and so often recognise conformational epitopes (Blanchin et al., 2002; Guo et al., 2001) and therefore studies on denatured protein may underestimate their true frequency. In this work attempts to demonstrate antibodies to T cell costimulatory molecules using two other methodologies are described. Radiolabelled CTLA4 and B7.2 were synthesised by in vitro transcription-translation and used for immunoprecipitation studies. Additionally, in order to analyse reactivity to native protein attempts were made to express the

extracellular domain (ECD) of CTLA-4 at high levels on the surface of eukaryotic cells using a glycosylphosphatidylinositol (GPI) anchor. This technique has been successfully used to display the thyrotropin receptor extracellular domain at approximately 10 times higher density at the cell surface than standard methods (Da Costa and Johnstone, 1998) and was chosen because the high expression levels may allow the problem of non-specific binding of human sera to cells to be overcome (Matsui et al., 1999).

5.2 Materials and Methods

5.2.1 Construction of CTLA4, B7.1, B7.2 and CD28 ecd expression plasmids

Complementary DNA for CTLA-4 was obtained by RT-PCR from peripheral blood mononuclear cells taken from a healthy volunteer which had been stimulated for 3 days with 5μg/ml of concanavalin A. Complementary DNA for the remaining three molecules could be readily amplified from unstimulated PBMCs. Primers were designed to incorporate *BamH I* (or *Kpn I* in the case of CTLA4) and *Xho I* restriction sites into the PCR product to allow cloning. The plasmid pcDNA3-TSHr-gpi, which carries an insert corresponding to the extracellular domain of the thyrotropin receptor cloned in frame with a GPI anchor sequence (Coyne et al., 1993) was used as the parent vector (figures 20 and 21). The final constructs were all verified by sequencing.

Figure 20. The sequence directing the attachment of a polypeptide to a GPI anchor

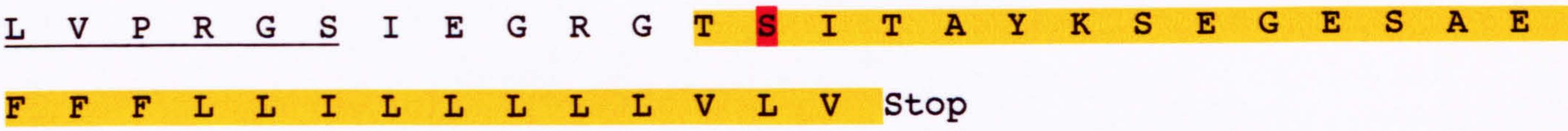


Figure 20. The artificial amino-acid sequence shown is that which was engineered into the parent construct and begins immediately following residue 412 (isoleucine) of the TSHr. The underlined sequence is a thrombin cleavage site (not employed in this work) and the sequence highlighted in gold is the sequence directing the attachment to the GPI anchor.

The serine residue highlighted in red represents the putative covalent attachment site of the anchor.

Figure 21. Construction of the plasmid pcDNA3-CTLA4-ecd

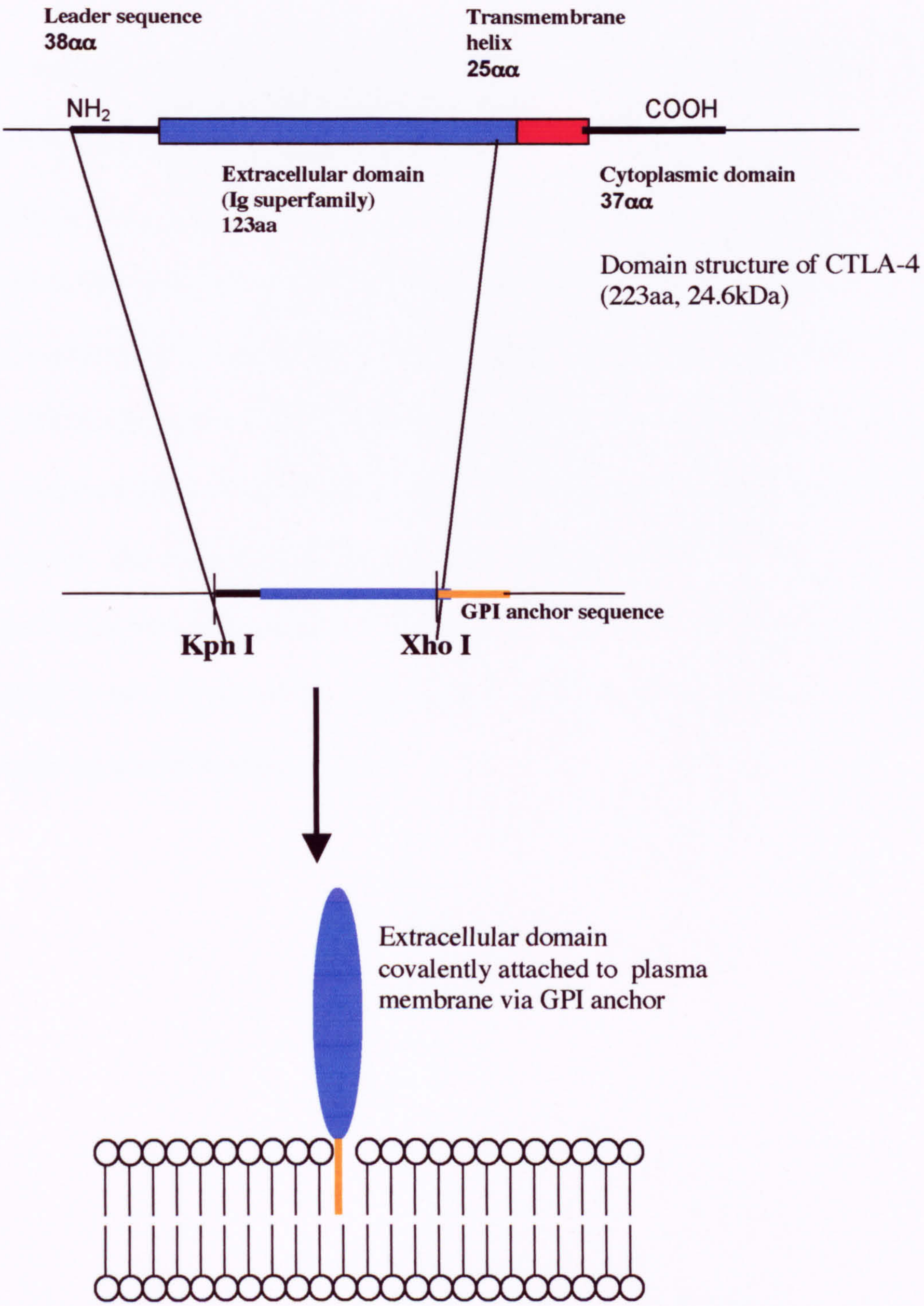


Figure 21. Construction of the CTLA4ecd expression plasmid is illustrated, all 4 molecules were cloned using the same system.

5.2.2 Production of 35S-labelled CTLA-4 and B7.2 by in vitro transcription-translation, and immunoprecipitation assays

The plasmids constructed above were used as templates for *in vitro* transcription-translation (IVTT) as described previously, however a polypeptide product was obtained only from the B7.2 construct (figure 22). In further attempts to express CTLA4, B7.2 and CD28 an alternative method was used. Full-length cDNA was amplified using a sense oligonucleotide primer containing the T7 polymerase promoter and ribosome binding site. The linear PCR product was then used as template for IVTT. This approach was successful for CTLA4 (figure 23) but not for B7.1 or CD28, and no further attempts to express these molecules were made. Immunoprecipitaion assays were subsequently performed as described previously.

Figure 22. IVTT products from pcDNA3/CTLA4 ecd, B7.1 ecd, B7.2 ecd and CD28 ecd expression plasmids

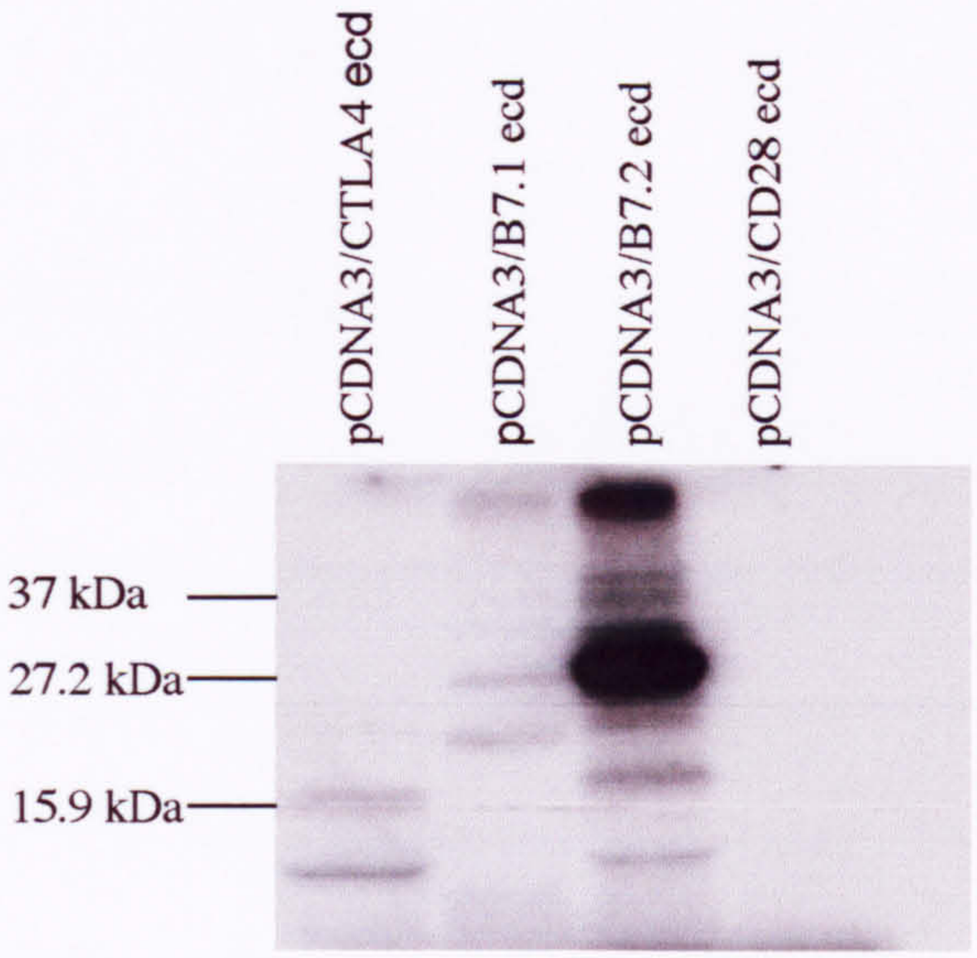
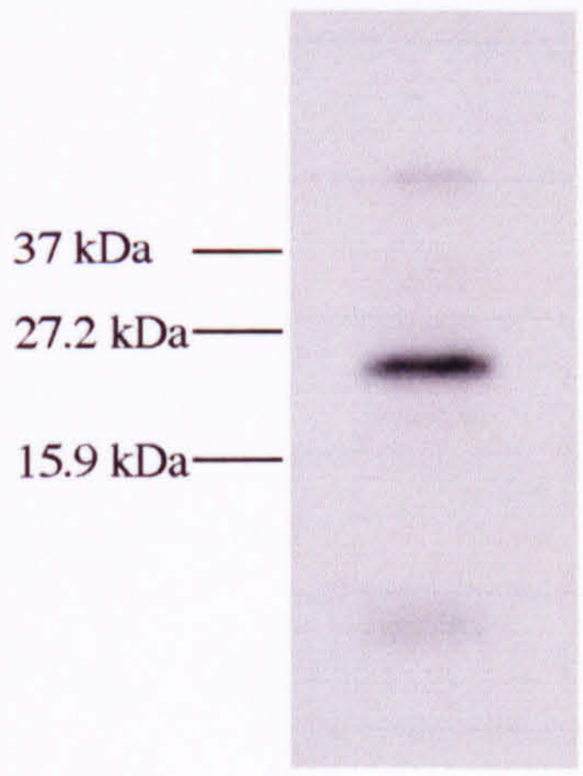


Figure 23. IVTT of full-length CTLA-4



5.2.3 Transfection of Chinese Hamster Ovary (CHO) cells and selection of stable transformants

CHO cells were grown in monolayer cultures in 6 well plates to 60-70% confluence. Each well was transfected with 1µg of plasmid DNA, prepared using the Qiagen Maxi plasmid prep kit according to the manufacturer's instructions. Transfection was performed using Fugene 6 (Roche) according to the manufacturer's protocol. After 48 hours the cells were removed from the well with trypsin and transferred to a 10cm culture dish with complete medium (Ham's F12 supplemented with 10% fetal calf serum, 2mM L-glutamine, penicillin, streptomycin) supplemented with Geneticin© at 800µg/ml. The culture was continued with changes of medium every 48 hours until the majority of the cells had died and visible clumps had begun to form. When clumps of 30-50 cells were established 40 cell clumps were picked and cultured in 24 well plates until confluent. Clones were screened for cell surface CTLA4 ecd expression by FACS analysis.

5.2.4 Indirect immunofluorescence and FACS analysis

Sub-confluent cells in monolayer culture were removed from the plate using Cell Dissociation Medium (Sigma) and after washing in PBS were resuspended in 100µl complete medium to which was added anti-CTLA4 monoclonal antibody (Clone BN13, BD Pharmingen) or IgG2A isotype control (Serotec) at 2µg/ml and incubated for 30 minutes at 4°C. After washing once in complete medium the cells were incubated with goat anti-mouse IgG FITC conjugate (Sigma) 5µg/ml for a further 30 minutes at 4°C and

washed again. One colour flow cytometric analysis was performed using a Becton Dickinson FACScan and proprietary software.

5.3 Results

5.3.1 Recombinant CTLA-4 and B7.2 synthesised by IVTT are immunologically intact

In order to ensure that the recombinant molecules produced above were suitable for use in immunological assays titration immunoprecipitation experiments were performed with commercially produced antibodies (figure 24). The antibodies used were a rabbit affinity purified polyclonal anti-CTLA4 IgG, (Santa Cruz Biotechnology) and goat polyclonal anti-B7.2 IgG (Santa Cruz Biotechnology). Specific and titratable binding was seen for each molecule. For subsequent assays each antibody was used as a positive control at a concentration of 8ng/µl at which concentration binding was reliably higher than background but in the expected range of positive sera.

5.3.2 Absence of specific antibodies in RA patients to CTLA4 and B7.2 as assessed by immunoprecipitation assays

Thirty RA sera and 30 control sera were tested for binding to each molecule. A 1/10 dilution of serum in IP buffer (5µl of serum in a total volume of 50µl) was used. Binding data for individual sera are shown in figure 25. No specific binding to either CTLA4 or B7.2 was seen in any serum despite definite binding to control antibodies.

Figure 24. Binding of specific antibody to radiolabelled CTLA4 and B7.2

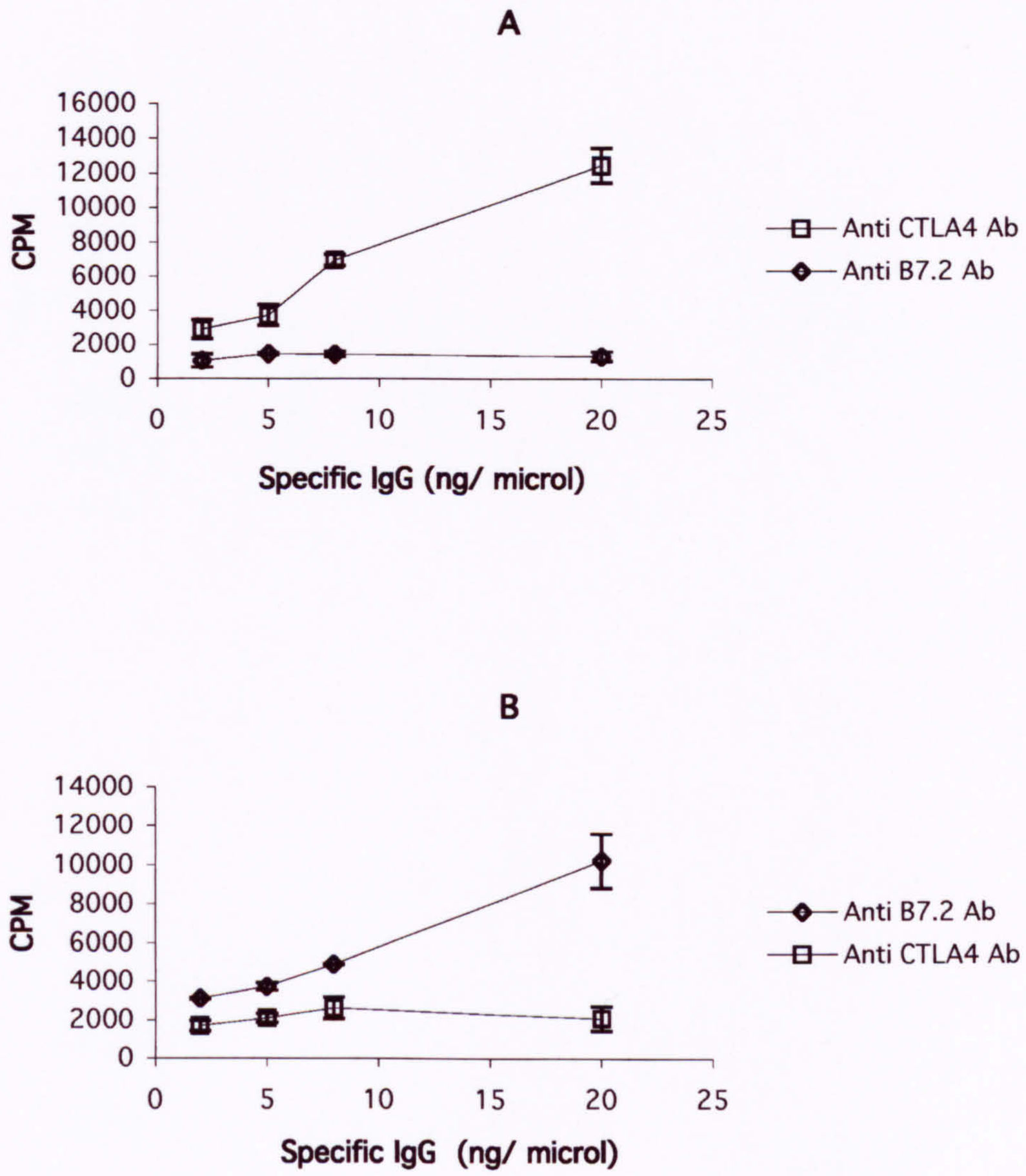


Figure 24. Panel (A) shows data for binding of each antibody to recombinant 35S-labelled CTLA4 and panel (B) for binding to recombinant 35S-labelled B7.2. In each case the other antibody was used as a negative control.

Figure 25. Reactivity of RA and control sera to 35S-labelled CTLA4 and B7.2

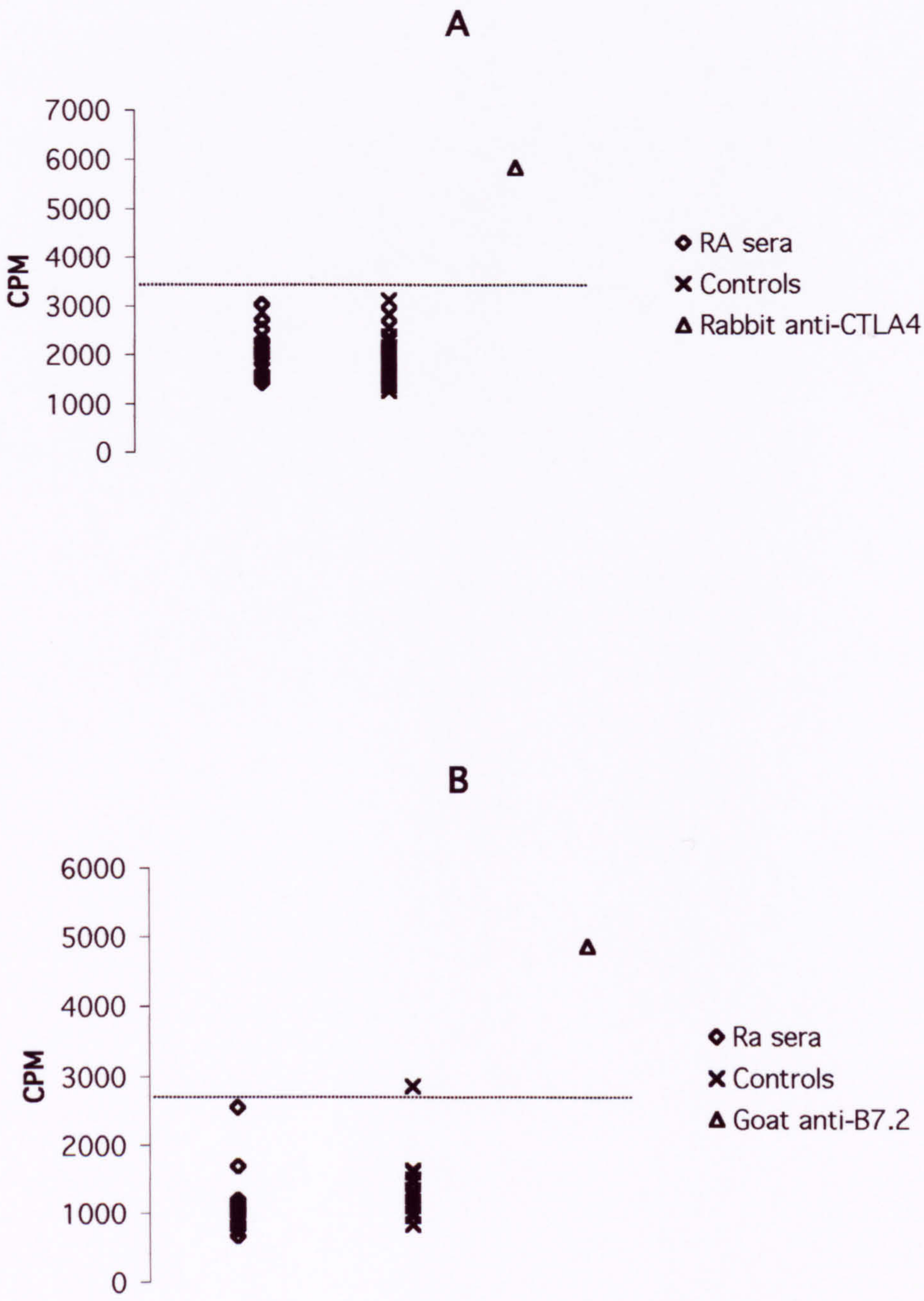


Figure 25. Binding of individual sera to CTLA4 (panel A) and B7.2 ecd (panel B) are shown. Each serum was tested at a dilution of 1/10 (5μl serum in a total volume of 50μl). The dashed horizontal line represents 3sd above the mean of the control group in each assay. Control antibodies were used at 8ng/μl.

5.3.3 Development of a flow cytometry assay for anti-CTLA4 antibodies

In total 40 clones were screened for surface CTLA4ecd expression by flow cytometry. On initial screening 2 clones appeared to demonstrate cell surface expression however this was not confirmed subsequently. Synthesis of CTLA4 by the transfected clones was also analysed by Western blotting using the anti-CTLA4 monoclonal antibody BN13 against cell lysates, however no expression was seen, and therefore in the time available further attempts to develop this assay were not carried out.

5.4 Discussion

5.4.1 Lack of reactivity to CTLA4 and B7.2 in immunoprecipitation assays

In this work ^{35}S-labelled CTLA4 and B7.2 were synthesised *in vitro* and binding to specific antibodies demonstrated. Despite the production of immunologically intact recombinant polypeptides we were unable to corroborate the results of Matsui *et al* who demonstrated antibodies to CTLA4 using β galactosidase fusion proteins in 9/48 patients. In our study 30 RA sera were assayed and therefore approximately 5 or 6 sera would be expected to be positive. This may reflect a genuine difference in the populations studied however there are a number of other possible explanations for these divergent observations.

5.4.1.1 Assay sensitivity.

It is possible that the assay described above was relatively insensitive and the titres of antibody present were below the limit of detection. Without a direct comparison of assays this is very difficult to establish. From the titration experiments for CTLA4, a specific IgG concentration of 5ng/μl did give a signal which could be reliably differentiated from background. Considering a value of 10μg/μl as the average human serum IgG concentration, specific IgG would therefore need to comprise approximately 0.05% of the total IgG to be detected (assuming human antibodies are of similar affinity to the rabbit antibody, a condition which may not be fulfilled). This figure is certainly comparable to the titre of other autoantibodies. In the study of Matsui *et al* antibodies were detected using ELISA at dilutions between 1 in 500 and 1 in 1000, suggesting they

are of relatively high titre and it is therefore surprising they were not detected in this assay. Some idea of sensitivity could have been gained by concomitant assay of a ubiquitous antibody in these patients such as antibodies to tetanus toxoid and may have helped to answer this question.

5.4.1.2 Nature of the antigenic determinants.

Immunoprecipitation assays rely on the formation of multivalent immune complexes and therefore require a polyclonal serum. It is well established that individual monoclonal antibodies often do not immmunoprecipitate their cognate antigen for this reason although "cocktails" often do. It is therefore possible that anti-CTLA4 antibodies because of their fine specificities or other properties do not efficiently form immune complexes in solution. Epitope mapping studies performed in the report of Matsui *et al* suggest that multiple regions of the CTLA4 molecule are recognised, however these studies were all performed on denatured polypeptides using ELISA and it is possible therefore that predominantly linear or even denatured epitopes were demonstrated, which may be different from those required for efficient immunoprecipitation.

5.4.1.3 Presence of a soluble inhibitor of binding.

A soluble form of CTLA4 (sCTLA4) lacking the transmembrane and cytoplasmic domains has been described (Magistrelli et al., 1999; Oaks et al., 2000) and can be detected in normal sera at concentrations ranging from 5 to 100ng/ml. A contrasting report suggests that levels of sCTLA4 in normal individuals is low or undetectable, and found elevated levels of up to 100ng/ml in patients with autoimmune thyroid disease

(Oaks and Hallett, 2000). This form of CTLA4 is synthesised by resting T cells and therefore a role as a paracrine negative regulator of T cell activation has been suggested. To date levels of sCTLA4 have not been assessed in other autoimmune diseases. It is a theoretical possibility therefore that sCTLA4 in sera may have interfered in the immunoprecipitation assay, although it is not clear whether sCTLA4 at up to 100ng/ml serum concentration (and therefore a ten-fold lesser concentration in the assay) would have this effect. Attempts were made to measure sCTLA4 levels in our sera. Both studies described above used home-made capture ELISAs with monoclonal antibody pairs; in each case the same monoclonal was used as capture antibody (BN13, BD Pharmingen) however neither of the other antibody was available and therefore the polyclonal rabbit IgG (Santa Cruz SC9094) was used as a secondary antibody, followed by a goat anti-rabbit IgG-alkaline phosphatase conjugate as detection reagent. Although an adequate standard curve was obtained using this system (figure 18), background levels with serum samples were too high for quantitation. Therefore in the absence of more specific reagents no further attempts to measure sCTLA4 levels were made in the current work. Clearly in addition the presence of autoantibodies in sera may also interfere with the measurement of sCTLA4 levels in ELISAs, and it is possible that this may account for the some of the variability in sCTLA4 levels measured in the reports above. Further attempts to search for antibodies to CTLA4 using these methods may therefore require the use of purified IgG from patients.

Figure 26. Standard curve of sCTLA4 ELISA

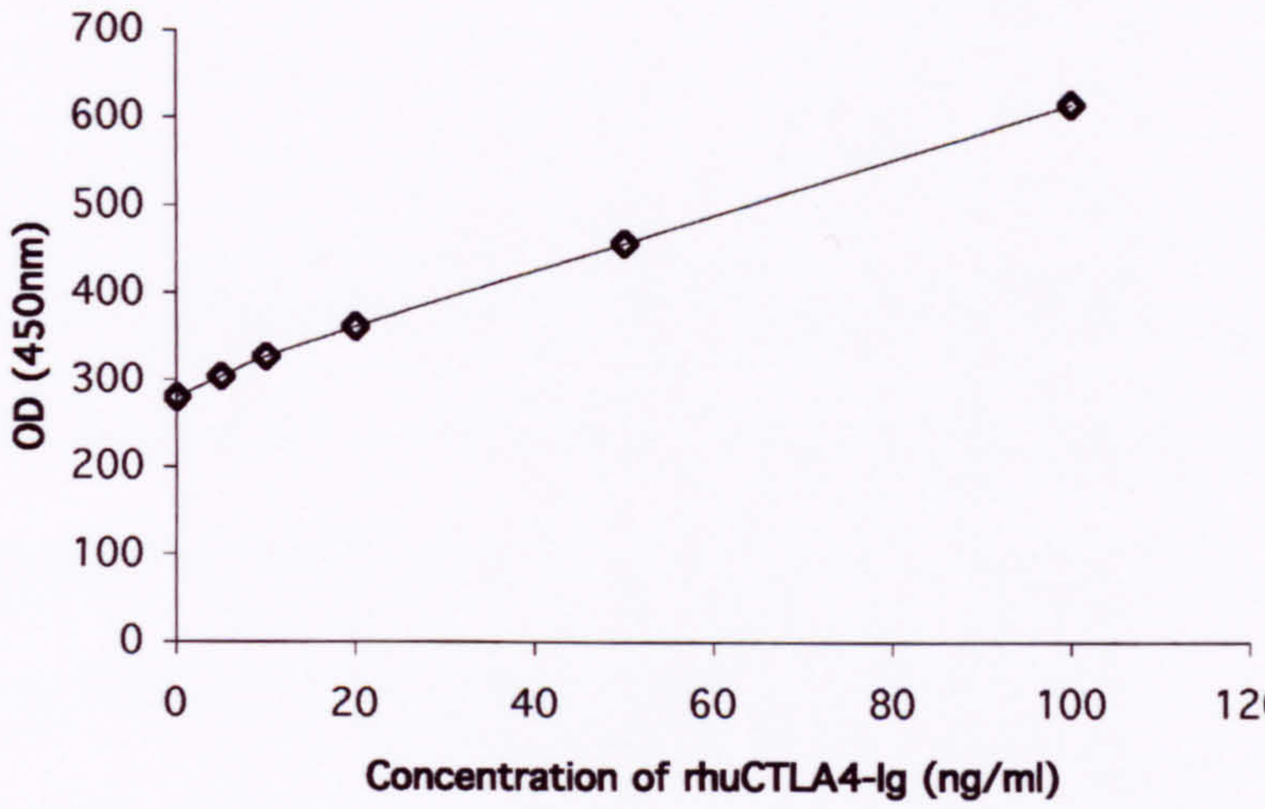


Figure 26. Recombinant human CTLA4-Ig fusion protein (rhuCTLA4-Ig, R&D Systems) was used as standard.

5.4.2 .Conclusions

Using radioimmunoprecipitation assays we were unable to demonstrate the antibodies to CTLA4 reported by another group of workers. Possible reasons for this are discussed. The importance of T cell costimulatory molecules in lymphocyte regulation is undisputed, and CTLA4-Ig fusion proteins are effective therapies for many animal models of disease. Clinical trials of CTLA4-Ig fusions in human disease, particularly SLE are underway. Clearly the presence of antibodies may therefore have clinical impact as well as providing information on diagnosis or pathogenesis. For these reasons we suggest that further studies are required in order to establish the frequency of anti-CTLA4 antibodies in systemic autoimmune diseases.

6 Discussion

This thesis describes the construction of a cDNA library from rheumatoid pannus, its expression in both a lambda and a phage display vector, and the development of strategies for screening of phage displayed libraries using rheumatoid sera. The validity of our approach was demonstrated by the successful enrichment of a clone encoding eEF1α, shown by others and ourselves to be a target of autoantibodies in patients with rheumatoid arthritis.

A large body of work over the last twenty years utilising many different approaches has sought to characterise novel targets of antibodies in rheumatoid arthritis. Autoantibodies such as rheumatoid factors, anti-CCP antibodies and anti-RA33 have been demonstrated by a number of techniques and their presence and frequency corroborated by the work of independent groups. Numerous single reports of autoantibodies also exist however many of these remain uncorroborated, such as the anti-CTLA4 antibodies that are the subject of chapter 5. What therefore is the value of using another approach to search for yet more antibodies in rheumatoid arthritis? In contrast to the majority of previous work, we have focussed on patients with early disease defined as within 3 months of onset of symptoms, on the premise that the profile of autoantibodies present early in the disease course may reflect earlier pathological events. Little information exists on the frequency of autoantibodies at such an early point in the disease course. Many studies have reported the presence of autoantibodies in patients with synovitis of recent onset (Goldbach-Mansky et al., 2000; Kroot et al., 2000) however all included patients with onset of

synovitis in the previous 12 months. In addition the methodology employed in our studies should allow the selection of targets of autoantibodies based on binding to non-denatured polypeptide, and is less dependent on the abundance of individual proteins in target tissue, both important differences from the majority of previous work in this field.

It is important to note that other than eEF1α 1 we did not isolate any other known antigens of which there are several. This is likely to reflect the constraints of the methods used in terms of expression of eukaryotic sequences in a prokaryotic system and folding of polypeptides as well as the methods for generating cDNA. Alternative methods that would attempt to overcome some of these constraints might include affinity chromatography on IgG columns generated from patient sera and identification of interacting polypeptides by mass spectrometry, or indeed cloning in a eukaryotic system.

To what extent are autoantibodies in patients with rheumatoid arthritis pathogenic, or do they occur as a consequence of pathologic events? Although the biological plausibility of antibody-mediated joint pathology is beyond doubt, little evidence for a causative role for antibodies in human disease exists, which would require the demonstrate of a highly specific autoantibody association with the disease as well as its ability to cause disease in adoptive transfer. To date only anti-CCP antibodies appear to be highly specific for rheumatoid arthritis, however no data exists on their pathogenicity in experimental models. Conflicting reports exist of antibodies in patients with rheumatoid arthritis to glucose-6-phosphate isomerase as found in the K/BxN transgenic model. Fundamentally the presence of autoantibodies must reflect the T/B cell interaction believed to be

important particularly in the early stages of the disease (Cope, 2002). There is evidence from the study of connective tissue diseases that the development of individual antibody specificities is influenced by a number of biological variables including MHC restriction (Wilson et al., 1994), pathways for post-translational modification and degradation of proteins (Casciola-Rosen et al., 1999) as well as clearance of apoptotic debris from cells and it is likely that similar variables influence the production of specific autoantibodies in individuals with rheumatoid arthritis. The dissection of such influences in patients with rheumatoid arthritis, as initiated in chapter 4 may provide valuable data regarding etiology and pathogenesis of rheumatoid arthritis.

Rheumatoid arthritis is a heterogeneous condition; hence a single characteristic autoantibody profile may not be expected. Because of the ease of measuring antibodies in blood and studies of other systemic diseases such as vasculitis and the connective tissue diseases, much interest remains in the ability of autoantibodies to define clinically relevant subgroups of patients. However the majority of the autoantibodies described in rheumatoid arthritis are present in less than 50% of individuals, and do not appear to have any prognostic value over and above traditional assessments such as CRP and tender/swollen joint counts. One study has suggested that although anti-CCP positive patients develop rather more radiologic damage than anti-CCP negative patients, the predictive value of anti-CCP positivity for radiologic damage at 6 years was moderate in multiple regression analyses and certainly much less than that for IgM rheumatoid factor (Kroot et al., 2000). Anti-eEF1α 1 is present in the majority of patients with Felty's syndrome, and data presented in chapter 4 suggests that this is not simply a reflection of

MHC restriction. Further work is required to test the hypothesis that these patients represent a clinically or pathologically distinct subgroup.

It has been recently been shown that anti-CCP antibodies predate the onset of rheumatoid arthritis by several years in some cases (Dahlqvist et al., 2002; Nielen et al., 2002) suggesting that the pathological events relevant to the formation of autoantibodies occur months or years before the onset of symptoms. Recent studies on the properties of T cells in patients with rheumatoid arthritis suggests the possibility of an early antigen-driven phase of T cell activation followed by a later phase in established disease dictated by cytokines (Cope, 2002). Detection of disease-associated autoantibodies might therefore allow the identification of asymptomatic individuals in whom the study of these earliest events may be possible.

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