ANTIGLOBULINS IN RELATION TO RHEUMATIC DISEASES: DEVELOPMENT OF ELISA ASSAYS AND THEIR APPLICATION IN PROSPECTIVE AND FAMILY SURVEYS

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A B S T R A C T

Enzyme-linked immunosorbent assays (ELISA) were developed for the measurement of rheumatoid factors (RF) of the IgM, IgA and IgG isotypes. The assays are based on the adsorption of rabbit IgG to a solid phase, followed by incubations of sample dilutions and enzyme-labelled isotype-specific anti-human immunoglobulins. Due to interference by IgM RF it was found essential to pepsin digest the rabbit anti-human IgA used for the IgA RF assay. By using a monoclonal mouse anti-human IgG and an enzyme-linked goat $F(ab')_2$ anti-mouse IgG, pepsin digestion of samples to be tested for IgG RF was only marginally beneficial. The results were expressed as units with reference to dilutions of an internal standard. The IgG RF and IgA RF assays are sensitive and specific but their reproducibility needs to be improved.

A low percentage of normals was found to have raised serum IgG antiglobulins which reacted preferentially with ruminant IgG. This prohibits the use of ruminant IgG as substrate in tests for autoreactive IgG antiglobulins. These antiglobulins may represent a deviation from the normal, acquired immunological unresponsiveness to bovine milk proteins. The IgM but not the IgG responses of 29 individuals to 14 pneumococcal polysaccharides correlated positively with their anti-ruminant IgG levels.

In a study of 181 members of an inbred Icelandic family, a significant aggregation of rheumatoid arthritis (RA) was found in those members whose parents both belonged to the family. These members also had significantly higher serum IgG and IgM levels than their relatives. Joint symptoms and IgM RF levels correlated

positively with the age of the family members while IgA RF levels showed no such association, perhaps indicating that different mechanisms may be involved in the production of these two RF isotypes.

Thirty-three patients with early inflammatory joint disease, 28 of whom developed RA, were followed prospectively for 2-4 years. Changes in serum IgA RF levels correlated significantly with concurrent changes in clinical activity, and those seven who presented with raised serum IgA RF developed significantly more bone erosions than the remaining cases. By contrast, none of the five patients who presented with raised IgM RF only, developed an erosive disease.

These observations are consistent with defective immunoregulation and excessive T helper effect in RA.

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C H A P T E R I

GENERAL INTRODUCTION

C H A P T E R I

GENERAL INTRODUCTION

I.1. HISTORICAL AND CURRENT VIEWS ON AETIOLOGICAL ASPECTS OF THE RHEUMATIC DISEASES

Rheumatic diseases are known to have affected mankind ever since the days of prehistoric homo sapiens. Advanced osteoarthritis of the femur has been demonstrated in an ancient pre-dynastic Nubian, and gout is described in Hippocratic writings from the 5th century BC under the name of Podagra, as "the most violent, tenacious and painful of joint affectations" (Copeman and Scott, 1978). It was not until fairly recently, however, that the present classification of the rheumatic diseases was developed. The credit for the first description of rheumatoid arthritis (RA) as a separate entity has been given to Dr. Landre-Beauvais, a Paris physician who described it in 1800 under the name "goutte asthénique primitive" and noticed that it mainly affected women (Copeman and Scott, 1978). In 1859, Garrod proposed the name rheumatoid arthritis, among other reasons to distinguish the condition from gout, which he realised was an unrelated entity (Garrod, 1859). In 1904 Osler delineated systemic lupus erythematosus (SLE) clinically and suggested a vascular basis for the disease (Osler, 1904).

Describing and defining the rheumatic diseases, however, was not equivalent to defining their aetiologies. Over the last few decades great effort has been devoted to finding the cause(s) of the various rheumatic diseases. A brief summary of this work is presented here,

with special emphasis on RA and SLE, including animal models for these diseases.

Any speculations about the aetiology of a disease like RA must be seen in the epidemiological and clinical context of the condition. It is widely recognised that the onset of RA can occur at any age but especially between 30 and 60 years (Wood, 1968) and the prevalence generally increases with age for both males and females (Masi and Medsger, 1979). There is a definite female predilection, especially in the younger age groups (Masi and Medsger, 1979; Kato *et al*, 1971) and some familial clustering, both of clinical disease and serum rheumatoid factor (RF) (Lawrence, 1970). RA occurs in all human races with a prevalence for definite RA in adults, as determined by the ARA-criteria (Ropes *et al*, 1958) from 0.12% of the total population in rural South Africa (Beighton *et al*, 1975) to 3.0% in Heinola, Finland (Laine, 1962). Climate does not seem to be a major factor in determining the prevalence of definite/classical RA (Lawrence *et al*, 1966).

The onset of RA is usually insidious but may be sudden. There are sometimes prodromal symptoms of fatigue and myalgias before the development of joint stiffness and swelling, which may be asymmetrical or monoarticular at first but usually evolves over weeks or months to a relatively symmetrical pattern of polyarthritis (Bywaters, 1975). The course is then highly variable but generally the arthritis runs a remittent but progressive course, with exacerbation followed by partial remission (Kellgren, 1969).

Turning to the question of the cause(s) of RA, many aetiological hypotheses have been developed and tested over the years. There does

not seem to be any strong evidence for generalised metabolic or biochemical defects in RA, although plasma concentrations of histidine have been reported to be low in RA (Gerber, 1975). Allergy to dietary constituents has also been suggested as a contributory factor, supported among other reasons by the finding that the absorption of food constituents may be associated with the development of circulating immune complexes in some IgA deficient and normal individuals (Paganelli *et al*, 1979; Cunningham-Rundles *et al*, 1978). Despite some striking case reports (Parke and Hughes, 1981), however, the evidence for an aetiological rôle of food allergy in RA seems limited.

In 1891, Bouchard pointed out that polyarthritis might be a sequel of various infections, e.g., gonorrhoea, dysentery and scarlet fever (Copeman and Scott, 1978). The number of infectious agents implicated in the aetiology of RA has been growing ever since but the rôle of infections has been difficult to prove. Earlier on in this century, the theory of "focal sepsis" enjoyed some popularity (Currey, 1978); as teeth and tonsils were thought to harbour chronic infection, their removal was encouraged. Later, diphtheroids were claimed to be likely aetiological agents and it was suggested that intracellular diphtheroids in the synovium might stimulate an immune response, damaging to the host cell (Duthie *et al*, 1967). The diphtheroids, however, have now been largely discredited (Person and Sharp, 1976). Mycoplasma infections can, following an acute septic phase, produce a chronic proliferative synovitis in mice. The demonstration of shared antigens between mycoplasma and normal mouse synovium has been suggested as a possible explanation for this (Harwick *et al*, 1976). In the human situation, however, most attempts to isolate mycoplasmas from joint

tissues or to demonstrate specific anti-mycoplasma immunity have been negative (Person and Sharp, 1976).

Over the last fifteen years or so, much attention has been paid to viruses as possible aetiological agents in RA. Arthritis after infection or vaccination with rubella is a well known complication and rubella virus has occasionally been isolated from synovial fluid in such cases (Weibel *et al*, 1969). This arthritis is usually self-limited, however, lasting for 1-3 weeks on average (Steere and Malawista, 1979). Attempts to isolate rubella and other viruses from synovium have generally been unsuccessful (Person and Sharp, 1976) and the resistance of rheumatoid synovial cell cultures to infection with viruses (Smith, 1971) was found to be due to increased hyaluronic acid synthesis by the rheumatoid cells which prevented the attachment of added virus (Clarris *et al*, 1974). Recently, there has been renewed interest in the possible rôle of Herpes viruses, especially Epstein-Barr virus (EBV), in the aetiopathogenesis of RA. This followed the discovery of Alspaugh and Tan (1975) that patients with Sjoegren's syndrome associated with RA had precipitating antibodies against an antigen called SS-C. This antigen, now known as rheumatoid arthritis-associated nuclear antigen (RANA), appears restricted to human B lymphocytes transformed by EBV (Ferrell and Tan, 1981). Anti-RANA antibodies have been reported to occur in up to 94% of patients with sero-positive RA (Catalano *et al*, 1979) but these antibodies can also be demonstrated in a high proportion of normals, most of whom have evidence of a previous EBV infection as shown by raised titres of antibodies to Epstein-Barr viral capsid antigen (Catalano *et al*, 1980). Furthermore, Catalano *et al* (1980) followed up 11 young individuals with infectious mononucleosis and demonstrated rising anti-RANA titres for more than a year after the

recovery. A primary EBV infection does always seem to be followed by a life-long carrier state (Nilsson *et al*, 1971).

Viruses have also been implicated in the aetiopathogenesis of SLE which is a multisystem disease, predominantly of young women, characterised by vasculitis, synovitis and serositis with a marked tendency to exacerbation and remission (Hughes, 1982). The interest in viruses and SLE has been stimulated by the finding that the New Zealand Black (NZB) mouse, a thoroughly studied animal model of SLE, spontaneously produces high levels of an endogenous type C RNA virus (Quimby and Schwartz, 1982), which is, however, xenotropic, i.e., it can only infect cells of other species. A viral envelope glycoprotein with a molecular weight of 70,000 daltons (gp70) is present in considerable quantities in the sera of these mice and forms rapidly sedimenting immune complexes as gp70- anti-gp70 in older mice of the (NZBxW)F_1 hybrid (Dixon *et al*, 1980). However, although these immune complexes may contribute to the nephritis in the NZBxW mice, Datta *et al* (1978) showed recently that the genes that determine the expression of infectious xenotropic virus in NZB mice segregate independently from those that are involved in the autoimmune disease of these mice. Thus, the xenotropic virus and its products do not seem to be an essential feature of the autoimmunity in NZB mice.

As for human SLE, extracts of SLE tissues, as well as some other human tissues, were found by competitive radioimmunoassay to contain a protein of possibly type C viral origin (Strand and August, 1974). Other workers, however, have failed to demonstrate these viruses in human tissues (Charman *et al*, 1976; Todaro *et al*, 1976) but more

recently type C-related antigen was found by immunofluorescence in the glomeruli of SLE patients (Mellors and Mellors, 1978). However, C-type antigens have also been found in non-SLE tissues and attempts to isolate type C viruses from SLE tissues have virtually always been unsuccessful (Phillips, 1978).

In summary, all the available evidence seems to suggest that the aetiopathogenesis of rheumatic diseases like RA and SLE cannot be explained on the basis of single causes, such as metabolic derangements, adverse reactions to dietary constituents or infections with any known micro-organisms. Rather, it seems likely that the pathogenesis can best be explained as a complex interplay of multiple genetic and environmental factors, leading eventually to termination of self-tolerance. Our current understanding of some of these factors is reviewed in the latter three sections of this chapter.

I.2. GENETIC ASPECTS OF RA AND SLE

Hereditary factors have long been claimed to be of importance in the rheumatic diseases. In 1806, William Heberden published a clinical description of rheumatoid arthritis and ended it with the sentence, "Is this not in some degree hereditary?" (Lawrence, 1970).

In the late 1920's Kroner reported four generations of RA in one family (Lawrence, 1970) and the number of reported families with clusters of RA and/or other rheumatic diseases is still increasing. However, many of the earlier studies, claiming to show familial aggregation of RA, suffered from methodological defects. Before the publication of the ARA-criteria for RA (Ropes *et al*, 1958) the definition of RA tended to be imprecise, and estimates of RA-prevalence among relatives of index cases and controls were often based upon subjective comments about any history suggestive of RA (Masi and Shulman, 1965). Also, a rheumatic sufferer is more likely to know of rheumatic complaints among relatives than a random healthy control.

In a survey in the town of Leigh in Northern England, Lawrence and Ball (1958) studied 183 first degree relatives of patients with either clinical RA or positive rheumatoid factor (RF) as measured by the sheep cell agglutination test (SCAT). An age and sex matched control group of 822 first degree relatives of a healthy control group was also studied. Clinical, radiological and serological evidence of RA was found four times more frequently in the families of RF positive index cases than in the control group. Minimal clustering of clinical RA was seen around the seronegative RA index cases. However, in a survey of 1100 Blackfeet and 969 Pima Indians in Montana and Arizona, U.S.A., no

convincing familial aggregation of probable and definite RA as judged by the ARA-criteria (Ropes *et al*, 1958) was observed and the authors concluded that there was no evidence for any important rôle of genetic mechanisms in the aetiology of RA and RF in these Indians (O'Brien *et al*, 1967). The results of these two studies appear contradictory, but a later comprehensive survey from Manchester, Leigh and Wensleydale in England where 645 first degree relatives of RA patients were examined, showed a clear excess of clinical RA in the families of the seropositive probands (Lawrence, 1970), and there was an even greater familial aggregation of severe erosive RA around the seropositive probands as 17 cases of grade 3-4 erosive arthritis were found in a group of 342 such relatives where only 2.5 would have been expected by chance. However, there was no aggregation of clinical or erosive RA among the relatives of seronegative RA probands (Lawrence, 1970).

If aggregation of RA occurs in families, what are its mechanisms? Hellgren (1969) found that the prevalence of clinical RA among 223 spouses of Swedish RA patients did not differ from that of an age and sex matched control group, and most other published reports agree with this (Lawrence, 1970). The opposite might be expected if RA resulted from infections passed around in the family. If the infection requires genetic susceptibility, however, this does not exclude infection as an important factor in the aetiopathogenesis. Twin surveys have also provided important clues here and a British study conducted by the Arthritis and Rheumatism Council of 382 index twins and 374 co-twins revealed that the concordance of seropositive arthritis was 30% in monozygous twins but only 5% in dizygous twins, furthermore, no concordance was observed in twins with seronegative polyarthritis

(Lawrence, 1970). Taken together, these data clearly favour the importance of heredity in seropositive RA, but the pattern of inheritance seems complex and it has been concluded that it is neither compatible with classical Mendelian dominant nor recessive traits (Bennett, 1976). For instance, in the case of a monogenic dominant trait, dizygous co-twins and first degree relatives should have about half as great concordance for RA as monozygous co-twins of affected individuals but the concordance in the first group is only about one-sixth of that in the monozygous group (Lawrence, 1970).

Turning to the question of a possible association of RA with genetic markers, it was realised some twenty years ago that there was no convincing association with erythrocyte markers like the ABO, MN or Kell blood groups (Cohen *et al*, 1963). It has been reported that haptoglobin 2-2 is more common in RA, especially seronegative, than in the general population (Nettelbladt and Sundblad, 1965) and more recently, a small but significant increase in the frequency of the Z allele of alpha 1-antitrypsin has been reported in RA patients (Cox and Huber, 1976). That association is potentially interesting as individuals possessing the Z allele have lowered levels of alpha 1-antitrypsin; the function of which is to inhibit enzymes like collagenases and neutral proteases which again may be of importance in joint destruction in RA (Arnaud *et al*, 1977). Also, the frequency of the F allele of C3 has been reported to be increased in RA (Broennestam, 1973).

Over the last ten years real effort has been put into the search for an association between RA and products of the major histocompatibility complex (MHC) which is situated on the short arm of the 6th

chromosome (see Figure I.1.). The genes of the MHC can be grouped as classes I, II and III (Brent and Batchelor, 1982). Class I genes code for the HLA-A, B and C antigens, these are glycoproteins which are found on the surface of all nucleated human cells and platelets. Class II genes code for cell surface glycoproteins of a different structure and function, the best known of which are the DR antigens. These are found, for example, on B lymphocytes, macrophages, Langerhans' cells and possibly some endothelial cells. The class II genes, which in the human MHC occupy the D-DR region, are considered analogous to the I-region genes of the murine MHC. The DR-antigens are thought to be homologous to the murine Ia antigens which are considered of prime importance in the regulation of specific immune responsiveness and cellular interactions in the immune response (Winchester and Kunkel, 1979). The MHC class III genes code for some components of the complement system (C2, C4 and Bf), including both classical and alternative pathways.

A common feature of all known MHC genes is their polymorphism which is most marked in the case of class I genes, recognised by the allogeneic features of the gene products (Brent and Batchelor, 1982). The first definite associations between class I genes (HLA-A, B, C) and disease were published in 1972, followed a year later by the linking of HLA-B27 with ankylosing spondylitis (Brewerton *et al*, 1973; Schlosstein *et al*, 1973) which is the strongest HLA-disease association found so far, with a relative risk of 87.4 (Svejgaard *et al*, 1980).

Despite intensive search, no definite association has been found between alleles of the HLA-A or B loci and RA in white populations (Winchester, 1981). However, the findings of Astorga and Williams (1969)

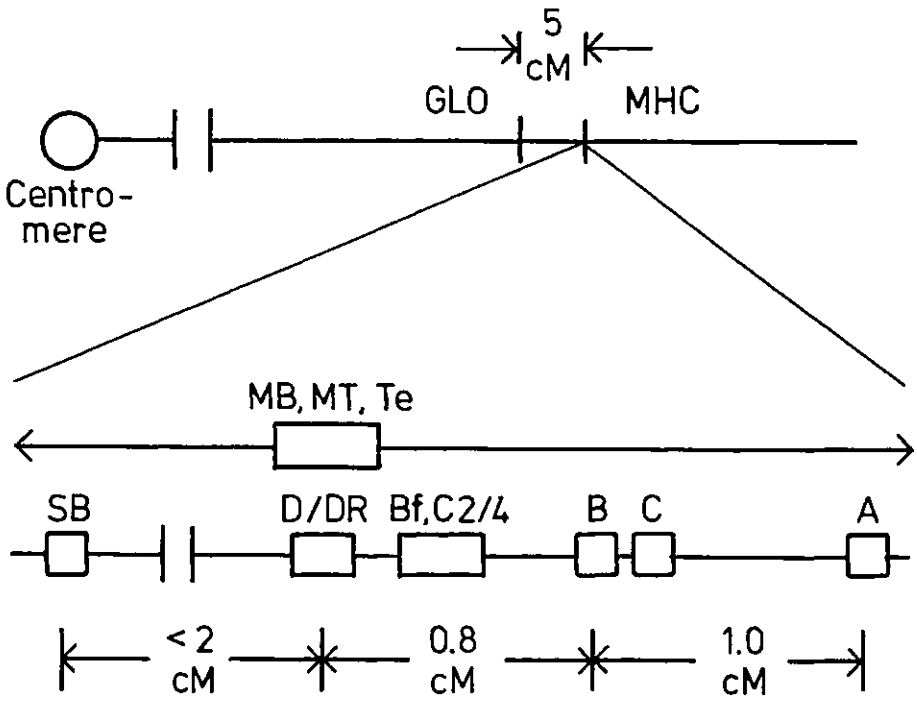


Figure I.1: The human MHC complex on the short arm of the 6th chromosome.

The upper part of the figure shows the position of the MHC, relative to the glyoxalase (GLO) marker and the centromere. The approximate distance in centimorgans (cM) between the MHC and GLO loci is shown.

The lower part of the figure shows the finer details of the MHC complex: A, B, C, D and DR refer to the main HLA gene loci. The genes for the complement components (Bf, C2 and C4) map between HLA-B and HLA-DR. The position of the class II genes MB, MT and Te within the MHC is uncertain. (Modified from Schwartz, 1982a).

that lymphocytes from RA patients failed in 64% (14/22) of experiments to produce mutual stimulation in one-way mixed leucocyte culture (MLC) suggested that RA patients might to some extent constitute a homogenous group. It was thought unlikely that this was an overall defect in RA lymphocytes as these responded normally to stimulation with phytohaemagglutinin, and normal lymphocytes produced stimulation of RA lymphocytes in 73% (16/22) of experiments. It was later found that the mutually shared diminished reactivity of RA lymphocytes in MLC has a genetic basis and is due to their sharing of the w4 allele of the HLA-D locus in up to 54% of cases (Winchester, 1981).

This issue was further clarified by the findings of Panayi and Wooley (1977) that a particular serologically defined B lymphocyte alloantigen occurred in 74% (32/43) of RA patients, compared with 27% (10/37) of controls. It was soon found that these allelic B cell alloantigens correlated significantly with the HLA-D antigens, and accordingly the former were designated HLA-D related or HLA-DR (Panayi *et al*, 1978). It has since been widely confirmed that the frequency of the HLA-DR4 allele is significantly higher in RA patients than controls, ranging from 55-70% in different series (Winchester, 1981). Furthermore, it has been shown that it is mainly seropositive RA which is associated with DR4 and during the VIIIth International Histocompatibility Workshop it was shown that whereas seronegative RA was not associated with DR4, there was a positive correlation between the association of RA with DR4 and the titre of RF (Winchester, 1981).

Apart from the general association of RA with DR4 it has been shown that the risk of RA patients developing complications from gold and penicillamine treatment may also be under genetic control.

Wooley *et al* (1980) found that 79% (19/24) of those RA patients who developed proteinuria while on gold or penicillamine were positive for HLA-B8 and DR3, and DR2 has also been indicated as a risk factor here (Panayi *et al*, 1978) even though DR2 may be associated with a favourable prognosis in RA (Griffin *et al*, 1981).

Familial aggregation has also been reported in SLE. The reported prevalence of SLE in relatives of affected individuals has varied from 0.4 to 3.4% (Hughes, 1982) and that by itself would imply significant aggregation, as the reported population prevalence ranges from 1/250 to 1/20000 (Siegel and Lee, 1973; Fessel, 1974; Serdula and Rhoads, 1979). The mouse models of SLE have lent further support to the importance of heredity in SLE-like disorders. It has been calculated, for instance, that at least three autosomal dominant genes seem to be required for the expression of the lupus-like nephritis in the (NZBxW)F_1 hybrid, two of which are derived from the NZW and one from the NZB strain (Knight and Adams, 1978). It has also been shown that expression of the early lupus-like disease in the MRL/l mouse strain which has 50% mortality at the age of 5-6 months, is dependent on a recessive autosomal gene, lpr, for lymphoproliferation (Dixon *et al*, 1980).

In human SLE there does seem to be familial aggregation of serological abnormalities, as well as clinical disease. Thus, Lowenstein and Rothfield (1977) found that consanguineous household and nonhousehold contact relatives of SLE patients had significantly higher mean titres of antinuclear antibodies than spouses or controls. Block *et al* (1975) showed that the concordance for SLE in monozygous twins was approximately 60% (4/7) and the concordance for ANA and hypergammaglobulinaemia was 71% and 87% respectively, in monozygous twins. Furthermore, the concordance

for SLE in dizygous twins was not raised relative to that of first degree relatives, lending further support to the contribution of heredity.

Early HLA studies in SLE suggested an association with HLA-B8 (Grumet *et al*, 1971) but later several authors have reported a significant increase of HLA-DR3 in SLE (Celada *et al*, 1980; Scherak *et al*, 1980). Furthermore, Reinertsen *et al* (1978) and Gibofsky *et al* (1978) found an association between SLE and both DR2 and DR3. This deserves a few comments. First, HLA-B8 is known to be in linkage disequilibrium with HLA-DR3, thus lending support to the early observation of Grumet and his coworkers (Celada *et al*, 1980). Furthermore, inherited deficiency of various complement components is known to predispose to SLE-like disease, and in one study 5.9% (8/137) of SLE patients had heterozygous C2 deficiency (Glass *et al*, 1976), but the C2 deficiency gene occurs preferentially in association with Dw2/DR2 (Svejgaard *et al*, 1980) and that may contribute to the association between SLE and DR2.

In summary, the definite association of HLA-DR4 with seropositive RA lends strong support to the observations of Lawrence and others that heredity plays a rôle in the aetiopathogenesis of seropositive RA. The HLA findings would also support the view that seronegative and seropositive RA are indeed aetiologically separate (Winchester, 1981). Furthermore, the DR4 association clearly separates seropositive RA from SLE and other DR3-related "autoimmune" diseases like sicca syndrome, idiopathic Addison's disease and Grave's disease (Svejgaard *et al*, 1980). Also the observation that treating DR3/DR2 positive RA patients with remission inducing drugs tends to cause reactions like

proteinuria, rash or neutropenia is of particular interest in view of the well known association of these features with DR3-related autoimmune syndromes.

I.3. RHEUMATOID FACTORS

Rheumatoid factors have been defined as "antibodies specific to antigenic determinants on the Fc fragments of human or animal immunoglobulin G" (Mannik, 1979). These antibodies belong to the family of antiglobulins which are antibodies to antigenic determinants on immunoglobulins. Antiglobulins to IgM, IgA, IgE and IgD have been reported to occur (Mannik, 1979; Pope *et al*, 1982) but anti-IgG antibodies have been studied much more thoroughly. Apart from RFs these include "pepsin agglutinators" or antibodies to determinants on $F(ab')_2$ of IgG which are revealed by pepsin digestion of the whole molecule (Carrico *et al*, 1974; Mellbye and Natvig, 1971). Autoantibodies to the Fab fragment of IgG have also been reported to occur (Waller *et al*, 1969).

In 1922 Meyer reported that the sera from some individuals, affected for example, by cirrhosis and chronic bronchitis, contained a heat-stable factor which could agglutinate red cells from sheep and guinea pigs but only if these had first been sensitised with antibody (Meyer, 1922). Later, Waaler (1940) found this "agglutination activating factor" in 30% (23/77) of sera from RA patients but in only 1% (2/202) of controls. Rose *et al* (1948) later reported similar association of the agglutination factor with RA, and laid the basis for the differential agglutination test. The factor was named rheumatoid factor because of the strong association with RA, and its high prevalence in patients with rheumatoid nodules was noticed early (Jacobson *et al*, 1956). Further it was found that the RF responsible for the agglutination reactions was mainly a macroglobulin (IgM), having a corrected sedimentation coefficient of 19S (Kunkel *et al*, 1959). Later, however, Kunkel

et al (1961) reported the occurrence of "intermediate components" in several rheumatoid sera, with sedimentation coefficients ranging from 9-17S. Kunkel *et al* suggested that the active portion of these complexes represented the 7S counterpart of the 19S group of rheumatoid factors and Schrohenloher (1966) later demonstrated anti-IgG activity in pepsin digests of the intermediate complexes, thus supporting the hypothesis that the intermediate complexes consisted of self-associated IgG RF or complexed IgG-IgG RF.

In 1966 Heimer and Levin reported the presence of IgA RF in some rheumatoid sera and also in the saliva of one RA patient. More recently several workers have reported the occurrence of IgE RF in RA (Zuraw *et al*, 1981). Thus, the RFs exist in all the major classes of immunoglobulins and it is therefore likely that their production is to some extent T-cell dependent (Marchalonis, 1978).

The specificity of rheumatoid factors has been the subject of many studies, which have shown that RFs represent a heterologous mixture of antibodies which may show primary specificity for rabbit gammaglobulin, human gammaglobulin, or both human and rabbit gammaglobulins (Williams and Kunkel, 1963). IgM RFs have been most thoroughly investigated in this respect and these have been reported to react to a greater or lesser extent with IgG from a wide range of animal species, including baboon, pig, horse and sheep (Johnson and Faulk, 1976; Hannestad *et al*, 1981). Further, Torrigiani and Roitt (1975) showed that the substrate specificity of IgG RF may depend on the patient's condition, as IgG antiglobulins from seronegative RA patients and patients with Sjoegren's syndrome reacted preferentially with insoluble horse gammaglobulin, whereas IgG antiglobulins from

seropositive RA patients reacted comparably with rabbit and horse gammaglobulin.

In addition to preferential reactivity with IgG from different species, RFs also react with various determinants on the human Fc-gamma. Natvig *et al* (1972) provided evidence by haemagglutination inhibition techniques, where pepsin and papain digestion products were used as inhibitors, that RFs probably interact with four separate determinants, one on the C_H3- and three on the C_H2-domain of human IgG. Some of these determinants may be close to or identical with the allotypic determinants of IgG. Thus, RFs with specificities for the antigens Gm(a) (on IgG1), Gm(b^1) (on IgG3), and Gm(g) (on IgG3) have been described (Natvig *et al*, 1972). However, it has also been found that native or heat-aggregated IgG lacking the specific a, b^1 and g allotypic determinants may react with RFs of the anti-Gm(a), -Gm(b^1) and -Gm(g) varieties respectively (Gaarder and Natvig, 1974). This supports the suggestion that the anti-Gm activity of RFs may result from close proximity of the RF antigenic site to the Gm site (Williams and Kunkel, 1965). Some nonallotypic determinants have also been implicated as likely RF-substrates, the best known of these is the Ga antigen, first described by Allen and Kunkel (1966) in relation to an RA patient (Ga) whom they had studied. This antigen, which is found on the C_H2-domain of IgG 1, IgG 2, and IgG 4, constitutes a frequent antigenic determinant for IgM RF (Gaarder and Natvig, 1970) and the report of Abraham *et al* (1972) that a monoclonal IgA RF reacted with IgG 1, IgG 2 and IgG 4 would also support the rôle of the Ga antigen as a general RF substrate.

Apart from reacting with the Fc portion of IgG, some RFs also display reactivity with nuclear antigens. Hannestad and Johannessen

(1976) passed the pepsin digested gammaglobulin fraction of an RA patient serum through a Sepharose-human IgG column and subsequently eluted an immunoglobulin fraction with considerable RF and anti-nuclear factor (ANF) activity. As this eluted fraction was only 0.1% of the total applied to the column, and did not contain any detectable Fc-gamma determinants, it was concluded that the ANF activity could neither be attributed to nonspecific adherence of ANF to the column nor to the binding of ANF by RF (Hannestad and Johannessen, 1976). It was later reported that approximately 40% of sera from 62 cases of seropositive classical RA and some sera from patients with SLE and mixed cryoglobulinaemia display this cross-reactivity of RF with cell nuclei (Hannestad *et al*, 1981). Furthermore, there is evidence that the ANF activity of these cross-reactive RFs is directed against determinants present on nucleosomes consisting of DNA and inner histones (Hannestad *et al*, 1981). This might imply that not only IgG but also nuclear antigens stimulate production of the antibody which is collectively called RF (Aitcheson *et al*, 1980).

The interactions of RFs with IgG have been investigated in considerable depth. As IgM RF shows enhanced precipitating reactivity with aggregated or denatured IgG, it has been postulated that aggregation of IgG might lead to the unmasking of specific RF-reactive groups (Henney and Stanworth, 1965). Moreover, the failure of native IgG to inhibit the agglutination by RF of erythrocytes coated by aggregated IgG suggested preferential reactivity of RF with aggregated IgG (Normansell, 1971). However, it has been shown that reduced and alkylated RF-subunits (sedimentation coefficient 7S) react equally strongly with native and heat-aggregated IgG, with an equilibrium constant of 4×10^5 l/mole (Normansell, 1971). Also, Eisenberg (1976)

showed that whereas intact IgM RF bound 1.2×10^6 more effectively than Fabμ of RF (on a molar basis) to aggregated IgG, the Fabμ bound equally strongly to monomeric and aggregated human IgG. It seems likely, therefore, that the apparent specificity of RF for aggregated IgG reflects polyvalent interactions rather than new antigenic specificities revealed on the IgG aggregates (Eisenberg, 1976).

IgG RF is in the unusual position of having antibody activity against itself. Indeed, there is evidence that the "intermediate complexes" found in RA sera, sedimenting between 7S and 19S, mainly consist of self-associated IgG RF (IgG-IgG complexes) (Schrohenloher, 1966). Pope *et al* (1974) showed that the equilibrium constant for the association of $F(ab')_2$ of IgG RF with monomeric IgG was about 1.5×10^5 l/mole, which is similar to the value obtained for IgM RF by Normansell (see above) and indicates low affinity (Soothill and Steward, 1971). However, the calculated association constant for the formation of a cyclic dimer of IgG RF-IgG RF is an high as 10^{10} (Pope *et al*, 1974). Recently it has been demonstrated that the antigenic valency of intact normal IgG for IgG RF is only one (Nardella *et al*, 1981). Thus, the binding of native IgG to IgG RF complexes could effectively terminate further polymerisation (see Figure I.2). This again supports the hypothesis that the lower the "ambient" concentration of native IgG the larger the self-associated IgG RF complexes, as evidenced, for example, by the finding that gammaglobulin complexes in synovial effusions tend to be larger than the corresponding serum complexes (Winchester *et al*, 1970). This again may be of pathogenetic significance as large gammaglobulin complexes fix complement much more readily than small ones (Agnello *et al*, 1970).

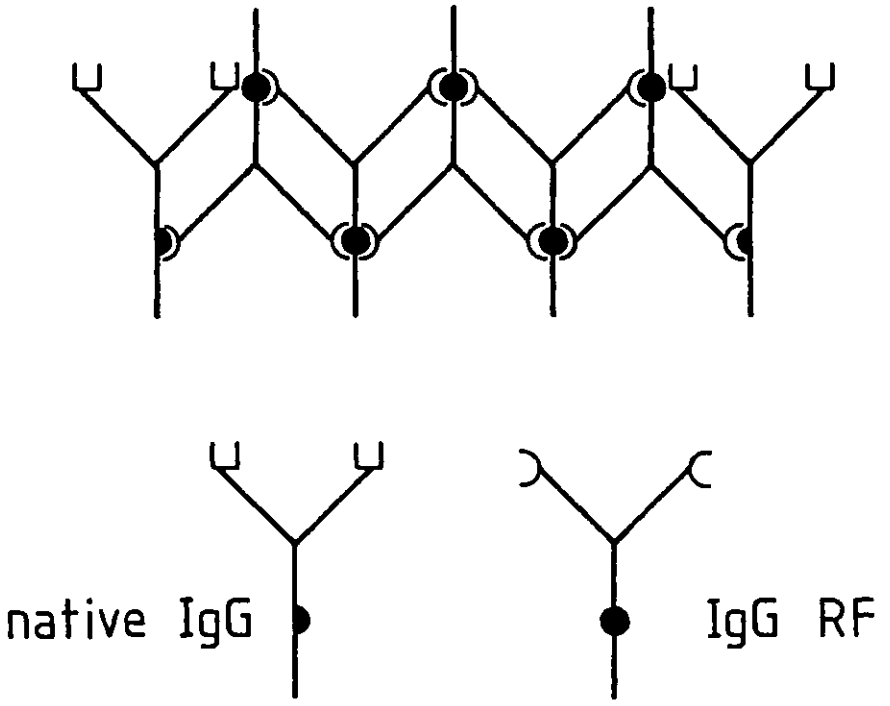


Figure I.2: The self-association of IgG RF, giving rise to stable complexes, the enlargement of which is terminated by native IgG.

While the Fc portion of IgG RF (bottom right hand side) is bivalent for the binding of IgG RF, the valency of native IgG (bottom left hand side) is apparently only one for the association with IgG RF.

Raised levels of RFs as measured by standard agglutination tests are said to occur in the sera of over 75% of RA patients (Fye *et al*, 1978) but RFs are by no means confined to RA. Apart from their frequent occurrence in rheumatic diseases like Sjoegren's syndrome and SLE they are also found in various chronic diseases like pulmonary fibrosis, leprosy, tuberculosis and subacute bacterial endocarditis (SBE), as well as asymptomatic elderly individuals (Fye *et al*, 1978; Heimer *et al*, 1963). However, if the underlying disease (e.g., infections) can be cured, the RF levels tend to diminish or disappear (Carson *et al*, 1978). It has been demonstrated that SBE patients generally develop circulating immune complexes early on in their disease, with appearance of IgM RF and IgG RF normally occurring some nine days later (Carson *et al*, 1978). This might suggest that the biological rôle of RFs is to aid in recovery from some infectious diseases, as IgM RF bound to immune complexes may hasten their elimination (Carson *et al*, 1981). It is not clear, however, why the RF production sometimes seems to become autonomous, as in RA and old age. Genetic factors may be involved: it has been shown that the MRL/l strain of mice is highly prone to develop RF-like antiglobulins, especially of the IgG class, as well as a chronic destructive synovitis of the hind limb joints (Eisenberg *et al*, 1979). In humans, however, the importance of heredity is controversial despite several reports suggesting familial aggregation of RF reactive against human and/or rabbit IgG (Ziff *et al*, 1958; Lawrence *et al*, 1970).

The traditional tests for RF are based on the agglutination of particles coated by IgG. Thus, the latex fixation test (Singer and Plotz, 1956) uses latex particles coated with human Cohn fraction II and the Rose Waaler test uses sheep erythrocytes sensitised with

rabbit anti-sheep erythrocyte antibody (Rose *et al*, 1948). As IgM RF has an average valence of five in the interaction with IgG (Mannik, 1979) but IgG and IgA RFs only a valence of two (Nardella *et al*, 1981; Abraham *et al*, 1972), IgM RF is obviously far more effective in cross-linking and agglutinating particles than the other two RF classes. Thus, agglutination tests will primarily detect IgM RF, and different approaches must be made for detection of IgG and IgA RFs.

The most widely used alternative is that of solid-phase immuno-adsorbents, where human or animal IgG is solidified, either by cross-linking (Torrigiani and Roitt, 1967), by covalent linking to an insoluble matrix like Sepharose (Hannestad and Johannessen, 1976) or by non-covalent adsorption to polystyrene surfaces (Hay *et al*, 1975). This is then followed by incubation with the test sample, when any RF with free antigen-binding sites should bind to the IgG. Where the IgG substrate is covalently bound, the RF can then be eluted by acid buffer, urea or potassium thiocyanate and assayed in an agglutination or precipitation system. In the case of polystyrene surfaces, the RF-incubation is followed by incubation with radio- or enzyme-labelled class-specific anti-human antiglobulin (Hay *et al*, 1975; Gripenberg *et al*, 1979).

All the solid-phase techniques have their limitations and disadvantages, however. The cross-linked and covalently bound IgG is liable to bind IgG nonspecifically which may then be eluted and give rise to false positive reactions (Nardella and Mannik, 1978). These immunosorbents are also rather cumbersome and poorly suited for large-scale testing of samples.

In the polystyrene solid-phase assays, IgM RF may, theoretically, give rise to false positive reactions in tests for IgG and IgA RFs. The pentavalency of IgM RF means that it may bind one or more molecules of native IgG and still have free antigen binding sites, through which it can attach to the IgG on the solid phase. There is indeed evidence that some of the IgG RF in serum may circulate as IgM RF-IgG, having a sedimentation coefficient of 22S (Mannik, 1979). This native IgG, being linked to the solid phase through IgM RF, might then be falsely recognised as IgG RF by the labelled antibody used for the detection of IgG RF (see Figure I.3.A.). A second possible cause of false positive reactions might be the binding by IgM RF on the solid phase of the Fc portion of the labelled antibody used for the detection of IgG RF or IgA RF (see Figure I.3.B.). However, the practical importance of these possible interactions has been debated (Nineham *et al*, 1976; Carson *et al*, 1977). Furthermore, as much of the IgG RF in serum and synovial fluid is probably in the form of self-associated or "hidden" IgG RF complexes (Winchester *et al*, 1970; Pope *et al*, 1974) it is uncertain to what extent IgG RF can be measured without first dissociating these complexes (Hay *et al*, 1976b). Further clarification of these methodological questions is clearly needed in order that the significance of IgG RF and IgA RF can be more precisely defined. These considerations will be elaborated further in chapters III and V of this thesis.

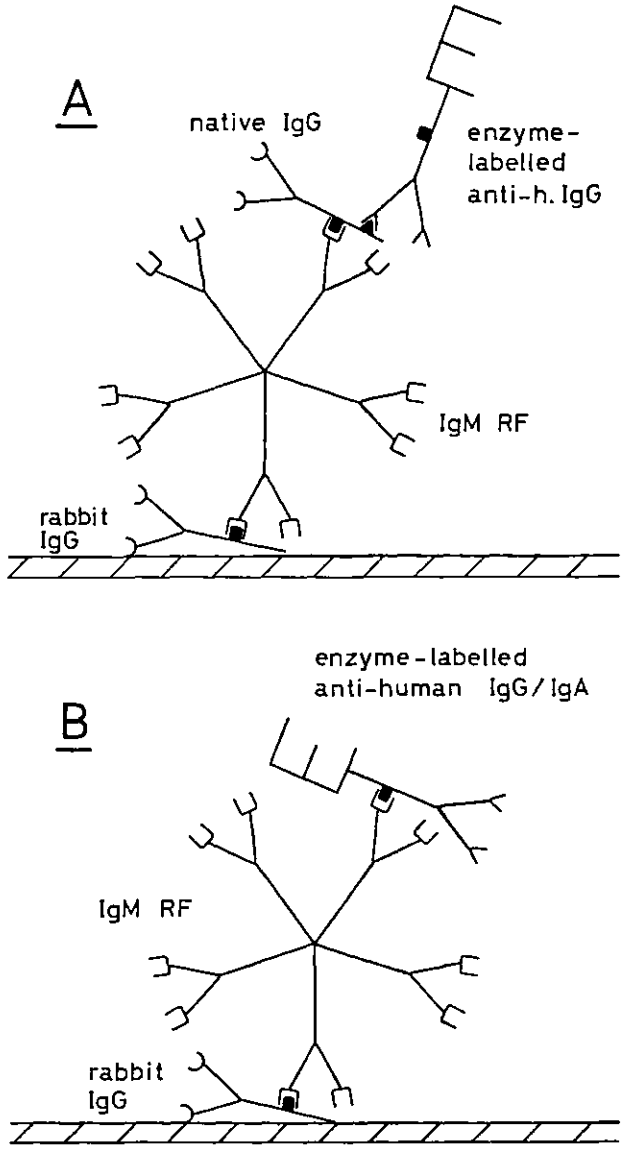


Figure I.3: IgM RF may cause false positive results in ELISA for IgG RF and IgA RF.

A: IgM RF, complexed with native serum IgG, binds to rabbit IgG on the solid phase. Subsequently, enzyme-labelled anti-human IgG recognizes the native IgG, yielding a false positive IgG RF test.

B: IgM RF, bound to rabbit IgG on the solid phase, recognizes Fc determinants of an enzyme-labelled antibody (directed e.g. against human IgG or IgA), causing a false positive test, e.g. for IgG RF or IgA RF.

I.4. THE IMMUNOPATHOLOGY OF RHEUMATOID ARTHRITIS

From the clinical point of view, RA is primarily a disease of joints, and there is firm evidence that in RA the joint is a site of considerably heightened immunological reactivity (Roitt *et al*, 1982). Accordingly, this review will mainly focus on the immunopathology of the joint, mentioning only briefly the extra-articular apsects of RA.

The normal synovial membrane that lines diarthrodial joints has several structural characteristics. Facing the joint cavity is a layer of lining cells of synoviocytes, normally rather loosely arranged in a bed of intercellular ground substance. Two cell types predominate in the lining cell layer, type A (macrophage-like) cells, and type B (fibroblast-like) cells. In addition, there are type C (intermediate-type) cells, showing both macrophage- and fibroblast-like features. Under the cellular lining there is a layer of loose connective tissue with an abundant capillary network. Normal synovial membrane contains only a few lymphoid cells, and its surface is relatively smooth and unfolded (Zvaifler, 1973).

The earliest synovial changes in RA include perivascular infiltration of neutrophil polymorphs and lymphocytes, together with perivascular oedema and increased gaps between the lining cells. The early exudate in the joint cavity is rich in mononuclear cells.

Later, the synovium becomes thickened by large infiltrates of lymphocytes and plasma cells, the latter often forming follicles, especially around small blood vessels, with occasional germinal centres, resembling those in active lymph nodes, which may indicate the presence of a strong and persistent antigenic stimulus in the synovium (Glynn,

1978). The synovial lining becomes multilayered in established RA, reaching a depth of 10-20 cells (Zvaifler, 1979a) with increasing prominence of macrophage-like cells (van Furth, 1981). The hypertrophied synovium forms villi and folds which protrude into the joint cavity, and now the predominant synovial exudate cell is the neutrophil polymorph.

Later on, the synovial picture is dominated by pannus formation: vascular granulation tissue composed of proliferating fibroblasts, small blood vessels, variable numbers of inflammatory cells and some collagen fibres. The pannus extends out from the synovium, encroaching upon the edges of the articular cartilage, and the general view has been that the pannus is responsible for the observed cartilage destruction (Zvaifler, 1973).

Although the synovial exudate protein levels in active RA tend to reflect those of the corresponding plasma, the hypertrophied synovium shows various features of an independent immunoglobulin-producing organ. In one study, ^{125}I-IgG was injected i.v. and it was found that the subsequent ratio of ^{125}I-IgG/IgG in synovial effusions was lower than expected, suggesting that 12-24% of the synovial effusion IgG was derived from local production. No evidence for such local production was found in patients with Reiter's disease or osteoarthritis. The authors estimated that as much as 95 mg IgG might be produced daily in an active rheumatoid knee (Zvaifler, 1971). Herman *et al* (1971) showed that synovial membrane lymphocytes from RA patients, recently boosted with tetanus toxoid, produced significantly more IgG *in vitro* than peripheral blood lymphocytes (PBL). However, only a negligible amount of the synovial cell immunoglobulin was

anti-toxoid, whereas almost 50% of the PBL immunoglobulin had such activity.

By immunofluorescence, IgG, IgM, complement components (C3, C4) and fibrin can be demonstrated in the rheumatoid synovium, both in lining cells, vessel walls and the interstitium. Even in early lesions IgG tends to predominate, and is often found in association with complement (Zvaifler, 1973). Furthermore, intracellular IgG can be demonstrated in a large proportion of rheumatoid synovial plasma cells, and Munthe and Natvig (1972a) demonstrated that 30-60% of all RA - synovial plasma cells contained IgG RF, possibly as intracellular complexes, as the antiglobulin activity of these cells could generally only be demonstrated after pepsin digestion of tissue sections. These intracellular IgG RF complexes were particularly prominent in synovia from seronegative patients (Munthe and Natvig, 1972a) and it has been suggested that the intracellular neutralisation of antibody may circumvent the normal homeostatic mechanisms controlling antibody production (Roitt *et al*, 1982).

In 1967 Hannestad reported that certain rheumatoid synovial effusions contained altered IgG, which formed precipitates upon the addition of an RF+ serum (Hannestad, 1967). Subsequently, many workers have demonstrated the presence of immune complexes (ICs) in RA synovial effusions, and Winchester *et al* (1970) noted that these complexes seem to consist mostly of IgG, some of which has anti-IgG activity. The complexes range in size from 7S to over 19S, as determined by analytical ultracentrifugation. The authors noted that the intermediate sized complexes (7S-19S) tended to be larger than

those from the corresponding sera, and the heavier complexes were easily precipitated with isolated C_{1q} or IgM RF. Furthermore, there was an inverse relation between the presence of ICs and the levels of C3 and C_{1q} in the synovial effusions. Later, Munthe and Natvig (1972b) provided evidence that intracellular synovial IgG RF complexes can fix fresh serum complement. However, a certain minimal complex size seems to be required for effective C-fixation and phagocyte clearance of IgG-ICs, as Mannik and Arend (1971) showed that albumin-IgG complexes will only fix complement effectively (*in vitro*) if they exceed a lattice size of Ag_1Ab_2 or Ag_2Ab_2. Thus, IgG RF complexes which in the presence of excess native IgG (in serum) will theoretically not exceed the Ag_2Ab_2 size (Nardella *et al*, 1981) should not fix complement readily. That would, in itself, agree with the report of Halla *et al* (1978) where no C_{1q}-binding activity was observed in RA-sera even though such activity was present in 71% (34/49) of the corresponding synovial fluids, containing presumably larger-size IgG RF complexes.

The presence and binding of IgM RF is also thought to enhance C-fixation and phagocytosis of synovial fluid IgG-ICs as Hurd *et al* (1971) showed that neutrophils would only develop inclusions in IgM RF-negative synovial effusions if purified IgM RF was added. The same authors, however, demonstrated intracellular IgG and C3 in synovial lining phagocytes in seronegative RA, thus, the presence of IgM RF is not an absolute requirement for the C-fixation and removal of synovial fluid IgG-ICs.

Considerable effort has been expended on looking for specific antibodies in synovial fluid complexes, and the presence of antibodies against $F(ab')_2$-gamma (pepsin agglutinators), viral components and type I and II collagen have been reported (Mellbye and Natvig, 1971;

Cremer *et al*, 1974; Andriopoulos *et al*, 1976). Male *et al*, (1980), however, analysed synovial fluid immune complexes from seropositive RA patients and found, in addition to IgG and IgM, some C_{1q} and moderate amounts of activated C4 and C3 but only trace amounts of DNA, factor B or other proteins. The authors concluded that their data were consistent with IgG being at least the main antigen involved in the pathogenesis of RA (Male *et al*, 1980) and suggest a predominant rôle for the classical complement pathway in the pathogenesis of RA. Others, however, have found depressed levels of factor B and properdin in synovial fluids of seropositive RA (Ruddy *et al*, 1975). This might suggest activation of the alternative complement pathway, possibly by IgA RF, as IgA is known to be an alternative pathway activator (Cooper, 1978).

The uptake of ICs by synovial fluid phagocytes is followed in turn by the release of lysosomal enzymes, many of which have been identified in synovial effusions. These include collagenase, cathepsins and various proteases, all of which are potentially important mediators of joint inflammation and cartilage destruction (Zvaifler, 1979a). The cartilage breakdown may then in some cases of RA lead to autosensitisation to type II collagen, as antibodies to this collagen type are now known to exist in a low percentage of RA patients (Beard *et al*, 1980).

Various lymphokines, the products of sensitised lymphocytes, are regularly present in RA-synovial effusions (Zvaifler, 1979a). This is not surprising, in view of the heavy mononuclear infiltration in RA, and there is also evidence that activated cells in the rheumatoid pannus are involved in the local production and activation of collagenase which in turn may participate in the destruction of the adjacent cartilage

(Werb *et al*, 1977). Furthermore, several reports of dramatic clinical improvement in RA following prolonged thoracic duct drainage (Wegelius *et al*, 1970; Akatsu *et al*, 1981) would lend further support to the importance of cellular immunity in the pathogenesis of RA.

In conclusion, it is fair to say that we do not yet know what triggers off the reaction that eventually leads to autosensitisation to IgG and the inflammatory processes so characteristic of classical RA. As outlined in I.2. above, there is now strong evidence for the importance of heredity in the aetiopathogenesis of seropositive RA and - as outlined in I.3. and I.4. above - the central rôle of the rheumatoid factors, and possibly other antiglobulins, in the pathogenesis of RA seems quite convincing.

The main objectives of the study described in this thesis were:

Firstly to establish satisfactory assays for the detection of the various types of rheumatoid factors.

Secondly to apply these assays in a prospective survey of early rheumatoid joint disease.

Thirdly to study the relationship between genetic and immunological factors in a large inbred family with high incidence of rheumatic diseases.

C H A P T E R I I

GENERAL ELISA METHODOLOGY AND TECHNICAL VARIABLES

C H A P T E R I I

GENERAL ELISA METHODOLOGY AND TECHNICAL VARIABLES

II.1. INTRODUCTION

II.1.1. Selecting a method for RF tests

From 1963-1979 several methods were reported, purporting to be measures of RFs of the different immunoglobulin classes. These methods can be categorised broadly as follows:

(a) Fractionation of samples by ultracentrifugation or Sephadex/DEAE chromatography, followed by latex testing of the fractions for RF activity (Chodirker and Tomasi, 1963; Schrohenloher, 1966; Norberg, 1976).

(b) Adsorption of RFs onto polymerized rabbit/human IgG, followed by elution in acid and quantification of the eluate by immunodiffusion methods using antisera against human IgA, IgG or IgM (Heimer and Levin, 1966; Torrigiani and Roitt, 1967).

(c) Selective removal of IgM RF from test samples by pepsin digestion or incubation with Con A, followed by reversed radial immunodiffusion, haemagglutination tests or latex test (Theofilopoulos *et al*, 1974; Tanimoto *et al*, 1976).

(d) Immunofluorescence tests, using insolubilised sensitised red cells, followed by test sample and labelled class-specific anti-human immunoglobulin (Ig) (Estes *et al*, 1973; Kallerup *et al*, 1979).

(e) Solid phase immunosorbent assays where rabbit/human IgG (or Fc-fragments thereof) is adsorbed onto plastic surface, followed by test sample and radio- or enzyme-labelled class- or subclass-specific anti-human Ig antibody (Hay *et al*, 1975; Nineham *et al*, 1976; Carson *et al*, 1977; Shakib and Stanworth, 1978; Willems and Klaassen de Kort, 1978; Dunne *et al*, 1979; Gripenberg *et al*, 1979).

The first aim of the current study was to establish a method of quantifying RFs of IgG, IgA, and IgM classes, which should ideally be sensitive, specific, safe, objective, reproducible and suitable for large-scale testing of samples. Most of the methods quoted above seemed to fall somewhat short of these objectives:

1. Any agglutination method for IgG RF is bound to be rather insensitive as IgG is up to 750 times less efficient an agglutinator than IgM (Greenbury *et al*, 1963).

2. IgG polymers are known to adsorb native IgG non-specifically (Nardella and Mannik, 1978) and this IgG may then cause false positive reactions when it is eluted by acid.

3. Immunodiffusion tests are relatively insensitive, the lower limit of detection being around 3-11 µg/ml for human Ig

classes (Torrigiani and Roitt, 1967).

4. The interpretation of immunofluorescence tests is highly subjective.

5. Rapid decay of many radioisotopes, potential biomedical hazards and expensive counting equipment are major drawbacks to the widespread application of radioimmunoassay.

Taking all this into account, it was decided in early 1979 that enzyme-linked immunosorbent assay (ELISA) might be the most appropriate technique for the current project, and the rest of chapter II will deal with the background and basic methodology of ELISA, with special reference to antiglobulin tests.

II.1.2. ELISA: Historical background

In 1966, Van Oss and Singer showed that immunoglobulin binds quite strongly, yet non-specifically, to polystyrene latex particles and it was postulated that the binding was hydrophobic, of the van der Waals-London type. These authors furthermore showed that the presence of the non-ionic detergent polyoxyethylenesorbitan mono-oleate (Tween 80) decreased the binding drastically, possibly by its hydrating action on the protein (Van Oss and Singer, 1966). A year later, Catt and Tregear (1967) described a solid-phase radioimmunoassay for human placental lactogen and growth hormone where antibody-coated polystyrene/polypropylene tubes were incubated with ^{125}I-labelled hormones and test samples. However, enzymes were then already being used as labels

in immunohistology and the simple coupling method of Avrameas (1969) made enzymes a feasible alternative to radioisotopes.

In 1971, Engvall and Perlmann, and van Weemen and Schuurs, independently described immunoassays where antigen-enzyme conjugates were used, and Engvall and Perlmann coined the term, "enzyme-linked immunosorbent assay (ELISA)". In their original ELISA, Engvall and Perlmann used CNBr-activated microcrystalline cellulose as immunosorbent but they soon adapted the assay to disposable polystyrene tubes (Engvall *et al*, 1971). Later, Voller and Bidwell introduced the use of 96-well disposable polystyrene microtitre plates (microplates) in ELISA, "micro-ELISA" (Voller and Bidwell, 1975). Since then, the popularity of ELISA has increased rapidly, especially within microbiology (Voller *et al*, 1976) and the introduction of semi-automatic spectrophotometers for reading and printing the microplate results (Heimer *et al*, 1981) has contributed substantially to the ease and objectivity of ELISA.

II.1.3. Indirect ELISA for RFs

Several varieties of ELISA techniques have been developed for the detection of antibodies and antigens, one of which is the "indirect method" for measuring antibody (Voller *et al*, 1976). As shown in Figure II.1., the method consists of four successive steps, the first of which is the adsorption of the relevant antigen to the plastic of the microplate-well. After incubation and subsequent washing, dilutions of the antibody-containing test sample are incubated in the microplate, followed by further washing. In the third step, enzyme-labelled, class- or subclass-specific anti-Ig is incubated in the

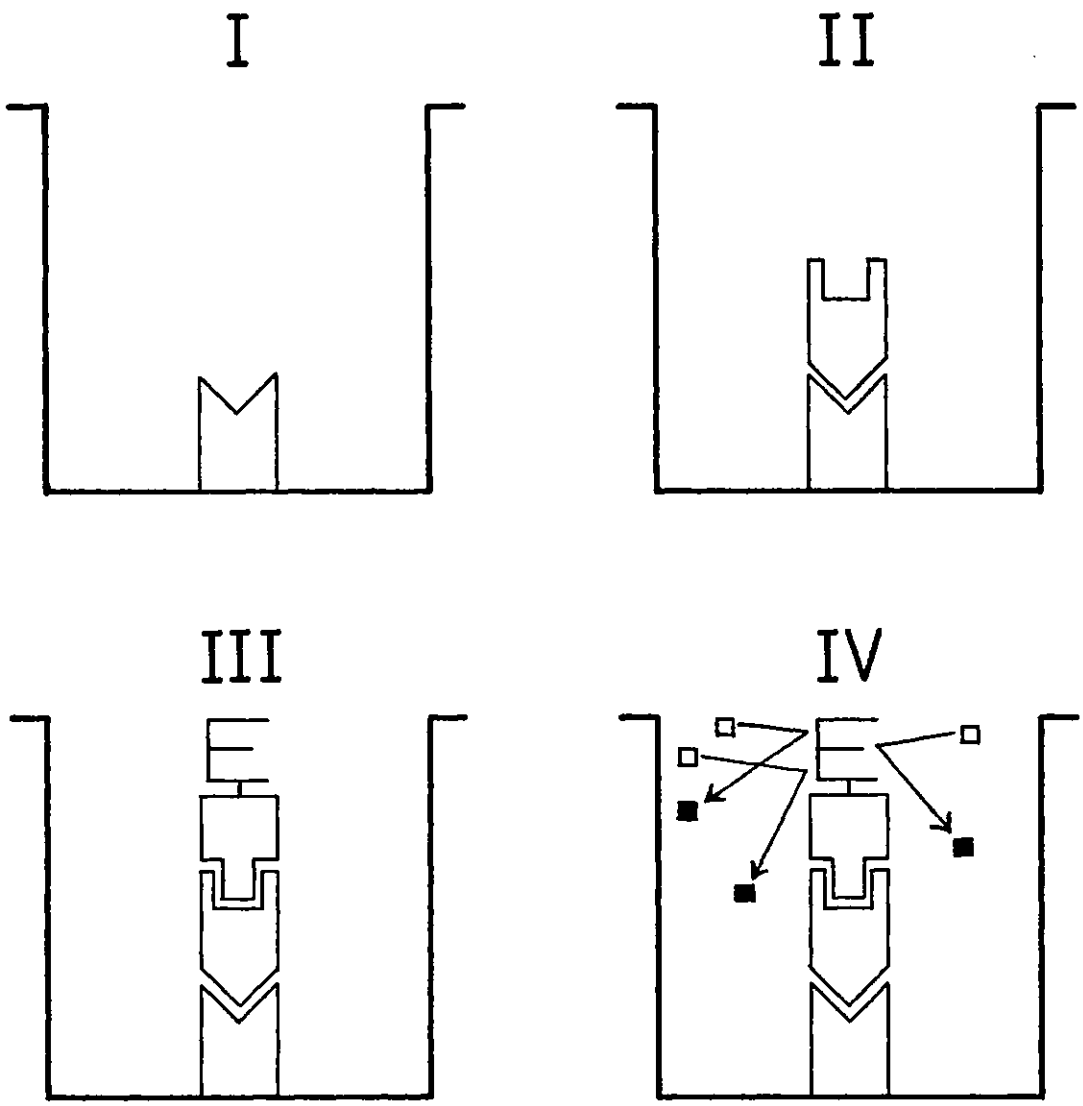


Figure II.1: The basic principles of indirect ELISA:

I. Antigen adsorption to solid phase.
II. Antibody binding to antigen.
III. Addition of enzyme-labelled (E) anti-immunoglobulin.
IV. Conversion of enzyme substrate (open squares) to coloured product (closed squares).

microplate, followed by washing and incubation (step 4) of a solution containing an enzyme substrate, the cleavage product of which can be measured spectrophotometrically.

As the antigen for RFs is readily available (i.e., IgG of human or animal origin) the indirect ELISA would seem a promising technique for the detection of RFs of the various Ig classes. Obviously, its principle is very similar to that of the solid phase radioimmunoassay (SPRIA) developed by Hay *et al* (1975). While radioimmunoassay is highly sensitive, Engvall and Perlmann (1972) showed that ELISA is also very sensitive as they could detect less than 1 ng/ml of specific antibody by indirect ELISA. That is comparable to the sensitivity of the radioimmunoassay reported by Koopman and Schrohenloher (1980) for detection of rheumatoid factors.

II.2. EQUIPMENT, MATERIALS AND REAGENTS

II.2.1. Basic equipment

1. Dispensing pipette. An adjustable 50-200 microl, 12-channel pipette (EFLAB OY, Finland) was used to deliver all reagents into the microplate wells. Filling time per plate was approximately 30 seconds. Generally, 100 microl/well were the standard volume throughout in all the assay steps.

2. Suction pipette. A home-designed 8-channel suction pipette attached to a water-driven vacuum pump, was used to clear the microplates of reagents.

3. Microplate reader. A Titertek Multiskan microplate reader (Flow Laboratories) was used throughout the project to read the colour reaction in the microplates. The Multiskan has 8 detectors, each of which reads one row of the microplate. During routine operation, the detectors first read all the 8 wells in column 1 simultaneously, then the plate is moved one step in order that column 2 can be read, etc. until all the 12 columns have been read and the results printed. This normally takes 60-70 seconds per plate.

II.2.2. Basic materials and reagents

1. Microplate. Flat-well polystyrene microplates were used throughout. Figure II.2. shows the basic features of such a plate which measures 12.8 x 8.6 x 1.4 cm^3 and contains 96 wells, arranged in a pattern of 8 x 12; 8 horizontal 12-well rows (A-H) and

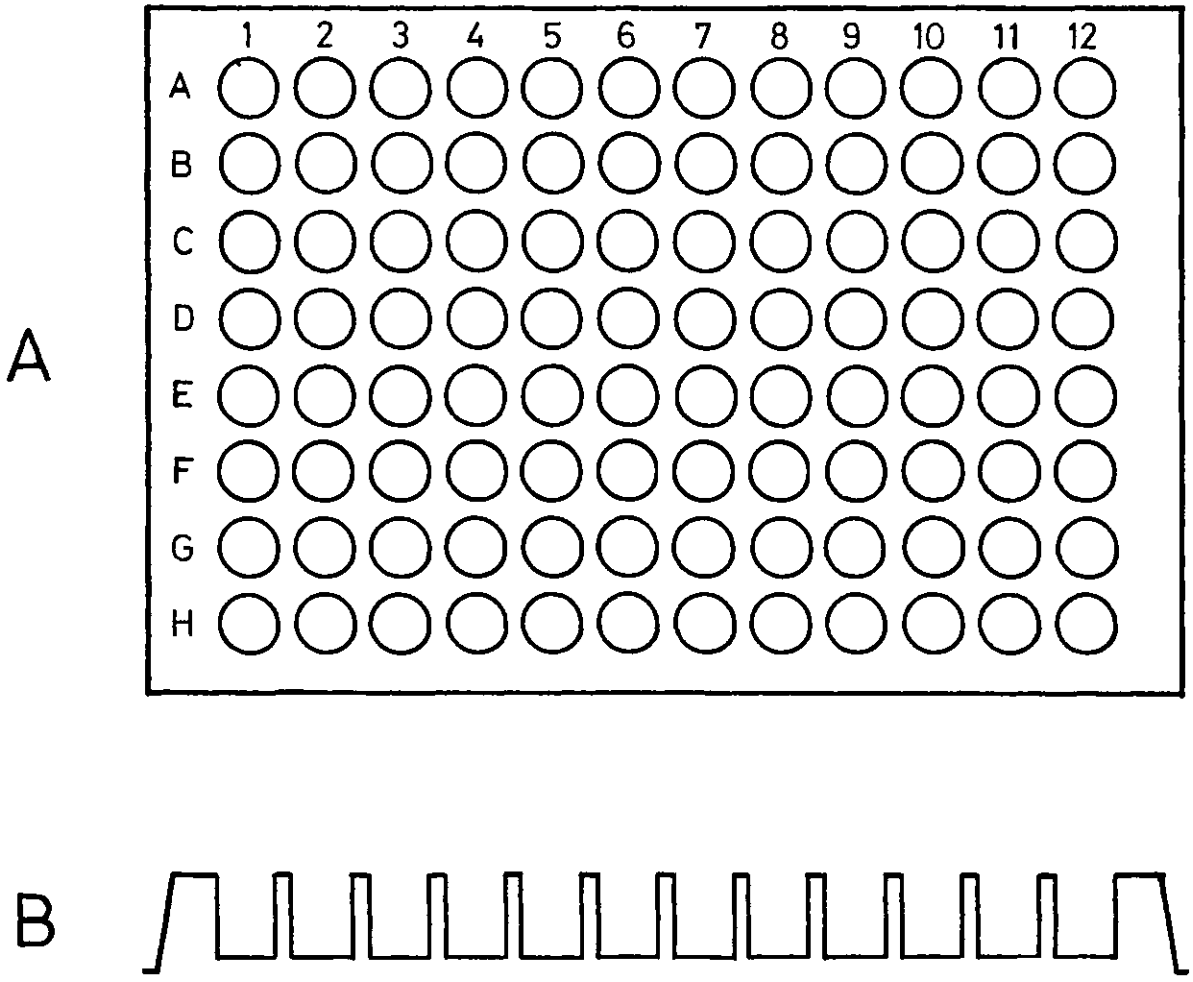


Figure II.2: A standard flat-well microplate.

A: Viewed from above, showing how the 96 wells are arranged in a pattern of 8 horizontal rows (A-H) and 12 vertical columns (1-12).

B: Cross-sectional view, through the wells of one row.

12 vertical 8-well columns (1-12). Maximum capacity of each well is approximately 350 microl, about 75 microl being the minimum needed to cover the bottom of the well.

2. Coating buffer. A 0.05 M carbonate/bicarbonate buffer, pH 9.6, containing 0.02% sodium azide ($Na N_3$) as a preservative, was used to dilute and incubate the immunoglobulins used for coating the microplate wells.

3. PBS-Tween. Phosphate-buffered saline, pH 7.4, containing 0.05% polyoxyethylenesorbitan monolaurate (Tween 20, Sigma, London) and 0.02% of $Na N_3$ was used throughout for washing of the microplates and for dilutions of BSA and antibody-enzyme conjugates.

4. Hypertonic serum diluent. PBS-Tween containing a final concentration of 0.5 M Na Cl and 0.5% Tween 20 was used throughout for the dilution of test sera.

5. Diethanolamine buffer. Diethanolamine (BDH, Poole, U.K.) was diluted to a final concentration of 10% in distilled water and the pH adjusted to 9.8 with hydrochloric acid. This buffer, containing 0.02% $Na N_3$, was used for all enzyme substrate incubations.

6. BSA. Bovine serum albumin (Miles Laboratories Ltd.) dissolved in PBS-Tween to a final concentration of 1% was used to saturate the microplates after the coating steps.

7. Alkaline Phosphatase. Alkaline phosphatase (AP), (Type VII-S, Sigma, London) was used throughout for labelling of the anti-immunoglobulins

applied in the ELISA systems.

8. pNPP. Para-nitrophenyl phosphate (Sigma, London) was used throughout as the enzyme substrate.

II.3. STANDARD METHODOLOGY

The following standard procedures were applied throughout the project, unless otherwise stated:

1. Coating of microplates. All immunoglobulins used for coating the microplates were diluted in the coating buffer, then dispensed (100 microl/well) into the microplates and incubated overnight under cover at 4°C. Generally, only rows B-G were coated, the remaining outer rows serving as blanks.

2. Washing of microplates. After sucking the microplates free of reagents, the plates were washed by filling the wells up with a wash-bottle, containing PBS-Tween, after which the plates were left on the bench for at least 3 minutes. Then the plates were emptied by inversion and two shakings over the sink, blotted x 2 on blotting paper and refilled with PBS-Tween or when washed sufficiently, sucked dry with the suction pipette before the next assay step. Generally the plates were washed x 2 after the coating and BSA saturation steps but x 3 after test sample and enzyme conjugate incubations.

3. BSA saturation. Following the coating step and 2 washes the plates were either sucked dry and stored under cover at 4°C or saturated with BSA for immediate use. A 1% solution of BSA in PBS-Tween (see II.5.3.) was incubated in the plates for 2 hrs. at room temperature (r.t.), followed by 2 washes. Afterwards the plates were generally used on the same or following day.

4. Sample incubation. Sera and synovial fluids were routinely diluted in hypertonic serum diluent and incubated at r.t. for 2 hrs.,

unless otherwise specified, followed by 3 washes, and immediate incubation with the appropriate enzyme conjugate.

5. Enzyme conjugate incubation. The stock solutions of enzyme conjugates were diluted in PBS-Tween to a final concentration which varied from 1/250 to 1/2000, depending on the conjugate. The plates were incubated for 2½ hrs. at r.t., followed by 3 washes and immediate incubation with the enzyme substrate.

6. Enzyme substrate incubation. Freshly made 1 mg/ml solution of pNPP in diethanolamine buffer was incubated in the microplates for periods of up to 60 minutes. Afterwards, the plates were usually read immediately at 405 nm in the Titertek Multiskan, but sometimes the reaction was stopped by the addition of 50 µl of 3 M Na OH per well and the plates kept overnight at 4°C before reading.

II.4. PREPARATION OF ENZYME CONJUGATES

Generally, antibodies to human immunoglobulins were purchased unconjugated and were coupled to alkaline phosphatase (AP) using a modification of the one step glutaraldehyde method of Avrameas (Voller *et al*, 1976).

Briefly, the coupling of AP to immunoglobulins was performed as follows:

Immunoglobulin was mixed with AP at a ratio of approximately 2:5 (w/w) in a small volume (1-2 ml) of PBS and was dialysed overnight against several litres of PBS in order to remove any ammonium sulphate from the AP preparation. The immunoglobulin was then linked to the AP by adding glutaraldehyde to a final concentration of 0.2%, this was allowed to react for 1-2 hrs. at r.t. The excess glutaraldehyde was then removed by dialysis overnight against PBS, following which the conjugate was dialysed overnight against 0.05 M TRIS buffer (pH 8.0). Finally, the conjugate was diluted to approx. 4 ml in the TRIS buffer, and BSA and Na N_3 added to a final concentration of 1% and 0.02% respectively. The conjugates were then stored in the dark (aluminium foil covered) at 4°C.

The anti-human IgG and anti-human IgA conjugates were always quality-tested on microplates coated with increasing dilutions of human IgG and IgA. The results of one such testing are shown in Figure II.3. where two conjugates, one freshly made, the other 14 months old, are compared. Both conjugates were coupled under identical conditions, 1.0 mg of affinity purified goat anti-human IgG, gamma chain specific (TAGO Inc.) being used.

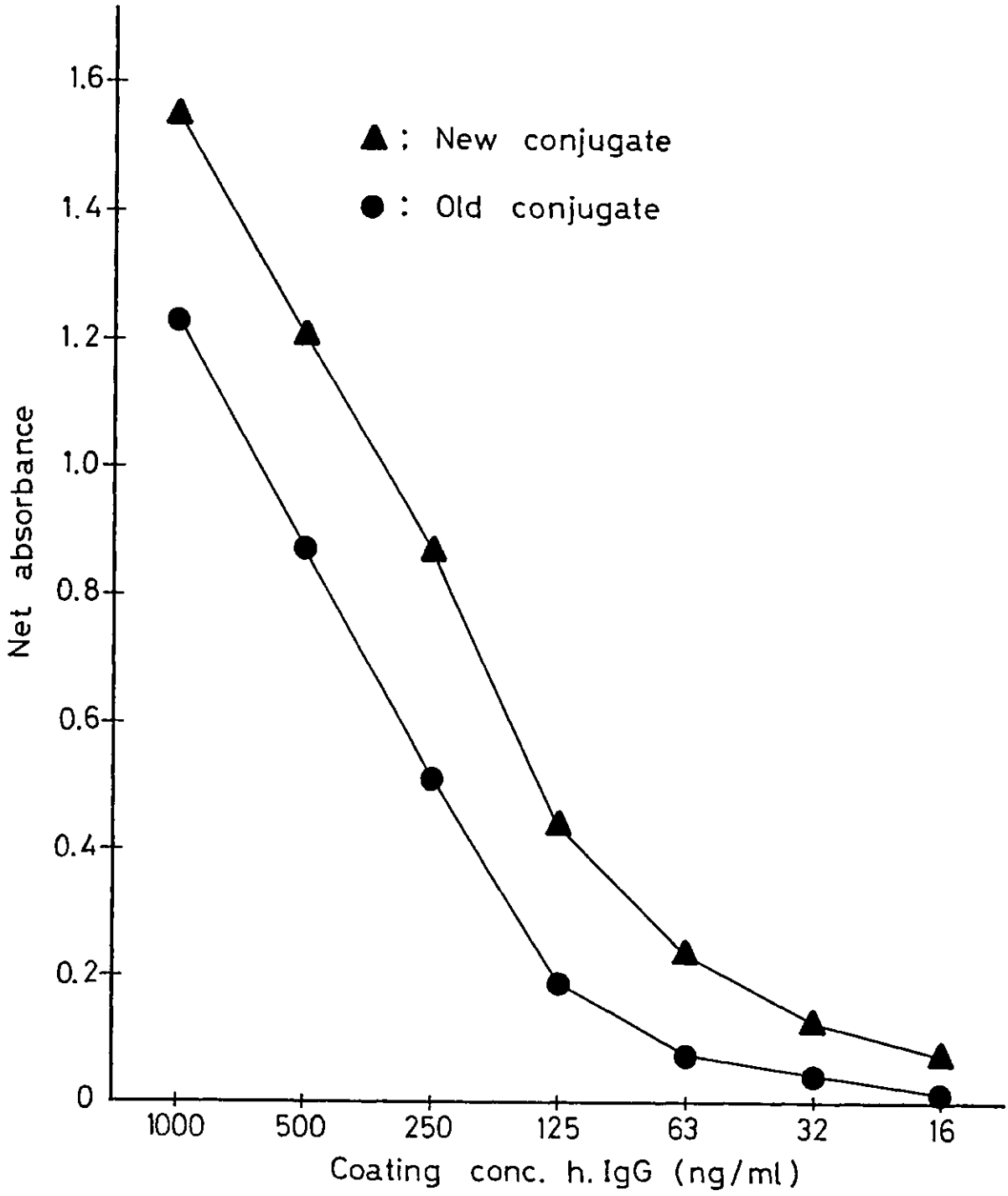


Figure II.3: Comparison between two anti-human IgG-AP conjugates, one freshly made, the other 14 months old.

Conjugate dilutions: 1/500.

The new conjugate detected human IgG adsorbed from the 15 ng/ml coating buffer while the old conjugate had on average 2-4 times less anti-IgG activity.

These results show the high sensitivity of the ELISA system, the lower limit of detection being at or below 15 mg/ml. Also, this indicates the the conjugates retain much of their activity after being kept for over a year at 4°C.

II.5. VARIABLES TESTED

II.5.1. Coating concentrations

The optimal concentration of IgG used for coating in the RF assays was determined separately for each of the three RF isotypes. This subject is dealt with in chapter III in association with the specific RF assays.

II.5.2. Shelf life of coated microplates

In order to test the shelf life of coated plates, half of one plate (rows A-D) (type M29AR) was coated with a 10 µg/ml solution of goat IgG, then washed, sucked dry and stored in a desiccator at 4°C for 7½ months. It was then compared with a freshly-coated M29AR plate from a different batch. After BSA saturation and incubation with doubling dilutions of two sera containing different levels of IgG anti-ruminant IgG (see chapter IV) the plates were incubated with an anti-human IgG-AP conjugate.

The dose-response curves, obtained by subtracting the average values for blank wells from the average values for coated wells, are shown in Figure II.4. The curves are quite similar for both plates, indicating that antigenically intact goat IgG remained on the stored plate throughout the 7½ months storage period. Rather surprisingly, however, the sera reacted more strongly with the old plate. This might possibly be due to batch-to-batch variation which is known to affect the reproducibility of ELISA (Denmark and Chessum, 1978). These results nevertheless show that storage of coated microplates, even for several months, is quite acceptable.

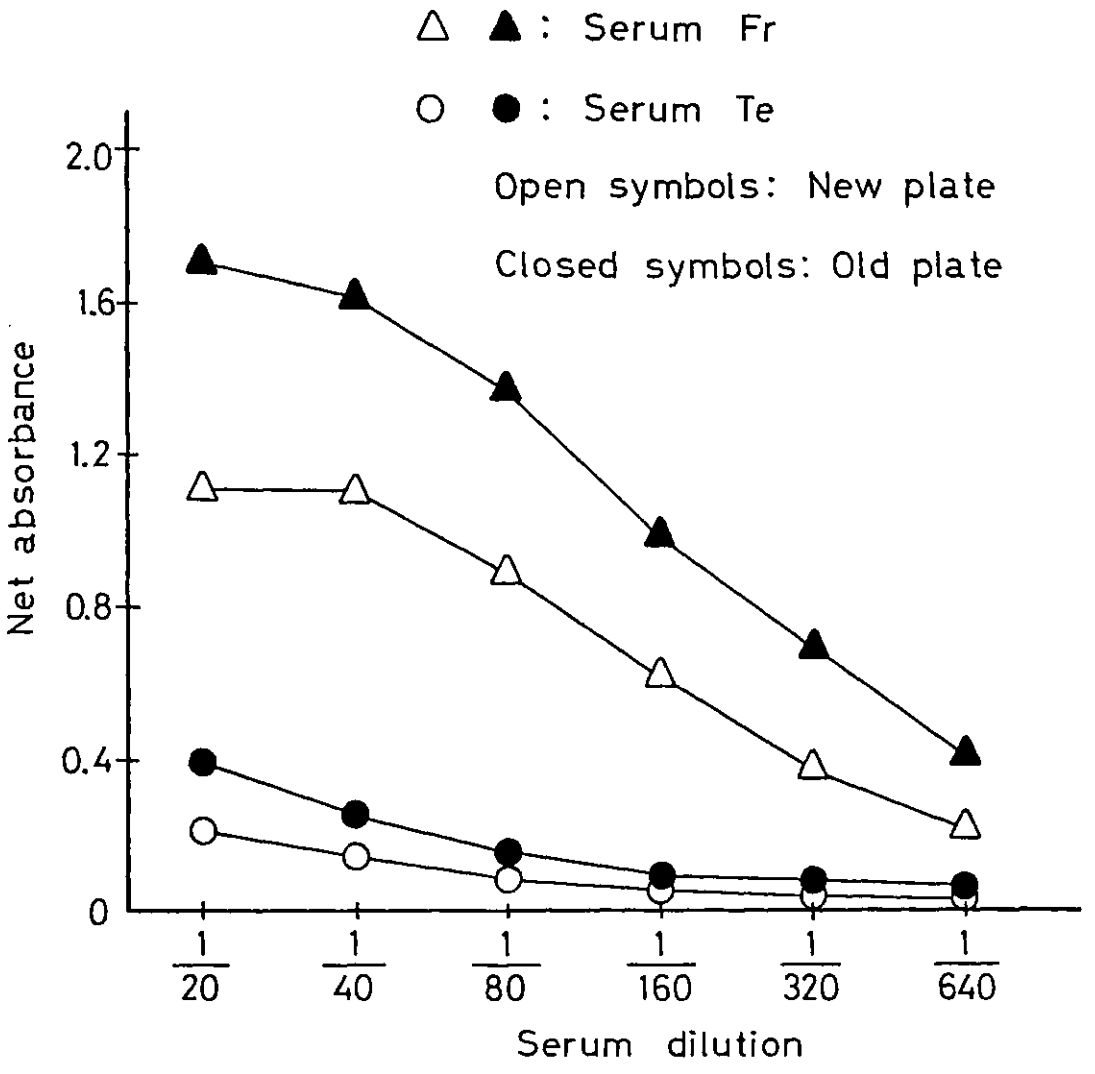


Figure II.4: The shelf life of coated microplates: comparison of a freshly coated plate to one kept in a desiccator at 4°C for 7½ months.

Two sera (Fr and Te) were tested for IgG anti-ruminant IgG levels (chapter IV.2).

II.5.3. Saturation of microplates with BSA

As the optimal concentration of proteins used for coating in ELISA/SPRIA-systems is often fairly low (1-10 µg/ml) (Salonen and Vaheri, 1981; Hay *et al*, 1975), there will generally be some free binding sites left on the solid phase, to which Ig from the test sample or the anti-Ig preparation may subsequently adhere. In order to block such free sites many workers have incubated the solid phase with solutions of either BSA or gelatine after the primary coating (Voller *et al*, 1976; Hay *et al*, 1976a; Carson *et al*, 1977).

In order to investigate the effect of BSA saturation, the following experiments were performed.

(a) The effect of BSA saturation on the binding of antibody-enzyme conjugate:

Two rows of a microplate were coated overnight with a 10 µg/ml solution of rabbit IgG while two adjacent empty rows served as blanks. After washing x 3, 50% of the wells were saturated for 2 hrs. at r.t. with a 1% solution of BSA in PBS-Tween. After a further 3 washings, the plate was incubated for 3 hrs. at r.t. with dilutions in PBS-Tween of goat anti-human IgM antibody-AP conjugate (Miles Laboratories). After washing x 3, the plate was incubated for 60 mins. at r.t. with pNPP in diethanolamine buffer. The reaction was then stopped by the addition of 3 M Na OH and the plate was read at 405 nm.

The gross average absorbance figures are shown in Figure II.5. and while these clearly indicate some cross-reactivity of the conjugate with the rabbit IgG, they also show that the BSA saturation diminishes

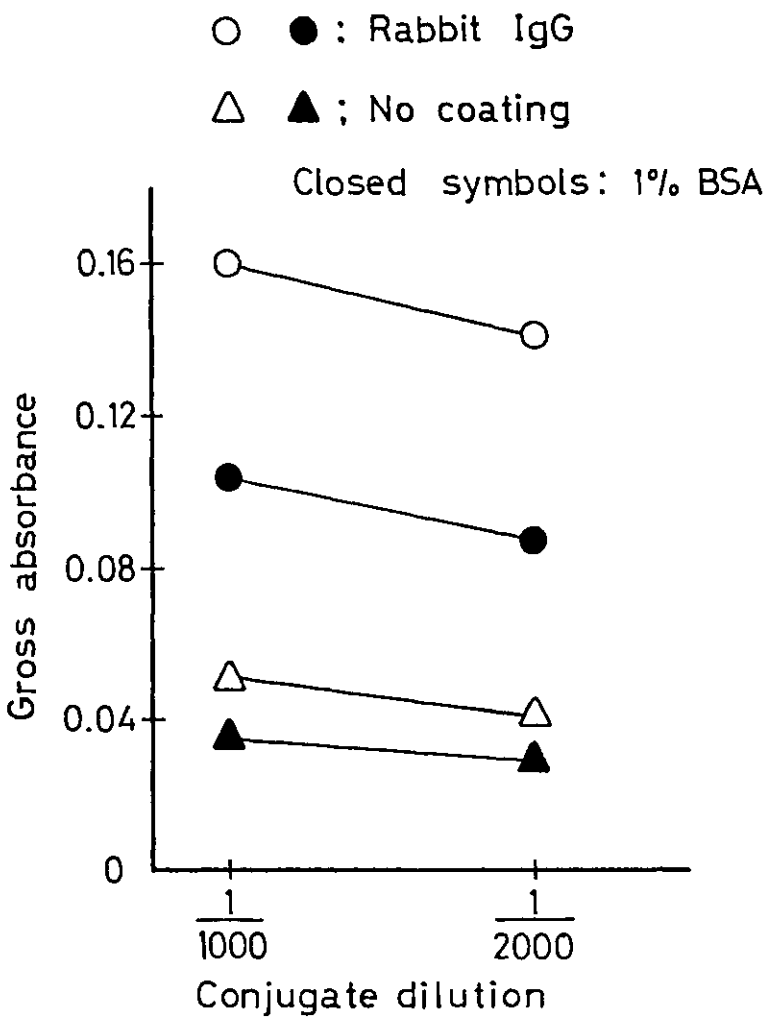


Figure II.5: The inhibition by BSA of conjugate adsorption to microplates.

Two rows of a microplate were pre-coated with rabbit IgG (circles). Half the plate was saturated with 1% BSA (closed symbols), then the whole plate was reacted with anti-human IgM-AP conjugate and pNPP.

Mean BSA inhibition: 33%.

the non-specific adherence of the conjugate to the microplate. On average, the values for the BSA saturated wells were 33% lower than those for the unsaturated wells.

(b) The effect of BSA saturation on the binding of serum Ig:

An uncoated microplate was saturated with BSA as follows:

Rows A-B: 0 BSA; rows C-D: 1% BSA; rows E-F: 2% BSA; rows G-H: 4% BSA. After washing as in (a) above, the rows were incubated with 10-fold dilutions of a serum in PBS-Tween for 2 hrs. at r.t. followed by a 1/1000 dilution of the goat anti-human IgM conjugate and pNPP as in (a) above.

The results, shown in Figure II.6., demonstrated that the absorbance values were on average 42% lower when BSA saturation was used, thus suggesting that BSA saturation prevents adsorption of serum proteins (IgM) to the microplate. However, there was not any marked difference between 1%, 2% and 4% BSA.

II.5.4. Heat inactivation of test sera

There is some uncertainty in the literature about whether heat inactivation of sera (at 56°C for 30 mins.) is essential or not when testing for antiglobulins in ELISA/SPRIA systems. Hay *et al* (1975) and Vejtorp *et al* (1979) used heat inactivation while Carson *et al* (1977) and Pope and McDuffy (1979) found it unnecessary.

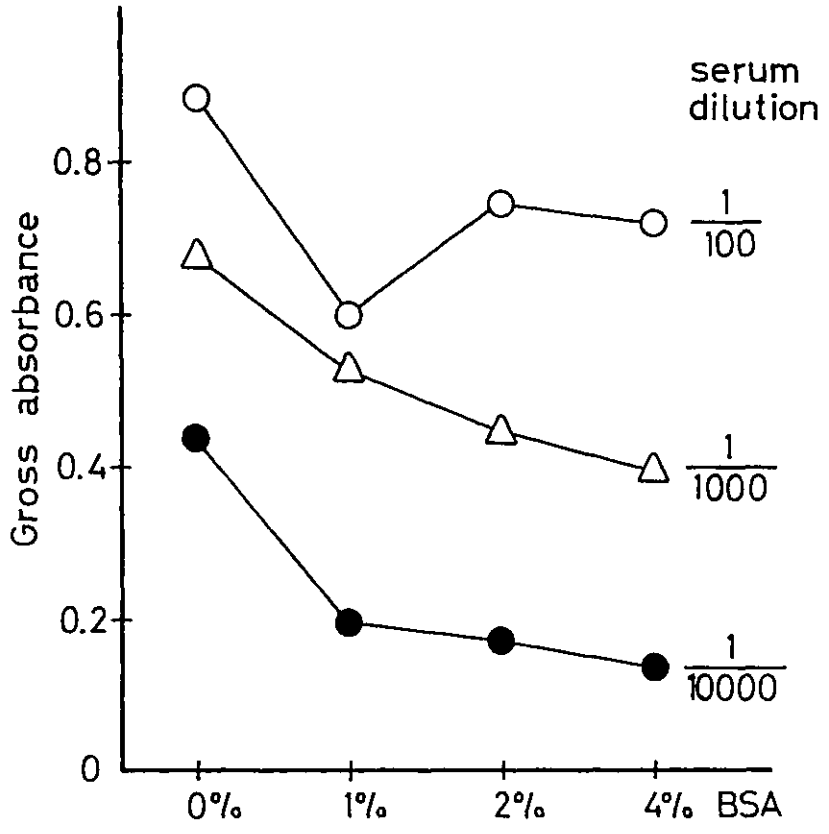


Figure II.6: The inhibition by BSA of serum IgM adsorption to microplates:

Three tenfold serum dilutions were incubated in a plate previously saturated with varying concentrations of BSA, followed by anti-human IgM-AP conjugate and pNPP. Mean BSA inhibition: 42%.

An experiment was therefore performed in which 19 sera with a wide range of IgG anti-ruminant IgG (see chapter IV) were tested, with and without heat inactivation.

Microplates were coated (rows A-D) with a 10 µg/ml solution of goat IgG, saturated with BSA, and incubated with the sera. Each serum dilution was tested in duplicate, both in coated and blank wells. Both the heated and non-heated samples from each individual were tested on the same plate.

Following incubation with a goat anti-human IgG-AP conjugate, the average net absorbance (abs.) value for each serum dilution (heated and non-heated) was calculated by subtracting the average for the blank wells from the average for the coated ones. The net average abs. values at serum dilution 1/40 are shown in Figure II.7. where the non-heated values (the abscissa) were plotted against the heated ones (the ordinate). The values correlated very closely, $r = 0.986$, and the slope of the regression line was 0.933, which indicates practically identical values for heated and non-heated sera.

II.5.5. Storage of microplates before reading

Sometimes it was impracticable to read the microplates immediately after the incubation with pNPP. Instead, the reaction was stopped by 3 M Na OH and the plates kept overnight, then read the following day.

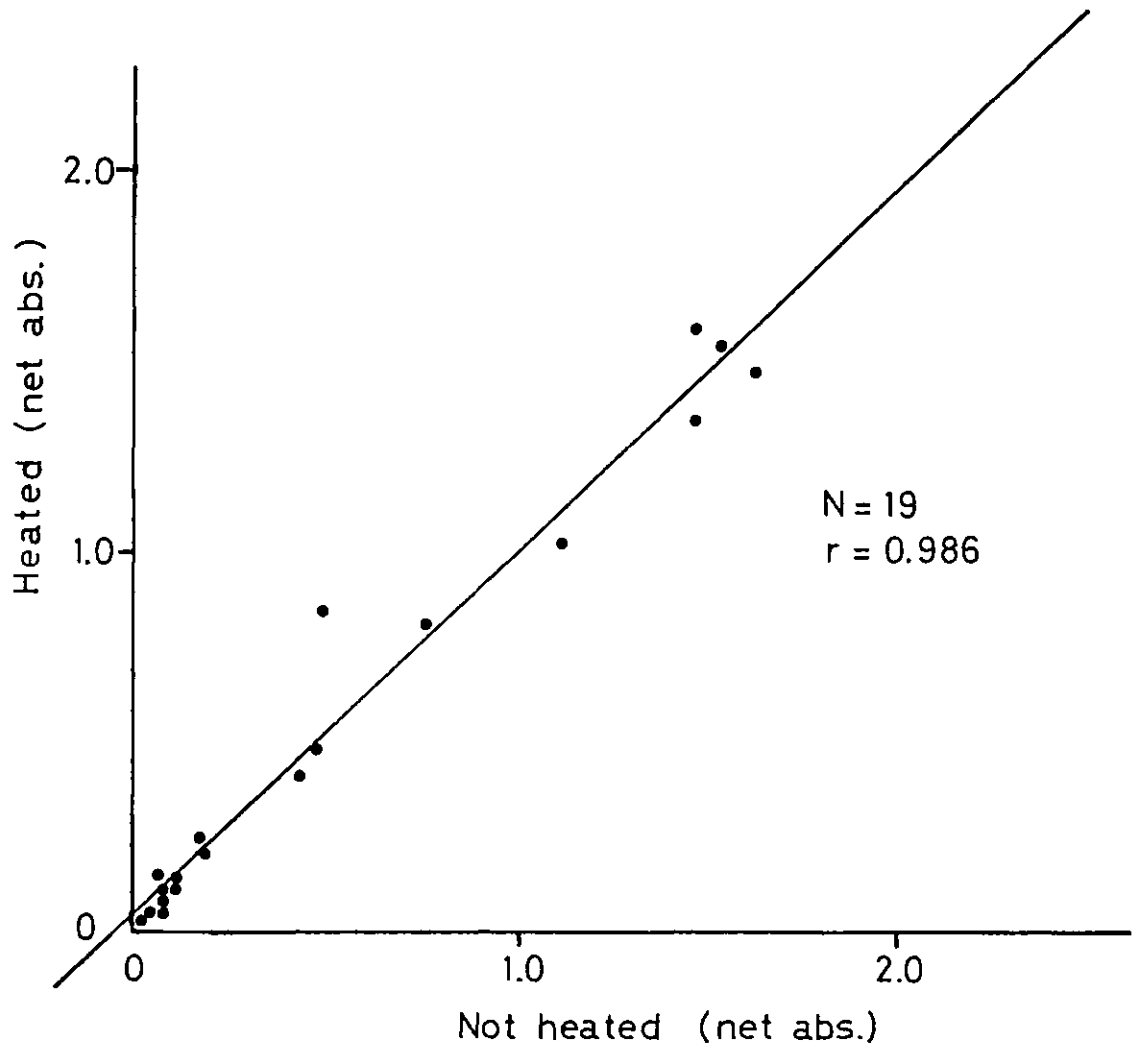


Figure II.7: The effect of heat-inactivation of sera for antiglobulin ELISA:

Nineteen sera were tested for IgG anti-ruminant IgG levels (chapter IV.2.), both heat-inactivated (56°C for 30 min.) and non-heat-inactivated.

Serum dilution: 1/40.

The regression equation: $y = 0.933x + 0.064$.

In order to test the effect of the overnight storage, six different sera with a wide range of IgM RF as measured by a sheep cell agglutination test (RAHA) were incubated for 2 hrs. at a dilution of 1/100 in two different batches of M29AR microplates (two plates from each batch) which had been coated with a 10 µg/ml solution of rabbit IgG, and saturated with 1% BSA. Each serum was tested in 16 coated and 16 blank wells in each of the two batches. Subsequently the plates were incubated for 2½ hrs. with a 1/1000 dilution of goat anti-human IgM-AP conjugate. Finally, the plates were incubated for 30 mins. at r.t. with pNPP solution and the reaction stopped by adding 3 M Na OH. The plates were read immediately in the microplate reader (day 1), then kept under cover overnight at r.t. and read again (day 2).

The mean net abs. values for each serum on day 1 and day 2 were calculated (see Table II.1.). The mean net increase in absorbance from day 1 to day 2 for the two batches of plates was 0.071 ± 0.010. Furthermore, when the mean abs. values for day 1 were plotted against those for day 2, a very close correlation was observed (r = 0.9997) (see Figure II.8.). As the slope of the regression line is very close to 1.0 (0.983), the small increase in abs. overnight is relatively independent of the day 1 abs. values. Therefore, the overnight storage of microplates for later reading seems acceptable.

II.5.6. Comparison of microplates and their pre-treatment

II.5.6.1. Adsorption and well-to-well variation

Since the introduction of "micro-ELISA" by Voller and Bidwell (1975),

Table II.1: The effect of storing fully developed microplates overnight.

	Batch 1 (plates D47 and D48)			Batch 2 (plates D45 and D46)		
Serum	$\bar{X}_1$	$\bar{X}_2$	$\bar{X}_2-\bar{X}_1$	$\bar{X}_1$	$\bar{X}_2$	$\bar{X}_2-\bar{X}_1$
Ha	0.187	0.267	0.079	0.191	0.270	0.079
Te	0.220	0.297	0.077	0.197	0.277	0.080
Bo	0.721	0.793	0.072	0.763	0.817	0.054
Th	0.852	0.916	0.064	0.740	0.821	0.081
We	0.902	0.966	0.064	0.825	0.904	0.079
Em	1.111	1.166	0.055	1.041	1.115	0.074

$\bar{X}_1$: Mean net abs. values on day 1 (plates read immediately after stopping enzyme substrate conversion).

$\bar{X}_2$: Mean readings of the same plates after overnight storage at r.t. The mean difference $(\bar{X}_2-\bar{X}_1)$ ± 1 SD: 0.071 ± 0.010.

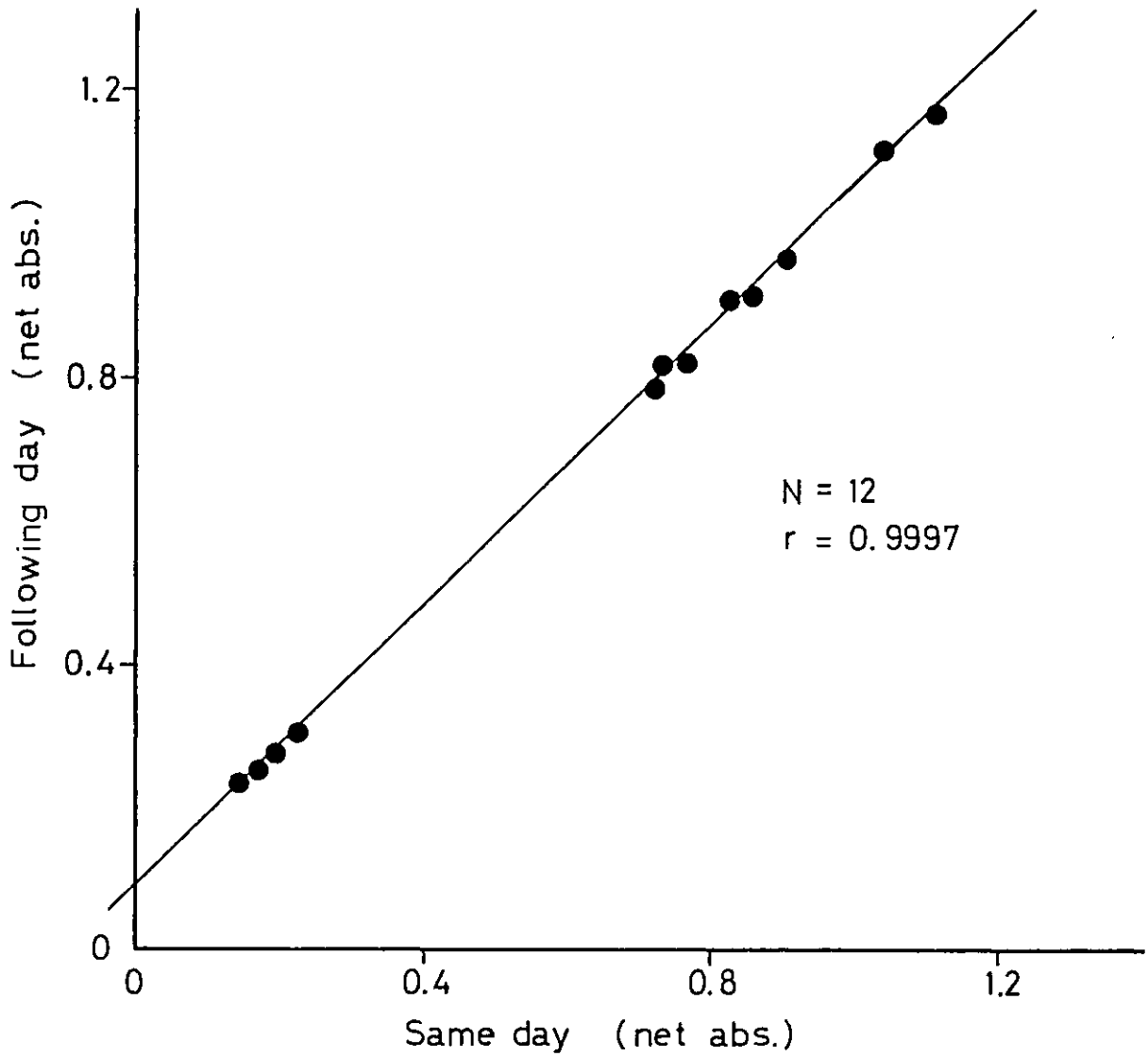


Figure II.8: The effect of overnight storage of microplates after termination of the enzyme reaction.

Twelve sera were tested for IgM RF levels (chapter III.1); incubated at a dilution of 1/100, followed by anti-human IgM-AP conjugate, pNPP and 3 M NaOH.

The plates were read immediately (abscissa values) and after overnight storage at r.t. (ordinate values).

Regression equation: $y = 0.983x + 0.083$.

it has been realised that the available microplates exhibit marked variability in adsorptive properties. Not only may microplates from different manufacturers adsorb protein to a varying extent, but there is also considerable batch-to-batch variation and well-to-well variation within the same plate (Denmark and Chessum, 1978; Kricka *et al*, 1980). Furthermore, the wells at the microplate edges tend to give higher readings than those towards the centre - "the edge effect" - and this may seriously affect the accuracy of ELISA (Burt *et al*, 1979).

In order to select a suitable microplate for the currect project, three different types of flat-well microplates were compared with respect to general affinity for immunoglobulins. The uniformity of adsorption or well-to-well variation of each plate, expressed as the coefficient of variation (CV) (i.e., the standard deviation of the absorbance, multiplied by 100, divided by the mean absorbance), was compared for the different plates.

Design of the Experiment:

Three different types of microplates (all flat-well) were tested:

1. M29AR (Sterilin Ltd.) polystyrene "Microtiter" plate, not tissue culture grade (TCG).

2. Linbro E.I.A. (Flow Laboratories) polystyrene micro-titration plate, cat. no. 76-381-04, not TCG.

3. M29ARTL (Sterilin Ltd.) polystyrene "Microtest" plate, TCG.

Evaluation Procedure A:

One plate of each type was incubated overnight at 4°C (96 wells/plate) with a 1/250 dilution in PBS of a goat anti-human IgG-AP conjugate, then incubated with pNPP for 90 mins. at r.t. and read in the Titertek Multiskan.

Evaluation Procedure B:

One plate of each type was coated overnight (96 wells/plate) with a 10 µg/ml solution of goat IgG in carbonate/bicarbonate buffer, followed by BSA saturation and incubation with a 1/80 dilution of a reference serum (positive for anti-ruminant IgG antibodies) for 2 hrs. at 37°C (waterbath) and overnight at 4°C. Subsequently, the plates were incubated for 3½ hrs. with a 1/1000 dilution of a goat anti-human IgG-AP conjugate, followed by pNPP for 40 mins.

Evaluation Procedure C:

Rows A-D of four M29AR plates and four Linbro plates were coated with 10 µg/ml goat IgG, rows E-H serving as blanks. The plates were then saturated with BSA and incubated with 1/50 dilutions of four sera with different levels of anti-ruminant IgG antibodies (each serum incubated in the whole of an M29AR plate and a Linbro plate) for 1 hr. at 37°C (waterbath) and overnight at 4°C. This was then followed by a 1/500 dilution of a goat anti-human IgG-AP conjugate at r.t. for 3½ hrs. and pNPP for 24 mins.

Findings:

Table II.2. shows the mean abs. values, standard deviations and

Table II.2: The binding of an antibody-enzyme conjugate by three different types of microplates.

Plate	mean abs.	SD	CV
Linbro	0.157	0.030	19.1%
M 29 AR	0.128	0.017	13.3%
M 29 ARTL	0.697	0.104	14.9%

The plates were treated as described under procedure A, by incubation with antibody-enzyme conjugate, followed by pNPP.

coefficients of variation (CV) for the three plates tested with procedure A. The tissue culture grade plate (M29ARTL) clearly bound the conjugate a lot more efficiently than the other plates. The CV values are rather similar for all three plates (13.3-19.1%).

Table II.3. shows the mean abs., SD, and CV values for procedure B. As previously, the M29ARTL type gave considerably higher readings than the other two, but again the CV values were similar in all three types of plates (13.6-15.4%).

Table II.4. shows the mean abs., SD, and CV values for the binding of sera to the coated and blank wells described under procedure C. The well-to-well variation was considerably less for the coated than the blank wells, as judged by the CV values. Furthermore, the reproducibility of the M29AR plates was somewhat better than that of the Linbro plates; however, Table II.5. shows that this was not statistically significant. On the other hand, the results in Table II.5. indicate that the non-specific background of the M29AR plates was significantly less than that of the Linbro plates.

Comment:

It is of interest that the Linbro plates, claimed to be specifically designed for ELISA, were similar or even inferior to the M29AR plates, as judged by the above findings.

Taken together, these results show that all three types of microplates displayed rather large well-to-well variation, as the CV values in Tables II.2. and II.3. and in the "blank" section of

Table II.3: Comparison of results from three different microplate types during an IgG anti-goat IgG assay.

Plate	mean abs.	SD	CV
Linbro	0.678	0.098	14.5%
M 29 AR	0.801	0.123	15.4%
M 29 ARTL	1.209	0.165	13.6%

The plates were treated as described under procedure B, by coating, BSA-saturation and incubations with serum, enzyme conjugate and pNPP.

Table II.4: Comparison of IgG anti-goat IgG absorbance values obtained in two different types of microplates

		Coated (48 wells)			Blank (48 wells)		
Serum	Plate	mean abs.	SD	CV	mean abs.	SD	CV
Sa	M 29 AR	1.494	0.101	6.8%	0.073	0.008	11%
	Linbro	1.599	0.121	7.6%	0.108	0.019	18%
N-20	M 29 AR	0.292	0.026	8.9%	0.078	0.010	13%
	Linbro	0.356	0.029	8.1%	0.118	0.020	17%
N-39	M 29 AR	0.247	0.022	8.9%	0.076	0.009	12%
	Linbro	0.279	0.034	12%	0.112	0.021	19%
N-41	M 29 AR	0.631	0.050	7.9%	0.074	0.013	18%
	Linbro	0.738	0.060	8.1%	0.137	0.021	15%

The plates were treated as described under procedure C, half of each plate coated with goat IgG, the remaining half left uncoated (blank).

Table II.5: Comparison between well-to-well variation and assay background of two types of microplates.

	M 29 AR	Linbro	Significance
Mean CV for coated wells	8.1	9.0	NS
Mean CV for blank wells	13.5	17.3	NS
Mean background	18.7 ± 12.3%	24.7 ± 14.8%	$p < 0.05$

The plates were treated by procedure C, see Table II.4.

The mean blank for each plate, expressed as % of the corresponding coated abs., was the mean background for that plate.

Paired student's t test was used for the statistical comparisons.

Table II.4. were always between 10 and 20%. The following attempt was therefore made to improve on this.

II.5.6.2. Pre-treatment of microplates with jewellery cleaning concentrate (JCC)

Recently, it has been suggested that pre-treatment of polystyrene surfaces with agents like glutaraldehyde, hydrochloric acid and ethanol might improve the reproducibility of ELISA systems (Salonen and Vaheri, 1979). This might affect the adsorption in the coating-step and also remove putative impurities from the polystyrene, thus diminishing the batch-to-batch and well-to-well variation.

One of the pre-treatment schedules claimed to diminish batch-to-batch and well-to-well variation of ELISA microplates involves sonicating the plates in a strong solution of an ammonia-containing detergent, jewellery cleaning concentrate (JCC), followed by thorough rinsing in distilled water (Mr. B. S. Chessum, personal communication). In view of the rather high well-to-well variation demonstrated in II.5.6.1., it was decided to test the effect of JCC-treatment on several different types of microplates, using different protocols.

Design of the experiment:

Jewellery cleaning concentrate (JCC) treatment:

JCC, purchased from L & R Ultrasonic Ltd., Harrow, London, was diluted to a final concentration of 15% (v/v) in distilled water. The microplates were completely immersed in this solution in an ultrasonic

bath (Soniprobe Cleaning Tank Type 1130/2A, Series No. 195, Dawe Instruments Ltd., London) and sonicated for 6 mins. Afterwards, the plates were rinsed many times in tap water and distilled water, then blotted on paper and sucked dry.

Types of microplates tested (all flat-well):

1. M29AR (Sterilin Ltd.), polystyrene "Microtiter" plate, not tissue culture grade (TCG).

2. M129A (Dynatech Laboratories Ltd.) "Microelisa" immulon plate, not TCG.

3. M129B (Dynatech Laboratories Ltd.) "Microelisa" immulon plate, cobalt gamma sterilised, not TCG.

4. M29 (Dynatech Laboratories Ltd.) flexible polyvinyl chloride "Microtiter" plate, not TCG.

5. Linbro E.I.A. (Flow Laboratories) polystyrene microtitration plate, cat. no. 76-381-04, not TCG.

6. Nunc Immunoplate I (Gibco Europe Ltd.) polystyrene plate, cat. no. 2-39454, not TCG.

7. Falcon, Microtest II, no. 3040, polystyrene plate, TCG.

8. Costar, cat. no. 3596, polystyrene plate, TCG.

Evaluation Procedure A:

Seven different types of microplates (types 1-8 above, except the Falcon plate) were tested. Two plates of each type, one JCC-treated, the other not, were incubated overnight at 4°C (96 wells/plate) with a 1/500 dilution in PBS of a goat anti-human IgA-AP conjugate, then incubated with pNPP for about 90 mins. at r.t. Finally, the reaction was stopped by 3 M Na OH.

Evaluation Procedure B:

Two plates of each of six different types (M29AR, M129A, M129B, M29, Nunc and Falcon), one JCC-treated, the other not, were coated overnight at 4°C with a 0.25 µg/ml solution of human IgG in carbonate/bicarbonate buffer. Subsequently, the plates were incubated with a 1/1000 dilution in PBS-Tween of a goat anti-human IgG (TAGO Inc.) - AP conjugate for 3½ hrs. at r.t. After incubation with pNPP for 30 mins., the reaction was stopped by 3 M Na OH.

The mean abs., SD, and CV values for each plate were then calculated.

Findings:

Table II.6. shows the results for the plates treated as described under procedure A. Table II.7. shows the analogous results for the plates treated by procedure B.

The individual microplate types had widely different affinities for immunoglobulins. Gamma irradiation of microplates apparently increases markedly the protein-binding affinity of the plastic as the

Table II.6: The effect of JCC-treatment on adsorption of an antibody-enzyme conjugate to different microplates.

	JCC-treated				No pre-treatment			
Type of plate	Plate no.	mean abs.	SD	CV	Plate no.	mean abs.	SD	CV
M 29 AR	D-17	0.176	0.031	17.4%	D-18	0.134	0.014	10.1%
M 129 A	D-23	0.231	0.018	7.9%	D-24	0.177	0.013	7.6%
M 129 B	D-21	0.432	0.023	5.3%	D-22	0.769	0.056	7.3%
M 29	D-29	0.210	0.022	10.4%	D-30	0.279	0.024	8.6%
Linbro	D-19	0.208	0.025	11.8%	D-20	0.161	0.014	8.7%
Nunc	D-25	0.501	0.033	6.6%	D-26	0.779	0.064	8.2%
Costar	D-31	0.379	0.038	10.0%	D-32	0.372	0.029	7.9%
Mean		0.305		9.9%		0.382		8.3%

The plates were treated as described under procedure A in II.5.6.2.

The table shows the mean abs. values and their SD and the coefficients of variation (CV) of the abs. values for the seven paired different microplates.

Table II.7: The effect of JCC-treatment on adsorption of human IgG to different microplates.

	JCC-treated				No pre-treatment			
Type of plate	Plate no.	mean abs.	SD	CV	Plate no.	mean abs.	SD	CV
M 29 AR	D-43	0.462	0.051	11.1%	D-44	0.331	0.046	13.8%
M 129 A	D-33	0.710	0.096	13.5%	D-34	0.670	0.053	7.9%
M 129 B	D-35	1.175	0.098	8.4%	D-36	1.559	0.092	5.9%
M 29	D-37	0.578	0.090	15.5%	D-38	0.381	0.074	19.4%
Nunc	D-39	1.090	0.092	8.4%	D-40	1.282	0.068	5.3%
Falcon	D-41	0.331	0.034	10.8%	D-42	0.269	0.021	7.9%
Mean		0.724		11.3%		0.749		10.0%

The plates were treated as described under procedure B in II.5.6.2.

The table shows the mean abs. values and their SD and the CV of the abs. values for the six paired different microplates.

M129B always gave higher abs. readings than the M129A. Also, it appears that JCC-treatment decreases the protein-binding affinity of the plates slightly, as the mean abs. values were slightly lower for the JCC-treated plates. Table II.8. shows, however, that this difference was not statistically significant.

Regarding the well-to-well reproducibility, the JCC-treatment was not beneficial. In fact, it increased the overall well-to-well variation as the CV values were slightly higher for the JCC-treated plates. However, Table II.8. shows that this was not statistically significant.

While these results do not support the claim that JCC-treatment improves the reproducibility in ELISA they still do not explain the large well-to-well variation seen in many of the plates tested here. The possibility that some of this variation is due to instrumental error of the microplate readers is explored in the following section.

II.5.7. Spectrophotometers for ELISA: advantages and limitations

II.5.7.1. The optimum working range of the Titertek Multiskan

The Beer-Lambert law states that $A = ExCxl$ (where A = absorbance ($\log \frac{I^{\circ}}{I}$); E = molar absorptivity ($cm^{-1}\ mol^{-1}$); C = concentration of absorbing substance (mol^{-1}/l) and l = length of light path (cm)), (Suovaniemi and Jarnefelt, 1982). In order to test how well the optical system of the Multiskan obeys this law, the following experiment was undertaken.

Table II.8: The effect of JCC-treatment of microplates on the amount and uniformity of protein adsorption.

	mean abs.	CV
No. of pairs	13	13
$\bar{X}_{\text{treated}} - \bar{X}_{\text{untreated}}$	-0.052	1.4%
t	1.02	1.57
Level of significance	p >0.1	p >0.1

The table summarises the results from Tables II.6 and II.7. where the mean abs. and CV values for the untreated plates were subtracted from the corresponding JCC-treated ones.

Paired student's t test was used to assess the significance of the differences observed.

Design of the experiment:

Fifty µl of an antibody-AP conjugate was added to a 1 mg/ml solution of pNPP in 10% diethanolamine buffer. This was then kept stirring at r.t. for 1 hr. in order to convert the pNPP to para-nitrophenol. Then, the strongly yellow solution was diluted serially by transferring 4 ml through a row of tubes, each containing 2 ml of diethanolamine buffer, giving a dilution factor of 1.5. Then, 200 µl aliquots were pipetted into flat-well microplates, 12 aliquots for each dilution. Ninety-six well were filled with diethanolamine buffer alone (buffer blank).

The plates were then read at 405 nm in the Multiskan and the mean buffer blank abs. value substracted from the mean abs. value for each dilution. For the sake of simplicity, the log of the abs. value was plotted against the log of the pNPP concentration.

Findings:

Figure II.9. shows the log-transformed dilution curve. It can be seen that the first five dilutions were beyond the Multiskan range and the sixth had a mean net abs.of 1.910. That observation *per se* indicates that only $1/1.5^5$ = 13% of the total pNPP need be hydrolyzed to p-nitrophenol in order to give an abs. of 1.9. This is reassuring, because it means that within the Multiskan abs.-range (<2.0), there will always be a pNPP excess. Furthermore, the regression analysis revealed a highly significant correlation (r = 0.997) between the log (conc.) and the log (abs.) values for dilutions 6-21, dilution 21 had a net abs. of 0.004. Also, the equation for the regression line was: $y = 1.00x - 1.729$, which is in good agreement with the Beer-Lambert law after log transformation: log (A) = log (C) + log (E x l), where log (E x l) is a constant

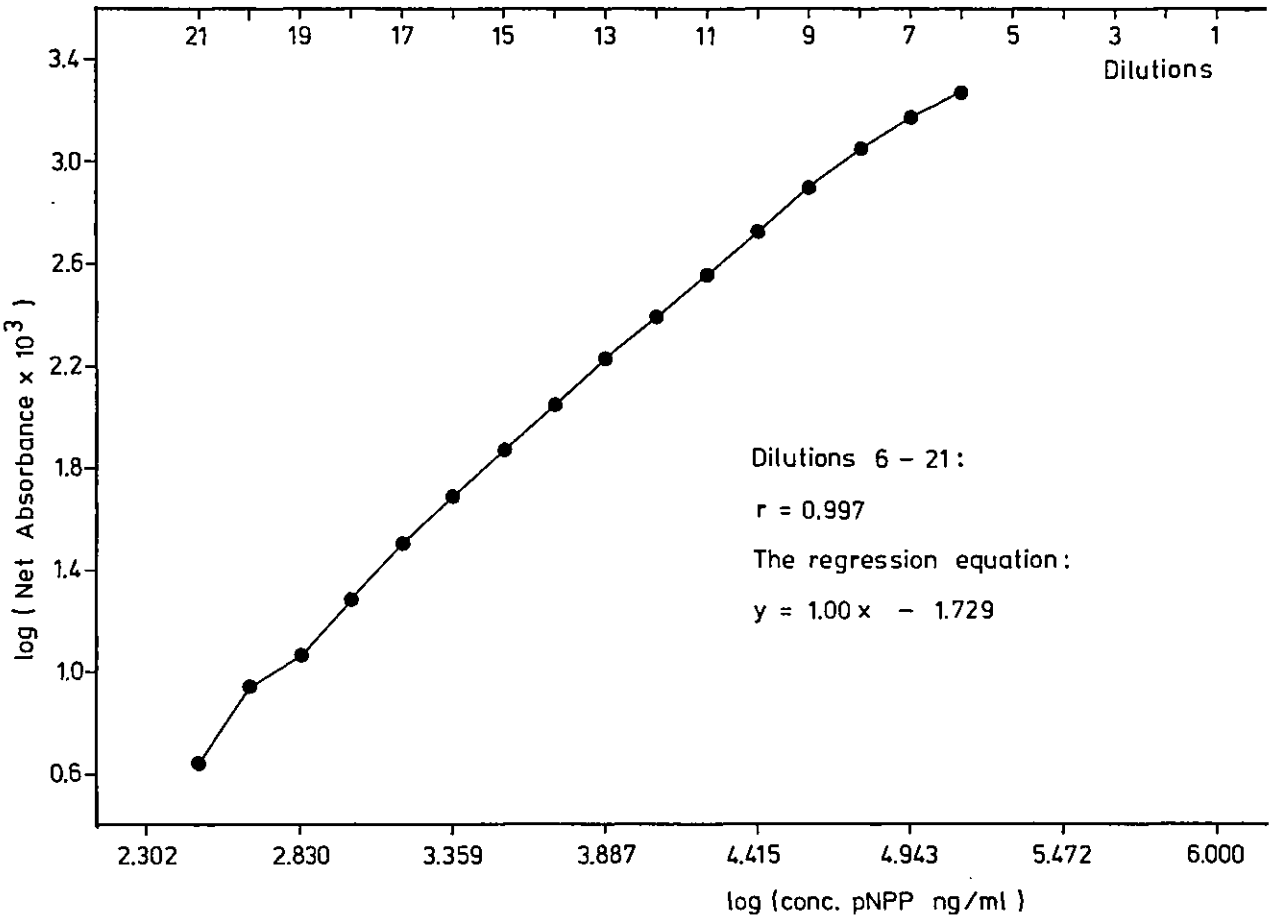


Figure II.9: The linearity of a log-transformed 1:1.5 dilution curve of p-nitrophenol, read in the Titertek Multiskan.

The regression equation for dilutions 6-21: y = 1.00x - 1.729.

The abs. values for dilutions 6 and 21 were 1.910 and 0.004, respectively.

(-1.729). Thus, these results imply that the optical system of the Multiskan was reliable within virtually the whole of its working range (0.004-1.910).

II.5.7.2. Reproducibility of readings: comparison between the Titertek Multiskan and the MR 580 Microelisa Autoreader

Recently, several manufacturers have introduced automated microplate readers which use dual wavelength systems for measuring absorbance in microplate wells. The basis of this system is that within the vertical light beam, going through the microplate, there are two alternating wavelengths, e.g., 410 nm for the detection of p-nitrophenol (sample-wavelength) and a reference-wavelength (e.g. 630 nm) which allows the operator to compensate for such effects as plastic artefacts, fingerprints, etc., as the abs. of the reference wavelength is automatically subtracted from the abs. of the sample-wavelength.

One of these "dual wavelength" microplate readers, MR 580 Microelisa Autoreader (Dynatech Laboratories Ltd.) which only uses one detector for reading the microplates (instead of the 8 in the Multiskan) was compared with the Multiskan.

Design of the experiment:

One microplate was incubated for 3 hrs. at r.t. with a 1/250 dilution in PBS of an antibody-AP conjugate. Subsequently the plate was incubated with a standard pNPP solution and left at r.t. overnight, then the reaction was stopped by 3 M Na OH.

The plate was read x 10 in both the Multiskan and the MR 580. Then, 20 wells were selected at random and the mean abs., SD, and CV values were calculated for the 10 readings of each well in both microplate readers.

Findings:

Table II.9. shows the summarized results of the within-well reproducibility testing of the two microplate readers. The mean abs. values for each of the 20 wells ranged from 0.332 and 0.372 to 0.470 and 0.521 for the MR 580 and the Multiskan, respectively. Interestingly, the reproducibility of the MR 580 readings was much poorer than that of the Multiskan. The mean CV value for the MR 580 (1.84%) was significantly higher than that (0.68%) for the Multiskan ($p < 0.001$, student's t test).

Thus, in terms of reproducibility of readings the Multiskan seemed better than the MR 580. The actual difference was small, however, and may not be of much practical significance.

II.5.7.3. Differing sensitivity of the Multiskan detectors: a contribution towards the edge effect?

During the early stages of the current project it was repeatedly observed that the values obtained (from the Multiskan) for rows A and B on the microplates seemed disproportionately high when compared with the rest of the rows. Sometimes, a stronger colouration in row A could be visualized, thus lending support to the general view that the wells at the edges tend to give higher readings than those towards

Table II.9: Comparison of the reproducibility of readings of two microplates readers.

	MR 580	Multiskan
Range of mean abs. for single wells	0.332-0.470	0.372-0.521
Range of SD for single wells	0.002-0.016	0.001-0.005
Range of CV for single wells	0.80-4.6 %	0.29-1.04%
CV: mean ± SD	1.84% ± 0.90%	0.68% ± 0.20%

Twenty selected wells of a microplate were read 10 times each in the MR 580 and the Titertek Multiskan.

The table shows the ranges of the mean abs., SD and CV values for individual wells.

the plate centre (Burt *et al*, 1979). Then, however, it was observed that if the microplate was read "the wrong way round", i.e., starting on column 12, the "edge effect" in row A seemed to diminish, whereas the "edge effect" in row H increased. In order to test the hypothesis that the 8 detectors of the Multiskan might be mal-adjusted, relative to each other, the following experiment was undertaken.

Design of the experiment:

The 26 microplates described in II.5.6.2. and Tables II.6. and II.7. above were read both in the Titertek Multiskan and in the MR 580 Microelisa Autoreader described in II.5.7.2. In the MR 580, the sample-wavelength was 410 nm and the reference-wavelength 630 nm. Subsequently, the mean abs. values for each row (A-H) and each column (1-12) were calculated and these means were compared with the mean abs. for the whole plate.

The deviation of the row/column means from the plate mean was expressed as a percentage of the plate mean. Then, the results for the two microplate readers were compared, using paired student's t test to assess the signficance of the differences observed.

Findings and comments:

Table II.10. shows the deviation of the individual row-means from the plate-means and the standard deviation of these. This is depicted graphically in Figure II.10.A. where the striking difference between the two microplate readers is obvious. In fact, using paired student's t test, the results for the two microplate readers differed significantly from each other for all the 8 rows.

Table II.10: Deviation of row-means from plate-means in 26 microplates, measured by two different microplate readers.

	MR 580		Multiskan		Difference between readers (DF = 25)	
Row	Mean Deviation	SD	Mean Deviation	SD	t	Significance
A	0.67%	10.4%	7.67%	8.68%	5.88	p <0.001
B	0.63%	8.01%	4.69%	6.89%	5.79	p <0.001
C	-2.49%	5.02%	0.03%	3.92%	3.28	p <0.01
D	-1.13%	4.84%	-2.83%	3.12%	-3.95	p <0.001
E	0.25%	4.69%	-3.36%	4.68%	-6.52	p <0.001
F	0.37%	3.91%	-2.77%	4.68%	-4.80	p <0.001
G	-0.30%	5.79%	-3.37%	4.97%	-4.30	p <0.001
H	1.99%	8.44%	-0.38%	4.94%	-2.45	p <0.05

Statistical comparisons between the two microplate readers: paired student's t test.

DF: degrees of freedom.

However, Table II.11. and Figure II.10.B. show the different situation for the plate columns. Even though the column-means differed as much as 4.6% from the plate-means, the results for the two microplate readers closely resembled each other and the difference between the two was nowhere significant at the 5% level.

These results clearly imply that at least some of the 8 detectors of the Titertek Multiskan were seriously mal-adjusted. The detectors reading rows A and B stood out as giving disproportionately high overall readings.

As the MR 580 uses only one detector for reading the whole plate, this comparison seems theoretically valid, a view very much strengthened by the finding that the column-readings of the two microplate readers differed in an identical way from the plate-means.

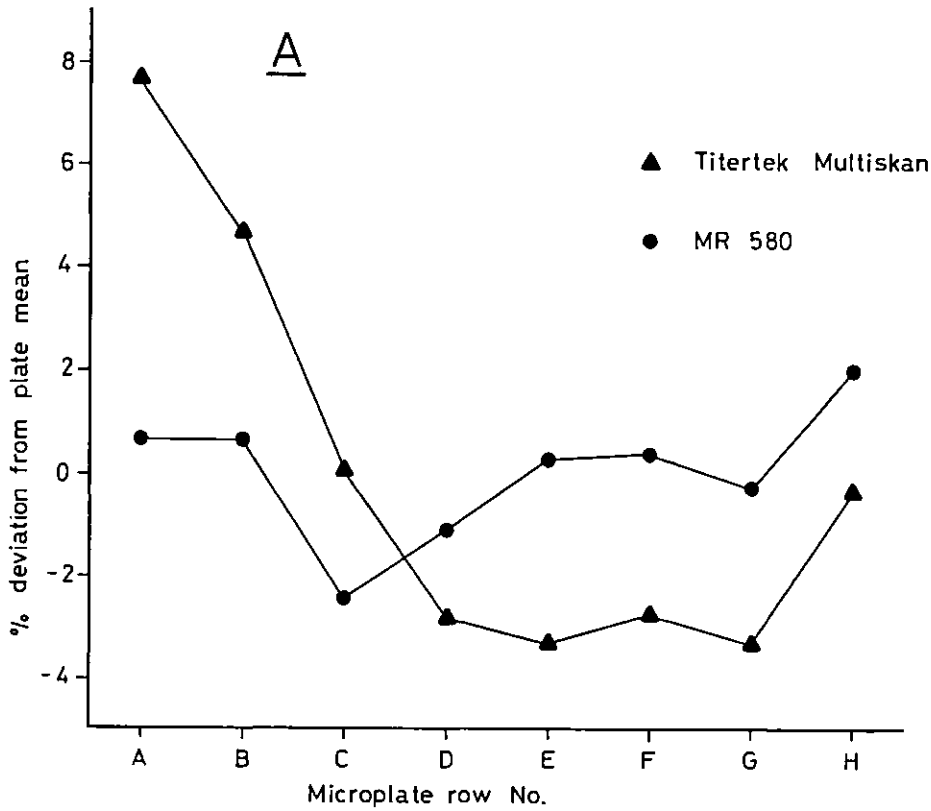
This study showed that on the whole the outermost columns of the 26 microplates tested did not exhibit a positive edge effect. On the contrary, column 5 gave on average the highest readings. However, some edge effect was apparent in row H, and row C tended to give the lowest row-results.

Figure II.10: The deviation of row- and column-readings from plate-means:

Twenty-six microplates were read in both the Titertek Multiskan and the MR 580 Microelisa Autoreader.

A: The mean % deviation of the row-means from the plate-means.

B: The mean % deviation of the column-means from the plate-means.


A
Titertek Multiskan
MR 580
% deviation from plate mean
8
6
4
2
0
-2
-4
A
B
C
D
E
F
G
H
Microplate row No.

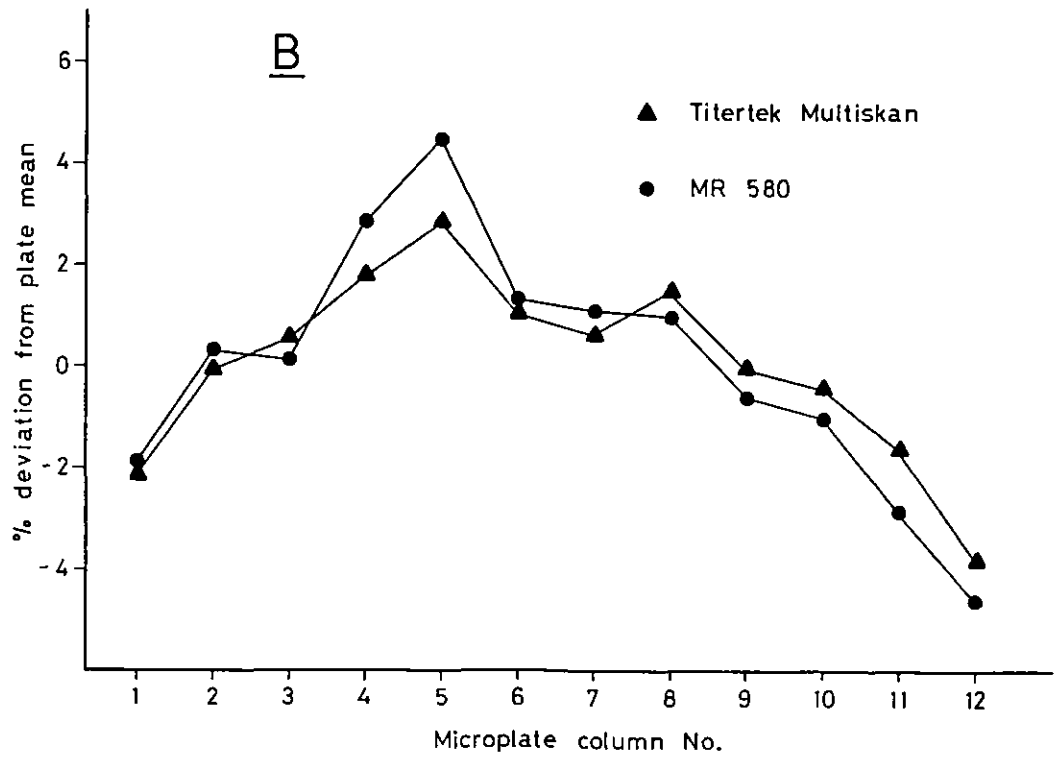

B
Titertek Multiskan
MR 580
% deviation from plate mean
6
4
2
0
-2
-4
1
2
3
4
5
6
7
8
9
10
11
12
Microplate column No.

Table II.11: Deviation of column-means from plate-means in 26 microplates, measured by two different microplate readers.

	MR 580		Multiskan		Difference between readers (DF = 25)	
Column	Mean Deviation	SD	Mean Deviation	SD	t	Significance
1	-1.88%	7.13%	-2.12%	6.04%	0.24	NS
2	0.33%	5.98%	-0.02%	4.53%	0.67	NS
3	0.11%	5.62%	0.56%	4.07%	-0.73	NS
4	2.83%	6.01%	1.79%	4.20%	1.92	NS
5	4.47%	6.73%	2.83%	3.70%	2.05	NS
6	1.33%	5.64%	1.04%	4.53%	0.69	NS
7	1.07%	5.99%	0.63%	4.54%	1.18	NS
8	0.96%	6.23%	1.47%	4.54%	1.24	NS
9	-0.62%	4.49%	-0.07%	3.17%	0.18	NS
10	-1.01%	4.88%	-0.43%	4.25%	0.96	NS
11	-2.89%	7.39%	-1.64%	5.27%	1.67	NS
12	-4.61%	13.67%	-3.88%	10.71%	0.84	NS

Statistical comparison between the two microplate readers: student's t test.

II.6. DISCUSSION

The basic equipment, materials, and reagents described in section II.2. were used throughout all the ELISA work described in this thesis.

The outlines of the standard methodology, as described in section II.3., also apply to the whole of the ELISA work. However, the finer details of the individual assays, e.g., types and concentrations of coating materials, sample dilutions used and expression of results, are dealt with in the individual assay sections (chapters III and IV).

The basis of the ELISA methodology used in the current project is well established, for a review, see Voller *et al* (1976). As the total number of technical variables in ELISA (e.g., buffers, temperature and timing of assay steps, washing procedures, the choice of enzymes, microplates and microplate readers) is astronomical, it was impractical in the current project to test but a few of these.

One of the variables which has been a matter of controversy in ELISA/SPRIA systems is the choice of diluent for sera. While Gripenberg *et al* (1979) used PBS containing 0.05% Tween 20, various authors have used 0.5-1% BSA in either PBS-Tween or TRIS buffer (Hay *et al*, 1975; Vejtorp *et al*, 1979; Pope and McDuffy, 1979). However, Ziola *et al* (1978) diluted their sera in PBS containing 20% pig serum and 2% Tween 20, which must be a rather expensive option. A very much cheaper method is that of Saunders (1979) who found that a hypertonic buffer with a pH of 8.0, containing 0.5 M NaCl and 0.5% Tween 20, was as effective in reducing non-specific background as a diluent containing 4-10% BSA.

This method was adopted here, and samples were routinely diluted in a 0.01 M phosphate buffer (pH 7.4) containing 0.5 M NaCl, 0.5% Tween 20 and 0.02% Na N_3 (referred to as hypertonic serum diluent).

In section 5 of this chapter, some of the variables involved in an indirect micro-ELISA were studied. The standard methodology, as described in II.3. is to some extent based on the results in II.5.

The observation that antigen-coated microplates may safely be stored for months (desiccated at a low temperature) agrees with the findings of Vaheri and Salonen (1980) who found that plates precoated with rubella virus antigen retained all their antigenic activity for at least two months if stored at -20°C or +4°C. This stability has obvious practical implications, e.g., the possibility of shipping precoated microplates to distant locations.

The finding that heat inactivation of sera is unnecessary for ELISA antiglobulin tests agrees with the findings of Carson *et al* (1977), and this is clearly different from the agglutination tests for anti-globulins where sera are routinely heat inactivated (Rose *et al*, 1948) due to the agglutinating activity of C_{1q}. It has even been reported that heat inactivation of sera (at 56°C for 30 mins.) may lead to greatly increased non-specific binding of Ig in ELISA systems (Saunders, 1979), and some of my early experiments with tissue culture grade (TCG) microplates (M29ARTL), which have high affinity for Ig, support this (data not shown).

The observation that microplates with pNPP solution can safely be kept overnight for later reading, after stopping the reaction with

3 M Na OH, agrees with the findings of Vaheri and Salonen (1980) who found that after stopping the pNPP cleavage by alkali, the absorbances did not change within 24 hrs. This is one of the advantages of using AP for ELISA, as the horseradish peroxidase substrates may continue to develop colour, even after Na OH is added (Bullock and Walls, 1977).

Considerable effort was made to select a suitable type of microplate for the project. From the limited number of comparisons shown in Tables II.2., II.3. and II.4., it seemed that the cheap M29AR plate was at least as good as, if not better than, the more expensive Linbro E.I.A. plate. However, the data shown in Tables II.6. and II.7. indicate that while the M29AR, the M129A and the Linbro plates all have similar affinities for protein, the M29AR exhibits slightly greater well-to-well variation than the others. Generally, the protein-binding affinity of tissue culture grade (TCG) plates, M129B and the Nunc plate is so high that this would inevitably cause background problems in antiglobulin tests with low dilutions (1/10) of test sera. The results of some of my earliest experiments where TCG plates (M29ARTL) were used for antiglobulin tests, indeed support this (data not shown). Thus, it was eventually decided to use the M29AR polystyrene "Microtiter" plates (Sterilin Ltd.) because these plates were the cheapest and at least as good as any of the other varieties.

The "edge effect" problem has been the source of much discussion amongst ELISA workers. Yet, no-one seems to know exactly what causes it nor how it can be "cured". Many factors might contribute to this effect, such as different internal dimensions of the peripheral wells, surface irregularities at the edges, strain and fine cracks in the edge wells during incubation and inter-well variation in the thermal history

of the microplate (Kricka *et al*, 1980). My experience does not suggest that pre-treatment with jewellery cleaning concentrate will "cure" the edge effect.

If the microplates are incubated on the bench, the edge wells tend to become warmer than the central wells (Burt *et al*, 1979) and this might be especially important during the enzyme-substrate incubation which is known to be highly temperature dependent (Porstmann *et al*, 1981; Hausamen *et al*, 1967). In order to allow for this and fluctuations in room temperature, the microplates were routinely incubated in a 37°C waterbath during the enzyme substrate incubation (pNPP step) of the RF tests (see chapter III).

In addition to the obvious mal-adjustment of the A- and B-row detectors of the Titertek Multiskan (see Figure II.10.A.) it can be seen from the SD values in both Tables II.10. and II.11. that the outermost rows and columns tend to show greater well-to-well variation than the central wells. Also, some edge effect seems to be present in row H (see Figure II.10.A.).

In view of this, rows A and H were generally only used as uncoated blanks and the edge columns (especially 1 and 12) were preferentially only used for high serum dilutions. This agrees with Kricka *et al* (1980) who recommended that the wells at the edges of a plate should not be used.

II.7. CONCLUSION

On the basis of the literature published (1963-1979) it was decided that enzyme-linked immunosorbent assay (ELISA) would probably offer the best approach for quantitative estimations of rheumatoid factors of IgG, IgA and IgM classes. Alkaline phosphatase was found to be a convenient enzyme for this ELISA work. The polystyrene microplate M29AR was on balance found to be the best type available despite somewhat poor reproducibility which was not improved by sonication in a strong detergent. The edge wells of the microplate were avoided, as these tended to give high and variable readings, perhaps partly due to faulty optics of the microplate reader used.

C H A P T E R I I I

THE MEASUREMENT OF RHEUMATOID FACTORS BY ELISA

C H A P T E R I I I

THE MEASUREMENT OF RHEUMATOID FACTORS BY ELISA

III.1. IgM RHEUMATOID FACTOR

III.1.1. Introduction

Traditionally, rheumatoid factor (RF) has been measured by agglutination tests, using either erythrocytes sensitised with IgG, or IgG-coated latex particles as the RF substrate (Rose *et al*, 1948; Singer and Plotz, 1956). As reviewed in chapter I.3., agglutination tests primarily detect IgM-class antibodies, but over the last twenty years, many different methods for RF-testing have been published, some of which seem to be capable of distinguishing between RFs of the various immunoglobulin classes (Torrigiani and Roitt, 1967; Hay *et al*, 1975; Kallerup *et al*, 1979; March *et al*, 1981). Some of the newer tests, however, are based on methods like nephelometry (Virella *et al*, 1978) or the precipitation in fluid phase of aggregates of radiolabelled gammaglobulin and RF (Yamagata *et al*, 1979). These methods are unlikely to give much information on the class- and subclass-distribution of RFs.

The agglutination methods have been used in RF-testing for over forty years (Waaler, 1940) and give a reasonable indication of the IgM RF-levels, but they are not without disadvantages. Sheep erythrocytes, traditionally used in the sheep cell agglutination (Rose Waaler) test, contain many antigens which may react with components of human serum and synovial fluid. This necessitates the pre-test absorption of samples (Sato *et al*, 1977) or parallel testing of sensitised and

unsensitised cells (Rose *et al*, 1948). Furthermore, standardisation of the amboceptor (rabbit anti-sheep red cell antibody) can be difficult, and determination of the end-dilution (titre) in agglutination tests is subjective (Karsh *et al*, 1980).

An international reference preparation of RA serum has been available for more than ten years (Anderson *et al*, 1970) and it has been shown that its use can reduce the variance of between-laboratory results in RF tests by as much as 81% (Fulford *et al*, 1978). Although this reference preparation was originally used to standardise the sheep cell agglutination test, various workers have since used it for different IgM RF assays (Kallerup *et al*, 1979; Vejtorp *et al*, 1979; Halbert *et al*, 1980; Karsh *et al*, 1980; Ziola and Tuokko, 1980).

As mentioned in chapter II, it was decided early in the course of the current project that ELISA tests for IgG, IgA and IgM RFs should be established. Subsequently, Willems and Klaassen de Kort (1978) reported an indirect ELISA for IgM RF, which correlated well with the conventional latex fixation and Rose Waaler tests. Tests for IgM and IgG RFs, based on solid phase radioimmunoassay (SPRIA) had already been described in 1975-1977 (Hay *et al*, 1975; Nineham *et al*, 1976; Carson *et al*, 1977). As the SPRIA tests are very similar in principle to the indirect ELISA, the experience of the SPRIA workers was directly relevant to the current project. The remaining sections of chapter III.1. will deal with the establishment of an ELISA test for IgM RF.

III.1.2. Different assay conditions tested

III.1.2.1. Initial observations - dilution curves

Guided by recent publications dealing with indirect ELISA and the testing of IgM RF by SPRIA and ELISA (Voller *et al*, 1976; Willems and Klaassen de Kort, 1978) it was relatively easy to obtain test-responses which clearly distinguished between Rose Waaler positive and negative sera. The standard assay procedure, as described in chapter II, was used throughout. The antibody-enzyme conjugate was goat anti-human IgM antibody (affinity purified, μ-chain specific, TAGO Inc.) coupled to alkaline phosphatase (AP).

In accordance with the method of Carson *et al* (1977), human IgG (isolated by Dr. H. Valdimarsson, using normal human sera and sodium sulphate precipitation followed by DEAE chromatography) was one of the first substrates tried for the IgM RF assay. As some preliminary experiments suggested that sera were more strongly reactive with heat-aggregated (aggregated for 30 mins. at 63°C) than native IgG, the aggregated preparation was initially used for microplate coating.

When testing sera from several RA patients on microplates coated with a 20 μg/ml solution of aggregated human IgG, the dilution curves shown in Figure III.1. were obtained. All the sera were tested on the same day without heat-inactivation. These curves revealed several interesting points:

Firstly, the curves tended to reach an upper plateau at the lowest dilutions tested, and there was another "background"-plateau at high dilutions.

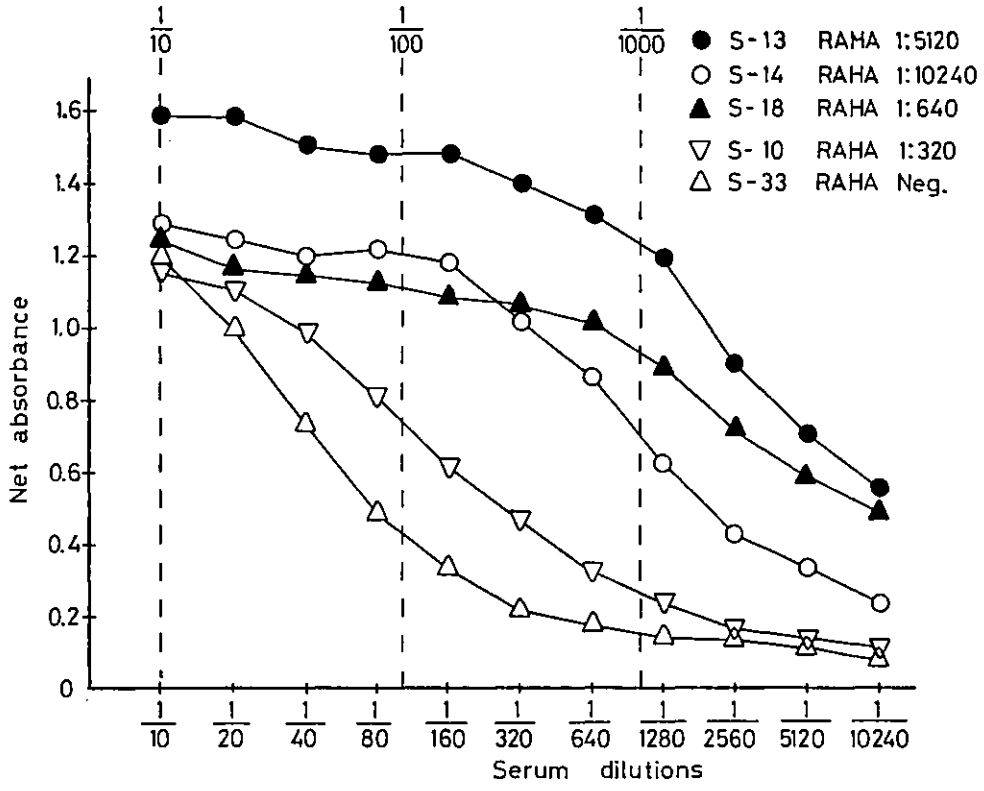


Figure III.1: ELISA dilution curves for five sera from RA patients, tested for IgM RF.

Heat-aggregated human IgG, at a concentration of 20 µg/ml, was used to coat the microplates.

The hypothetical 1/100 and 1/1000 serum dilution points are indicated.

Secondly, the rate at which individual sera fell from the upper plateau towards the lower varied considerably. This rate correlated roughly with the serum IgM RF level as measured by the RAHA-test (RAHA: a commercial agglutination test kit which employs sheep red cells coated with denatured rabbit gamma globulin, Finnie *et al* (1974)), e.g., serum S-33 (RAHA-negative) reached the lower plateau at dilutions 1/320-1/640, but at that point serum S-14 (RAHA >1/10,240) and serum S-13 (RAHA 1/5,120) were just starting to descend.

Thirdly, it would seem that the plateaus reached by individual sera differ in absolute level, e.g., the plateau of S-14 is considerably lower than that of S-13. Although this could partly be due to experimental factors such as plate-to-plate variation, this might also be due to different affinities of the rheumatoid factors involved.

Fourthly, Figure III.1. raises the question of whether it might be feasible to express the IgM RF results simply as the net absorbance at one particular dilution. If so, which dilution should be selected? Would it perhaps be more quantitative to use the sum of more than one dilution, e.g., the net absorbance at dilution 1/1000 and 1/100 and 1/10? Suffice it to say at this stage that the sum of the net abs. values at dilutions 1/100 and 1/1000 appears to give a fairly quantitative reflection of the individual dilution curves shown in Figure III.1.

III.1.2.2. The effect of heat-aggregating the RF-substrate

As mentioned in III.1.2.1., it had been observed that heat-aggregated human IgG gave consistently higher IgM RF readings than

non-aggregated human IgG. This increased reactivity might not necessarily improve discrimination between sera with low and high levels of IgM RF. In order to clarify this, the effect of heat-aggregation was assessed as follows:

Materials, methods: Human IgG (source, see III.1.2.1.) was aggregated by heating a solution containing 8.6 mg/ml IgG at 63°C for 30 mins. Heat-aggregated and non-aggregated preparations were then diluted to a concentration of 20 µg/ml and used for the coating of five microplates. Doubling dilutions of 10 sera with varying IgM RF levels (in the RAHA test) were tested. The mean net abs. value for each dilution on each substrate was calculated.

Findings: Table III.1. shows that the combined mean net abs. values at dilutions 1/10 and 1/160 for the 10 sera tested on each substrate. The values for the aggregated substrate were on average 50% higher than those for the non-aggregated IgG, and this is expressed in Table III.1. as the ratio of the absorbancies for the aggregated versus non-aggregated substrates. However, Figure III.2. indicates that this average 50% increase in binding did not improve the discrimination between high- and low-response sera as there was no significant correlation ($r = -0.33$, not significant) between the absorbance on the non-aggregated substrate and the increase after aggregation.

III.1.2.3. Reactivity of rheumatoid IgM with IgG from different species

It has been known for many years that RF may react, not only with

Table III.1: Comparison of IgM reactivity with heat-aggregated and non-aggregated human IgG

	Combined net abs. at dil. 1/10 + 1/160		Ratio
Serum	Aggregated substrate	Non-aggregated substrate	Aggregated/ non-aggregated
No 6103	1.382	0.926	1.49
No 6077	3.185	2.929	1.09
Lu	0.574	0.455	1.26
In	0.677	0.407	1.66
N-3	1.037	0.712	1.46
N-2	0.685	0.394	1.74
Te	0.922	0.612	1.51
F.Fr.	0.465	0.357	1.30
McD	2.840	1.430	1.99
Sa	0.891	0.526	1.69
Mean ± SD	1.266 ± 0.960	0.875 ± 0.792	1.52 ± 0.26

Ten sera with widely varying IgM RF levels (RAHA test) were assayed, coating concentration 20 µg/ml human IgG, see III.1.2.2.

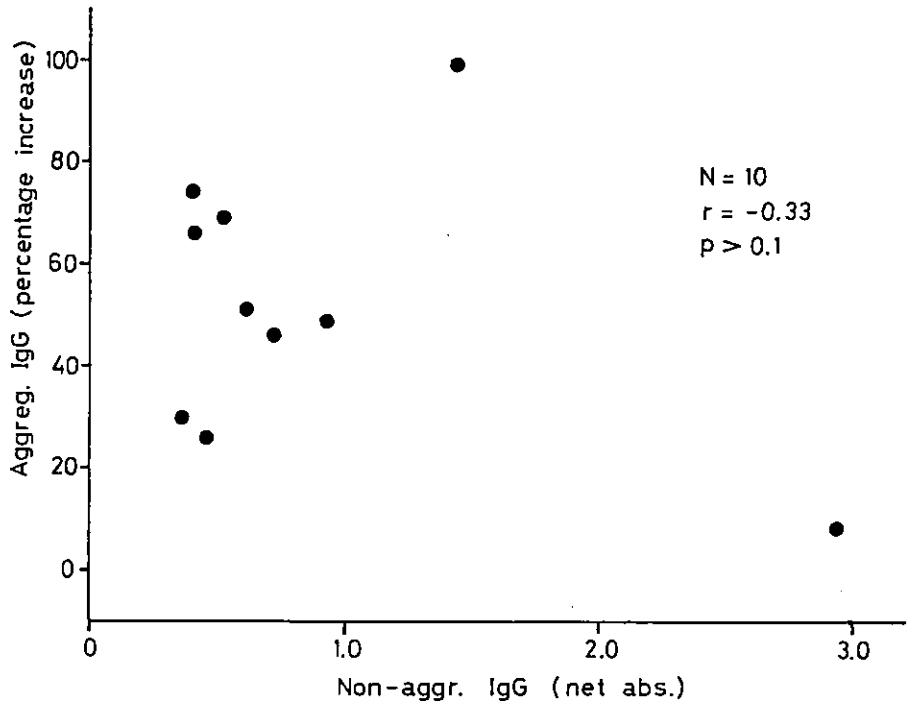


Figure III.2: The increase in IgM-reactivity after heat-aggregation of the human IgG substrate.

The 10 sera tested were those listed in Table III.1., see also III.1.2.2.

The correlation was non-significant, r = -0.33, p >0.1, product-moment correlation method.

human and rabbit IgG (Waaler, 1940; Singer and Plotz, 1956) but also with IgG from a wide range of animal species, including swine, baboon, horse, and sheep (Hannestad *et al*, 1981; Johnson and Faulk, 1976). However, RFs may show variable IgG reactivity depending on the species of origin, and *in vitro* produced RF has been reported to react with human and rabbit IgG but not goat, mouse or swine IgG (Gilliland *et al*, 1978; Steinitz and Tamir, 1982). In view of this, the reactivity of IgM RF against IgG from different species was compared in the following experiment.

Materials, methods: IgG from six different animal species was studied: rabbit, goat, sheep and bovine IgG (obtained from Miles Laboratories Ltd.), mouse IgG (a gift of Dr. I. V. Hutchinson, St. Mary's Hospital) and human IgG (source, see III.1.2.1. above) were used.

Twelve microplates (two for each species) were coated with a 10 µg/ml solution of IgG and incubated with 10-fold dilutions (1/10, 1/100 and 1/1000) of sera from 14 RA patients. These sera had a wide range of RF-levels, RAHA-titres ranging from negative (<1:40) to 1:10,240. Each serum dilution was tested in duplicate in coated and adjacent blank wells for each of the six IgG preparations.

The plates were then reacted with a 1/1000 dilution of the anti-human IgM conjugate. This conjugate was also tested for cross-reactivity with the IgG in coated wells which had not been exposed to serum.

After pNPP incubation for 45 mins., the plates were read and the mean net absorbance value for each serum dilution on each substrate was

calculated. The RAHA-titres for the sera were converted to rank values (negative = 0; 1:10,240 = 9) and these were then correlated (using the "product-moment" correlation) with the net absorbance values from serum dilution 1/10 and various combinations of the three dilutions tested.

Findings: The conjugate did not react with any of the six substrates used for coating.

Figure III.3. shows the combined net absorbance values for serum dilutions 1/10, 1/100 and 1/1000 for each of the 14 sera tested on the six different substrates. Clearly, the sera reacted on the whole most strongly with rabbit and human IgG. However, some correlation between the RAHA titres (top of Figure III.3.) and the ELISA responses was present for all six substrates tested. Indeed, Table III.2. indicates (in the right hand end column) that the results for all the six substrates correlated to a similar extent with the RAHA-titres.

Somewhat surprisingly, of the six substrates, bovine IgG gave the best correlation with RAHA-titres. However, the rabbit IgG also gave a good correlation with RAHA, especially the combined net absorbance values for dilutions 1/100 plus 1/1000 and for dilutions 1/10 + 1/100 + 1/1000.

One striking feature in Figure III.3. is the close similarity of RF reactivity of all the three types of ruminant IgG (goat, sheep and bovine). This is further underlined in Table III.3. where the net absorbance values for dilutions 1/10 + 1/100 + 1/1000, obtained on sheep IgG, are correlated with the analogous results for the other five

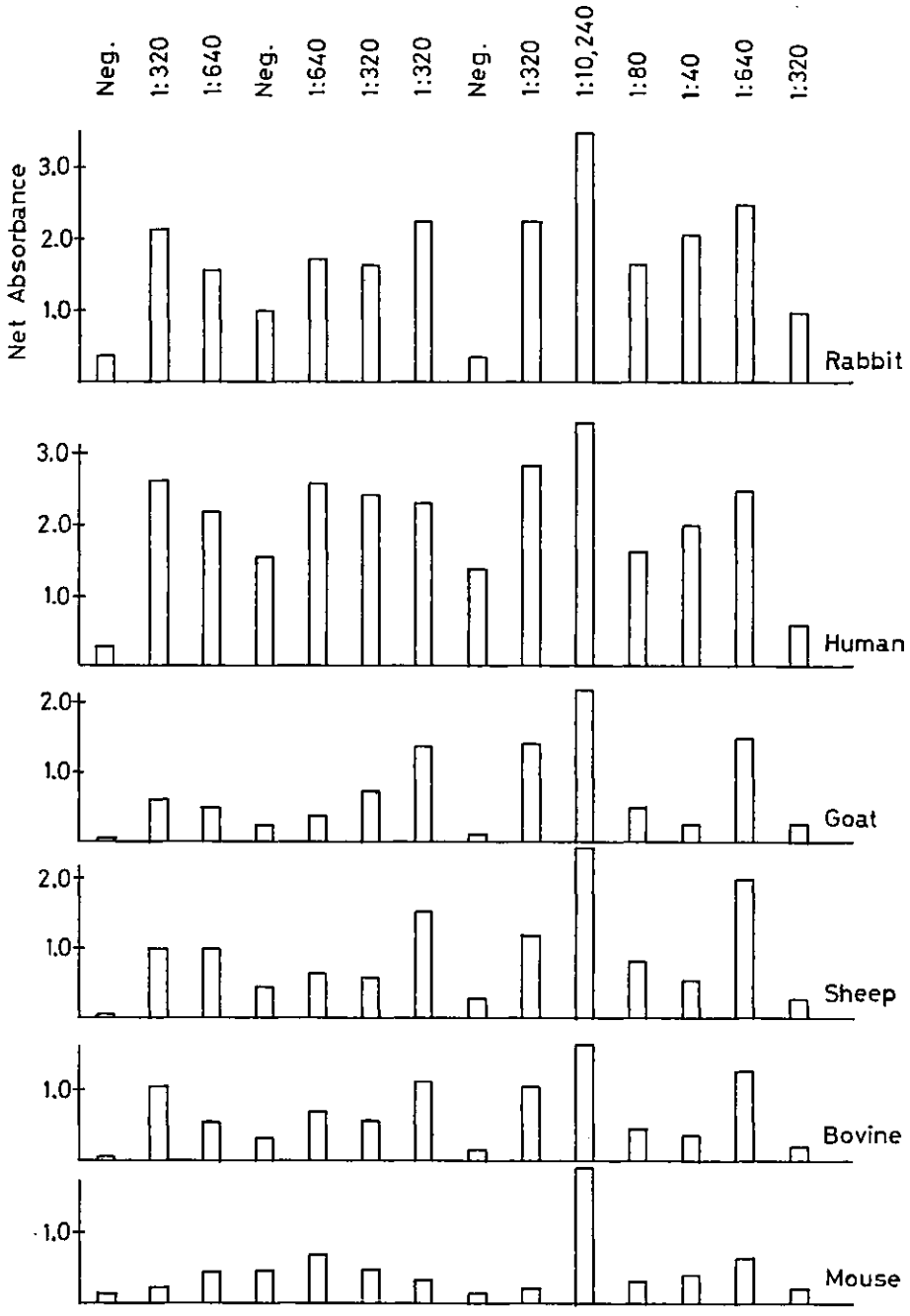


Figure III.3: IgM anti-IgG levels in sera from 14 RA patients with RAHA titres (top of figure) ranging from negative (<1/40) to 1/10,240.

The results represent the combined net absorbance (CNA) at serum dilutions 1/10 + 1/100 +1/1000 on plates coated with IgG from the six animal species shown, cf. III.1.2.3.

Table III.2: The association between ranked RAHA titres and IgM anti-IgG activity by ELISA.

RF-substrate		serum - dilution combination					
		1/10	1/100	1/1000	1/10 + 1/100	1/100 + 1/1000	1/10 + 1/100 + 1/1000
Rabbit IgG	r	0.700	0.744	0.789	0.740	0.806	0.798
	p<	0.01	0.01	0.001	0.01	0.001	0.001
Human IgG	r	0.627	0.643	0.810	0.641	0.749	0.724
	p<	0.02	0.02	0.001	0.02	0.01	0.01
Goat IgG	r	0.742	0.685	0.756	0.774	0.765	0.780
	p<	0.01	0.01	0.01	0.01	0.01	0.001
Sheep IgG	r	0.767	0.768	0.760	0.784	0.772	0.791
	p<	0.01	0.01	0.01	0.001	0.01	0.001
Bovine IgG	r	0.816	0.746	0.798	0.802	0.786	0.814
	p<	0.001	0.01	0.001	0.001	0.001	0.001
Mouse IgG	r	0.760	0.724	0.575	0.755	0.688	0.735
	p<	0.01	0.01	0.05	0.01	0.01	0.01
Mean r		0.735	0.718	0.748	0.749	0.761	0.774

Fourteen rheumatoid sera were tested, see Figure III.3. and III.1.2.3.

The association is expressed as the product-moment correlation coefficient (r) for IgG from the six different species indicated at six different combinations of serum dilutions

Table III.3: The antigenic similarity between sheep IgG and IgG from five other species.

Sheep IgG vs.	Antigenic similarity (correlation coefficient r)	Level of Significance
Mouse IgG	0.727	$p < 0.01$
Human IgG	0.776	$p < 0.01$
Rabbit IgG	0.900	$p < 0.001$
Goat IgG	0.951	$p < 0.001$
Bovine IgG	0.952	$p < 0.001$

The ELISA IgM anti-IgG responses of 14 rheumatoid sera at serum dilutions 1/10 + 1/100 + 1/1000 were determined.

The antigenic similarity was expressed as the r-value for the association between the responses to sheep IgG vs. the other types of IgG.

substrates. Clearly, the anti-sheep IgG activity correlated more closely with that of bovine and goat IgG than with the other three substrates.

III.1.2.4. Correlation between RAHA-titres and IgM RF measured by ELISA

In order further to evaluate the suitability of the ELISA system described in III.1.2.3. for quantitative estimations of serum IgM RF, the following experiment was performed:

Materials, methods: Rabbit IgG (Miles Laboratories Ltd.) was used for coating at a concentration of 10 µg/ml. The sera from 116 patients (107 with classical/definite RA, 9 with osteoarthritis), diluted to 1/10, 1/100 and 1/1000, were tested in duplicates, simultaneously and without heat-inactivation.

The mean net absorbance values for the individual dilutions were calculated, and the combined net absorbance (CNA) values for serum dilutions 1/100 + 1/1000, and also for dilutions 1/10 + 1/100 + 1/1000, were plotted against the RAHA-titres of the individual sera.

The "product-moment" correlation coefficients (r) for the association of the ranked RAHA values (see III.1.2.3. above) with the ELISA results were then calculated, using a programmable calculator (TI 59, Texas Instruments).

Findings: Figure III.4. shows the combined net absorbance values for dilutions 1/10 + 1/100 + 1/1000 of the 116 sera, plotted against the RAHA-titre. The r-value, 0.800, indicates a highly significant correlation ($p < 0.001$). However, in agreement with the results in Table III.2. it was found that the CNA value at dilutions 1/100 + 1/1000 correlated even better with the RAHA results ($r = 0.842$). Thus, if it is assumed that the RAHA test gives a reasonably quantitative estimation of the serum IgM RF levels, this ELISA test does so as well, although individual sera may behave quite differently in the two systems.

III.1.3. Expression of data and the use of internal and international standards

III.1.3.1. Introduction

In order to reduce inter- and intra-assay variation, the use of well-defined standards or reference sera is imperative. This applies especially to techniques like ELISA, where very many variables can affect the results. Indeed, international reference preparations have been used in ELISA work, e.g., in the assessment of anti-tetanus antibody levels (Nieminen *et al*, 1979) and in IgM RF tests (Vejtorp *et al*, 1979; Halbert *et al*, 1980; Karsh *et al*, 1980). The procedure used to express the IgM RF ELISA results in terms of international units will now be discussed.

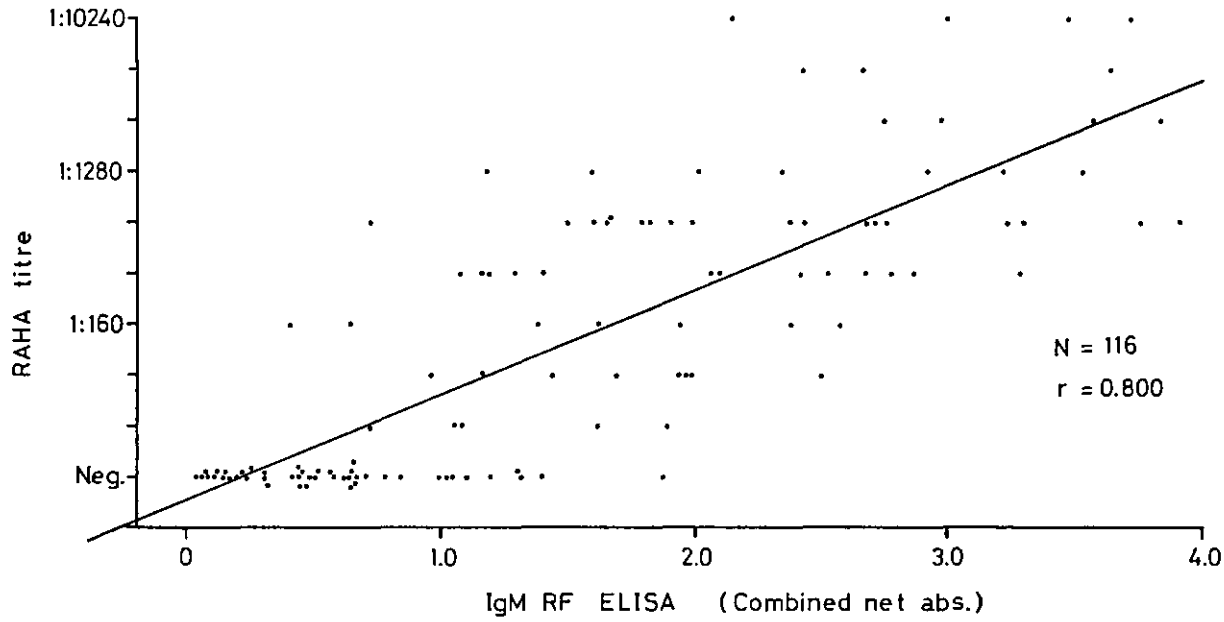


Figure III.4: The association between IgM RF ELISA results and the RAHA test.

Sera from 116 patients (107 with classical/definite RA, 9 with osteo-arthritis) were tested at dilutions 1/10 + 1/100 + 1/1000, see III.1.2.4.

The ELISA results correlated highly significantly with the ranked RAHA results ($r = 0.800$, $p < 0.001$).

III.1.3.2. The standards used

The serum from a patient with classical RA (nodular, erosive disease) having a RAHA-titre of 1:2560, was used as an internal day-to-day standard in the IgM RF tests.

This serum was calibrated against the "1st British Standard for Rheumatoid Arthritis Serum" (Anderson *et al*, 1970). One ampoule of this standard, containing 100 international units (IU) of freeze-dried material, was obtained from the National Institute for Biological Standards and Control, Holly Hill, Hampstead, London, N.W.3.

III.1.3.3. Calibration of the internal standard

The internal standard serum was calibrated against the British Standard in the following way. The freeze-dried standard was dissolved in 1.0 ml of PBS, to 100 IU/ml. This solution and the internal standard serum were then tested in doubling dilutions, starting at 1/10, on a microplate coated (rows B-G) with 10 µg/ml rabbit IgG using the standard assay procedure for IgM RF (see III.1.4.1.).

The mean net absorbance values for each dilution were calculated and the dilution curves for both standards plotted (Figure III.5.). The internal standard was clearly stronger than the undiluted (100 IU/ml) British (international) standard. However, the shapes of the dilution curves were very similar for both standards, and by measuring the horizontal distance from the seven first points on the curve for the British standard (dilutions 1/10-1/640) to the curve for the internal

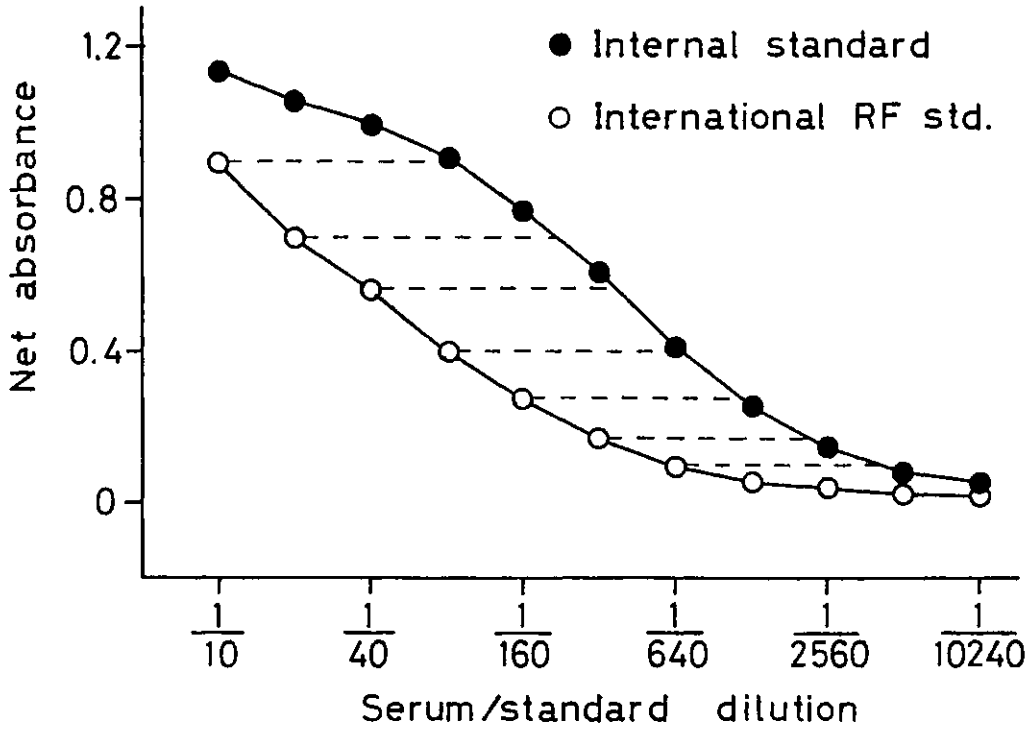


Figure III.5: Dilution curves from simultaneous IgM RF ELISA of the international RF standard and the internal IgM RF standard.

The internal standard was on average 3.13 dilutions (or 8.75 times) stronger than the international standard, see III.1.3.3.

standard, it was concluded that the internal standard was on average 3.13 dilutions stronger than the British standard. The internal standard is therefore $2^{3 \cdot 13} = 8.75$ times stronger than the British standard. It follows that the undiluted internal standard serum should contain 875 IU/ml of RF.

III.1.3.4. The practical application of the internal standard

In III.1.2.3. and III.1.2.4. it was shown that the combined net absorbance (CNA) at dilutions 1/10 + 1/100 + 1/1000 and at dilutions 1/100 + 1/1000 gave a reasonably quantitative measure of the serum IgM RF. As the tests for IgM RF and IgA RF were generally run in parallel, and as it was found beneficial to include the 1/10 dilution in the IgA RF test (see chapter III.2.), it was technically easier to include the 1/10 dilution in the IgM RF test as well.

Figure III.6. illustrates how the internal standard was diluted for IgM RF testing. In order to cover a wide range of IgM RF levels, yet use only a limited number of dilutions, the internal standard was diluted by a factor of 1:4 from the first 1/10 dilution onwards. Thus, a system of six different dilutions, each of which in turn contained three 10-fold dilutions, was obtained. These dilutions were then tested in parallel with unknown samples, the strengths of which were determined from the CNA dilution curve for the internal standard. Figure III.7. shows a typical CNA dilution curve for the internal standard, from which values of between 0.85 and 875 IU/ml can be read.

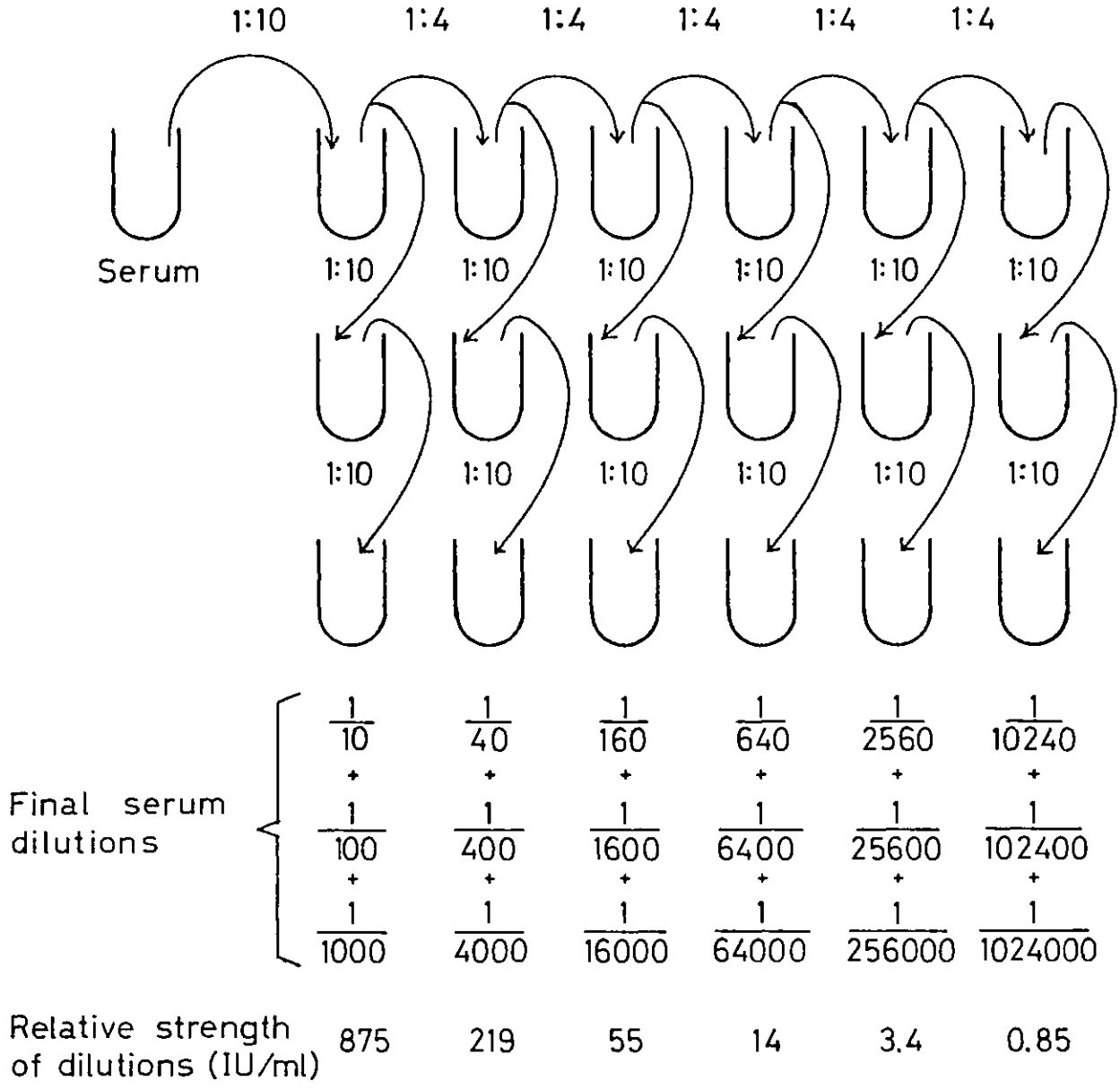


Figure III.6: The dilution system of the internal IgM RF standard as applied for the routine IgM RF ELISA.

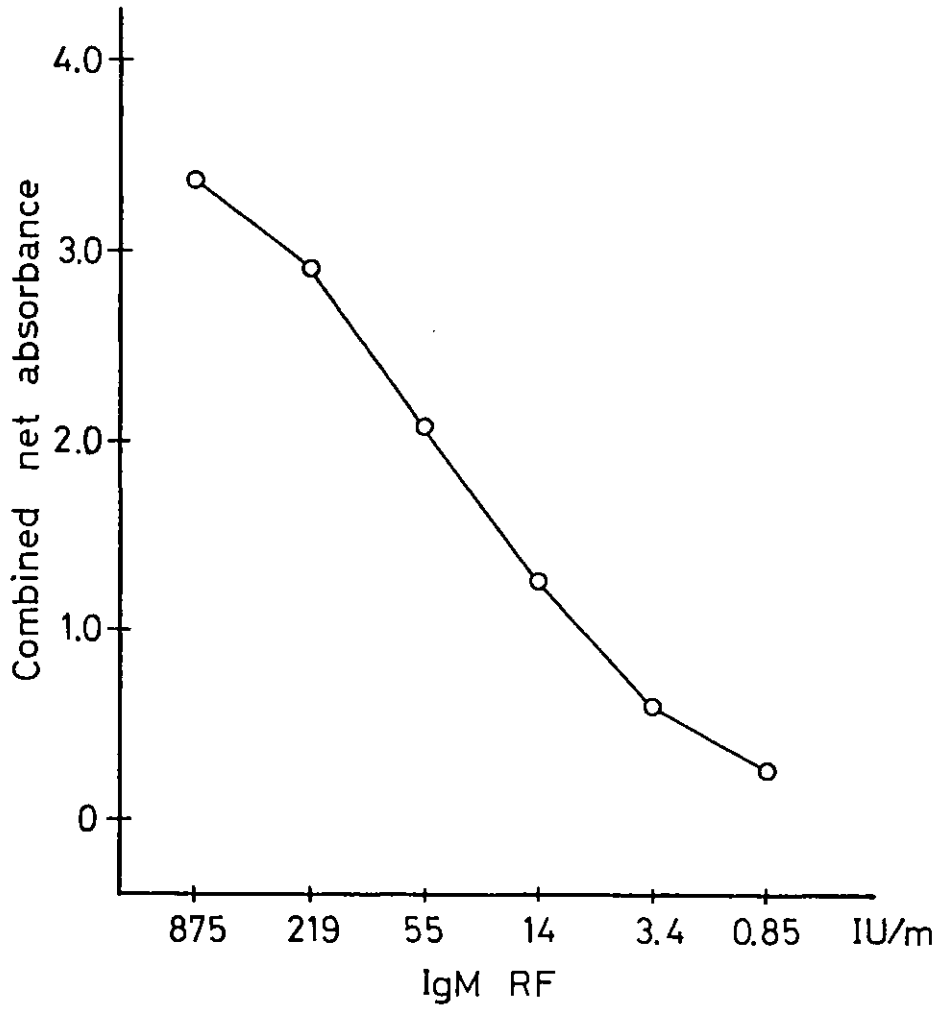


Figure III.7: A typical dilution curve for the internal IgM RF standard, diluted as shown in fig. III.6.

III.1.4. The IgM RF assay as applied:
reproducibility and normal ranges

III.1.4.1. The standard assay procedure for IgM RF:

Coating of microplates: Rows B-G of M29AR microplates were incubated overnight at 4°C with 100 µl/well of coating buffer containing 10 µg/ml normal rabbit IgG (Miles Laboratories Ltd.). Rows A and H served as uncoated blanks. Following the incubation the plates were sucked dry and washed x 2 with PBS-Tween, then either saturated with 1% BSA in PBS-Tween (100 µl/well) for 2-3 hrs. at r.t. or stored under cover at 4°C.

Sample incubation: After the BSA saturation, the plates were incubated with dilutions of test samples and the internal standard. Each serum/synovial fluid sample was tested unheated, at dilutions 1/10 and 1/100 and 1/1000. The sample dilutions were incubated in triplicates (100 µl/well) for 2 hrs. at r.t. in coated wells plus in one adjacent blank well. The plates were then sucked dry and washed x 3 with PBS-Tween.

Conjugate incubation: Following the serum incubation, the plates were incubated (rows A-H) with 100 µl/well of a 1/1000 dilution of a goat anti-human IgM antibody (TAGO Inc.) coupled to alkaline phosphatase. After 2½ hrs. at r.t. the plates were sucked dry and washed x 3 with PBS-Tween.

Enzyme substrate: Following the conjugate incubation the plates were incubated with a 1 mg/ml solution of pNPP in 10% diethanolamine-HCl buffer, pH 9.8. After filling with 100 µl/well, the plates were

immediately transferred to a 37°C waterbath where they were kept floating (without a lid) for exactly 16 mins. The reaction was then stopped by adding 50 µl of 3 M NaOH to each well.

Expression of results: After wiping the bottom of the micro-plates with soft tissue, they were read at 405 nm in a Titertek Multiskan which had been blanked on a clean, empty plate. The absorbance value for the blank (uncoated) well of each dilution was then subtrated from the triplicate mean, giving the mean net absorbance for that dilution. The IgM RF content (IU/ml) of each sample was then determined by reading the CNA value against the CNA dilution curve for the internal standard, assayed on the same day.

III.1.4.2. Day-to-day reproducibility of the IgM RF assay

Two rheumatoid sera (La and Tw) were tested repeatedly on different days. Prior to testing, the sera were aliquoted into 0.2 ml portions in order to avoid repeated freezing and thawing.

The results are shown in Figure III.8., expressed as both CNA values and RF units (IU/ml). The CNA values for the two sera exhibited somewhat parallel fluctuations, and these fluctuations were also very similar to the same-day fluctuations of the internal standard (at dilutions 1/10 + 1/100 + 1/1000). Accordingly, these fluctuations were eliminated to some extent by conversion to RF units, as shown in the RF unit-graph of serum La. Table III.4. also shows that the day-to-day variation of serum La (expressed as the coefficient of variation, CV) was less (12.3%) when the results were expressed as RF units than when expressed as CNA (18.6%). However, the opposite was true for serum Tw

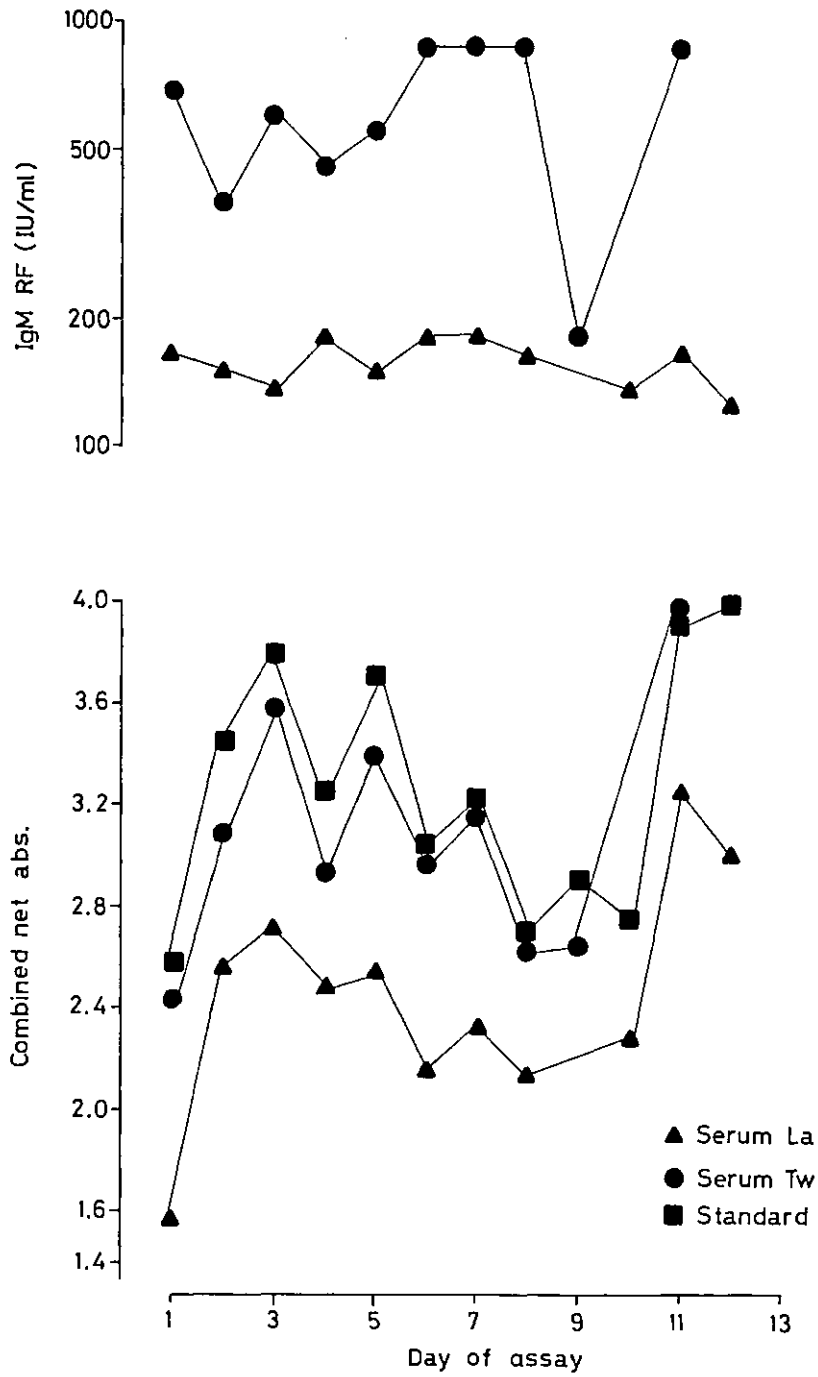


Figure III.8: Day-to-day reproducibility of the IgM RF ELISA.

The day-to-day fluctuations of the CNA values for the internal IgM RF standard and two rheumatoid sera (La and Tw) are shown in the lower half of the figure).

The upper half shows the day-to-day fluctuations of the IU/ml-values for the two rheumatoid sera.

Table III.4: The day-to-day reproducibility of the IgM RF ELISA

Serum	Mode of expression	No. of test days	Mean	CV
La	CNA	11	2.456	18.6%
	IU/ml	11	159	12.3%
Tw	CNA	10	3.080	15.0%
	IU/ml	10	637	38.6%

The means of the CNA and IU/ml values for each of two rheumatoid sera tested on 10 and 11 different days are shown, as well as their coefficient of variation (CV).

which exhibited a 38.6% day-to-day variation in the unit values but only 15% CNA variation.

III.1.4.3. Normal ranges

In order to establish a normal range for serum IgM RF levels, sera from 102 healthy individuals were tested (see Figure III.9.). These normal sera were derived from three sources:

1. Laboratory staff, a total of 23, age range 18-45 yrs.

2. Non-Icelandic Caucasian workers in Icelandic fisheries plants, a total of 41, age range 19-28 yrs.

3. Healthy Icelanders, a total of 38, age range 7-80 yrs.

The RF unit values for this group were mostly between 2 and 10 IU/ml (see Figure III.9.). Only 5 sera exceeded 20 units, and none reached 50 IU/ml. The highest serum level, 41 IU/ml, was from a healthy 80 year old Icelandic female.

In keeping with the suggestions of Ropes *et al* (1958), it was decided that levels above 20 IU/ml should be considered raised, as less than 5% (5/102) of the normal group exhibited such levels.

Thus standardised, the IgM RF ELISA test has been applied to about 830 serum and synovial fluid samples from the groups described in chapters V and VI. The results for groups of Icelandic patients

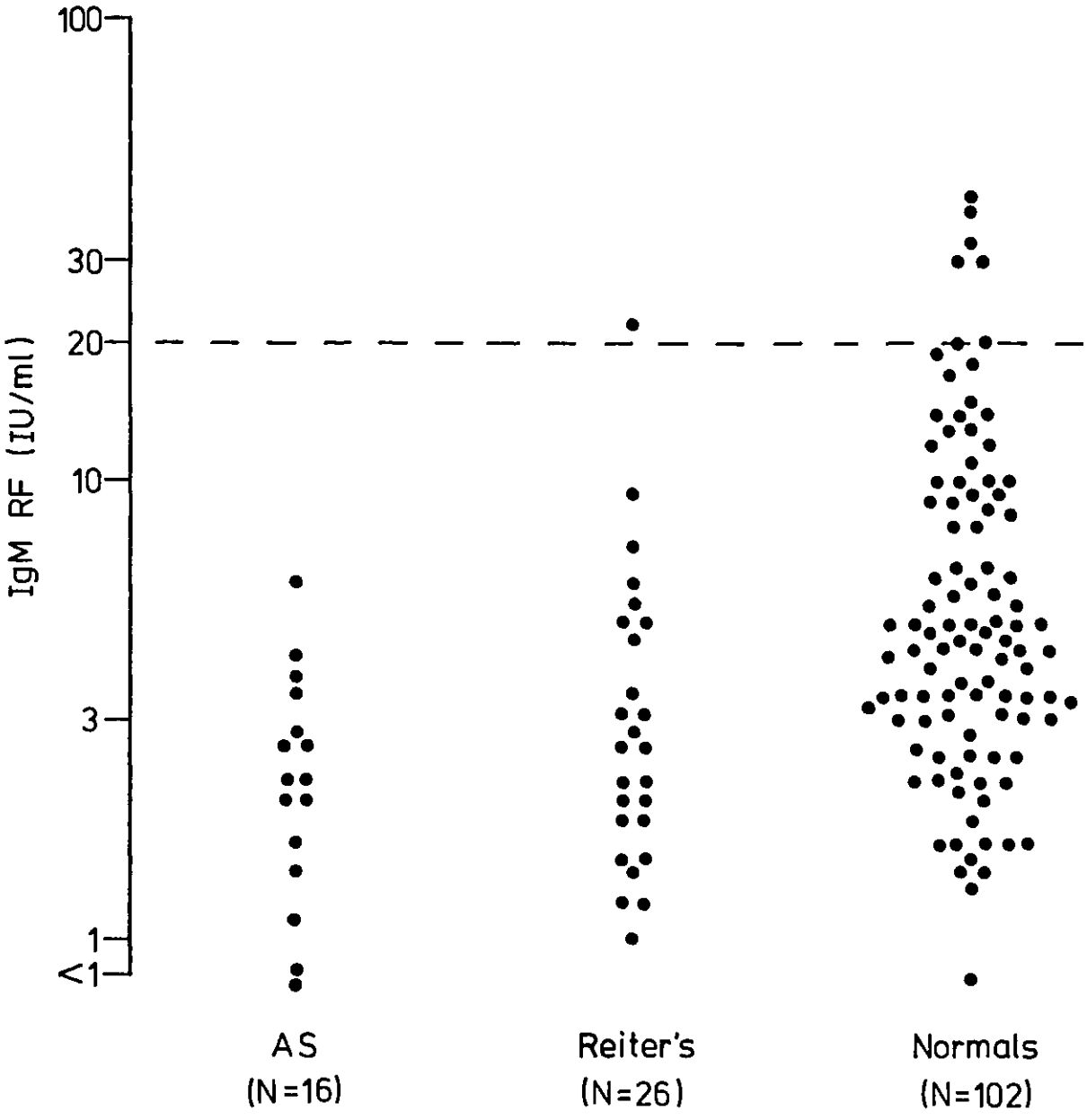


Figure III.9: IgM RF levels as measured by ELISA in 102 sera from normal individuals and in sera from Icelandic patients with ankylosing spondylitis (N = 16) and Reiter's disease (N = 26).

Raised IgM RF was defined as activity >20 IU/ml.

with Reiter's disease (N = 26) and ankylosing spondylitis (N = 16) are also shown in Figure III.9. Clearly, the distribution of these is very similar to that of the normal controls, only one patient exceeding 20 IU/ml.

III.1.5. Discussion

The observation (Table III.1.) that heat-aggregation of the IgM RF-substrate increases the binding of IgM RF, is not new. Indeed, Henney and Stanworth (1965) showed that RF (IgM RF) forms precipitates more easily with aggregated than native IgG. Koritz *et al* (1980) reported that heat-aggregated human and rabbit IgG preparations coupled to erythrocytes by chromic chloride, were far more effective than the corresponding native preparations in promoting agglutination with RF. However, Karsh *et al* (1980) who used goat IgG as substrate in their IgM RF ELISA, found that heat-aggregation of the substrate did not make any marked difference. That agrees with a second report from the same group (Halbert *et al*, 1980) where human IgG was used as substrate in IgM RF ELISA. Thus, it would seem that contrary to the agglutination tests, but in agreement with the results in chapter III.1.2.2., heat-aggregation of the IgM RF substrate in an ELISA/SPRIA system is unnecessary.

The findings that IgM RF reacts much more strongly with rabbit and human IgG than with IgG from the other four tested species (see Figure III.3.) is in agreement with the report of Koritz *et al* (1980) who found that erythrocytes coated with rabbit IgG were agglutinated more effectively than cells coated with swine IgG, and although sheep

IgG-coated cells were readily agglutinated by rheumatoid sera, the titration pattern was markedly different from that of the Rose Waaler test. However, the results presented in Figure III.3. and Table III.2. would suggest that the differences in binding of IgM RF to the six different substrates are quantitative rather than qualitative, as evidenced by the very similar ranking of the binding strength of the 14 sera to the six different substrates, e.g., the serum at the left hand end of Figure III.3. (RAHA negative) did not bind to any extent to any of the six substrates, whilst the serum with a RAHA-titre of 1:10,240 bound strongly to all six substrates.

The avid binding of IgM RF to IgG from species such as rabbit, goat, sheep and mouse may have direct practical consequences, as antibodies used in ELISA/SPRIA systems are often raised in these animal species. This might then lead to false positive reactions if the IgM RF, stuck to the solid phase, picked up the Fc-portion of a labelled IgG antibody. This problem will be discussed further in chapter III.2.

The correlation data presented in Table III.3. indicate that sheep IgG is antigenically more closely related to bovine and goat IgG than to IgG from rabbit, human or mouse. This is not surprising as the 3 first named animals are all ruminants. The implications of this will be discussed further in chapter IV.

Turning to the question of which IgG to select as RF substrate, the correlation data in Table III.2. indicate that the overall best correlation with the RAHA test is obtained by using bovine IgG as substrate, and testing the sera at a 1/10 dilution only. However, for reasons to be discussed in chapter III.3., ruminant IgG seems a poor

substrate for IgG RF tests. Moreover, for the purpose of comparison it is advantageous to use the same substrate for all three classes of RFs. Rabbit IgG was selected because:

(a) it reacted quite strongly with IgM RF; and

(b) it gave CNA results which correlated quite closely with the RAHA results.

However, different patterns of reactivity may well be observed with different substrates, e.g., human, horse or sheep IgG (Williams and Kunkel, 1963; Torrigiani and Roitt, 1975; Koritz *et al*, 1980). This could well be the subject of a separate ELISA study.

The expression of results in ELISA systems has been the source of considerable discussion in the literature. Frequently, the absorbance (optical density) value at one particular sample dilution has been taken, and either compared directly with the positive/negative reference samples or alternatively the results have been expressed as the ratio of the absorbance of the test sample to that of negative reference samples. Other methods have also been reported, such as titrations of samples where the titre is expressed as the dilution at which the test sample gives the same absorbance value as the negative reference sample (Voller *et al*, 1978). That, however, might require up to 10 dilutions of each sample, and is therefore inevitably both time consuming and expensive.

Bullock and Walls (1977), using an ELISA for the detection of antibodies to toxoplasma, found that no single dilution of serum could

measure all serum titres. These authors suggested that measurements at sample dilutions 1/10, 1/100 and 1/1000 should cover the levels of antibodies encountered in most patients.

Most SPRIA/ELISA workers have used a single sample dilution in IgM RF tests, varying from 1/10-1/1000 (Hay *et al*, 1975; Carson *et al*, 1977). However, the shape of the curves shown in Figure III.1. indicates that, in agreement with Bullock and Walls (1977), a combined net absorbance (CNA) value of more than one dilution would give a more quantitative expression of the RF levels. The correlation data in Table III.2. support this. Indeed, the CNA values at dilutions 1/10 + 1/100 + 1/1000 correlate, on the whole, better with the ranked RAHA-titre than any of the single dilutions or other dilution combinations shown in Table III.2. Thus, all samples were routinely tested at dilutions 1/10, 1/100 and 1/1000, and the CNA value was used to quantitate the IgM RF level. There are certain problems here, however. The use of three different dilutions is quite time consuming and there is evidence from Figure III.1. that dilution curves of different high-titre sera may not reach the same saturation (plateau) level. This may perhaps reflect different avidity of the RF populations involved, as it has been shown (Zollinger and Boslego, 1981) that dilution curves for low avidity monoclonal antibodies may have a much lower plateau than similar curves for antibodies of higher avidity. This might contribute to discrepancies between CNA values and RAHA titres for individual sera.

Several SPRIA workers have used monoclonal or even polyclonal RFs for standardisation of RF tests, whereupon the results can be expressed as µg antibody (IgM RF) per ml (Koopman and Schrohenloher, 1980). However, it may be even more meaningful to express the results

with reference to an internationally available and accepted standard. Hopefully, such standards will be more generally applied in the years to come, e.g., for estimations of different isotypes of rheumatoid factors.

A technical point of interest is that in the IgM RF test described in III.1.4.1. several sera exhibited rather high non-specific binding to the blank well, especially at dilution 1/10. This phenomenon, which was not observed in the IgA RF and IgG RF tests, necessitated the subtraction of each blank well from its corresponding test triplicate. Salonen and Vaheri (1979) reported that IgM seems to have a far greater tendency than IgG or IgA to adhere non-specifically to polystyrene, and my observation agrees with that.

It has been suggested (McLaren *et al*, 1981) that a quantitative ELISA should have an inter-assay variation of less than 10%. Table III.4. shows that the current ELISA falls short of that objective. However, my inter-assay CV is comparable to the 19% reported by Ziola *et al* (1978) for their IgM RF SPRIA. It is marginally better in terms of day-to-day variation to use the internal standard, but for inter-laboratory comparison, it is essential to use an internationally calibrated standard.

As to the sensitivity of the current assay, the calibration curve extends down to 0.85 IU/ml, and in practice, very few sera were found to exhibit lower levels than that (see Figure III.9.). Thus, the assay is quite sensitive and it could probably be adapted for IgM RF estimations in tissue culture supernatants.

The upper limit of normal (20 IU/ml) in the current assay agrees well with the report of Ziola *et al* (1978), who found only 4.9% (13/267) of blood donors and staff controls to have levels above 20 IU/ml. Also, Halbert *et al* (1980) found 4% (7/174) of normals above this level.

Although the amount of mathematics involved in the calculation of test results is considerable, this should pose no problem, as microplate readers can be linked directly with computers. Also, written programmes for ELISA work are beginning to appear in the literature (Ritchie *et al*, 1981) and no doubt the availability of these will further stimulate ELISA workers to make use of computer technology.

III.1.6. Conclusion

During the establishment of ELISA for IgM RF measurement, heat-aggregation of the substrate was found unnecessary. IgM RF generally seems to react with murine and ruminant IgG, albeit not as strongly as with human or rabbit IgG. Using rabbit IgG as substrate and expressing the results as the combined net absorbance at sample dilutions 1/10 + 1/100 + 1/1000, the RF levels correlate closely with those of the RAHA test. The assay is sensitive and quantitative, and when the results are expressed as CNA, the day-to-day reproducibility was between 10 and 20%. After converting the results to international RF units, the upper limit of normal for serum was 20 IU/ml, which is in good agreement with previous reports.

III.2. IgA RHEUMATOID FACTOR

III.2.1. Introduction

In 1966 Heimer and Levin reported the results of their experiments with sera from six, and salivary fluids from a large number of RA patients, where they absorbed the sera with a solid, heat denatured human fraction II (Heimer and Levin, 1966). After eluting the solids in acid, the authors demonstrated by immuno-electrophoresis (IEP) the presence of IgA-like proteins in the eluate, and by density gradient ultracentrifugation they showed that some of the IgA which sedimented with a rate of 7S had rheumatoid factor activity as judged by the latex test. Furthermore, this agglutinating activity was not abolished by reduction with 2-mercapto-ethanol (2-ME). IgA RF activity was found in one of the salivary samples.

This is apparently the first definite report of IgA having RF activity, but Williams and Kunkel (1963) had previously observed the development of a beta-2A arc on IEP when testing fractions of rheumatoid sera, eluted off columns with human or rabbit IgG. Thus, their report is suggestive of the presence of IgA RF.

Torrigiani and Roitt (1967), using insoluble rabbit γ-globulin to absorb rheumatoid antiglobulins, followed by acid elution and Ig-estimation by immunodiffusion, detected raised levels of IgA anti-globulins in approximately 50% of RA patients.

Estes *et al* (1973) and Kallerup *et al* (1979), using indirect immunofluorescent techniques, demonstrated IgA RF activity in some

rheumatoid sera. Kallerup *et al* further assessed the IgA RF content of the international RF-standard and found that whereas IgM RF could be demonstrated in dilutions of the international standard containing 1.0 IU/ml, IgA RF activity could not be demonstrated in dilutions containing under 7.5 IU of IgM RF/ml, thus indicating a low IgA RF content of the standard.

Dunne *et al* (1979), using a SPRIA, similar to the IgM and IgG antiglobulin assay of Hay *et al* (1975), showed that IgA RF reacts, not only with human and rabbit, but also with bovine and guinea pig IgG, in a similar fashion to IgM RF. These authors also found that IgA RF reacts more strongly with IgG1, IgG4, and IgG2 than with IgG^3, which agrees with the study of Abraham *et al* (1972) on a monoclonal IgA RF. Dunne *et al* furthermore found that groups of patients with RA, Sjoegren's syndrome (SS) with RA, SS without RA, and SLE all had higher mean IgA RF levels than normal controls. These authors also noted increased salivary fluid levels of IgA RF and IgM RF in patients with RA and SS with and without RA.

IgA class antiglobulins are not restricted to humans as Van Snick and Masson (1980) demonstrated the occurrence of IgA and IgM anti-globulins in various mouse strains, including 129/Sv, CH3 and DBA/2. Interestingly, it was found that the IgA anti-IgG was restricted in specificity, reacting only with mouse IgG2a. These authors also found evidence that environmental, as well as genetic factors, were involved in the induction of anti-IgG because when assaying the sera, marked intra-strain differences were observed, depending on which laboratory the mice had been raised in.

III.2.2. Preparation of anti-human IgA reagents

III.2.2.1. Introductory remarks

It has been realised, since the introduction of solid phase immunoassays for antiglobulin measurements, that the presence of IgM RF may, theoretically at least, give rise to false positive or exaggerated responses in IgG antiglobulin tests (Nineham *et al*, 1976; Carson *et al*, 1977). One of the possible mechanisms is that IgM RF, bound to IgG (or Fc-gamma) on the solid phase, may still have free binding sites which can fix radio- or enzyme-labelled antibody via the Fc portion of the latter (see Figure 1.3.B.). This can give rise to a false positive IgG RF test, and the same may obviously apply to IgA RF tests.

In order to evaluate the theoretical advantage of pepsin-digesting the anti-IgA antibody used in IgA RF assays, it was decided to include the preparation of anti-IgA antibody in the current project.

III.2.2.2. Materials and methods

Human IgA, isolated from a myeloma serum by DEAE chromatography, was kindly provided by Dr. Pamela W. Ewan, St. Mary's Hospital. IgA-deficient plasma (IgA-DP) was a gift of Dr. O. Jensson, Director of the Blood Bank, Reykjavik, Iceland. After defibrination with 1500 U.S. (N.I.H.) units of thrombin (Topical thrombin, Parke-Davis) per 400 ml of plasma, the plasma contained 1100 mg IgG/100 ml, 77 mg IgM/100 ml and approximately 1 mg IgA/100 ml, as measured with a Hyland Laser Nephelometer using commercial nephelometric kits (Hyland LAS-R, Travenol Laboratories Ltd., U.K.).

The IgG fraction of a sheep anti-human IgA antiserum was a gift of Dr. A. R. Bradwell, Birmingham University, U.K. This serum had been rendered specific for the alpha-chain of human IgA by absorption with insolubilised IgA deficient human serum. This preparation contained 28.6 mg/ml of protein, measured in a UV spectrophotometer at 280 nm.

Immunisation schedule. Four New Zealand White rabbits (numbered I-IV) were each immunised on day 0 with approximately 50 µg IgA emulsified in Freund's complete adjuvant (FCA) (Difco Laboratories, Michigan, U.S.A.). The injections were always given subcutaneously into four different sites. The animals were then test-bled from an ear vein and boosted, first on day 28 with 50 µg IgA in FCA, then on days 42, 56, and 81, with 1.8-2.0 mg IgA in incomplete Freund's adjuvant (Difco). Rabbit No. I (r I) was exsanguinated on day 68, rabbit No. II (r II) on day 88 and rabbits III (r III) and IV (r IV) on day 92, each rabbit yielding on average 90 ± 12 g serum.

Assessment of anti-IgA response. Sera from the test-bleedings were analysed for anti-human serum activity by IEP and reverse single radial immunodiffusion (RSRID), using a protocol provided by Dr. A. R. Bradwell (personal communication). Briefly, the IEP testing was done by electrophoresing 5 µl normal human serum (NHS) on slides containing 1% agarose, 3% PEG, at approximately 110 volts for 60 mins. (barbitone buffer, pH 8.4). The slides were subsequently incubated overnight in a humid chamber with the rabbit serum to be tested in the troughs.

One trough on each IEP slide always contained the untreated rabbit serum, but the other contained serum pre-absorbed by incubation

(30 mins. at r.t.) with 30 µl IgA-DP per 200 µl rabbit serum.

The RSRID slides were prepared by mixing 25 µl NHS with 3 ml 1% agarose, 3% PEG. Subsequently, 5 µl of absorbed and non-absorbed rabbit serum were put into each well, followed by diffusion in a humid chamber overnight. The sheep anti-human IgA antibody served as a reference on the IEP and RSRID slides.

Isolation and purification of anti-human IgA antibody. Gamma-globulin fractions were isolated from the rabbit sera by the ammonium sulphate precipitation method (Hudson and Hay, 1980). These fractions (up to 20 mg at a time) were adsorbed with an affinity column containing IgA-DP. Briefly, this column was prepared by coupling 0.71 ml of the defibrinated IgA-DP, containing approximately 30 mg protein (OD at 280 nm) to 3 g of CNBr-activated Sepharose 4B (Pharmacia Fine Chemicals, Uppsala, Sweden), using the protocol of the manufacturer. The coupled Sepharose was packed into a 5 ml glass pipette and washed with PBS containing 0.02% Na N_3.

Between runs the column was eluted with 0.1 M glycine-HCl buffer, pH 2.5.

The adsorbed fractions were concentrated using an Amicon ultra-filtration system (filter type XM 100 A) under a pressure of 15 lbs/in^2 (psi) to a protein concentration of 5-10 mg/ml.

Preparation of $F(ab')_2$ from the anti-human IgA antibody. Having ensured the specificity of the adsorbed rabbit anti-human IgA gamma globulin by IEP, several Amicon-concentrated fractions from rabbit III

were pooled. This gamma-globulin was then pepsin-digested as described by Garvey *et al* (1977). Briefly, about 35 mg of gamma-globulin were dialysed overnight against 0.07 M acetate buffer, pH 4.0, then centrifuged to remove precipitate. About 14.5 mg of this protein was mixed with 0.5 mg pepsin (porcine, x 2 crystallised, Sigma, London), yielding an enzyme/protein ratio of 3/100. This mixture was incubated in a 37°C waterbath for 18 hrs. The rest of the gamma-globulin (14.5 mg) was also incubated as a pepsin-free control.

Following the incubation, the digest and the control were centrifuged to remove precipitate, and neutralised to pH 8.0 with a 1 M solution of TRIS.

The digest and control were passed separately through a Sephadex G-150 Superfine chromatography column (void volume 35-40 ml) in order to separate $F(ab')_2$/IgG from smaller peptides, and fractions of 1.1 ml were collected sequentially.

The first two protein peaks of the digested fraction were collected separately, Amicon-concentrated and tested by IEP for reactivity with NHS.

The completion of pepsin digestion determined by ELISA. Microplate wells were coated with 20 µg/ml of rabbit gamma-globulin from three sources:

(a) the first Sephadex G-150 peak of the pepsin-digested antibody;

(b) the undigested control;

(c) normal rabbit IgG.

Following BSA saturation, two different protocols were used:

1. Some of the wells were incubated with dilutions of a rheumatoid serum containing a high titre of IgM RF (RAHA test), followed by a dilution of anti-human IgM-AP conjugate.

2. Other wells were incubated with dilutions of the IgA used for immunisation (final concentrations 63, 31 and 16 µg IgA/ml), followed by anti-human IgA-AP conjugate.

Finally, the whole of the plate was incubated with pNPP.

The preparation of enzyme-conjugates, and inter-calibration of these. Having demonstrated that the first Sephadex G-150 peak of the pepsin digest ($F(ab')_2$) was unreactive with IgM RF in the ELISA system, yet could bind IgA, 4.8 mg of this protein were coupled to 2500 units of alkaline phosphatase, using the procedure described in chapter II. This conjugate will henceforth be referred to as $F(ab')_2$-A-AP.

Also, 5.0 mg of the undigested antibody were conjugated to 2,500 units of alkaline phosphatase, henceforth referred to as IgG-A-AP. These two conjugates were then tested on microplates coated with increasing dilutions of human IgA in order to compare their activities.

III.2.2.3. Results

Relative anti-human IgA titres of the rabbit sera. Table III.5. shows the comparative titres of the sera from rabbits I, III and IV, before and after absorption with IgA-DP. The serum from rabbit III clearly had the highest titre, and was therefore used exclusively for the making of the $F(ab')_2$-A-AP and IgG-A-AP.

Figure III.10. shows that the anti-human IgA activity of the adsorbed gamma-globulin fraction of r III was somewhat stronger than that of the reference sheep anti-human IgA antibody.

Specificity of the rabbit serum fractions. Figure III.11. shows three IEP slides (anode: right hand end), where the specificity of several preparations was tested. It can be seen that after the IgA-DP adsorption both the anti-human IgA antibody (slide "III", lower trough) and the digested $F(ab')_2$ preparation (slide "5") were specific as judged by IEP.

Sephadex chromatography of the digest/control. Figure III.12. shows the OD profiles (at 280 nm) for the 1.1 ml fractions collected from the Sephadex G-150 column. In Figure III.12.A., the profile for pepsin-digested rabbit anti-human IgA is shown. Clearly, there are two main protein peaks (1 and 2) with some trace of protein in between. Figure III.12.B. shows the analogous profile for the non-digested control of the rabbit anti-human IgA gamma-globulin. As expected, only one peak is visible here, presumably containing IgG. Peak 1 in Figure III.12.A. would be expected to contain $F(ab')_2$ plus possibly some undigested IgG (Stanworth and Turner, 1973). It can be seen that

Table III.5: The anti-human serum activity of rabbit sera, after immunisation with human IgA.

Source of serum	Pre-test absorption with IgA-DP	Ring diameter (mm)
Rabbit I	yes	5.1
	no	5.4
Rabbit III	yes	5.8
	no	6.0
Rabbit IV	yes	4.8
	no	5.1

The test system used was reverse single radial immunodiffusion (RSRID) with normal human serum (NHS) in the gel.

The rabbit sera were tested before and after absorption with IgA-deficient human plasma (IgA-DP).

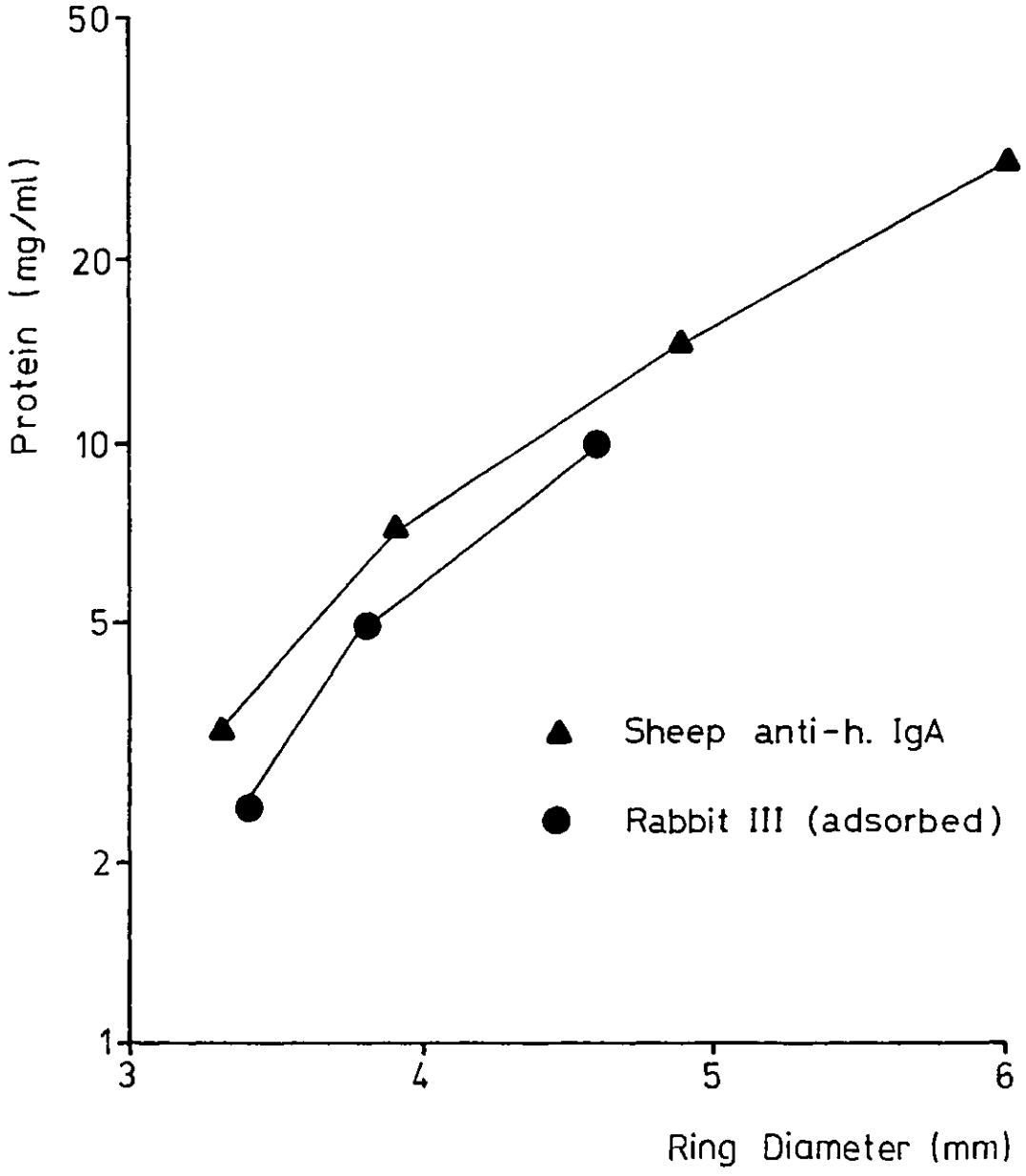


Figure III.10: Comparison of anti-human IgA titres of the reference sheep anti-human IgA and the serum γ-globulin fraction from rIII, after adsorption on the IgA-DP column.

The γ-globulin concentration (O.D. at 280 nm) is expressed as a function of the ring diameter in the RSRID system (see III.2.2.2.).

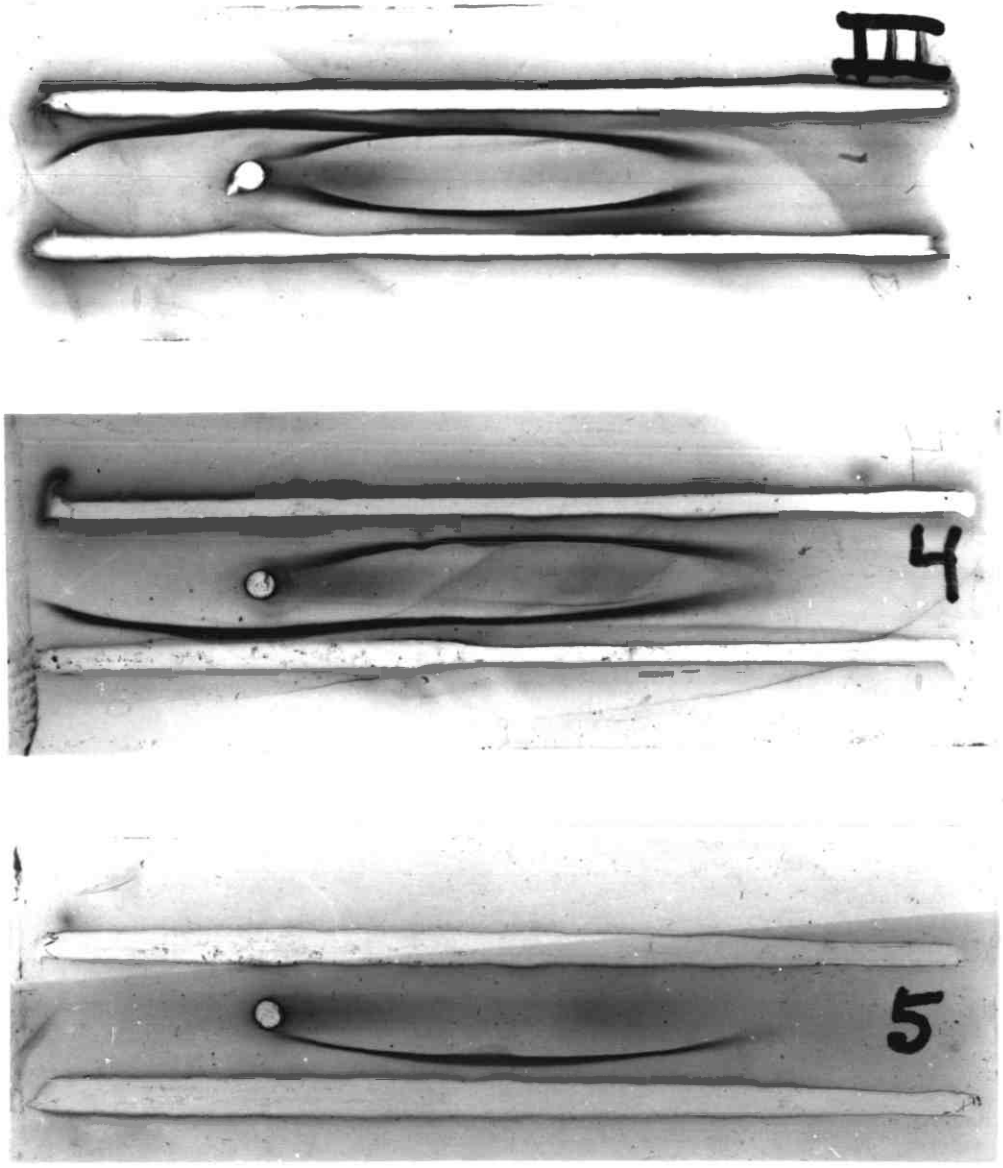


Figure III.11: Anti-NHS activity of rabbit γ-globulin fractions before and after adsorption with IgA-DP. (IEP slides; anode: right hand end).

Slide "III" shows the anti-NHS reactivity of rIII γ-globulin fractions, before (upper trough) and after (lower trough) adsorption on the IgA-DP column.

Slide "4" shows the analogous reactivity of IgA-DP-adsorbed rI γ-globulin (upper trough) and the reactivity of the acid eluate of the IgA-DP column (lower trough, presumably anti-light chain antibody).

Slide "5" shows the anti-NHS reactivity of the 1st protein peak shown in fig.III.12.A.,i.e. the $F(ab')_2$ fraction of the IgA-DP-adsorbed γ-globulin from rIII.

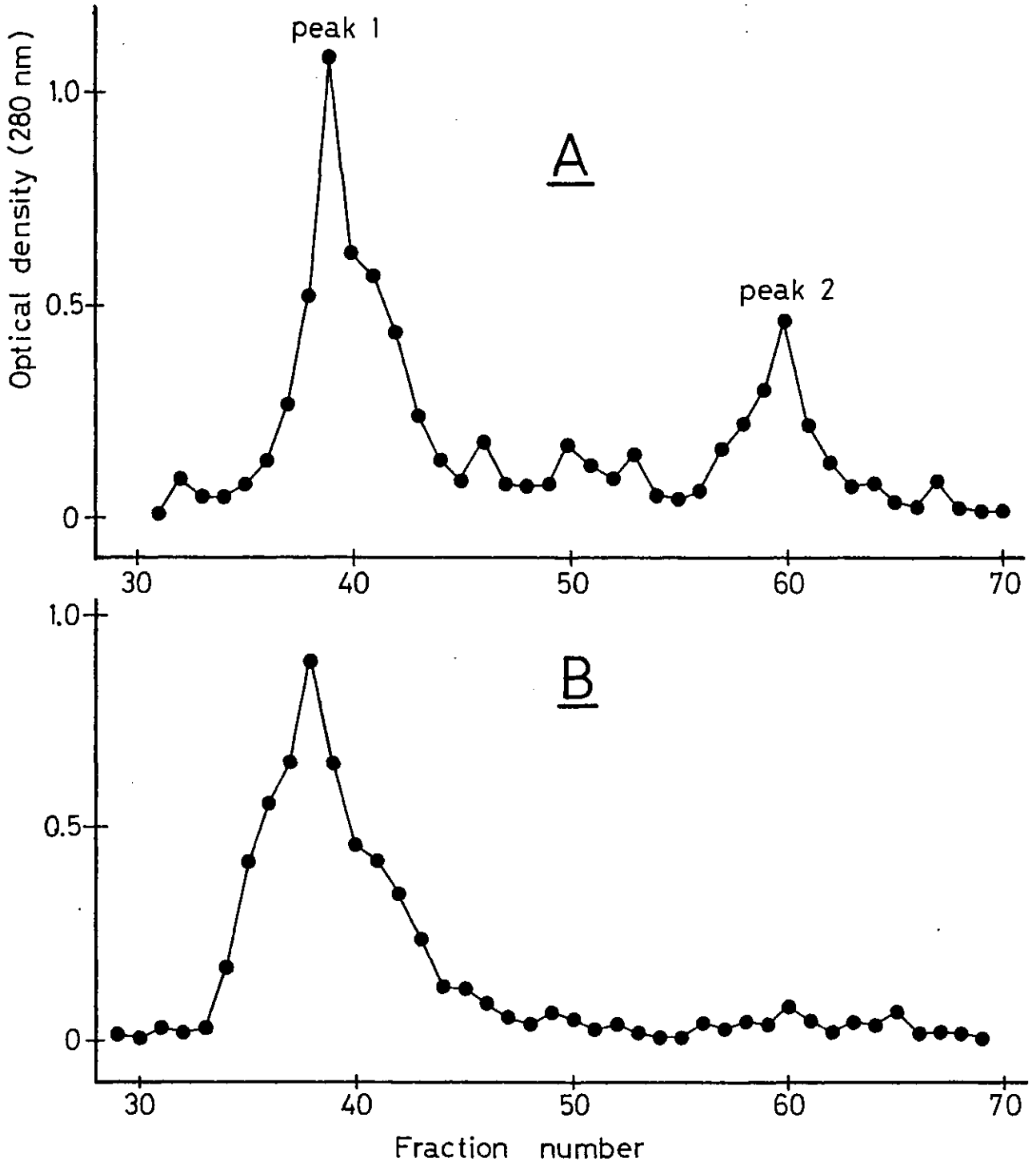


Figure III.12: Elution profiles of pepsin-digested and undigested rIII γ-globulin from Sephadex G-150 S chromatography column.

A: Pepsin digested preparation

B: Undigested control

Fractions of 1.1 ml were collected, the first 30 fractions eluted did not contain any significant amount of protein.

both peak 1 in Figure III.12.A. and the peak in Figure III.12.B. appear approximately at the void volume of the column, 40 ml.

IgM RF and IgA reactivity of the digested anti-human IgA. Figure III.13. shows the results of simultaneous ELISA-testing of three protein preparations for reactivity with IgM RF and human IgA. The undigested control was from the main protein peak shown in Figure III.12.B., and the pepsin digest from the first protein peak shown in Figure III.12.A. The third was normal rabbit IgG (Miles Laboratories Ltd.). Briefly, Figure III.13.A. shows that both native rabbit IgG and the undigested rabbit gamma-globulin bound IgM RF quite avidly, whereas the digest ($F(ab')_2$) totally failed to do so. However, Figure III.13.B. shows that at the coating concentration used (20 µg/ml) the $F(ab')_2$ bound IgA somewhat more effectively than the undigested preparation. As expected, the native rabbit IgG bound almost no IgA.

In a separate experiment, normal rabbit IgG was mixed with thoroughly pepsin-digested rabbit gamma-globulin ($F(ab')_2$) and a final concentration of 80 µg/ml was then used to coat microplate wells, followed by dilutions of the rheumatoid serum used in the experiment in Figure III.13. Figure III.14. shows that as little as 0.5-1% contamination of the digest with normal IgG could be detected in this system.

Comparison of anti-human IgA enzyme conjugates. Six serial dilutions (1/500-1/3800; a dilution factor of 1:1.5) were made of both the $F(ab')_2$-A-AP and IgG-A-AP conjugates. These were then tested on plates coated with doubling dilutions of IgA. Figure III.15. shows four of the dilution curves obtained. These are representative of the

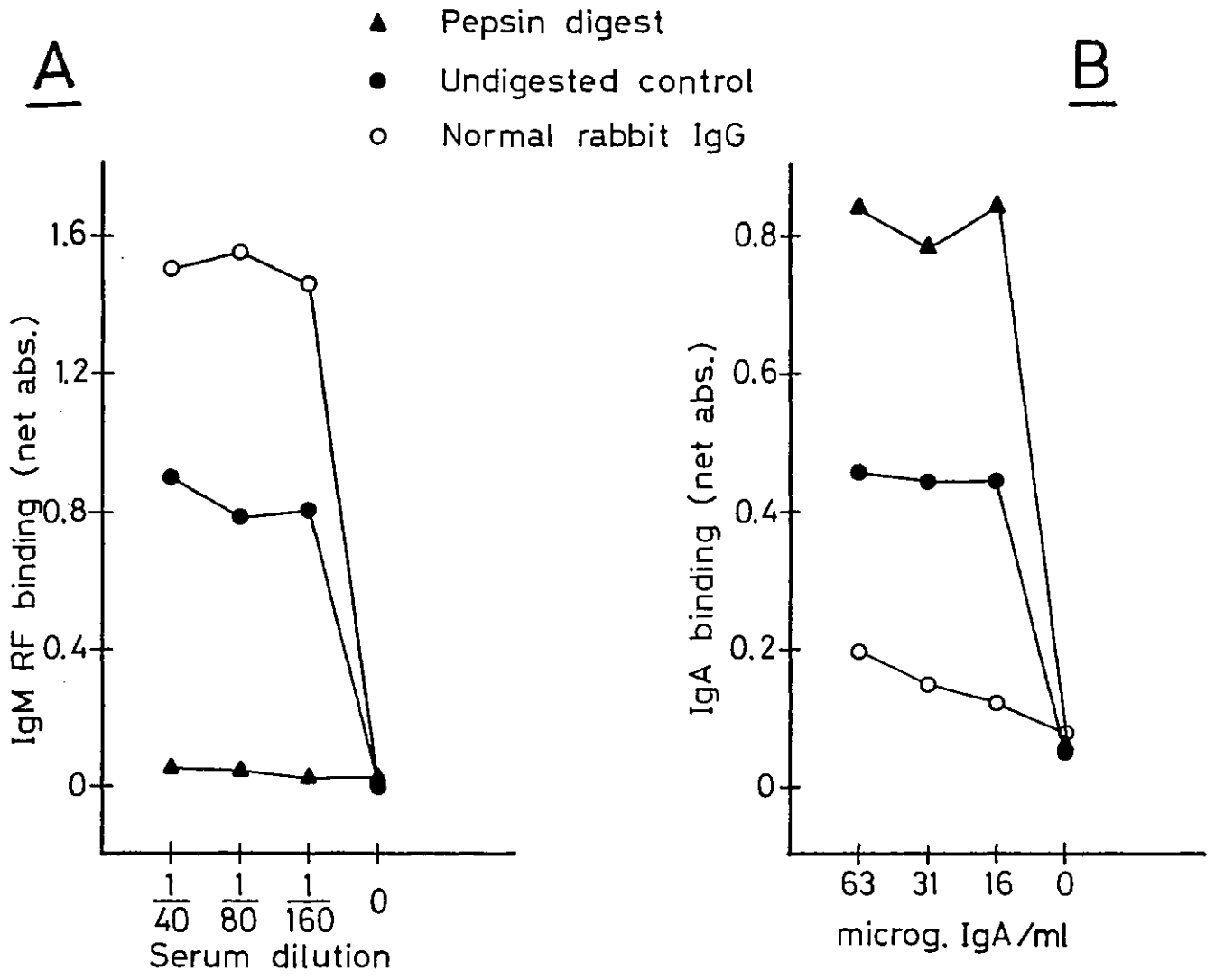


Figure III.13: The reactivity of three rabbit γ-globulin preparations with IgM RF and human IgA.

A: The binding of IgM from a rheumatoid serum to the protein from peak 1 in fig.III.12.A. (triangles), to the protein from the peak in fig.III.12.B. (closed circles) and to normal rabbit IgG (open circles).

B: The binding of human IgA to the same three preparations as in A.

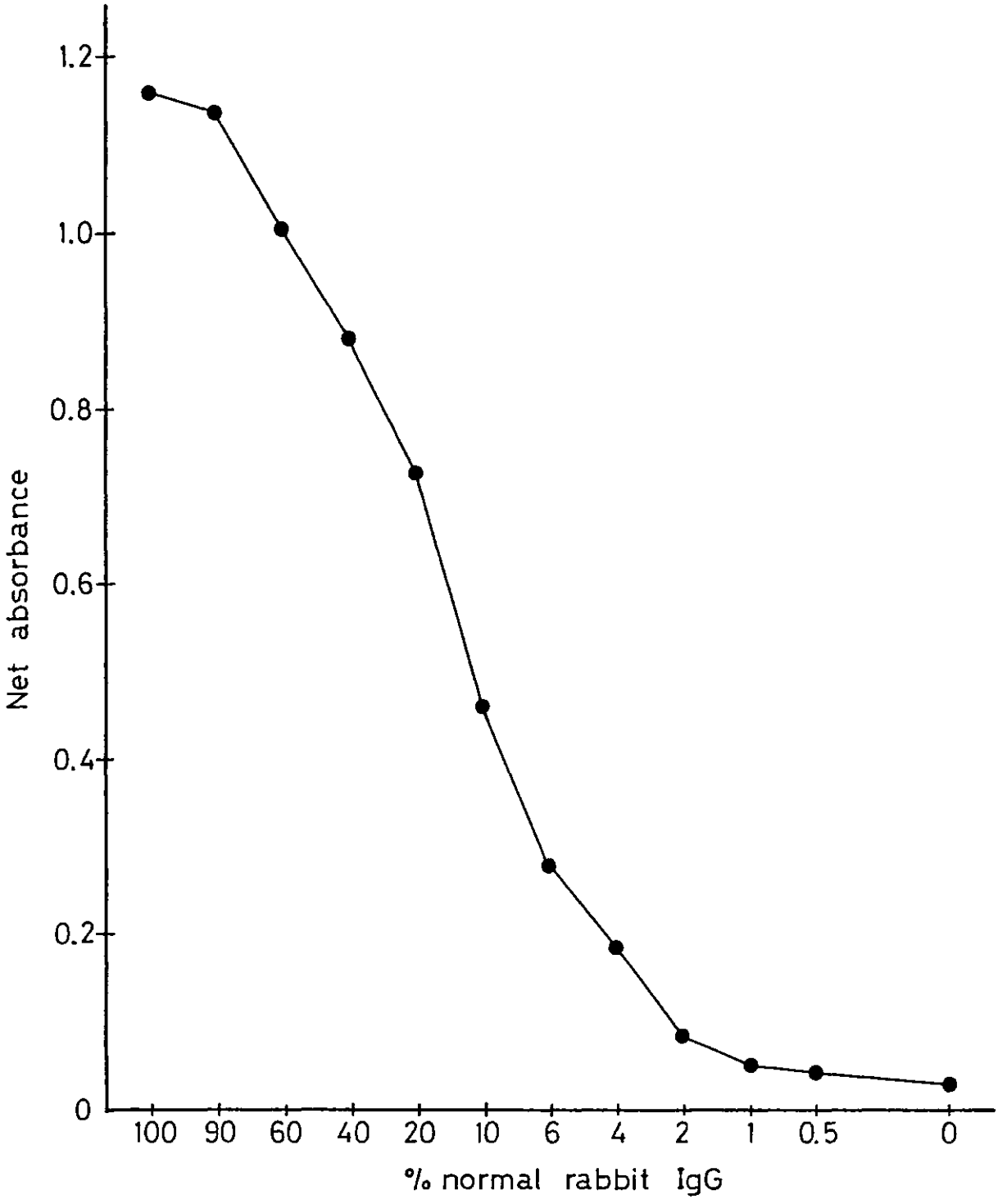


Figure III.14: The binding of IgM from a rheumatoid serum to mixed pepsin-digested rabbit γ-globulin and normal rabbit IgG.

Micro-ELISA plates were coated with a final conc. of 80 µg/ml of a mixture of the digest and normal rabbit IgG, followed by a dilution of rheumatoid serum and anti-human IgM-AP conjugate.

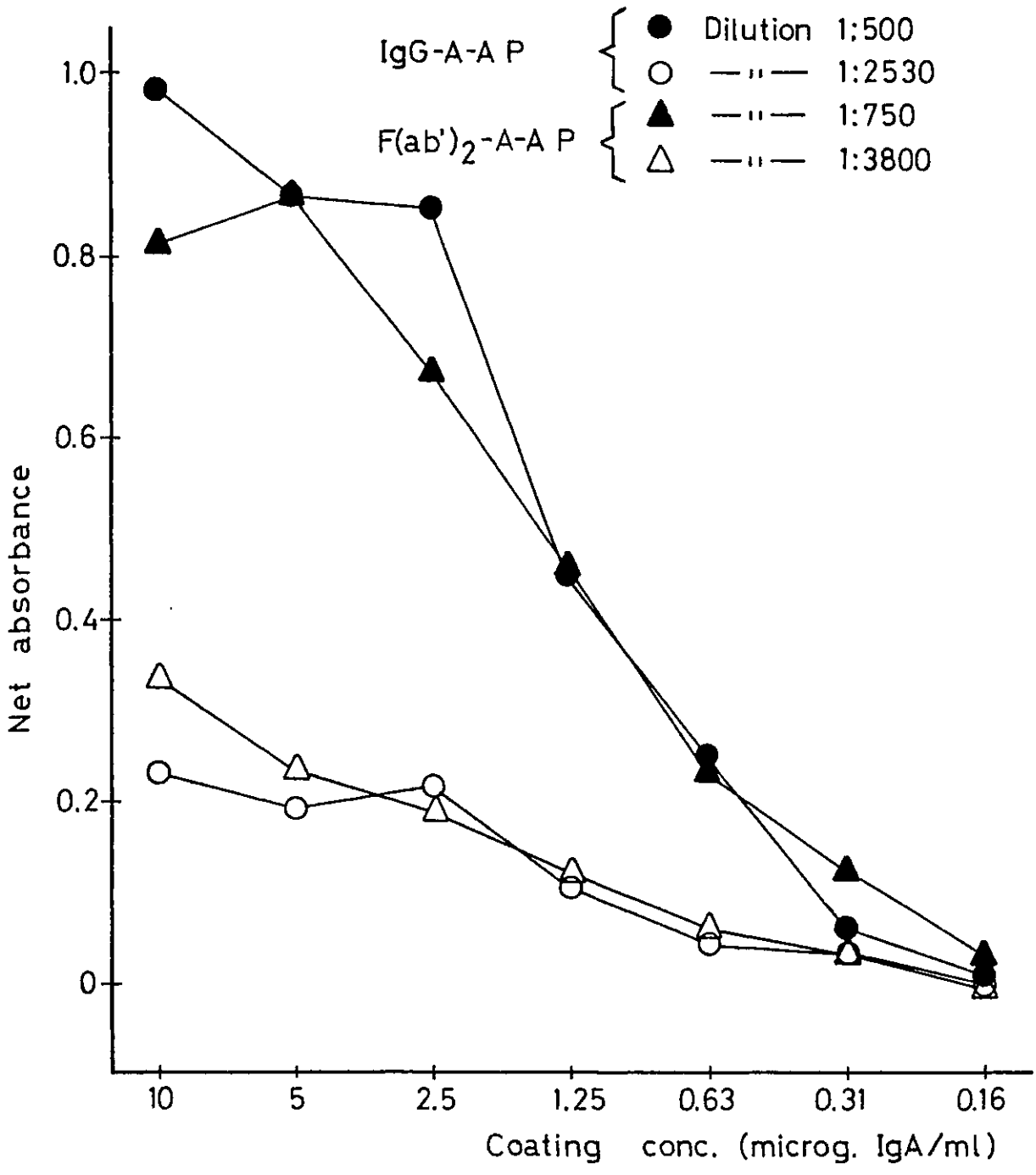


Figure III.15: Simultaneous testing of two anti-human IgA-AP conjugates for reactivity with human IgA.

The pepsin-digested antibody-AP conjugate ($F(ab')_2$-A-AP) had 1.5 times greater activity than the undigested conjugate (IgG-A-AP).

rest, indicating that irrespective of the absolute dilution, if the $F(ab')_2$-A-AP was 1.5 times more dilute than the IgG-A-AP, the dilution curves were similar. The graphical impression was further strengthened by calculating the difference between the five similar pairs of graphs, each having seven pairs of absorbance values. For the 35 absorbance pairs, the IgG-A-AP values (dilution strength IgG-A-AP:$F(ab')_2$-A-AP = 1.5:1.0) were on average 0.007 ± 0.063 absorbance units higher than the ones for $F(ab')_2$-A-AP. The paired student's t test indicated that this difference was not significant, $p > 0.1$.

Accordingly, in comparative serum antiglobulin tests, the $F(ab')_2$-A-AP and IgG-A-AP were used at final dilutions of 1/750 and 1/500, respectively.

III.2.3. Different assay conditions tested.

III.2.3.1. Comparison of $F(ab')_2$-A-AP and IgG-A-AP

At the time when the two conjugates were made, as described in chapter III.2.2., very little information was available in the literature as to the practical advantage of using pepsin-digested antibody for IgA antiglobulin detection. The following experiment was performed to shed some light on the issue.

Materials and methods: Serum samples from 27 rheumatic patients, most of whom had RA, were studied. The RAHA titres for 25 of these sera were available, ranging from negative (<1:40) to 1:1280.

The antiglobulin assay was performed in microplates coated with 10 µg/ml rabbit IgG as described for IgM RF (chapter III.1.4.1.). All

sera were incubated in doubling dilutions, 1/10-1/160, followed by 1/500 dilution of IgG-A-AP and 1/750 dilution of $F(ab')_2$-A-AP. Each conjugate was tested in a duplicate of coated and blank wells for each serum dilution.

Each serum was tested on one plate only, in order to enable better comparison of the two conjugates.

In order to test the reproducibility of the experiment, it was repeated, using identical conditions, on a different day.

Results: Using $F(ab')_2$-A-AP, the inter-assay reproducibility, expressed as the correlation between the CNA values on day 1 and day 2, is shown in Figure III.16. The CNA values on day 2 were on average 37% higher than those on day 1, but their ranking was very similar ($r = 0.953$) on the two days.

The CNA values for dilutions 1/10-1/160 of each individual serum on day 2 are shown in Table III.6. The mean CNA for the digested conjugate was only 33% of the CNA obtained with the undigested conjugate.

When the dilution curves for the individual sera were plotted, some striking features emerged:

(a) The absorbance values obtained with the pepsin-digested conjugate were almost always lower than those obtained with the undigested conjugate. Nevertheless, several sera showed considerable reactivity with the digested conjugate (see Figure III.17. and Table III.6.).

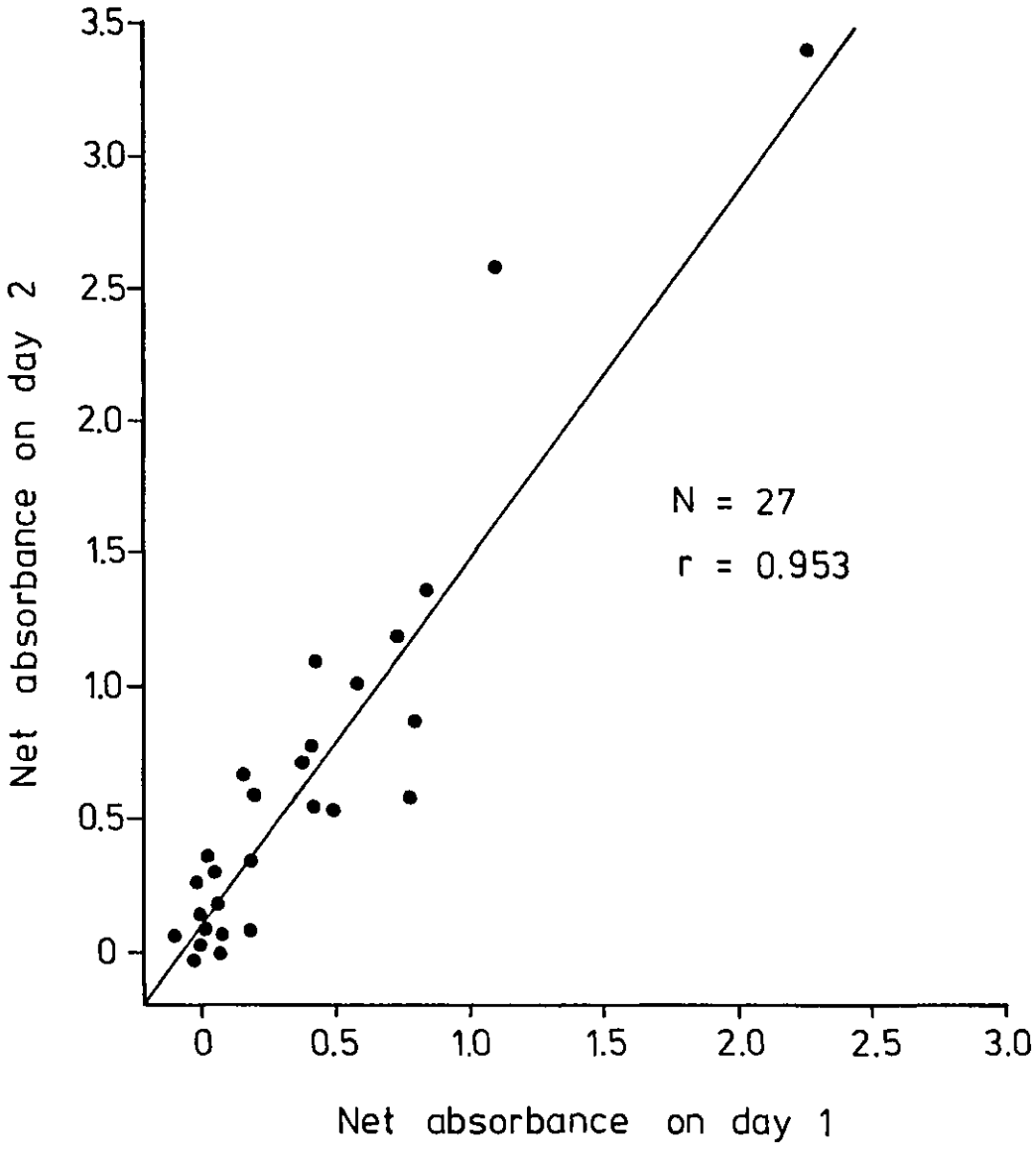


Figure III.16: Day-to-day reproducibility of an IgA RF ELISA, using the $F(ab')_2$-A-AP conjugate.

Twenty-seven sera were tested on two different days, the results expressed as the CNA values at dilutions 1/10 + 1/20 + 1/40 + 1/80 + 1/160.

There was a highly significant correlation between results from the two different days ($r = 0.953$, $p < 0.001$, the regression equation: $y = 1.37X + 0.098$).

Table III.6: Comparison between digested $(F(ab')_2$ -A-AP and non-digested (IgG-A-AP) conjugates for IgA RF ELISA.

		CNA (1/10 - 1/160)	
Serum	RAHA titre	IgG-A-AP	$F(ab')_2$-A-AP
O'Br	1:80	1.721	0.572
Pe	Neg	0.338	0.027
Ve	1:1280	4.413	3.383
La	1:160	3.701	0.870
Co	-	2.601	0.259
Se	1:640	2.404	2.575
At	Neg	0.400	0.048
Hu	Neg	1.195	0.296
Mi	1:640	2.463	0.352
Br	Neg	0.852	0.066
Ha	Neg	1.852	1.098
Co	1:640	2.757	0.643
Wi	1:80	1.101	0.163
Bro	1:40	1.874	0.333
Ba	1:40	1.647	0.540
Ro	Neg	1.417	0.077
Ru	1:320	4.182	1.005
Te	1:40	2.844	1.184
Re	Neg	0.216	0.030
Ki	-	0.262	0.060
Dr	1:320	3.009	0.526
Up	Neg	0.939	0.101
Bo	1:80	1.573	0.755
Roz	1:160	2.732	1.353
Bar	1:160	2.909	0.722
Th	1:640	2.929	0.601
Wo	Neg	0.975	0.018
Mean		1.974	0.656
SD		1.176	0.784

Twenty-seven sera from rheumatic patients were tested, the IgM RF titres (RAHA) for 25 of these are shown.

Each serum was tested in one microplate for reactivity with both conjugates, the results expressed as the CNA values at serum dilutions 1/10 + 1/20 + 1/40 + 1/80 + 1/160.

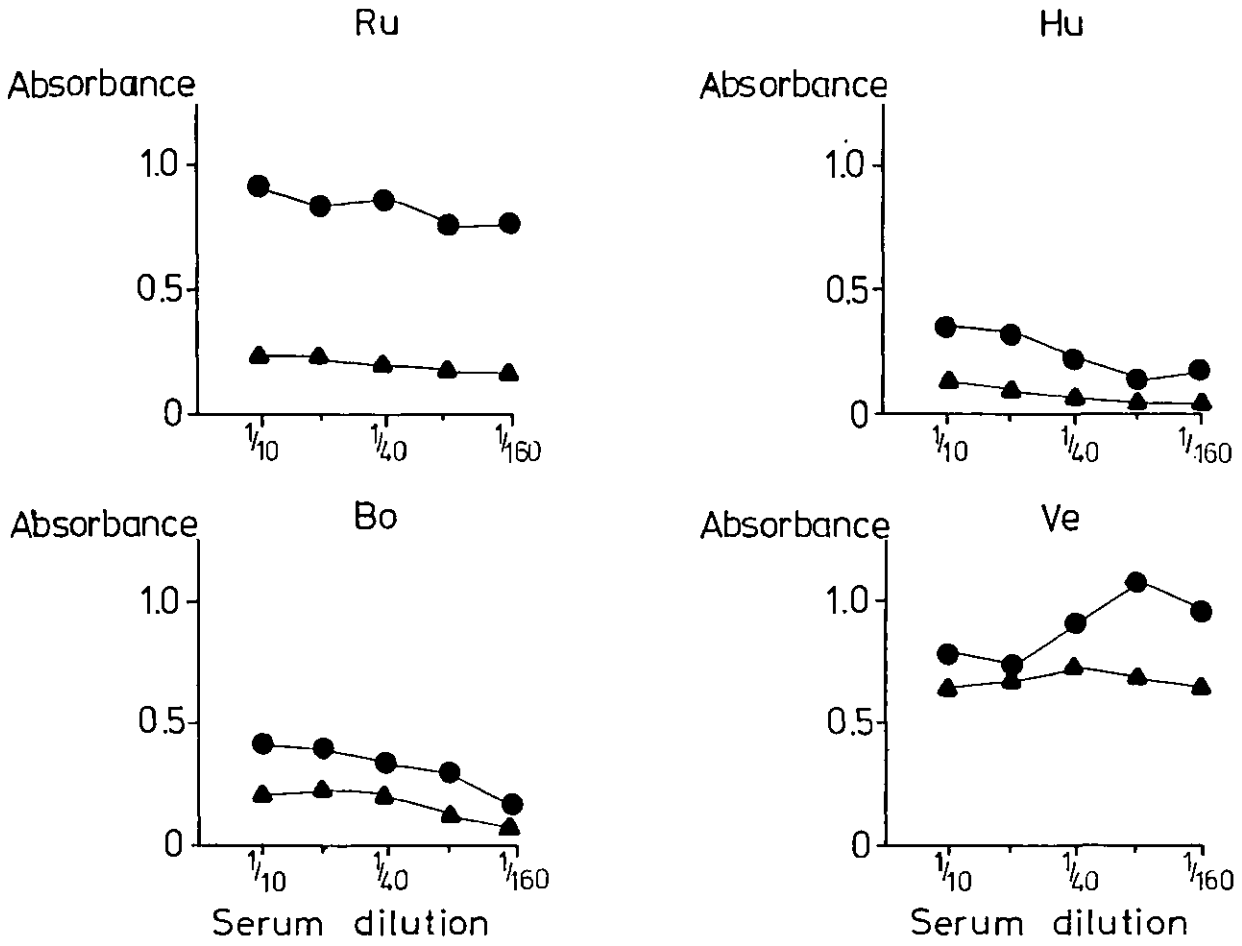


Figure III.17: Comparison between pepsin-digested and non-digested conjugates for IgA RF ELISA.

Dilution curves for four rheumatoid sera are shown, for both the digested conjugate ($F(ab')_2$-A-AP, dilution 1/750, closed triangles) and the non-digested conjugate (IgG -A-AP, dilution 1/500, closed circles) under otherwise identical conditions, see III.2.3.1.

(b) When the net CNA decreases (i.e., CNA for IgG-A-AP minus CNA for $F(ab')_2$-A-AP) were plotted against the RAHA titre (Figure III.18.), a significant positive correlation was observed ($r = 0.503$, $0.01 < p < 0.02$), suggesting that the observed difference between the two conjugates was indeed related to the level of IgM RF.

These results clearly indicate the advantage of using pepsin-digested, rather than undigested, antibody in IgA antiglobulin tests, at least under conditions similar to those described here.

In all the IgA RF assays described below, $F(ab')_2$-A-AP was the conjugate used.

III.2.3.2. Selection of coating concentration for the IgA RF ELISA

Although most SPRIA/ELISA workers have used coating concentrations of 5-10 µg/ml in antiglobulin tests (Hay *et al*, 1975; Gripenberg *et al*, 1979), Dunne *et al* (1979) used 60 µg human IgG/ml for coating in their IgA RF SPRIA. However, these authors did not present any data to show the advantage of this rather high concentration.

In order to assess the possible advantage of using 60 rather than 10 µg/ml for coating, the following experiment was performed.

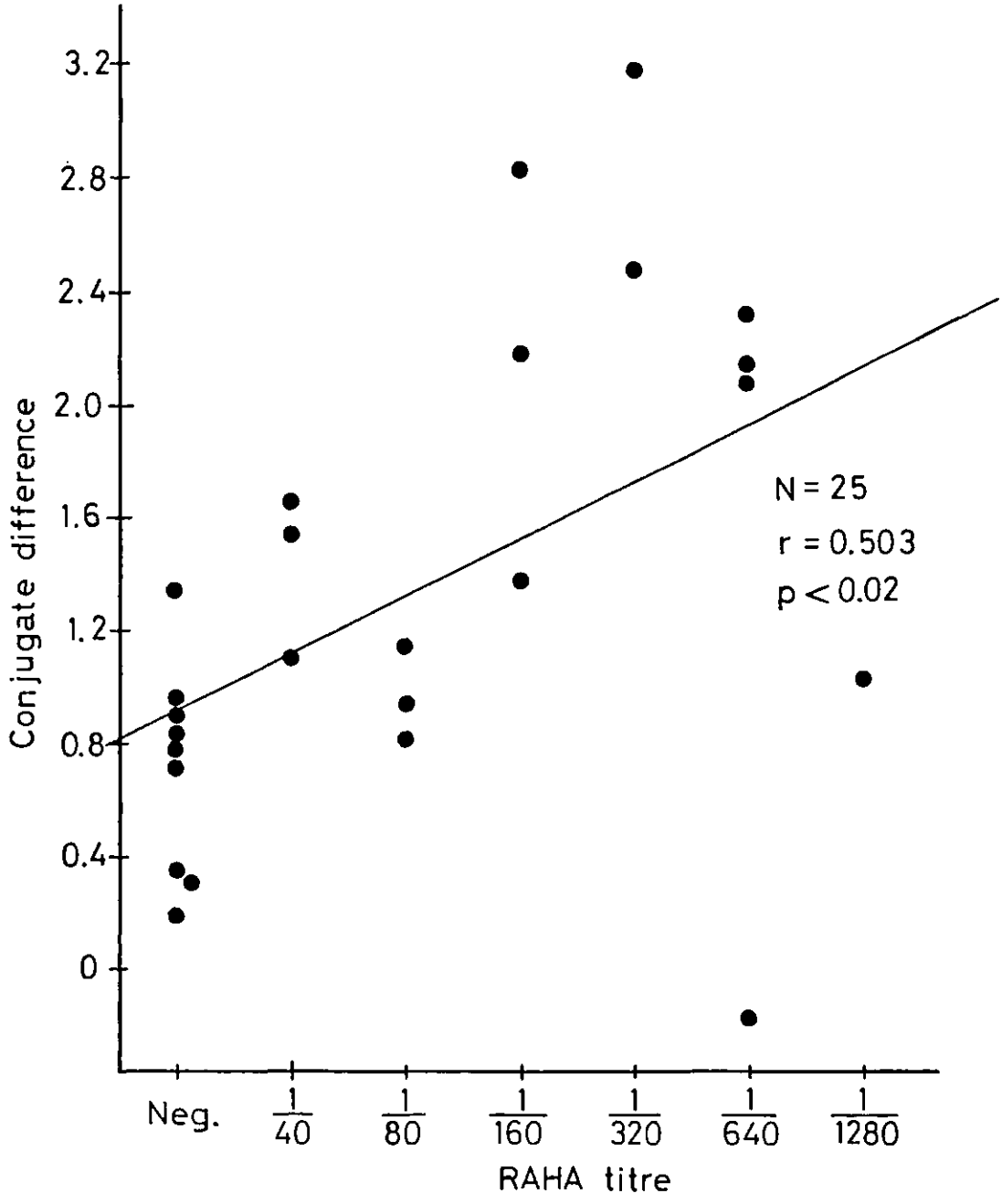


Figure III.18: The association in 25 sera between IgM RF levels and the difference between the responses to non-digested and pepsin-digested anti-human IgA-AP conjugates.

The CNA value obtained with the IgG-A-AP conjugate minus the corresponding value for the $F(ab')_2$-A-AP conjugate (see table III.6.) was plotted against the RAHA titre of the serum.

The association was statistically significant ($r = 0.503$, $0.01 < p < 0.02$).

Materials and methods: Sera from 16 patients attending the rheumatology clinic at the Royal National Orthopaedic Hospital, Stanmore, were tested. These patients all had classical/definite RA and their RAHA titres ranged from negative (<1:40) to 1:5120.

Microplate wells were coated with 10 or 60 µg normal rabbit IgG/ml. Sera were diluted 1/10, 1/100 and 1/1000, and were incubated at r.t. for 2 hrs., followed by application of 1/1000 dilution of $F(ab')_2$-A-AP.

Results: Table III.7. shows the results of the experiment in brief. The CNA values for the 60 µg/ml concentration were always at least as high or higher than the CNA values for the lower concentration, although the 60 µg/ml:10 µg/ml ratio was quite variable, ranging from 1.00-2.79. On the basis of this experiment, it was decided to use the higher coating concentration in the standard procedure for the IgA RF ELISA.

III.2.4. The use of standards, and expression of results

III.2.4.1. Selection of sample dilutions

On the basis of the data shown in Figure III.19., and by analogy with the data for IgM RF in chapter III.1., it was assumed that the CNA value for more than one serum dilution would be quantitatively superior to the net absorbance value for any one single dilution. As the IgM and IgA antiglobulin assays were generally performed simultaneously, it was convenient to use the same dilution system for both. Consequently, samples were routinely diluted 1/10, 1/100 and 1/1000

Table III.7: A comparison between coating with 60 and 10 µg/ml rabbit IgG for the IgA RF ELISA.

	CNA (dilutions 1/10 + 1/100 + 1/1000)		Ratio
	60 µg/ml	10 µg/ml	60/10
Number tested	16	16	
Mean	0.650	0.523	1.40
SD	0.454	0.378	0.43
Range	0.053 - 1.578	0.019 - 1.364	1.00 - 2.79

Sixteen RA sera were tested at dilutions 1/10 + 1/100 + 1/100.

The means, SD values and ranges for the CNA values are shown, together with the CNA ratios for the two concentrations.

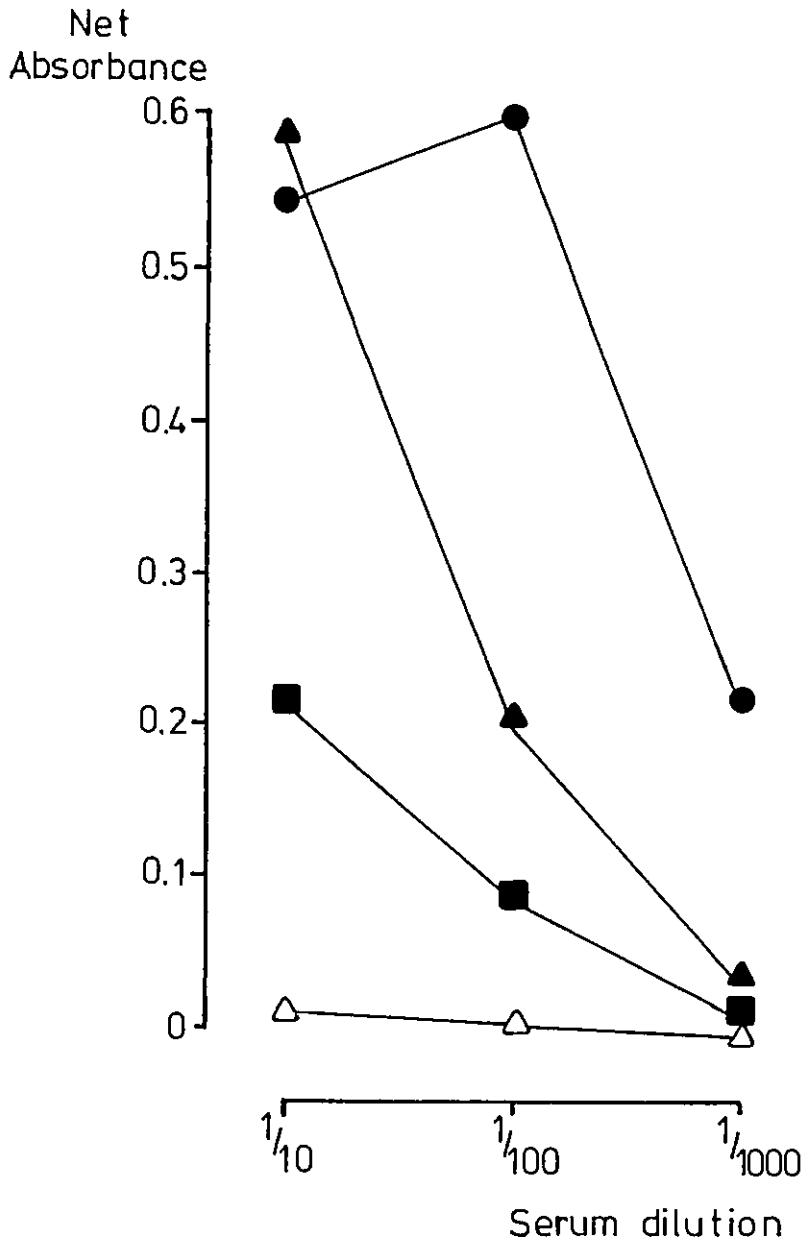


Figure III.19: IgA RF ELISA dilution curves of four rheumatoid sera.

The sera were tested at three ten-fold dilutions, in microplates coated with 60 µg/ml rabbit IgG, using the $F(ab')_2$-A-AP conjugate.

for the IgA class ELISA.

III.2.4.2. Selection of an internal standard, and comparison with the international RF standard

One of the rheumatoid sera shown in Figure III.19, serum Ve (●), from a patient with classical, nodular erosive RA, was found to react very strongly in the IgA RF assay. This serum, which had a RAHA titre of 1:1280, was selected as an internal standard and included every day in the IgA RF assay, diluted in an identical fashion to the IgM RF standard as indicated in Figure III.6. The IgA RF levels of the individual samples were then determined by reading the CNA values at dilutions 1/10 + 1/100 + 1/1000 from the standard curve. The IgA RF activity in the undiluted internal standard was arbitrarily defined as 1000 U/ml, and one typical standard curve is shown in Figure III.20.

Comparison with the international RF standard. The international (British) RF standard was reconstituted as described in chapter III.1.3.3. and diluted in parallel with the internal standard. Subsequently, both were assayed on the same microplate. Each dilution was tested in triplicate in coated wells plus in one adjacent blank well. Following incubation with a 1/1000 dilution of the $F(ab')_2$-A-AP and a 30 min. incubation with pNPP in a 37°C waterbath, the plates were read and the dilution curves in Figure III.21. plotted. Using the calculation method described in chapter III.1.3.3., it was found that the internal standard was $2^{4.74} = 26.7$ times stronger than the international standard. Accordingly, if the internal standard serum contains 1000 U/ml, the IgA RF

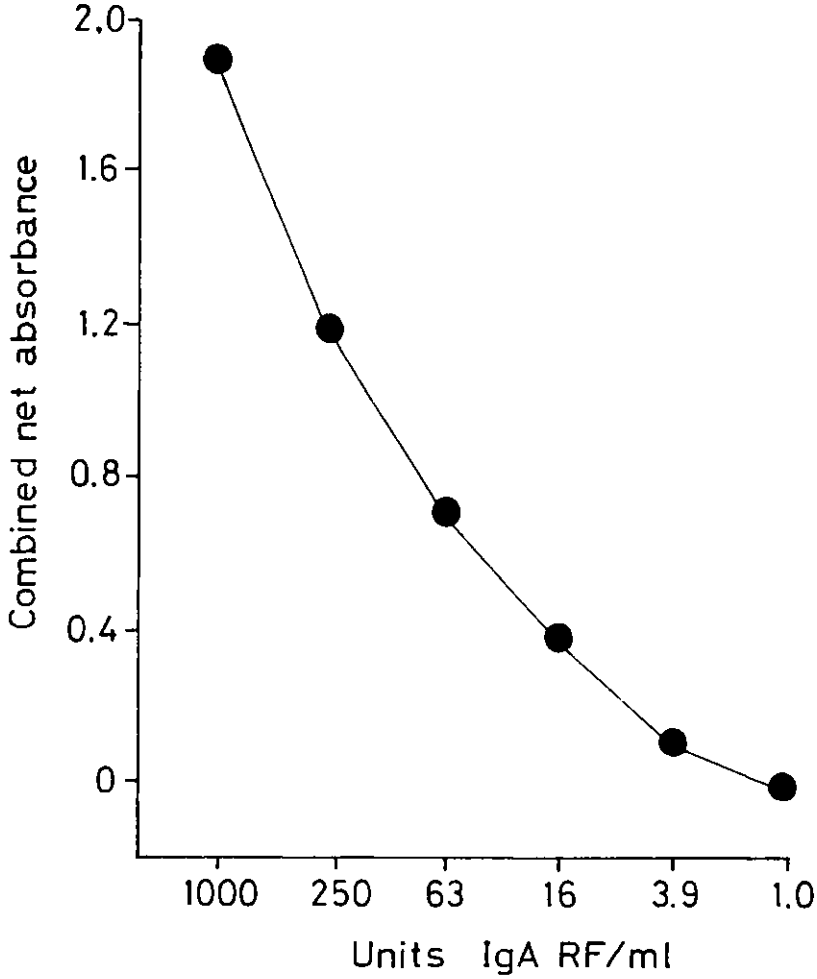


Figure III.20: A typical dilution curve for the internal IgA RF standard, diluted as shown in fig. III.6.

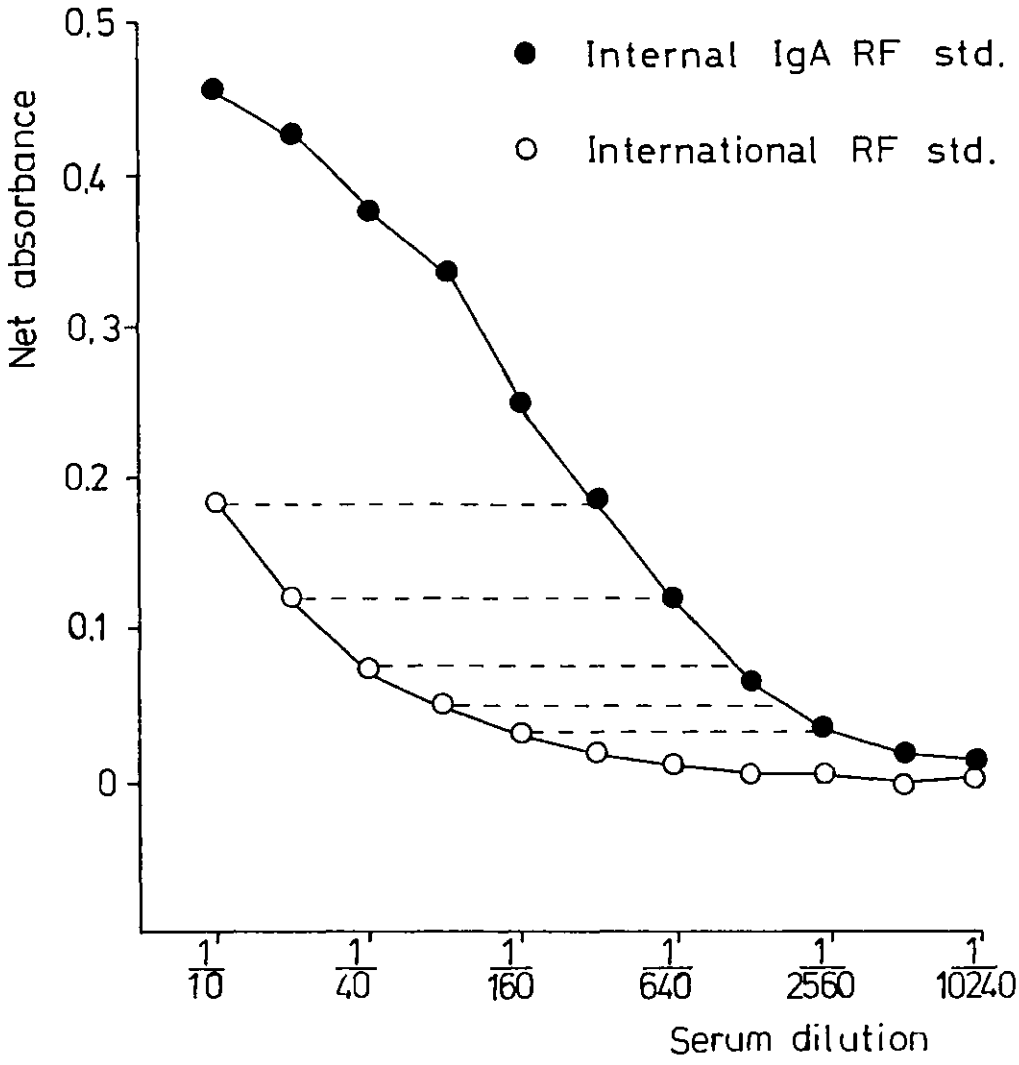


Figure III.21: Dilution curves from simultaneous IgA RF ELISA of the international RF standard and the internal IgA RF standard.

The internal standard was on average 4.74 dilutions (or 26.7 times) stronger than the international standard, see III.2.4.2.

content of one ampoule of the international RF standard is 37 units.

III.2.5. The IgA RF assay as applied: reproducibility and normal ranges

III.2.5.1. The standard assay procedure for IgA RF

Coating of microplates. This was identical to the procedure described in III.1.4.1. for IgM RF, except that coating volumes were 75 µl/well, and the coating concentration was 60 µg/ml of normal rabbit IgG.

Sample incubation. This was identical to the IgM RF ELISA, three dilutions, 1/10, 1/100 and 1/1000, for each sample, incubated for 2 hrs. at r.t. in triplicates in coated wells and one adjacent blank well.

Conjugate incubation. Plates were incubated for 2½ hrs. at r.t. with 1/1000 dilution of the $F(ab')_2$-A-AP conjugate (see chapter III.2.2.2.), otherwise the procedure was identical to the IgM RF ELISA.

Enzyme substrate. The plates were incubated with 100 µl/well of pNPP for exactly 30 mins. in a 37°C waterbath (see the procedure for IgM RF for further details).

Expression of results. After reading the plates as described for the IgM RF ELISA, the mean background readings for rows A and H were calculated. This background value was then subtracted from the mean of

the triplicates at each dilution. After calculating the CNA value for all three dilutions of each sample, the IgA RF content (U/ml) of the sample was determined by reading off the dilution curve for the internal standard, as described for the IgM RF assay.

III.2.5.2. Reproducibility of the IgA RF assay

Within-day reproducibility. Two sera (Fi and La) were assayed on nine different plates on the same day. Figure III.22. shows the reproducibility of the results, expressed both as the CNA value and as U/ml. The coefficients of variation (CV) were 19.7% and 33.4% for the CNA and 26.5% and 46.3% for the unit values.

Day-to-day reproducibility. Two sera (Wo and Fi) were assayed on 10 and 13 different days, respectively. Figure III.23. shows the CNA and unit graphs for these, and it can be seen that the CV values range from 34.3% to 55.0%, a considerably poorer reproducibility than for the IgM RF ELISA (see Table III.4. and Figure III.8.).

III.2.5.3. Normal ranges

In order to establish a normal range for serum IgA RF, the 102 normal sera used to obtain the normal range for IgM RF (see chapter III.1.4.3.) were assayed. The results shown at the right hand end of Figure III.24. indicate that 4.9% of the normals (5/102) had 10 U/ml or above. Accordingly, an IgA RF level of 10 U/ml or above was considered to be raised.

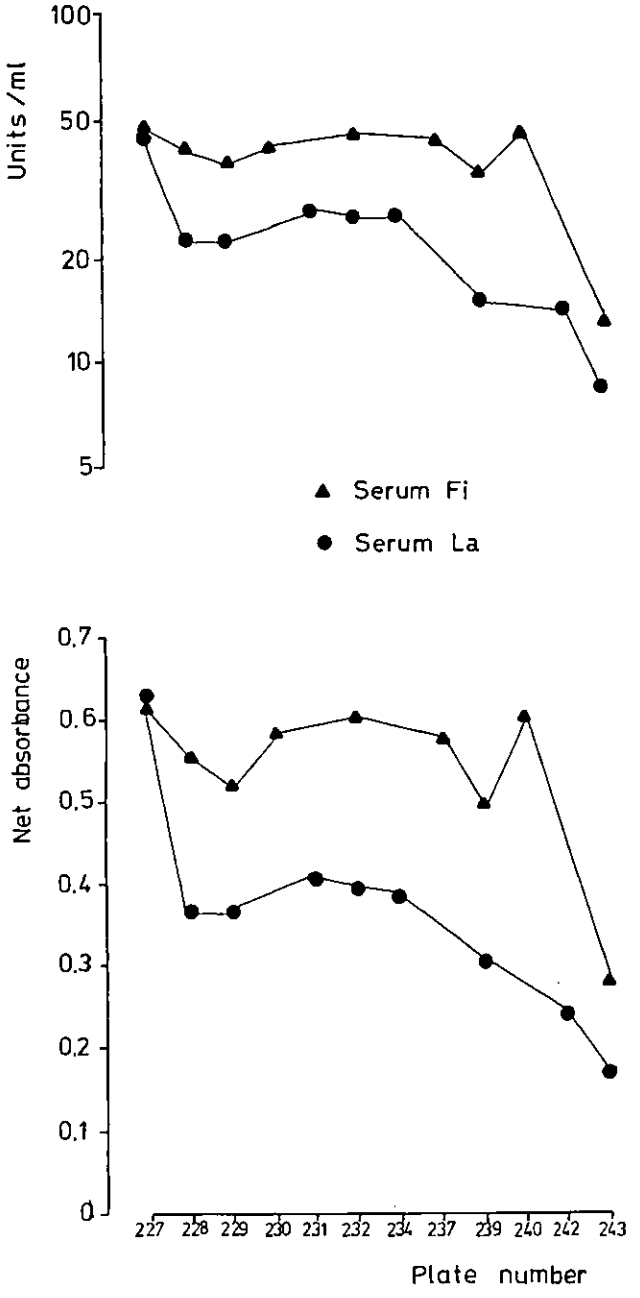


Figure III.22: The within-day reproducibility of the IgA RF ELISA.

Two rheumatoid sera (Fi-triangles and La - circles) were each assayed in 9 different plates.

The CV figures for the CNA and U/ml values were 19.7% and 26.5% and 33.4% and 46.3% for Fi and La, respectively.

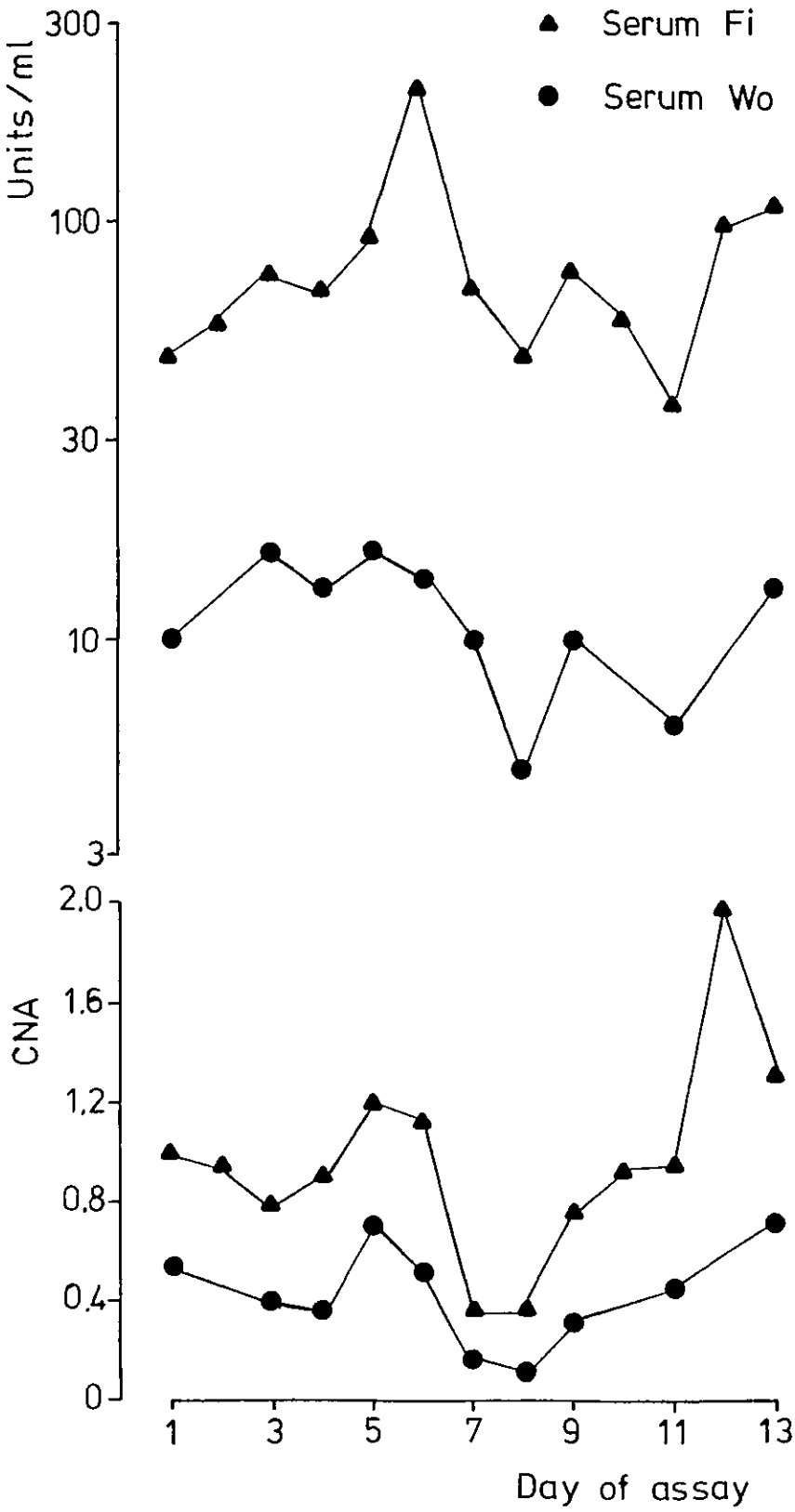


Figure III.23: Day-to-day reproducibility of the IgA RF ELISA.

Two rheumatoid sera (Wo-circles and Fi-triangles) were each assayed on 10 and 13 days, respectively.

The CV values for the CNA and U/ml values were 48.8% and 34.3% and 42.9% and 55.0% for Wo and Fi respectively.

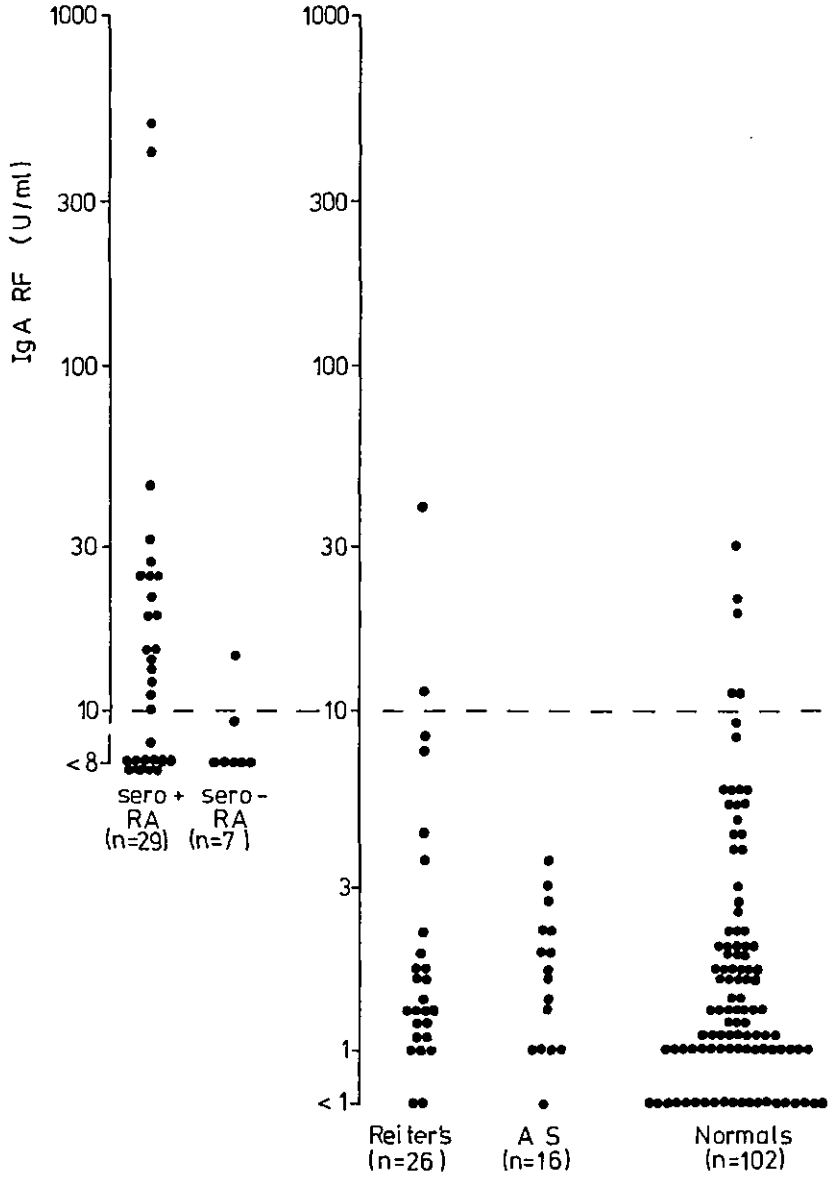


Figure III.24: IgA RF levels in 102 sera from normal individuals and in sera from Icelandic patients with ankylosing spondylitis (AS) (N = 16) and Reiter's disease (N = 26). Also shown are IgA RF levels in 29 seropositive and 7 seronegative RA patients.

Raised IgA RF was defined as activity $\geq$ 10 U/ml.

Figure III.24. also shows the IgA RF levels in groups of Icelandic ankylosing spondylitis and Reiter's patients. These patients had mostly normal levels of IgA RF.

On the other hand, the group of sero positive (RAHA titre $\geqslant$1:40) RA patients shown at the left of Figure III.24. is clearly different, as 55% (16/29) had raised IgA RF levels, some up to 500 U/ml.

This obviously brought up the question of whether the observed IgA RF levels simply reflect the IgM RF levels, possibly due to reaction of IgM RF with some residual Fc on the pepsin-digested $F(ab')_2$-A-AP conjugate. In Figure III.25. the IgA RF levels of 177 members of the family dealt with in chapter VI are plotted against their IgM RF levels. Whilst there was a significant positive correlation between the two RFs as evidenced by a Spearman's rank correlation coefficient (r_s) of 0.349 ($p < 0.001$), the association is by no means close. It can be seen, for instance, that all the five family members who had IgM RF in excess of 500 IU/ml, had less than 8 U/ml of IgA RF. Moreover, as shown in Figure III.13., using a sensitive ELISA system, no Fc-gamma contamination could be detected in the $F(ab')_2$ preparation.

Furthermore, the total serum IgA levels of 173 members of the family (chapter VI, Table VI.9.) did not show any significant correlation with the IgA RF (r_s = 0.067, not significant). Thus, it seems unlikely that the responses in the IgA RF test are due to non-specific adherence of serum IgA to the microplate.

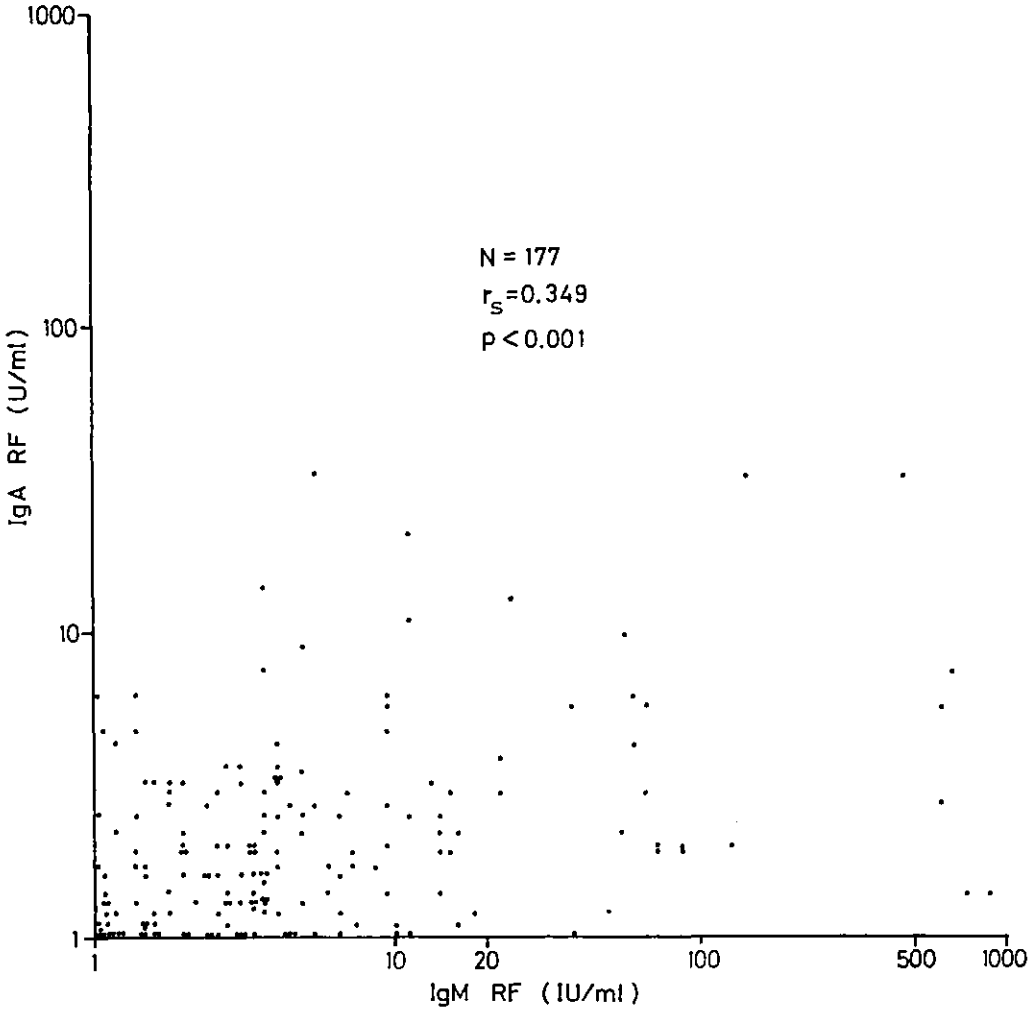


Figure III.25: The relationship between serum levels of IgM RF and IgA RF in 177 members of the Icelandic Adalvik family dealt with in chapter VI.

The correlation was statistically significant (Spearman's rank correlation coefficient r_S = 0.349, p <0.001).

III.2.6. Discussion

Various authors have used polyacrylamide gel electrophoresis in SDS to assess the end products of papain/pepsin digestion of immunoglobulins (Rousseaux *et al*, 1980; Beeson and Wissler, 1977). The ELISA described here for the assessment of the completion of the pepsin cleavage is a feasible alternative. The ELISA offers an opportunity to check not only that the Fc portions of the IgG have been destroyed, but also that the antibody ($F(ab')_2$) is functionally intact. Also, Figure III.14. indicates that the assay is very sensitive as it detects very slight contamination with native IgG. However, the selection of rheumatoid sera for this assay may be critical as some such sera contain IgM-class pepsin agglutinators that could react with the $F(ab')_2$ fragments (Heimer *et al*, 1982).

The results of the comparison between pepsin-digested and undigested anti-human IgA-AP conjugates show rather strikingly the importance of using $F(ab')_2$. This agrees with a recent report of Elkon *et al* (1981) who found that ^{125}I-labelled normal rabbit and sheep IgG bound much more avidly than ^{125}I-$F(ab')_2$ to rheumatoid or systemic sicca sera on the solid phase.

The importance of pepsin-digesting the antibody used in solid phase antiglobulin (of IgG-, IgA, or even IgM-isotype) assays may vary, however, depending on a number of factors:

(a) if the IgM RF of the sample is destroyed, e.g., by pepsin digestion or 2-ME reduction, the need for the use of $F(ab')_2$ should become less;

(b) if the labelled antibody used is raised in animals such as ruminants or mice, rather than rabbits, it may well be less "attractive" for IgM RF, cf. results in chapter III.1.2.3. and Figure III.3. However, a strongly IgM RF-positive serum may react with Fc gamma from any mammalian species;

(c) the one-step glutaraldehyde method for coupling alkaline phosphatase to antibody is known to result in high molecular weight complexes (Engvall *et al*, 1971) which may be especially "attractive" for IgM RF on the solid phase. Thus, pepsin digestion of the antibody may be even more important for ELISA than SPRIA systems;

(d) the use of affinity-purified antibody may diminish the need for pepsin digestion of the antibody, as the specific antibody activity per unit of labelled immunoglobulin will then be much higher.

The results in III.2.3.1. raise the question of whether it might be possible to design a solid phase assay for IgM RF where the solid phase is coated with rabbit or human IgG, followed by incubation with the sample to be tested and enzyme-labelled normal rabbit IgG. The sandwich assay of Maiolini *et al* (1978) where RF was adsorbed onto heat-aggregated cellulose-bound IgG and detected by the addition of enzyme-labelled aggregated IgG, certainly suggests that this approach may be feasible.

It was somewhat unexpected, therefore, to find that the ranked RAHA titres of the sera in Table III.6. did not correlate with the CNA values for the undigested conjugate ($r = 0.168$, not significant) but showed a highly significant correlation ($r = 0.634$, $p < 0.001$) with the $F(ab')_2$-A-AP CNA values. The explanation for this is not clear. It seems unlikely that it is due to contamination of the $F(ab')_2$-A-AP with undigested IgG-A-AP, in view of the data presented in Figure III.13.A. A possible explanation is that the undigested conjugate may be bound by IgM RF-IgG RF complexes. Such complexes, which are known to exist (Hay *et al*, 1976b) would not be capable of binding the digested $F(ab')_2$-A-AP.

Koopman *et al* (1982) recently reported a close correlation ($r = 0.673$) between IgA RF and IgM RF in seropositive RA patients, whereas Elkon *et al* (1981) and March *et al* (1981) found little or no correlation between the levels of the two RFs. It may be of interest in this context that Koopman *et al* used undigested (goat) antibody in their assay.

In agreement with Kallerup *et al* (1979) the international (British) standard for RF was found to have a low IgA RF content. One ampoule of the standard contains 100 IU IgM RF, which is five times the upper limit of normal per ml of serum. On the other hand, the upper normal limit for IgA RF in serum is 10 U/ml, and one ampoule of the standard only contains 3.5-4.0 times that amount.

Obviously, a new international standard with more IgA RF activity is needed.

The rather poor reproducibility of the IgA RF assay is a source of concern. The within-day reproducibility curves show that the values get progressively lower towards the right hand side of the figure (see Figure III.22.). This might possibly be related to the fact that the plates yielding the results on the left were filled up first during the serum incubation, but the plates yielding the results on the right were filled up last. Therefore, the left hand plates may have had 1.0-1½ hrs. longer serum incubation than the right hand ones, since serum was removed from all the plates virtually simultaneously.

III.2.7. Conclusion

A high-titre anti-human IgA antibody was raised in rabbits and cleared of reactivity with other immunoglobulins by an affinity column technique. After pepsin digestion and chromatographic isolation of the $F(ab')_2$ fragments, an ELISA showed that the product did not react with IgM RF, yet bound IgA avidly. The pepsin digested antibody was shown to be superior to its undigested analogue, as the former did not react with IgM RF in antiglobulin assays.

The digested antibody was used to establish an IgA RF ELISA which was found to be sensitive and specific. The results were expressed as units with reference to an internal standard.

III.3. IgG RHEUMATOID FACTOR

III.3.1. Introduction

Since the report by Kunkel *et al* (1961) that some rheumatoid sera had IgG complexes that probably contained IgG RF, many assays have been developed for the measurement of IgG RF. The principles of some of these were reviewed briefly in chapter II.1. and the numerous publications dealing with this topic would indeed suggest that none of the pre-existing assays were considered entirely satisfactory. Since the original description by Hay *et al* (1975), however, most attention has been focused on assays of the solid phase type, especially SPRIA, for antiglobulins of the IgG and IgA isotypes. Whilst these assays are simple and attractive in principle, they are not without problems. As shown in Figure I.3., IgM RF attached to the solid phase may, theoretically at least, cause false positive results in IgG RF assays by binding to either native IgG or the Fc portion of the radio/enzyme-labelled anti-IgG. However, the practical importance of these possibilities has been the subject of considerable controversy. Nineham *et al* (1976) found that radio-labelled undigested rabbit anti-ovalbumin did not bind to the solid phase, even though rabbit anti-IgG did so, suggesting that the use of $F(ab')_2$ for antiglobulin detection was not necessary.

Carson *et al* (1977) found that reduction of sera with dithiothreitol (DTT) prior to testing, not only abolished about 91% of the IgM RF response, but this treatment also reduced the observed IgG RF levels by as much as 50%, especially in sera with high titres of IgM RF. Carson *et al* used pepsin-digested antibody ($F(ab')_2$) in their IgG RF test, therefore their experience with DTT would suggest that IgM RF

does cause false positive reaction by binding to native IgG and the solid phase simultaneously. Also, Pope and McDuffy (1979) found that by adding monoclonal IgM RF to normal sera, these gave a false positive IgG RF test result. These authors also provided evidence that native IgG might bind non-specifically to the solid phase, and cause false positive reactions, especially in the presence of raised total serum IgG.

Theofilopoulos *et al* (1974) pepsin-digested their serum samples prior to testing for IgG RF. As the IgM molecule is highly susceptible to degradation by pepsin (Mihaesco and Seligmann, 1968; Kishimoto *et al*, 1968) but IgG generally retains its antibody activity as $F(ab')_2$ after digestion, this is theoretically attractive in IgG RF assays. The pepsin treatment, furthermore, has the advantage that degrading the Fc portion of IgG releases IgG RF (as $F(ab')_2$ fragments), thus revealing "hidden" IgG RF. Munthe and Natvig (1971) revealed RF activity by pepsin-digesting IgG complexes from rheumatoid tissue eluates, suggesting that this is of more than theoretical importance.

When it comes to large-scale testing of samples by pepsin-digestion, a convenient method is essential. As the method of Theofilopoulos *et al* (1974) involves dialysis of samples, it is quite cumbersome and requires relatively large amounts of sera. Quite recently, however, a convenient method was published by the same group (Wernick *et al*, 1981) where 50 µl aliquots of sera were digested prior to the IgG RF assay.

In the following sections of chapter III.3., attempts to establish an IgG RF ELISA are described.

III.3.2. Different assay conditions tested

III.3.2.1. Initial observations; goat IgG as IgG RF substrate

Due to the ready availability of goat anti-human IgG (affinity purified, gamma chain specific, TAGO Inc.), goat IgG (Miles Laboratories Ltd.) was initially selected as substrate for IgG RF. Tests for IgM antiglobulins were performed in parallel on the same substrate, using the goat anti-human IgM-AP conjugate described in chapter III.1. The principle of these assays was very similar to that described in chapter III.1. and III.2. Briefly, plates were coated with 10 µg/ml goat IgG, followed by BSA and sera diluted 1/10 and 1/100 in hypertonic serum diluent. After incubations with the appropriate antibody (anti-human IgG or anti-human IgM) conjugated with alkaline phosphatase, the plates were developed with pNPP and the CNA values calculated.

Figure III.26. shows the correlation between IgG antiglobulins and IgM antiglobulins in sera from 29 RAHA-positive RA patients. The IgM anti-goat IgG levels in these sera also correlated closely with the ranked RAHA titre ($r = 0.772$, $p < 0.001$). Figure III.26. shows that there was a significant correlation between the IgG and IgM antiglobulin levels ($r = 0.447$, $0.01 < p < 0.02$).

However, various normal sera were observed to have very high levels of IgG anti-goat IgG without any significant IgM anti-goat IgG. Figure III.27. shows the results of IgG and IgM antiglobulin tests performed on sera from 120 healthy blood donors from the Edgware Blood Bank, London. Clearly, there was no correlation ($r = 0.004$) between the levels of IgG and IgM antiglobulins.

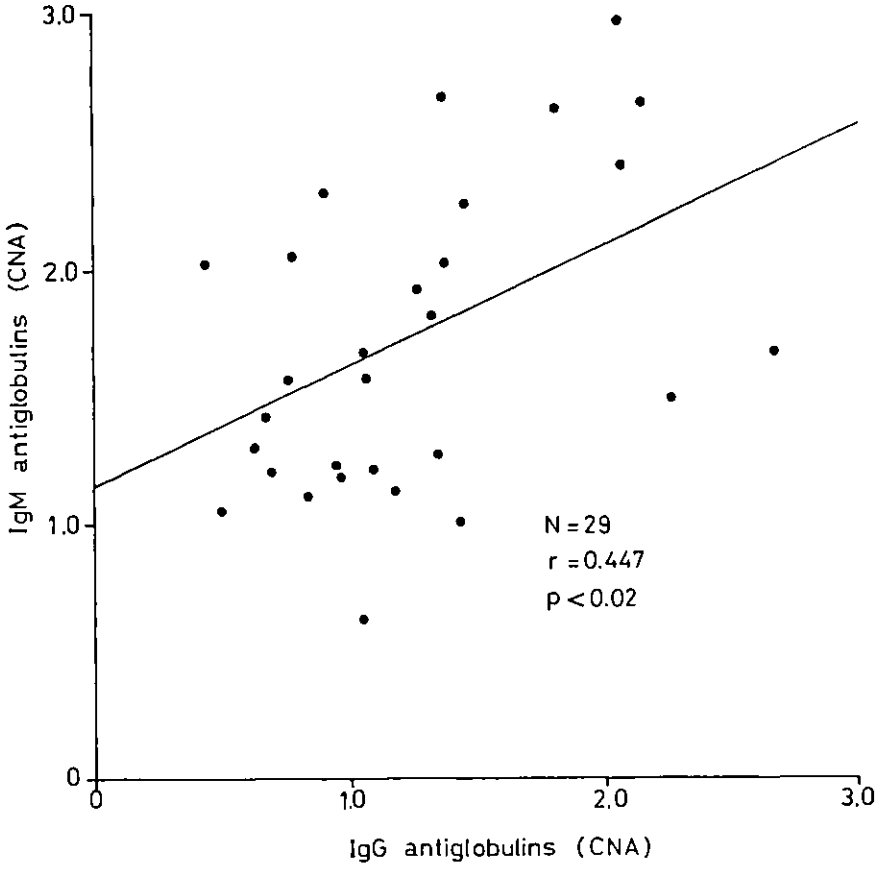


Figure III.26: The relation between IgG and IgM antiglobulin levels in sera from 29 RA patients, using goat IgG as substrate and goat anti-human Ig reagents.

The levels of the two isotypes correlated significantly ($r = 0.447$, $0.01 < p < 0.02$, the regression equation: $y = 0.463X + 1.157$).

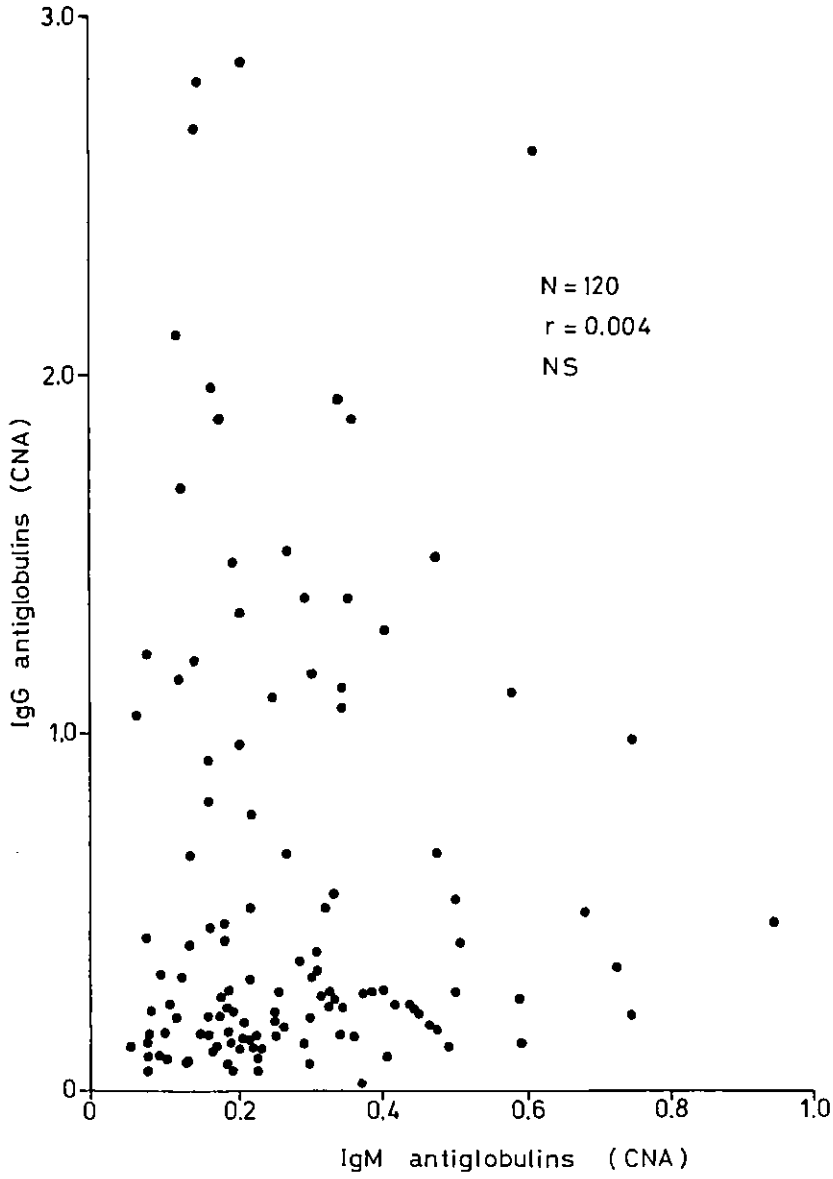


Figure III.27: The relation between IgM and IgG antiglobulin levels in 120 normal blood donors, using goat IgG as substrate and goat anti-human Ig reagents.

There was no significant correlation between the levels of the two antiglobulin isotypes (r = 0.004, NS).

The phenomenon of IgG antiglobulins against goat (ruminant) IgG, will be discussed further in chapter IV, but the idea of using goat IgG as substrate in RF tests was abandoned after these findings.

III.3.2.2. Rabbit IgG as IgG antiglobulin substrate: initial observations

Having reached the conclusion that goat IgG was unsuitable as substrate in IgG RF tests, rabbit IgG was tried next. A rabbit anti-human IgG antiserum (Fc-gamma specific) was a gift of Dr. F. C. Hay, Middlesex Hospital, London, and following ammonium sulphate precipitation (Hudson and Hay, 1980), the gamma globulin fraction was conjugated to alkaline phosphatase (see chapter II.4.). This conjugate (referred to as R-G-AP) was subsequently used in IgG RF assays.

Initially, sera were tested simultaneously for IgM and IgG antiglobulins, using plates coated with rabbit IgG and 1/10, 1/100 and 1/1000 dilutions of test sera. The R-G-AP was used at a dilution of 1/500 and the goat anti-human IgM-AP at a dilution of 1/1000.

Figure III.28. shows a close correlation ($r = 0.774$, $p < 0.001$) between the observed IgG and IgM antiglobulin levels in 100 patients, 90 of whom had RA and 10 had osteoarthritis.

When the 120 normal blood donor sera from the Edgware Blood Bank were tested in this system there was also a significant correlation ($r = 0.531$, $p < 0.001$) between the IgG and IgM antiglobulin levels.

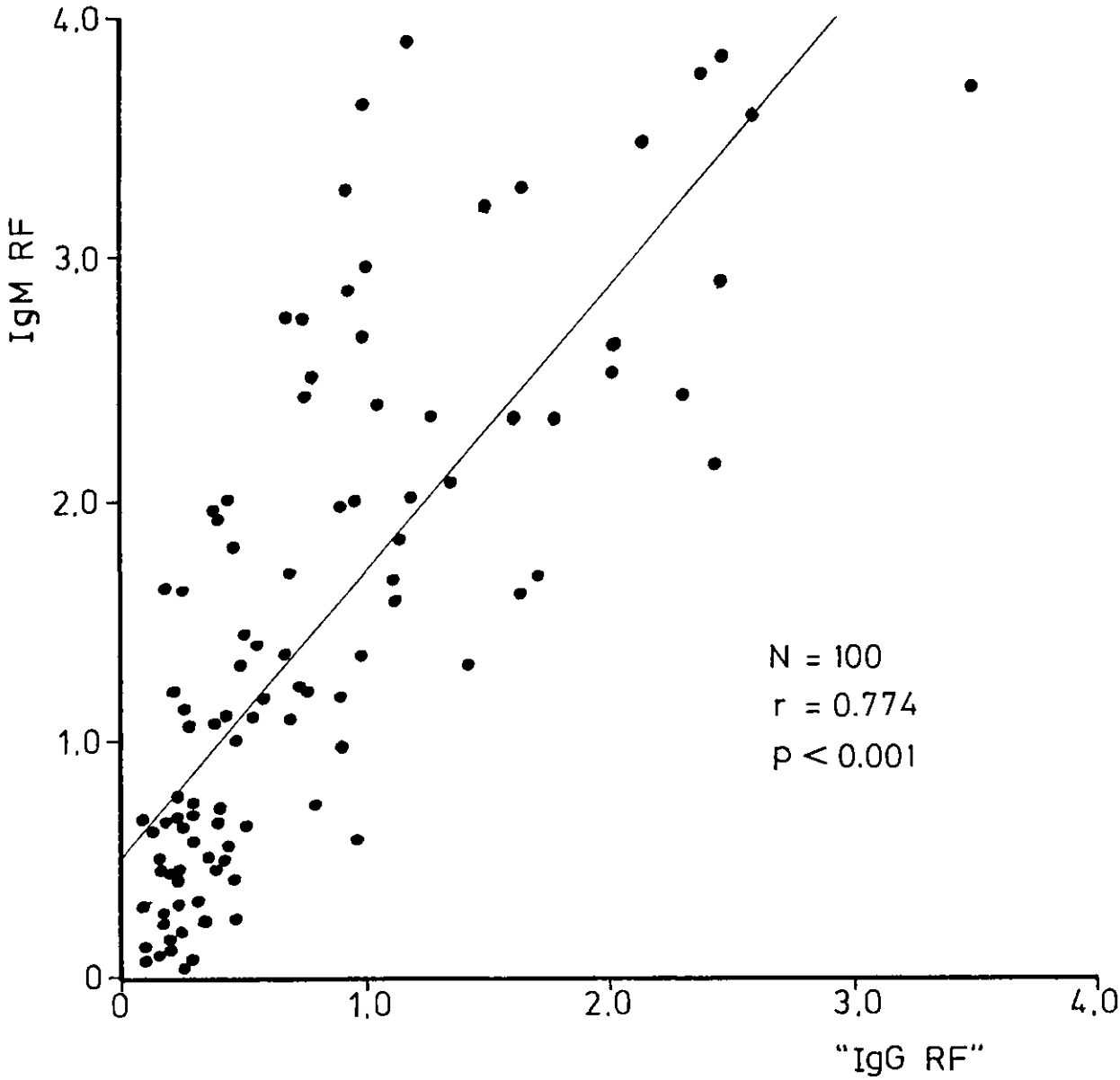


Figure III.28: The relation between IgG and IgM antiglobulin levels in sera from 90 RA patients and 10 patients with osteoarthritis.

Rabbit IgG was used as antiglobulin substrate and rabbit anti-human IgG-AP and goat anti-human IgM-AP as reagents, see III.3.2.2.

There was a highly significant correlation between the apparent levels of the two antiglobulin isotypes ($r = 0.774$, $p < 0.001$).

III.3.2.3. The effect of 2-ME on antiglobulin levels

In view of the possibility that the IgG antiglobulin levels shown in Figure III.28. merely reflected the presence of IgM antiglobulins (RF), the effect of 2-ME treatment was studied.

Materials and Methods: Twenty-seven sera from patients with RA were studied. Also, serum from one healthy PNEUMOVAX-immunised individual (Te) (see chapter IV.3.) was included as a negative control system.

The 2-ME (Sigma, London) was diluted in PBS, pH 7.4. Five aliquots of serum Te were mixed with equal volumes of doubling dilutions of 2-ME in PBS, yielding final concentrations of 0.063, 0.031, 0.016, 0.0078 and 0.0039 M of 2-ME. A 2-ME free PBS control was always included for each dilution.

Eight of the rheumatoid sera were mixed in a similar manner with dilutions of 2-ME, yielding final concentrations of 0.05, 0.025, 0.0125, 0.0063 and 0.0031 M 2-ME. The remaining 19 rheumatoid sera were mixed with PBS-diluted 2-ME, yielding a final concentration of 0.05 M 2-ME.

The 2-ME containing sera and the PBS-controls were incubated at 37°C for 60 mins., then diluted in PBS-Tween using doubling dilutions, 1/40-1/1280, and assayed immediately.

Serum Te was icubated for 2 hrs. at 37°C in microplates which had been coated with 8.8 µg/ml of PNEUMOVAX polysaccharide mixture. Afterwards, 48 wells of each plate were incubated with a 1/1000

dilution of a goat anti-human IgG-AP conjugate but the remaining 48 wells received a 1/1000 dilution of a goat anti-human IgM-AP conjugate.

The rheumatoid sera were incubated for 2 hrs. in plates coated with rabbit IgG (10 µg/ml). Afterwards, 48 wells of each plate were incubated with a 1/500 dilution of the R-G-AP, the remaining wells received a 1/2,000 dilution of goat anti-human IgM-AP.

After incubating the conjugates for 2-2½ hrs. at r.t., the plates were reacted with pNPP and the dose-response curves (using the mean net absorbance) plotted.

The relationship between the 2-ME induced decrease in IgG antiglobulins and the corresponding IgM antiglobulin levels was assessed in the following way: the mean net absorbance for dilution 1/80 of each 2-ME treated sample was expressed as a percentage of its untreated control. This percentage was then plotted against its corresponding value (dilution 1/80) for undigested IgM antiglobulins. The product-moment correlation coefficient (r) for the association was then calculated.

Results: Figure III.29. shows the effect of 2-ME upon the anti-PNEUMOVAX IgG and IgM levels in serum Te. Clearly, the IgM response was already partially destroyed by 0.0039 M 2-ME, and 0.016 M 2-ME did not leave any detectable IgM activity. However, even at 0.063 M 2-ME the IgG response was virtually unaffected.

Figure III.30. shows the analogous graphs for one rheumatoid serum,

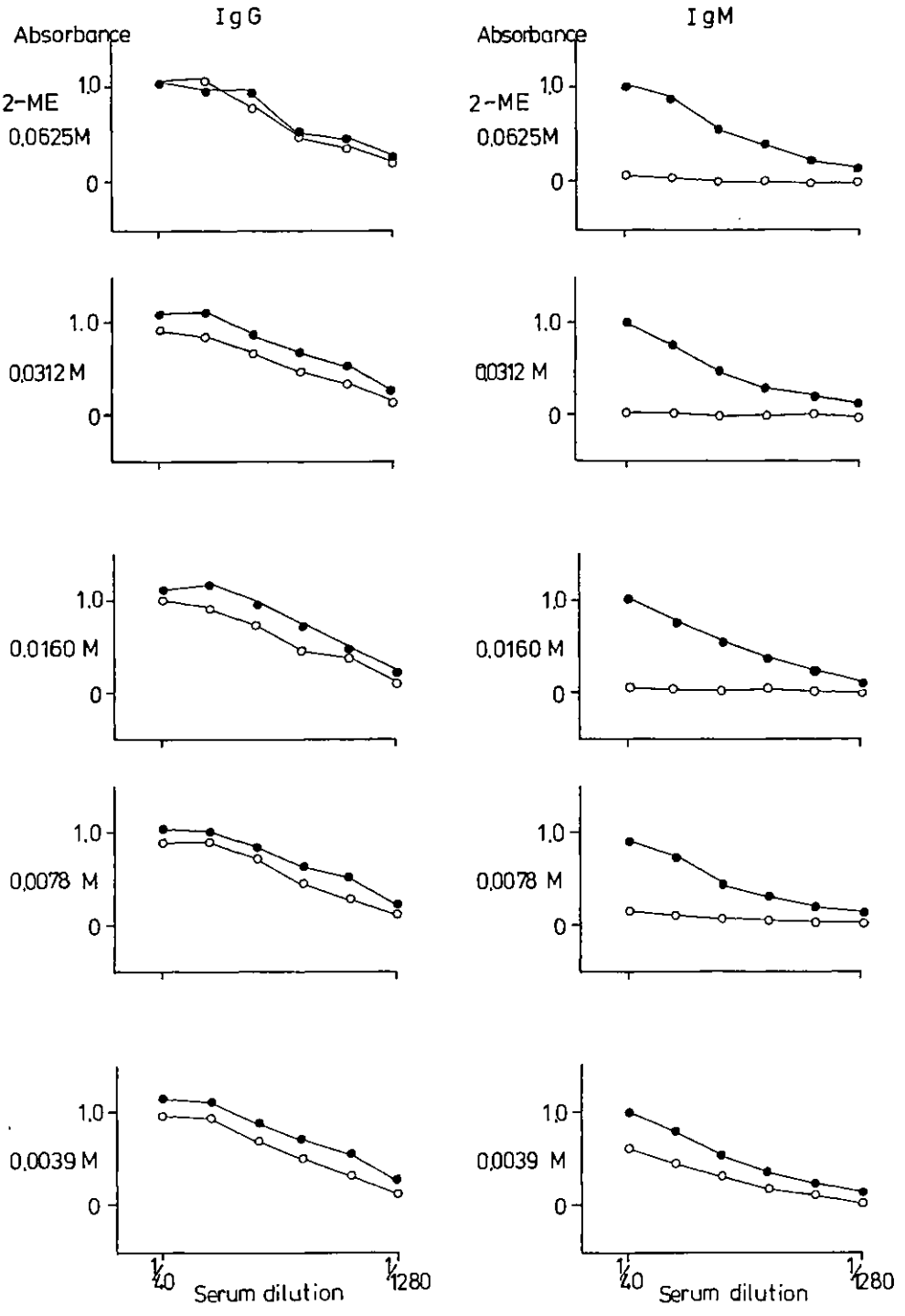


Figure III.29: The effect of 2-ME treatment on IgG and IgM anti-PNEUMOVAX antibodies (serum Te).

open circles: 2-ME
closed circles: PBS control
Left hand side: IgG antibodies
Right hand side: IgM antibodies

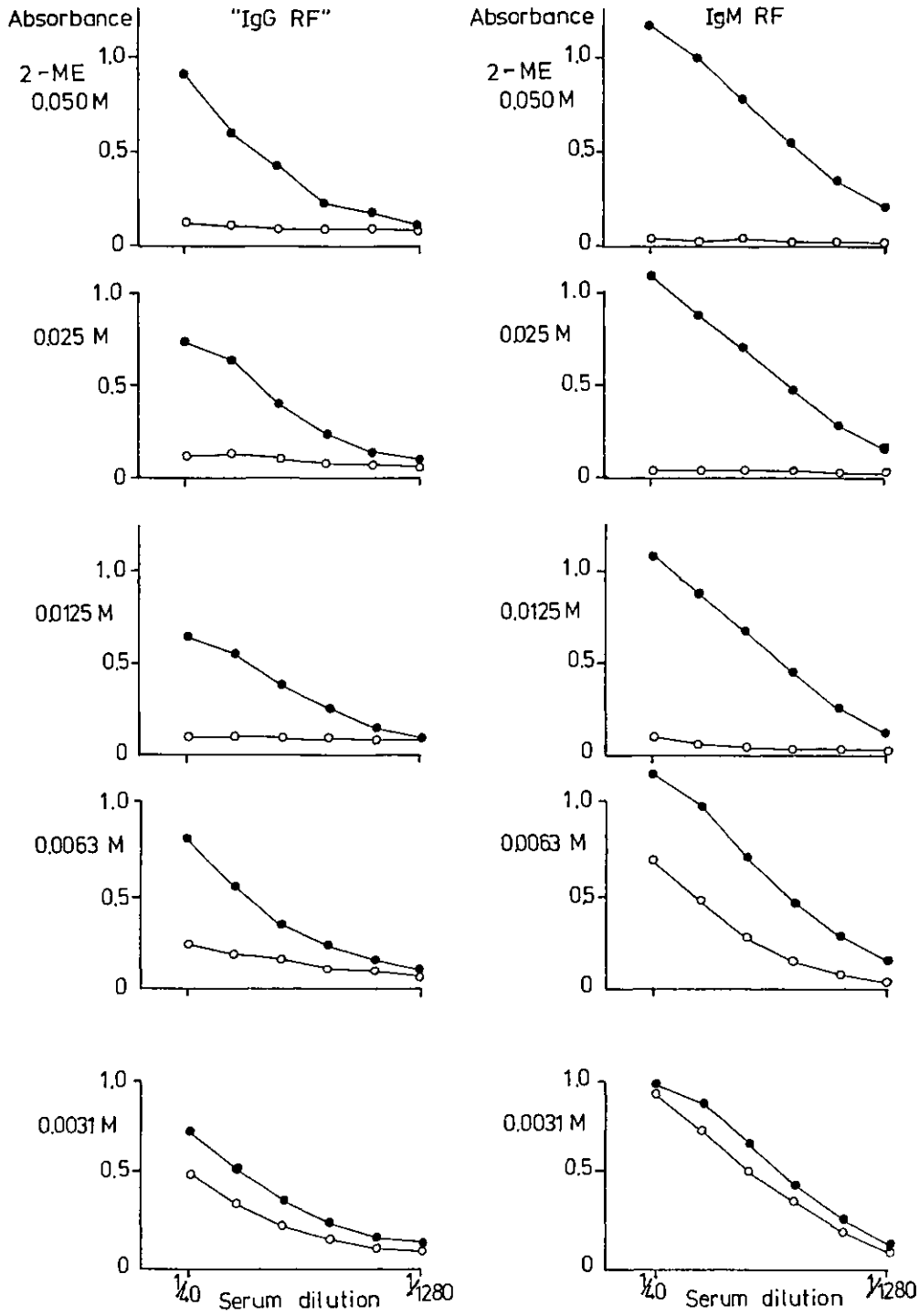


Figure III.30: The effect of 2-ME treatment of a rheumatoid serum on IgG and IgM antiglobulins as detected by the reagents described in III. 3.2.2. and III.3.2.3.

open circles: 2-ME
closed circles: PBS control
Right hand side: IgM antiglobulins
Left hand side: "IgG" antiglobulins

typical for the eight sera tested at five different 2-ME concentrations. At a 2-ME concentration of 0.0031 M the IgG and IgM curves were close to the unreduced PBS controls. However, with increasing 2-ME concentration both the IgG and IgM reactivities were reduced simultaneously, and at 0.025 M 2-ME no activity remained.

There was a clear association ($r = -0.874$, $p < 0.001$) between the serum IgM RF level (net IgM absorbance at dilution 1/80) and the extent of destruction by 2-ME of the "IgG antiglobulins" in the 27 rheumatoid sera (Figure III.31.). Thus, 2-ME destroyed 95.6% of the "IgG antiglobulins" in the serum having the highest IgM antiglobulin level, leaving only 4.4% (see the dot at the top left in Figure III.31.).

Briefly, these results indicate that the "IgG antiglobulins" in this test system were mostly a reflection of the IgM antiglobulin levels, and did not give any reliable picture of IgG antiglobulin levels.

However, this test system did not discriminate between binding of the Fc portion of the conjugate to IgM RF on the solid phase, and binding of the conjugate to human IgG attached to IgM RF on the solid phase (see Figure I.3.A. and I.3.B.).

III.3.2.4. Pepsin-digestion of sera

Although Figure III.29. indicates that it is feasible to inactivate IgM antibodies by 2-ME without affecting the IgG levels, pepsin-digestion has the theoretical advantage that it breaks up

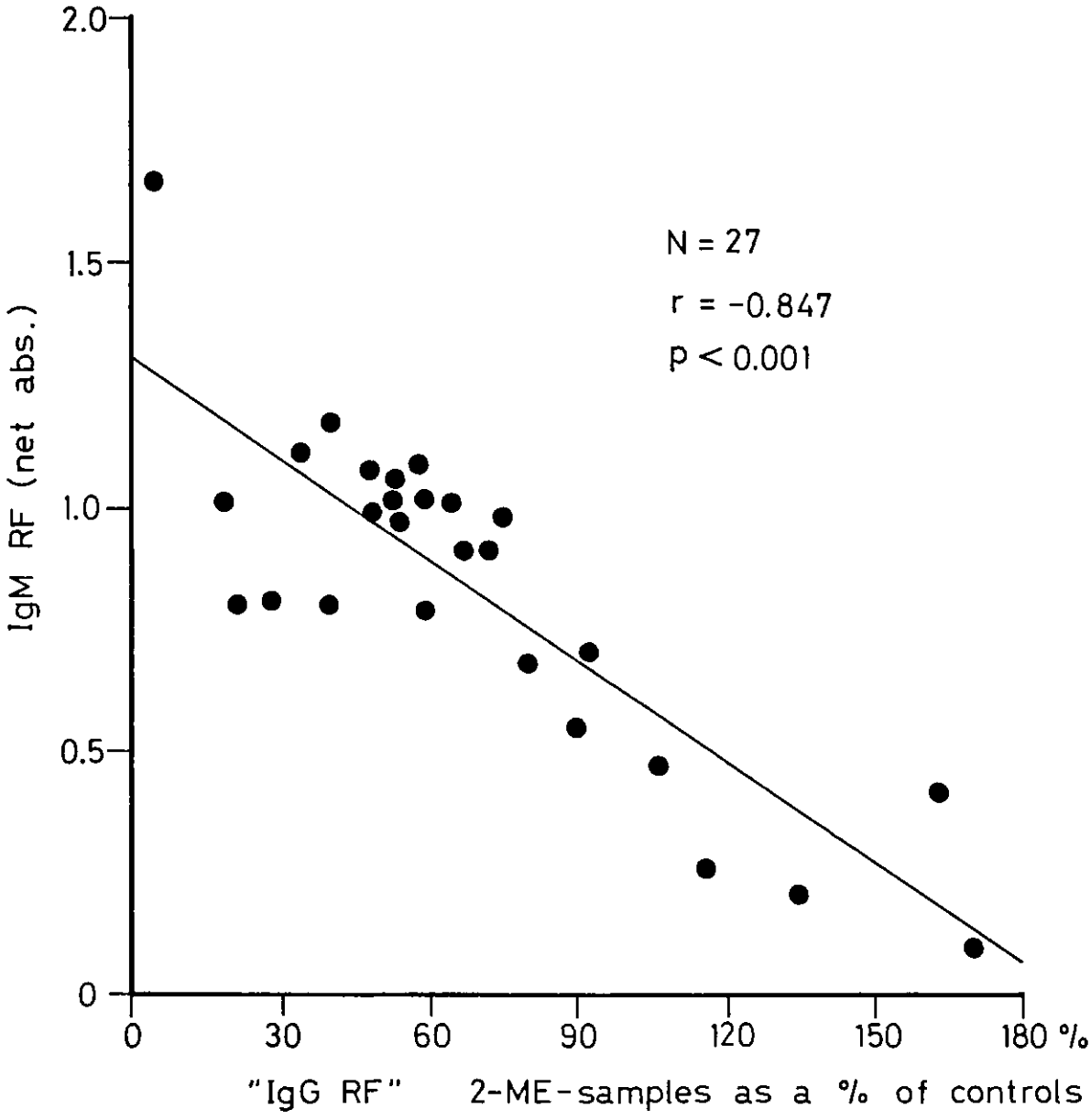


Figure III.31: The relationship in 27 rheumatoid sera between IgM RF levels and the 2-ME induced decrease in the "IgG" antiglobulin levels, as measured by the reagents described in III.3.2.2.

The "IgG" antiglobulin levels after 2-ME treatment (serum dilution 1/80) are expressed as the percentage of the corresponding PBS control.

The correlation was highly significant ($r = -0.847$, $p < 0.001$, the regression equation: $y = -0.0069X + 1.31$).

self-associated ("hidden") complexes of IgG RF. In order to investigate the effect of various pepsin concentrations on the levels of IgM, IgG and IgA antibodies, the following experiments were performed.

Materials and Methods: Aliquots of serum Te (PNEUMOVAX-immune, see chapter III.3.2.3.) were incubated at 37°C with double volumes of 0.1 M acetate buffer, pH 3.6, containing 0.02% Na N_3 and pepsin (porcine, x 2 crystallised, Sigma, London) at concentrations ranging from 0.20-3.38 mg/ml. A pepsin-free control (serum diluted in the acetate buffer) was included. The buffer/serum ratio of 2/1 gave a pH of approximately 4.1.

After 16 hrs. incubation the samples were centrifuged at 2000 g for 20 mins. at 4°C to remove precipitates, and the supernatant neutralised to pH 8.0 with a 1 M solution of TRIS. The digested/undigested samples were then diluted immediately in the hypertonic serum diluent and incubated in doubling dilutions (final serum dilution 1/15-1/120) in microplates coated with 10 µg/ml PNEUMOVAX polysaccharide mixture. After 2 hrs. incubation at r.t. the plates were incubated with dilutions of goat anti-human IgG-AP and goat anti-human IgM-AP and subsequently reacted with pNPP. The absorbance values for IgM and IgG at serum dilution 1/30 were plotted as a function of the pepsin concentration used.

One strongly RAHA-positive serum (Ve) was also pepsin-digested in a similar fashion, using pepsin concentrations of 0.67-3.38 mg/ml. The digests and undigested samples were diluted 1/25 - 1/50 - 1/100 and incubated in plates coated with 10 µg/ml rabbit IgG. The serum dilutions were first incubated for 1 hr. at 37°C, then overnight at 4°C.

The following day the plates were then incubated with three different conjugates:

(a) the standard goat anti-human IgM-AP, diluted 1/1000;

(b) the $F(ab')_2$-A-AP conjugate described in chapter III.2. diluted 1/500;

(c) the alkaline phosphatase conjugate of an $F(ab')_2$ fraction of a sheep anti-human Fab-gamma antibody, diluted 1/500. This antibody was a gift from Miles Laboratories Ltd.

After pNPP incubation the mean net absorbance values for each of the three conjugates at serum dilution 1/50 were plotted as a function of the pepsin concentration in the acetate buffer.

Results: Figure III.32. shows the effect of pepsin-digestion on the anti-PNEUMOVAX IgG and IgM antibodies. At a pepsin concentration of 1-1.5 mg/ml the IgM antibodies were completely inactivated. These concentrations reduced the mean net absorbance for the IgG antibodies to about 30-35% of the undigested value.

Figure III.33. shows the analogous results for the rheumatoid serum. As for the anti-PNEUMOVAX antibodies, a pepsin concentration of 1.0-1.5 mg/ml destroyed the IgM completely, but had minimal effect on the results obtained with the anti-IgA and IgG conjugates.

Briefly, these results indicated that:

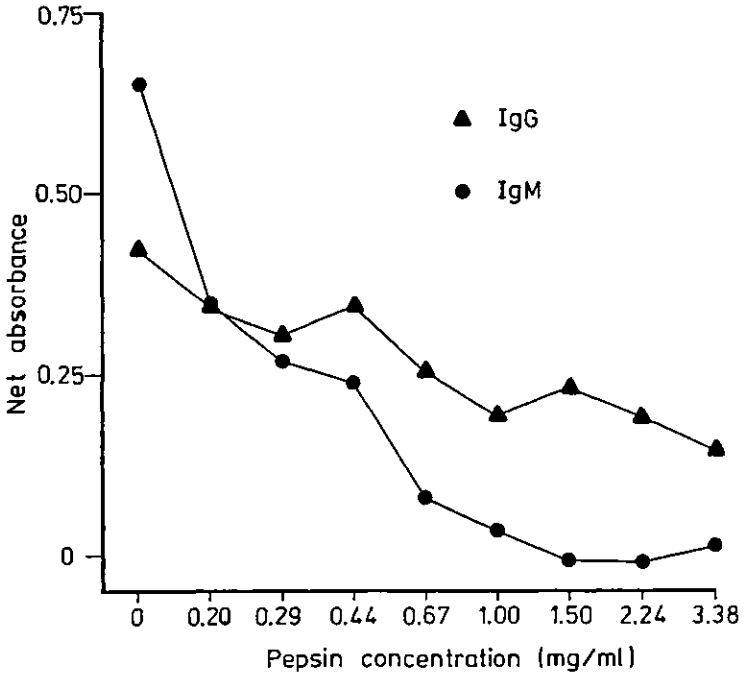


Figure III.32.

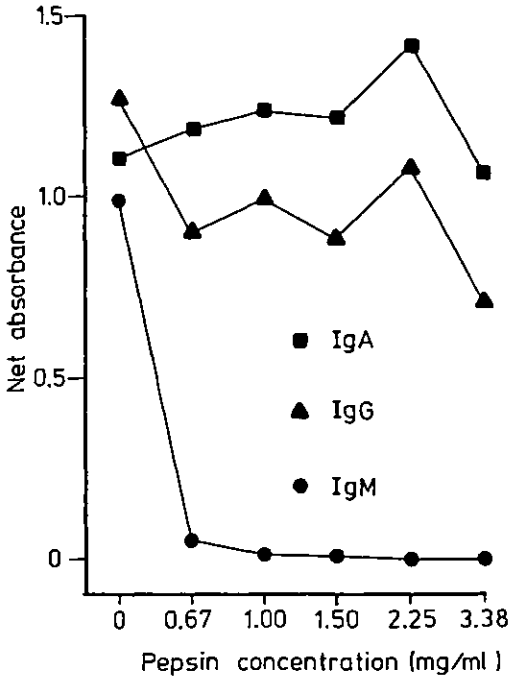


Figure III.33.

Figure III.32: The effect of pepsin digestion on serum IgG and IgM anti-PNEUMOVAX antibody levels.

A PNEUMOVAX-immune serum (Te) was incubated (16 hrs, 37°C) with increasing conc. of pepsin, then diluted and incubated in microplates, followed by anti-human IgG-and IgM-AP conjugates and pNPP.

The mean net abs. for the IgG (triangles) and IgM (circles) istypes at serum dilution 1/30 was plotted as a function of the pepsin concentration used.

Figure III.33: The effect of pepsin digestion on the antiglobulin levels in a rheumatoid serum (Ve).

The anti-human IgM, IgG and IgA AP conjugates used were those specified in III.3.2.4.

The mean net abs. for the IgM (circles), IgG (triangles) and IgA (squares) isotypes at serum dilution 1/50 was plotted as a function of the pepsin concentration used.

(a) IgM antibodies were almost completely inactivated by a pepsin concentration of 1.0 mg/ml, and this concentration also reduced the IgG activity to some extent.

(b) Pepsin-digestion did not affect IgA class antibodies under the conditions described here. This is the more interesting for the fact that this particular serum (Ve) had the highest IgA antiglobulin levels ever found in the IgA RF testing. Also, it was later found, when using monoclonal antibody for the IgG RF testing (Table III.8.) that serum Ve did not react at all. This indicates that the sheep anti-human Fab-gamma was cross-reacting with IgA in the above test, possibly because of anti-light chain reactivity.

III.3.2.5. Monoclonal antibody for IgG antiglobulin ELISA

All the three anti-human IgG conjugates described above were found to be poorly suited for an IgG RF ELISA. It was therefore fortunate that, in early 1982, a monoclonal anti-human Fab-gamma (Miles Laboratories Ltd.) became available.

This antibody (mouse IgG1, Fab-gamma specific, code no. 63-070) was purchased, together with a goat $F(ab')_2$ anti-mouse IgG (TAGO Inc., code no. 4250). The goat antibody was conjugated to alkaline phosphatase (referred to henceforth as $F(ab')_2$-m-G-AP). This double antibody system

of monoclonal anti-human IgG plus goat anti-mouse IgG was tested for reactivity with microplates coated with 5 µg/ml of human, rabbit, dog, horse, goat, sheep and bovine IgG.

Briefly, the reactivity (mean net absorbance) of the mouse monoclonal antibody with IgG from the latter six species was always less than 1% of the anti-human IgG reactivity. The reactivity of the goat antibody with human and rabbit IgG was only 2.4 and 1.7%, respectively, of the anti-mouse IgG reactivity.

Preliminary testing of several rheumatoid sera, digested with 1 mg/ml pepsin, using the protocol of Wernick *et al* (1981) (see chapter III.3.2.4. above) indicated that a 1/2,500 dilution of the monoclonal antibody, followed by a 1/250 dilution of the $F(ab')_2$-m-G-AP, would give a satisfactory reaction after a 60 min. pNPP incubation.

III.3.2.6. Testing of different serum dilutions and the use of standards

In order to establish which serum dilution or dilutions would be best suited for quantitative IgG RF testing, 13 sera and 3 synovial fluids from RA patients were incubated with 1 mg/ml pepsin in acetate buffer or buffer alone for 22 hrs., then centrifuged, neutralised, and diluted 1/10, 1/20 and 1/40 in hypertonic serum diluent. The samples were then incubated for 1 hr. at 37°C and overnight at 4°C in plates coated with rabbit IgG. Following incubation for 2½ hrs. with the mouse monoclonal antibody (diluted 1/2,500), the plates were incubated with a 1/250 dilution of the $F(ab')_2$-m-G-AP and subsequently reacted with pNPP.

Briefly, no prozone effect was observed in any of the 16 samples, the 1/10 dilution generally giving the strongest response, ranging from -0.019 to 0.670 (see Table III.8., where the mean net absorbance values for the digests and non-digests are listed). All the 13 sera were Rose Waaler positive.

Generally, the pepsin-digestion reduced the reaction considerably, 9 of the 16 digested samples exhibited 50-80% of the absorbance value of the pepsin-free non-digest (Table III.8.). However, there was no obvious relation between the undigested value and the per cent reduction after digestion.

In view of the overall rather low responses, even at sample dilution 1/10, and very high reagent cost, it was decided to test all samples only at dilution 1/10.

One of the sera in Table III.8. (Cl) which was available in a reasonable quantity was used as an internal standard and assigned an arbitrary IgG RF content of 1000 units/ml. This serum was digested in parallel with the test sera on each day of assay and diluted 4-fold, yielding dilutions containing 1000, 250, 63, 16, 3.9, and 1.0 U/ml. Figure III.34. shows one example of dilution curves for this digest and undigested control.

Serum Cl was compared with the international (British) standard for RA serum in order to assess the suitability of the latter as an IgG RF standard. By treating aliquots of the reconstituted 1.0 ml (see chapter III.1.3.3.) in the same way as sera, the dilution curves in Figure III.35. were obtained. Serum Cl was run in parallel.

Table III.8: The IgG RF levels in rheumatoid sera and synovial fluids with and without pepsin digestion.

		Mean net abs. at dil. 1/10	
Patient	Sample	Pepsin-free buffer	Pepsin
Cl	serum	0.419	0.247
Wa	serum	0.670	0.387
Li	serum	0.160	0.089
Ha	serum	0.460	0.233
Mu	serum	0.341	0.087
Whi	serum	0.075	0.037
Co	serum	0.126	0.100
Sh	serum	0.464	0.345
Who	serum	0.067	0.074
Tu	serum	0.092	0.031
Bl	serum	0.255	0.159
Da	serum	0.252	0.130
Ve	serum	-0.006	-0.019
Be	syn. fl.	0.141	0.108
Lu	syn. fl.	0.004	-0.004
Mu	syn. fl.	0.001	0.076

Thirteen sera and three synovial fluid (syn. fl.) samples were reacted with pepsin or acetate buffer alone, then incubated in microplates, followed by mouse monoclonal anti-human Fab-gamma and goat anti-mouse IgG-AP conjugate.

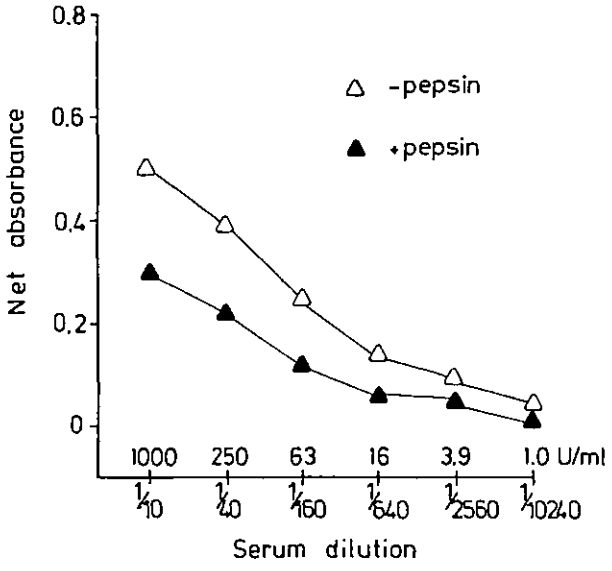


Figure III.34.

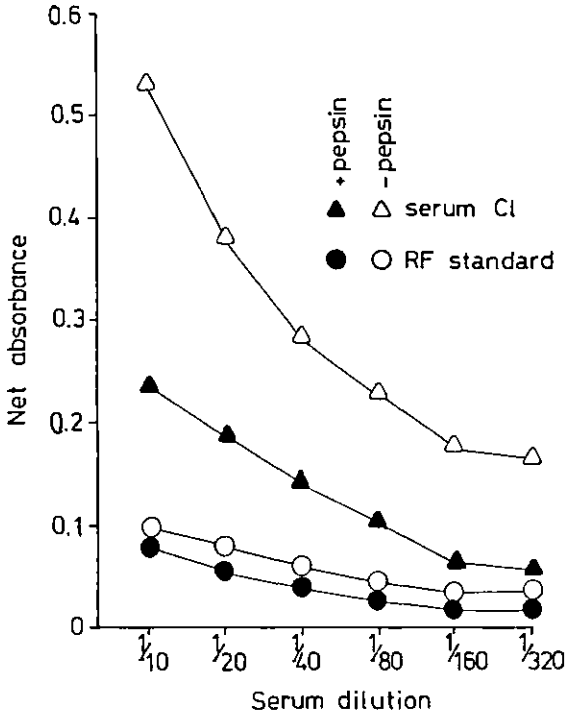


Figure III.35.

Figure III.34: Dilution curves (closed triangles: pepsin; open triangles: acetate buffer alone) for the internal IgG RF standard (serum Cl) on one day of assay.

Figure III.35: Dilution curves from simultaneous IgG RF ELISA of the international RF standard (circles) and the internal IgG RF standard (serum Cl; triangles).

Closed symbols: pepsin digests

Open symbols: Acetate buffer controls.

Briefly, the international standard was very much weaker than serum Cl. The curves for the undigested samples cannot be compared quantitatively as the RF standard is more than $2^5 = 32$ times weaker than serum Cl. The digested standard was about $2^{3 \cdot 4} = 11$ times weaker than the digested Cl serum.

On the basis of these observations it was decided that the international RF standard could not be used for the IgG RF assay.

III.3.3. The IgG RF assay as applied: reproducibility and normal ranges

III.3.3.1. The standard assay procedure for IgG RF

Coating of microplates. This was done as described for IgM RF in chapter III.1.4.1. Rows B-G were coated with rabbit IgG in coating buffer (10 µg/ml, 100 µl/well), followed by saturation with 1% BSA.

Pepsin-digestion and incubation of samples. Sample aliquots of 100 µl were incubated for 22 hrs. in a 37°C waterbath with 200 µl of 0.1 M acetate buffer, pH 3.6, containing 0.02% Na N_3 and either no pepsin or 1 mg/ml pepsin (porcine, x 2 crystallised, Sigma, London). A fresh pepsin solution was made on each day of assay. Following incubation, the samples were centrifuged at 2000 g for 20 mins. at 4°C and the supernatants neutralised with 0.3 M Na OH to pH 7.2-7.8 (monitored by a pH meter) and diluted in hypertonic serum diluent, final serum dilution 1/10.

After this the samples were immediately incubated in triplicates

(100 µl/well) in coated wells plus in one adjacent blank well. The digested and undigested samples were placed next to each other in the microplate. Following incubation for 1 hr. in a 37°C waterbath, the plates were incubated overnight at 4°C.

Monoclonal antibody and conjugate incubations. Mouse monoclonal anti-human Fab-gamma antibody (Miles Laboratories Ltd., code no. 63-070) was diluted 1/2,500 in PBS and incubated (100 µl/well, 96 wells/plate) at r.t. for 2½ hrs. Following that, the plates were incubated with a 1/250 dilution of the $F(ab')_2$-m-G-AP conjugate (75 µl/well, 96 wells/plate) for 2½ hrs. at r.t.

Enzyme substrate. The plates were incubated with 100 µl/well of pNPP for exactly 40 mins. in a 37°C waterbath, see otherwise the procedure for IgM RF in chapter III.1.4.1.

Expression of results. After reading the plates as described for IgM RF, the mean background readings for rows A and H were calculated. This background value was then subtracted from the mean of the triplicate for each sample. The IgG RF content (U/ml) was then determined by reading off the dilution curves for serum C1 in the same assay.

III.3.3.2. Day-to-day reproducibility of the IgG RF assay

Figure III.36. shows the day-to-day variation of dilution 1/10 of the internal standard (serum C1) on 15 successive days. The

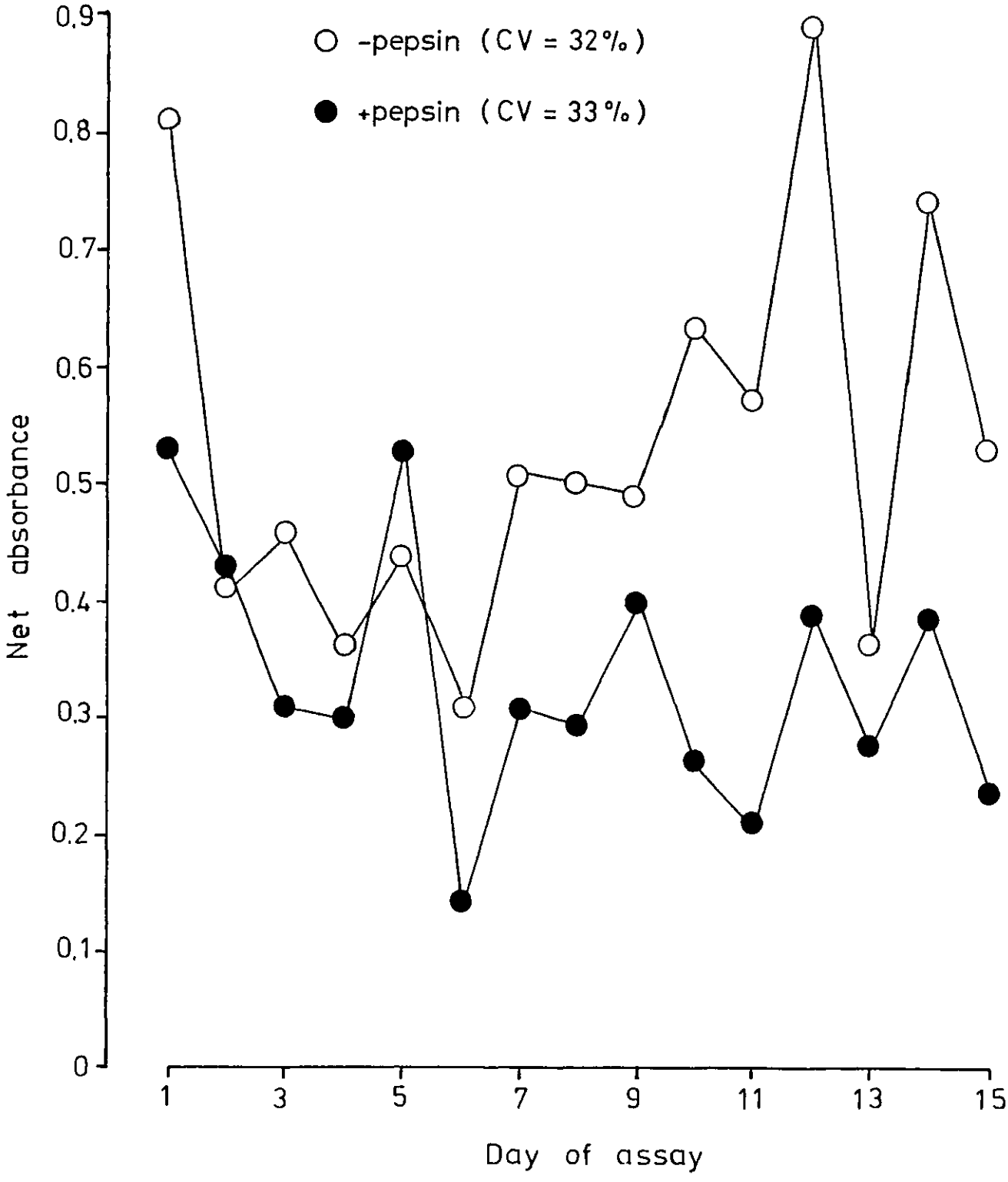


Figure III.36: Day-to-day variation of the internal IgG RF standard (serum C1, serum dilution 1/10).

Closed circles: Pepsin digests

Open circles: Acetate buffer alone

The coefficients of variation (CV) were 33% and 32% for the pepsin digests and acetate buffer controls, respectively.

coefficients of variation (CV) for the digested and non-digested samples were 33% and 32% respectively.

Figure III.37. shows the day-to-day variation for 2 sera, Ha and Co, where the absorbance values have been transformed to U/ml. The CV values were disturbingly high; 47% and 80% (Ha) and 88% and 101% (Co) for digests and undigested samples, respectively. However, the corresponding CV figures for the untransformed abs. values were considerably lower; 14% and 25% (Ha) and 50% and 54% (Co) for digests and undigested samples, respectively.

III.3.3.3. Normal ranges

In order to establish a normal serum range for the IgG RF ELISA, the 102 normal sera described in chapter III.1.4.3. were assayed. The results are shown in Figure III.38. and suggest a wide scattering of normal values. It was decided that $\geqslant$80 U/ml and $\geqslant$ 50 U/ml should be considered raised levels for digested and undigested samples, respectively.

The IgG RF levels in Icelandic groups of 16 ankylosing spondylitis and 26 Reiter's disease patients were very similar to those of the normal controls (see Figure III.38.).

Figure III.39. shows the serum IgG RF levels of 18 seropositive and 24 seronegative patients with classical/definite RA. Five of the 18 seropositive patients (28%) had raised levels, which differed significantly from the normal group ($p < 0.01$, chi-square test with

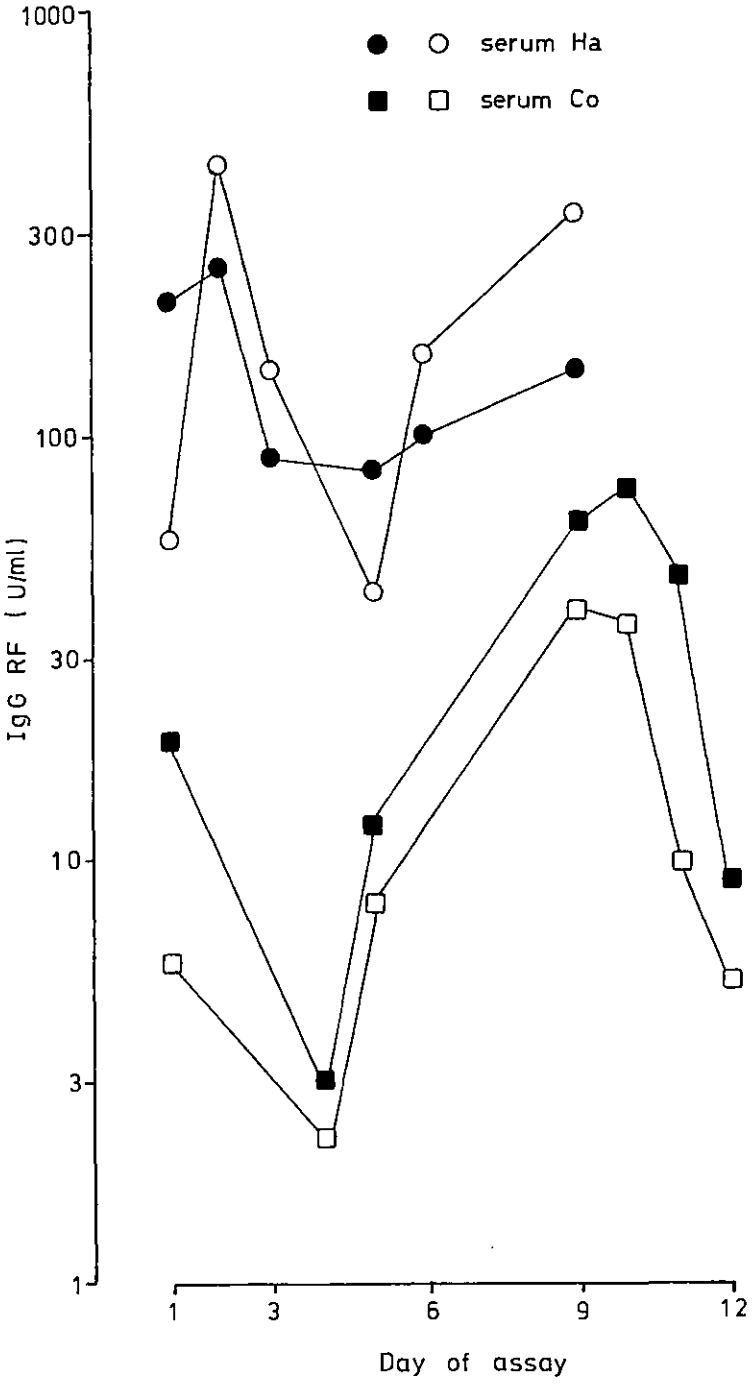


Figure III.37: The day-to-day reproducibility of the IgG RF ELISA.

Two rheumatoid sera, Ha (circles) and Co (squares) were tested on 6 and 7 different days, respectively.

The U/ml values for the digested (closed symbols) and undigested (open symbols) samples are depicted.

The coefficients of variation (CV) were 47% and 80% (serum Ha) and 88% and 101% (serum Co) for digested and undigested samples, respectively.

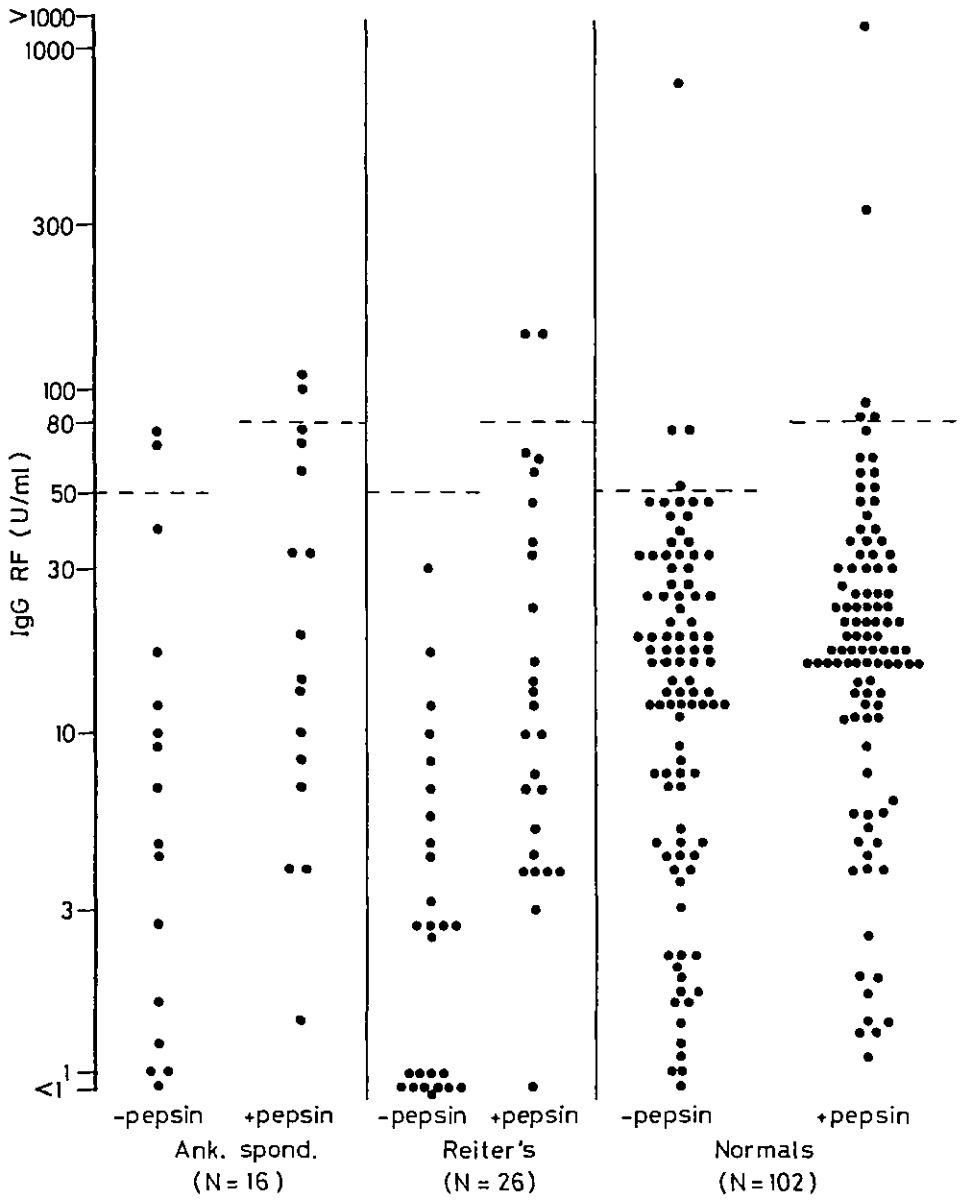


Figure III.38: The IgG RF levels (digested and undigested samples) in 102 sera from normal individuals and in sera from Icelandic patients with ankylosing spondylitis (Ank. spond.) (N = 16) and Reiter's disease (N = 26).

Raised IgG RF was defined as activity $\geqslant$ 80 U/ml and $\geqslant$ 50 U/ml for digested and undigested samples, respectively.

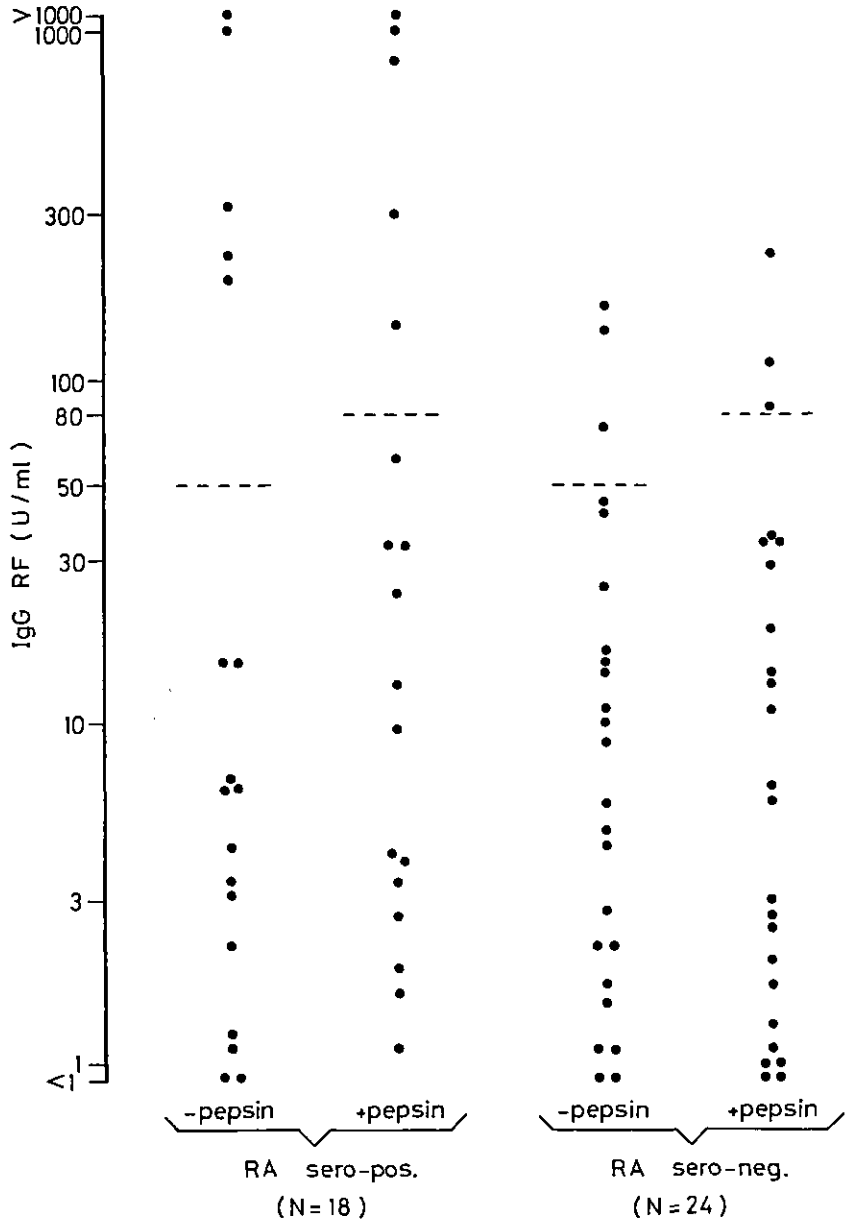


Figure III.39: The serum IgG RF levels (digested and undigested samples) in 18 seropositive and 24 seronegative RA patients.

The upper limits of normal for digested and undigested samples are indicated.

Yates's correction). Also, 3/24 (13%) of the seronegative RA patients had raised IgG RF levels.

When assaying 643 samples of sera/synovial fluids from the prospective study group discussed in chapter V, it was found that the mean net absorbance values for the non-digested samples correlated significantly ($r = 0.778$) with the values for the pepsin digests. Furthermore, regression analysis revealed that the mean net absorbance values for the digests were on average 58% of the corresponding values for the undigested samples, indicating that pepsin-digestion reduced the absorbance values by just over 40%.

The CNA values (dilutions 1/10 + 1/100 + 1/1000) for IgM RF in the 643 samples of the prospective group correlated significantly ($r = 0.289$, $p < 0.001$) with the undigested IgG RF levels. However, the IgM RF correlated even better ($r = 0.407$, $p < 0.001$) with the digested IgG RF values (Table V.2.).

There was no significant correlation between the digested/undigested sample values for IgG RF and the total serum IgG levels in a group of 173 members of the family discussed in chapter VI (digested IgG RF vs. total serum IgG:$r_s = -0.036$, undigested IgG RF vs. total serum IgG:$r_s = 0.104$, $p > 0.05$, Table VI.9.).

III.3.4. Discussion

The observation that some normal individuals have markedly raised levels of IgG antibodies reactive with ruminant IgG agrees with a recent

paper by Palosuo and Milgrom (1981). In agreement with the data presented in Figure III.27., these authors noted that the IgG anti-globulins in the sera of normal blood donors were independent of IgM antiglobulins. This subject is dealt with further in chapter IV. However, this indicates that ruminant (goat, sheep, bovine) IgG is unsuitable as substrate in IgG RF assays, as it would not discriminate between "rheumatoid" IgG anti-globulin and the "allergic" IgG class anti-ruminant IgG.

The results in Figure III.26. give the impression that in the presence of raised levels of IgM RF, IgG antiglobulins, possibly of autoreactive nature, are usually present as well. However, this could be a false positive reaction, caused by glutaraldehyde-aggregated antibody being bound by IgM RF on the solid phase (see discussion in III.2.6.).

The striking 2-ME induced decrease in "IgG antiglobulin" levels in IgM RF positive rheumatoid sera (Figures III.30. and III.31.) illustrates quite clearly the potential pitfalls in solid phase antiglobulin assays. The exact mechanism for the "IgM dependence" of the "IgG anti-globulin" levels is not quite clear. The most likely explanation is that as the rabbit gammaglobulin fraction used for R-G-AP was not affinity purified, a high proportion of the alkaline phosphatase labelled rabbit IgG molecules were non-specific. On top of that, the glutaraldehyde probably cross-linked the antibody to some extent, giving rise to complexes of rabbit Fc-gamma, an ideal RF-substrate (see Figure III.3.). These cross-linked antibody complexes in ELISA reagents may also explain why interference by RF seems to be a greater problem in ELISA than SPRIA systems.

The PNEUMOVAX antibody system (Figures III.29. and III.32.) serves as a valuable control here, ensuring that "IgM-independent" IgG antibodies are indeed largely resistant to 2-ME and pepsin.

The observation shown in Figure III.33. that IgA RF seems to be largely resistant to pepsin digestion has important implications. As IgA RF may occur in a substantial proportion of rheumatoid sera, (March *et al*, 1981) it is important in IgG RF assays to use antisera which do not react with light chains or the alpha chain of IgA. Indeed, this possibility was acknowledged in a recent paper by Faith *et al* (1982) where their commercial anti-human Fab-gamma antiserum was lacking in specificity. As the anti-human IgA reagent described in III.2. was affinity-purified, anti-light chain reactivity should not have been a problem in the IgA RF assays.

Turning to the IgG RF assay as applied, it was decided to incubate the sera in the microplates, both for 1 hr. at 37°C and overnight at 4°C. This was done in order to facilitate the detection of low-avidity antiglobulins, as low-avidity antibodies may be more effectively detected by incubation at a low temperature (Lindström and Wager, 1978).

The very high day-to-day coefficients of variation shown in Figure III.37. indicate that the IgG RF assay as applied is at best semiquantitative. It should be noted, however, that the undigested samples are no better than the digested ones in this respect. Also, as the unit values are logarithmic, the CV values for them will be higher than those for the corresponding absorbance values (see Figure III.22.). It might also be expected that the within-day

variability of the IgG RF assay is less than that shown in Figure III.37., cf. figures III.22. and III.23. for IgA RF.

The distribution of normal vs. patient serum levels of the IgG RF is markedly similar (see Figure III.38.). However, IgM RF positive RA sera (Figure III.39.) do exhibit significantly higher levels than the normals. It should be also noted that very few of the 42 RA patients whose serum IgG RF is depicted in Figure III.39. had any evidence of vasculitis, but the vasculitic subgroup of RA patients has been especially associated with high IgG RF levels (Allen *et al*, 1981). Also, various authors have reported considerable overlap between IgG antiglobulin levels of normals and RA patients (March *et al*, 1981; Faith *et al*, 1982) but the explanation for this remains speculatory.

Generally, the pepsin-digestion lowered the absorbance value by 30-50% (see Table III.8. and the results of the regression analysis for the prospective RA group in III.3.3.3.). This reduction was variable, however, and contrary to what might have been expected, it was often less marked for rheumatoid sera with high IgM RF levels. Some rheumatoid sera even showed a convincing absolute increase in absorbance after pepsin-digestion, indicating perhaps the digestion and release of IgG RF from IgG-IgG complexes. The observation that the IgG RF levels of digested samples show a better correlation with IgM RF than the IgG RF levels of the undigested samples do, supports this hypothesis.

The difference between the IgG RF ELISA as applied and the system where the R-G-AP conjugate was used (III.3.2.2. and III.3.2.3.) is very

striking. The fact that the IgG antiglobulin levels in the undigested samples do not show any marked association with IgM RF is clearly a different story altogether from the 2-ME experiments. The following is likely to account for this difference.

As the monoclonal anti-human Fab-gamma was used unlabelled, it was not subjected to cross-linking by glutaraldehyde and may therefore have had less tendency to react with IgM RF on the solid phase. Furthermore, IgM RF has lower affinity for mouse IgG than rabbit IgG (see Figure III.3.), and due to the exquisite specificity of the monoclonal antibody, the absolute concentration of mouse IgG in the monoclonal incubation was very low indeed. Moreover, as the antibody in the conjugate ($F(ab')_2$-m-G-AP) was pepsin-digested, its addition should not have led to any binding to IgM RF on the solid phase.

Taking these observations together, it would seem that when monoclonal mouse antibody is used, pepsin-digestion of the serum samples is only of marginal benefit which probably does not justify the considerable extra effort involved.

III.3.5. Conclusion

The selection of RF substrate and anti-RF antibody system were found to be critical factors for the establishment of the IgG RF ELISA. When using highly specific non-aggregated monoclonal antibody of non-rabbit origin, reduction or digestion of test samples is

probably unnecessary. Despite poor reproducibility and a wide normal range, raised levels of IgG antiglobulins reactive with rabbit IgG were detected significantly more often in sera from seropositive RA patients than normals.

C H A P T E R I V

ANTIBODIES TO RUMINANT IgG AND PNEUMOCOCCAL POLYSACCHARIDES

C H A P T E R I V

ANTIBODIES TO RUMINANT IgG AND PNEUMOCOCCAL POLYSACCHARIDES

IV.1. INTRODUCTION

As outlined in chapter III.3.2.1., it was observed during experiments with goat IgG as IgG antiglobulin substrate that several healthy blood donors had markedly raised IgG anti-goat IgG levels, yet did not show any significant IgM anti-goat IgG response. Indeed, some of the individuals who showed the greatest IgG anti-goat IgG responses in the system described in chapter III.3.2.1. were young healthy laboratory controls.

Thus, these IgG antiglobulins did not seem to bear any relation to RA or rheumatic diseases in general. However, literature search on anti-ruminant protein antibodies revealed the following:

In 1906, German workers described antibodies in the blood of "marasmic" infants that reacted with protein antigens in milk (Rothberg and Farr, 1965). Antibodies to cow's milk have since been widely studied and these have been found to be reactive with bovine serum albumin (BSA) and bovine gamma globulin, both of which are present in normal cow's milk (Holland *et al*, 1962). Rothberg and Farr (1965) tested 900 human sera for anti-BSA activity and found that whereas 75% of children up to 15 years of age showed anti-BSA activity, only 25% and 8% of adults aged 16-40 years, and over 40 years, respectively, showed such activity. Delire *et al* (1978) furthermore showed that infants who were fed on cow's milk invariably developed circulating immune complexes, consisting of maternal IgG and milk

proteins. When these authors tested sera from 50 healthy members of their staff, all the sera gave a precipitin line against a milk protein preparation.

Kletter *et al* (1971) showed that after infants started on cow's milk, they generally developed increasing levels of IgG-class anti-milk antibodies, reaching a maximum at about three months of age. Thereafter, the anti-milk IgG titre gradually declined. Kletter *et al* furthermore showed that while IgA anti-milk antibodies did occur, IgM class antibodies were only rarely encountered.

Hemmings and Williams (1978) showed that radio-labelled bovine IgG was taken up by gastrointestinal epithelial cells of the rat and broken down into fragments with a molecular weight of 20,000-50,000 daltons. Subsequently, these IgG-fragments became scattered throughout the rat tissues where they could still be precipitated by anti-bovine IgG antiserum. Thus, it seems clear that the gastrointestinal tract of man and animals is permeable to antigenically active fragments of heterologous IgG and this absorption of food antigens may be especially marked in patients with selective IgA deficiency (Cunningham-Rundles *et al*, 1979). However, the general clinical implications of this have been the subject of some controversy. Heiner *et al* (1962) reported a group of 7 patients suffering from poor weight gain and severe gastrointestinal and respiratory tract symptoms, all of whom had precipitins to cow's milk. On the other hand, Firer *et al* (1982) recently reported that healthy children had even higher IgG anti-milk antibody levels than children allergic to cow's milk.

In the light of this evidence from the literature, it seemed

quite likely that some of the IgG antiglobulins found by my ELISA system had arisen in response to milk proteins. The fact that these antiglobulins were reactive with goat IgG does not necessarily imply exposure of the individuals to goat proteins as it was shown in chapter III.1.2.3. (Table III.3.) that goat, sheep and bovine IgG are probably closely related antigenically. Also, goat's milk proteins have been shown to be antigenically similar to cow's milk proteins (Heiner *et al*, 1962).

The experiments described in the following section were performed in order further to characterise the IgG anti-ruminant antiglobulins and their possible relation to rheumatic diseases and immune responsiveness.

IV.2. ANTIBODIES TO RUMINANT IgG: PROPERTIES AND OCCURRENCE

IV.2.1. Immunochemical properties and specificity of anti-ruminant IgG

Sensitivity to 2-ME. It was shown in chapter III.3.2.3. (Figures III.30. and III.31.) that IgM antiglobulins in rheumatoid sera may cause false positive tests for IgG antiglobulins. In order to test whether the observed IgG anti-ruminant IgG antibodies were related to IgM antiglobulins in a similar manner, two sera from healthy laboratory workers which reacted strongly with goat IgG were incubated with 2-ME, using the protocol described in chapter III.3.2.3. These sera were then incubated for 2 hrs. at r.t. in plates coated with 10 µg/ml goat IgG, followed by 1/1000 dilutions of goat anti-human IgG (TAGO Inc.)-AP and goat anti-human IgM (TAGO Inc.)-AP conjugates. After incubation with pNPP and reading of the plates, the dilution curves for 2-ME treated sera and controls were plotted (see Figures IV.1. (serum Sa) and IV.2. (serum Fr)).

Briefly, it can be seen that these two sera contained only very low levels of IgM antibodies reactive with goat IgG, and the IgG antibody responses were not affected by 2-ME.

Inhibition by aggregated IgG. In order to test whether the IgG antiglobulins in healthy individuals differed from "rheumatoid" antiglobulins in terms of reactivity with IgG from different species, the following experiment was performed:

The two normal sera described above (Sa and Fr) and two sera from RA patients (Fi and Ru) with high RAHA titres were selected for

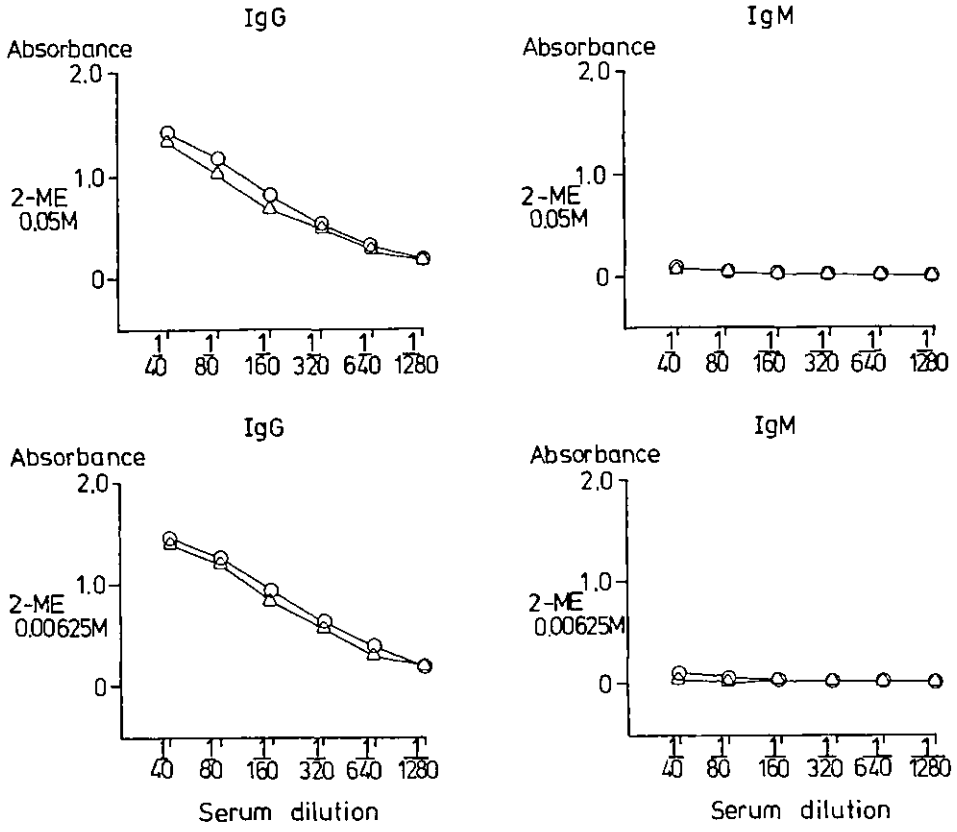


Figure IV.1: The effect of 2-ME treatment on serum antiglobulins reactive with ruminant IgG (serum Sa).

The 2-ME treatment was analogous to that described in III.3.2.3., see fig. III.30.

Left hand side: IgG antiglobulins
Right hand side: IgM antiglobulins
Triangles: 2-ME treated samples
Circles: PBS control

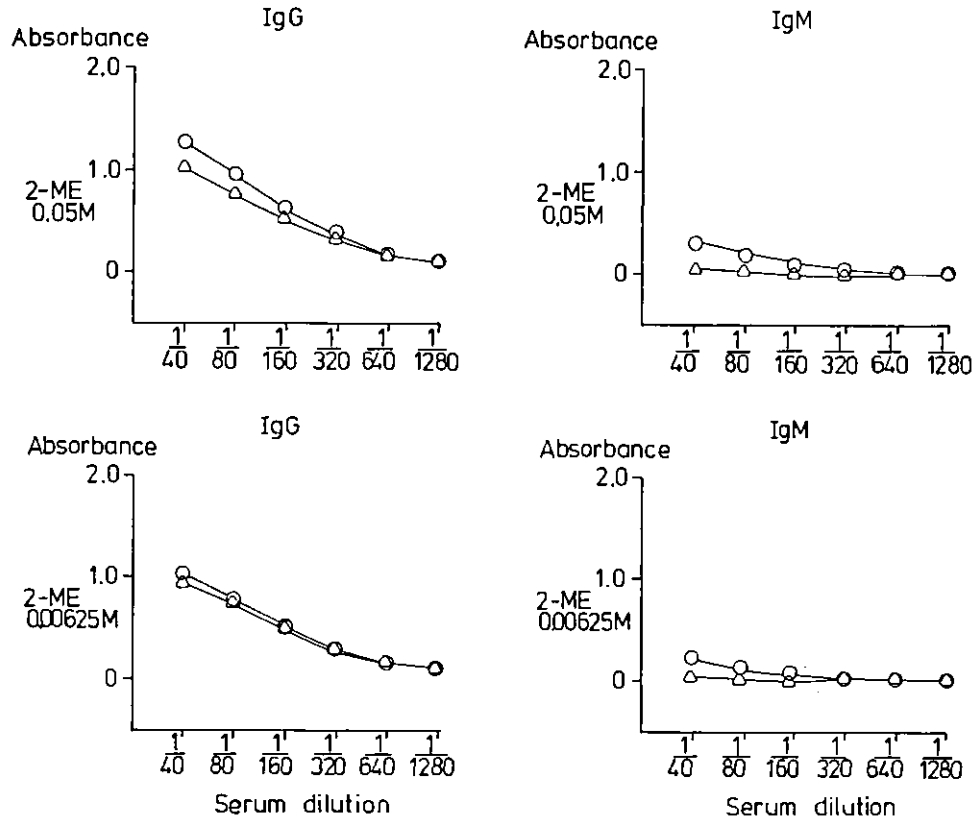


Figure IV.2: The effect of 2-ME treatment on serum antiglobulins reactive with ruminant IgG (serum Fr).

Experimental design and symbols were as shown for serum Sa in Fig. IV.1.

the experiment. Normal IgG of human, sheep and rabbit origin was diluted in PBS to a concentration of 10 mg/ml. These IgG solutions were aggregated by incubation in a 63 °C waterbath for 15 mins. (Halbert *et al*, 1980) and then diluted in hypertonic serum diluent, yielding a final concentration of 60 µg IgG/ml.

The four sera were diluted (final dilutions 1/50, 1/500 and 1/5000) in the hypertonic diluent containing either aggregated IgG or no IgG (control). After incubation in test tubes for 40 mins. at 37 °C, the serum dilutions were aliquoted into microplates coated with 10 µg/ml goat IgG. Following incubations for 1 hr. at 37 °C and for another hour at 4 °C, the microplates were reacted for 2½ hrs. at r.t. with a 1/1000 dilution of a goat anti-human IgG-AP conjugate. This was then followed by pNPP incubation for 55 mins., after which the plates were read.

Figure IV.3. shows the combined net abs. (CNA) values for the four sera pre-incubated with either aggregated rabbit, sheep or human IgG or diluent alone. Clearly, the incubation with sheep IgG has abolished the IgG antiglobulin response of the normals completely, whereas human and rabbit IgG had little effect. The rheumatoid sera, however, behaved very differently in that these were significantly inhibited by human IgG but much less by rabbit or sheep IgG. Although it may seem presumptuous to draw general conclusions from experiments with only four sera, this indicates that the IgG anti-goat IgG antiglobulins of healthy individuals indeed react preferentially with IgG of ruminant, rather than rabbit or human, origin.

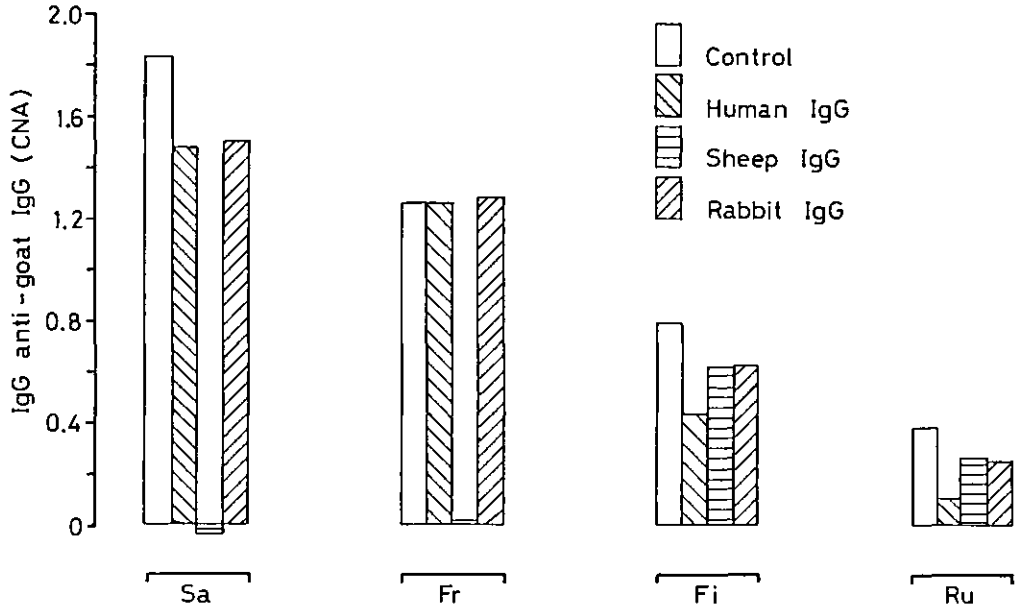


Figure IV.3: The reactivity of IgG antiglobulins in normal and rheumatoid sera with IgG from different species.

Two normal (Sa and Fr) and two rheumatoid (Fi and Ru) sera were pre-incubated with aggregated human, sheep or rabbit IgG and subsequently tested for IgG antiglobulin levels on plates coated with goat IgG (IV.2.1.).

The results were expressed as the CNA values for serum dilutions 1/50 + 1/500 + 1/5000.

IV.2.2. The occurrence of IgG anti-ruminant IgG and its relation to rheumatic diseases

IV.2.2.1. The ELISA for IgG anti-ruminant IgG

The assay for IgG anti-ruminant IgG was performed in a similar manner to the ELISA for IgM RF and IgA RF. Briefly, rows C-F of microplates were coated (overnight at 4°C) with a 10 µg/ml solution of goat IgG (Miles Laboratories Ltd.), followed by saturation with BSA. The sera to be tested were diluted in hypertonic serum diluent (final dilutions 1/10 and 1/100), then incubated for 2 hrs. at r.t. in duplicates in coated and adjacent blank wells.

After incubation for 2½ hrs. at r.t. with a 1/1000 dilution of a goat anti-human IgG (TAGO Inc.)-AP conjugate, the plates were reacted for 60 mins. with pNPP, then read immediately. The results were expressed as the combined net abs. (CNA) values for serum dilutions 1/10 + 1/100.

IV.2.2.2. IgG anti-ruminant IgG in the Icelandic family

The IgG anti-ruminant IgG levels of 177 members of the Icelandic family dealt with in chapter VI were measured. For the first 104 family sera assayed, sera from age and sex matched healthy Icelanders (obtained from the Blood Bank, Iceland) were used as controls. Figure IV.4. shows the distribution of the CNA values in the two groups. The values are clearly very similar, thus, the family members as a group do not seem to differ in this respect from the general Icelandic population.

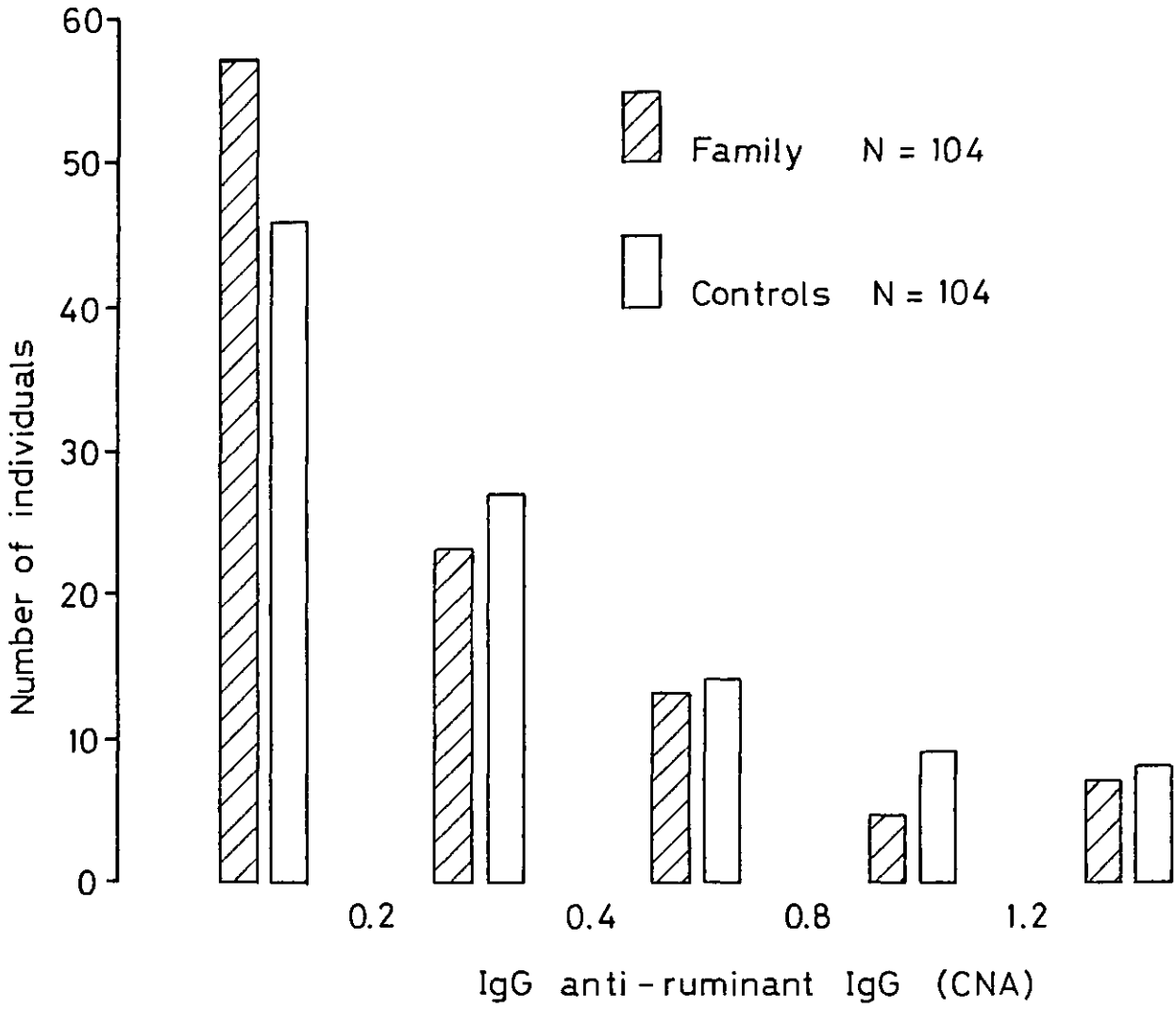


Figure IV.4: The IgG anti-ruminant IgG levels in 104 members of the Icelandic Adalvik family (see chapter VI) and an Icelandic age and sex matched control group.

The results were expressed as the CNA for serum dilutions 1/10 + 1/100.

Furthermore, it was found that the IgG anti-ruminant IgG levels of the 177 family members did not correlate with the total serum IgG levels (r_S = 0.090, not significant, see Table VI.9.). Thus, the test for IgG anti-ruminant IgG is not just measuring non-specific concentration-dependent sticking of native IgG to the solid phase.

There was a just significant negative correlation between age and IgG anti-ruminant IgG levels in the family (r_S = -0.150, $p < 0.05$, Table VI.11.), implying that the older people generally had less IgG anti-ruminant IgG than the younger family members.

During the family survey (see chapter VI), a questionnaire (Table VI.1.) was used in which the respondents were asked whether they had, during the previous 12 months, been affected by:

1. pain in $\geqslant$ one joint;

2. joint swelling;

3. morning stiffness of the joints; or

4. pain in $\geqslant$ three joints.

The questionnaire was analysed by determining the number of positive responses for each individual (ranging from 0-4). When these were correlated with the IgG anti-ruminant IgG levels, a negative correlation was observed (r_S = -0.160, $p < 0.05$, see Figure IV.5.)

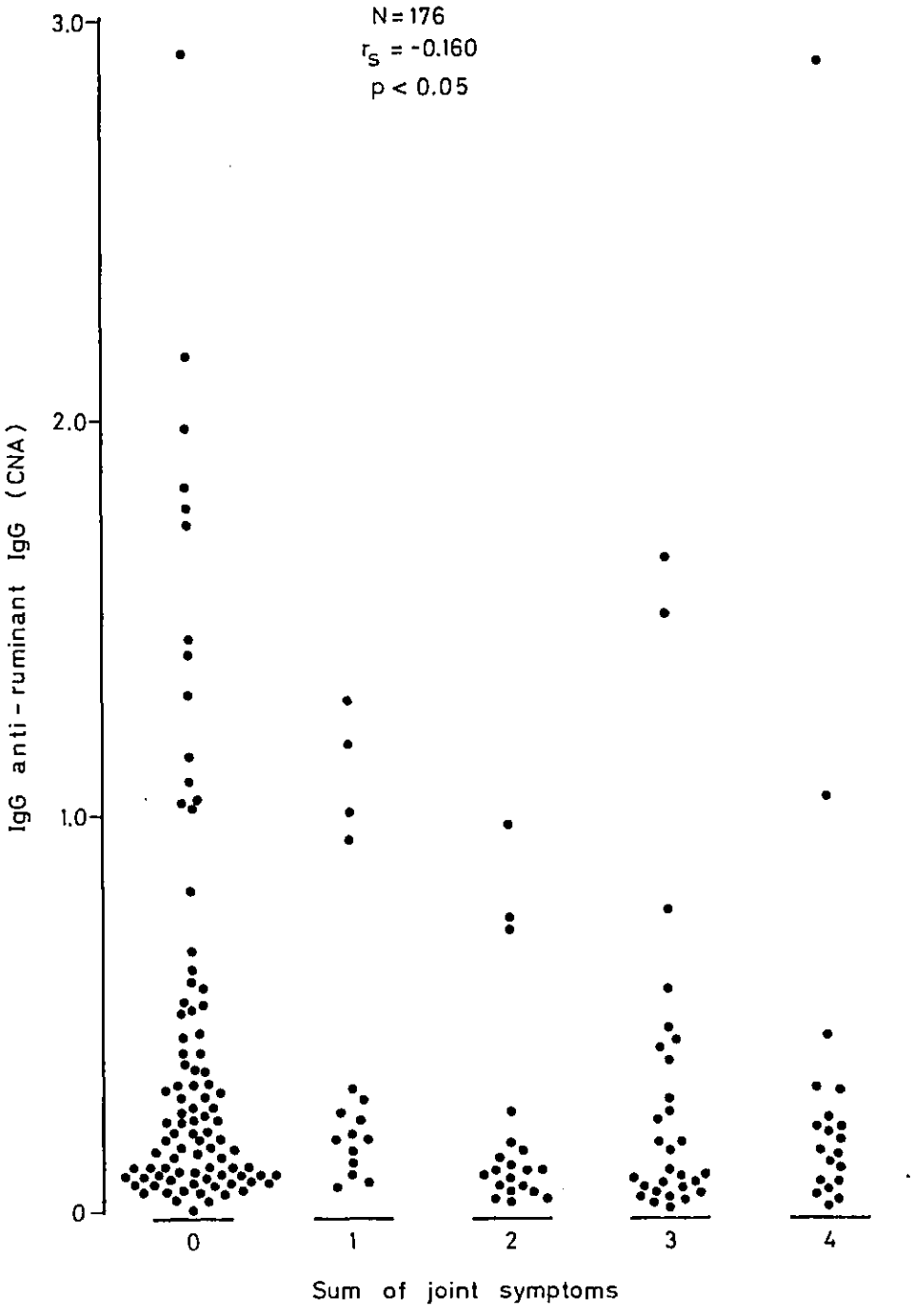


Figure IV.5: The relationship between joint symptoms and IgG anti-ruminant IgG levels in 176 members of the Icelandic Adalvik family (chapter VI).

The sum of joint symptoms refers to the response to questions 8-11 in table VI.1., see also VI.2.4 and VI.3.2.

The negative correlation was significant at the 5% level (r_s = -0.160, Spearman's rank correlation method).

Furthermore, when the IgG anti-ruminant IgG levels of 35 family members who were given a rheumatic diagnosis were compared with those of 142 healthy family members, the following was observed;

When CNA values >0.500 were taken as raised, significantly more healthy than affected family members had raised levels of IgG anti-ruminant IgG (see Table IV.1.).

IV.2.2.3. IgG anti-ruminant IgG in IgA deficient individuals

Twenty-six IgA deficient sera collected during a survey in Iceland (Ulfarsson *et al*, 1982) were kindly provided by Dr. O. Jensson, Director of The Blood Bank, Iceland. The IgG anti-ruminant IgG levels of these were determined by the ELISA assay. As shown in Figure IV.6., the levels were very similar in distribution to those of the 104 normal Icelandic controls (see Figure IV.4.).

IV.2.3. Discussion

The finding that the IgG anti-ruminant IgG levels were not affected by 2-ME agrees with a recent paper by Palosuo and Milgrom (1981). These authors who attempted to use goat IgG (complexed with human serum albumin) as IgG RF substrate in an ELISA system noted that some of their healthy blood donors had raised serum levels of IgG

Table IV.1: Comparison of IgG anti-ruminant IgG levels in healthy and affected members of the Icelandic Adalvik family.

Level of IgG anti-ruminant IgG	Healthy	Affected	Significance
Raised (CNA >0.500)	33	2	$p < 0.05$
Not raised	109	33	$\chi^2 = 4.39$

Sera from 177 family members were tested, 35 of whom were affected by rheumatic disease (see Tables VI.3. and VI.4.).

CNA values above 0.500 were considered raised.

Chi-square test with Yates's correction was used to compare the groups.

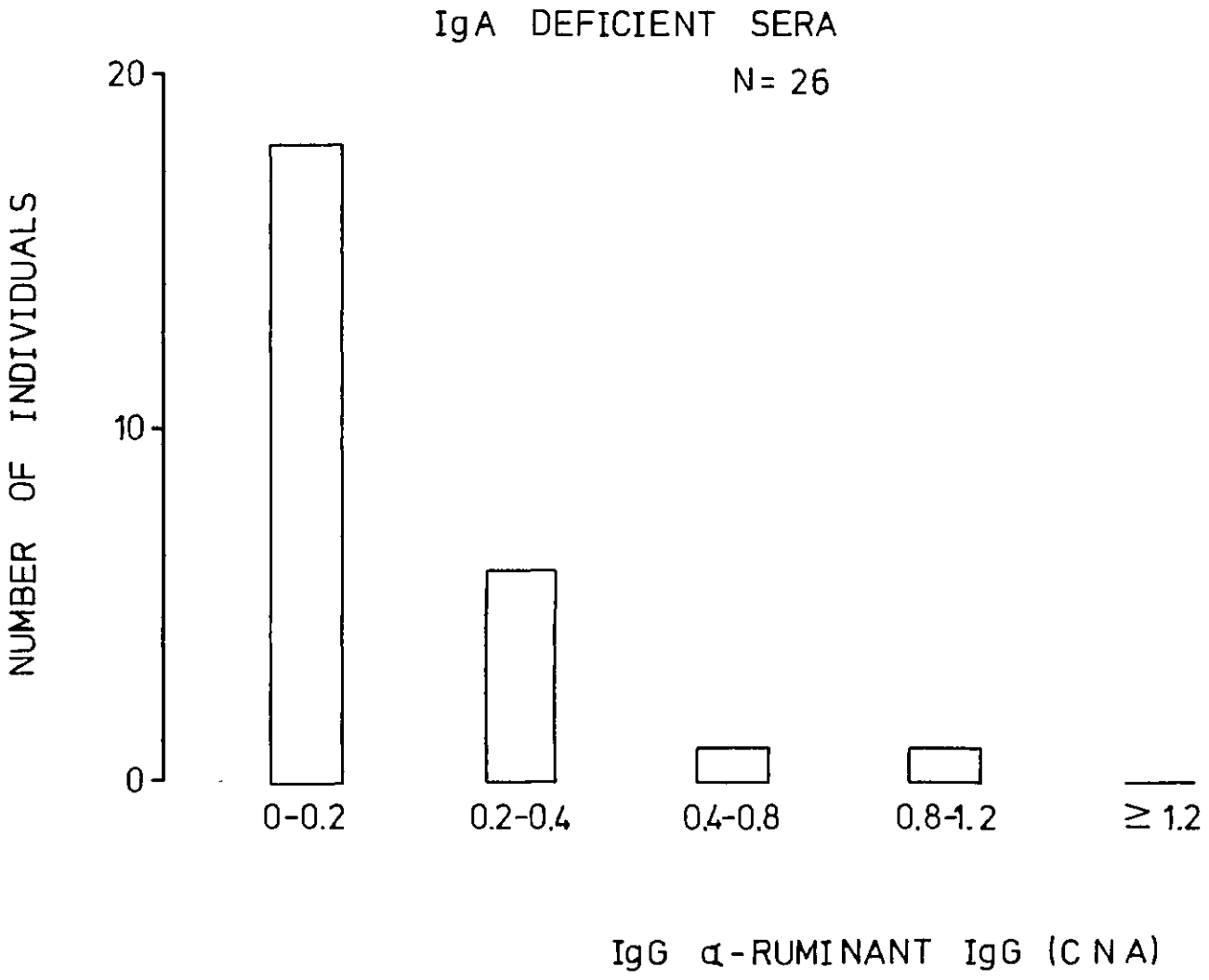


Figure IV.6: The distribution of IgG anti-ruminant IgG levels in 26 IgA deficient Icelandic individuals.

The antiglobulin levels were determined in the same manner as described for the 104 family members and their controls (fig IV.4.).

antiglobulins, but did not have any IgM RF. However, in contrast to the rheumatoid sera (Fi and Ru) shown in Figure IV.3., Palosuo and Milgrom noted that the IgG antiglobulins of four rheumatoid sera which they tested were almost completely absorbed by pre-incubation with ruminant IgG. This might mean that the distinction between IgG anti-ruminant IgG and IgG antiglobulins of "rheumatoid" nature is not always as clear as the results shown in Figure IV.3. seem to indicate.

Also, strongly IgM RF positive sera may display IgG anti-goat IgG responses, cf. Figure III.26., even though it was noted that only 3 of 27 Rose Waaler positive sera from the members of the Icelandic Adalvik family (chapter VI) showed enhanced reactivity (CNA value >0.500) in the IgG anti-goat IgG assay. Furthermore, there was no overall correlation in sera from 173 of the family members between the IgM RF levels (IU/ml) and the IgG anti-ruminant IgG (r_S = 0.072, not significant).

The results of Pope and McDuffy (1981) suggested that sheep and goat IgG is an even better substrate for IgG RF in a SPRIA system than human Fc-gamma. However, my results and those of Palosuo and Milgrom (1981) would strongly suggest that in an ELISA or SPRIA system for IgG RF, ruminant IgG should be avoided as substrate because of the frequent occurrence in the normal population of IgG antiglobulins which react preferentially with ruminant IgG.

Recently, there has been increasing interest in the rõle of anti-milk antibodies in eczema and milk allergy in children (Dannaeus and Inganas, 1981; Firer *et al*, 1982). While no information is available on allergy/eczema in the Icelandic family, total serum IgE levels in

123 of the family members (measured by Phadebas IgE PRIST kits, Pharmacia Diagnostics AB, Uppsala, Sweden) did not show any correlation with the IgG anti-ruminant IgG levels (r = 0.025, not significant).

Huntley *et al* (1971) and Leikola and Vyas (1971) reported the occurrence of antibodies to ruminant IgM in IgA deficient sera. Cunningham-Rundles *et al* (1978) found circulating immune complexes and precipitating antibodies to bovine milk in 50% of 22 IgA deficient patients. It was somewhat surprising, therefore, that only one of the 26 Icelandic IgA deficient individuals had a CNA value for IgG anti-ruminant IgG in excess of 0.5, and generally their levels closely mimicked the values for the Icelandic family and controls.

How can the negative correlation between IgG anti-ruminant IgG antibody levels and rheumatoid symptoms/diseases in the Icelandic family be explained? Three possibilities will be discussed here.

(a) There was a clear positive correlation within the family between the occurrence of joint symptoms and age (see chapter VI.3.5. and Table VI.11.) (r_s = 0.230, $p < 0.01$). Also, the majority of those 39 who had a rheumatic diagnosis were elderly (mean age 50.6 years, compared with a mean age of 37.9 years for the rest). As there was a tendency for the IgG anti-ruminant IgG levels to be higher in the younger family members, and anti-milk protein antibody levels show negative correlation with age (Rothberg and Farr, 1965), the negative association in the family might be purely age-dependent and not a cause-and-effect phenomenon.

(b) Butler *et al* (1978) found when comparing various immunoassays that ELISA results correlated better with changes in antibody affinity than absolute antibody concentration, as measured by the Farr assay. If this can be extrapolated to the ELISA for IgG anti-ruminant IgG levels, it could be argued that while most, if not all, humans would be expected to show a humoral immune response against ruminant IgG, absorbed, e.g., from ingested cow's milk, only those individuals who are able to produce high affinity antibody would show an enhanced response in the ELISA system.

Alpers *et al* (1972) showed that mice producing high affinity antibody eliminate antigen more effectively than those producing low affinity antibody. Soothill and Steward (1971) furthermore showed that mice that produce low affinity antibody were more prone to develop nephritis following a viral infection than mice of strains producing high affinity antibody.

Thus, it could be postulated that those family members who are prone to produce antibody of low affinity would show diminished ELISA responses to ruminant IgG. These same individuals might also be expected to be less able to eliminate foreign antigens, e.g., viruses, which in turn might lead to chronic, perhaps subclinical infections and RF production and arthritis.

(c) Those family members who fail to mount an IgG response to ruminant IgG from the diet, might be generally poor at producing IgG (and IgM) antibodies against forein antigens. That, again, might lead to prolonged, perhaps subclinical, infections and then arthritis.

It might be argued that those individuals who are poor IgG antibody producers should have relatively low total serum IgG levels. The finding that the IgG anti-ruminant IgG levels in the family did not correlate with total serum IgG, therefore, seems to argue against possibility (c), and in favour of possibility (b).

It was therefore decided to test whether those who exhibited elevated IgG levels directed against ruminant IgG would mount enhanced *in vivo* antibody responses to foreign antigens, as detected by the ELISA system. This issue is the subject of the following section, chapter IV.3.

IV.3. ANTIBODIES TO PNEUMOCOCCAL POLYSACCHARIDES

IV.3.1. Introduction

A number of antigens have been used to assess the *in vivo* humoral immune response in humans. These include keyhole limpet haemocyanin (KLH) (Curtis and Hersh, 1972), the bacteriophage ΦX 174 (Lopez *et al*, 1975), helix pomatia haemocyanin (Weits *et al*, 1978) and flagellin from salmonella adelaide (Rowley and MacKay, 1969), all of which are claimed to elicit a primary antibody response.

However, for ethical reasons, approved vaccines are preferable to antigens like KLH for the *in vivo* assessment of antibody responses in volunteers. Various vaccines have been used for this purpose, including tetanus toxoid (Stevens and Saxon, 1978) and vaccines containing pneumococcal polysaccharides (Jarrett *et al*, 1980). The recent development of ELISA assays for the antibody responses to pneumococcal polysaccharides facilitates the use of these for the assessment of immune responsiveness (Barrett *et al*, 1980a; Russell *et al*, 1980; Melville-Smith and Sheffield, 1980; Hosea *et al*, 1981). Furthermore, the pneumococcal polysaccharide vaccines are generally safe, although 40-98% of vaccinees report local reactions, including discomfort, erythema and induration or swelling (Schwartz, 1982b). The clinical efficacy of the vaccination is a matter of controversy, however, but it has been shown to offer good protection in special groups of young gold miners and patients with sickle cell anaemia (reviewed by Schwartz, 1982b).

Various preparations of pneumococcal polysaccharide vaccines are available, the most frequently used one in the U.K. is a tetradecavalent

vaccine, "PNEUMOVAX", from Merck, Sharp and Dohme, containing a mixture of polysaccharides from 14 of the most clinically relevant serotypes of diplococcus pneumoniae (types 1, 2, 3, 4, 6, 8, 9, 12, 14, 19, 23, 25, 51 and 56). A recently discovered disadvantage associated with "PNEUMOVAX" was its tendency to induce antibodies that cross-reacted with blood group substance A (Boyer *et al*, 1981). It was found that the agent in "PNEUMOVAX" responsible for this was a macromolecular A-like antigen, probably derived from the culture medium and not associated with the bacterial polysaccharides themselves (Siber *et al*, 1982).

In view of both ethical and technical considerations PNEUMOVAX seemed to be well suited for testing the hypothesis that a raised serum IgG anti-ruminant IgG level might reflect the individual's general capacity to mount an antibody response to foreign antigens.

IV.3.2. Selection of vaccinees; immunisation protocol

Thirty-two members of the family dealt with in chapter VI were selected on the basis of accessibility (resident in Reykjavik or neighbourhood) and levels of IgG anti-ruminant IgG. Because of the possibility that PNEUMOVAX contained blood group A-like substance, females of child-bearing age were accepted only if they possessed the blood group A.

A total of 20 family members were visited and given one dose (0.5 ml) of PNEUMOVAX subcutaneously in the left upper arm. These 20 individuals had been shown to have widely varying levels of IgG

anti-ruminant IgG. Twenty ml of blood were taken from each individual immediately before the vaccination. Subsequently, 19 of the vaccinees were bled again 17-24 days later, but it proved impossible to contact one of those vaccinated.

The age range of the group was 26-69 years, mean 47.2 years, 9 females and 10 males. One suffered from definite SLE, another had suspected SLE, one had probable RA, one had palindromic rheumatism and one was judged to have either probable RA or suspected SLE. None of the 19, however, were taking disease modifying drugs (gold, penicillamine or chloroquine) or steroids.

A second group of 10 laboratory workers was selected from among the staff in the Department of Immunology, St. Mary's Hospital. These were four females and six males, all healthy, age range 20-45 years, mean age 31.1 years. After primary bleeding, each individual received 0.5 ml of PNEUMOVAX subcutaneously. Subsequently, the laboratory group was bled on days 7, 14 and 28.

All sera were separated soon after collection and stored at -80°C until tested.

IV.3.3. ELISA for IgG and IgM antibodies to pneumococcal polysaccharides

IV.3.3.1. Materials

The 14 individual polysaccharides in PNEUMOVAX were purchased separately (lyophilised, 2 mg of each polysaccharide) from "American Type Culture Collection", Maryland, U.S.A.

Standard flat-well microplates (M29AR) were used throughout. Antibody-enzyme conjugates were made by coupling alkaline phosphatase (see chapter II.4.) to goat anti-human IgG and goat anti-human IgM antibodies (affinity purified, TAGO Inc., U.S.A.). Other ELISA reagents were the same as those used in the RF assays.

IV.3.3.2. Method

Essentially, the method of Hosea *et al* (1981) was followed throughout. Briefly, rows B-G of the microplates were incubated (sealed) overnight at 37°C with 75 µl/well of polysaccharide dissolved in PBS containing Na N_3. After saturation with 1% BSA, the plates were incubated for 2½ hrs. at r.t. with paired pre- and post-vaccination sera. Each serum was tested in double dilutions from 1/10 to 1/320 for IgM antibodies and double dilutions, from 1/40 to 1/1280 for IgG antibodies. All dilutions were tested in triplicates in coated wells plus in one adjacent blank well.

Following serum incubations, the plates were reacted for 3 hrs. at r.t. with 1/1000 dilutions of the antibody-enzyme conjugates, then incubated for 45 mins. with pNPP and read immediately.

IV.3.3.3. Assay variables tested

Coating concentration. In order to test the effect of using increasing concentrations of polysaccharides for microplate coating,

the following experiment was performed;

Three microplates were each coated with increasing concentrations of a polysaccharide; 5, 10, 20 and 40 µg/ml respectively. The polysaccharides selected were types 1, 4 and 51. Following BSA saturation, each plate was incubated with a 1/20 dilution of one serum. Three different sera were selected for the study, preliminary experiments having shown that these reacted relatively strongly with the corresponding polysaccharides. Thus, serum G.H. was tested against polysaccharide type 1, serum S.G.F. against type 4, and serum Wi against type 51. After incubation with anti-human IgG and anti-human IgM conjugates, the plates were reacted with pNPP and read immediately.

Figure IV.7. shows that there was a tendency for the lower coating concentrations to give lower abs. readings. However, this was not consistent, e.g., serum Wi reacted more strongly with 5 µg/ml than 20 µg/ml coated plates.

On the basis of these findings and for economic reasons it was decided to use a coating concentration of 10 µg/ml throughout, for all the polysaccharides.

Repeated use of the same polysaccharide solution. In order to test whether it might be possible to use the same polysaccharide solution for repeated coating of different plates, the following experiment was performed:

A microplate was coated with 75 µl of PBS containing 20 µg/ml of polysaccharide type 1 and type 2 (12 wells each). After incubation

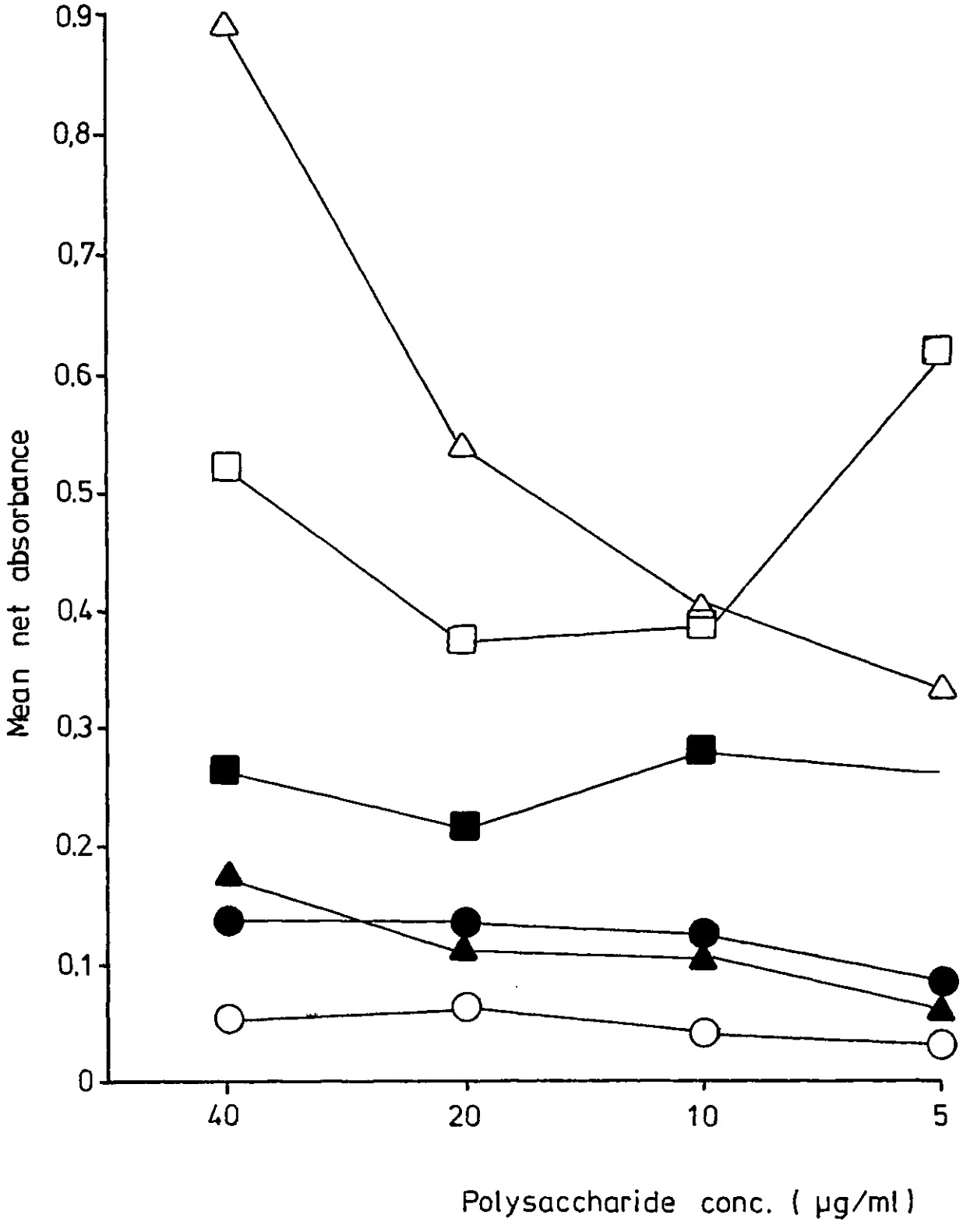


Figure IV.7: The effect of increasing coating concentrations of pneumococcal polysaccharides in anti-polysaccharide antibody ELISA.

squares: polysaccharide Type 51 (serum Wi)
triangles: polysaccharide Type 4 (serum S.G.F.)
circles: polysaccharide Type 1 (serum G.H.)
open symbols: IgG antibody
closed symbols: IgM antibody

overnight, the wells were sucked dry and the volume of the coating solution reconstituted to its original value with PBS. The polysaccharide solution was then re-used x 4 to coat microplates in the same manner, after which all the five plates were saturated with BSA and tested simultaneously for reactivity with one post-vaccination serum (Te, diluted 1/20).

Figure IV.8. shows that both the IgG and IgM responses fell dramatically after the coating solution had been used once, suggesting that a considerable proportion of the polysaccharide was bound to the microplate wells during the first incubation.

Accordingly, no further attempt was made to re-use the polysaccharide coating solutions.

IV.3.3.4. Time-response pattern of anti-pneumococcal antibodies

In order to determine when to bleed the vaccinees, the following experiment was performed:

Sera from two of the laboratory workers were tested; Wi against type 8 and Ga against type 1. Standard conditions were used; coating conc. of 10 µg/ml polysaccharide, followed by BSA, serum dilutions, anti-h. IgG and IgM conjugates and pNPP.

Figure IV.9. shows the time-response curves for the two sera on days 0, 7, 14 and 28, at serum dilution 1/20. Clearly, the IgG level was rising slowly throughout whereas the IgM response peaked at day 7 and then fell gradually.

These data indicate that optimal bleeding time for measuring IgG and IgM responses is between days 14 and 28.

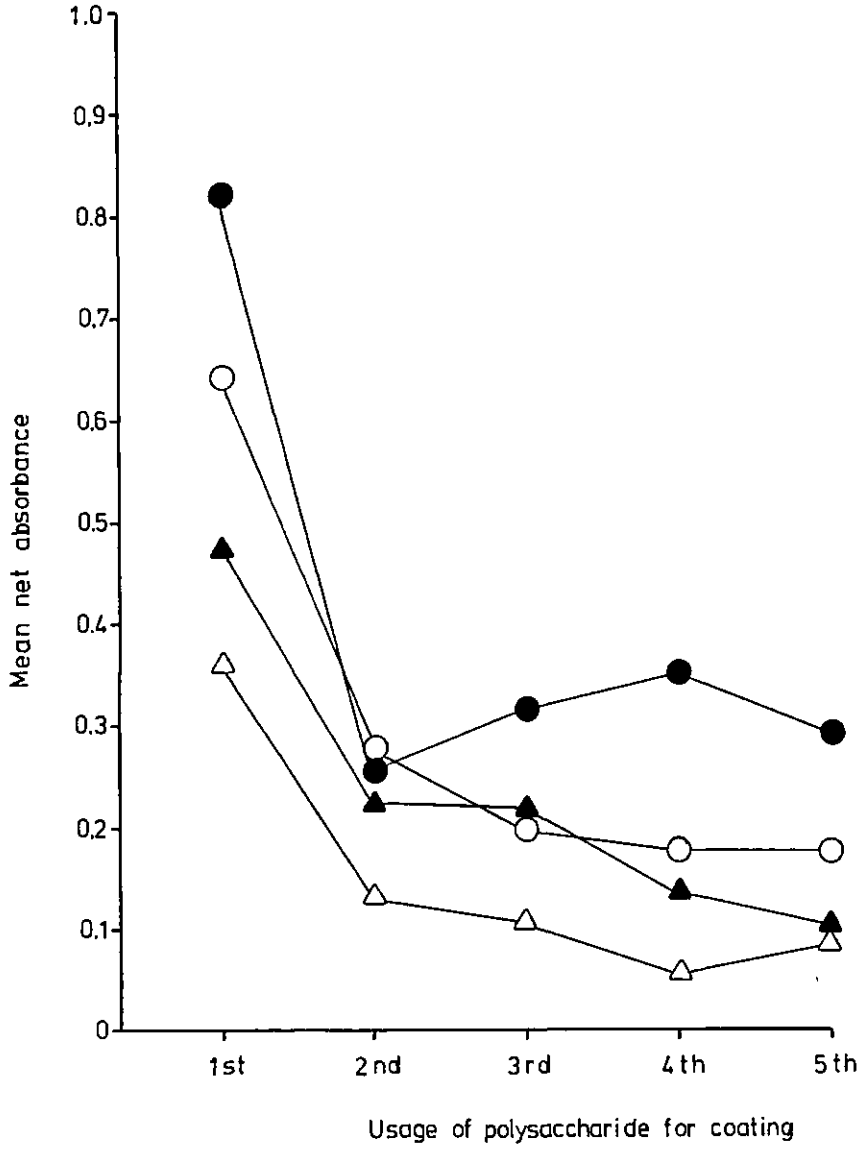


Figure IV.8: The effect of re-using polysaccharide solutions for microplate coating.

circles:	polysaccharide Type 1
triangles:	polysaccharide Type 2
Open symbols:	IgG antibody
closed symbols:	IgM antibody

One PNEUMOVAX-immune serum (Te) was used throughout, dilution 1/20.

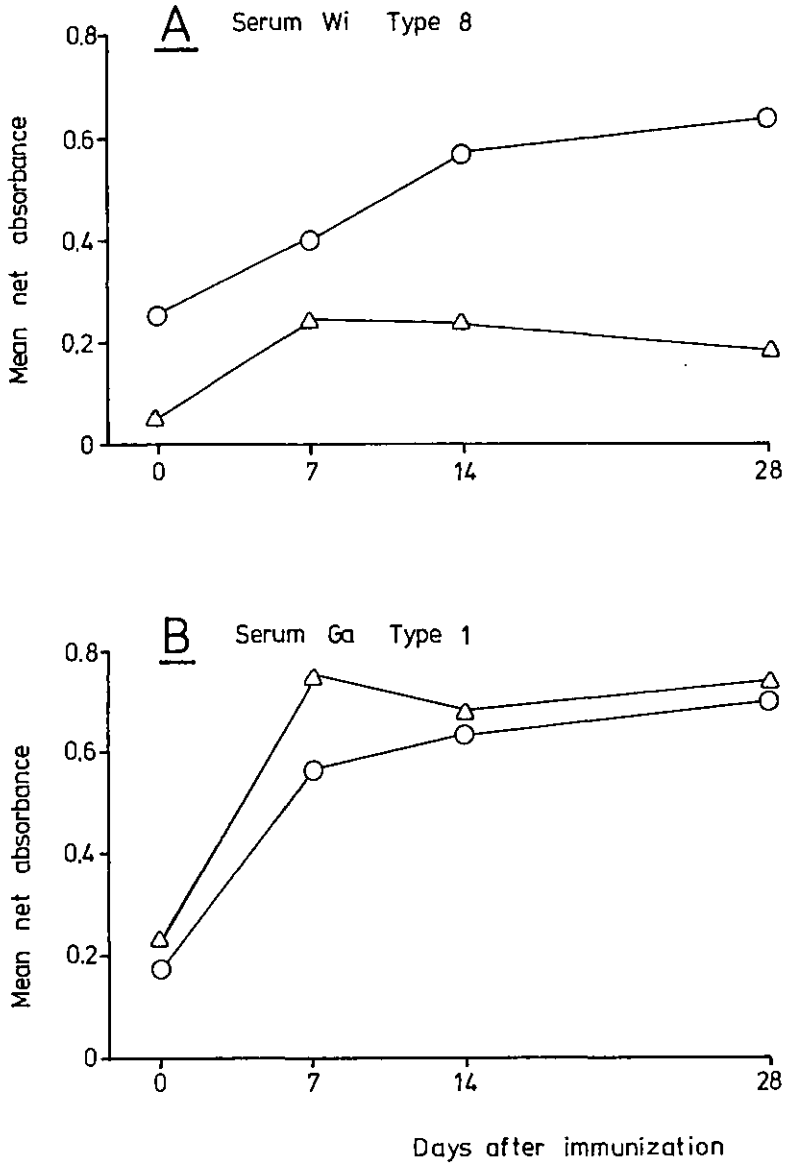


Figure IV.9: The time-response patterns of anti-polysaccharide antibody levels following PNEUMOVAX immunization.

The anti-type 8 responses of serum Wi (A) and anti-type 1 responses of serum Ga (B) are depicted.

Triangles: IgM antibodies

circles: IgG antibodies

IV.3.4. Expression of results

In order to diminish the effect of well-to-well variation in the microplates (see chapter II), the mean net abs. values for all the six serum dilutions for IgM and IgG were added together yielding a combined net abs. (CNA) value for each isotype, pre- and post-vaccination.

Additionally, the per cent increase (%Inc) in CNA after vaccination was calculated by the following formula:

$$\frac{(\text{CNA-post} - \text{CNA-pre}) \times 100}{\text{CNA-pre}} = \%\text{Inc}$$

As no standard for the anti-polysaccharide antibody content of sera was available and as it is very difficult to find individuals who have definitely not had contact with pneumococci, no attempt was made to standardise the current ELISA in terms of units/ml or absolute amount of antibody.

As the main aim of the PNEUMOVAX-study was to investigate whether the IgG anti-ruminant IgG levels may reflect the antibody-forming capacity of each individual, the paired sera from all the 29 vaccinees were assayed simultaneously for IgG anti-ruminant IgG, using serum dilutions 1/10, 1/100 and 1/1000 (see otherwise IV.2.2.1.). Table IV.2. shows the CNA values for the anti-ruminant IgG levels of the 29 individuals, both for the pre- and post-vaccination samples. Although there was some variation between the levels observed in the two samples from each individual (mean CV of 23.4%), there was no significant overall difference between the pre- and post-vaccination levels ($t = -1.58$, $p > 0.1$, paired student's t test).

Table IV.2: IgG anti-ruminant IgG levels in paired pre- and post-vaccination sera from the 29 PNEUMOVAX vaccinees.

	CNA		
Individual	Pre	Post	CV (%)
E.K.	0.113	0.093	13.7
S.G.	0.334	0.358	4.9
A.O.	0.164	0.232	24.3
G.H.	0.053	0.040	19.8
TH.H.	0.064	0.070	6.3
S.A.	0.716	0.648	7.1
G.J.B.	0.040	0.086	51.6
J.H.B.	0.076	0.108	24.6
TH.F.	0.007	0.020	68.1
L.V.	0.118	0.143	13.5
G.M.F.	0.197	0.234	12.1
J.G.F.	0.017	0.031	41.2
B.S.F.	0.186	0.173	5.1
E.J.	0.043	0.025	37.4
S.J.	0.079	0.017	91.3
O.B.H.	0.143	0.419	67.2
S.G.F.	0.137	0.195	24.7
O.H.K.	0.409	0.440	5.2
B.G.	0.418	0.427	1.5
Mu	0.036	0.064	39.6
Da	0.103	0.107	2.7
La	0.178	0.123	25.8
Va	0.069	0.057	13.5
Hu	0.129	0.173	20.6
Te	0.173	0.189	6.3
Ga	0.164	0.169	2.1
Sa	0.396	0.539	21.6
Wi	0.160	0.123	18.5
San	0.247	0.221	7.9
Mean ± SD	0.171 ± 0.155	0.190 ± 0.165	23.4 ± 22.4

The coefficients of variation (CV) between the pre- and post-vaccination levels are shown. The overall difference between the pre- and post-vaccination levels was not significant ($p > 0.1$, paired student's t test).

The means of the two CNA values thus obtained were subsequently used in a multivariate correlation analysis where the IgG anti-ruminant IgG level and age were correlated with the IgG and IgM pre-/post-/%Inc-values for all the 14 individual polysaccharide types. Also, the multivariate analysis included correlation analysis between the pre-/post-/%Inc-values for each polysaccharide type, both for IgG and IgM isotypes.

Furthermore, some correlation analyses were performed, comparing the responses to the individual polysaccharides (inter-type comparison).

The Pearson's product-moment correlation method was used in the multivariate analysis, applying a "Minitab" statistical package.

IV.3.5. Results

IV.3.5.1. Reproducibility of the anti-polysaccharide assay

In order to test the reproducibility of the assay system, two paired sera (Wi and La) were tested against type 19 on three successive days, using the standard assay conditions.

The results in Table IV.3. show both the pre-/post-CNA values and the %Inc values, both for IgG and IgM. The day-to-day variation of the CNA values was considerable (up to 67%), but the variation in the %Inc was much less, on average, 30%.

Table IV.4. shows the ratios between the individual CNA values

Table IV.3: The day-to-variation of the ELISA for anti-polysaccharide antibodies.

			Day 1	Day 2	Day 3	CV (%)
Serum La	IgG	Pre	0.184	0.546	0.885	65
		Post	0.191	0.640	1.025	67
		%Inc	3.8	17	16	60
	IgM	Pre	0.387	0.183	0.484	44
		Post	0.495	0.248	0.680	46
		%Inc	28	36	41	19
Serum Wi	IgG	Pre	0.627	1.839	1.602	47
		Post	1.607	3.894	4.217	44
		%Inc	156	112	163	19
	IgM	Pre	0.489	0.275	0.445	28
		Post	1.260	0.644	1.371	36
		%Inc	158	134	208	23

The anti-type 19 responses of two sera (La and Wi), measured on three different days, are shown, together with the CV values for the day-to-day variation.

Table IV.4: Day-to-day fluctuations in the between-patient ratios of the anti-polysaccharide ELISA.

			Day 1	Day 2	Day 3	CV (%)
Ratio Wi/La	IgG	Pre	3.41	3.37	1.81	32
		Post	8.41	6.08	4.11	35
		%Inc	41.1	6.59	10.2	98
	IgM	Pre	1.26	1.50	0.92	24
		Post	2.55	2.60	2.02	13
		%Inc	5.64	3.72	5.07	21
Mean						37

The anti-type 19 responses of serum Wi divided by the corresponding responses for serum La (Table IV.3.) are shown, together with the coefficients of variation (CV) for these.

(Table IV.3.) for serum Wi and La. There was considerable fluctuation in the day-to-day ratios, the mean CV being 37%.

IV.3.5.2. Responses to individual polysaccharides

Table IV.5. shows the means and standard deviations of the pre-/post-CNA values for the IgG responses to the 14 polysaccharides. Also shown in Table IV.5. is the % increase (%Inc) in CNA for the IgG responses.

Table IV.6. shows the analogous results for the IgM responses to the 14 individual polysaccharides.

The responses to the different polysaccharides varied widely, e.g., the IgG responses to type 9 increased on average by 343.7% after vaccination whereas the IgG responses to type 3 increased only by an average of 21.4% after the vaccination.

The IgM responses were similar. Again, the IgM responses to type 9 showed the greatest mean increase after vaccination: 354.1%, whereas the IgM responses to types 4 and 14 only increased by 18.6%.

On average, the IgM increased slightly more than the IgG after vaccination; 125.8% vs. 105.8%.

There was an overall highly significant correlation between the anti-polysaccharide antibody levels before and after vaccination.

Table IV.5: The IgG anti-polysaccharide responses.

	Pre		Post		%Inc	
Type	Mean	SD	Mean	SD	Mean	SD
1	0.736	0.324	1.202	0.766	64.7	77.7
2	0.499	0.240	0.861	0.403	84.1	70.0
3	1.110	0.542	1.286	0.597	21.4	38.8
4	2.340	1.047	2.905	1.167	29.5	25.3
6	0.822	0.341	1.045	0.467	29.3	24.6
8	0.532	0.311	1.055	0.469	134.0	94.3
9	0.443	0.342	1.714	1.053	343.7	229.4
12	1.012	0.379	1.526	0.783	54.7	57.9
14	0.790	0.460	1.188	0.630	61.4	60.4
19	0.461	0.193	1.078	0.816	123.8	110.6
23	0.655	0.431	1.262	0.779	113.9	128.3
25	1.807	0.574	2.355	0.712	31.9	17.9
51	0.450	0.263	1.249	0.900	171.3	119.2
56	0.771	0.548	1.984	1.045	217.3	261.0
Mean					105.8	

The means and standard deviations of the pre- and post-vaccination CNA and the %Inc values for the 29 vaccinees are shown.

Table IV.6: The IgM anti-polysaccharide responses.

	Pre		Post		%Inc	
Type	Mean	SD	Mean	SD	Mean	SD
1	0.491	0.291	1.093	0.917	120.5	113.4
2	0.831	0.613	1.332	0.852	125.8	291.3
3	0.792	0.429	1.162	0.566	66.2	74.3
4	1.258	0.740	1.454	0.852	18.6	25.9
6	0.391	0.252	0.530	0.327	54.1	57.6
8	0.346	0.242	0.916	0.745	224.3	274.6
9	0.350	0.219	1.268	0.889	354.1	539.4
12	0.680	0.406	1.290	0.743	104.8	91.0
14	0.545	0.345	0.662	0.467	18.6	25.9
19	0.655	0.462	1.450	0.894	145.9	146.7
23	0.234	0.227	0.340	0.453	207	945
25	0.833	0.368	1.008	0.481	20.9	31.9
51	0.614	0.425	1.478	0.741	170.2	113.9
56	0.441	0.264	0.814	0.479	130.4	226.3
Mean					125.8	

The means and standard deviations of the pre- and post-vaccination CNA and the %Inc values for the 29 vaccinees are shown.

Table IV.7. shows the results of the correlation analysis between the pre-/post-vaccination values for all the 14 polysaccharide types, both IgG and IgM.

With the notable exception of the IgM responses to type 9, all the post-vaccination values correlated significantly ($p < 0.01$ or 0.001) with the pre-vaccination values. Figure IV.10. shows the association of the pre-vaccination values of the 29 vaccinees with the corresponding post-vaccination values for the IgG anti-type 8 antibodies.

Generally, there was a negative correlation between the pre-vaccination antibody levels and the observed increase after vaccination. Table IV.8. shows that while this was not without exception, the overall association was significantly negative, both for the IgG and IgM isotypes.

IV.3.5.3. Relation between the responses to different polysaccarides

Some correlation analyses were performed to compare the responses (pre-/post-/%Inc) to the different polysaccharides. The responses to types 4 and 6 were generally closely related (see Table IV.9.).

Similar significant association patterns were observed between the IgG responses to types 4 and 8 and between the IgG responses to 51 and 56.

Also, there were significant association patterns between the IgM responses to types 1 and 2, between the IgM responses to types 8 and 12,

Table IV.7: The relationship between the pre- and post-vaccination anti-polysaccharide responses.

Polysacch. Type	IgG		IgM	
	r	p <	r	p <
1	0.609	0.001	0.731	0.001
2	0.833	0.001	0.810	0.001
3	0.815	0.001	0.703	0.001
4	0.928	0.001	0.957	0.001
6	0.921	0.001	0.894	0.001
8	0.852	0.001	0.642	0.001
9	0.749	0.001	0.302	NS
12	0.664	0.001	0.803	0.001
14	0.817	0.001	0.958	0.001
19	0.790	0.001	0.562	0.01
23	0.556	0.01	0.920	0.001
25	0.943	0.001	0.893	0.001
51	0.819	0.001	0.728	0.001
56	0.732	0.001	0.684	0.001
Mean ± SD	0.788 ± 0.116		0.756 ± 0.179	
Significance	p <0.001		p <0.001	

The relationship is expressed as the product-moment correlation coefficient (r) for the 29 paired pre-/post-vaccination sera.

The mean r-values for IgG and IgM differed significantly from zero (p <0.001, student's t test).

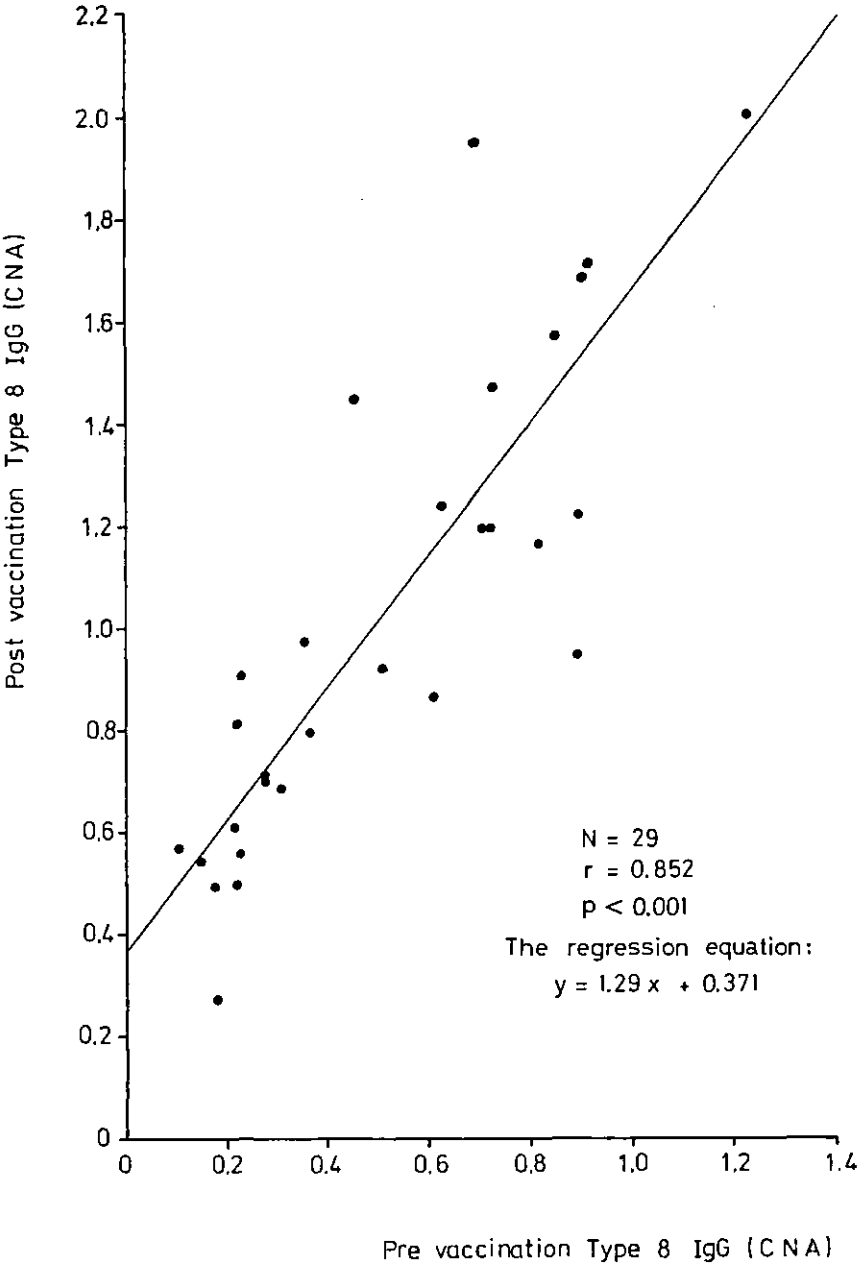


Figure IV.10: The relationship between the pre- and post-vaccination IgG anti-type 8 responses of the 29 vaccinees.

The correlation is highly significant, ($r = 0.852$, $p < 0.001$).

Table IV.8: The relationship (correlation coefficients, r) between the pre-vaccination responses and the %Inc values for the 29 vaccinees.

Polysaccharide Type	IgG	IgM
1	-0.038	0.032
2	-0.348	-0.315
3	-0.315	-0.500
4	-0.516	-0.203
6	-0.221	-0.443
8	-0.669	-0.323
9	-0.336	-0.282
12	-0.188	-0.293
14	-0.324	0.137
19	0.288	-0.247
23	-0.261	-0.182
25	-0.286	0.010
51	0.096	-0.389
56	-0.363	-0.351
Mean ± SD	-0.253 ± 0.230	-0.239 ± 0.185
$p <$	0.01	0.001

The mean r values were significantly negative ($p < 0.01$ for IgG and $p < 0.001$ for IgM, student's t test).

Table IV.9: The relationship between the anti-type 4 and anti-type 6 responses of the 29 vaccinees.

				Type 4			
				IgG		IgM	
				pre	post	pre	post
Type 6	IgG	Pre	r	0.653	0.595		
			p <	0.001	0.001		
		Post	r	0.646	0.603		
			p <	0.001	0.001		
	IgM	Pre	r			0.670	0.731
			p <			0.001	0.001
		Post	r			0.478	0.537
			p <			0.001	0.001

There was a highly significant correlation between the responses to the two serotypes (both pre- and post-vaccination IgG and IgM responses), $p < 0.001$.

and between the IgM responses to types 25 and 56.

IV.3.5.4. Responsiveness in relation to age

Generally, there was no significant correlation between the age of the vaccinees and their pre- and post-vaccination anti-polysaccharide antibody levels.

Table IV.10. shows the correlation coefficients for the association of age with the %Inc values for IgG and IgM. While there was a significant positive correlation between age and the increase in IgM anti-type 51, there was an even stronger negative correlation ($p < 0.01$) between age and the %Inc in IgM anti-type 8 and the %Inc in IgG anti-type 2.

However, the mean correlation coefficient for the association between age and %Inc in IgG did not differ significantly from zero and the same applied to the %Inc in IgM.

IV.3.5.5. The association between IgG anti-ruminant IgG and anti-polysaccharide antibodies

The IgG anti-ruminant IgG levels of the 29 vaccinees were expressed as the mean CNA of the pre- and post-vaccination sera (Table IV.2.). These values were correlated by multivariate analysis with the pre-/post-/%Inc values for the IgG and IgM levels against all the 14 polysaccharide types.

Table IV.10: The relationship between the responses to PNEUMOVAX immunisation and the age of the 29 vaccinees.

Polysaccharide Type	IgG		IgM	
	r	Significance	r	Significance
1	0.188	NS	-0.398	p <0.05
2	-0.487	p < 0.01	0.276	NS
3	0.157	NS	-0.247	NS
4	0.097	NS	-0.273	NS
6	0.162	NS	0.034	NS
8	-0.242	NS	-0.498	p <0.01
9	0.311	NS	0.147	NS
12	0.219	NS	-0.395	p <0.05
14	-0.251	NS	-0.322	NS
19	0.266	NS	0.170	NS
23	0.280	NS	0.293	NS
25	0.044	NS	-0.299	NS
51	0.132	NS	0.373	p <0.05
56	0.177	NS	0.212	NS
Mean	0.072		-0.066	
SD	0.233		0.307	
Diff. from 0	NS		NS	

The correlation coefficients (r) for the relation between the %Inc values and the age of those vaccinated are shown.

The mean r-values for the IgG and IgM responses did not differ significantly from zero (student's t test).

Table IV.11. shows the correlation coefficients for the association with IgG anti-polysaccharide antibodies. All the r-values were numerically low and none of them reached significance at the 5% level *per se*.However, the mean r-value for the association between IgG anti-ruminant IgG and pre-vaccination anti-polysaccharide IgG was -0.102 ±0.121 which is significantly different from zero ($0.001 < p < 0.01$, student's t test).

Also, the mean r-value for the association of IgG anti-ruminant IgG with post-vaccination anti-polysaccharide IgG was -0.085, again significantly negative ($0.02 < p < 0.05$). However, no such overall association was seen between the %Inc values for IgG and the IgG anti-ruminant IgG, $p > 0.05$.

Table IV.12. shows the analogous results for the association between IgG anti-ruminant IgG and IgM anti-polysaccharide antibodies. Here, there was a very significant association between the IgG anti-ruminant IgG and the post-vacc. IgM anti-type 9 levels, as well as between the IgG anti-ruminant IgG and the %Inc in IgM anti-type 9. Incidentally, it can be seen from Table IV.6. that the %Inc in IgM anti-type 9 was the greatest IgM increase observed.

There was also a significant positive correlation ($p < 0.05$) between IgG anti-ruminant IgG and the %Inc in the IgM anti-type 4 and anti-type 25.

Moreover, when all the correlation coefficients for the pre, post and %Inc columns, respectively, were analysed together, it was found that they differed significantly from zero, implying an overall

Table IV.11: The relationship (correlation coefficients, r) between the IgG anti-polysaccharide responses (pre-/post-CNA and %Inc values) and the IgG anti-ruminant IgG levels of the 29 vaccinees.

Polys. Type	CNA				%Inc	
	Pre		Post			
	r	Significance	r	Significance	r	Significance
1	-0.254	NS	0.029	NS	0.249	NS
2	-0.133	NS	0.018	NS	0.237	NS
3	-0.193	NS	-0.079	NS	0.174	NS
4	-0.158	NS	-0.160	NS	0.119	NS
6	0.007	NS	-0.063	NS	-0.290	NS
8	-0.126	NS	-0.191	NS	-0.095	NS
9	-0.245	NS	-0.358	NS	0.053	NS
12	0.161	NS	-0.079	NS	-0.210	NS
14	0.007	NS	0.103	NS	0.165	NS
19	0.011	NS	0.026	NS	-0.116	NS
23	-0.150	NS	-0.156	NS	-0.138	NS
25	-0.246	NS	-0.250	NS	-0.043	NS
51	-0.051	NS	0.025	NS	-0.057	NS
56	-0.060	NS	-0.052	NS	-0.210	NS
Mean	-0.102		-0.085		-0.012	
SD	0.121		0.127		0.178	
p <	0.01		0.05		NS	

The significance of the deviation from zero of the mean r-values is shown at the bottom of the table (student's t test).

Table IV.12: The relationship (correlation coefficients, r) between the IgM anti-polysaccharide responses (pre-/post-CNA and %Inc values) and the IgG anti-ruminant IgG levels of the 29 vaccinees.

Polys. Type	CNA				%Inc	
	Pre		Post			
	r	Significance	r	Significance	r	Significance
1	0.117	NS	0.180	NS	0.193	NS
2	0.233	NS	0.283	NS	0.261	NS
3	0.098	NS	0.018	NS	-0.084	NS
4	0.031	NS	0.146	NS	0.385	p <0.05
6	0.171	NS	0.102	NS	-0.109	NS
8	0.017	NS	0.157	NS	0.184	NS
9	-0.054	NS	0.472	p <0.01	0.522	p <0.01
12	0.257	NS	0.351	NS	0.270	NS
14	0.264	NS	0.271	NS	0.142	NS
19	0.079	NS	-0.050	NS	-0.214	NS
23	-0.121	NS	-0.083	NS	0.292	NS
25	-0.011	NS	0.147	NS	0.367	p <0.05
51	0.127	NS	0.130	NS	-0.050	NS
56	-0.034	NS	0.055	NS	-0.076	NS
Mean	0.084		0.156		0.149	
SD	0.120		0.152		0.221	
p <	0.05		0.01		0.05	

The significance of the deviation from zero of the mean r-values is shown at the bottom of the table (student's t test).

positive correlation between the IgG anti-ruminant IgG and the pre/post/%Inc IgM responses (see Table IV.12.).

IV.3.6. Discussion

The findings that the IgM anti-polysaccharide responses reach a maximum at about day 7 after vaccination (Figure IV.9.) are in keeping with the results of Hosea *et al* (1981). The continuing rise in the IgG anti-polysaccharide response until at least day 28 (Figure IV.9.) contrasts sharply with the IgM response. This would indicate that the current assay is indeed both isotype specific and at least semi-quantitative, although the day-to-day reproducibility is rather poor (Tables IV.3. and IV.4.).

The data presented in Tables IV.5. and IV.6. demonstrate the marked variation in the mean IgG and IgM responses to the individual polysaccharides. Clearly, the greatest relative increase in antibody levels after vaccination (both IgG and IgM) is against polysaccharide type 9, and this is in keeping with the findings of Melville-Smith and Sheffield (1980) and Pedersen *et al*, (1982).

The limited increase in IgG and IgM anti-type 3 is in conflict, however, with the report of Ammann *et al* (1977) where the anti-type 3 responses increased markedly after immunisation.

While the reason(s) for this discrepancy is not apparent, it can be postulated that regional variations in the prevalence of type 3

pneumococcus may account for differences in the pre-vaccination levels of anti-type 3. High pre-vaccination antibody levels may in turn lead to apparently lower response to the vaccination. The finding (Table IV.8.) that the pre-vaccination antibody levels were generally inversely related to the increase after vaccination would seem to support this.

This apparent regional variation in anti-polysaccharide responses may make the selection of a polysaccharide for immune response testing more difficult, however. If PNEUMOVAX is to be used for the assessment of immune responsiveness it would seem sensible to select a polysaccharide for the antibody assay which is intermediate, i.e., one that can differentiate between good and poor antibody producers.

The data presented in Table IV.9. may indicate an antigenic similarity between types 4 and 6. Other factors could influence these results, however, e.g., if the type-specific polysaccharides are contaminated by C polysaccharide, an apparent reaction of identity may arise (Latif and Webster, 1982). The finding that the IgM responses to type 1 correlated significantly with the IgM anti-type 2 responses, seems to agree with the findings of Russell *et al* (1980) who found that polysaccharide type 1 cross-reacted with isolated antibody fraction to type 2. Furthermore, it can be seen that the %Inc values for IgM against types 1 and 2 are very similar 120.5 and 125.8%,respectively. This seems to indicate that it is unnecessary to assess the responses to both polysaccharides following immunisation as one is likely to reflect the other. A similar feature is apparent for type 4 vs. type 6 where the %Inc values in IgG for both polysaccharides are 29%.

On the basis of these results it can be argued that the measurements of the IgG and IgM responses to a few polysaccharides, e.g., types 2, 4, 12 and 25, may yield the optimal information on the humoral immune responsiveness of those vaccinated with PNEUMOVAX.

The findings that there was no overall correlation between the pre-/post-/%Inc-responses to the polysaccharides and the age of the vaccinees may seem surprising, as it is generally recognised that the elderly show decreased antibody production to various antigens (Barrett *et al*, 1980b). However, only 3 of the 29 vaccinees had reached the age of 60, the oldest being 69. Thus, the majority were between 30 and 60 years of age.

Tables IV.11. and IV.12. indicate a poor correlation between the IgG anti-ruminant IgG levels and the responses to the polysaccharides. It is interesting, however, to see the discrepancy between the IgG and IgM responses to the polysaccharides, in so far as there is a tendency towards negative correlation between the IgG anti-ruminant IgG and the IgG anti-polysaccharide responses, whereas the association with IgM is a positive one. This issue deserves a few comments:

The ruminant IgG is probably presented to the immune system via the gut whereas the polysaccharides were injected subcutaneously. The route of administration may be of fundamental importance in deciding the type and magnitude of the immune response. Indeed, the work of Asherson *et al* (1977) suggested that different immune modulatory mechanisms may be operative in the mouse, depending on whether an antigen is presented via the gut or the skin.

Possibly, the responses to an oral vaccine, e.g., polio, might bear more resemblance to the IgG anti-ruminant IgG response than the anti-PNEUMOVAX responses.

IV.4. CONCLUSION

Antibodies, mainly of IgG class, with apparent specificity for IgG of ruminant origin, were detectable at varying levels in most individuals. Those individuals with the highest levels tended to be free of rheumatic symptoms and diseases. These antibodies probably represent an antibody response to gut-derived food antigens. The antibody levels did not show any strong correlation with antibody responses against percutaneously administered pneumococcal polysaccharide antigens.

C H A P T E R V

A PROSPECTIVE STUDY OF EARLY RHEUMATOID JOINT DISEASE: RHEUMATOID FACTOR PROFILES

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V.1. INTRODUCTION

The course of inflammatory polyarthritides, of which rheumatoid arthritis (RA) is the most common and thoroughly studied, is known to be highly variable. RA can vary in severity, from one mild attack, lasting only a few months, to severely progressive, destructive disease with early death from systemic complications (Rasker and Cosh, 1981).

As there is good evidence that aggressive therapy with potentially toxic agents like gold compounds may favourably alter the course of RA (reviewed by Zvaifler, 1979b), it is important to identify as early as possible those patients who are likely to develop severe disease.

Several authors have studied groups of RA patients prospectively, among other reasons, to try and identify disease features of prognostic importance.

Duthie *et al* (1964) studied a group of 307 RA patients who required hospital admission because of disease activity. These authors found that those who had an acute onset of disease and were admitted within a year of onset, had a more favourable prognosis. These authors also noted that those patients who repeatedly had positive sensitised sheep cell tests (IgM RF positive), showed significantly poorer

functional capacity at the end of the study than the RF negative patients. However, the group of Duthie *et al* is obviously selective in that only RA patients who needed hospitalisation were studied.

Otten and Boerma (1959) followed up a group of 141 patients with early RA, for at least one year. The presence of RF (Rose Waaler test), especially in high titres, was significantly associated with poor prognosis. Once seropositive, the patients tended to remain so, even after a period of five years of complete remission.

Jacoby *et al* (1973) followed 100 patients with definite or classical RA from within one year of onset of the arthritis. Poor functional capacity and high RF titre at first visit tended to predict poor prognosis.

Fleming *et al* (1976) followed 102 patients who were seen within the first year of rheumatoid disease. Old age at onset, early progression to symmetrical involvement and insidious onset were indicators of a poor prognosis.

Feigenbaum *et al* (1979) studied the course of 50 newly diagnosed RA patients by multivariate analysis. The authors designed a prognostic index composed of those six out of 400 entry items which proved to be of greatest prognostic value: Positive RF, two or more swollen upper extremity joints on examination, history of Raynaud's like symptoms, malaise or weakness at the onset of arthritis, white race and female sex.

Thus, most authors agree that the presence of RF (measured by agglutination) early in RA is a poor prognostic sign, when the outcome

at the end of the study period is measured either in terms of functional class (Steinbrocker *et al*, 1949) or the presence of erosions and joint swelling (Feigenbaum *et al*, 1979). All the RF data in the studies above, however, are limited to RFs of the IgM isotype, as the agglutination tests are poor at detecting other RFs.

In view of the potentially pathogenic rôle of IgG RF (and IgA RF) (see chapter I), it might be expected that serum/synovial fluid levels of RFs other than IgM RF may be of prognostic importance as well. Furthermore, while it has been shown that fluctuations in IgM RF do not correlate closely with disease activity (Otten and Boerma, 1959) no such information has been available for other RF isotypes.

The aim of the study presented in this chapter was mainly two-fold:

1. Do the RF profiles of the three main RF isotypes early on in rheumatoid joint disease have a prognostic value superior to that of IgM RF alone?

2. Do the fluctuations in the levels of the three main RF isotypes show association with fluctuations in clinical parameters indicating disease activity?

V.2. METHODOLOGY

V.2.1. Selection of patients and assessment protocol

All patients in this study presented to the Department of Rheumatology, St. Mary's Hospital, London, from 1979 to 1982. The patients were invited to enter the study if they were suspected of having rheumatoid arthritis and their symptoms had lasted less than one year. Patients who had already been treated with disease modifying drugs such as gold or penicillamine, were excluded.

The patients were seen regularly by Dr. R. H. Withrington, Senior Registrar in Rheumatology, and myself in a special outpatient clinic. Each patient was assessed every three months according to a special protocol, but the interval between visits was sometimes extended to six months if the patient remained asymptomatic.

The assessment protocol included the following:

1. Estimation of duration of morning stiffness (MS) of the joints (minutes).

2. The patient marked the severity of his/her overall pain on a 10 cm long horizontal pain visual analogue scale (PVAS) (Huskisson, 1974).

3. The overall joint tenderness was assessed according to the method of Ritchie *et al* (1968), yielding the Ritchie index.

4. The grip strength (GS) was measured by a specially designed grip bag (Savage, 1966) which was connected to a mercury sphygomanometer and inflated to 30 mm Hg. Three successive readings were taken for each hand (outstretched, unsupported) and the mean of the six readings (mm Hg) was taken as the grip strength.

5. On each day of assessment, a blood sample was taken for estimation of erythrocyte sedimentation rate (ESR) and rheumatoid factor levels, as measured by both conventional methods (latex and RAHA tests) and ELISA. Those patients who were RAHA positive on at least one occasion (RAHA titre $\geq$1:40) were considered to meet the 8th ARA-criterion for seropositivity (Ropes *et al*, 1958).

6. X-ray pictures of: (a) hands and wrists; (b) feet; and (c) cervical spine in flexion and extension were taken at the first visit and annually thereafter.

At the end of the study, all hand and wrist X-ray pictures were read by two rheumatologists. This was done in a blind fashion in so far as the names on the films were covered, but all films for each patient were read in sequence and the dates when the pictures were taken were known to the readers. The total defect score (erosions) and the total joint space narrowing score were determined for each film, using the method of Sharp *et al* (1971).

V.2.2. ELISA RF measurements

Blood samples were taken from the participants during every protocol assessment and often more frequently. Synovial fluid samples were also collected when knee effusions were present. Serum aliquots were kept at -80°C until late 1982 when all serum and synovial fluid samples were assayed for IgM RF, IgA RF and IgG RF (with and without pepsin digestion), using the ELISA protocols described in chapter III. All the serum and synovial fluid samples from each patient were tested on the same day in order to minimise errors related to day-to-day variations in the assays.

V.2.3. Data analysis

At the end of the study, the clinical assessment data and the ESR results were used to construct an overall index of disease activity (IDA) and mean disease activity grade (MDAG) for each visit, using a modification of the method of Mallya and Mace (1981). Briefly, the morning stiffness, the PVAS results, the Ritchie index, the mean grip strength and the ESR were each assigned a grade of 1-4, using the table of Mallya and Mace (1981) where grade 1 indicates normal state and grade 4 extreme affection by disease. The mean grade of the five parameters constitutes the index of disease activity (IDA) at that visit (ranging from 1.0-4.0) and this was then rounded off to mean disease activity grade (MDAG) I, II, III and IV, in order of increasing disease severity (Mallya and Mace, 1981).

The rheumatoid factor levels (measured by ELISA) were expressed

as units (chapter III). The IgM RF and IgA RF results were used unmodified for the statistical analysis but a different approach was adopted for IgG RF. As shown in chapter III the reproducibility of the IgG RF assay was considerably poorer than that of the IgM RF and IgA RF assays. Also, it became obvious that the between-visit fluctuations in IgG RF levels for each patient far exceeded the IgM RF and IgA RF fluctuations. As this was assumed to be at least in part due to technical, rather than biological variation, a three-point moving average (Erricker, 1981) was calculated for each serum IgG RF value of the time-response curve for that patient.

The clinical/serological parameters from the first visit were then correlated (using Pearson's product-moment correlation) with the data from the last visit, and with the total defect score of the last available hand and wrist X-ray picture. The maximal IgM RF, IgA RF and IgG RF levels observed for each patient were also correlated with these parameters.

Furthermore, the number of hand and wrist erosions (the total defect score) of those who presented with raised levels of the various RF isotypes was compared with the outcome of those with normal RF levels at onset. The student's t test was used to assess the significance of the difference between the groups.

Finally, the profiles of clinical activity (MS, PVAS, Ritchie index, GS, ESR and IDA) and RF levels for each patient during the study period were correlated, using the Spearman's rank correlation method and multivariate analysis. The distribution of the correlation coefficients (r_s) thus obtained was then assessed for the group as a

whole. The significance of the deviation from zero of the mean r_s value was determined by the student's t test.

When the profile of a clinical parameter or RF remained constant throughout the study period, the computer excluded that parameter from the multivariate analysis. In order to allow for this, when assessing the distribution of the r_s values, missing correlation coefficients were assigned a value of zero.

V.3. RESULTS

V.3.1. Clinical and radiological features of the study group

A total of 54 patients were entered into the study. Thirty-three of these had been followed for at least two years, including X-rays, by the end of 1982. The following results apply exclusively to this group of 33, unless otherwise stated.

Those patients who had met at least five of the ARA-criteria for RA (Ropes *et al*, 1958) were classified as having RA, the remaining ones as non-RA. Table V.1. shows some of the features of these two groups, 28 RA and 5 non-RA patients.

Briefly, the RA group was considerably older (mean age 55.9 years) at onset than the non-RA group (mean age 35.8 years). The female/male ratio of the RA group was 2.1/1 and that of the whole group, 1.8/1.

After at least a two year follow-up, 17 (61%) of the RA group had developed erosions of their hands and wrists and a total of 14 (50%) had been treated with steroids, gold, penicillamine or azathioprine.

The follow-up from onset of disease until the date of the most recent X-ray varied from 24-48 months. However, there was no significant correlation in the RA group between the number of erosions and the duration of disease ($r = 0.37$, NS).

Table V.1: Summary of patients with at least two years follow-up.

	RA	non-RA
Total number	28	5
Females/males	19/9	2/3
Mean age at onset ± 1 SD	55.9 ± 16.5 yrs.	35.8 ± 9.3 yrs.
Age range at onset	22-80 yrs.	26-51 yrs.
No. of ARA-criteria for RA: 0	-	1
" " " " " " " 4	-	4
" " " " " " " 5	7	-
" " " " " " " 6	13	-
" " " " " " " 7	8	-
Erosive disease (hands and wrists)	17 (61%)	0
Treated with: steroids	14 (50%)	0
" " " gold	8 (29%)	0
" " " penicillamine	6 (21%)	0
" " " azathioprine	1 (4%)	0
" " " gold/penicil./azathi.	8 (29%)	0
" " ster./gold/penicil./azathi.	14 (50%)	0

V.3.2. ELISA RF findings in the prospective group

A total of 651 serum and synovial fluid samples were collected during 1979 to 1982 from the 54 patients of the prospective study group. These samples were assayed for IgM RF, IgA RF and IgG RF, with and without pepsin digestion. Table V.2. shows that the IgA RF levels showed an overall highly significant association with the IgM RF levels ($r = 0.676$). Furthermore, there was a very close overall association between the levels of IgG RF with and without pepsin digestion ($N = 643$, $r = 0.778$, $p < 0.001$).

Figure V.1. shows the relationship between synovial fluid and serum levels of IgA RF in paired samples, both samples from each patient generally being taken on the same day. After performing logarithmic transformation of the unit/ml-values for the 25 pairs shown in Figure V.1., a close correlation was observed between the serum and synovial fluid levels ($r = 0.711$, $p < 0.001$). The slope of the regression line, 1.06, indicates that the absolute concentration of IgA RF was much the same in both serum and synovial effusions at any given time. Similar features were observed for the other RF isotypes in the paired synovial fluid/serum samples (see Table V.3.). However, digested IgG RF, and especially undigested IgG RF, tended to be lower in the synovial effusions than in the corresponding serum samples.

Analysis of the group of 33 who were followed up for at least two years showed that a total of 13 (39%) had raised IgM RF levels on one or more occasion(s) during the follow-up (see Table V.4.). The corresponding figure for IgA RF was 9 (27%) and a total of 7 (21%) presented with raised IgA RF (Table V.4.). Even fewer showed raised levels of IgG RF (with/without pepsin digestion).

Table V.2: The relationship between levels of IgM RF and the other RF isotypes in serum and synovial fluid samples from the whole prospective group of 54.

IgM RF vs.	No. of Samples	Correlation (r)	Significance (p <)
IgA RF	651	0.676	0.001
IgG RF + pepsin	643	0.407	0.001
IgG RF - pepsin	643	0.289	0.001

+ pepsin: samples were pepsin digested before the IgG RF assay.

- pepsin: samples were not pepsin digested (incubated in pepsin-free acetate buffer).

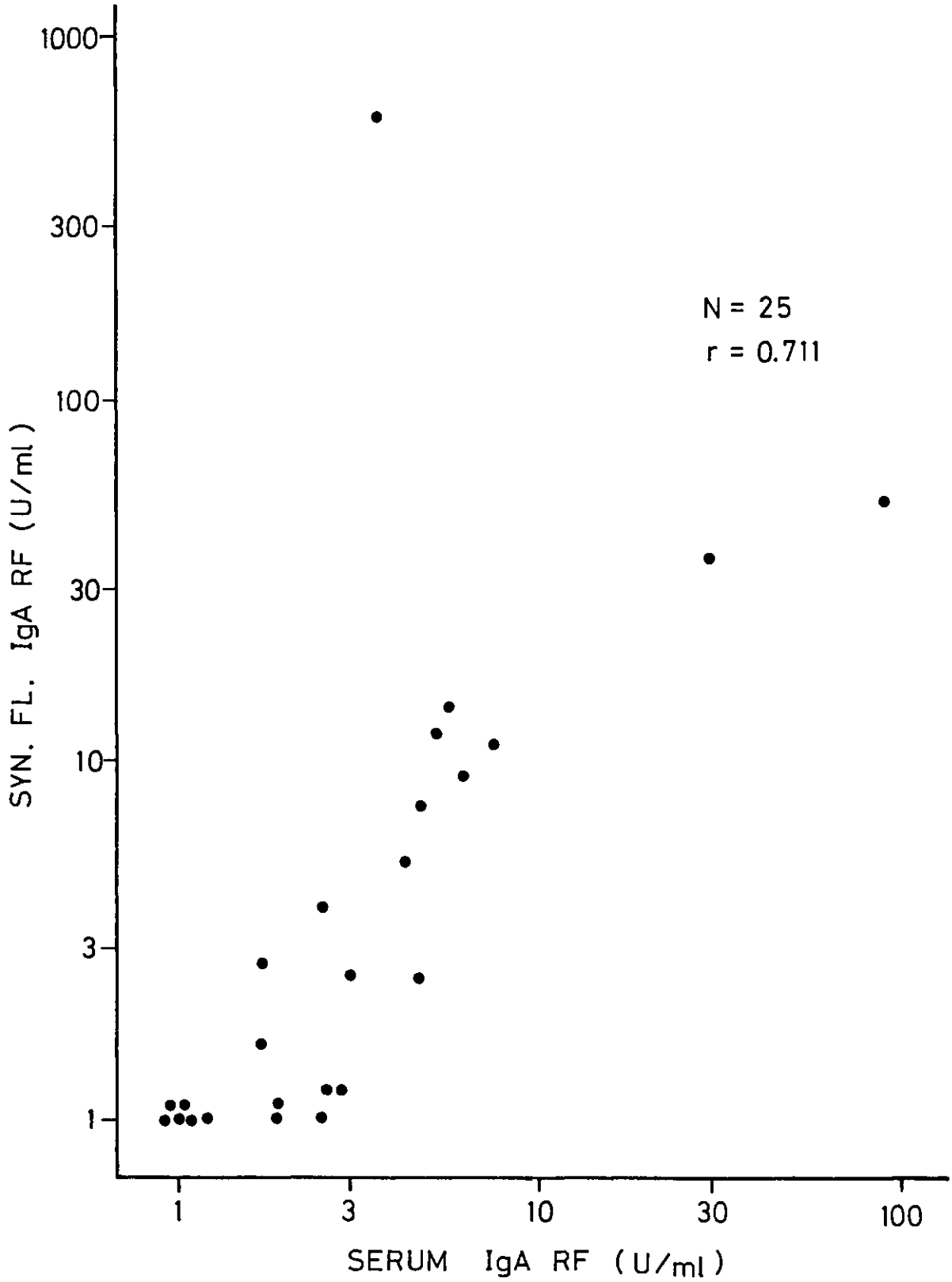


Figure V.1: The relationship between serum and synovial fluid IgA RF levels (paired samples) in the prospective group.

The log-transformed values showed a significant correlation ($N = 25$, $r = 0.711$, $p < 0.001$, the regression equation: $y = 1.06X + 0.036$).

Table V.3: RF levels in paired serum and synovial fluid samples from the prospective group.

	No. of pairs	r	p <	y = a x + b
IgA RF	25	0.711	0.001	y = 1.06X + 0.036
IgM RF	25	0.849	0.001	y = 1.06X - 0.124
IgG RF + pepsin	25	0.772	0.001	y = 0.700X + 0.286
IgG RF - pepsin	25	0.597	0.01	y = 0.518X + 0.175

The regression equation is based on the log-transformed paired serum (X-values) vs. synovial fluid (y-values) levels.

Table V.4: Raised serum RF levels in the group of 33 (ELISA results).

	At onset	During follow-up
IgM RF	10 (30%)	13 (39%)
IgA RF	7 (21%)	9 (27%)
IgG RF + pepsin	4 (12%)	8 (24%)
IgG RF - pepsin	4 (12%)	7 (21%)

The IgG RF results represent those where pepsin digestion was applied (+ pepsin) as well as the pepsin-free control (- pepsin).

The upper limits of normal were (see chapter III):
IgM RF $\leqslant$ 20 IU/ml, IgA RF < 10 U/ml, IgG RF + pepsin < 80 U/ml and IgG RF - pepsin < 50 U/ml.

V.3.3. RF profiles and clinical/radiological outcome of the group of 33

Table V.5. shows the results of a correlation analysis for the group of 33 where the serum IgM RF and IgA RF levels at first visit were correlated with the clinical outcome (as measured at the last visit during the follow-up). The IgM RF levels at onset were significantly associated with the final MDAG value ($p < 0.05$) and the last PVAS reading ($p < 0.05$).

Table V.6. shows how the number of hand and wrist erosions on the last X-ray picture correlated with the clinical features (at the first and last visit) and the RF levels at onset.

There was a striking correlation between the number of erosions and both grip strength and Ritchie index at onset and the last visit ($p < 0.001$). However, no significant association was seen between the number of erosions developed and the RF levels at onset.

Table V.7. shows a comparison of the radiological outcome of those who presented with raised vs. normal levels of RFs. Also, the outcome of those who ever developed raised RF levels is compared with that of those who did not.

Briefly, those who either presented with raised IgA RF or developed raised IgA RF later, developed significantly more erosions of their hands and wrists than the rest of the group ($p < 0.01$). However, there was no such correlation between erosions and the onset/maximal levels of IgM RF, IgG RF with pepsin or IgG RF without pepsin.

Table V.5: The correlation (r) between RF levels (IgA RF and IgM RF) at onset and the clinical measurements after at least two years follow-up of the group of 33.

		IgA RF	IgM RF
Last EMS	r	0.03	-0.06
	p <	NS	NS
Last PVAS	r	-0.02	0.39
	p <	NS	0.05
Last Ritchie	r	-0.05	0.33
	p <	NS	NS
Last GS	r	-0.07	-0.22
	p <	NS	NS
Last MDAG	r	-0.02	0.36
	p <	NS	0.05

NS: not significant, p >0.05.

Table V.6: The association in the group of 33 between the number of hand and wrist erosions developed and some of the clinical/laboratory parameters at the first and last visit.

	r	Significance
Age at onset	0.08	NS
Initial EMS	0.18	NS
" PVAS	-0.10	NS
" Ritchie	0.45	p <0.01
" GS	-0.46	p <0.01
" ESR	0.09	NS
" IgM RF	-0.06	NS
" IgA RF	0.24	NS
" IgG RF + pepsin	-0.13	NS
" IgG RF - pepsin	-0.11	NS
Last Ritchie	0.49	p <0.01
Last GS	-0.54	p <0.01
Last MDAG	0.35	p <0.05

Table V.7: Comparison of the radiological outcome between those with raised vs. normal RF levels in the group of 33.

		RF levels at first visit			Max. RF levels observed		
RF isotype	RF level	No. of subjects	No. of erosions mean ± SD	Significance (p <)	No. of subjects	No. of erosions mean ± SD	Significance (p <)
IgA RF	⩾10 U/ml	7	14.3 ± 15.3	0.01	9	12.4 ± 14.1	0.01
	<10 U/ml	26	3.0 ± 5.5		24	2.7 ± 5.4	
IgM RF	>20 IU/ml	10	6.6 ± 12.6	NS	13	7.1 ± 11.2	NS
	⩽20 IU/ml	23	4.8 ± 8.0		20	4.3 ± 8.3	
IgG RF + pepsin	⩾80 U/ml	4	3.8 ± 5.2	NS	8	4.9 ± 5.0	NS
	<80 U/ml	29	5.6 ± 10.0		25	5.6 ± 10.6	
IgG RF - pepsin	⩾50 U/ml	4	5.3 ± 10.5	NS	7	5.1 ± 8.2	NS
	<50 U/ml	29	5.4 ± 9.5		26	5.4 ± 9.9	

Student's t test was used to assess the significance of the difference between the groups.

NS: not significant, p >0.05.

Table V.8., furthermore, shows that when the 5 non-RA patients were eliminated from the group of 33, those with raised IgA RF still developed a significantly greater number of erosions.

Eleven of the 28 RA patients presented with raised serum levels of either IgA RF, IgM RF or both isotypes. Of these, four presented with raised serum IgM RF only, none of whom developed any erosions during the follow-up period. However, all the remaining seven who presented with raised serum IgA RF (with/without raised IgM RF) developed erosive disease of their hands and wrists. This difference was statistically significant ($p = 0.00303$, Fisher's exact probability test).

V.3.4. Fluctuations in disease activity and RF levels

Using multivariate analysis and the Spearman's rank correlation method, the association between fluctuations in disease activity and RF levels were determined for each of the 33 individuals studied. The number of clinical assessments for each individual upon which the multivariate analysis was based varied from 5-12, mean 7.6. As an example, the correlation coefficients (r_s) for patient G.W. are shown in Table V.9. These were based on nine assessments and it follows that $|r_s|$ greater than 0.597 indicated a statistically significant association at the 5% level. This male patient presented at the age of 42 to the rheumatology clinic. At that time (August 1979) he gave a six week history of pain, swelling and stiffness of the joints, especially of the left wrist and right ankle. He has now developed classical

Table V.8: Comparison between those with raised vs. normal IgA RF in the RA group of 28, with respect to the number of erosions developed.

IgA RF	Level	No of. Patients	Erosions		Significance
			Mean	1 SD	
At first visit	⩾ 10 U/ml	7	14.3	15.3	$p < 0.02$
	< 10 U/ml	21	3.7	5.9	
Maximum Levels	⩾ 10 U/ml	9	12.4	14.1	$p < 0.05$
	< 10 U/ml	19	3.4	5.9	

Student's t test was used to assess the significance of the difference between the groups.

Table V.9: Associations between fluctuations in clinical and serological parameters for patient G.W.

		EMS	PVAS	Ritchie	GS	ESR	IDA	IgM RF	IgA RF	IgG RF + pepsin
PVAS	r_s $p <$	0.779 0.01								
Ritchie	r_s $p <$	0.733 0.02	0.740 0.02							
GS	r_s $p <$	-0.229 NS	-0.643 0.05	-0.542 NS						
ESR	r_s $p <$	0.350 NS	0.778 0.01	0.577 NS	-0.744 0.02					
IDA	r_s $p <$	0.809 0.01	0.873 0.01	0.949 0.001	-0.564 NS	0.703 0.05				
IgM RF	r_s $p <$	0.365 NS	0.418 NS	0.544 NS	-0.285 NS	0.570 NS	0.532 NS			
IgA RF	r_s $p <$	0.550 NS	0.361 NS	0.824 0.01	-0.092 NS	0.239 NS	0.674 0.05	0.611 0.05		
IgG RF + pepsin	r_s $p <$	0.274 NS	0.711 0.02	0.310 NS	-0.561 NS	0.851 0.01	0.464 NS	0.600 0.05	0.092 NS	
IgG RF - pepsin	r_s $p <$	0.137 NS	0.268 NS	0.234 NS	-0.134 NS	0.519 NS	0.245 NS	0.900 0.001	0.360 NS	0.650 0.05

The values shown are the Spearman's rank correlation coefficients (r_s) from a multivariate analysis, based on nine follow-up assessments on patient G.W.

The significance of the correlation is indicated (NS = not significant).

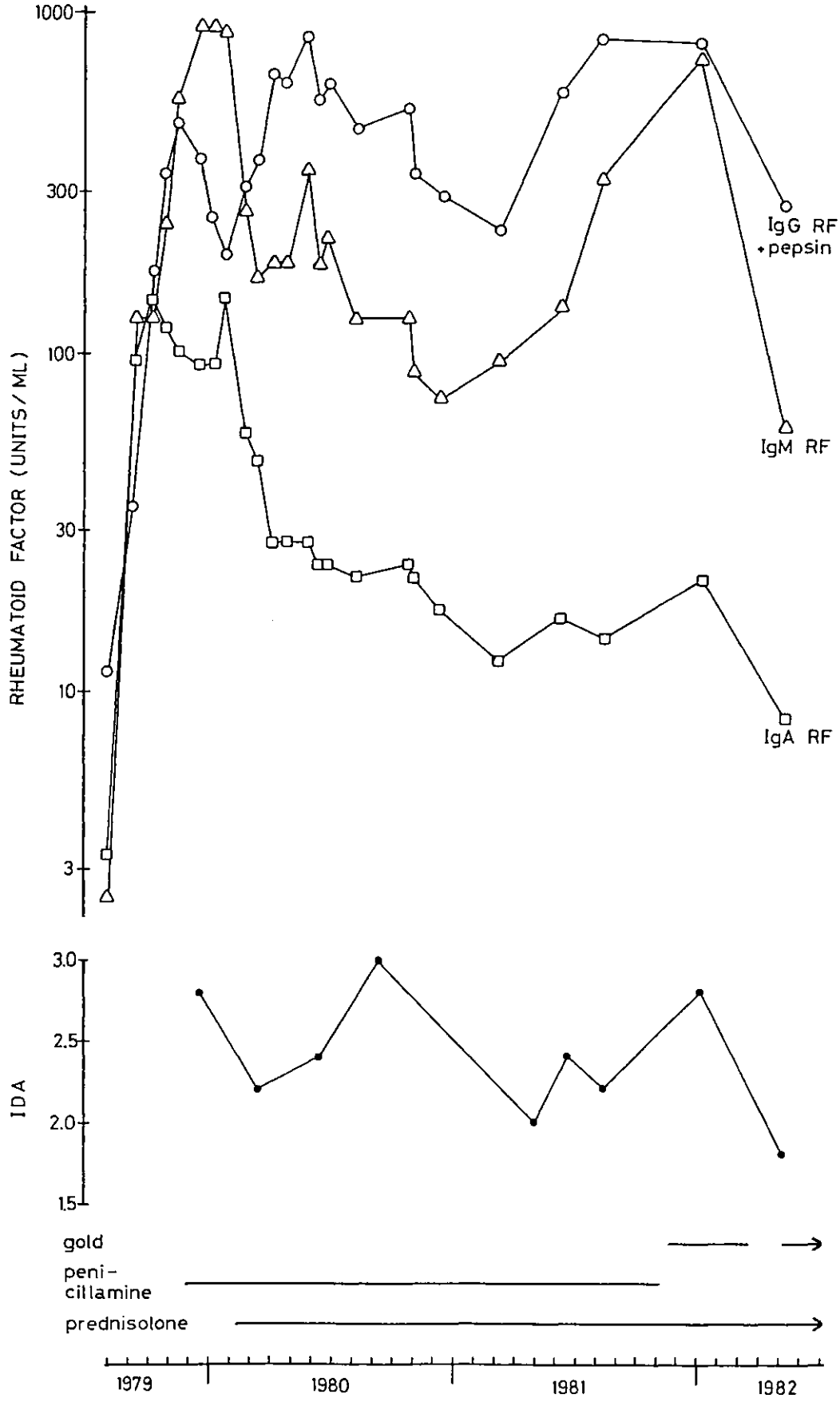
erosive RA and Figure V.2. shows his RF profiles from 1979 to 1982, together with his IDA profile and a brief outline of his drug treatment.

The three RF profiles of patient G.W. shown in Figure V.2. clearly exhibited a somewhat parallel course. This, however, was not always the case, as exemplified by patient F.K. in Figure V.3. This female patient presented (at age 64) to the rheumatology clinic in September 1980 with a six month history of pain and stiffness in her left shoulder, right arm and both knees. She has now developed definite RA with erosions of her wrist joints, but additionally she was found to have splenomegaly and neutropenia (neutrophil count x 2 less than 0.2 x 10^9/1) in 1982. Her neutrophil count was normal in 1980 (3.9 x 10^9/1), thus, her neutropenia and splenomegaly coincided with a rise in IgA RF (Figure V.3.) but the IgM RF and IgG RF remained within normal limits. Repeated bone marrow smears (in September and October 1982) showed myeloid hyperactivity with a deficit of mature neutrophils, thus compatible with Felty's syndrome (Decker and Plotz, 1979).

Table V.10. shows the overall association in the RA group of 28 between the fluctuations of the clinical parameters vs. the ESR and the RFs. These results closely resembled those in Table V.11. where the analogous results for the whole group of 33 are shown. Briefly, the ESR showed a very significantly negative correlation with the GS values (mean r_S = -0.344, which differs from zero with a probability of <0.001). Also, the ESR showed a significantly positive association with the IgA RF (mean r_S = 0.243, p <0.01). Interestingly, there was a significant negative association between the GS and IgA RF (mean r_S = -0.179, p <0.05) whereas neither of the other RF isotypes exhibited such an overall association with the clinical parameters (Tables V.10. and V.11.).

Figure V.2: The profiles of IgM RF, IgA RF, IgG RF (pepsin digested samples) and the index of disease activity (IDA) for patient G.W. during 1979-1982.

An outline of his drug treatment ("second line" drugs and steroids) during this period is shown at the bottom of the figure.


1000
300
100
30
10
3
RHEUMATOID FACTOR (UNITS / ML)
IgG RF
+pepsin
IgM RF
IgA RF
3.0
2.5
2.0
1.5
IDA
gold
peni-
cillamine
prednisolone
1979
1980
1981
1982

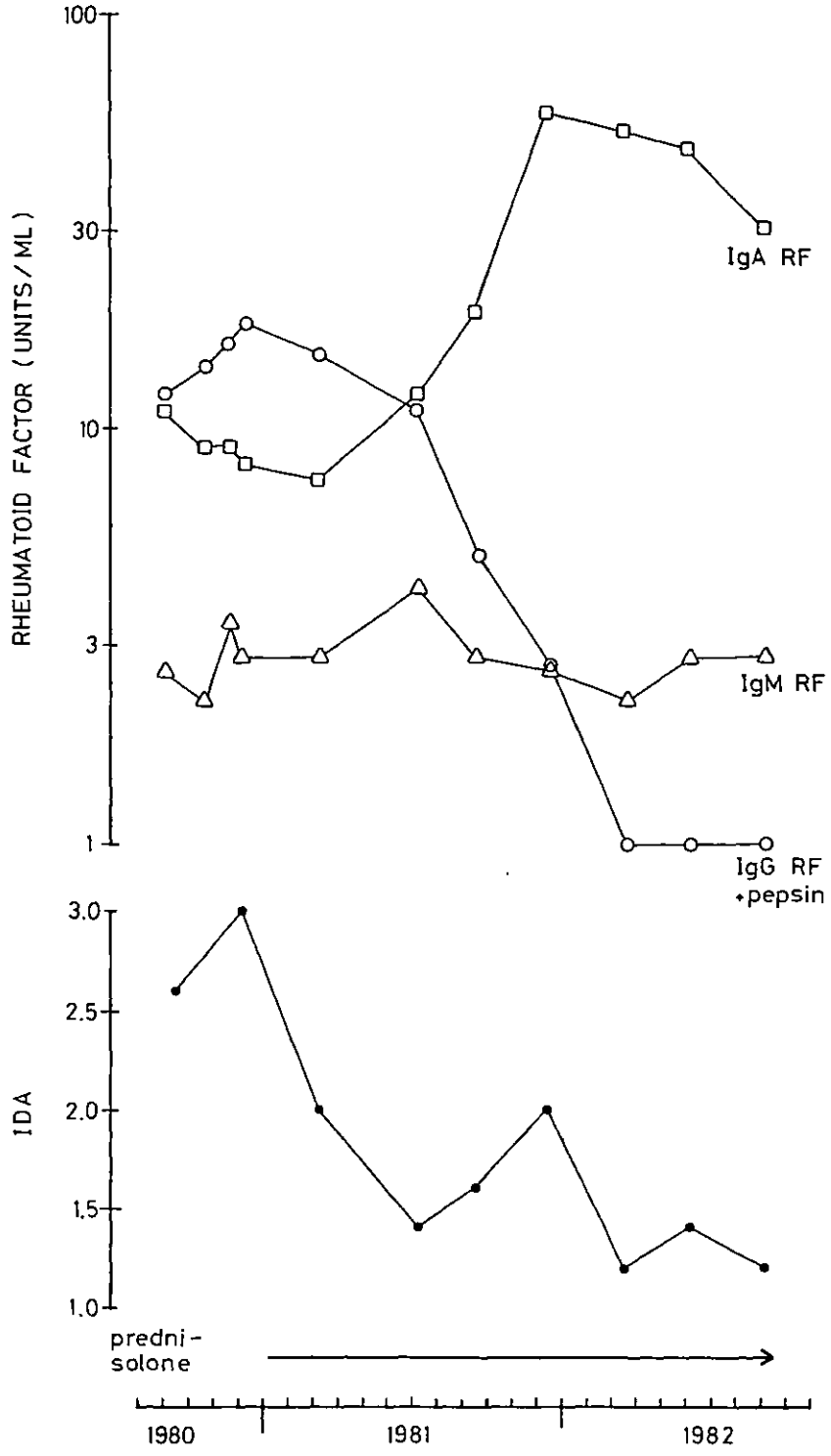


Figure V.3: The profiles of IgM RF, IgA RF, IgG RF (pepsin digested samples) and the index of disease activity (IDA) for patient F.K. during 1980-1982.

Her steroid treatment is indicated at the bottom of the figure.

Table V.10: The association between longitudinal fluctuations in clinical and serological parameters in the RA group (N = 28).

		ESR	IgM RF	IgA RF	IgG RF + pepsin	IgG RF - pepsin
EMS	$\bar{r}_s$ ± SD Significance	0.167 0.388 p <0.05	0.107 0.408 NS	0.121 0.372 NS	0.074 0.416 NS	0.051 0.320 NS
PVAS	$\bar{r}_s$ ± SD Significance	0.115 0.432 NS	0.023 0.503 NS	0.136 0.370 NS	0.132 0.467 NS	0.083 0.387 NS
Ritchie	$\bar{r}_s$ ± SD Significance	0.235 0.484 p <0.02	0.002 0.487 NS	-0.071 0.446 NS	0.152 0.484 NS	0.078 0.264 NS
GS	$\bar{r}_s$ ± SD Significance	-0.344 0.439 p <0.001	-0.049 0.506 NS	-0.179 0.415 p <0.05	-0.184 0.485 NS	-0.142 0.423 NS
IDA	$\bar{r}_s$ ± SD Significance	0.398 0.358 p <0.001	0.093 0.524 NS	0.180 0.363 p <0.02	0.122 0.498 NS	0.124 0.397 NS
ESR	$\bar{r}_s$ ± SD Significance		0.168 0.469 NS	0.243 0.396 p <0.01	0.168 0.444 NS	0.182 0.454 p <0.05
IgM RF	$\bar{r}_s$ ± SD Significance			0.248 0.478 p <0.02	0.062 0.453 NS	0.003 0.462 NS

Each $\bar{r}_s$ value represents the mean of the r_s values for each of the 28 RA patients.

The table also shows the standard deviations of the $\bar{r}_s$ values and the significance of the deviation from zero of these, as assessed by the student's t test.

Table V.11: The association between the longitudinal fluctuations in clinical and serological parameters in the whole prospective group followed up for at least two years (N = 33).

		ESR	IgM RF	IgA RF	IgG RF + pepsin	IgG RF - pepsin
EMS	$\bar{r}_s$ ± SD Significance	0.129 0.372 NS	0.108 0.383 NS	0.085 0.358 NS	0.082 0.385 NS	0.068 0.309 NS
PVAS	$\bar{r}_s$ ± SD Significance	0.071 0.421 NS	-0.002 0.484 NS	0.106 0.366 NS	0.080 0.466 NS	0.036 0.390 NS
Ritchie	$\bar{r}_s$ ± SD Significance	0.201 0.495 p <0.05	0.009 0.461 NS	-0.037 0.442 NS	0.082 0.507 NS	0.060 0.264 NS
GS	$\bar{r}_s$ ± SD Significance	-0.317 0.424 p <0.001	-0.028 0.477 NS	-0.173 0.402 p <0.02	-0.164 0.486 NS	-0.120 0.395 NS
IDA	$\bar{r}_s$ ± SD Significance	0.357 0.368 p <0.001	0.104 0.487 NS	0.161 0.367 p <0.02	0.082 0.491 NS	0.112 0.367 NS
ESR	$\bar{r}_s$ ± SD Significance		0.159 0.454 NS	0.210 0.387 p <0.01	0.167 0.430 p <0.05	0.194 0.432 p <0.02
IgM RF	$\bar{r}_s$ ± SD Significance			0.219 0.467 p <0.02	0.076 0.424 NS	0.057 0.440 NS

Each $\bar{r}_s$ value represents the mean of the r_s values for each of the 33 patients.

The table also shows the standard deviations of the $\bar{r}_s$ values and the significance of the deviation from zero of these, as assessed by student's t test.

V.4. DISCUSSION

Sharp *et al* (1971) designed a score for the rate of progression of erosions and joint space narrowing in their RA group of 90 patients, in which disease duration was taken into account. It must be borne in mind, however, that the time from onset of the disease in their RA group ranged from 1 month to over 40 years. As noted earlier, there was no significant association between the duration of diease in our RA group and the number of erosions which developed. Also, the range of disease duration was rather short, 24-48 months. Therefore, I felt justified to use the unmodified erosion count as an indicator of disease severity.

An association of paired serum and synovial fluid RF levels has been previously reported by Hay *et al* (1979) who found a strong correlation between the IgG antiglobulin levels in synovial fluid and serum. Also, these authors found a strong correlation between serum and synovial fluid IgM antiglobulins. However, in contrast to the results shown in Table V.3., Hay *et al* (1979) noted that the synovial fluid levels of IgG antiglobulins were nearly always higher than in the corresponding serum samples. Higher synovial fluid than serum levels of RFs have been taken as evidence for local production in the joint tissues. It has been argued that it is necessary to correct the synovial fluid/serum ratios of RFs with the corresponding albumin ratios (Jones *et al*, 1982) but no such corrections were performed here. Consequently, the data shown in Figure V.1. and in Table V.3. should be interpreted with caution.

The highly significant correlation observed between the number of erosions developed and the GS and Ritchie values, both at the

beginning and the end of the study (Table V.6.) is interesting. These results would imply that those who develop early involvement of their hands (reduced GS) or widespread polyarthritis (high Ritchie index) tend to do badly. Sharp *et al* (1971) reported a positive relationship between persistent inflammation of the hands and wrists and both defect score and rate of progression of defects. These authors also noted a striking association between limitation of fist formation and the radiological evidence of joint destruction, thus linking directly the objective X-ray evidence of destruction with the loss of function and disability. It is of interest here, however, that there was only a weak overall correlation between the IgM RF level at onset and the last PVAS reading (Table V.5.) and no significant correlation between the RF levels at onset and the number of erosions (Table V.6.). This contrasts with the findings of Sharp *et al* (1971) who noted a positive relation between the titre of RF and the rate of progression of X-ray defects.

Even more interesting are the findings shown in Tables V.7. and V.8. that those who developed raised levels of IgA RF early tended to do badly. However, neither raised IgM RF nor IgG RF were associated with adverse radiological outcome. None of the five patients (four RA, one non-RA) who presented with raised serum IgM RF and normal IgA RF and IgG RF developed erosive disease of their hands and wrists. This is in sharp contrast to the seven patients who presented with raised serum IgA RF as all these developed erosive disease within 2-4 years from the onset.

Apart from a brief recent report (March *et al*, 1983), very little has been published on IgA RF measurements in relation to prospective

studies in joint disease but the observations presented in Tables V.7. and V.8. clearly warrant further study. Possibly, the presence in serum of IgA RF reflects general RF production of pathogenic importance whilst the IgM and IgG isotypes are rapidly removed because of their inherent properties of fixing complement and/or forming self-aggregating complexes. Consequently, the IgA RF could be a marker for heightened RF production, albeit relatively harmless in itself.

The observation that the IgA RF levels in serum fluctuate in a closer harmony with the clinical parameters than the other RF isotypes (Tables V.10. and V.11.) lends further support to the hypothesis that IgA RF may at least be a marker of disease activity if not directly pathogenic. To my knowledge, no published studies have addressed the question of whether fluctuations in rheumatoid disease activity may be associated with fluctuations in IgA RF levels. This issue needs to be studied in a larger group of patients with early/established joint disease.

Finally, the observation that rising levels of IgA RF may coincide with the development of extra-articular rheumatoid features like those of Felty's syndrome is most interesting. Dunne *et al* (1979) and Elkon *et al* (1981) have previously shown an association between IgA RF and the extra-articular features in systemic sicca syndrome, and my observations seem to lend further support to the importance of IgA RF in the context of rheumatoid arthritis.

V.5. CONCLUSION

Thirty-three patients with early rheumatoid joint disease were followed up prospectively for at least two years, and their RF profiles related to the clinical/radiological course and outcome. Those patients who showed raised serum levels of IgA RF, either at presentation or later on, developed significantly more erosions of their hands and wrists than those with normal serum IgA RF. Furthermore, fluctuations of serum levels of IgA RF showed a closer association with clinical parameters and ESR than did any of the other RF isotypes. An isolated rise in serum IgA RF and the simultaneous development of Felty's syndrome was noted in one patient. Together, these findings support the hypothesis that IgA RF may be a disease marker, or even of direct pathogenic importance in rheumatoid arthritis.

C H A P T E R V I

STUDIES ON AN INBRED ICELANDIC FAMILY WITH CLUSTERING OF RHEUMATIC DISEASES

C H A P T E R V I

STUDIES ON AN INBRED ICELANDIC FAMILY WITH CLUSTERING OF RHEUMATIC DISEASES

VI.1. INTRODUCTION

VI.1.1. Familial clustering of rheumatic diseases

As reviewed in chapter I.2., it has long been observed that various rheumatic diseases are aggregated in families. This kind of aggregation is not confined to rheumatic diseases like RA. It has been stated that almost all human disorders are more likely to afflict someone with an affected relative than someone with an equivalent set of unaffected relatives (Edwards, 1969). Furthermore, familial aggregation may be due to either shared genetic background or shared environment, or a combination of both.

The relative contribution of nature and nurture to familial aggregation of RA has been the subject of much speculation. The results of the ARC twin survey where the concordance of seropositive arthritis was six times as great in monozygous as in dizygous twins, led Lawrence (1970) to conclude that the aggegation of arthritis in families did not depend on shared environment.

It was later shown that seropositive RA is strongly associated with the presence of the HLA-DR4 allele (reviewed in chapter I.2.) and this allele seems to be associated with RA rather than RF *per se* (Karr *et al*, 1980). It has also been shown recently that families with multiple cases of RA may have more than one DR4-containing MHC haplotype (Stroem and Moeller, 1981), suggesting that the association may be with

DR4 itself or a gene which is in a strong linkage disequilibrium with DR4. Recently, O'Neill *et al* (1982) reported an association between RA and a rare variant of complement C4; C4B2.9 (Perth). However, Rittner and Bertrams (1981) who investigated the polymorphism of C2, C4, Bf and C3 in 107 patients with RA did not find any such association but these authors noted a clear association of RA with HLA-DR4, the genes for which are thought to be very close to the loci for C4/C2/Bf (Rittner and Bertrams, 1981).

As mentioned in chapter I.2., SLE has frequently been reported to be aggregated in families. Cleland *et al* (1978) investigated 103 members of four separate kindreds where multiple cases of SLE occurred. The authors postulated that the HLA antigens B8, A11, and B35 might have direct effect on disease expression but DR-typings were not performed. Furthermore, high incidence of raised serum ANA was found, both in non-consanguineous (spouses) and consanguineous relatives of the SLE patients, suggesting the involvement of a transmissible agent. Bell and Maddison (1980), however, found in a survey of 64 patients with SLE and mixed connective tissue disease, that raised levels of antibodies to the cytoplasmic glycoprotein Ro were associated with the presence of HLA-B8 and DR3, suggesting a genetic basis for the autoantibody production. Others have reported the presence of hypergammaglobulinaemia in relatives of SLE patients, supposedly reflecting a genetic influence (Leonhardt, 1964; Siegel *et al*, 1965).

Familial clustering of rheumatic diseases other than RA and SLE has frequently been reported. The B27-related disease like ankylosing spondylitis (AS) and Reiter's disease are among these.

It has been shown that as many as 22% of the 1st degree relatives of AS patients may be affected, a susceptibility closely associated with the presence of HLA-B27 (Daneo *et al*, 1977). Yunus *et al* (1981) who studied 45 1st degree relatives of 12 Reiter's patients, suggested considerable familial aggregation of this disease, especially in adult male 1st degree relatives carrying the B27 antigen.

Most authors consider the susceptibility to rheumatic diseases and serological abnormalities to be inherited as a dominant trait, rather than recessive (Lawrence, 1970). Steiner *et al* (1966) studied the prevalence of positive RF in an inbred population on the coastal island of Marken in the Netherlands. The prevalence was only a little higher than in a control population on the mainland, contrary to what might have been expected if the tendency to produce RF was inherited as a recessive trait.

VI.1.2. The Icelandic family: background and collaborative studies

The population of Iceland (234,980 on the 1st December 1982) has remained fairly stable, geographically and ethnically, for centuries, being isolated in the Atlantic Ocean. This stability and small total number of inhabitants make family studies on the population relatively easy, and several such studies have been reported (Arnason, 1935; Gudmundsson *et al*, 1972; Jensson *et al*, 1967). Furthermore, various parts of the country were very isolated and considerable inbreeding has occurred there in the past.

Dr. J. Thorsteinsson, a consultant rheumatologist in Reykjavik, noted in the early 1970's that several of his patients, suffering from SLE, RA and other rheumatic diseases, originated from an isolated community called Adalvik, in North-West Iceland. The Adalvik community is mountainous and practically inaccessible except by sea. The population there reached a maximum of about 500 just before 1930 but major emigration started soon after that and the last inhabitants left in the early 1950's. The majority emigrated to neighbouring villages in the North-West and to the Reykjavik area in the South-West of Iceland (see Figure VI.1). The story of the Adalvik community and its inhabitants 1702-1952 has been published, providing important genealogical information about this population (Kristmundsson and Bjarnason, 1971).

Dr. Thorsteinsson noted that most of the patients he saw from Adalvik belonged to a large and highly inbred family, henceforth referred to as the Adalvik family. These people were the descendants of a local sheriff who was born in 1784 and died in 1866. The aggregation of rheumatic diseases in this family prompted Dr. Thorsteinsson to start a clinical and genetic study in 1975-1976. The genetic part of the study has been carried out by Dr. A. Arnason and his colleagues in the Genetic Division of the Blood Bank, Reykjavik, Iceland. Preliminary results of these studies have been reported (Thorsteinsson and Arnason, 1977; Arnason, 1977; Arnason and Thorsteinsson, 1978a; Arnason and Thorsteinsson, 1978b; Thorsteinsson *et al*, 1979).

When the project described in this thesis was started, it was felt that the Adalvik family might offer interesting material in relation to the immunogenetic aspects of RA and other rheumatic



Figure VI.1: A map of Iceland, showing Adalvik in the North-West corner (top left) and the main routes of migration from there.

diseases. A major effort was therefore made to continue the studies on the Adalvik family, in close collaboration with Dr. Thorsteinsson and the Genetic Division of the Blood Bank, Iceland, where most of the genetic marker studies have been performed. The genetic studies will be the subject of a separate publication (Arnason *et al*, in preparation), but these include the determination of the HLA-A, B, and C haplotypes of 117 family members relevant to this thesis and studies of the genetic polymorphism of erythrocyte surface markers, the complement components C2, C4, C3 and Bf and other serum and red cell proteins. HLA-DR typing has been performed on 22 selected family members by Dr. K. I. Welsh, Guy's Hospital, London.

Briefly, HLA-typing of the 117 individuals showed that 69 (59.0%) possessed the HLA-B27 antigen (heterozygous or homozygous). Understandably, in view of the high degree of inbreeding in the Adalvik family, a striking clustering of a few MHC haplotypes was found among these 117 individuals. By inference from the limited number of HLA-DR and C4 typings done so far, the five following MHC-haplotypes (HLA-A;B;C;DR;Bf;C4) made up a total of 63.3% of the 234 haplotypes determined:

1. 9;27;w2;2;F;A3A2,BQO: 16.7% (39/234)
2. 2;27;w1;1;S;A3,B1: 13.7% (32/234)
3. 2;12; ;4;S;A3,BQO: 12.0% (28/234)
4. 1;8; ;2;S;A3,B1: 11.5% (27/234)
5. 28;35;w4;5 or 6;F;A3,B1: 9.4% (22/234)

This chapter deals with the clinical and immunological, and some of the genetic features of this family, although a detailed genetic analysis is beyond the scope of this thesis.

VI.2. METHODOLOGY

VI.2.1. Selection of family members for study

In 1975-1978, approximately seventy members of the Adalvik family had been interviewed, examined and bled. In relation to the project described in this thesis, all available individuals from the 1975-78 survey were contacted again in 1980 and 1981. These mostly belonged to the oldest surviving generations of the family, the majority of whom were born and brought up in Adalvik. Moreover the survey was extended to include as many adult members of the family as could be easily traced. A special priority was given to those adults whose parents were both of the family. These will henceforth be referred to as the inbred group.

It proved impossible to contact all surviving adult descendants of the family ancestor (the sheriff in Adalvik). It was even very difficult to estimate their total number accurately, but it is probably of the order of several hundreds, scattered over most of Iceland, and several are known to be living abroad. The selection therefore depended much on Dr. J. Thorsteinsson's personal knowledge of the family, as well as on information obtained from some of the family members about their relatives. However, more complete information was available on the inbred individuals who are only a small fraction of the estimated total and every effort was made to contact as many of these as possible.

VI.2.2. Interview protocol, clinical assessment, other sources of clinical information

The selected family members were either visited at home or asked to attend for an interview and examination at the National Hospital, Reykjavik, or at their local health centre. A questionnaire relating to rheumatic symptoms and diseases, adopted from the Heart Preventive Clinic, Reykjavik, was used (Table VI.1.). The examining physician (most often myself, but sometimes Dr. J. Thorsteinsson) explained the questions and helped with filling in the answers. Those interviewed were asked about past major illnesses and hospital admissions, as well as symptoms of photosensitivity, alopecia, skin rashes and Raynaud's phenomenon.

A routine clinical examination of peripheral joints was performed on those interviewed. When suggested by history, the spine and sacroiliac joints were examined as well.

All available case records for the family members at the National Hospital, Reykjavik, were reviewed and used, together with the interview data, for establishing the diagnoses. Furthermore, almost all those who suffered to any extent from rheumatic complaints had been under the care of Dr. J. Thorsteinsson, whose knowledge was therefore invaluable.

The ARA-criteria for RA (Ropes *et al*, 1958, see Table VI.2.) and SLE (Cohen *et al*, 1971) and the New York criteria for AS (Bennett and Burch, 1967) were used to establish the diagnoses.

Table VI.1: The standard interview questionnaire used during the family survey, adopted from the Heart Preventive Clinic, Reykjavik.

1. Do any of your relatives (relating only to first degree relatives) have arthritis?

2. Have you ever consulted a doctor because of arthritis in childhood (under 15 years of age)?

3. Have you ever consulted a doctor because of back problems?

4. Have you ever consulted a doctor because of muscular rheumatism?

5. Have you ever consulted a doctor because of arthritis?

6. Are you now being seen regularly by a doctor because of arthritis?

7. Do you now take any drugs on a regular basis because of arthritis?

8. Have you had pain in the joints? (refers to the last 12 months)

9. Have you had swelling of the joints? - ditto -

10. Have you had morning stiffness of the joints? - ditto -

11. Have you had pain in three or more limb joints? - ditto -

Table VI.2: The revised diagnostic ARA-criteria for rheumatoid arthritis (Ropes *et al*, 1958).

1. Morning stiffness.
2. Pain on motion or tenderness in at least one joint.
3. Swelling in at least one joint.
4. Swelling in at least one other joint.
5. Symmetrical joint swelling.
6. Subcutaneous nodules.
7. X-ray changes typical of RA.
8. Demonstration of rheumatoid factor.
9. Poor mucin precipitate from synovial fluid.
10. Synovial histology characteristic for RA.
11. Characteristic histology of rheumatoid nodules.

VI.2.3. Immunological parameters studied

At the time of the interview/assessment, blood samples were taken for immunological and genetic analyses. Sera were separated on the day of collection, aliquoted and stored at -80°C, and transported between Iceland and London on dry ice (-80°C).

Serum RF levels were determined both by conventional agglutination tests (Rheumaton, Rose Waaler and latex, performed in the Department of Bacteriology, University of Iceland) and by ELISA (IgM RF, IgA RF, IgG RF with and without pepsin digestion). Only Rose Waaler positive sera (titre $\geq$ 1:10) were considered to fulfil the 8th ARA-criterion for seropositivity.

Serum anti-nuclear factor (ANF) levels were measured in the Department of Immunology, University of Iceland, using a standard immunofluorescence test, with rat liver as a substrate. Reactivity at serum dilution $\geq$ 1:10 was recorded as positive ANF.

Total serum IgG, IgA and IgM was measured, using a laser nephelometer and commercial nephelometric kits (Hyland LAS-R, Travenol Laboratories Ltd., U.K.).

Total serum IgE was measured using solid phase radioimmunoassay kits (Phadebas IgE PRIST, Pharmacia Diagnostics AB, Uppsala, Sweden).

Serum IgG anti-ruminant IgG levels were determined (see chapter IV.2.).

VI.2.4. Data analysis

The relationship between the various clinical/immunological parameters in the family as a whole was assessed by multivariate analysis using Spearman's rank correlation method.

The clinical/immunological characteristics of the inbred family members vs. their age and sex matched internal control group were compared by the Mann Whitney rank sum test.

Wilcoxon's signed ranks test was used to compare the clinical/immunological features of those family members carrying the MHC haplotype HLA-A2; Bl2;BfS with an age and sex matched internal control group.

For the statistical analysis the results of questions 8-11 on joint symptoms in Table VI.1. were ranked, yielding values ranging from 1-5; 1 = no symptoms, 5 = yes to all four questions. Also, the results of the Rheumaton, latex, Rose Waaler and ANF tests were ranked for the analyses (Rheumaton: neg. = 1, (+) = 2, + = 3, ++ = 4; latex: neg. = 1, (+) = 2, + = 3, ++ = 4; Rose Waaler: neg. = 1, pos. at titre 1:10 = 2, 1:20 = 3 etc.; ANF: neg. = 1, pos. at titre 1:10 = 2, 1:20 = 3 etc.).

VI.3. RESULTS

VI.3.1. Age and sex characteristics, the pedigree and diagnoses

A total of 181 family members, 89 females and 92 males, were included in the 1980-1981 study. Figure VI.2. shows the age and sex distribution of these (age as on 31 December 1980), age range 6-90 years.

Figure VI.3. shows the family pedigree. It covers nine generations to a greater or lesser extent but for the sake of simplicity, primarily those family members who are relevant to this study are depicted. The great number of consanguineous marriages (a total of 12, indicated by double lines) is quite striking. Most of these occurred before the emigration from Adalvik. Also included in the pedigree is a sidebranch (descendants of II,5) that interconnects extensively with the main pedigree. Gossip, supported by genetic marker evidence, suggests that this branch may, in fact, descend directly from the common ancestor, I,1 (the sheriff in Adalvik).

The diagnoses of the affected family members are shown in the pedigree, together with their pedigree numbers. Thirty-nine out of the 181 family members studied were judged to be affected by disease: 10 classical/definite RA, 10 probable RA, 3 definite SLE, 10 suspected SLE, 2 definite AS, 1 possible AS, 2 reactive arthritis, 1 Reiter's syndrome, 1 palindromic rheumatism and 1 psoriatic arthritis. The 10 individuals classified as suspected SLE met only 2 or 3 of the ARA-criteria for SLE. One lady (VII,56) who suffered from chronic inflammatory disease of the dorsal and lumbar spine was labelled as possible AS despite lack of X-ray evidence of sacroiliitis. Two

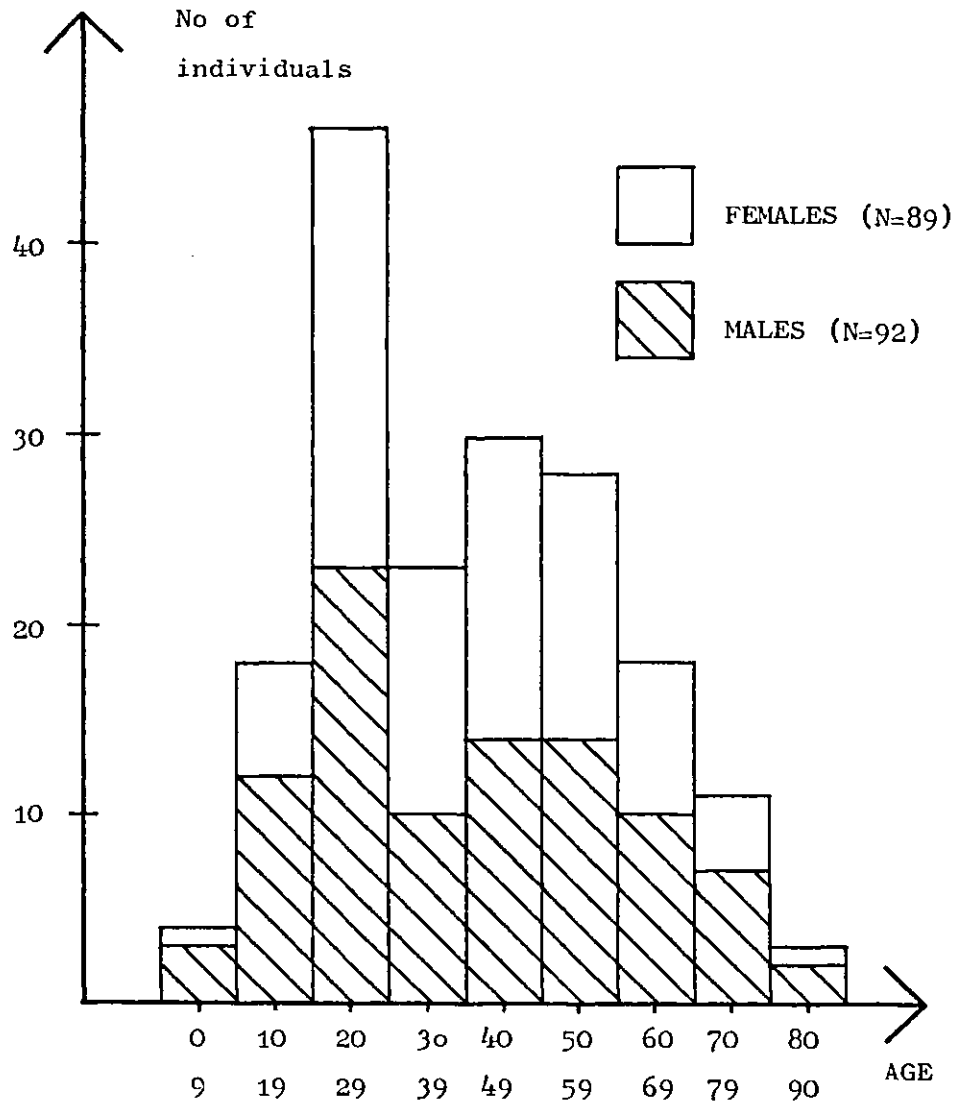
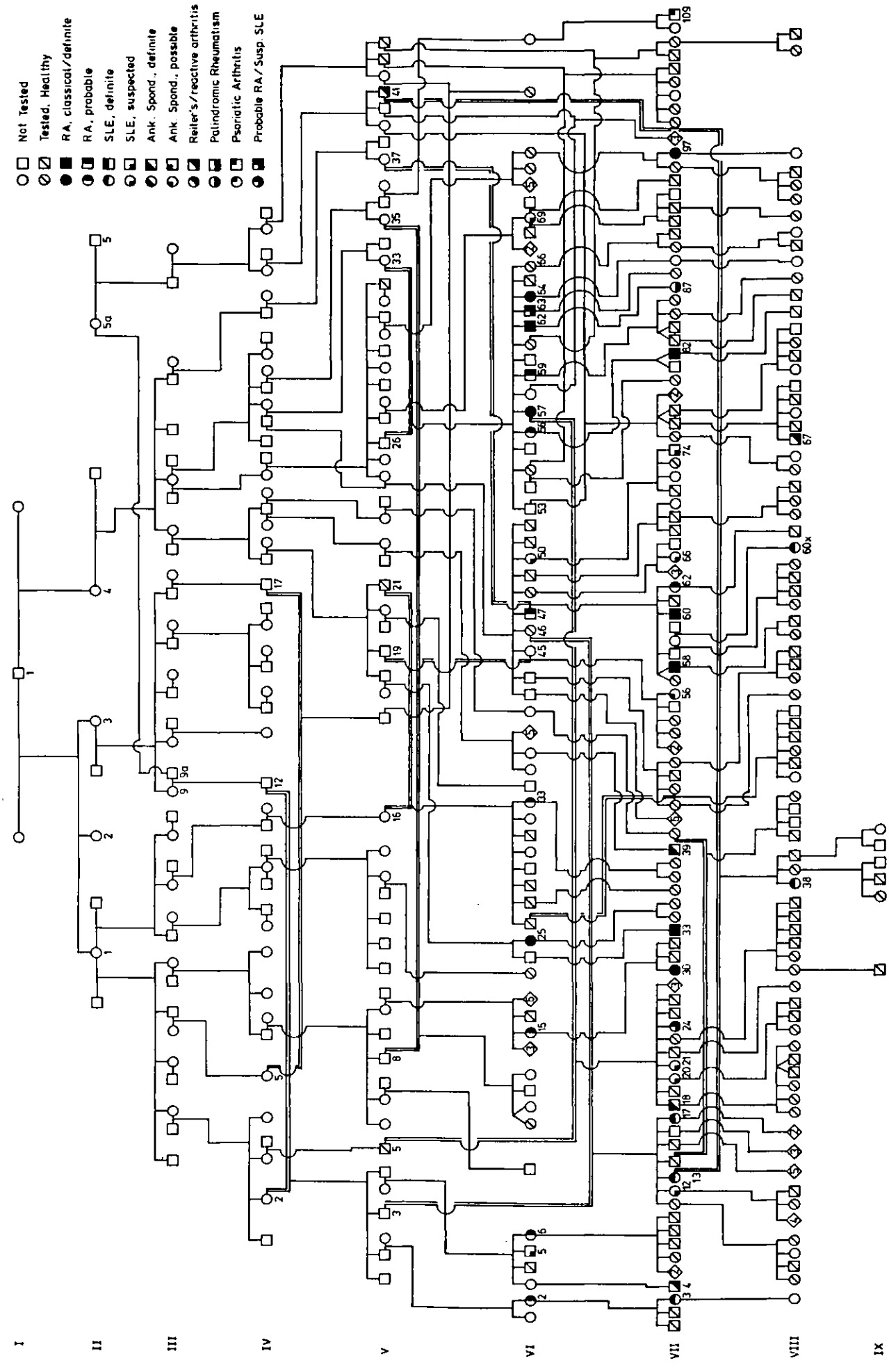


Figure VI.2: The age and sex distribution of the 181 family members studied.

Figure VI.3: The pedigree of the Adalvik family.

circles: females
squares: males
double lines: consanguineous marriages

The pedigree numbers of those who were affected by rheumatic disease or married their relatives are shown.


Not Tested
Tested, Healthy
RA, classical/definite
RA, probable
SLE, definite
SLE, suspected
Ank. Spond., definite
Ank. Spond., possible
Reiter's/reactive arthritis
Palindromic Rheumatism
Psoriatic Arthritis
Probable RA/Susp. SLE
I
II
III
IV
V
VI
VII
VIII
IX

family members (VI,63 and VII,24) were classified as having either probable RA or suspected SLE.

The pedigree also depicts as SLE No. VIII,38, a daughter of VII,13 and V,41. This female died in 1956 from what was considered definite SLE.

Furthermore, the pedigree also shows a stepdaughter of No. VII,59, No. VIII,60x, who has definite SLE. This female was brought up within the family but is not of known blood relation to it.

TablesVI.3. and VI.4. present a brief clinical summary of the 39 family members who were judged to be affected by a rheumatic disease. The ARA-criteria for RA met by each of the 20 classical/definite/probable RA patients are shown in Table VI.3., cf. Table VI.2. It should be noted that for reasons already stated, individuals VI,63 and VII,24 are shown both in Tables VI.3. and VI.4.

VI.3.2. Results of the interview questionnaire

The results of the interview questionnaire on rheumatic symptoms/diseases are shown in Table VI.5. It is noteworthy that 42 (47%) of the females had had pain in at least three limb joints over the last 12 months prior to the interview. Similarly, 40 (45%) of the females had seen their doctor because of muscular rheumatism and 29 (33%) gave a positive history of joint swelling over the last 12 months. Few, however, were on regular drug therapy for arthritis (only 13 females and 6 males).

Table VI.3: Summary of the 20 family members who had classical/definite/probable RA.

Pedigree number	Age	Sex	ARA-criteria for RA	Diagnosis
VI,2	60	F	1, 2, 3, 8	Probable RA
VI,6	50	F	1, 2, 8	Probable RA
VI,15	58	F	1, 2, 3, 8	Probable RA
VI,25	75	F	1, 2, 3, 4, 5	Definite RA
VI,33	46	F	1, 2, 8	Probable RA
VI,47	78	M	1, 2, 8	Probable RA
VI,56	73	F	2, 3, 4, 5	Probable RA
VI,57	71	F	1 - 10	Classical RA
VI,59	68	M	1, 2, 8	Probable RA
VI,62	62	M	1, 2, 3, 4, 5, 8	Definite RA
VI,63	60	M	1, 2, 3	Probable RA
VI,64	59	F	1, 2, 3, 4, 5, 8, 10	Classical RA
VII,24	39	F	1, 2, 8	Probable RA
VII,30	28	F	1 - 5, 7 - 10	Classical RA
VII,33	43	M	2, 3, 4, 5, 8	Definite RA
VII,58	54	M	1, 2, 3, 4, 5, 7	Definite RA
VII,60	50	M	1, 2, 3, 4, 5, 8	Definite RA
VII,62	47	F	1, 2, 3	Probable RA
VII,82	44	M	1, 2, 3, 4, 5	Definite RA
VII,97	31	F	1, 2, 3, 4, 5	Definite RA

Table VI.4: Summary of the 21 family members who were given diagnoses other than RA.

Pedigree number	Age	Sex	Summary of findings	Diagnosis
V,41	62	M	Polyarthritis, prostatitis, iritis	Reiter's syndrome
VI,5	53	M	Discoid LE, photosensitivity	Suspected SLE
VI,50	72	F	Arthralgias, pos. ANF (1:80)	Suspected SLE
VI,63	60	M	Photosensitivity, Raynaud's phenomenon	Suspected SLE
VI,69	73	F	Photosensitivity, Raynaud's, arthralgias	Suspected SLE
VII,3	24	F	Seven ARA-criteria for SLE	Definite SLE
VII,4	33	M	Polyarthritis with fever	Reactive arthritis
VII,12	59	F	Raynaud's, photosensitivity, ANF (1:80)	Suspected SLE
VII,13	58	F	Arthritis, photosensitivity, Raynaud's, pos. LE-cells	Definite SLE
VII,17	40	F	Butterfly rash, photosensitivity, ANF (1:80), thrombocytopenia	Definite SLE
VII,18	51	M	Progressive spondylitis from age 18	Definite AS
VII,20	48	F	Arthralgias, leukopenia, pos. ANF	Suspected SLE
VII,21	46	F	Photosensitivity, alopecia, ANF (1:40)	Suspected SLE
VII,24	39	F	Polyarthralgias, Raynaud's phenomenon	Suspected SLE
VII,39	32	M	Progressive spondylitis from age 22	Definite AS
VII,56	49	F	Clinical spondylitis, arthralgias	Possible AS
VII,66	45	F	Arthralgias, photosensitivity, ANF (1:40)	Suspected SLE
VII,74	36	M	Arthralgias, pos. ANF (1:80)	Suspected SLE
VII,87	37	F	Recurrent polyarthritis, pos. Rose Waaler	Palindromic rheumatism
VII,109	29	M	Seronegative erosive polyarthritis, psoriasis	Psoriatic arthritis
VIII,67	29	M	Polyarthritis following diarrhoea	Reactive arthritis

Table VI.5: The results of the interview questionnaire.

	Females (N = 89)			Males (N = 92)		
	yes	no	not answered	yes	no	not answered
1. Relatives with arthritis	51 (57%)	30 (34%)	8 (9%)	48 (52%)	37 (40%)	7 (8%)
2. Arthritis in childhood	9 (10%)	77 (87%)	3 (3%)	3 (3%)	87 (95%)	2 (2%)
3. Back problems	30 (34%)	55 (62%)	4 (4%)	36 (39%)	53 (58%)	3 (3%)
4. Muscular rheumatism	40 (45%)	44 (49%)	5 (6%)	18 (20%)	71 (77%)	3 (3%)
5. Arthritis	27 (30%)	58 (65%)	4 (4%)	21 (23%)	70 (76%)	1 (1%)
6. Medical care due to arthritis	13 (15%)	73 (82%)	3 (3%)	6 (7%)	85 (92%)	1 (1%)
7. On drugs due to arthritis	13 (15%)	72 (81%)	4 (4%)	6 (7%)	85 (92%)	1 (1%)
8. Pain in $\geqslant$ 1 joint	50 (56%)	37 (42%)	2 (2%)	35 (38%)	57 (62%)	0 (0%)
9. Joint swelling	29 (33%)	58 (65%)	2 (2%)	13 (14%)	79 (86%)	0 (0%)
10. Morning stiffness	26 (29%)	60 (67%)	3 (3%)	20 (22%)	72 (78%)	0 (0%)
11. Pain in $\geqslant$ 3 joints	42 (47%)	45 (51%)	2 (2%)	20 (22%)	72 (78%)	0 (0%)

The questions were those listed in Table VI.1.

VI.3.3. Results of conventional RF and ANF estimations

Table VI.6. shows the results of conventional RF and ANF estimations on sera from 180 of the family members. Fourteen (16%) of the females and 13 (14%) of the males were Rose Waaler positive. More than 25% of the whole group gave a positive reaction in the Rheumaton screening test. Furthermore, 13 (15%) of the females were ANF positive.

Table VI.7. shows that positive latex and Rheumaton tests were significantly associated with a recent history of joint swelling (question 9 in Table VI.1.). The frequency of positive Rose Waaler was also increased in the group with a history of joint swelling. However, this trend was not statistically significant (Table VI.7.).

It can be read from Table VI.3. that 13 of the 20 (65%) individuals with classical/definite/probable RA were Rose Waaler positive.

VI.3.4. ELISA: RF and IgG anti-ruminant IgG levels in the family

Table VI.8. summarises a few features of those family members who had raised RF levels in the ELISA tests. The frequency of raised IgM RF was 12.9% (23/178) and 19 out of these 23 IgM RF positive individuals were also Rose Waaler positive. That association is highly significant (X^2 = 87.4, $p << 0.001$, chi-square test with Yates's correction).

Table VI.6: The results of conventional RF tests and ANF tests on 180 family members.

	Females (N = 88)		Males (N = 92)	
	no.	%	no.	%
Rheumaton test	24	27%	24	26%
Latex test	11	13%	16	17%
Rose Waaler (titre $\geq$ 1:10)	14	16%	13	14%
ANF (titre $\geq$ 1:10)	13	15%	2	2%

Table VI.7: The relationship between the presence of rheumatoid factor (conventional tests) and a recent history of joint swelling (question 9 in Table VI.1.).

RF test		Joint swelling		Significance
		yes	no	
Latex	pos.	12	15	$p < 0.01$, $\chi^2 = 6.78$
	neg.	29	121	
Rheumaton	pos.	17	32	$p < 0.05$, $\chi^2 = 4.63$
	neg.	23	104	
Rose Waaler	pos.	10	17	NS, $\chi^2 = 2.82$
	neg.	30	119	

As the interview and serology data were not complete, the grand total for the three 2x2 tables was only 177 (Latex), 176 (Rheumaton) and 176 (Rose Waaler), respectively.

Chi-square test with Yates's correction was used for the comparisons.

Table VI.8: Summary of family members with raised RF levels (ELISA).

	IgM RF	IgA RA	IgG RF + pepsin[2)]	IgG RF - pepsin
No. of family members tested[1)]	178	178	178	178
No. with raised levels	23 (12.9%)	7 (3.9%)	6 (3.4%)	10 (5.6%)
Max. RF levels observed	>875 IU/ml	33 U/ml	691 U/ml	397 U/ml
Mean age of those with raised RF	55 yrs	44 yrs	54 yrs	49 yrs
Raised RF and affected by disease	11	1	2	1

1) Blood samples were not available from three of the family members included in the study.

2) + pepsin and - pepsin refer to the IgG RF tests with and without pepsin digestion of samples, respectively.

The data in Table VI.8. show furthermore that those who were IgM RF positive were on average older and more affected by disease (Tables VI.3. and VI.4.) than those who had raised levels of the other RF isotypes.

The distribution of the IgG anti-ruminant IgG levels and the relation of these antibodies to rheumatic symptoms and diseases in the family members was dealt with in chapter IV.2.

Table VI.9. shows the results of a multivariate analysis where the RF levels (ELISA and ranked Rose Waaler results), the total serum immunoglobulin levels and the IgG anti-ruminant IgG levels were intercorrelated. Interestingly, total serum IgG showed a significant positive correlation, not only with total serum IgA and total serum IgM, but also with IgM RF (ELISA). Furthermore, total serum IgM showed a definite association with both IgM RF (ELISA and Rose Waaler) and IgA RF. The Rose Waaler results showed a close association with IgM RF (ELISA), and IgA RF was overall significantly associated with IgM RF (both by ELISA and Rose Waaler).

Although Table VI.9. does not reveal any overall significant correlation between IgG anti-ruminant IgG and IgG RF with/without pepsin digestion, those family members who had raised levels of either IgG RF + pepsin or IgG RF - pepsin, showed a significant tendency to have raised IgG anti-ruminant IgG as well ($p < 0.02$, see Table VI.10.). Also, a significant proportion of those with raised IgG RF - pepsin had raised IgG anti-ruminant IgG as well ($p < 0.05$, see Table VI.10.).

Table VI.9: Interrelation between immunological parameters in family members.

		Total ser. IgG	Total ser. IgA	Total ser. IgM	IgG anti-rum. IgG	Rose Waaler	IgM RF ELISA	IgA RF ELISA	IgG RF ELISA - pepsin
Total ser. IgA	r_s $p <$	0.283 0.001							
Total ser. IgM	r_s $p <$	0.277 0.001	0.109 NS						
IgG anti-ruminant IgG	r_s $p <$	0.090 NS	-0.032 NS	0.072 NS					
Rose Waaler	r_s $p <$	0.082 NS	0.072 NS	0.205 0.01	0.090 NS				
IgM RF ELISA	r_s $p <$	0.185 0.02	0.035 NS	0.335 0.001	0.072 NS	0.511 0.001			
IgA RF ELISA	r_s $p <$	0.063 NS	0.067 NS	0.195 0.02	0.085 NS	0.260 0.001	0.349 0.001		
IgG RF ELISA - pepsin	r_s $p <$	0.104 NS	-0.060 NS	0.037 NS	0.087 NS	0.065 NS	0.074 NS	0.141 NS	
IgG RF ELISA + pepsin	r_s $p <$	-0.036 NS	-0.150 0.05	-0.029 NS	0.021 NS	-0.071 NS	-0.131 NS	0.039 NS	0.398 0.001

N = 173, some data were missing for 8 family members. Spearman's rank correlation coefficients (r_s) were calculated by multivariate analysis. Ranked Rose Waaler results were used for the analysis.
+ pepsin and - pepsin: for explanation, see Table VI.8.

Table VI.10: The association between raised IgG RF and raised IgG anti-ruminant IgG in the family.

	IgG anti-ruminant IgG (CNA)		Level of Significance
	⩾ 0.500	< 0.500	
Raised IgG RF +/- pepsin	6	6	p <0.02
Normal " " " "	29	136	χ^2 = 5.51
Raised IgG RF - pepsin	5	5	p <0.05
Normal " " " "	30	137	χ^2 = 4.25

Chi-square test with Yates's correction was used for the comparisons.

VI.3.5. Age in relation to clinical and immunological parameters

Table VI.11. shows the results of a correlation analysis where the age of the family members was correlated, using Spearman's rank correlation method, with joint symptoms and immunological parameters. Both joint symptoms, expressed as the number of positive replies to questions 8-11 in Table VI.1., and IgM RF levels, expressed as either ranked latex and Rose Waaler results or IU/ml (ELISA), showed a highly significant positive correlation with age, and the same applied to the ranked ANF results and total serum IgA. However, there was no such correlation between IgA RF and age, and the IgG anti-ruminant IgG levels showed a significant negative correlation with age ($r_s = -0.150$, $p < 0.05$).

The mean age of the 39 affected family members was 50.6 ± 14.6 years, which was significantly different from the 142 healthy family members, whose mean age was 37.9 ± 18.7 years ($p < 0.001$, student's t test). The 20 individuals with classical/definite/probable RA were even older than the rest of the affected group, mean age 54.8 ± 14.2 years.

Table VI.12. presents a brief summary of those five family members who had serum IgM RF in excess of 500 IU/ml. Their high age (mean 68 years) is quite striking, also, their IgA RF was normal without exception. Furthermore, the three oldest were totally asymptomatic and none of the five had severe peripheral joint disease. This raises the question of whether the RF observed here may be of a different nature from that of the patients discussed in chapter V.3.2. and V.3.3.

Table VI.11: The association between age of family members and their clinical/immunological characteristics.

	r_s	Significance
Sum of joint symptoms	0.230	p <0.01
Latex test	0.372	p <0.001
Rose Waaler test	0.232	p <0.01
ANF	0.174	p <0.05
IgG anti-ruminant IgG	-0.150	p <0.05
Total serum IgG	0.046	NS
Total serum IgA	0.188	p <0.02
Total serum IgM	0.121	NS
ELISA IgM RF	0.221	p <0.01
ELISA IgA RF	0.040	NS
ELISA IgG RF - pepsin	0.105	NS
ELISA IgG RF + pepsin	-0.005	NS

N = 173, data were missing for 8 family members.

Spearman's rank correlation method was used to assess the association between the age and the various parameters shown.

Ranking of joint symptoms and latex, Rose Waaler and ANF as described in VI.2.4.

Table VI.12: Summary of the five family members who had IgM RF >500 IU/ml.

Pedigree number	V,32	VI,2	VI,52	VI,54	VII,18
Age (years)	90	60	64	75	51
Sex	Male	Female	Male	Female	Male
Rheumatic disease[1)]	Nil	Prob. RA	Nil	Nil	AS
Joint symptoms[2)]	Nil	++++	Nil	Nil	+++-
IgM RF (IU/ml)	>875	605	727	605	663
IgG RF + pepsin (U/ml)	2.2	7.5	3.2	3.0	1.9
IgG RF - pepsin (U/ml)	57	<1.0	3.0	3.2	2.5
IgA RF (U/ml)	1.3	5.7	1.4	2.7	7.5

1) Prob. RA = probable rheumatoid arthritis;
AS = ankylosing spondylitis.

2) "Joint symptoms" refer to responses to questions 8-11 in Table VI.1., e.g., family member VI,2 answered "yes" to all four, whereas VII,18 answered questions 8-10 by "yes" and question 11 by "no".

VI.3.6. The inbred group: characteristics and comparison with other family members

The family pedigree (Figure VI.3.) illustrates 12 consanguineous marriages in the Adalvik family. A total of 65 first generation descendants of these marriages (i.e. the inbred group) were known to be alive in 1980, all above 15 years of age. Of these, 47 (72%) were traced and blood samples obtained from a total of 46 (71%).

There were 24 females and 23 males in the inbred group of 47, mean age 49.5 ± 13.7 years. Of these, 4 had probable RA, 5 classical/definite RA, 2 probable RA/suspected SLE, 2 definite SLE, 3 suspected SLE, 1 definite AS and 1 possible AS. Consequently, 38% (18/47) of those contacted suffered from one rheumatic disease or another, the 11 RA cases giving a minimal RA prevalence of 17% (11/65) in the inbred group as a whole.

Eleven of the 46 (24%) were Rose Waaler positive, a minimal prevalence of 17% in the inbred group as a whole.

In order to see whether the inbred group differed clinically or immunologically from the rest of the family, a group of "non-inbred" family members was selected, age and sex matched with the inbred group of 46 for whom blood samples were available. This group was selected at random from the 134 "non-inbred" ones, i.e., without regard to their clinical/immunological features.

Thirteen of the 46 controls (28%) were affected by disease. Of these, 2 had probable and 3 definite RA, 5 suspected SLE, 1 Reiter's disease, 1 reactive arthritis and 1 psoriatic arthritis.

Table VI.13. shows the results of a comparison between the inbred group and the controls. The groups were almost identical with respect to age. Subjective joint symptoms were slightly more frequent in the inbred group. There was an overall tendency for the inbred group to have higher RF levels (of all three isotypes), although the difference was only significant for the ranked results of the Rheumaton test ($p < 0.05$, Mann Whitney test). Furthermore, the inbred group had significantly higher serum levels of IgG and IgM ($p < 0.05$, Mann Whitney test).

Table VI.14. shows the results of MHC haplotype determinations on the inbred group and its control group. While these results are incomplete as only 74 haplotypes were determined with certainty in the 46 inbred individuals and 65 haplotypes in the 46 controls (several individuals were not tissue typed and other typings were only partially complete), there was a striking excess of the five most common "family haplotypes" in the inbred group as compared with the controls. Table VI.14. shows that the five "family haplotypes" made up a total of 81% (60/74) and 52% (34/65) of the total in the inbred and control groups, respectively. This difference was statistically significant ($X^2 = 11.81$, $p < 0.001$, chi-square test with Yates's correction).

VI.3.7. Relationship between clinical/immunological features and genetic markers

Five of six family members with AS/Reiter's disease/reactive arthritis were tissue typed. All were HLA-B27 positive. However,

Table VI.13: Comparison between clinical/immunological features of the inbred group and its internal control group (age and sex matched).

	Inbred group (N = 46) (mean ± SD)	Control group (N = 46) (mean ± SD)	Significance of difference
Age	49.5 ± 13.9 years	49.5 ± 14.2 years	NS
Sum of joint symptoms	2.78 ± 1.67	2.55 ± 1.63	NS
Latex test	1.78 ± 1.25	1.50 ± 1.01	NS
Rheumaton test	2.15 ± 1.23	1.67 ± 1.01	p <0.05
IgG anti-ruminant IgG	0.268 ± 0.339	0.259 ± 0.326	NS
Total serum IgG	1019 ± 195 mg/dl	938 ± 141 mg/dl	p <0.05
Total serum IgA	199 ± 63 mg/dl	214 ± 67 mg/dl	NS
Total serum IgM	145 ± 67 mg/dl	116 ± 60 mg/dl	p <0.05
ELISA IgM RF	51.4 ± 144 IU/ml	41.0 ± 139 IU/ml	NS
ELISA IgA RF	4.1 ± 6.7 U/ml	2.1 ± 1.2 U/ml	NS
ELISA IgG RF - pepsin	18.0 ± 66.1 U/ml	7.6 ± 14.2 U/ml	NS
ELISA IgG RF + pepsin	29.4 ± 109.9 U/ml	6.9 ± 12.6 U/ml	NS

The Mann Whitney rank sum test was used to compare the two groups. Ranking of joint symptoms and latex and Rheumaton tests: see VI.2.4. The IgG anti-ruminant IgG levels are expressed as CNA at ser dilutions 1/10 plus 1/100.

Table VI.14: The distribution of the five "family haplotypes" in the inbred group and its control group, compared with the family as a whole.

Haplotype (A; B; Bf)	Inbred group (N = 46)	Control group (N = 46)	Family as a whole (N = 117)
9; 27; F	12/74 (16%)	11/65 (17%)	16.7%
2; 27; S	19/74 (26%)	4/65 (6%)	13.7%
2; 12; S	13/74 (18%)	6/65 (9%)	12.0%
1; 8; S	8/74 (11%)	7/65 (11%)	11.5%
28; 35; Cw4; F	8/74 (11%)	6/65 (9%)	9.4%
Other haplotypes	14/74 (19%)	31/65 (48%)	36.7%
Not determined	18	27	-

only 27 of those 37 affected individuals who were HLA-A, B-typed possessed the B27 antigen. This did not differ significantly from the B27 frequency in the family (69/117).

All the 10 family members with classical/definite RA were typed for HLA-A, B and C, as well as C4 and Bf polymorphism. Six of these were found to possess the haplotype HLA-A2;B12;Bfs;C4A3,C4BQO which within the family carries the DR4 antigen. DR-typing of three of these six RA patients confirmed the presence of DR4. By contrast only one of the 10 who were classified as probable RA possessed this haplotype.

Table VI.15. shows the results of C4 phenotyping in relation to clinical features, rheumatoid factors and IgG anti-ruminant IgG. Briefly, while this typing was not complete, as only 81 family members were fully typed, the results did not show any significant association between silent C4 alleles and either RA, all rheumatic diseases, raised RF levels or raised levels of IgG anti-ruminant IgG in the family.

Those family members (N = 26) who carried the haplotype A2;B12;BfS (and so presumably DR4) were compared with an age and sex matched group of family members lacking this haplotype. The results are shown in Table VI.16. where Wilcoxon's signed ranks test was used to compare the groups. Briefly, there was a non-significant trend for the 2;12;S group to have higher levels of IgM RF and IgA RF than the controls. However, the control group had significantly higher levels of ANF ($p = 028$).

When a similar analysis was performed for the other four main

Table VI.15: The distribution of silent C4B alleles in the family in relation to disease and serological abnormalities.

	No. not tested	No. with no silent allele	No. with 1 silent allele	No. with 2 silent alleles	Significance of difference from total
Total group	100	30	40	11	-
Affected by disease	12	10	15	2	NS
Classical/def./prob. RA	3	5	10	2	NS
Positive Rheumaton	20	10	16	3	NS
Positive ANF	5	4	6	0	NS
Raised ELISA IgM RF	9	3	10	1	NS
Raised ELISA IgA RF	3	2	2	0	NS
Raised ELISA IgG RF - pepsin	7	0	3	0	NS
Raised ELISA IgG RF + pepsin	2	2	2	0	NS
Raised IgG anti-rum. IgG	24	5	2	4	NS

The significance of the difference between the total group and the subgroups was assessed by the chi-square test.

Raised IgG anti-ruminant IgG: CNA at serum dilutions 1/10 plus 1/100 >0.500.

Table VI.16: Comparison between family members possessing the haplotype A2; B12; BfS and an age and sex matched control group.

	A2; B12; BfS (N = 26) (mean ± SD)	Control (N = 26) (mean ± SD)	Significance of difference
Age	48.0 ± 17.9 years	48.0 ± 17.9 years	NS
Sum of joint symptoms	3.00 ± 1.67	2.50 ± 1.68	NS
Latex test	1.85 ± 1.32	1.35 ± 0.89	NS
Rheumaton	1.96 ± 1.22	1.69 ± 1.09	NS
ANF	1.27 ± 0.83	2.12 ± 1.84	p = 0.028
Total serum IgG	963 ± 151 mg/dl	970 ± 180 mg/dl	NS
Total Serum IgA	194 ± 84 mg/dl	204 ± 67 mg/dl	NS
Total Serum IgM	106 ± 60 mg/dl	129 ± 77 mg/dl	NS
ELISA IgM RF	74.1 ± 217 IU/ml	11.4 ± 25.7 IU/ml	NS
ELISA IgA RF	2.7 ± 6.2 U/ml	2.4 ± 1.8 U/ml	NS
ELISA IgG RF - pepsin	18.5 ± 44.6 U/ml	31.9 ± 94.6 U/ml	NS
ELISA IgG RF + pepsin	18.2 ± 58.3 U/ml	29.8 ± 135 U/ml	NS

Wilcoxon's signed ranks test was used to compare the two groups.
Ranking of joint symptoms, latex, Rheumaton and ANF: see VI.2.4.

family haplotypes (see chapter VI.1.2.) no significant association was found between these and levels of RF or IgG anti-ruminant IgG.

When looking at the MHC haplotype distribution of those family members who had raised levels of IgM RF in the ELISA test (Table VI.17.), a strikingly similar distribution to that of the total group was observed. By chi-square test, the two groups did not differ significantly.

Nineteen of the 35 family members who had raised levels of IgG anti-ruminant IgG (CNA $\geqslant$ 0.500) were tissue typed. A total of 37 MHC haplotypes were identified in these and the distribution of the five most common is shown in Table VI.17. Clearly, this distribution closely mimics that of the total group.

The distribution of MHC haplotypes in those six family members who had raised IgA RF did not differ significantly from the distribution in the family as a whole. However, all these six were homozygous for BfS while only 92 of the 177 family members tested were so. This association between BfSS and raised IgA RF was significant ($p = 0.027$, Fisher's exact probability test).

Table VI.17: Distribution of MHC haplotypes in family members with raised IgM RF ELISA and raised IgG anti-ruminant IgG.

Haplotype (A;B;Bf)	Raised IgM RF ELISA (N = 23)	Raised IgG anti-ruminant IgG (N = 35)	Family as a whole (N = 117)
9;27;F	8/37 (22%)	7/37 (19%)	16.7%
2;27;S	5/37 (14%)	7/37 (19%)	13.7%
2;12;S	5/37 (14%)	3/37 (8%)	12.0%
1;8;S	5/37 (14%)	5/37 (14%)	11.5%
28;35;Cw4;F	3/37 (8%)	3/37 (8%)	9.4%
Other haplotypes	11/37 (30%)	12/37 (32%)	36.7%
Not determined	9	33	-

Of the 23 family members with raised IgM RF ELISA, four were not tissue typed and only one haplotype could be identified in one of the remaining 19, thus only 37 haplotypes were identified.

Of the 35 with raised IgG anti-ruminant IgG (CNA >0.500), 16 were not tissue typed and only one haplotype could be identified in one of the remaining 19 (37 haplotypes identified).

VI.4. DISCUSSION

One of the fundamental questions in relation to the Adalvik family is bound to be: Is there a convincingly raised prevalence of rheumatic diseases in these people, as compared with the normal population?

The answer is not a simple one. There is, unfortunately, no reliable information available on the actual size of the population from which the 181 members studied here were drawn. Accordingly, no accurate disease-prevalences can be calculated for this family as a whole since the selection of members for this study was not random. However, the inbred group within the family is well defined and the finding that at least 17% of its members had classical/definite/probable RA does indicate aggregation of RA, as the prevalence in adult Caucasion populations has been estimated to be 1-5% and 0.6-2.0% for females amd males respectively (Wolfe, 1968; Allander, 1970; Hellgren, 1970; Linos *et al*, 1980).

In 1976 we reported an overall prevalence of one case of definite SLE per 11.500 in the Icelandic population (Teitsson and Thorsteinsson, 1977). The occurrence of four cases (three alive, one dead) of definite SLE in the family far exceeds what would be expected by chance alone, even if it is assumed that the family members may be a total of several hundreds.

The reported population prevalence rates of AS in adult Caucasian populations vary from 0.1-0.5% (Carter *et al*, 1979). Furthermore, at least 90% of AS patients possess the HLA-B27 antigen, the frequency of which in Europeans is about 7% (Migone

et al, 1978). As 59% of the family members tested were B27 positive, it might be anticipated that the prevalence of AS in the Adalvik family was about 8 times that of the normal population. Accordingly, the finding of two cases of definite AS in the family is not unexpected. Similarly, other B27-associated diseases like Reiter's disease and reactive arthritis might be expected to be more common in the Adalvik family than in the general population.

The findings that RA and joint symptoms occurred predominantly in middle aged or elderly members of the Adalvik family is not surprising. Linos *et al* (1980) showed a definite rise in the prevalence of RA with increasing age in the population of Rochester, Minnesota. The population studies in Leigh, Wensleydale and Watford in England broadly agree with that (Wood, 1968). Also, the British population studies suggested an increase with age in the prevalence of RF as measured by the sensitised sheep cell agglutination test (Wood, 1968). Furthermore, it has been recognised for twenty years that agglutinating antiglobulins may occur in the sera of elderly asymptomatic individuals (Heimer *et al*, 1963) although the significance of these antibodies is uncertain. It was therefore not surprising to find that the serum IgM RF levels in the family showed a significant positive correlation with age.

More surprising, however, was the finding that the IgA RF levels did not show any significant association with age, although this RF isotype was on the whole associated with IgM RF. It is possible that the raised IgM RF levels in apparently healthy elderly people (Table VI.12.) reflect a fundamentally different process from that operating in RA patients where IgM RF and IgA RF are often elevated

simultaneously (chapter V). This topic will be further elaborated in chapter VII, the general discussion.

The finding that 11 of the inbred group were Rose Waaler positive indicates considerable aggregation of seropositivity as this yields a minimal prevalence of 17% in the group as a whole. In comparison, the frequency of Rose Waaler positivity (titre $\geqslant$1:10) among 4297 Icelanders (a population survey) aged 34-61 years was 2.15% and 1.06% for females and males, respectively (Allander *et al*, 1972). It should be stressed that the sera from this population survey and those of the Adalvik family were tested in the same laboratory.

The RF levels and the frequency of joint symptoms did not differ markedly between the inbred group and the internal control group. However, it must be remembered that the selection of the "non-inbred" family members was even more biased than that of the inbred group. Accordingly, it can be assumed that the control group may have had considerably more symptoms and RFs than the average Icelandic population of the same age and sex.

The results shown in Table VI.10. indicate that some of the IgG class antiglobulins found in the family members may be reactive both with goat and rabbit IgG. This need not be surprising in view of the reactivity profiles of IgM class antiglobulins (see III.1.2.3.) and the work of Pope and McDuffy (1981). However, the findings in the family that IgG RF with/without pepsin digestion is not associated with age, IgA RF or IgM RF (as measured by ELISA or Rose Waaler) may cast some doubt on its relation to rheumatic diseases in this family.

Possibly, these antiglobulins represent a response to some non-self antigens similar to the IgG anti-ruminant IgG.

The finding that six of the ten family members with classical/definite RA had the MHC haplotype A2;B12;BfS, and so in all probability DR4, agrees with the findings of others that RA, especially seropositive, is associated with the presence of the HLA-DR4 antigen (Doublog *et al*, 1980; Winchester, 1981; Khan *et al*, 1983). However, the absence of the A2;B12;BfS haplotype in nine of those who were classified as probable RA is in marked contrast to the classical/definite group. Possibly, some of these nine may have non-family haplotypes containing the DR4 antigen although DR-typing of two of the probable RA cases did not reveal its presence.

The preliminary results of the complement C4 phenotyping of the family members do not suggest any association of silent C4 alleles with rheumatic diseases or raised levels of RF or ANF in the family. The opposite might have been expected in view of the newly reported associations of silent C4 or C2 with SLE (Fielder *et al*, 1983).

Those family members who possess the haplotype A2;B12;BfS (and DR4) did not have significantly higher RF levels than an age and sex matched control group. This is in line with a recent report of Rodriguez *et al* (1983) who did not find any association between the presence of DR4 and serum RF levels in healthy individuals. These authors furthermore investigated the *in vitro* production of IgM RF after stimulation of peripheral blood lymphocytes by pokeweed mitogen and Epstein-Barr virus (EBV). Again, no association between IgM RF production and DR4 was found (Rodriguez *et al*, 1983).

The association between raised serum IgA RF and the genotype BfSS is potentially very interesting in view of the recently reported association between autoimmunity and products of the MHC class III genes (Hughes and Batchelor, 1983). Further studies on the possible association of IgA RF with genetic variants of the complement system are clearly indicated in order to clarify this issue.

In summary, evidence is presented that an inbred subgroup of the Adalvik family has higher frequency of RA and higher IgM RF levels than the general population. There is also clustering of SLE in the family. Further studies are needed in order to clarify the possible association between autoimmunity and products of the MHC and immunoglobulin genes in this family. The *in vitro* production of immunoglobulin and RFs by lymphocytes from the inbred group should be compared with that of a carefully matched control group from the normal population. Furthermore, it would be interesting to study the idiotypes of the rheumatoid factors found in the Adalvik family, as Carson *et al* (1981) have shown that the individually specific idiotypes on IgM and IgG rheumatoid factors from a single kindred may be inherited. Such a study might, for example, throw some light on the question of whether the IgG class antiglobulins found in the Adalvik family are likely to be autoreactive.

VI.5. CONCLUSION

Clinical and immunological studies on members of a highly inbred Icelandic family revealed significant clustering of RA and IgM RF in those 46 individuals whose parents both belonged to the family. One MHC haplotype which contains the DR4 antigen was associated with the presence of classical/definite RA but not with raised RF or immunoglobulin levels. Joint symptoms and IgM RF were positively associated with age of the family members but IgA RF failed to show such association despite an overall association with IgM RF. Very high IgM RF levels without a concomitant rise in IgA RF in healthy elderly family members may indicate that these RFs are of a different nature from those traditionally associated with RA.

C H A P T E R V I I

GENERAL DISCUSSION

C H A P T E R V I I

GENERAL DISCUSSION

Enzyme-linked immunosorbent assay (ELISA) has become a widespread and popular technique in many different branches of medicine over the last twelve years. The amount of published literature relating to the different applications of ELISA is now vast. As ELISA has been claimed to be likely to replace radioimmunoassay to a considerable extent, it is surprising how few workers seem to have made serious attempts to investigate and improve on the precision and reproducibility of ELISA.

Most ELISA workers use microplates that are advertised by the manufacturers as being specially designed for the technique, with respect to protein binding and optical properties. My observations, however, seem to cast doubt on the superiority of the "special" plates over the general purpose (and cheaper) ones.

It is unclear as to how the "edge effect" which has bothered many ELISA workers can be improved. As reviewed in chapter II, several factors may contribute to it, one of which is temperature difference between the edge and the central wells during the enzyme substrate incubation. This may be improved by immersing the plates in a waterbath during the temperature-critical enzyme-substrate step, as described in chapter III. Possibly, some form of pretreatment of the microplates may improve the uniformity of protein-binding to the plates, although my results do not suggest that sonication of the

plates in jewellery cleaning concentrate is of any help.

Micro-ELISA work cannot be performed efficiently without access to a microplate reader. The various available microplate readers may differ in quality, and my results suggest that it is essential to check them, probably at regular intervals, in order to ensure that the precision is satisfactory. My findings that the eight different detectors of the Titertek Multiskan may be markedly maladjusted, relative to each other, agree with the recent report of Genta and Bowdre (1982) who found that the Multiskan detectors differed highly significantly from each other ($p < 0.001$). These authors, however, pointed out that the actual difference between the detectors in their study was very small and non-significant in relation to the other sources of variability in ELISA systems.

It has been apparent since the original description by Hay *et al* (1975) that solid phase immunoassays are well suited for quantitative estimations of rheumatoid factors (RF). However, great care must be exercised if false positive reactions are to be avoided in ELISA/SPRIA tests for RFs of the various immunoglobulin classes/ subclasses. One of the most likely sources of error in IgG and IgA RF assays is the binding by IgM RF on the solid phase of Fc portions of the labelled anti-immunoglobulin (Figure I.3.B.). My work on IgA RF assays where rabbit antibody was used suggests that it is essential to use fragments ($F(ab')_2$) of the RF-detecting antibody rather than the intact immunoglobulin molecule, in order to avoid non-specific binding to IgM RF. This agrees with the recent work of

Tarkowski *et al* (1983) who used a new variant, diffusion-in-gel ELISA, for their RF assays. These workers found that enzyme-labelled normal rabbit IgG gave virtually identical results to labelled rabbit anti-human IgM in IgM RF assays and it was essential to use $F(ab')_2$ or Fab fragments of the anti-immunoglobulins for IgG RF and IgA RF assays. My experience with labelled rabbit anti-human IgG in IgG RF ELISA does indeed agree with the opinion of Tarkowski *et al*, that enzyme-labelled rabbit IgG cannot be used unmodified in assays for IgG RF.

However, the situation may be different if radiolabelled or unlabelled monoclonal antibody is used for the RF assay. The results presented in III.3.3.3. and Table VI.9. do suggest that when unlabelled monoclonal mouse antibody and pepsin-digested labelled anti-mouse IgG are used for the detection of IgG anti-globulins, the problems created by the presence of IgM RF are minimised as the results were much the same with/without pepsin digestion of samples. As discussed in III.3.4., this may be due to lower affinity of IgM RF for mouse Fc-gamma than rabbit/human Fc-gamma, together with the fact that the cross-linking agents used to conjugate enzymes to antibodies (e.g., glutaraldehyde) are known to cause aggregation of the antibody, thus yielding aggregates of Fc-gamma. The agents used to radiolabel antibodies probably cause less aggregation than, for example, glutaraldehyde. Consequently, the presence of IgM RF may cause less problems in SPRIA systems, although the work of Elkon *et al* (1981) suggests that SPRIA is not free from the problems of interference by IgM RF.

It seems very likely that the use of monoclonal antibodies,

either radiolabelled or followed by a second labelled and digested antibody, may constitute the optimal approach to the establishment of class- and subclass-specific RF assays. It is essential, though, to test carefully the reactivity - and cross-reactivity - of the monoclonal antibody used. If, for instance, a monoclonal anti-human IgG only reacts with one or a few IgG subclasses, false negative results may arise in an IgG RF assay.

The complicated sample dilution system used in the IgM and IgA RF assays in chapter III was rather time-consuming. However, Figure III.1. indicates that no one single sample dilution will yield optimal information: one low dilution fails to discriminate between high titre sera whereas one high dilution may fail to distinguish sera with low to moderate titres. It does therefore seem essential to use more than one dilution as the data presented in Table III.2. indicate. Although the amount of calculation involved is considerable, it will become easier as computer-linked microplate readers become more widely available.

It remains to be seen whether the micro-ELISA system of chapter III can be adapted for RF estimations in tissue culture supernatants. Koopman *et al* (1980) developed a sensitive SPRIA for the detection of IgM RF produced *in vitro* but the detection of *in vitro* produced IgG RF and IgA RF has proved to be a great problem. Egeland *et al* (1982), using an indirect haemolytic plaque assay, could demonstrate the secretion of IgG RF and IgA RF by mononuclear cells from RA synovial membranes and it should be possible to develop indirect ELISA for the demonstration of *in vitro* produced RFs, especially if monoclonal antibodies are applied.

Poor reproducibility was a matter of considerable concern in the IgG RF assays. Variables associated with the pepsin digestion may have contributed to this, although the reproducibility was not much better in the pepsin-free variant of the assay. This problem clearly needs to be sorted out, and a possible approach to that is to omit the incubation in acid altogether. Conceivably, the incubation in acid may have dissociated complexes containing IgG RF and IgM RF, as the pH of the buffer (approximately 4.1) is similar to that used by various workers to fractionate sera containing immune complexes (Hay *et al*, 1976b). However, this incubation alone should not destroy IgM RF (see Figure III.33.) which might be expected to reassociate with native IgG or IgG RF following neutralisation of the samples. It would be easy to sort out whether the buffer incubation gives different results to incubation in PBS alone.

The results of the experiments described in III.3.2.1. underline the importance of substrate selection in antiglobulin assays, especially for IgG antiglobulins. The presence in normal individuals of IgG antiglobulins with an apparent preferential reactivity with ruminant IgG, has not been generally acknowledged by RF workers although Palosuo and Milgrom (1981) recently reported findings similar to those in III.3.2.1. As discussed in IV.1., the presence of anti-milk antibodies in humans has been known for over seventy years although the clinical significance of this remains unknown.

Assuming that the IgG anti-ruminant IgG represents a response to gut-derived antigens(s), how do these antibodies relate to other immunological/clinical features of the individuals? Why do some

individuals mount a strong antibody response against a gut-derived antigen, whilst others fail to do so?

The gastro-intestinal immune responses and their regulation have recently been the subject of intensive research. It seems likely that the regulatory mechanisms of the mucosal system differ from those of the systemic immune system (reviewed by Strober *et al*, 1981). That such differences may exist is not unexpected, in view of the constant exposure of the mucosal surfaces to a myriad of substances with known immunostimulatory properties. Accordingly, it might be expected that the mucosal systems possess mechanisms which allow suppression of responses to antigenic challenges which, parenterally presented, would elicit a strong response. The experimental evidence for this comes, for example, from the work of Asherson *et al* (1977), who studied tolerance induced in animals by oral feeding of skin-sensitising agents. These workers found that the unresponsiveness was associated with suppressor B cells capable of suppressing the skin reactivity, and suppressor T cells capable of suppressing the DNA proliferation of primed lymph node cells. The exact mechanism of action of these suppressor populations is unclear, however, and it has been reported that the types of unresponsiveness after feeding or injection of human gammaglobulins both have the same kinetics and affect the same cell compartments (Vives *et al*, 1980).

Korenblat and his coworkers gave 17 human adults large doses of BSA by mouth and subsequently immunised seven of these intradermally with BSA. Generally, only those who had circulating anti-BSA antibodies prior to the BSA-feeding showed a significant

antibody response to the BSA. It was concluded that this probably represented an acquired hyporesponsive state to BSA in the non-responders (Korenblat *et al*, 1968).

More recently, Vives *et al* (1980) showed that mice fed with human gamma-globulin became consistently unresponsive to subsequent parenteral challenge with this antigen. Interestingly, de-aggregated human gamma-globulin was far more efficient than aggregated preparations in inducing the unresponsiveness.

On the basis of this and my observations on IgG anti-ruminant IgG, it could be argued that while most human adults are constantly exposed to bovine gamma-globulin through the drinking of cow's milk, only a small percentage fails to become unresponsive to bovine gamma-globulin. The precise abnormalities underlying this lack of unresponsiveness are, of course, unknown but this seems to warrant a further study. Possibly, those adults who show an anti-ruminant IgG response have defective immune suppressor mechanisms and so would be expected to show a generally enhanced immune response to antigenic challenge.

The findings (IV.3.5.5.) that the pre-vaccination antibody levels and the responses to immunisation with PNEUMOVAX did not show any consistent correlation with the levels of IgG anti-ruminant IgG need not mean that these antiglobulins are unrelated to immune responsiveness. The route of the antigen presentation (subcutaneous vs. oral) is different and the immunogenic properties of the polysaccharides and the immunoglobulins may be crucially different. In contrast to most soluble proteins, polysaccharides are often able to

elicit antibody responses in mice that are deficient in mature T cells but these antibody responses are generally of low magnitude, affinity and isotype diversity (Kehrl and Fauci, 1983). There is circumstantial evidence, however, that pneumococcal polysaccharides may elicit a T cell dependent antibody response in man. Nurmi *et al* showed that pre- and post-vaccination antibody levels were lower in 16 patients with trisomy-21 than in age and sex matched controls. As the trisomy-21 patients have been reported to have defects in the T cell system, these authors suggested that T cells might have a rôle in anti-polysaccharide responses in man (Nurmi *et al*, 1982). More recently, however, Kehrl and Fauci (1983) showed that although humans responded to polysaccharide immunisation by production of specific plaque-forming cells, polysaccharide binding cells and anti-polysaccharide immunoglobulin, a booster immunisation four months later failed to reproduce this. Furthermore, stimulation *in vitro* with polysaccharide failed to elicit antibody production or blastogenic (T cell) responses of lymphocytes from those vaccinated whereas pokeweed mitogen stimulation of these cells resulted in an anti-polysaccharide antibody response. Thus, these anti-polysaccharide responses differed fundmentally from those to T dependent protein antigens like KLH and tetanus toxoid.

It has been shown that the murine antibody responses to pneumococcal polysaccharides are predominantly of the IgM class and the responses to types 1, 3 and 14 at least are T lymphocyte independent (reviewed by Schiffman, 1981). The results of Kehrl and Fauci (1983) and Schiffman (1981) indeed suggest that the human antibody responses to pneumococcal polysaccharides resemble those seen in the mouse model. It might therefore be expected that the predominant human

antibody responses to pneumococcal polysaccharides are of the IgM isotype. As shown in IV.3.5.5. (Table IV.12.), the post-vaccination IgM anti-polysaccharide antibody levels in the 29 PNEUMOVAX vaccinees correlated significantly with their IgG anti-ruminant IgG levels. Consequently, although there are obvious dissimilarities between the persistent antigenic challenge provided by the (presumably) gut-derived protein ruminant IgG and that of the subcutaneously administered polysaccharides in PNEUMOVAX, it can perhaps be argued that the responses observed in the two different systems are logically related.

Six of the ten members of the Icelandic Adalvik family who suffered from classical/definite RA had the MHC haplotype A2;B12;BfS, which in the family carries DR4. This is in line with the generally high frequency of DR4 among RA patients (Svejgaard *et al*, 1980). The rôle of the DR4 antigen in the pathogenesis of RA remains unknown but the recent report of Solinger *et al* (1981) that 100% (21/21) of DR4 positive individuals exhibited cellular immunity (increased production of leukocyte inhibition factor) against collagen, whereas only 21% (8/38) of DR4 negative individuals did so, is most interesting. It should be noted, however, that 11 of the 21 DR4 positive individuals did not have any evidence of arthritis, consequently the anti-collagen reactivity is not in itself sufficient for the expression of disease.

The failure to find definite associations between genetic markers and the production of autoantibodies in members of the Icelandic family is perhaps not surprising. Reveille *et al* (1983) studied the relation of HLA haplotypes and hereditary complement

deficiencies to disease and serological abnormalities in people from eight kindreds with multiple cases of SLE. These workers failed to find any striking associations, although HLA DR2 and/or DR3 were found in five of the six subjects having anti-Ro (SSA) antibodies. Similarly, Rodriguez *et al* (1983) failed to demonstrate an association between the presence of HLA DR4 and *in vitro* production of IgM RF by lymphocytes from healthy individuals. Aho *et al* (1982) studied the occurrence of RF, ANF and thyroid autoantibodies in 76 monozygotic and 82 dizygotic same-sex twin pairs. Only eight of the twin pairs (four monozygotic and four dizygotic) were concordant for the occurrence of these autoantibodies and the authors suggested that these antibodies were in most instances determined by environmental factors.

Although autoimmune manifestations in humans are generally not considered to be inherited as a recessive trait, it is interesting that the inbred group of the Icelandic family had significantly higher levels of serum IgG, IgM and RF (as measured by the Rheumaton test) than an age and sex matched control group (Table VI.13.). This may be interpreted as evidence for the importance of recessive genes in the production of immunoglobulins and RF but for obvious reasons, opportunities to study this in human populations are rarely encountered. However, in the MRL/l mouse, which expresses multiple autoimmune abnormalities including RA-like arthritis, RF production and vasculitis, the predominant immunological abnormalities are determined by a single autosomal recessive lpr gene (Hang *et al*, 1982). My observations on the family, and the MRL/l

story thus seem to suggest a possible rôle for recessive gene(s) in the pathophysiology of RA.

This question of whether recessive heredity plays a rôle in the clinical/laboratory manifestations of autoimmunity in the Icelandic family could be further investigated by a long-term follow-up of the family members. As the inbreedings in the family practically ceased after the emigration from Adalvik, the effect of recessive genes would soon be expected to disappear. It is the aim of those who have been involved in the family study to follow the family members prospectively over the years to come in order to test this hypothesis.

A notable feature of the Adalvik family was the lack of correlation between IgA RF levels and age, opposed to IgM RF, which showed the expected positive correlation with age (Table VI.11.). Furthermore, although the IgA RF and IgM RF levels correlated closely in the prospective group ($r = 0.676$, Table V.2.) and less closely, yet significantly, in the Adalvik family ($r_s = 0.349$, Table VI.9.), those five family members who had the highest IgM RF levels all had normal serum IgA RF (Table VI.12.). The high mean age and absent/mild peripheral arthritis of the group shown in Table VI.12. immediately raises the question of whether this apparently single isotype rise in RF is of a different nature from, for example, the RF responses seen in Figure V.2., where parallel rises in all three isotypes accompanied a severe course of RA.

If it is postulated that the RF production which often accompanies RA is poly-isotypic, i.e., involving IgM RF, IgA RF and

possibly IgG RF in a considerable proportion of RA cases, cf. Table V.2. and Figure V.2., how could the single isotype (IgM) RF production observed in Table VI.12. be explained?

One possible explanation for this will be explored: Epstein-Barr virus (EBV) is known to cause selective infections of human B lymphocytes, and latent EBV may survive intracellularly throughout life. While EBV can act as a T cell independent activator of B cell antibody secretions (Fong *et al*, 1981), specific anti-EBV T lymphocytes normally hold EBV-infected B cells in close check. This T cell control may, however, decline with increasing age and during malignant disease (Britton, 1982).

Fong *et al* (1981) showed that EBV inducible IgM anti-IgG producing B cells increase in number from birth to old age. More recently, the same group showed that EBV inducible IgM RF producing cells preferentially belong to an immature B lymphocyte subset which carries receptors for mouse red blood cells (Fong *et al*, 1983).

Conceivably, therefore, the raised RF levels observed in healthy elderly individuals are related to activation and subsequent IgM RF production of EBV infected cells. Being a T cell independent response, an isotype switch would be unlikely to occur and the RF produced would be predominantly of the IgM isotype. The report by Thorsteinsson *et al* (1975) that three non-arthritic individuals with high RF levels all died within five years from cancer, might reflect "malignancy-induced" loss of B cell control by T cells and subsequent EBV induced IgM RF production.

The data presented in V.3.2. and Table V.3. and Figure V.1. suggest that the paired serum and synovial fluid levels of IgM RF and IgA RF in the group with early inflammatory joint disease were generally similar. Also, the analogous data for IgG RF did not show excess IgG RF in the synovial fluid.

There is now growing evidence for the rôle of the synovium as an active lymphoid organ in RA. The synovium may not only produce RFs but also exhibit features of delayed-type hypersensitivity. Janossy *et al* (1981) demonstrated the presence of large inter-digitating cells (ID cells) in the rheumatoid synovia. These cells, thought to be antigen-presenting cells, were morphologically similar to those found in the paracortical areas of lymph nodes. Furthermore, the synovial ID cells were surrounded by T lymphocytes which were shown to be predominantly helper/inducer cells, and the synovial ratio of helper/inducer to suppressor/cytotoxic T cells was considerably higher than the analogous ratio in the lymph nodes (Janossy *et al*, 1981). Klareskog *et al* (1982) showed that the close relationship in the RA synovia between macrophages and T helper cells resembled the delayed-type hypersensitivity picture seen in human skin after injection of PPD. More recently, however, Poulter *et al* (1983) showed that the majority of macrophages in such skin lesions stain positively for acid phosphatase and negatively for ATP-ase, but the reverse is true for RA synovial ID cells. The authors considered the extensive accumulation of ID cells and lack of suppressor T cells in RA synovia to be a clear indication of an imbalance of immunoregulatory mechanisms, and to be contributory to the progressive nature of RA (Poulter *et al*, 1983).

The observation that saliva from patients with RA (and Sjoegren's syndrome) may contain IgA RF was first reported by Heimer and Levin (1966) and later confirmed by Dunne *et al* (1979) and Elkon *et al* (1981). It has also been shown that a considerable percentage of RA patients may have serum IgA RF levels above that of controls, but apart from a recent report by March *et al* (1983), the question of whether serum levels of IgA RF may yield any information on disease prognosis or severity seems to have been left largely unexplored. My observations that those RA patients who develop raised IgA RF early in their disease tend to do badly (V.3.3.), definitely warrants further study in a larger group of patients.

It was perhaps rather surprising that only raised IgA RF and not IgM RF, showed an association with adverse outcome (Table V.7.) since raised IgM RF has long been thought to imply a bad prognosis in RA (Duthie *et al*, 1964).

Further evidence in support of the potential importance of IgA RF is the observation (V.3.4.) that fluctuations in IgA RF levels showed a significant negative correlation with the grip strength of both the RA group (N = 28) and the whole group with early inflammatory joint disease (N = 33) (see Tables V.10. and V.11.) whereas neither IgM RF nor IgG RF with/without pepsin digestion showed such an association.

Any explanations for the association between IgA RF and disease severity and activity in RA are bound to be somewhat speculative. IgA responses in man and higher vertebrates are

thought to be highly thymic dependent (reviewed by Kiyono *et al*, 1982). Recently, Kawanishi *et al* (1983) demonstrated the existence of T cells in Peyer's patches of the murine gut which could cause direct switching of B cells expressing IgM on their surface to B cells expressing surface IgA. This isotype switch, which did not involve B cells expressing IgG, could perhaps explain the simultaneous occurrence of IgM and IgA antibodies without any demonstrable IgG of the same specificity.

IgA is classically associated with the secretory immune system, and at least a portion of the serum IgA may be produced in mucosal tissues (Vaerman *et al*, 1973). Recently, Koopman *et al* (1983) reported that >75% of serum IgA RF activity sedimented at a rate intermediate between IgM and monomeric IgA. As J chain was present in the IgA RF peak, these workers concluded that IgA RF is predominantly polymeric. This agrees with the work of Elkon *et al* (1982) who showed that whereas only 14-26% of total serum IgA was polymeric, 64-86% of IgA RF sedimented at a rate greater than 10S and these workers could deomonstrate the presence of J chain in four of five rheumatoid factor eluates. Taken together, this evidence seems to favour the mucosal production of IgA RF. Also, the results of Van Snick (1981) favour the mucosal origin of autoreactive IgA antiglobulins as he provided evidence that IgM and IgA anti-IgG2a antiglobulins were predominantly produced in mesenteric and caudal lymph nodes of young 129/Sv mice. However, this leaves open the question of whether these mucosa-associated antiglobulins are produced as a response to a local (gut-derived) antigen.

It is uncertain why serum IgG RF often appears normal despite raised levels of IgM RF and IgA RF. Possible explanations would include:

(a) rapid clearance of IgG RF as self-associated complement-fixing complexes;

(b) limited production of IgG RF due to direct IgM to IgA isotype switch; and

(c) technical problems with the IgG RF assay.

More interesting, however, is the question as to why IgA RF seems to be far superior to IgM RF as an indicator of disease severity and activity in RA. Possibly, this reflects a systemic derangement of T cell function in RA. Isolated IgM RF production may occur in apparently healthy individuals, perhaps because of T-independent B cell activation by EBV. However, the production of IgA RF and active collaboration between ID cells and T helper cells in the RA synovium (Janossy *et al*, 1981) may imply a generalised T helper cell overactivity or a deranged balance between suppressor and helper T cells, leading to dominance of the latter. Such an immuno-regulatory imbalance might lead to immune complex-mediated and cell-mediated tissue destruction of the kind characteristically seen in rheumatoid arthritis.

A B B R E V I A T I O N S

abs.	absorbance
ANA	anti-nuclear antibodies
ANF	anti-nuclear factor
anti-h.	anti-human
AP	alkaline phosphatase
ARA	American Rheumatism Association
AS	ankylosing spondylitis
ATP-ase	adenosine triphosphatase
BSA	bovine serum albumin
CNA	combined net absorbance
Con A	concanavalin A
CV	coefficient of variation
DTT	dithiothreitol
EBV	Epstein-Barr virus
ELISA	enzyme-linked immunosorbent assay
ESR	erythrocyte sedimentation rate
$F(ab')_2$-A-AP	The $F(ab')_2$ fraction of a rabbit anti-human IgA antiserum coupled to alkaline phosphatase
$F(ab')_2$-m-G-AP	The $F(ab')_2$ fraction of an affinity purified goat anti-mouse IgG antibody coupled to alkaline phosphatase
gp70	a glycoprotein of viral origin with a molecular weight of 70,000 daltons
GS	grip strength
HLA	human leukocyte antigen
ICs	immune complexes
IDA	index of disease activity
ID cells	interdigitating cells

IEP	immunoelectrophoresis
Ig	immunoglobulin
IgA-DP	IgA-deficient plasma
IgG-A-AP	the undigested gammaglobulin fraction of a rabbit anti-human IgA antiserum coupled to alkaline phosphatase
%Inc	percent increase in combined net absorbance after immunization
IU	international units
JCC	jewellery cleaning concentrate
KLH	keyhole limpet haemocyanin
MDAG	mean disease activity grade
2-ME	2-mercaptoethanol
MHC	major histocompatibility complex
MR 580	a microplate reader: MR 580 Microelisa autoreader
MS	morning stiffness
N	number of subjects in a sample
NaN_3	sodium azide
NHS	normal human serum
NS	not significant
NZB	New Zealand Black mouse
NZW	New Zealand White mouse
OD	optical density
p	statistical probability
PBL	peripheral blood lymphocytes
PBS	phosphate buffered saline (pH 7.4)
PBS-Tween	phosphate buffered saline (pH 7.4) containing 0.05% Tween 20

PEG	polyethylene glycol
pNPP	para-nitrophenyl phosphate
PPD	purified protein derivative (tuberculin)
PVAS	pain visual analogue scale
r	the Pearson's product-moment correlation coefficient
RA	rheumatoid arthritis
RANA	rheumatoid arthritis-associated nuclear antigen
RF	rheumatoid factor
R-G-AP	undigested gammaglobulin fraction of a rabbit anti-human IgG antiserum coupled to alkaline phosphatase
r_s	the Spearman's rank correlation coefficient
RSRID	reverse single radial immunodiffusion
r.t.	room temperature
S	S value or sedimentation coefficient
SCAT	sheep cell agglutination test
SD	standard deviation
SDS	sodium dodecyl sulphate
SLE	systemic lupus erythematosus
SPRIA	solid phase radioimmunoassay
SS	Sjoegren's syndrome
syn. fl.	synovial fluid
TCG	tissue culture grade
TRIS	tris (hydroxymethyl) methylamine
vs.	versus

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R E F E R E N C E S

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