M.Sc. Faculty of Graduate Studies and Research

SPECIES SPECIFICITY OF HUMAN IgM RHEUMATOID FACTOR

by

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ABSTRACT

Nephelometry has been used to study the reaction of rheumatoid factor (RF) with IgG. This nephelometric assay, validated by comparisons with standard agglutination assays, showed that RF reacted with human or rabbit but not with chicken aggregated IgG. IgM-globulin was responsible for nephelometric RF activity. In over 250 sera, there were no differences in reactivity between human and rabbit aggregated IgG. IgM-RF, isolated from sera, also reacted with human and rabbit native IgG, failing to show species specificity. A monoclonal IgM-RF showed that this methodology was capable of detecting such specificities. Further evidence for RF-native IgG reaction was the complete absorption of RF by native IgG and that RF-native IgG complexes fixed complement (C1q). These results show that RF does not exhibit species specificity for human or rabbit IgG; that RF reacts with native IgG, as well as with aggregated IgG; and that RF-native IgG complexes can bind C1q.

M. Sc. Microbiology & Immunology

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SPECIFICITE D'ESPECES DU FACTEUR RHUMATOIDE-IgM HUMAIN

RESUME

On a utilisé le néphelomètre pour étudier la réaction du facteur rhumatoide (FR) avec des IgG. L'épreuve néphelometrique, validée par des comparaisons avec des épreuves d'agglutinations standardisées, a démontré que le FR réagit avec des IgG agrégées de l'homme et du lapin mais non avec ceux du poulet. L'immunoglobuline responsable de cette activité du FR est de la classe IgM. Dans plus de 250 serums, on n'a noté aucune différence entre la réactivité des IgG agrégées de l'homme et ceux du lapin. Le FR, isolé des serums, a réagi aussi avec des IgG natives de l'homme et du lapin, sana montrer de spécificité quant a l'espèce. Un FR monoclonal a démontré que cette méthodologie peut déceler de telles spécificités. Le fait que l'absorption des FR par des IgG natives soit complète et que les complexes de FR-IgG natives fixent le complément (Clq), témoignent en plus d'une réaction entre le FR et les IgG natives. Ces résultats démontrent donc que le FR n'est pas spécifique de l'espèce, pour l'homme ou pour le lapin, et que le FR réagit avec les IgG natives, ainsi les IgG agrégées, et enfin, que les complexes FR-IgG natives peuvent fixer le Clq.

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ABBREVIATIONS AND TERMINOLOGY

ABBREVIATIONS

Abbreviations commonly used in this thesis include:

C1q	Complement Component C1q
C3	Complement Component C3
EACA	ε-Amino-n-Caproic Acid
F_{ab}	F_{ab} Fragment of an Immunoglobulin Molecule
F_c	F_c Fragment of an Immunoglobulin Molecule
F II	Cohn Fraction II Gamma Globulin (IgG)
gARGG	Glutaraldehyde-Aggregated Rabbit Gamma Globulin (IgG)
hAHGG	Heat-Aggregated Human Gamma Globulin (IgG)
hARGG	Heat-Aggregated Rabbit Gamma Globulin (IgG)
HGG	Human Gamma Globulin (IgG)
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
LFT	Latex Fixation Test
NeRF	Nephelometric Rheumatoid Factor Assay
Net LS	Net Light Scatter
NHS	Normal Human Serum

ABBREVIATIONS (cont.)

NHGG	Human Native Gamma Globulin (IgG)
NRGG	Rabbit Native Gamma Globulin (IgG)
PEG	Polyethylene Glycol
RA	Rheumatoid Arthritis
RF	Rheumatoid Factor (see Terminology)
IgA-RF	IgA Class Rheumatoid Factor
IgG-RF	IgG Class Rheumatoid Factor
IgM-RF	IgM Class Rheumatoid Factor
mRF	Monoclonal Rheumatoid Factor
mRF_{mi}	Monoclonal Rheumatoid Factor Isolated from a Patient (MI) (IgM)
mRF_{og}	Monoclonal Rheumatoid Factor Isolated from a Patient (OG) (IgM)
pRF	Polyclonal Rheumatoid Factor
RGG	Rabbit Gamma Globulin (IgG)
sARGG	Spontaneously-Aggregated Rabbit Gamma Globulin (IgG)
SCAT	Sheep Cell Agglutination Test
SRBC	Sheep Red Blood Cells

TERMINOLOGY

RF	RF (rheumatoid factor) is a general term, used here, to denote human antibodies with antiglobulin activity, reactive with IgG.
RF Activity	RF activity denotes the capacity of RF to react with IgG. The use of this term is restricted to RF of the IgM class of immunoglobulins, unless specified otherwise.

TERMINOLOGY (cont.)

NeRF Activity	NeRF Activity denotes RF activity measured nephelometrically.
RF-Serum	RF-serum is a term designating serum which contains RF activity, or to which RF has been added.

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CHAPTER I

INTRODUCTION

Rheumatoid factor (RF) is a class of serum proteins, which are among the most common autoantibodies in man. RF is usually defined as an antibody which reacts with antigenic determinants on the F_c portion of human immunoglobulin G. Rheumatoid factors belonging to each immunoglobulin class have been described, and of these IgM-RF is the most extensively characterized. In addition to its reaction with human IgG, RF can react with IgG of other species, such as rabbit IgG. The reactions of IgM-RF with human and with rabbit IgG appear similar, but direct comparisons of these reactions have been limited since different methods have been employed to study the reaction of RF with each of these antigens. Most methods used to study the reaction of RF with IgG have used non-native IgG (i.e. aggregated, complexed or denatured IgG). It is generally assumed that RF preferentially reacts with such antigens rather than with native IgG. This has been due in part to the paucity of methods which can directly demonstrate the reaction of RF with native IgG.

The availability of nephelometry to quantitate antigen-antibody reactions in fluid-phase, avoiding possible antigen denaturation, has provided a new method to analyze the RF-IgG reaction. As a result, the reaction of human RF with IgG has been examined in detail in a new nephelometric rheumatoid factor assay (NeRF). This has necessitated:

1. The development of new methods for the aggregation of IgG, providing aggregated IgG useable in the nephelometer;
2. Establishing the validity of this nephelometric rheumatoid factor assay by standardizing assay conditions and by comparisons with accepted RF assays (i.e. the sheep cell agglutination test and the latex fixation test);
3. Identifying the immunoglobulin class responsible for the NeRF reaction;
4. Assaying a wide sample of sera containing RF to determine their reactivity with human, with rabbit, and with chicken aggregated IgG antigens;
5. Analyzing the effects of reacting native IgG with rheumatoid factors isolated from sera, directly by reacting native IgG with RF and indirectly by absorbing activity with native IgG;

6. Analyzing the kinetics of the reaction of RF with aggregated and with native IgG from humans and from rabbits by measuring the rates of the RF-IgG reaction; and
7. Demonstrating biological activity in RF-IgG complexes by investigating the reaction of C1q with these complexes.

CHAPTER II

HISTORICAL REVIEW

1 SEROLOGICAL MANIFESTATIONS OF RHEUMATOID FACTOR : HISTORICAL

In 1912, Dean described a thermostable substance present in the globulin fraction of normal guinea-pig serum which caused the agglutination of sheep erythrocytes sensitized with a sub-agglutinating concentration of rabbit anti-SRBC antisera (Dean, 1912). Meyer, during routine testing of over 100,000 sera for the Wasserman reaction, noted 10 sera which contained a factor which augmented the agglutination of the sensitized sheep red blood cells used in the test (Meyer, 1922). This substance has been regarded as what we would now designate rheumatoid factor or RF (Bartfeld, 1969a).

1.1 AGGLUTINATION REACTIONS WITH RABBIT GAMMA GLOBULIN

Waaler, in 1940, found a serum from a patient with rheumatoid arthritis which was capable of inhibiting the hemolysis of sheep erythrocytes in a complement fixation

test and furthermore caused agglutination of the same cells sensitized with rabbit hemolysin (Waaler, 1940). This substance was termed 'agglutination activating factor' and was reported to be present to a significant degree in 27 of 77 rheumatoid arthritis patients whereas only 10 of 202 control sera (non-RA) expressed this agglutinating activity. The factor was thermostable (56°C. for 30 minutes), was globulin in nature and could not be adsorped from the serum with unsensitized sheep cells. This phenomenon of agglutination enhancement was rediscovered by Rose and co-workers in 1948 who noted that the factor was found almost exclusively in the sera of patients with active rheumatoid arthritis (Rose et al., 1948). Subsequently, the Waaler-Rose test in various modifications including the widely used sheep cell agglutination test or SCAT (Ball, 1950) were used routinely for the detection of agglutinating factor in sera.

1.2 AGGLUTINATION REACTIONS WITH HUMAN GAMMA GLOBULIN

In addition to the use of sheep cells sensitized with rabbit anti-SRBC antibody, other systems employing human gamma globulin (Cohn fraction II) as reactant were devised. The first of these tests to employ human IgG was the FII tanned sheep cell agglutination test (Heller et al., 1954). In this method, sheep cells previously treated

with tannic acid were coated with Cohn fraction II and the cells were agglutinated by RA sera. The authors devised this test after observing the inhibition of sheep cell agglutinating activity by the addition of human gamma globulin. Waller and Vaughn used human Rh positive red blood cells sensitized with human anti-Rh serum as markers for the agglutination factor (Waller and Vaughn, 1956). Latex-polystyrene particles coated with human gamma globulin were agglutinated in the now widely used latex fixation test of Singer and Plotz (Singer and Plotz, 1956; Plotz and Singer, 1956). Bentonite silicate particles served as inert carriers of human gamma globulin in the bentonite flocculation test (Bozicevich et al., 1958).

The term 'rheumatoid factor' was first used to describe the circulating factor responsible for the agglutination reactions obtained in the sensitized or coated cell tests by Epstein and associates (Epstein et al., 1956). The authors devised a cell or particle free system in which Cohn fraction II was added directly to a heat-decomplemented serum sample and the precipitation reaction quantitated visually after 48 hours of incubation. The rheumatoid factor activity responsible for the observed precipitation reaction was localized in the euglobulin fraction of the serum.

Using these agglutination tests, researchers investigated other diseases in which rheumatoid factor was

produced, the nature of the factor itself and the nature of the "reactant" in the observed agglutination reactions.

2 RHEUMATOID FACTOR IN HUMAN DISEASE

With the development of more sensitive and reproducible agglutination assays in the early 1960's, attention was focused on the distribution of RF activity with regard to disease states. Rheumatoid factor activity had been primarily associated with rheumatoid arthritis since the pioneer work of Waller and of Rose and associates in the 1940's. Low levels of RF activity were none-the-less found in normal individuals (Aho, 1961; Waller et al., 1964), in older persons (De Blécourt et al., 1965) and even in psychiatric populations (Oreskes et al, 1968).

High levels of rheumatoid factor activity are not confined to rheumatoid arthritis but have also been demonstrated in other chronic conditions such as: subacute bacterial endocarditis (Williams and Kunkel, 1962); syphilis (Peltier and Christian, 1959); infectious mononucleosus (Bradford, 1965); leprosy (Cathcart et al., 1961); liver diseases (Bonomo et al., 1963); lung diseases (Bonomo et al., 1966); sarcoidosis (Kunkel et al., 1958) and in other conditions too numerous to mention (reviewed by Bartfeld, 1969b; Waller, 1969).

It is apparent from this evidence that rheumatoid factor activity is not confined to the serum of rheumatoid arthritis patients, however, interpretation of these observations has been made difficult by the variety of assay systems employed and by the conflicting results thereby obtained. Although low RF activity is not disease specific and can be seen during an acute immune response to a number of infectious agents and with advanced age, there is little doubt that the highest titers of RF activity are associated with the disease rheumatiod arthritis (Finley et al., 1979).

3 RHEUMATOID FACTOR IN RHEUMATOID ARTHRITIS

3.1 RHEUMATOID ARTHRITIS

3.1.1 ETIOLOGY

Rheumatoid arthritis (RA) is a systemic disease which is characterized by articular inflammation and destruction with accompanying muscular pain and stiffness. The precise etiology of RA is unknown, however, there is evidence which would suggest that environmental factors, such as bacteria, mycoplasmas and viruses (Person and Sharp, 1976), as well as a genetic predisposition (Stastny, 1978) may play a role in the initiation of this disease.

3.1.2 IMMUNE ABBERATIONS

Evidence for altered immune functions include hypergammaglobulinemia, decreased in vivo and in vitro T-lymphocyte reactivity and possibly a functional defect in the T-cell population (Waxman et al., 1973). Lymphocytes from patients with RA stimulated and responded to other rheumatoid lymphocytes in a mixed lymphocyte culture reaction less well than lymphocytes from normal subjects (Astorga and Williams, 1969). McDuffie and Bunch (1977b) pointed out that this observation might have been due to the sharing of the HLA-DR4 alloantigens by RA patients as first described by Stastny (1976). Stastny (1978) found that the B-cell alloantigen HLA-DRw4 occurred in 70% of RA patients while a normal control group showed only a 28% incidence of this genetic marker.

3.2 RHEUMATOID FACTOR IN RHEUMATOID ARTHRITIS

The most characteristic feature for RA is the production of autoantibodies to IgG. These antiglobulins are collectively called rheumatoid factors, are found in all classes of immunoglobulins and will be discussed in detail in subsequent sections.

3.2.1 CIRCULATING IMMUNE COMPLEXES

Franklin and co-workers (1957) first described evidence for a circulating 22S complex composed of IgM rheumatoid factor and IgG from fractionation studies of RA sera by ultracentrifugation. In addition, numerous researchers have since demonstrated the presence of immune complexes in RA sera (Kunkel et al., 1961; Kunkel, 1963; Heimer and Levin, 1966; Winchester et al., 1970; Luthra et al., 1975; Zubler et al., 1976; Gabriel and Agnello, 1977). Furthermore, it has been shown that these complexes are capable of binding C1q (Winchester et al., 1971; Zubler et al., 1976; Gabriel and Agnello, 1977) and C3 as evidenced by binding in the Raji cell test (Lambert et al., 1978).

In addition to these large 22S complexes, smaller, 10-17S, 'intermediate complexes' as first described by Chodirker and Tomasi (1963) have been found to be made up of two molecules of 'self-associating' IgG class RF (Pope et al., 1974; Hay et al., 1976; Pope and McDuffy, 1979). These intermediate complexes have been found to be capable of binding IgM-RF (Winchester et al., 1971; Luthra et al., 1975; Gabriel and Agnello, 1977).

3.2.2 PATHOPHYSIOLOGICAL CORRELATIONS

3.2.2.1 CLINICAL SIGNIFICANCE OF RHEUMATOID FACTOR

The clinical significance of RF in the context of RA is indicated by the correlation of positive serological tests with more severe articular and systemic disease (Epstein and Engelman, 1959; Cats and Hazevoet, 1970; Cats and Klein, 1970) and by the poorer long-term prognosis in seropositive patients in terms of disease activity and functional capacity (Duthie et al., 1964). These correlations seem to apply to mainly the IgM-RF levels which are preferentially detected by the standard clinical test procedures, the sheep cell agglutination test and the latex fixation test. With the development of assays which allowed for the detection of IgG- and IgA- rheumatoid factor, elevated levels of these rheumatoid factors, as well as IgM-RF levels, were found to be associated with more severe disease in adult and juvenile patients with RA (Bianco et al., 1971; Panush et al., 1971).

3.2.2.2 SYNTHESIS OF RHEUMATOID FACTOR

Local synthisis of RF by synovial tissue has been shown (Smiley et al., 1968) and the joint is thought to be the major site of RF synthesis with most if not all the increased RF in serum being attributable to spillover (Hay et al., 1979). RF has also been shown to be synthesized by normal B-Cells following stimulation with a polyclonal B-Cell mitogen, pokeweed mitogen (Koopman and Schroenloher, 1980; Karsh, 1980). These data would suggest that B-Cells capable of synthesizing RF commonly occur in normal individuals but are not ordinarily activated.

3.2.2.3 COMPLEMENT ACTIVATION BY RHEUMATOID FACTOR

IgM-RF bound to aggregated IgG can activate complement via the classical pathway (Tanimoto et al., 1975) and IgG-RF, under some conditions (Bianco et al., 1974), may also be capable of complement activation. Not all the complement binding sites on aggregated IgG are blocked by RF, however, most of the complement fixing activity of the aggregate was mediated by RF rather than by IgG (Tesar and Schmid, 1970; Natvig et al., 1972). Local consumption of complement in the joint space of patients with RA has also been observed (Ruddy and Austen, 1970). There is also

significant correlation between serum levels of RF and consumption of C4 implying that RA is a systemic disease in which immune complexes containing RF play a significant role in the pathogenesis of the disease (Kaplan et al., 1980).

3.2.2.4 ROLE OF RHEUMATOID FACTOR IN THE PATHOGENISIS OF RHEUMATOID ARTHRITIS

The ability of RF to form complexes with IgG, fix complement and promote phagocytosis of IgG aggregates (Hurd et al., 1970) points to involvement of RF in the inflammatory process associated with RA. It has been postulated that the biological basis for this inflammatory response in the rheumatoid joint is activation and release of lysosomal enzymes from phagocytic cells after ingestion of RF complexes and complement (Hollander et al., 1965). Although rheumatoid factors are often thought to be deleterious, they may, however, have a positive or beneficial affect upon the host, by accelerating elimination of immune complexes through phagocytosis (Ilter and Turner, 1973; Ziola et al., 1978) or through other mechanisms such as viral neutralization (Stage and Mannik, 1972).

While it is true that high titers of RF are generally associated with the disease rheumatoid arthritis,

seropositivity per se does not determine the severity of this disease in view of the occurrence of high titers in a number of other, unrelated chronic illnesses (Bartfeld, 1969b). It has been concluded that the pathogenic mechanism in RA cannot be attributed solely to the presence of RF. Furthermore, factors such as the synthesis, alteration in the quantity and avidity or specificity of the RF produced may also be of critical importance (Hay et al., 1976). However, in view of the physicochemical properties associated with RF, there is little doubt that RF contributes significantly to the pathogenesis of rheumatoid arthritis (Kaplan et al., 1980; Koopman and Schroenloher, 1980).

4 PHYSICOCHEMICAL CHARACTERISTICS OF RHEUMATOID FACTOR

4.1 ANTIBODY NATURE OF RHEUMATOID FACTOR

Until 1957, RF had been described as a number of things including an accessory agglutinating factor, a conglutinin, a fraction of complement (Bartfeld, 1969a) and something between a component of the complement system and an antibody in its own right (Epstein, 1969). The notion that RF was indeed an antibody was suggested by analytical ultracentrifugation studies of sera by Franklin and coworkers (Franklin et al., 1957). The authors demonstrated

the presence of a circulating 22S complex in the sera of some rheumatoid arthritis patients. Upon disassociation of this material with urea, 19S and 7S fractions were recovered. The 19S fraction was found to be reactive in the Cohn fraction II precipitation method of Epstein (Epstein et al., 1956) and was apparently gamma globulin in nature by electrophoretic studies. Christian furthered these studies by demonstrating an equilibrium reaction seen in the classical antigen-antibody systems (Christian, 1959). The author added heat-aggregated human IgG to the isolated 22S complexes and found that after precipitation occurred, 7S gamma globulin was recovered in the supernatant. This observation was suggestive of an equilibrium reaction whereby the addition of excess antigen resulted in the release of soluble IgG after centrifugation to remove the precipitable rheumatoid factor-IgG complexes.

Fractionation of a RF containing serum by diethylaminoethylcellulose (DEAE-cellulose) chromatography resulted in two fractions, one of which contained rheumatoid factor activity (Epstein and Tan, 1962). The material which was retained in the anion-exchange column was found to be identical with euglobulin protein, had a sedimentation coefficient of 19S and was termed a gamma macroglobulin. This identification was based on earlier fractionation studies by the same authors of normal serum (Epstein and Tan, 1961).

In addition to the identification of RF as an antibody, the authors suggested the following definition for RF, "The ability of a class of γ-macroglobulins to combine with 7S globulin defines these proteins as RF".

4.2 IMMUNOGLOBULIN CLASSES OF RHEUMATOID FACTOR

Until 1963, RF was thought to be mainly a macroglobulin with a sedimentation coefficient of 19S and precipitating as a euglobulin. In todays terminology this would be a reasonable definition of an IgM class rheumatoid factor or IgM-RF. The presence of 10-17S, intermediate complexes, in the sera of patients with rheumatoid arthritis was found to contain RF activity (Chodirker and Tomasi, 1963), although they were unable to demonstrate RF activity in the 7S gamma globulin isolated from these complexes. Heimer and Nosenzo in 1965 found a number of serum from patients with RF whose pseudoglobulin fractions were able to agglutinate latex particles coated with human gamma globulin (Heimer and Nosenzo, 1965). The majority of this fraction was found to be made up of 19S IgM-RF, however, some of the pseudoglobulin fractions were found to be insensitive to 2-mercaptoethanol treatment and by electrophoresis appeared to be IgG. Further evidence for a 7S IgG-RF was found during the analysis of intermediate complexes by Schrohenloher (Schroenloher, 1966).

The development of specific antiglobulin serum allowed Torrigiani and Roitt in 1967 to demonstrate conclusively RF in the IgM, IgG and IgA classes of immunoglobulin directed against rabbit IgG (Torrigiani and Roitt, 1967). IgM and IgG rheumatoid factors were found in 100 per cent and IgA rheumatoid factor in 50 per cent of seropositive RA sera by this method based on quantitation by radial immunodiffusion. Numerous assays were developed and the levels of IgM-, IgG- and IgA-RF were measured in both seropositive and seronegative RA, normal and nonrheumatic disease sera (Torrigiani et al., 1970; Panush et al., 1971; Abraham et al., 1972; Schur et al., 1975). The IgM and IgG factors are the most common but the standard serological tests detect mainly IgM type RF which is a more 'efficient' agglutinator than IgG with the same specificity (Klein et al., 1966; Torrigiani and Roitt, 1967; Cats and Klein, 1970; Estes et al., 1973; Stage and Mannik, 1973; Carson et al., 1977). IgM was shown to be a more 'efficient' binder of an antigen with multiple antigenic sites, such as aggregated IgG or IgG bound to cells, than IgG due to the influence of the polyvalency of the pentameric IgM molecule (Crothers and Metzger, 1972; Hornick and Karush, 1972).

Following the first solid evidence for IgG-RF in 1967, a plethora of assays were developed to measure minute quantities of this class of RF (Estes et al., 1973; Abruzzo

and Heimer, 1974; Lyet and Normansell, 1974; Norberg, 1976; Pope and McDuffie, 1979). IgG-RF has been demonstrated in all normal sera to some degree as well as in most disease states. However, there are serious questions as to the clinical value (McDuffie and Bunch, 1977a) and validity (Lea and Ward, 1972) of these assays. In addition, there is the possibility of false positives due to the nature of the assay (Stage and Mannik, 1973), and to the interference in the quantitation of the IgA- and IgG-RF classes by IgM-RF (Hay et al., 1976; Carson et al., 1977; Singh and Francis, 1978).

4.3 IgM-RHEUMATOID FACTOR

Classical IgM rheumatoid factor does not seem to differ in structure from other 19S IgM molecules and has a molecular weight of 900,000 daltons. IgM-RF is a pentamer consisting of 5 subunits each with a molecular weight of 180,000 daltons (Miller and Metzger, 1965) which are linked into a stable circular arrangement by the J chain (Mestecky et al., 1971). Each of the IgM monomeric subunits consists of two light and two heavy chains (Miller and Metzger, 1966) and the light chains are predominantly of the kappa subclass, 90% as compared to approximately 60-70% kappa light chains in pooled normal human IgM (Carson and Lawrence, 1978).

4.4 VALENCY OF IgM-RHEUMATOID FACTOR

With a purified preparation of IgM-RF which reacted with both rabbit and human IgG, it was shown that each of the subunits of the RF was able to bind either rabbit or human IgG and that there were no subunits which failed to react with either antigen (Chavin and Franklin, 1969). As each subunit of IgM-RF contains two potential binding sites for human IgG (Miller and Metzger, 1966; Stone and Metzger, 1968), it would be expected that the valency of the pentameric molecule would be 10. It was shown that each of the F_{ab} fragments was able to combine with IgG (Stone and Metzger, 1968), however, the same authors noted that the monomeric subunit of the IgM-RF was univalent and not bivalent for IgG as expected. Similarly, the valency of IgM-RF was determined to be 5 by ultracentrifugation studies (Chavin and Franklin, 1969) and by binding experiments with monomeric IgM-RF subunits (Normansell, 1970). The notion that one of the two binding sites on the monomeric subunit may be blocked by steric hindrance following the binding of an IgG molecule was strenghthened by the work of Edberg and co-workers on the valency of Rabbit IgM anti-dextran antibody as a function of the size of the dextran molecule (Edberg et al., 1972). The authors calculated that the valency of IgM was 5 for dextran molecules of molecular weights of

7,100 to 237,000 daltons. IgG with a molecular weight of approximately 160,000 daltons would be expected to give an apparent valency for IgM-RF of 5 based on these results.

4.5 BINDING CONSTANTS

The association constants for the binding of IgM-rheumatoid factor and IgG have been determined by numerous researchers. The values obtained for both polyclonal (p-IgM-RF) and monoclonal (m-IgM-RF) rheumatoid factors with a variety of gamma globulin antigens are summarized in Table 2.1. Although the exact figures given for the binding constants for the reaction between IgM rheumatoid factor and IgG are not all the same, the range of 10^4 - 10^5 1/M is quite low when compared to the affinities, 10^6 - 10^8 1/M, of corresponding anti-IgG antibodies produced by hyperimmunizing animals (Steward et al., 1973; Wager and Teppo, 1978) and in other well defined IgM anti-hapten systems (Williams, 1979). The binding constants for the reaction between IgG-RF and IgG were also found to be of the same magnitude as those obtained for the IgM rheumatoid factors (Lyet and Normansell, 1974). The low association constants would account for the ready dissociation of RF-IgG complexes in acid buffers, pH 3.5 - 4.0 (Allen and Kunkel, 1966; Normansell and Stanworth, 1966; Hay et al., 1976). Low affinity

auto-reactive antibodies are commonly found in autoimmune diseases (Eisenberg, 1976; Winfield et al., 1977; Wager and, Teppo, 1978). It has been established that low affinity

TABLE 2.1

BINDING CONSTANTS FOR THE REACTIONS OF POLYCLONAL AND MONOCLONAL IgM-RF WITH VARIOUS GAMMA GLOBULIN ANTIGEN PREPARATIONS

RF Tested	Antigen*	Binding Constant (liters/Mole)	Reference
p-IgM-RF	7S HGG	3-5 X 10^5	Normansell, 1970
"	"	4 X 10^5	Normansell, 1971
"	"	1-3 X 10^5	Normansell and Young, 1975
"	"	1 X 10^4	Dissanayake et al., 1977
"	"	1-5 X 10^5	Wager and Teppo, 1978
"	hAHGG	3-5 X 10^5	Normansell, 1970
"	"	5 X 10^5	Normansell, 1971
"	"	0.9 X 10^5	Dissanayake et al., 1977
"	F_c-HGG	4-7 X 10^4	Steward et al., 1973
"	"	2 X 10^5	Lyet and Normansell, 1974
"	"	6 X 10^3	Dissanayake et al., 1977
"	7S RGG	8 X 10^5	Normansell, 1971
"	"	1-9 X 10^4	Dissanayake et al., 1977
m-IgM-RF	7S HGG	5 X 10^4	Stone, 1973
"	"	1-9 X 10^3	Wager and Teppo

* Abbreviations used for the antigens are: 7S HGG - native human IgG; hAHGG - heat-aggregated human IgG; F_c-HGG - F_c fragment of human IgG; 7S RGG - native rabbit IgG

antibody achieves little or no immune elimination of antigen and therefore there is also little elimination of antibody (Steward et al., 1973). High affinity antibodies would be expected to be rapidly cleared from the circulation after combination with antigen and the presence of high titers of circulating rheumatoid factors may be indicative of a low affinity antibody (Wager and Teppo, 1978).

Another property of some IgM-RF and IgG complexes in rheumatoid arthritis sera is insolubility at temperatures less than 37°C. which is also termed cryoprecipitation (Heimer et al., 1962; MacKenzie et al., 1968; Johnston and Abraham, 1979; Johnston et al., 1979). The molecular basis for the cryoprecipitation of these complexes is unknown.

5 SPECIFICITY OF RHEUMATOID FACTOR

The discussion on the specificity of rheumatoid factors will be divided into three sections based on the heterogeneity that these antibodies exhibit, conformational specificity, structural specificity and finally species specificity.

5.1 CONFORMATIONAL SPECIFICITY

The specificity of RF in the classical sheep cell

agglutination and the latex fixation tests is directed towards the F_c portion of the IgG molecule which has become denatured by the process of coating the latex particles or by combination with antigen, SRBC, in the SCAT assay. Heller and coworkers demonstrated that Cohn fraction II could inhibit the agglutinating activity of serum (Heller et al., 1954) and subsequently it was shown (Epstein et al., 1956) that RF could precipitate with Cohn fraction II. This would seem to indicate that native 7S IgG was able to react with RF as well as denatured IgG but it was shown that aggregated IgG was needed to give a precipitin reaction with RF (Christian, 1958) and this author also showed that the procedures used by Heller and Epstein resulted in aggregation of the 7S IgG. Christian demonstrated that heat-aggregation of IgG (63°C. for 10 minutes) produced a conformational form of IgG which was highly reactive with RF in a precipitin reaction and also was a powerful inhibitor of the FII tanned sheep cell agglutination test (Heller et al., 1954). RF was described as an autoantibody against altered gamma globulin which shows cross reactivity with native gamma globulin (Aho and Simon, 1963). The idea that RF reacted with native IgG had been suggested by the inhibition studies of Grubb (1956) and also by the experiments describing the 22S complex formed by the interaction of 19S IgM-RF and autologous IgG (Franklin et al.,

1957; Christian, 1959). Aho and Simon (1963) showed that the interaction between native 7S IgG and RF was much weaker than the reaction of RF with aggregated or denatured IgG. Haemagglutination titers obtained with RF sera showed that RF gave higher titers with aggregated rather than unaggregated human IgG coated cells eventhough rabbit anti-human IgG antibody reacted similarly with both types (Butler and Vaughn, 1964). These observations spawned numerous experiments to establish the conformational form of the antigen, IgG, which reacted with RF.

5.1.1 REACTION WITH DENATURED OR AGGREGATED IgG

Ultracentrifugation and optical rotation studies revealed that the degree of reactivity of IgG with RF depended upon the extent of denaturation irrespective of the type of denaturing agent (heat, alkali or detergent) employed (Henney and Stanworth, 1965). It was established that these denaturation procedures led to the rupture of the disulphide bonds in the F_c region of the IgG molecule and that the interaction of these ruptured bonds exposed new antigenic determinants which were recognized by RF (Henney and Ishizaka, 1968). This change in the F_c portion of the IgG molecule was also thought to occur when IgG combined with antigen in an immune complex (Gell and Kelus, 1967).

Gell and Kelus defined RF as an IgM anibody induced by "damaged" IgG. This damage was caused by the binding of IgG with antigen and it was postulated that heat-aggregation and other forms of denaturation simulated complex formation (Christain, 1958; Henney and Stanworth, 1965; Gell and Kelus, 1967; Henney,1969). It was also shown by gel fractionation of heat-aggregated IgG that the aggregate population was reactive with RF in a quantitative precipitin reaction and that native , 7S, IgG separated from the aggregates was essentially non-reactive in this system (Henney, 1969). Henney demonstrated that heating caused a change in the rotary dispersion constant of gamma globulin and that this change was related to the ability of these aggregated preparations to react with RF.

5.1.2. REACTION WITH NATIVE IgG

Although it may be accepted that some rheumatoid factors react only with antigenic determinants present on conformationally altered IgG, there is also overwhelming evidence which suggests that RF also reacts with native IgG. Early evidence was, as described previously, confined to the existence of 22S complexes in RA serum (Franklin et al., 1957; Christain, 1959) and to inhibition studies (Grubb, 1956). A thermostable inhibitor of the FII tanned sheep cell aggluti-

nation test was described by Oreskes and Plotz (1965). This inhibitor was found to be present in the fraction of RA serum corresponding to 7S IgG. Allen and Kunkel showed that the inhibitory substance present in the serum of RA patients could be removed from the serum by dissociation with acetate buffer, pH 4.0 during Sephadex G-200 chromatography of RA serum (Allen and Kunkel, 1966). This material was found to be autologous IgG and seemed to interfere with the anti-Rh human erythrocyte agglutination assay (Waller and Vaughn, 1956) by being complexed with RF in the serum and thereby not allowing the RF to agglutinate the cells coated with human anti-Rh antibody. The relative reactivities of the two forms of IgG could be explained on the basis of a hapten-antigen relationship, the unaggregated 'native' IgG failing to precipitate the RF but nevertheless being capable of inhibiting the reaction between RF with complete antigen (aggregated or complex bound IgG; Henney and Stanworth, 1965).

5.1.3 MULTIVALENCY

RF was found to react with native, 7S, IgG by the ultracentrifugation studies of Normansell (1970) and subsequently, this author demonstrated that the binding constants for the reaction between RF and either native or heat-aggregated human IgG were identical (Normansell, 1971). Normansell

suggested that the increased reactivity with RF attributed to aggregated gamma globulin appeared to be due to the multivalent nature of the antigen. This theory would seem to be in agreement with experiments which demonstrated that multideterminant antigens with a high density of antigenic sites bound more strongly to IgM antibodies than monovalent antigens (Crothers and Metzger,1972). Eisenberg concluded that the enhanced interaction of a monoclonal RF with aggregated IgG was due entirely to multiple bonds between individual antigen and antibody molecules (Eisenberg, 1976). The importance of polyvalent antibody-antigen interactions increases binding affinity and perhaps favors precipitation. In addition, Eisenberg found that there was no evidence for any increased specificity of single antibody active sites for new antigenic determinants revealed on the IgG by the process of aggregation. Dissanayake and co-workers demonstrated that RF isolated under neutral pH conditions exhibited binding constants with native human IgG which were substantially lower than for aggregated IgG (Dissanayake et al., 1977). This preparation of RF was found to contain autologous IgG and when this IgG was separated from the complex by acid dissociation, the RF reacted similarly with both native and aggregated human IgG. Recently, during studies on immune tolerance the heat-aggregated human IgG in rabbits, it was shown that no completely new antigenic

determinants are created on heat-aggregation of IgG, but there appeared to be some potentiation of immunodominance of an already existing determinant (Hunneyball and Stanworth, 1979).

5.2 STRUCTURAL SPECIFICITY

5.2.1 IgG FRAGMENTS

Rheumatoid factors have been shown to react with the F_c fragment of human IgG (Stone and Metzger, 1968; Cerrotini and Grey, 1969; Lyet and Normansell, 1974; Carson et al., 1977; Singh and Francis, 1978), heavy chains (Allen and Kunkel, 1966; Henry et al., 1968; Johnston and Abraham, 1978), pF_c' fragments (Ilter and Turner, 1973; Dissanayake et al., 1977) and specifically to one site on the Cγ3 region and three sites on the Cγ2 region (Natvig et al., 1972; Steward et al., 1973).

5.2.2 GENETIC AND NON-GENETIC IgG DETERMINANTS

The various rheumatoid factors reacting with native human IgG may be classified according to the antigen with which they interact (Natvig et al., 1972). RF was found to react with only a portion of anti-Rh antisera in

the anti-Rh human erythrocyte agglutination assay (Waller and Vaughn, 1956). The heamagglutinating activity of these rheumatoid factors could be inhibited by some but not all human serum or IgG preparations (Grubb, 1956). The IgG type producing inhibition in this system was designated Gm(a) and was subsequently shown to be genetically determined (Grubb and Laurell, 1956). Other Gm genetic antigens have been shown to be reactive with RF, namely Gm(x), (b^1) and (g) (Harboe and Lundevall, 1959; Harboe, 1959; Natvig, 1966). In addition, the 'non a', 'non b^1' and 'γ_4 non a' antigens which are antithetic to Gm(a) and Gm(b^1) have also been found to be reactive with some rheumatoid factors (Natvig et al., 1972). The 'γ_4 non a' antigen is confined to the IgG_4 subclass while the 'non a' and 'non b^1' antigens are allotypic within one given subclass but are also present in all proteins of other subclasses, predominantly in the IgG1 and IgG3 subclasses.

5.2.3 IgG SUBCLASS DETERMINANTS

Certain IgG subclass antigens have also been shown to react with RF and these are probably the most commonly encountered RF specificity. A major antigen in this group is the Ga antigen shared by the IgG1, IgG2 and IgG4 subclasses (Allen and Kunkel, 1966; Gaarder and Natvig, 1970). From

these data, it is apparent that RF is a heterogeneous population of anti-globulins but as it has been pointed out (Chavin and Franklin, 1969; Normansell and Young, 1975), the rheumatoid factors present in a typical RA serum show little distinction as a whole among reactivity with either different genetic γ-globulin types or subclasses, with the possible exception of IgG_3 (Normansell and Stanworth, 1968; Normansell and Young, 1975).

5.2.4 HETEROGENEITY: POLYCLONAL NATURE OF RHEUMATOID FACTOR

IgM rheumatoid factors which precipitated as cryoglobulins with their antigen IgG molecules attached were isolated by Johnston and Abraham (1979) and analyzed for IgG specificity. The class of the 'antigen IgG' isolated from these complexes was also analyzed. In some cases, the IgM-RF precipitated an IgG which was enriched for a certain subclass indicating a highly preferential activity, however, other rheumatoid factors showed that the selection of IgG was a random and non-specific process (Johnston and Abraham, 1979). The 'antigen-IgG' may not be a unique species in most instances (Johnston et al., 1979) and data were found which support the notion that the original antigenic stimulus for RF synthesis could be cross-reactive with IgG, or else was

no longer in the patient's serum when the IgG was isolated from the cryoprecipitate.

Certain IgM-rheumatoid factors show serologic specificity for genetic determinants on IgG not present in the host but present in others not afflicted with the disease (Williams, 1979). It is evident from the data which have been found by these numerous researchers that the classical RF found in the sera of most patients with RA, in a collective sense, represents a population of rheumatoid factors with heterogeneous anti-gamma globulin specificities (Bulter and Vaughn, 1965). The term polyclonal RF may also be applied to this population of rheumatoid factors isolated from patients with RA as opposed to monoclonal rheumatoid factors, which are monospecific (may bind with different antigens that share the same determinants) and are usually isolated from patients with Waldenstrom's macroglobulinemeia.

5.3 SPECIES SPECIFICITY

The classical rheumatoid factor assays demonstrated that rheumatoid factors were a group of anti-antibodies which were able to cross-react with human and rabbit IgG molecules. The three assays which were used to show that there may be differences in species specificity were the sheep cell agglu-

tination test (Waaler, 1940; Rose et al., 1948; Ball, 1950), employing rabbit anti-SRBC antibody, and two assays using human IgG as the antigen, the FII tanned sheep cell agglutination test (Heller et al., 1954) and the latex fixation test (Singer and Plotz, 1956).

5.3.1 CLINICAL SIGNIFICANCE

Plotz and Singer (1956) noted that the results of the latex fixation test compared favourably with the titers obtained in the SCAT. At higher titers, the sheep cell test was better, but those sera containing low titers in the latex test (1:20) were negative in the SCAT test (1:16). There has been agreement between various researchers doing comparative testing with these three assay systems that the SCAT test was the most specific for rheumatoid factors found in RA, the FII test was the most sensitive and that the latex fixation test in the form of a slide agglutination test was the most simple to perform with reasonable sensitivity (Ansell et al., 1969; Bartfeld, 1969b; Cathcart and O'Sullivan, 1969; Waller, 1969; Cathcart et al., 1975; Gall et al., 1978).

5.3.2 HUMAN REACTIVE AND BI-SPECIFIC RHEUMATOID FACTORS

Cross-reactivity between RF and gamma globulin from various species was first investigated by Vaughn in 1956, using an inhibition assay (Vaughn, 1956). In this method it was shown that immune precipitates, formed with egg albumin and rabbit anti-albumin antibody, were able to inhibit the agglutination of sensitized sheep cells by RF. Guinea-pig and horse immune precipitates could not inhibit this reaction between RF and the rabbit anti-SRBC antibody. Further studies by Vaughn and co-workers showed that absorption with rabbit immune precipitates selectively removed that portion of the rheumatoid factor responsible for agglutination of sheep cells coated with rabbit antibody, with minimal, but significant, reduction of agglutination titer for cells coated with human gamma globulin (Vaughn et al., 1958). It was also shown that absorption of serum with heat aggregated human gamma globulin could inhibit all agglutinating activity in rheumatoid sera. These data provided support for a theory that there were two types of RF, one of which reacted with only human IgG and the other which reacted with both human and rabbit IgG. The cross reactivity with rabbit gamma globulin, however, was thought to be weaker than with human gamma globulin (Edelman et al., 1958).

Anion exchange chromotography was found to yield

one 19S peak which could be absorped with Cohn fraction II and recovered by disassociation with urea to give a relatively pure IgM-RF preparation (Lospalluto and Ziff, 1959). This preparation of RF, when rechromatographed, yielded two peaks one of which agglutinated rabbit sensitized sheep cells and latex particles coated with human gamma globulin, termed factor I, and a second peak which agglutinated only the human coated particles, factor II. Factor II was found to be present in normal as well as RA serum and it was thought that this human-only reactive RF provided a possible explanation for the observations that sera from patients with sarcoidosis and syphilis agglutinate latex particles coated with human IgG but do not agglutinate rabbit sensitized-SRBC. Ultracentrifugation studies demonstrated that two distinct rheumatoid factor populations existed (Heimer and Schwartz, 1961). One population, sedimenting at 19.4S, was found to be reactive with cells coated with either human or rabbit gamma globulin while another, sedimenting at 19.5S, was reactive only with cells coated with human gamma globulin.

The viewpoint that all rheumatoid factors were reactive with human IgG while a subpopulation showed reactivity with both human and rabbit gamma globulin was supported by the endeavours of Butler and Vaughn (1964; 1965). These authors demonstrated that rheumatoid sera were re-

active with human, rabbit, bovine, equine, porcine and quinea-pig gamma globulin but were not reactive with chicken gamma globulin (Butler and Vaughn, 1964). With cells coated with animal gamma globulins, the respective animal gamma globulin was always considerably more effective than the other gamma globulins in inhibiting the agglutination reaction. For example, inhibition of the anti-Rh system (Waller and Vaughn, 1956) could be accomplished with the addition of human normal serum but not with rabbit normal serum (Allen and Kunkel, 1966) and native rabbit but not human IgG was found to inhibit the sheep cell agglutination test (Glynn, 1968; Normansell, 1971). It was shown nevertheless that it was possible to inhibit all RF agglutinating activity by absorption of the sera with insoluble human gamma globulin. The other animal gamma globulins when insolubilized inhibited only their respective agglutination reactions. Subsequently it was observed that insolubilized rabbit gamma globulin was capable of partially absorping reactivity with human gamma globulin coated cells (Butler and Vaughn, 1965).

5.3.3 MONOREACTIVE AND BI-SPECIFIC RHEUMATOID FACTORS

It was also thought that there were rheumatoid factors which reacted with only rabbit gamma globulin in

addition to the two reactivities previously described. Dispersion of agglutination and mixed agglutination studies, using rabbit sensitized alligator erythrocytes and human anti-Rh human erythrocytes, showed that three populations of rheumatoid factor existed: factor a combining with human gamma globulin only; factor b combining with both human and rabbit gamma globulin; and factor c combining with rabbit gamma globulin only (Milgrom et al., 1962). This theory is widely accepted today and has been confirmed by numerous researchers (Williams and Kunkel, 1963; Normansell and Stanworth, 1968; Skalba and Stanworth, 1969; Normansell , 1972; Stewart et al., 1975).

Human FII, aggregated FII and rabbit gamma globulin, diazotized to polyaminostyrene columns, were used to separate rheumatoid factors from sera of patients with RA (Williams and Kunkel, 1963). The rheumatoid factors which passed through human gamma globulin columns were unreactive in human agglutination tests but retained positive rabbit reactivity and conversely, fractions passing through rabbit gamma globulin columns were reactive with human IgG only.

Normansell showed that three populations of RF existed by absorption of the rheumatoid factor onto columns of human and rabbit gamma globulin (Normansell, 1972). The majority of the RF had specificity for both human and rabbit gamma globulin with smaller amounts specific for one

or the other species of gamma globulin. The monospecific rheumatoid factors which passed through the affinity columns were shown to have similar binding constants for their respective 7S rabbit or human IgG antigens in the ultracentrifuge. The rheumatoid factors absorped to the human or rabbit gamma globulin columns could be eluted with 0.05 M glycine-HCl buffer, pH 2.8, and when tested in the ultracentrifuge, were reactive with rabbit, human and horse gamma globulin (binding constants 1-5 X 10^5 liters/mole). The binding of RF with rabbit gamma globulin was found to be stronger than with human IgG. The binding constants were adjusted on the basis of experiments which showed that only 67% of the rabbit IgG was reactive with RF whereas all of the human IgG was found to be potentially reactive with a polyclonal IgM-RF (Normansell and Stanworth, 1968; Normansell, 1972). This was based on the results of Henney and Stanworth (1964) in which the amount of precipitation occurring between RF and heat-aggregated gamma globulin varied according to the species of gamma globulin employed and on the suggestion (Skalba and Stanworth, 1969) that one conformational form of rabbit IgG did not react with a pooled RF preparation. The conformation of rabbit IgG responsible for this species cross-reactivity was confined to those molecules which were sensitive to papain. Gamma globulins from species comprising mainly papain resistant

molecules, eg. horse, bovine, chicken, showed low reactivity with RF whereas gamma globulins which comprise predominantly papain sensitive molecules, eg. human, baboon, rabbit, showed relatively high reactivity.

Stewart, Hunneyball and Stanworth, employing a sheep cell agglutination assay with baboon and rabbit anti-SRBC sensitized sheep cells, showed that each of these systems was detecting a specific RF population, i.e. one which is specific for human (baboon) IgG and one which is specific for rabbit IgG (Stewart et al., 1975). A third population was found to react with both. The incidence of the monoreactive RF population in the sera of patients with suspected or confirmed RA was low, less than 9%. Although most if not all rheumatoid factors have been shown to react with either human or rabbit IgG, or both, the possibility that there may be a population of RF which reacts monospecifically with IgG from another species has also been suggested (Hansson and Winblad, 1978) but these would be expected to be quite rare.

Dissanayake and co-workers (1977) showed that the binding constants for the precipitation reaction between pooled polyclonal RF and rabbit or human 7S IgG were similar and were of the same order of magnitude (10^4 - 10^5 1/M) as those obtained by Normansell (1971; 1972). Once more, the binding constants, when adjusted for the potentially

nonreactive rabbit IgG, showed that RF reacted more strongly with rabbit gamma globulin than with human IgG.

The predominance of the RF reactive with both rabbit and human gamma globulin has been questioned (Milgrom et al., 1962). Normansell (1972) noted that it was not possible to isolate a reactive population of RF that was purely bi-specific and therefore, the existence of this class of RF could only be demonstrated by agglutination testing of rheumatoid factors eluted from affinity columns containing rabbit or human IgG. It would seem however, that there is general agreement between investigators that the bi-specific RF does exist and is the most predominant form occurring in RA sera.

5.4 GENESIS OF SPECIFICITY

It is apparent from these data that there are families of RF molecules, each with distinct antigenic specificities in rheumatoid factor containing sera, which would account for many of the observations that RF-sera or isolated RF react with IgG of different species. In addition, it has been suspected that there may be antigenic sites on gamma globulin molecules which are common to the IgG molecules from different species, accounting in part for the reactivity of RF with IgG from various species (Henney and

Stanworth, 1964). It has also been shown that there is a 66% sequence homology between the human and rabbit Cγ3 regions on the F_c portions of these IgG molecules (Steward et al., 1973). Furthermore, the observation that denatured or aggregated IgG blocked sensitized cell assays or RF assays in which aggregated IgG is used as antigen, regardless of the IgG species used, has been widely demonstrated. Here, aggregation is thought to unmask antigenic sites which are not readily available to RF in native IgG molecules (Williams and Kunkel, 1963; Henney and Stanworth, 1965; Skalba and Stanworth, 1969; Gaarder, 1973; Stewart et al., 1975; Dissanayake et al., 1977).

There is, however, another alternative explanation to account for these observations. Aggregation results in the formation of a macromolecular complex of IgG molecules, and the subsequent aggregate carries many antigenic determinants on a single macromolecule as opposed to a single or relatively few sites on each native IgG molecule. The binding of pentameric IgM-RF to such a complex would allow several binding sites to react with several antigenic sites on the same macromolecule, yielding a greater 'total' affinity, even though each individual affinity is the same as that for the native IgG. This would then account for the ability of native IgG to block assays in which aggregated IgG of the same species is used as antigen and account for

the capacity of aggregated IgG of any species to inhibit native IgG-RF interactions and assays utilizing aggregated IgG of dissimilar species. This would also explain the apparent greater affinity of RF for denatured IgG compared with native IgG. If such a mechanism does operate, then it is possible that RF activity could be obscurred in some sera due to the binding of RF with autologous native IgG in these sera which is not readily unmasked by aggregated IgG in the assays employed (so-called 'hidden' RF; Allen and Kunkel, 1966; Hansson and Winblad, 1978; Maiolini et al., 1979). Maneuvers, such as gentle acidification of serum, dilution or gel filtration at neutral pH (Abruzzo and Heimer, 1974; Hansson and Winblad, 1978) have been shown to dissociate RF-native IgG complexes, thereby making RF detectable. It should be noted, however, that examples of, 'hidden' RF are uncommon (McDuffie and Bunch, 1977a).

5.5 HETEROGENEITY OF RF SPECIFICITY

It is evident from the knowledge that has been accumulated on RF since its initial description in 1940, that it is a more heterogeneous population of molecules than Waaler's 'agglutination activation factor' (Waaler, 1940). The heterogeneity and specificities of RF, which have been described above, include specificity towards in-

tact IgG molecules (monomeric, aggregated, surface absorbed), towards IgG subclass determinants on IgG1 - IgG4, towards determinants on F_c fragments both genetic and non-genetic, towards antigen-antibody complexes and against heterologous IgG molecules, most notably towards rabbit IgG. Thus, rheumatoid factor is usually defined as a population of molecules directed primarily against determinants on the F_c portion of the human IgG molecule.

In addition to rheumatoid factors which react with IgG, antiglobulins which react with IgA (Fudenberg et al., 1968), IgM (MacKenzie et al., 1967), IgE (Williams et al., 1972), pepsin-digested IgG (Osterland et al., 1963) and light chains (Williams, 1963) have all been discribed but these are generally not regarded as rheumatoid factors. Monoclonal and polyclonal rheumatoid factors with anti-di- and anti-trinitrophenyl activity have been described (Hannestad, 1969a; 1969b) and furthermore, rheumatoid factors react with deoxyribonucleic acid (DNA) (McCormick and Day, 1963; Hannestad and Johannessen, 1976; Agnello et al., 1978; Johnson, 1979), double stranded DNA (Bell and Schur, 1975; Grennan et al., 1977), single stranded DNA (Lange et al., 1976), denatured DNA (Hannestad, 1969b), DNA-histone (Agnello et al., 1980) and nuclear histone (Aitcheson et al., 1980) have also been reported.

6 RHEUMATOID FACTOR ASSAYS

6.1 THE SHEEP CELL AGGLUTINATION AND LATEX FIXATION TESTS

The two most popular assays in clinical use today for the detection of RF are, the sheep cell agglutination test (SCAT) and the latex fixation test (LFT). The sensitivity and apparent simplicity of agglutination tests make them popular for the detection of abnormal levels of RF. The clinical specificity of the SCAT assay renders it a valuable diagnostic aid for rheumatoid arthritis (Lea and Ward, 1978). There have been, however, numerous criticisms of these two techniques in recent years.

6.2 DISADVANTAGES OF THE SCAT AND LFT TECHNIQUES

The reagents used in the SCAT assay, namely the sheep cells and the rabbit anti-SRBC antibodies, are labile and the method has been found to be relatively insensitive (Shakib and Stanworth, 1978; Gripenberg et al., 1979). In addition, both the SCAT and LFT methods suffer from difficulties of standardization (Klein et al., 1976; Lea and Ward, 1978; Gripenberg et al., 1979). Semi-quantitation is obtained in these assays by selecting the last positive reaction in a doubling dilution scale leading to low pre-

cision (Maiolini et al., 1978). The difficulty and inherent subjectivity in reading titration endpoints leads to poor reproducibility (Virella et al., 1978; Klein et al., 1979; Weinblatt and Schur, 1980). Using the latex or sensitized sheep cell tests, 46% of 208 clinical laboratories reported abberant and widely varying results in assaying 5 RF-containing samples (Singer, 1974). Another disadvantage in using these tests is the inability of agglutination assays to demonstrate the presence of IgA, IgG or other non-agglutinating rheumatoid factors with low avidity (Lea and Ward, 1978; Jones et al., 1979). These observations have led to the development of new RF assays which are more sensitive and more reproducible than the latex or sheep cell agglutination tests.

6.3 CURRENT RF ASSAYS

Some of the current methods for the measurement of RF in human serum are summarized in Table 2.2. These assays fall into four basic categories based on the methodology employed. These are: haemagglutination assays in which a sensitized human or animal erythrocyte is agglutinated by RF; latex agglutination assays in which latex particles coated with IgG are agglutinated by RF; precipitation assays in which complexes of RF and IgG are precipi-

tated in gel (by an auxillary antibody or by the use of polyethylene glycol); and solid phase assays which generally employ solid phase IgG to which RF is absorbed and the reaction quantitated with the addition of anti-human immunoglobulins labelled with radioactivity, flourocein or enzymes. In addition, nephelometric techniques have been described and these will be discussed in subsequent sections.

For 14 of these assays in which comparisons were done between the new assay results and the classical SCAT and LFT titers for the same sera, only one test (Shakib and Stanworth, 1978) did not show correlation with these standard RF assays. Although the results of the new tests were comparable with those obtained in the SCAT and LFT assays, most, if not all, of these new assays were shown to be more sensitive and more reproducible than the classical tests. Each of these tests was found to have advantages and disadvantages associated with the methodology employed, however, it is not within the scope of this discussion to consider the tests per se although comparisons may be made with the results obtained by these authors.

TABLE 2.2

CURRENT METHODS OF RHEUMATOID FACTOR ASSAY

Antigen	Quantitation	RF Classes[1]	Ref.
Haemagglutination Assays			
HGG, native or fragments	Inhibition of anti-Rh human erythrocyte agglutination	M	1.
HGG, covalently bound to formalinized SRBC	Agglutination read visually	M mainly[2]	2.
Latex Agglutination Assays			
HGG bound to latex beads	Turbidity in Spectrophotometer	M mainly	3.
NHGG or hAHGG bound to latex beads	Agglutination in Coulter Counter	"	4.
NHGG bound to latex beads	Agglutination in Coulter Counter	"	5.
HGG bound to latex beads	Light Scatter in Nephelometer	"	6.
HGG bound to latex beads	Agglutination read visually	"	7.
HGG bound to latex beads	Aggregation in Platelet Aggregometer	"	8.
Precipitation Assays			
hAHGG-^{125}I	Immunoprecipitation with anti-IgM	M	9.
hAHGG	Precipitation in Agarose	M mainly, G[3] after 2-ME	10.
HGG or EqGG, F_c coupled to FITC	Precipitation with 10% PEG	M,G,A in toto[4]	11.

(cont.)

TABLE 2.2 (cont.)

Antigen	Quantitation	RF Classes	Ref.
Precipitation Assays (cont.)			
hAHGG-^{125}I	Precipitation with 2.5% PEG	M mainly	12.
Solid Phase Assays			
RGG or HGG insolubilized with ethyl-chloroformate	Radial Immunodiffusion	M,G,A seperately[5]	13.
RGG bound to plastic	^{125}I-anti-IgM or anti-IgG	M,G seperately	14.
HGG, F_c bound to plastic	^{125}I-Rabbit F_{ab_2} anti-IgM or anti-IgG	M,G seperately	15.
hAHGG or gARGG bound to plstic	^{125}I-Goat anti-IgM	M	16.
RGG anti-SRBC bound to smeared SRBC	^{125}I-Rabbit anti-IgM or anti-IgG	M,G seperately	17.
RGG bound to plastic	FITC-Rabbit anti-IgM or anti-IgG	M,G seperately	18.
hAHGG or hARGG bound to cellulose	ELISA-hAHGG or hARGG	M,G,A in toto	19.
RGG bound to plastic	^{125}I-Rabbit anti-IgM or anti-IgG	M,G seperately	20.
RGG or HGG insolubilized with glutaraldehyde	Radial Immunodiffusion	M,G,A seperately	21.
HGG anti-viral antigen bound to latex particles	^{125}I-Pig anti-IgM	M	22.

(cont.)

TABLE 2.2 (cont.)

Antigen	Concentration	RF classes	Ref.
Solid Phase Assays (cont.)			
hAHGG or ARGG bound to plastic	ELISA-anti-IgM	M	23.
RGG bound to plastic	^{125}I-Rabbit anti-IgM or anti-IgG	M,G seperately	24.
RGG anti-SRBC bound to smeared SRBC	FITC-anti-IgM, anti-IgG, or anti-IgA	M,G,A seperately	25.
HGG bound to plastic	ELISA-anti-IgM	M	26.

[1] Immunoglobulin class of RF detected in these assays;
[2] Agglutination assays detect mainly IgM-RF but there may be some additional agglutination caused by the other classes of RF;
[3] IgG-RF detected after reduction of serum with 2-mercaptoethanol;
[4] Total contribution of IgM-, IgG, and IgA-RF detected;
[5] IgM-, IgG-, and IgA-RF detected seperately.

Abbreviations used in this Table were: HGG, Human IgG; SRBC, Sheep red blood cells; NHGG, Human Native IgG; hAHGG, Heat-Aggregated Human IgG; PEG, Polyethylene Glycol; RGG, Rabbit IgG; gARGG, Glutaraldehyde-Aggregated Rabbit IgG; ELISA, Enzyme-Linked Immunosorbent Assay; hARGG, Heat-Aggregated Rabbit IgG.

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6.4 NEPHELOMETRIC RHEUMATOID FACTOR ASSAYS

6.4.1 NEPHELOMETRY: DEFINITION AND APPLICATIONS

The quantitation of specific proteins in human serum by nephelometric measurement of immunoprecipitin reactions has gained importance in the past few years (Killingsworth and Savory, 1973). Nephelometry is defined as the detection of light energy scattered or reflected towards a detector which is not in the direct path of the transmitted light (Kusnetz and Mansberg, 1978). A nephelometer is capable of detecting soluble molecular aggregates in solutions which appear perfectly clear to the naked eye. The increase in light scattering due to the formation of antigen-antibody complexes has been examined extensively (Goldberg and Campbell, 1951; Tengerdy, 1967; Davies, 1971; Marrack and Richards, 1971). The parameters for the nephelometric quantitation of human IgG in serum with goat anti-human IgG antiserum were studied by Killingsworth and Savory (1973).

6.4.2 LASER NEPHELOMETRY

Until 1976, measurement of immunological reactions nephelometrically had determined light scattered at

an angle of 90° (Killingsworth and Savory, 1972). This angle limits the sensitivity of the technique and does not allow sample backgrounds to be well differentiated from the specific immunological reaction being quantitated (Buffone et al., 1975; Kusnetz and Mansberg, 1978). The development of a nephelometer employing a laser as the light source allowed detection of forward light scatter (31°) and increased the sensitivity of nephelometry for the detection of antigen-antibody reactions (Deaton et al., 1976). The quantitation of various serum proteins has been investigated using the laser nephelometer among which are: IgA; IgG; IgM; complement component C3; and albumin (Deaton et al., 1976). Comparisons of immunoglobulin determinations in pathological sera by radial immunodiffusion (RID) and laser nephelometry demonstrated that the nephelometer offered a considerable advantage over RID (Vitella and Fudenberg, 1977). Laser nephelometry was found to be more precise and was not limited by the molecular-size characteristics of the proteins assayed such as may occur with diffusion methods. It should be noted that most commercial nephelometric assays employ polyethylene glycol to improve or enhance these assays. The effect of polyethylene glycol on immunoprecipitation reactions will be discussed below.

6.4.3 THE EFFECT OF POLYETHYLENE GLYCOL ON IMMUNOPRECIPITIN REACTIONS

Polyethylene glycol (PEG) is an example of a water soluble polymer which has been used extensively to enhance the precipitation of immune complexes (Harrington et al., 1971; Creighton et al., 1973). Increasing concentrations of up to 5.0% added to agar gel progressively accelerated specific immunoprecipitation and promoted the formation of visible precipitin lines (Harrington et al., 1971). Soluble complexes as small as IgG trimers could be precipitated by the addition of PEG with a final concentration of 7.5% (Creighton et al., 1973). Most of the native immunoglobulins were precipitated by 20% PEG in this system, however, at concentrations of polyethylene glycol up to 3.5%, the decrease in the solubilities of IgM, IgG and IgA were negligible while 10% of a heat-aggregated HGG preparation was precipitated with this concentration.

The general mechanism of action of PEG is unknown, but the modification of protein solubilities observed in the presence of water soluble polysaccharides would suggest that a molecular solvent exclusion effect is involved. The effect of PEG on the precipitation of immune complexes was explained in terms of a steric exclusion of the antigen-antibody complexes from the domain of the polymer (Hellsing,

1969). It was shown that PEG could be used to precipitate soluble immune complexes in conditions in which either free antigen or antibody remain soluble (Creighton, 1973). The use of polymer enhancement was found to increase the sensitivity and to decrease the reaction time of the nephelometric method (Lizana and Hellsing, 1974a; 1974b). The overall effects of PEG in nephelometric assay systems are: increased sensitivity, increased reaction rate allowing short incubation times for quantitation, and permits antigen to be quantitated in either antigen or antibody excess.

6.4.4 NEPHELOMETRIC RF ASSAY

Nephelometry has been used for several years to quantitate the concentrations of serum proteins but has only recently been applied to the measurement of rheumatoid factor. The reaction between RF-containing serum and heat-aggregated human IgG in the laser nephelometer was first described in our laboratories (Lamberson et al., 1977). Subsequently the Hyland Division of Travenol Laboratories, Inc., developed a clinical nephelometric RF assay (Jones et al., 1979). In addition, this reaction has been studied by other researchers whose techniques are summarized in Table 2.3. It has been shown that the results of the commercially available Hyland nephelometric rheumatoid factor

assay correlate well with the RF titers obtained in the latex fixation test (Husmann et al., 1979; Weinblatt and Schur, 1980) and in the sheep cell agglutination test (Husmann et al., 1979).

TABLE 2.3

NEPHELOMETRIC RHEUMATOID FACTOR ASSAYS

Antigen	Quantitation	Reference
hAHGG, monomer free	Hyland Laser Nephelometer	Lamberson et al., 1977
hAHGG, unfractionated	Hyland Laser Nephelometer	Jones et al., 1979
hAHGG, unfractionated	Hyland Laser Nephelometer	Husmann et al., 1979
hAHGG, unfractionated	Behring Laser Nephelometer	Roberts-Thomson and Bradley, 1979
hAHGG, unfractionated	Hyland Laser Nephelometer	Whitsed et al., 1979
hAHGG, unfractionated	Beckman Rate Nephelometer	Finley et al., 1979
hAHGG, unfractionated	Hyland Laser Nephelometer	Weinblatt and Schur, 1980
hAHGG, monomer free; hARGG	Hyland Laser Nephelometer	Duke et al., 1980

6.4.5 ADVANTAGES OF NEPHELOMETRIC RF ASSAY

Although numerous assays have been developed for the detection of RF in human serum (reviewed in Sect. 6.3), nephelometry offers several advantages over other assay techniques. Short incubation times (less than 60 minutes) made possible by the enhancing effect of polyethylene glycol allow results to be obtained rapidly. The NeRF assay is simple and makes use of stable reagents which do not require labelling with radioactivity or with enzymes. A higher precision than with RID is obtained and the sensitivity, although not as great as the RIA or enzyme-linked assay systems, is respectable (60 ng/ml IgG may be quantitated in the Hyland nephelometer).

By avoiding the use of cellular or particulate reagents, the NeRF assay allows quantitation by rate or by endpoint analysis. With the cell or latex particle agglutination tests, quantitation is by titration, a notoriously inaccurate technique (95% confidence limits generally are plus or minus 2 dilutions; Finley et al., 1979). The variability of the NeRF assay is also less than the RIA (Jones et al., 1979) or the latex fixation test (Weinblatt and Schur, 1980). Since the reaction is carried out in fluid phase, the problems associated with gel diffusion assays (e.g. monomeric versus polymeric antigen quantitation) are

avoided. The only apparent advantage of any of the other RF assays over the NeRF assay is a function of the ability of the indirect RIA or enzyme-linked assays to distinguish between different subclasses of RF that participate in the test reaction (Jones et al., 1979).

7 GOALS OF THE RESEARCH

The goal of this research has been to increase the knowledge about antibody-antigen reactions, specifically the reaction of RF with IgG. These reactions have been investigated using a newly developed nephelometric assay. Experiments have been designed to examine both the species and conformational requirements for IgG to react with rheumatoid factor.

CHAPTER III

MATERIALS AND METHODS

1 CHEMICALS AND SUPPLIES

Reagents and supplies were obtained from the following sources: polyethylene glycol (PEG; 6000 M.W.), ε-amino-n-caproic acid (EACA), glycine-HCl, Folin-Phenol reagent, sodium dodecyl sulfate (SDS), Fisher Scientific Co., Dorval, Que.; Tris-hydroxyaminomethane (Tris), glutaraldehyde, ethylenediaminetetraacetic acid (Na_2EDTA), ethyleneglycol bis (aminoethyl)-tetraacetic acid (EGTA), Sigma Chemical Corp., St. Louis, Mo.; Freon-MF (trichloromonofluoromethane), Dupont Canada Ltd., Maitland, Ont.; 2-Mercaptoethanol, Eastman Organic Chemicals, Rochester, N.Y.; bovine serum albumin (5X crystaline), Pentex Laboratories, Kankakee, Ill.; 2-iodoacetamide, J.T. Baker Chemical Co., Phillipsburg, N.J.; Bio-Gel A-5m, Bio-Rad Laboratories, Richmond, Ca.; DEAE cellulose (DE-52), Whatman Ltd., Maidstone, Kent, England; Sephadex G200, Pharmacia Fine Chemicals, Uppsala, Sweden. All other chemicals were of reagent grade and were purchased from Fisher Scientific Co., Dorval, Que.

2 BUFFERS

0.015 M Tris-0.15 M NaCl buffer, pH 7.4, was used in all experiments unless otherwise noted in the Text. Buffers were filtered through a 0.45 µm filter (Millipore Corp., Bedkord, Mass.) and were used within 7 days of preparation. In some experiments polyethylene glycol (PEG) was added at concentrations noted in the Text. Concentrations of PEG are indicated as w/v, expressed as percent (%).

3 SERUM SAMPLES

Sera submitted for rheumatoid factor titration were obtained from the clinical laboratory of the Division of Clinical Immunology, Royal Victoria Hospital, Montreal. These sera were stored at -20°C for not more than one week prior to use. In addition, blood from selected patients with definite or probable rheumatoid arthritis, as well as from normals, were obtained. Blood samples were allowed to clot at room temperature (22°C), centrifuged at 1,000 x g (15 min.; 22°C) and sera stored at 4°C until used. Before being assayed for rheumatoid factor activity, all sera were heat-decomplemented (56°C; 30 min) and then clarified by centrifugation (13,000 x g; 10 min; 22°C).

4 SERUM MANIPULATIONS

4.1 DELIPIDATION OF SERUM AND BIOLOGICAL MATERIALS

Sera which were visually lipaemic were delipidated with Freon-MF. Two volumes of Freon-MF were vigourously mixed with one volume of heat-decomplemented serum for 10 minutes. Samples were then centrifuged (13,000 x g; 10 min; 22°C) and the delipidated serum carefully removed from the underlying lipid interface. Following this delipidation procedure, sera were immediately assayable in the nephelometer. Other biological materials, as indicated in the Text, were delipidated with Freon-MF using this same procedure.

4.2 EUGLOBULIN PRECIPITATION OF SERUM

Serum were dialyzed exhaustively against distilled water at 4°C and the euglobulin fraction separated from the pseudoglobulin by centrifugation (10,000 x g; 10 min; 4°C). The pellet was resuspended in Tris-NaCl buffer and clarified by centrifugation (10,000 x g; 10 min; 4°C). Both the resupended pellet and the supernatant were assayed for RF-activity in the nephelometer.

4.3 REDUCTION AND ALKYLATION OF SERUM

4.3.1 CYSTEINE AND IODOACETAMIDE

0.5 M cysteine was added to serum to give final concentrations from 0.025-0.25 M (see Results 3.1) and incubated at 22° for 10 minutes after which 1 M iodoacetamide was added to give final concentrations twice the cysteine concentrations. After reduction and alkylation, serum was dialyzed exhaustively against Tris-NaCl buffer.

4.3.2 2-MERCAPTOETHANOL AND IODOACETAMIDE

2-mercaptoethanol (final concentration 0.1 M) was added to serum, incubated for four hours at 22°C after which iodoacetamide was added (final concentration 0.2 M) and reacted for an additional 10 minutes. After reduction and alkylation, serum was exhaustively dialyzed against Tris-NaCl buffer.

4.4 PRODUCTION OF "ARTIFICIAL" RF-SERUM

Purified polyclonal rheumatoid factor (pRF) at concentrations indicated in the Text, was added to 1 ml heat-decomplemented human normal serum (no detectable NeRF-acti-

vity) and incubated with gentle mixing for 24 hours at 4°C. "Artificial" RF-serum was then assayed for NeRF activity.

5 PROTEIN DETERMINATION

Protein was measured by the Lowry method, using bovine serum albumin as standard (Lowry et al., 1951) or spectrophotometrically. For purified proteins, there was a close relationship between protein concentrations as measured by the Lowry method and by absorbancy at 280 nm, using known extinction coefficients. The extinction coefficients, expressed as $OD_{280\ nm}$ for a 10% protein solution and a 1 cm light path, used for protein concentration determination were: 11.85, IgM rheumatoid factors; 14.3, human native IgG; 14.6, rabbit native IgG (Little and Donahue, 1968).

6 GEL FILTRATION CHROMATOGRAPHY

Analytical and preparative gel filtration were done using 6% agarose gel (Bio-Gel A-5m). Tris-NaCl buffer was the elution buffer and fractions were collected representing approximately 1% of the total volume of the column. Sample volumes applied did not exceed 3% of the total volume of the column. Columns were calibrated with human IgG,

human IgM and heat-aggregated human IgG (MW greater than 5×10^6 daltons; Methods 7.4). Human IgG and IgM were kindly donated by Dr. C.K.Osterland. Eluates were monitored for protein content by the Lowry method or by absorbancy at 280 nm.

Nephelometric activity of the eluates was assayed by reacting aliquots of the eluates with a corresponding RF or antigen preparation. In these experiments, a final incubation volume of 1.0 ml Tris-NaCl buffer containing 1% PEG was used. RF in serum (final concentration 25 µl/ml; LFT 1:1024) was used to test for antigen activity in eluates while aggregated IgG (antigen; final concentration 50 µg/ml) was used to test for RF activity in eluates (see Results). Reactants were incubated for 60 min at 22°C and activity, quantitated in the nephelometer, was expressed as Net LS after subtraction of appropriate blanks.

7 PROTEIN ISOLATION

Three major groups of protein were purified: rheumatoid factors; gamma globulin (IgG); and C1q.

7.1 RHEUMATOID FACTOR ISOLATION

7.1.1 POLYCLONAL RHEUMATOID FACTOR (pRF)*

pRF was isolated from pooled sera of four patients with rheumatoid arthritis which had high titers of RF activity when assayed by the latex fixation test (LFT). Serum was fractionated by euglobulin precipitation and the precipitate, collected by centrifugation (5,000 x g; 10 min; 4°C) was resuspended in Tris-NaCl buffer containing 0.02% NaN_3 and chromatographed on Bio-Gel A-5m. Peak tubes corresponding to the molecular weight of purified IgM (900,000 daltons) were pooled, concentrated by ultrafiltration using Amicon P-10 membranes (Amicon Corp., Lexington, Mass.) to a final concentration of 7.6 mg/ml, and filtered through a 0.22 µm filter (Millipore) into sterile vials for storage at 4°C. Purity of the isolated pRF was established by analytical gel filtration chromatography (Bio-Gel A-5m), sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; after the method of Weber and Osborne, 1969), and by immunoelectrophoresis (IEP; as described in Ouchterlony and Nilsson, 1973) using goat anti-human IgM antiserum (Hyland Division, Travenol Laboratories, Costa Mesa, Ca.). Results of gel filtration of isolated pRF (Results 4.1) showed a single peak of protein with an elution volume cor-

responding to IgM. SDS-PAGE showed a single band of protein with a molecular weight estimated to be 900,000 daltons. IEP showed a single precipitin line characteristic of IgM.

7.1.2 MONOCLONAL RHEUMATOID FACTOR (mRF_{og})

Monoclonal rheumatoid factor was isolated from a patient (OG) with rheumatoid arthritis and Waldenstrom's macroglobulinemia. Plasma samples from this patient were a generous gift of Dr. J. DelCarpio of the RVH. Plasma was diluted with three volumes of 0.015 M Tris-0.20 M NaCl buffer, ph 7.6, and then fractionated with ammonium sulfate at 50% saturation. This precipitate was collected by centrifugation (5,000 x g; 10 min; 4°C), resuspended in Tris-NaCl buffer as before and dialyzed against distilled water. The precipitate was resuspended in this buffer, concentrated by ultracentrifugation using an Amicon P-10 membrane (Amicon) and subjected to gel filtration on Bio-Gel A-5m. Protein content ($OD_{280\ nm}$) and RF activity (Methods, 6) were measured on each fraction. Fractions with peak RF activity with heat-aggregated human IgG were pooled, concentrated by ultrafiltration, and rechromatographed on this column. Again, peak activity fractions were pooled, sterilized by passage through a 0.22 µm filter and stored at 4°C at a

final concentration of 0.7 mg/ml in 0.015 M Tris-0.20 M NaCl buffer, pH 7.6, containing 0.02% NaN_3. Purity of the isolated mRF_{og} was established by gel filtration (Bio-Gel A-5m), SDS-PAGE, and IEP. Results of gel filtration (Results, 5.1), showed a single peak of activity with an elution volume corresponding to IgM. SDS-PAGE showed a single band of protein with a molecular weight estimated to be 900,000 daltons. Immunoelectrophoresis showed a single precipitin arc, characteristic of IgM, when run against goat anti-human IgM antiserum (Hyland).

7.1.3 MONOCLONAL RHEUMATOID FACTOR (mRF_{mi})

A second monoclonal rheumatoid factor was isolated from a patient (MI) with rheumatoid arthritis and Waldenstrom's macroglobulinemia. mRF_{mi} was isolated from plasma by euglobulin precipitation followed by gel filtration chromatography using Sephadex G-200 (Pharmacia). Fractionation was done using 0.15 M acetate buffer, pH 4.5. This mRF_{mi} was isolated by C.K.Osterland and was a generous gift.

All isolated rheumatoid factor preparations were centrifuged at 90,000 x g (75 min; 4°C) in an ultracentrifuge and filtered through 0.22 µm filters prior to use in nephelometric assays.

7.2 GAMMA GLOBULIN (IgG) ISOLATION

7.2.1 HUMAN IgG

Human IgG was obtained from the Swiss Red Cross (γ-globulin i.v., S.R.K. 6%, The Swiss Red Cross Transfusion Service, Berne). This material was centrifuged to remove insoluble or denatured IgG (90,000 x g; 75 min; 4°C), then sterilized by passage through a 0.22 µm filter and stored at 4°C in buffer containing 0.02% NaN_3. This material was the source of human IgG for use as human native IgG (NHGG) and for heat-aggregated human IgG (hAHGG).

7.2.2 RABBIT IgG

Healthy rabbits were bled and IgG isolated from serum by the Cohn-ethanol fractionation method (Deutsch 1967). The Cohn fraction II material was lyophilized and resuspended in Tris-NaCl buffer prior to use, at a concentration of 75 mg/ml. After resuspension, the rabbit IgG was centrifuged (10,00 x g; 10 min; 4°C) to remove insoluble or denatured material. The soluble IgG was used for the production of spontaneously-aggregated rabbit IgG (sARGG) or glutaraldehyde-aggregated rabbit IgG (gARGG).

A second preparation of rabbit IgG was isolated from rabbit serum obtained from the Institut Armand Frappier (Laval, Que.). Serum was fractionated by ammonium sulfate (50% saturation), harvested by centrifugation (5,000 x g; 10 min; 4°C), and the precipitate resuspended in Tris-NaCl buffer. This material was then exhaustively dialyzed against 0.005 M phosphate buffer, pH 7.4, and then applied to a column of DEAE cellulose (after the method of Fahey and Terry, 1973). Material, not adsorbed to the column was pooled after determining protein content (absorbancy at 280 nm) and cellulose acetate electrophoretic mobility. Two pools were obtained as described in the Results section (1.1.13). These pools were then centrifuged (90,000 x g; 75 min; 4°C), sterilized by 0.22 µm pore filtration and stored at 4°C in buffer containing 0.02% NaN_3. This material was the source of rabbit IgG for use as rabbit native IgG (NRGG) or heat-aggregated rabbit IgG (hARGG).

7.2.3 CHICKEN IgG

Chicken IgG was isolated from freshly obtained chicken blood from an abbatoire. After blood was clotted and serum collected, IgG was isolated by ammonium sulfate fractionation. Ammonium sulfate was added (50% saturation) and the resultant precipitate harvested by centrifugation

(5,000 x g; 10 min; 4°C), resuspended in Tris-NaCl buffer, exhaustively dialyzed against this buffer and then stored at 4°C.

7.3 ISOLATION OF C1q

C1q was isolated from human serum by the method of Yonemasu and Stroud (1971) with the modifications used by Zubler and co-workers (1976). Throughout the isolation procedures, activity was monitored by the latex fixation test and purity established by the Ouchterlony technique (Ouchterlony and Nilsson, 1973), using rabbit anti-human C1q antiserum (Behringwerk Ag, Marburg W. Germany). Isolated C1q was sterilized by filtration through a 0.22 µm filter and stored at 4°C at a final concentration of 1.5 mg/ml in Tris-NaCl buffer containing 0.02% NaN_3.

8 AGGREGATION METHODS

8.1 SPONTANEOUSLY AGGREGATED RABBIT IgG (sARGG)

Lyophilized rabbit IgG was resuspended in Tris-NaCl buffer at a final concentration of 75 mg/ml by mixing at 22°C for 6 hours followed by 18 hours at 4°C. Insoluble material was removed by centrifugation (10,000 x g; 20 min;

4°C) and the rabbit IgG containing aggregates that had formed spontaneously was stored at 4°C at a final concentration of 6.0 mg/ml. The sARGG content of this rabbit IgG was determined by gel filtration chromatography using Bio-Gel A-5m. Protein content ($OD_{280\ nm}$) and RF activity (Methods, 6) of each fraction were measured. sARGG was only used within 7 days of preparation.

8.2 GLUTARALDEHYDE AGGREGATED RABBIT IgG (gARGG)

Lyophilized rabbit IgG (described above) was aggregated with glutaraldehyde, using a modification of the method of Avrameas and Ternynck (1969). Lyophilized rabbit IgG was resuspended in 0.015 M phosphate buffer, pH 7.0, and was reacted at a final concentration of 20 mg/ml with glutaraldehyde (final concentration 1 mg/ml). This mixture was incubated for up to 180 seconds at 22°C with vigorous stirring. Thereafter, an equal volume of 2 M ε-amino-n-caproic acid, or 2 M glycine-HCl, was added to stop the reaction. After incubation at 22°C for 60 minutes, insoluble aggregates were removed by centrifugation (10,000 x g; 20 min; 4°C). Soluble gARGG was exhaustively dialyzed against Tris-NaCl buffer and stored at 4°C at a final concentration of 2.0 mg/ml. gARGG was used within 2 weeks of preparation.

8.3 HEAT-AGGREGATED RABBIT IgG (hARGG)

Pool II rabbit IgG from ion-exchange chromatography of rabbit serum (Results 1.1.13) was centrifuged (90,000 x g; 75 min; 4°C) to remove insoluble and denatured material. Rabbit IgG was then heated at 71°C for up to 60 minutes. Insoluble material was then removed by centrifugation (10,000 x g; 20 min; 4°C) and the soluble hARGG were stored at 4°C at a final concentration of 2.0 mg/ml. hARGG was used within 3 months of preparation.

8.4 HEAT-AGGREGATED HUMAN IgG (hAHGG)

Human IgG at a concentration of 20 mg/ml was centrifuged (90,000 x g; 75 min; 4°C) and soluble IgG then heated at 63°C for 40 min. Aggregates were harvested by centrifugation (90,000 x g; 30 min; 4°C) and resuspended in Tris-NaCl buffer by stirring the aggregates with a spin-bar for 18 hours at 4°C. Insoluble aggregates were removed by centrifugation (10,000 x g; 20 min; 4°C) and the hAHGG, at a final concentration of 2.0 mg/ml was stored at 4°C. hAHGG was used within 3 months of preparation.

All aggregate preparations were clarified by centrifugation (10,000 x g; 20 min; 4°C) prior to use in nephelometric assays.

8.5 INSOLUBLE AGGREGATED IgG

Insoluble IgG aggregates for absorption studies were prepared from human IgG or pool II rabbit IgG (Results 1.1.13) by heating at 75°C for 60 min. The insoluble IgG aggregates were separated from the soluble aggregated or unaggregated IgG by centrifugation (3,000 x g; 10 min; 4°C) and were washed several times with Tris-NaCl buffer, followed by further slow speed centrifugation to separate the insoluble aggregates. The insoluble rabbit and human IgG aggregates at a final concentration of 2.0 mg/ml were stored at 4°C.

9 RHEUMATOID FACTOR ASSAYS

9.1 SHEEP CELL AGGLUTINATION TEST (SCAT)

The SCAT assay for rheumatoid factor activity was carried out according to the recommendations of the World Health Organization (Mackay and Ritts, 1979). In brief, Sheep erythrocytes (SRBC; Institute Armand Frappier, Laval, Que.) were sensitized with a sub-agglutinating concentration of rabbit anti-SRBC antibody (1:4 dilution of last dilution of anti-SRBC antibody causing agglutination of red cells; Difco Laboratories, Detroit, Michigan). Test sera were

heat-decomplemented (56°C; 30 min) and were absorped with unsensitized SRBC to remove heterophile antibodies. Doubling dilutions of the absorped sera were pipetted into V-bottomed microtiter plates and sensitized SRBC were added. Following incubation for 18 hours at 4°C, agglutination was visually determined, the SCAT titer defined as the last doubling dilution of serum causing definite agglutination of the sensitized SRBC.

9.2 LATEX FIXATION TEST (LFT)

LFT assays were done on microscope slides by mixing 50 µl of doubling dilutions of heat-decomplemented sera with 50 µl of human IgG coated latex polystyrene particles (Latex-Globulin reagent, Hyland Division, Travenol Laboratories, Costa Mesa, Ca.). After swirling for 1 minute, agglutination was visually determined and the LFT titer was defined at the last doubling dilution of serum causing definite agglutination.

9.3 NEPHELOMETRIC RHEUMATOID FACTOR ASSAY (NeRF)

9.3.1 HYLAND LASER NEPHELOMETER PDQ

9.3.1.1 PRINCIPLE OF USE

The Hyland laser nephelometer used in these studies is designed to measure the light scatter produced by the formation of antigen-antibody complexes. The measurement is made by passing a laser beam (wavelength 632.8 nm) through a solution containing the reactants (antigen and/or antibody) and detecting the intensity of the scattered light with a photomultiplier tube (PM tube). The amount of light scattered by the complex is proportional to the concentration of antigen being quantitated. The photomultiplier tube is mounted at such an angle (31^{o}) as to maximize the light scatter of the antigen-antibody complexes while minimizing the scatter of the individual reactants. Particulate material such as dust or other foreign contaminants which may cause fluctuations in the amount of light scattered are excluded from the final measurement by means of an electronic screening device which averages the light scatter over a preselected computing time (5-90 seconds). In experiments reported here, a 5 second computing time was used.

9.3.1.2 QUANTITATION OF LIGHT SCATTER

Antigen-antibody reactions are carried out in glass cuvettes (10 x 75 mm, borosilicate, test tubes; Fisher Scien-

tific, Dorval, Que.) and light scatter is reported as percent relative light scatter (%RLS). Light scatter is relative due to the lack of an absolute reference standard for light scatter. In the experiments reported here, the machine was calibrated daily by the reaction of a stable reference preparation of isolated rhuematoid factor (pRF) with heat-aggregated human IgG (hAHGG). The sensitivity of the PM tube is determined electronically by adjusting the amplification of the PM output signal. Controls for this amplification are expressed as sensitivity settings and all results presented here are corrected to a sensitivity setting of 3.3. Results of experiments are expressed as net light scatter (Net LS) which represents the total light scatter for a reaction mixture (eg. antigen and antibody mixture) less the sum of the light scatter of the individual reactants (eg. antigen or antibody alone).

9.3.2 NeRF ASSAY

The RF activity in human serum has been measured nephelometrically using a modification of the method of Lamberson, Gilmore and Osterland (1977).

9.3.2.1 BASIC NeRF ASSAY

25 µl of heat-decomplemented serum were added to Tris-NaCl buffer or Tris-NaCl buffer containing aggregated IgG to give a final volume of 1.0 ml. For some experiments, PEG was present in the incubation buffers, usually at a final concentration of 10 mg/ml (1%), which is specified in the Text. Aggregated IgG concentration is specified in the experiments reported in the Results section (Chapter IV) and was usually at a concentration of 50 µg/ml. Controls, containing identical concentrations of heat-decomplemented serum alone, aggregated IgG alone, and buffer alone were incubated concomitantly with the test mixture. Incubations for the routine assay of RF activity in serum were 60 minutes at 22°C. Other incubation times are indicated in the Text. Light scatter was measured for each test mixture and control mixture and the RF activity expressed as the net light scatter (Net LS) after subtraction of the light scatter of the individual constituents (serum; aggregates). The light scatter of buffer alone was used to zero the nephelometer. An example of a typical experimental design and results is shown in Table 3.1.

TABLE 3.1

TYPICAL NeRF ASSAY DESIGN AND RESULTS

Mixture	Constituents	Incubation Mixture		Typical LS (sensitivity 3.3)
Test	RF + Ag	RF-serum	25 µl	150
		hAHGG	25 µl	
		Buffer	950 µl	
			1000 µl	
Control	RF alone	RF-serum	25 µl	10
		Buffer	975 µl	
			1000 µl	
"	Ag alone	hAHGG	25 µl	12
		Buffer	975 µl	
			1000 µl	
"	Buffer alone	Buffer	1000 µl	0*

NeRF Activity (Net LS) = $LS_{RF + Ag} - (LS_{RF} + LS_{Ag} + LS_{Buffer})$

= 150 - (10 + 12 + 0*)

= 128

Ag = NeRF antigen (eg. hAHGG); * Buffer alone was used to zero the machine.

9.3.3.2 VARIATIONS OF THE NeRF ASSAY

9.3.3.2.1 NeRF ASSAY OF PURIFIED RHEUMATOID FACTORS

In some experiments, RF activity of purified RF was assayed by substituting this material for RF-serum. In these experiments, the basic methodology was used but the quantity of RF was expressed as a final concentration

(weight/volume) in the incubation mixture. The recovery of RF in absorption experiments was quantitated using concentration-RF activity curves. These curves were constructed by reacting known concentrations of purified RF with a constant concentration of aggregated IgG. The RF activity of samples of unknown concentrations were then quantitated by reference of these curves.

9.3.3.2.2 NeRF ANTIGEN ACTIVITY

9.3.3.2.2.1 NeRF ANTIGEN ACTIVITY OF AGGREGATED IgG

The basic NeRF assay was used to determine the activity of aggregated IgG antigens using either purified RF, heat-decomplemented RF-serum, or C1q. In these experiments, a constant concentration or RF or C1q was incubated with antigen at a concentration specified in the Text and NeRF activity quantitated.

9.3.3.2.2.2 NeRF ANTIGEN ACTIVITY OF NATIVE IgG

Native rabbit or human IgG was substituted for aggregated IgG in some experiments. In these experiments, the assay conditions were the same as the basic NeRF assay with the exception that only purified RF was reacted with

the native IgG.

9.3.3.2.3 VARIATIONS IN INCUBATION TIMES

The time course of the reaction of RF with native or aggregated IgG antigens was analyzed by measuring light scattering of incubation mixtures of RF and antigen as well as appropriate controls at specific time intervals. Experiments could be grouped according to the length of the time intervals at which light scatter was measured. These are: Routine Assays; Time Course Assays; and Kinetic Assays.

9.3.3.2.3.1 ROUTINE ASSAYS

In routine assays, RF activity was measured as an endpoint analysis, after the reaction between RF and antigen had stabilized. This type of assay was used mainly to quantitate RF activity in RF-serum and was generally observed after a constant incubation time of 60 minutes, or as noted in the Text. Results of the routine assays were used for comparison with the classical RF tests, the LFT and SCAT assays.

9.3.3.2.3.2 TIME COURSE ASSAYS

In addition to the end-point type of routine NeRF assay, time course assays were done in which Net LS was measured at discrete time intervals (10-120 min, as noted in the Text). Results of the time course studies were used to characterize antigen and isolated RF preparations, as well as, to determine the optimal times for end-point analysis used in the routine assays.

9.3.3.2.3.3 KINETIC ASSAYS

The third type of timed analysis that was used was the kinetic assay in which light scatter was measured continuously from the initiation of the RF-antigen reaction. This reaction generally measured during the first 10 minutes of the reaction. In brief, purified RF (final concentration 50 µg/ml) was mixed rapidly with a given concentration of antigen in Tris-NaCl buffer, with or without PEG as noted in the text, and the NET LS recorded continuously by a linear pen recorder. The peak rate of reaction of RF with each concentration of antigen was expressed as Net LS/min, and was obtained from the maximum slope of the individual pen recorder tracings. V_{max} and K_m values were derived by graphing the peak rate for each antigen concentration

against the antigen concentration used. V_{max} is the asymptote of the maximum rate obtained and K_m is the antigen concentration which produced half velocity ($V_{max}/2$).

9.3.3.3 SEQUENTIAL NeRF ASSAYS

Experiments to determine if RF-native IgG complexes would bind C1q were done as follows. Rheumatoid factor was mixed with native IgG in a final 1.0 ml volume of Tris-NaCl buffer containing 2% PEG and this mixture incubated at room temperature for 30 min. Thereafter, 25 ml C1q was added to give a final concentration of 25 µg/ml. After gentle mixing, this was incubated at room temperature for 30 min. Control incubations of the individual components and their combinations were made simultaneously at the same final concentrations as in the test mixture. In these experiments, the final concentrations of the reactants are detailed in the Results section. Light scattering of each incubation mixture was quantitated and Net LS of the added C1q was calculated. In other experiments 200 µl of heat-decomplemented serum that had no detectable RF activity, was substituted for the native IgG used above. The volume of buffer used was corrected to maintain a final incubation volume of 1.0 ml. Two different sera were used, one obtained from a healthy adult and a second from an adult

with acquired agammaglobulinemia.

10 ABSORPTION STUDIES

10.1 ABSORPTION OF RF WITH INSOLUBLE AGGREGATED IgG

pRF or mRF at a final concentration of 0.3 mg/ml were mixed with 1.0 gm of the insoluble rabbit or human aggregates in 5.0 ml. Tris-NaC buffer. Samples were mixed at 4°C for 24 hours and the RF-aggregate complexes were removed by centrifugation (90,000 x g; 30 min; 4°C). Supernatants were assayed for remaining RF activity by reacting 25 µl and 100 µl of the supernatants with a final concentration of 25 and 50 µg/ml of hARGG or hAHGG in 1.0 ml Tris-NaCl buffer. Net LS was determined after 60 minutes of incubation. The percent activity remaining was calculated by dividing the Net LS of RF remaining in the supernatant by the Net LS of the RF incubated without insoluble aggregates. In addition, the concentration of RF remaining in the supernatant was quantitated from standard activity-concentration curves and expressed as µg/ml of RF.

10.2 ABSORPTION OF RF WITH NATIVE IgG

For the absorption of RF, native rabbit or human IgG was mixed with a RF in buffers containing PEG, and removing the subsequent precipitates by centrifugation (13, 000 x g; 30 min; 4°C). Purified RF at final concentrations of 150 or 300 µg/ml was absorbed with native IgG at concentrations specified in the text, using PEG at concentrations which are also indicated in the text. The RF-native IgG mixture was mixed for 18 hours at 4°C. RF remaining in the supernatant, after removal of precipitates, was quantitated by reacting 100 µl of the supernatant with 25 µg hARGG or hAHGG for 60 min at room temperature in 1.0 ml Tris-NaCl containing no PEG. RF activity and RF concentration remaining in the supernatant after absorption were calculated as previously described (Methods 10.1). In some experiments, the precipitate was resolubilized, using Tris-NaCl buffer, to the original incubation volume, and RF activity and RF concentration similarly quantitated. IgG in the supernatants or in the resolubilized precipitates did not interfere appreciably with the assay of RF with aggregated IgG, since addition of native IgG to control NeRF assays, at concentrations present in the supernatants, changed Net LS by less than 16%.

11 STATISTICAL ANALYSIS

Data obtained from NeRF assays and from the SCAT and LFT[2] assays were compared using linear regression analysis and fitting of regression lines by the least squares method. Significance limits (p values) for the correlation coefficients (r values) were determined by reference to scientific tables in Documenta Geigy.

CHAPTER IV

EXPERIMENTAL PROCEDURES AND RESULTS

1 CONDITIONS FOR NEPHELOMETRIC RHEUMATOID FACTOR ASSAY (NeRF)

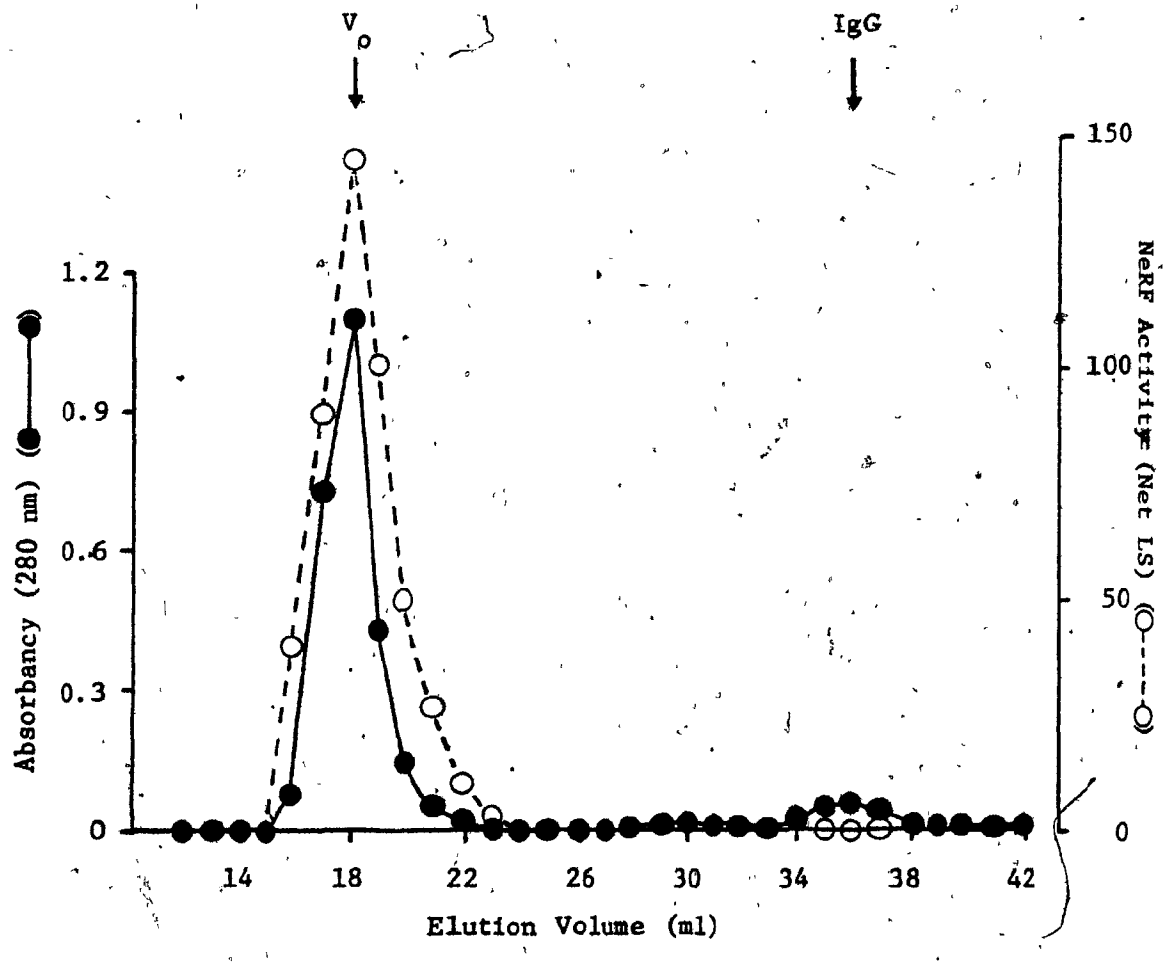
1.1 ANTIGEN PREPARATION

1.1.1 HEAT-AGGREGATION OF HUMAN IgG (hAHGG)

In order to determine the relative quantities of aggregated and unaggregated gamma globulin in the hAHGG preparation, the material was subjected to gel filtration. When these fractions were reacted with heat-decomplemented, RF-containing serum (Latex titer = 1:1024), only materials eluting in the void volume of the column were reactive, Fig. 4.1. Fractions which eluted corresponding to an estimated molecular weight of 7S IgG were unreactive in this assay system.

FIGURE 4.1

GEL FILTRATION CHROMATOGRAPHY OF hAHGG



1.0 ml sample of heat-aggregated human IgG (2.0 mg/ml) was chromatographed on Bio-Gel A-5m (0.9 x 90 cm.) and 1.0 ml fractions collected. Absorbancy (280 nm.) and NeRF antigen activity (Net LS) are illustrated. IgG, elution peak of native IgG chromatographed separately; V_o, void volume.

1.1.2 TIME COURSE OF THE REACTION OF hAHGG WITH RF-CONTAINING SERUM

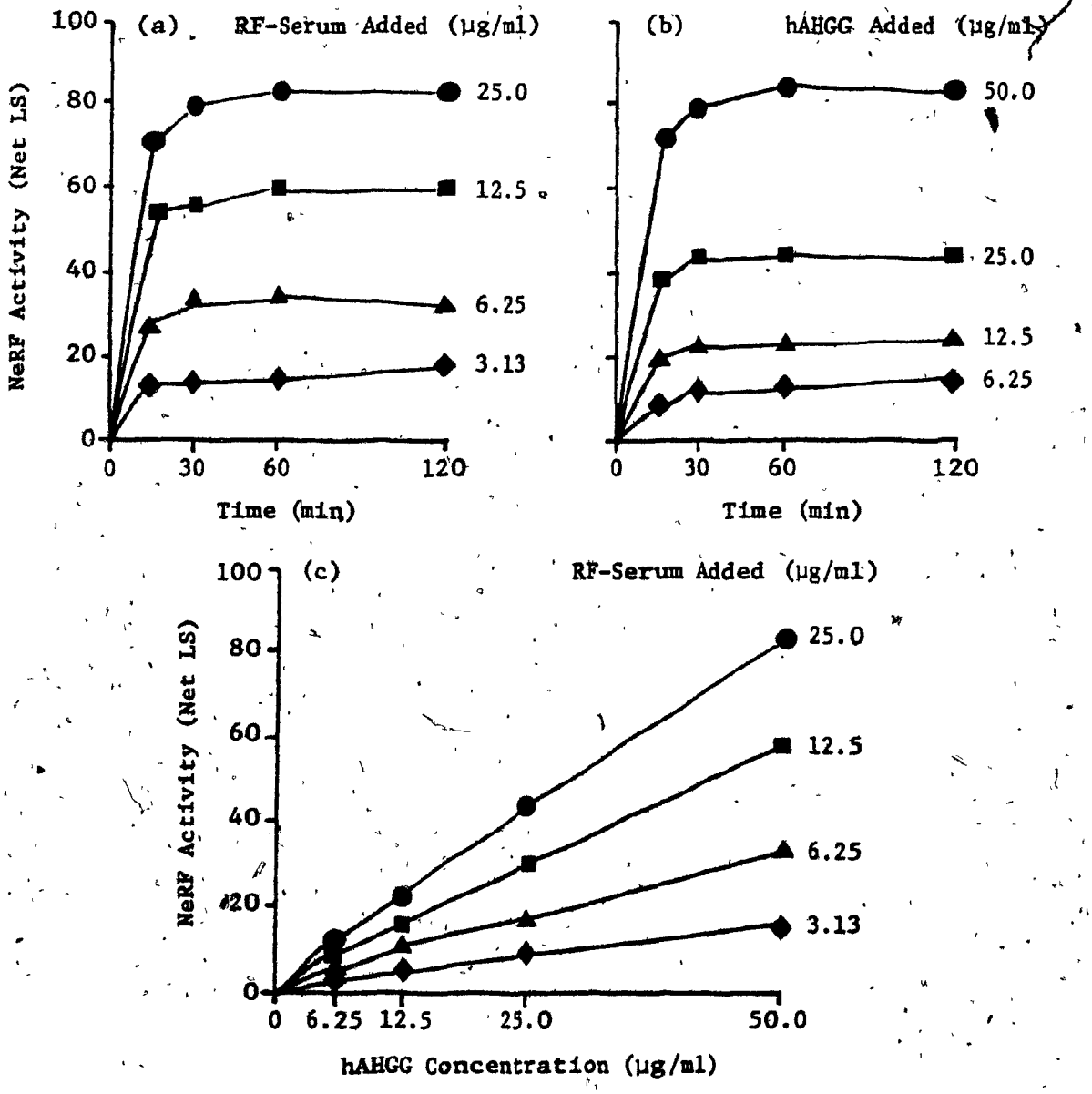
The time course of the reaction of 50 µg. of the human aggregates with various dilutions of a rheumatoid surum is shown in Fig. 4.2a. The amount of light scatter increased with the time of incubation and at 60 minutes the reaction reached a plateau phase and was constant for 60 minutes thereafter. Similar results were obtained when a constant amount of serum was reacted with various concentrations of the aggregated human IgG, Fig. 4.2b. When these results are plotted at the plateau time reached at 60 minutes, the amount of light scatter obtained is dependent on both the amount of RF-containing serum added as well as the concentration of the antigen, hAHGG, used, Fig. 4.2c

1.1.3 SPONTANEOUS AGGREGATION OF LYOPHILIZED RABBIT IgG UPON RESOLUBILIZATION (sARGG)

Having established the conditions for the nephelometric measurement of RF in serum using heat-aggregated human IgG as the antigen, our attention focused on the aggregation of rabbit IgG. Resolubilization of lyophilized rabbit IgG resulted in the formation of spontaneous aggregates which were fractionated on BioGel A5m, Fig. 4.3. un-

FIGURE 4.2

TIME COURSE FOR THE REACTION OF RF-SERUM WITH hAHGG



Heat-aggregated human IgG was reacted with RF-serum (LFT 1:1024) in 1.0 ml Tris-NaCl buffer containing 1% PEG and Net LS determined as indicated (a;b) or after 60 min incubation (c). hAHGG (50 µg/ml) was reacted with RF-serum at the concentrations indicated (a); serum (25 µl/ml) was reacted with hAHGG at the concentrations indicated (b); or hAHGG at the concentrations indicated was reacted with different concentrations of RF-serum (c).

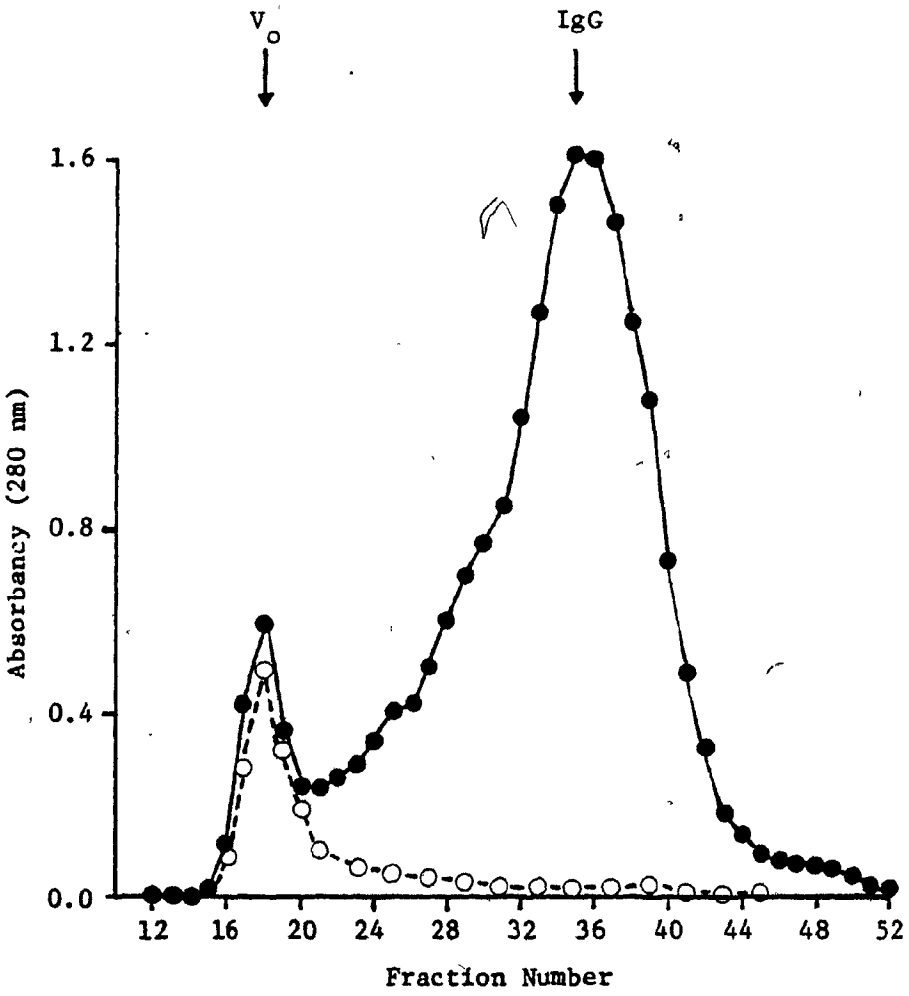
like the human aggregates, it was apparent from the elution profile that only a small amount of the rabbit IgG was aggregated yielding a small void peak. Aggregates with molecular weights of less than 5 x 10^6 daltons and greater than a 7S IgG molecule (160,000 daltons) were also obtained. When the fractions were tested for NeRF antigen activity, the majority of the activity was recovered in the void volume. The fractionated sARGG was pooled into six fractions for further analysis.

1.1.4 NeRF ANTIGEN ACTIVITY OF FRACTIONATED sARGG

The six pooled fractions, labelled I-VI, were assayed for protein content and adjusted to have a concentration of 2.0 mg/ml. The fractions were then assayed in the nephelometer for reactivity with a heat-decomplemented RF-serum. The results of this experiment are summarized in Table 4.1. Fraction I contained the most NeRF antigen activity while fraction II had only half of this activity and the remaining fractions contained almost no activity. Rechromatography of fraction I showed it contained aggregates, found in the void volume of the BioGel A5m column, Fig. 4.3.

FIGURE 4.3

GEL FILTRATION CHROMATOGRAPHY OF sARGG



1.0 ml sample of spontaneously aggregated rabbit IgG (●) (6.0 mg/ml) was chromatographed on Bio-Gel A-5m (2.5 x 48 cm) and 3.0 ml fractions collected. 1.0 ml sample of isolated Fraction I aggregates (○) (2.0 mg./ml.) were chromatographed seperately. IgG, elution volume of native IgG; V_o, void volume.

TABLE 4.1

NeRG ANTIGEN ACTIVITY OF POOLED FRACTIONS OF sARGG

Pool	Fraction Nos.	NeRF Activity (unfractionated = 100%)
I	16 - 20	110%
II	21 - 26	52%
III	27 - 31	6%
IV	32 - 38	2%
V	39 - 45	1%
VI	46 - 52	1%

Fractions from gel filtration of sARGG on BioGel A5m were pooled and adjusted to a final concentration of 50 µg/ml when assayed with a RF-containing serum for NeRF antigen activity. Activity was expressed as a percent of unfractionated sARGG assayed at a concentration of 150 µg/ml.

1.1.5 COMPARISON OF NeRF VALUES OBTAINED WITH UNFRACTIONATED sARGG AND FRACTION I sARGG

Fraction I aggregates and the unfractionated sARGG were compared by assaying 24 heat-decomplemented RF-sera with each of these antigens. Except for differences in the NeRF antigen concentrations used (Fraction I, 50 µg/ml; unfractionated sARGG, 150 µg/ml), the two rabbit antigen preparations gave similar nephelometric results. The r-value of 0.83 was significant ($p < 0.001$) and the relationship was linear. The Fraction I aggregates were not stable for more than three days and tended to become insoluble after this time rendering them too troublesome to prepare and use reliably. The unfractionated sARGG could be formed

quite simply and new batches could be made with reasonable reproducibility when needed. The unfractionated spontaneous rabbit aggregates were used for further studies.

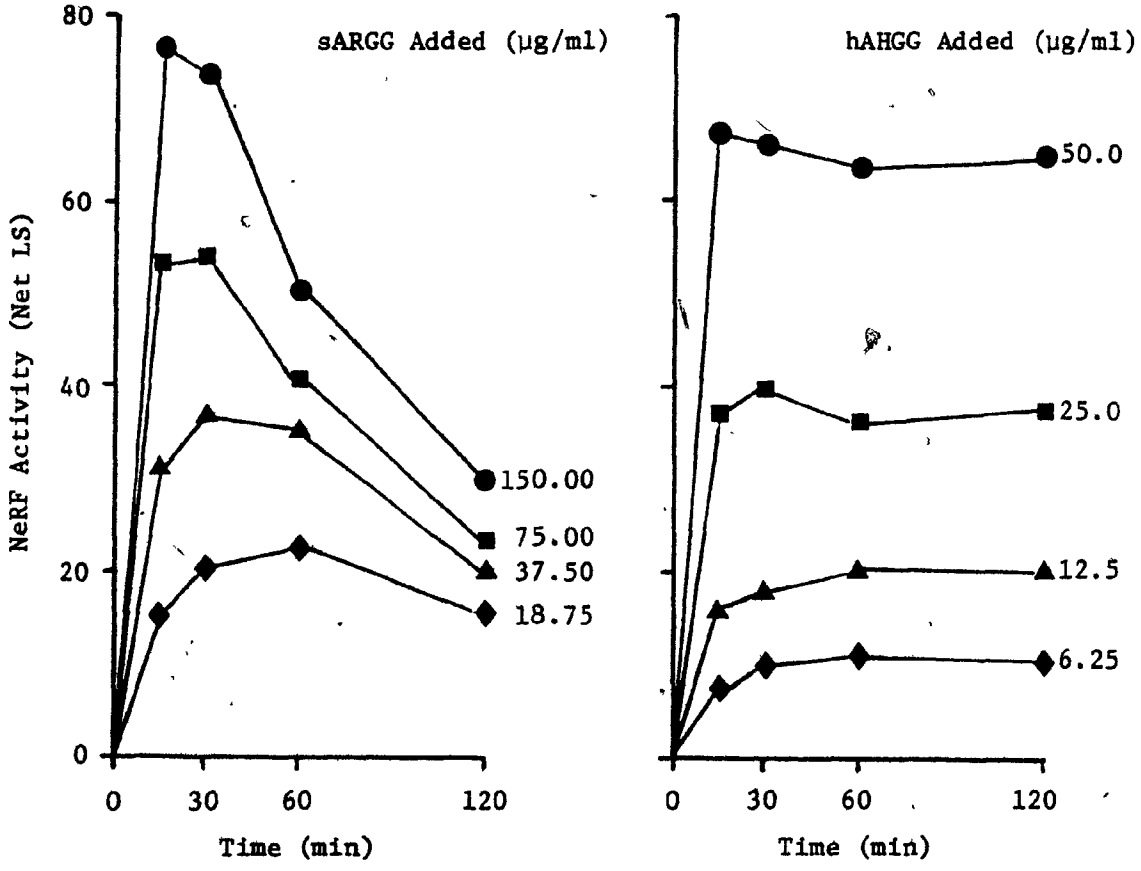
I.1.6 TIME COURSE OF THE REACTION OF sARGG WITH RF-CONTAINING SERUM

The results of the time course study of the nephelometric reaction between a constant amount of serum and various concentrations of either sARGG or hAHGG is shown in Fig. 4.4. For both antigens there was a concentration-dependent increase in the reaction. With sARGG, there was an initial increase in light scattering but after 30 minutes there was a decrease, while the reaction with hAHGG plateaued and remained stable throughout the incubation period. It was possible however to compare the reaction of RF with these two antigens after 30 minutes of incubation. The spontaneously aggregated rabbit aggregates tended to become insoluble with extended storage and failed to produce a plateau unlike their human counterpart. In order to fulfill the need for more reliable preparations of soluble aggregated rabbit IgG alternative methods of aggregation were investigated.

FIGURE 4.4

TIME COURSE OF THE REACTION OF RF-SERUM

WITH sARGG AND WITH hAHGG



Heat-decomplemented RF-serum (LFT 1:1024; final concentration 25 ul/ml was reacted with various concentrations of sARGG and hAHGG in 1.0 ml Tris-NaCl buffer containing 1.0% PEG. Net LS was measured after the incubation times as indicated.

1.1.7 ALTERNATIVE METHODS OF RABBIT IgG AGGREGATION

Although the literature mentions that rabbit IgG may be aggregated by heating at 71°C., it was found that the lyophilized and resuspended rabbit IgG did not form soluble aggregates upon heating. Aggregation with ethyl-chloroformate (Ilter and Turner, 1973) also produced insoluble aggregates. By decreasing the concentration of glutaraldehyde and the incubation time before addition of the amino-group donor to stop the reaction, a modified method of glutaraldehyde aggregation (Avrameas and Ternynck, 1969) produced soluble rabbit aggregates.

1.1.8 CONDITIONS FOR GLUTARALDEHYDE AGGREGATION OF RABBIT IgG (gARGG)

The final concentrations of 0.1% glutaraldehyde and 2.0% rabbit IgG were used and the reaction was carried out in Tris-NaCl buffer, ph 7.4. As the length of the incubation period appeared to be a determining factor in the glutaraldehyde cross-linking of free amino groups on the rabbit IgG molecules, experiments were done using incubation times of 30, 60 and 180 seconds. A further consideration was the choice of amino-donor used to stop the reaction. The results of these experiments are illustrated in Table 4.2.

TABLE 4.2

CONDITIONS FOR GLUTARALDEHYDE AGGREGATION OF RABBIT IgG

Incubation Time (sec.)	Amino-Donor	Centrifugation g	Centrifugation Time (min.)	NeRF Activity (sARGG = 100%)
30	Glycine	10,000	10	15%
30	"	10,000	30	3%
30	EACA	10,000	10	176%
60	"	uncentrifuged		311%
60	"	10,000	10	300%
180	"	10,000	10	220%

Rabbit IgG was reacted with glutaraldehyde for the times specified and the reaction terminated with the amino-donor indicated, after which insoluble material was removed by centrifugation. The aggregates were then assayed at a final concentration of 50 µg/ml, using an RF-serum (LFT 1:1024), and their activity expressed as a percent of the activity of sARGG, assayed at the same final concentration.

Glycine was found to be unsuitable as an amino group donor because the aggregates formed were insoluble. When ε-amino-n-caproic acid (EACA) was used, however, the aggregates remained soluble and it was possible to determine the optimal incubation period. 60 seconds of incubation followed by the addition of EACA to stop the reaction gave an increase of 300% in NeRF antigen activity with a RF-containing serum over the Net LS value obtained with the same concentration of sARGG.

1.1.9 COMPARISON OF NeRF ANTIGEN ACTIVITY OF sARGG vs gARGG

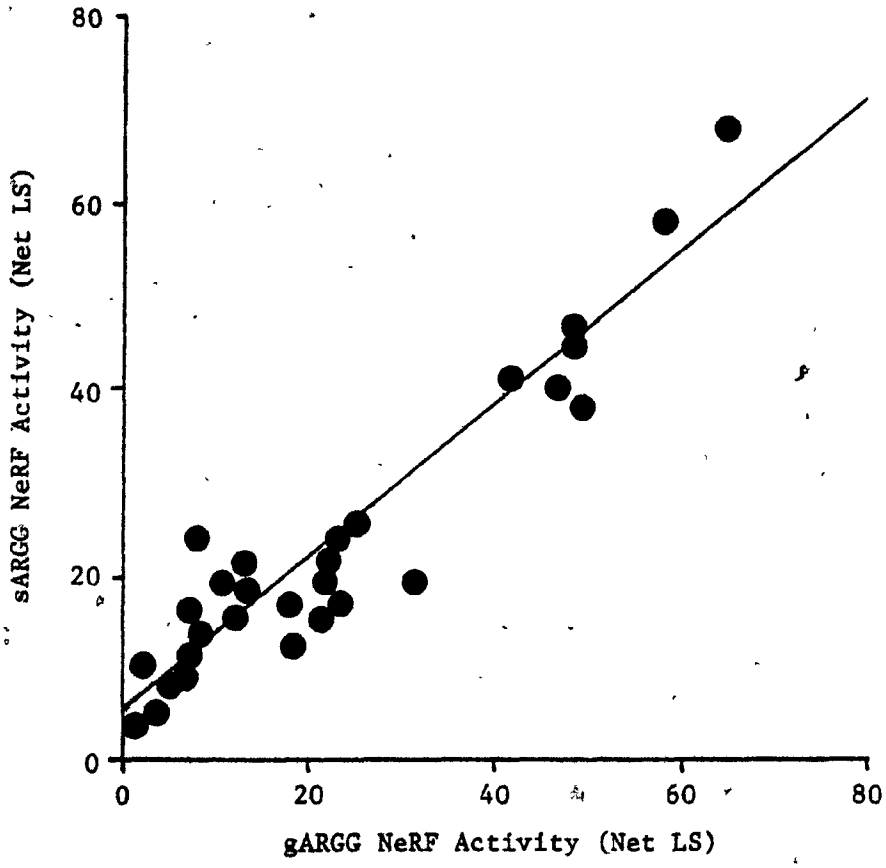
The Net LS values for 29 RF-containing sera obtained with sARGG and gARGG are compared in Fig. 4.5. The glutaraldehyde aggregates reacted in a similar fashion as the spontaneous aggregates and a linear relationship was found ($r = 0.94$; $p < 0.001$)

1.1.10 TIME COURSE OF THE REACTION OF gARGG WITH RF-CONTAINING SERUM

In addition to the similar reactivity of the glutaraldehyde and spontaneous rabbit aggregates, illustrated in Fig. 4.5, and the increased stability of the gARGG reagent, the time course of the reaction of the glutaraldehyde aggregates with a RF-containing serum was similar to that obtained with hAHGG. In Fig. 4.6, the time course for the nephelometric reaction of a RF-serum with either gARGG or hAHGG are compared. The glutaraldehyde aggregates were found to react in a similar manner to the human aggregates. The gARGG reaction plateaued and remained stable, differing from the sARGG reaction which demonstrated a decrease in net light scatter after 30 minutes of incubation.

FIGURE 4.5

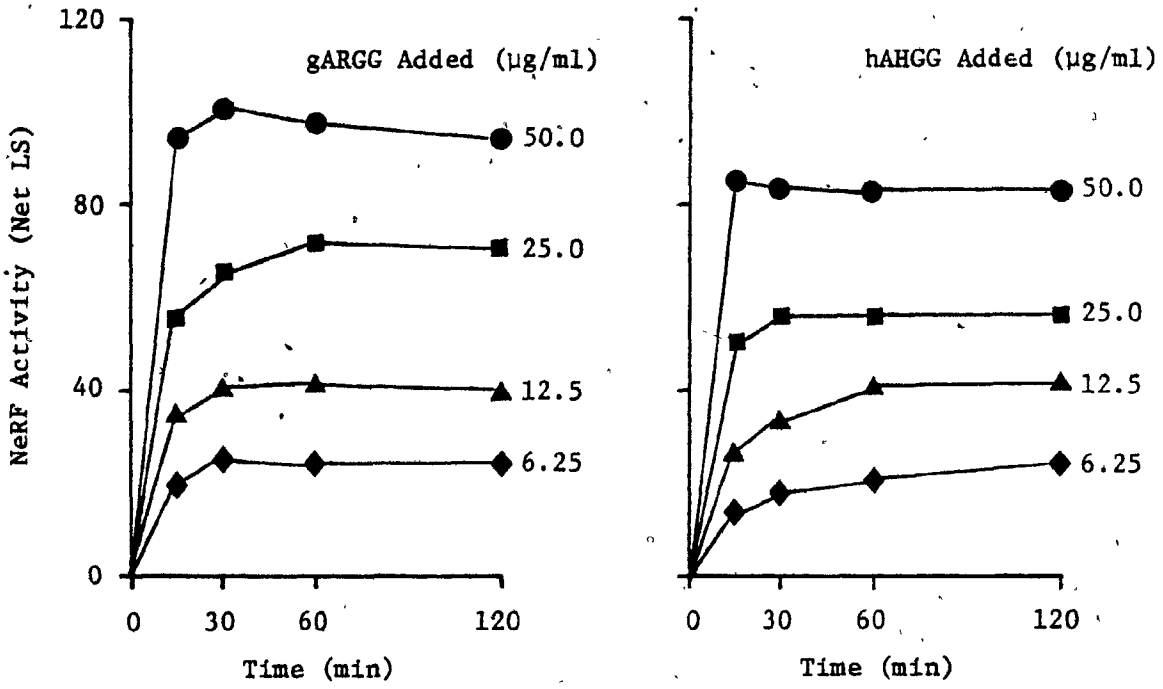
COMPARISON BETWEEN NeRF ACTIVITY OBTAINED WITH gARGG AND WITH sARGG



Net LS values for the nephelometric reaction of 29 heat-decomplemented RF-sera with gARGG and with sARGG were compared by linear regression analysis. Data obtained were: equation of the regression line, $Y = 0.81 X + 5.25$; $r = 0.94$; $p < 0.001$.

FIGURE 4.6

TIME COURSE OF THE REACTION OF RF-SERUM WITH gARGG AND WITH hAHGG



Heat-decomplemented RF-serum (LFT 1:1024; final concentration 25 µl/ml was reacted with various concentrations of gARGG and hAHGG in 1.0 ml Tris-NaCl buffer containing 1.0% PEG. Net LS was measured after the incubation times as indicated.

1.1.11 REPRODUCIBILITY OF AGGREGATE PREPARATIONS

An important consideration in developing an antigen for use in the NeRF assay is that new preparations must react in a manner indistinguishable from previous batches. New preparations of gARGG were shown to react in a similar fashion as older preparations when identical concentrations of both two week old and freshly prepared gARGG were tested in the NeRF assay with 15 RF-containing sera. In this experiment a regression coefficient of $r = 0.99$ ($p < 0.001$) emphasized the reproducibility of glutaraldehyde aggregate preparation.

1.1.12 WITHIN-RUN REPRODUCIBILITY OF gARGG AND hAHGG

Within-run reproducibility was used to characterize the gARGG and the results of quadruplicate tests of four different RF-sera are summarized in Table 4.3. From these data, the coefficients of variation for the NeRF assays with the rabbit and human antigens are 3.3% ± 0.3% ($\bar{X}$ ± S.D.) and 4.3% ± 1.6% respectively. With the preparation of the glutaraldehyde-aggregated rabbit IgG, we had produced an antigen which reacted quantitatively and qualitatively similar to a homologous preparation of human aggregated IgG in the NeRF assay. Preparation of heat aggregated rabbit gamma

globulin was undertaken to provide another antigen for comparative studies. As the lyophilized rabbit gamma globulin was unsuitable for these experiments, rabbit IgG was isolated from rabbit serum.

TABLE 4.3

WITHIN-RUN REPRODUCIBILITY OF gARGG AND hAHGG ANTIGENS

Antigen	Serum 1	Serum 2	Serum 3	Serum 4
gARGG	81.5 ± 0.7*	35.5 ± 0.3	65.6 ± 0.5	82.4 ± 0.7
hAHGG	75.1 ± 0.7	41.5 ± 0.6	64.7 ± 0.4	88.8 ± 1.2

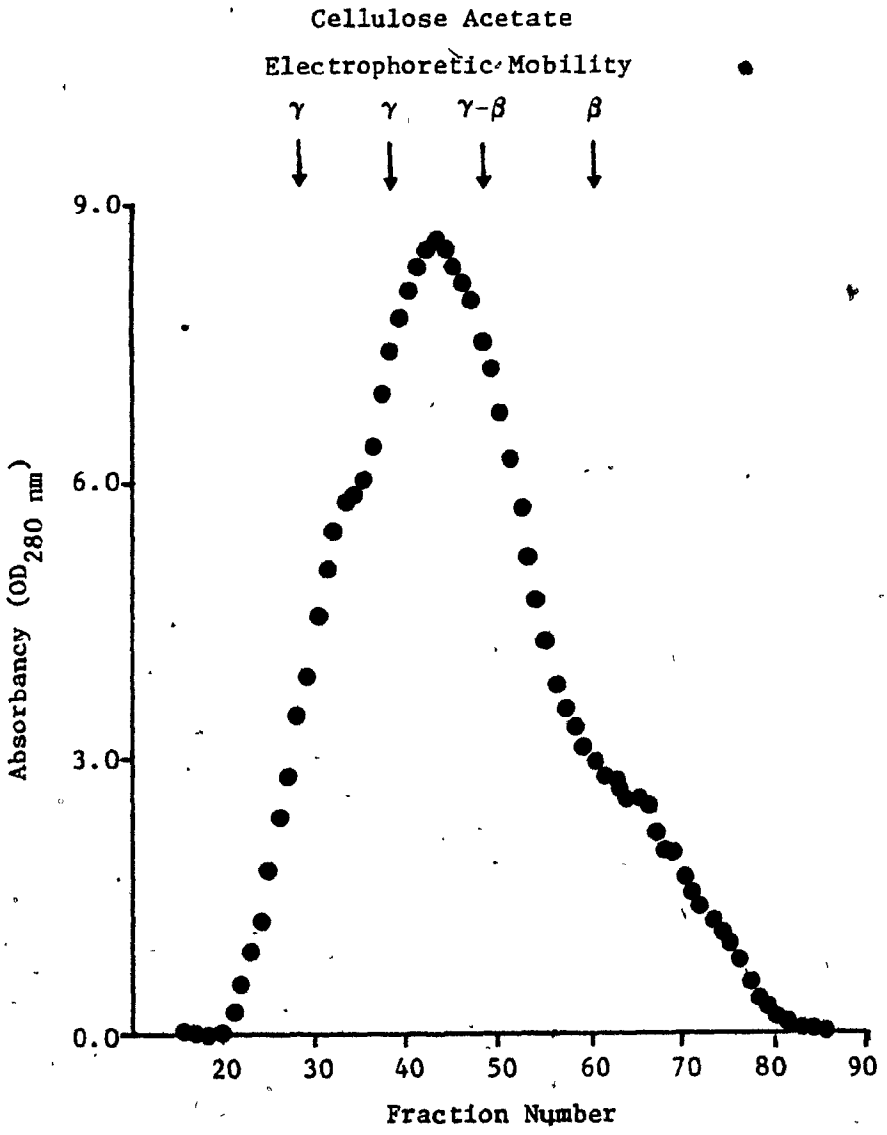
* All figures given are Net LS ± S.E.M. for quadruplicate assays.

1.1.13 ION-EXCHANGE CHROMATOGRAPHY OF RABBIT SERUM

The results of DEAE-cellulose chromatography of rabbit serum are shown in Fig. 4.7. Fractions 20-32 were relatively clear in appearance while the remaining tubes were turbid, containing a lipid material. Electrophoresis of fractions 28, 38, 51 and 60 showed tubes 28 and 38 contained protein with γ mobility. Fraction 51 contained material of predominantly γ mobility whereas fraction 60 contained predominantly β mobility material. Fractions 24 to 32, which were not turbid, were pooled (Pool I) and frac-

FIGURE 4.7

ION-EXCHANGE CHROMATOGRAPHY OF RABBIT SERUM



Rabbit serum was subjected to 50% ammonium sulfate fractionation, the precipitate harvested by centrifugation (5,000 x g; 10 min.; 4°C.) and resuspended in Tris - NaCl buffer. The resuspended precipitate was dialyzed ehaustively against 0.005 M phosphate buffer, pH 7.4, and 200 ml chromatographed on DEAE-cellulose, equilibrated with this buffer. 5.0 ml. fractions of the material not adsorbed to the column were collected. Absorbancy (280 nm) and cellulose acetate electrophoretic mobility of the fractions are illustrated.

tions 33 to 44, which were turbid, were pooled (Pool II).

1.1.14 FRACTIONATION OF POOLS I & II BY GEL FILTRATION

Pools I and II from the ion-exchange chromatography of rabbit serum were fractionated on BioGel A5m. Pool I eluted as a single peak containing 98% of the protein chromatographed. The elution volume of this peak corresponded to that of purified 7S IgG. Pool II, however, showed contamination with a high molecular weight substance which was turbid, presumably a lipid, accounting for the marked turbidity of this pool. This peak eluted near the void volume and accounted for 22% of the pool II material chromatographed.

1.1.15 DELIPIDATION OF RABBIT IgG POOL II

Pool II was delipidated by freon-treatment and was no longer turbid. When the delipidated rabbit IgG pool II was chromatographed on BioGel A5m, the material eluting near the void was reduced by 73% compared with non-delipidated pool II. The remainder of the delipidated pool II IgG (93%) eluted at the same position as purified 7S IgG.

1.1.16 HEAT AGGREGATION OF RABBIT IgG (hARGG)

Rabbit IgG was aggregated by heating at 71°C. With increasing incubation at 71°C., the percent of delipidated pool II IgG which aggregated, increased as shown in table 4.4. This increase was quantitated by gel filtration (BioGel A5m) and material eluting as aggregated and an unaggregated IgG was measured. Pool I when incubated at 71°C, formed insoluble aggregates unlike pool II IgG. Incubation of pool II Rabbit IgG for 30 minutes at 71°C. uniformly produced aggregates useful for nephelometry and was used for preparation of hARGG.

TABLE 4.4

EFFECT OF HEATING RABBIT IgG AT 71° C.

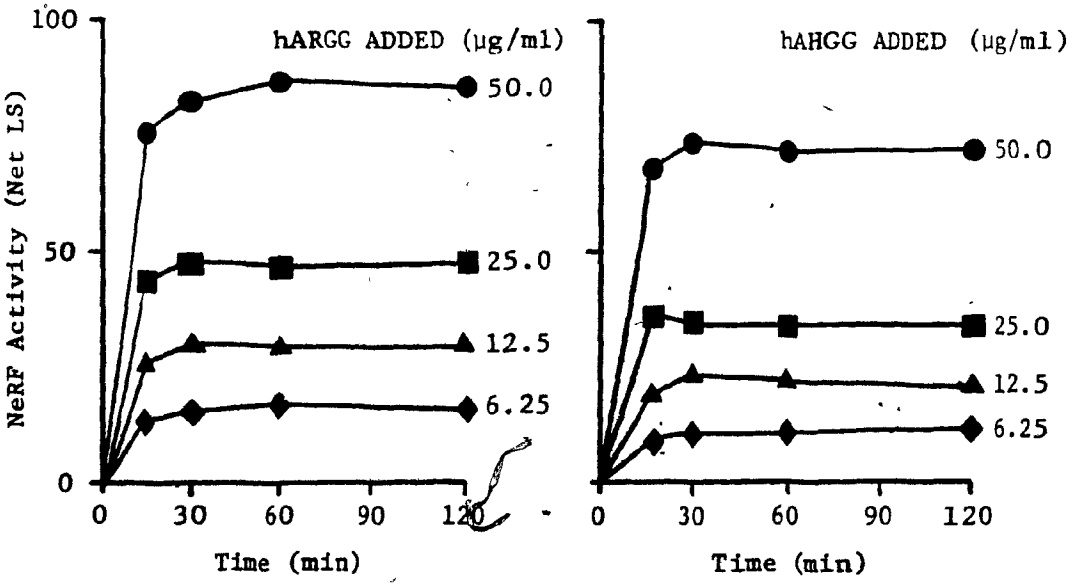
Incubation Time (min.)	Aggregated IgG (% of Total)	Unaggregated IgG (% of Total)
0	6%	94%
10	23%	77%
30	44%	56%
60	61%	39%

1.1.17 TIME COURSE OF THE REACTION OF hARGG WITH RF-CONTAINING SERUM

The time course for the reactions of a RF-serum with varying amounts of hARGG or hAHGG is illustrated in Fig. 4.8. Each antigen showed the same profile which is characterized by a concentration-dependent increase in light scattering with time, an eventual stabilization of the reaction and virtually indistinguishable reactions with

FIGURE 4.8

TIME COURSE OF THE REACTION OF RF-SERUM WITH hARGG AND WITH nAHGG



Heat-decomplemented RF-serum (LFT 1:1024) was reacted with various concentrations of hARGG and hAHGG. Net LS was measured after the incubation times as indicated.

the same serum.

1.1.18 REACTION OF CHICKEN IgG WITH RF-SERUM

Chicken IgG which had been aggregated by brief exposure to heating at 63°C was mixed at a final concentration of 50 µg/ml with 20 µl of heat-decomplemented RF-serum in 1.0 ml Tris-NaCl buffer containing 1.0% PEG. The reaction of aggregated chicken IgG was found to be negligible when compared to the reaction of hAHGG with RF-sera after 60 minutes of incubation. Net LS values for the NeRF reaction of chicken IgG with 18 RF-sera averaged 0.1 ± 1.6 ($\bar{X}$ ± SD) while these same sera reacted with hAHGG gave an average Net LS value of 41.7 ± 29.5.

1.2 ASSAY CONDITIONS

As the optimal incubation times and antigen concentrations had been established during the time course studies, buffer conditions for the NeRF assay were investigated.

1.2.1 EFFECT OF POLYETHYLENE GLYCOL (PEG)

In the previous experiments to establish the antigen concentrations and incubation times, polyethylene glycol (1.0% w/v) had been used as a complexing aid (after

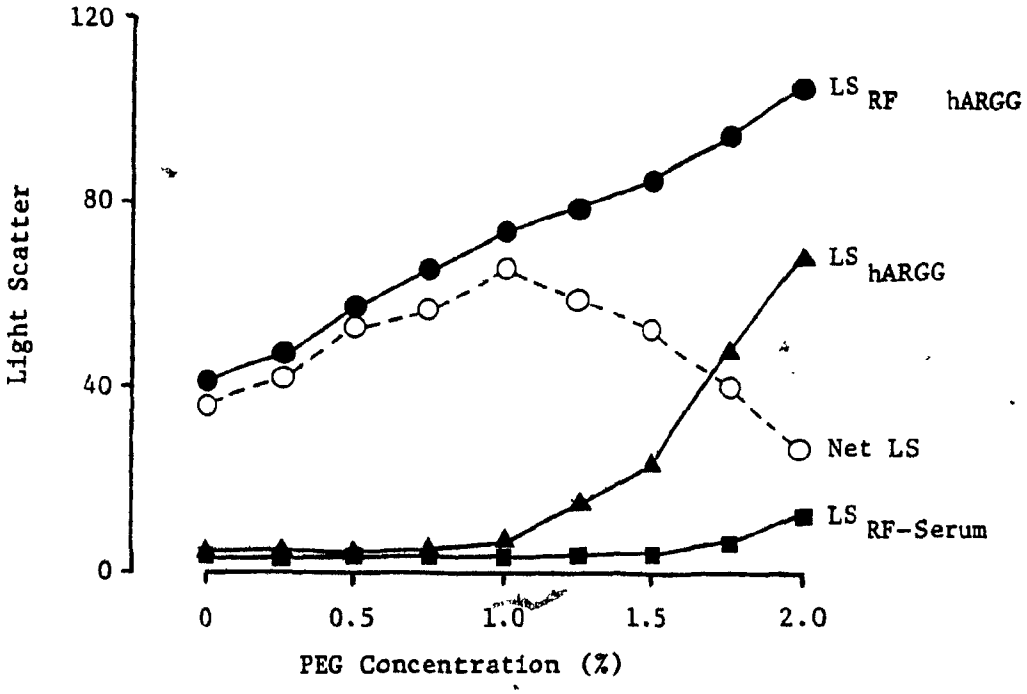
Lamberson et al., 1977). Although incubation of aggregated IgG with RF-containing serum results in an increase in light scatter in the absence of PEG, the addition of PEG results in the enhancement of the light scatter obtained. The effect of various concentrations of PEG (MW = 6000) on the reaction of rabbit aggregates with an RF-serum is shown in Fig. 4.9. Similar results were found with the human aggregates (data not shown). PEG concentrations between 0.25 and 1.0% resulted in an increase in net light scattering which was dependent upon the amount of PEG included in the reaction buffer. At concentrations of polyethylene glycol in excess of 1.0%, the Net LS decreased as a result of the massive increase in aggregate LS and subsequent formation of visible complexes which erratically scattered light. 1.0% PEG included in the reaction buffer resulted in a 79% enhancement of net light scattering without substantially increasing aggregate LS, and as a result, this concentration of PEG was used in routine assays for testing serum.

1.2.2* EFFECT OF pH

The effect of pH on the NeRF assay results is shown in Fig. 4.10. The reaction between a RF-serum and hARGG is maximal over the pH range 7.0 - 10.0.

FIGURE 4.9

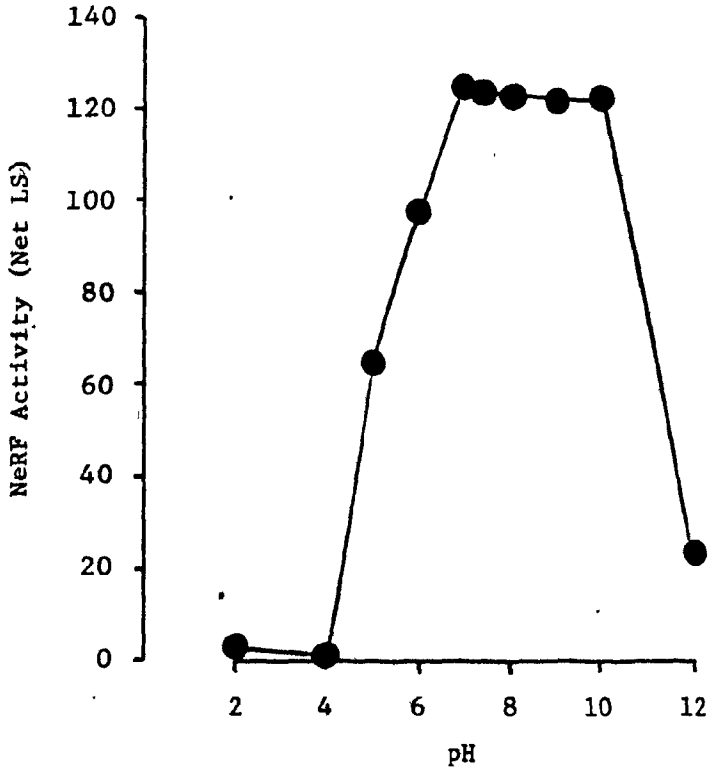
EFFECT OF PEG ON NeRF ACTIVITY



Heat-decomplemented RF-serum was reacted with hARGG in Tris-NaCl buffer containing no PEG or various concentrations of PEG as indicated. Light scatter of the individual reactants, as well as Net LS were determined after 60 minutes of incubation. Final concentrations of reactants in 1 ml buffer were: RF-serum, 25 µl/ml; hARGG 50 µg/ml.

FIGURE 4.10

EFFECT OF pH ON NeRF ACTIVITY



Heat-decomplemented RF-serum was reacted with hARGG in Tris-NaCl buffer containing 1.0% PEG, pH as indicated. Net LS was measured after 60 minutes of incubation. Final concentrations of reactants in 1.0 ml buffer were: serum, 25 µl/ml; hARGG, 50 µg/ml.

1.2.3 EFFECT OF NaCl CONCENTRATION

A concentration of 0.015 M NaCl was found to be optimal for the detection of RF by hARGG, Fig. 4.11. Since IgM is generally regarded to be a euglobulin, the possibility of loss of RF due to low ionic strength buffers was avoided by using buffers containing isotonic saline concentrations (0.15 M NaCl). The effect of decreasing concentrations of NaCl less than 0.15 M on the solubility of rheumatoid factor is shown in Table 4.5. The serum used for

TABLE 4.5

SOLUBILITY OF RF IN LOW IONIC STRENGTH BUFFERS

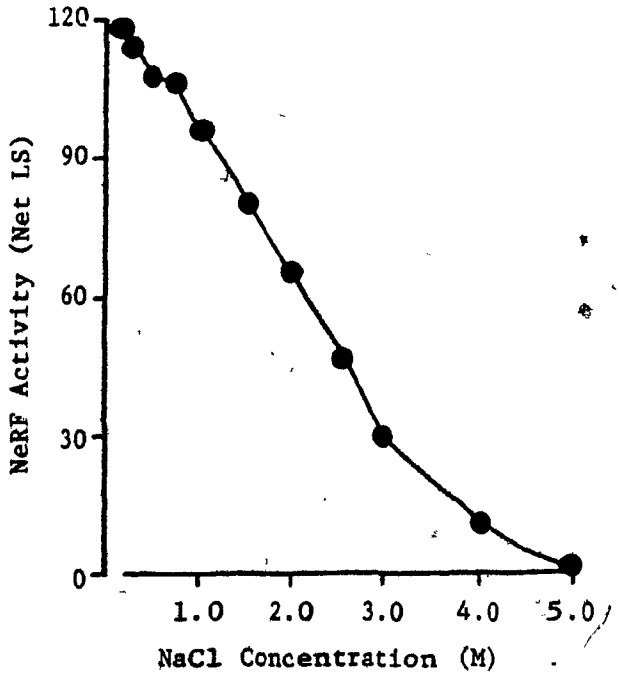
NaCl Concentration (M)	NeRF Activity (undialyzed = 100%)	
	Supernatant	Pellet
0.150	99%	0%
0.100	75%	13%
0.050	13%	50%
0.010	5%	68%
0.005	5%	72%

Following exhaustive dialysis of RF-containing serum against Tris-NaCl buffer (NaCl concentration as indicated), the samples were centrifuged and the pellets resuspended in Tris-NaCl buffer (0.15 M NaCl) to original volume. Supernatants and pellets were assayed for NeRF activity and results were expressed as percent of undialyzed activity.

this study showed a striking decrease in solubility of RF, as evidenced by its precipitation upon centrifugation, in buffers containing less than 0.15 M NaCl. For routine testing, the following buffer conditions were used: NaCl, 0.15 M; Tris, 0.015 M; Polyethylene glycol, 1.0% w/v; ph 7.4.

FIGURE 4.11

EFFECT OF NaCl CONCENTRATION
ON NeRF ACTIVITY



Heat-decomplemented RF-serum was reacted with hARGG in Tris-NaCl buffers containing 1% PEG, NaCl concentration as indicated. Net LS was measured after 60 minutes of incubation. Final concentrations of reactants were: serum, 25 µl/ml; hARGG, 50 µg/ml.

1.2.4 EFFECT OF FREON-TREATMENT OF SERUM SAMPLES

Lipid in serum samples can interfere with nephelometric assays due to the background light scatter of lipids. 36 RF-sera were tested in the NeRF assay before and after delepidation with Freon-12. Freon treatment was found to have no effect on the Net LS of these sera, when assayed before and after delipidation (correlation coefficient = 0.95; $p < 0.001$). Serum LS, however, decreased from 50.4 ± 15.3 ($\bar{X}$ ± S.D.) to 4.5 ± 1.6 for these sera. As a result, delipidation was performed routinely on samples which were visually lipemic. Freon treatment of these sera decreased their background scatter and allowed all samples to be tested at the same sensitivity settings in the nephelometer and eliminated the need to change the settings for individual samples during batch testing.

2 COMPARATIVE STUDIES OF RF ACTIVITY IN HUMAN SERA AS DETERMINED BY THE CLASSICAL METHODS AND BY NeRF ASSAY

Comparative studies have been done using the sheep cell agglutination test, the latex fixation test and the nephelometric RF assay with both rabbit and human antigens. Data have already been presented which show the similar

NeRF reactivity between the sARGG and gARGG antigens with 29 RF-sera (Sect. 1.1.9; Fig. 4.5).

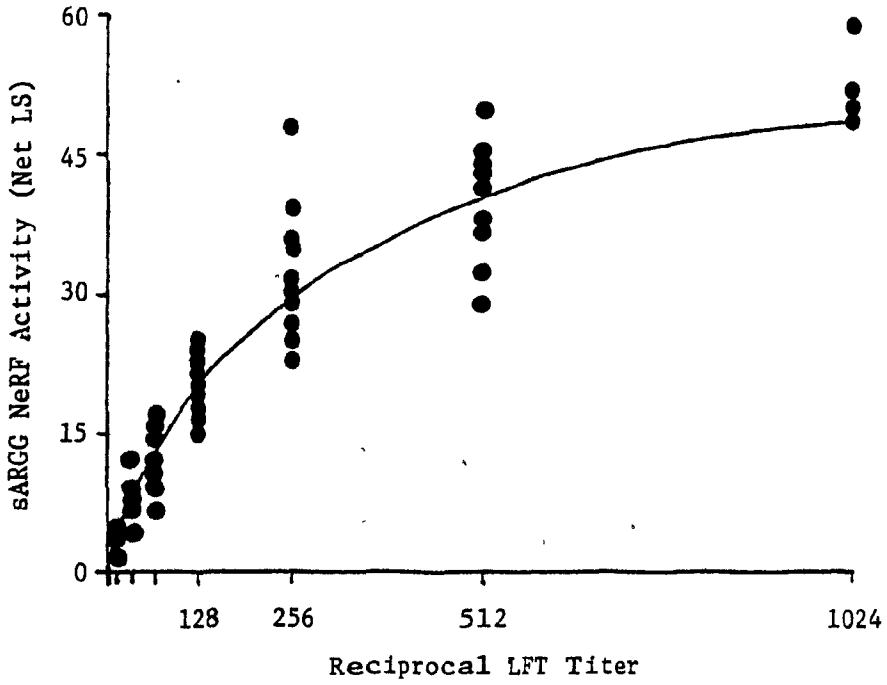
2.1 sARGG AND hAHGG vs LFT

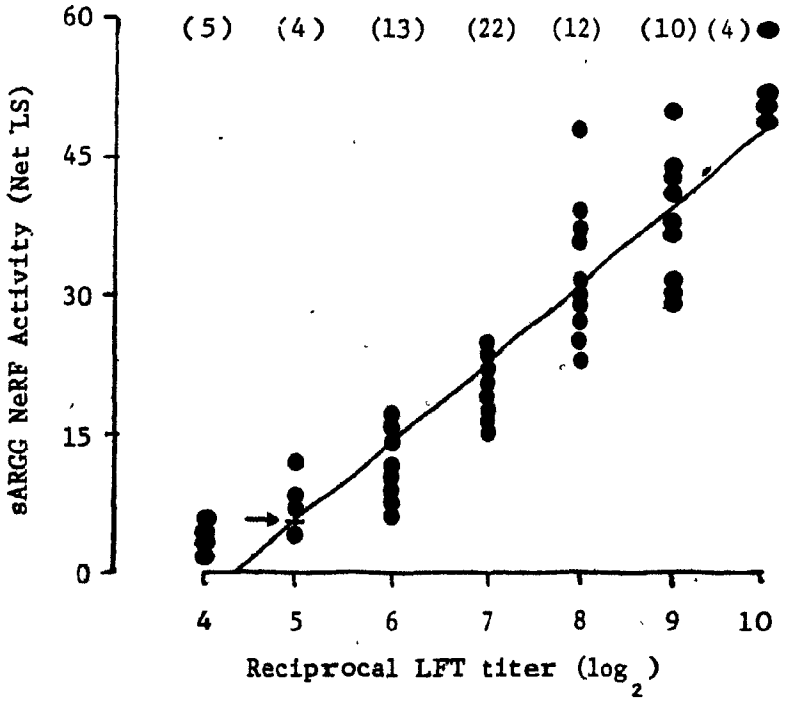
Latex titers of 67 heat-decomplemented sera were measured concommitantly with NeRF assays, using sARGG and hAHGG. The Net LS values obtained using sARGG and hAHGG are compared with the latex titers for these sera in Figs. 4.12a, 4.12b and 4.13. Log_2 values for the latex titers were used to transform curvilinear data to linear for regression analysis. This is illustrated by comparing Fig. 4.12a with Fig. 4.12b. Linear relationships were observed with correlations of $r = 0.93$ and 0.88 obtained when the NeRF values for the rabbit and human antigens respectively were compared with the $\log_2$ values for the latex titers. Sera with LFT titers less than 1:32 have been designated here as seronegative. From the comparison experiments it has been possible to establish seronegativity with each of the NeRF assays. This has been done by establishing the Net LS value for a given NeRF assay at which the linear regression line intersects the LFT titer of 1:32. Thus in Fig. 4.12, sARGG vs LFT, sera with Net LS less than 6 are defined as seronegative.

Fig. 4.12. 67 heat-decomplemented sera were tested for RF-activity with the LFT assay and with the NeRF assay using spontaneously-aggregated rabbit IgG. Upper figure (a): curvilinear relationship obtained when LFT titers are plotted as linear values; lower figure (b): linear relationship obtained when LFT titers are plotted as $\log_2$ values. $\log_2$ values for the LFT titers and the corresponding Net LS values were compared by linear regression analysis. Data obtained were: regression line equation, $Y = 8.3 \log_2 X - 36.0$; $r = 0.93$; $p < 0.001$. Figures in parentheses refer to number of samples tested. Arrow designates seronegative limit (LFT 1:32) for sARGG in this comparison (Net LS = 6).

FIGURE 4.12*

COMPARISON OF sARGG NeRF ACTIVITY WITH LFT TITER

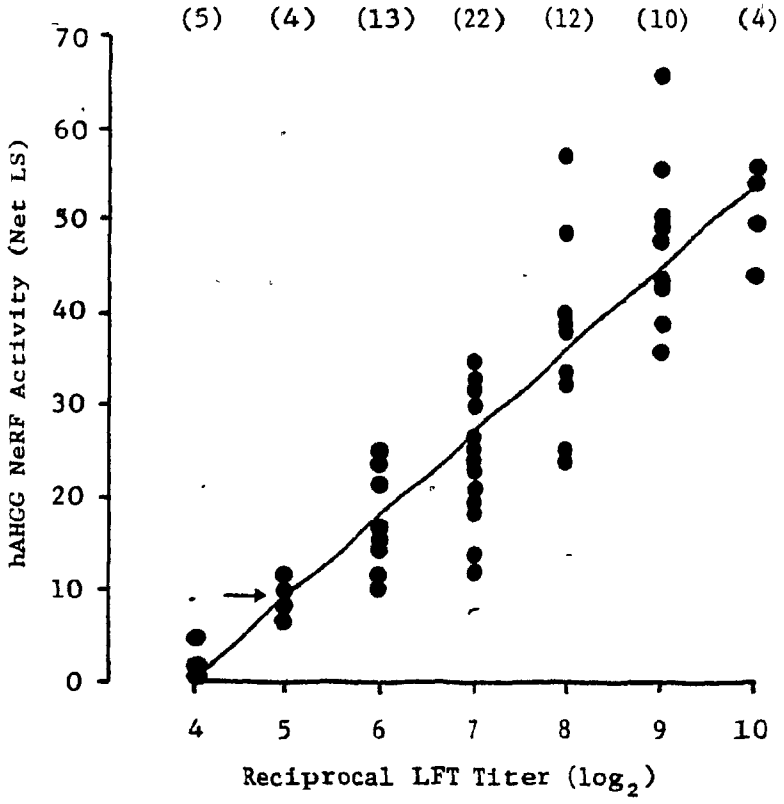




* Legend for Fig. 12 on page opposite.

FIGURE 4.13

COMPARISON OF hAHGG NeRF ACTIVITY WITH LFT TITER



67 heat-decomplemented sera were tested for RF-activity with the LFT assay and with the NeRF assay using heat-aggregated human IgG. Log_2 values for the LFT titers and the corresponding Net LS values were compared by linear regression analysis. Data obtained were: regression line equation, $Y = 8.8 \log_2 X - 34.8$; $r = 0.88$; $p < 0.001$. Figures in parenthesis refer to number of sera tested. Arrow designates seronegative value for hAHGG in this comparison (Net LS = 9).

2.2 sARGG vs hAHGG

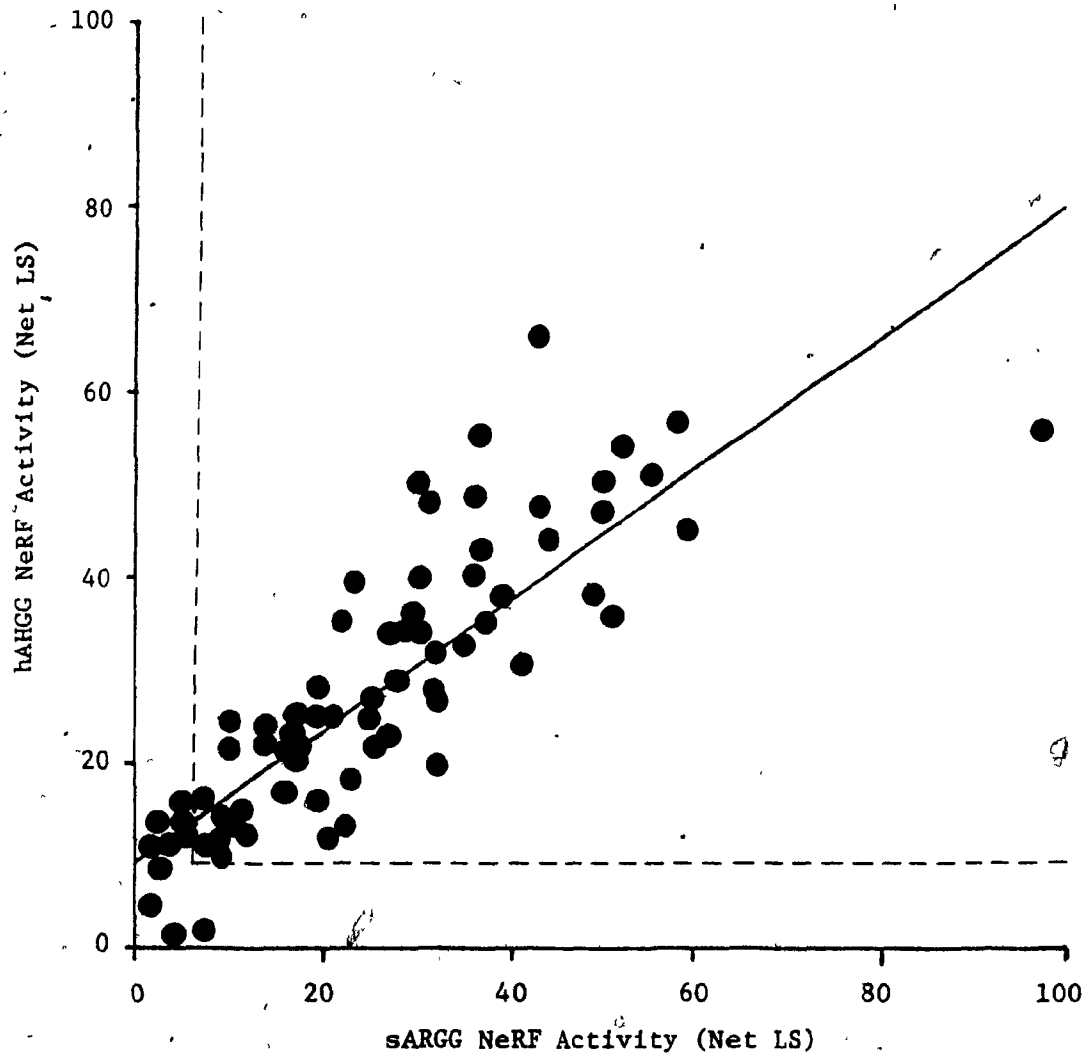
Reactions with sARGG and hAHGG were compared in the NeRF assay using these 67 sera. These results are shown in Fig. 4.14. A linear relationship was found ($r = 0.80$; $p < 0.001$) and the regression line intercepted the hAHGG axis at a Net LS value of 9.6. Only seronegative samples, as delineated by the seronegative limits for each antigen, failed to react with one or the other antigen.

2.3 SCAT vs LFT

The next set of data were obtained from comparative studies using the sheep cell agglutination test and the latex fixation test. Results of the SCAT and LFT are compared in Fig. 4.15 for a group of 29 RF sera. Three sera were found which differed in titer by two dilutions while the rest of the sera were found to differ by one dilution of had the same titer. An r value of 0.89 was found and the relationship was linear. Further evidence that freon-treatment of sera did not affect rheumatoid factor activity was demonstrated when 12 RF-sera were assayed for SCAT and LFT activity before and after freon-treatment. Only one sera gave a different titer after delipidation with freon and this was only a one doubling dilution decrease in

FIGURE 4.14

COMPARISON BETWEEN NeRF ACTIVITY OBTAINED WITH sARGG AND WITH hAHGG

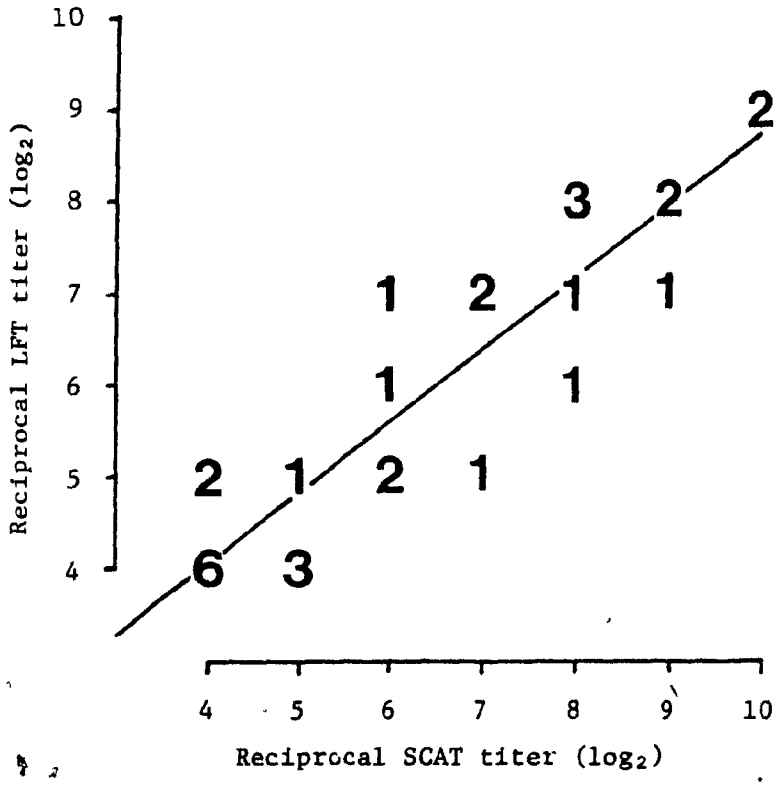


Net LS values for the nephelometric reaction of 67 heat-decomplemented RF-sera with sARGG and with hAHGG were compared by linear regression analysis. Data obtained were: regression line equation, $Y = 0.71 X + 9.6$; $r = 0.80$; $p < 0.001$. Hatched lines delineate seronegative limits as determined from Figs. 4.12 and 4.13.

FIGURE 4.15

COMPARISON BETWEEN TITERS OBTAINED

WITH THE LFT ASSAY AND WITH THE SCAT ASSAY



29 heat-decomplemented sera were tested for RF-activity with the LFT assay and with the SCAT assay. Log_2 values for the LFT and for the SCAT titers were compared by linear regression analysis. Data obtained were: regression line equation, $Y = 0.78 X + 0.91$; $r = 0.91$; $p < 0.001$. Numbers refer to number of sera tested.

SCAT titer.

2.4 gARGG AND hAHGG vs SCAT

The results of the SCAT testing and NeRF assays for these 29 sera are compared in Figs. 4.16 and 4.17. The $\log_2$ values were used to transform curvilinear data to linear for regression analysis as was the case with the latex titers in Sect. 2.2. For both gARGG data there is an increase in the average light scatter with each increase in SCAT titer. In addition, the linear regression analysis yielded r-values of 0.86 and 0.74 for the gARGG and hAHGG antigens respectively, and both values were significant ($p < 0.001$).

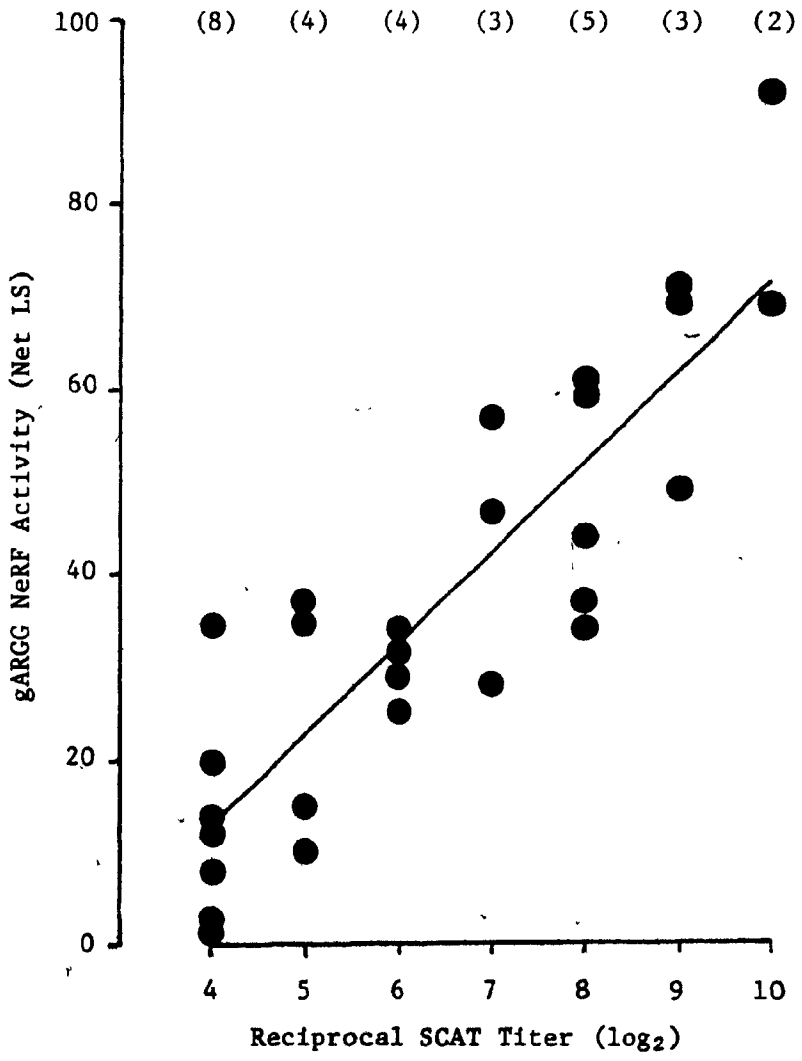
2.5 gARGG AND hAHGG vs LFT

Comparison of the results of NeRF assays and LFT titers for 47 sera are shown in Figs. 4.18 and 4.19. The $\log_2$ values for the latex titers gave linear relationships when compared with the Net LS values obtained nephelometrically. Light scatter clearly increased with increasing LFT titer. Results were found to be significant ($p < 0.001$) and linear with r-values of 0.84 and 0.89 for the rabbit and human antigens respectively.

FIGURE 4.16

COMPARISON OF gARGG NeRF ACTIVITY

WITH SCAT TITER

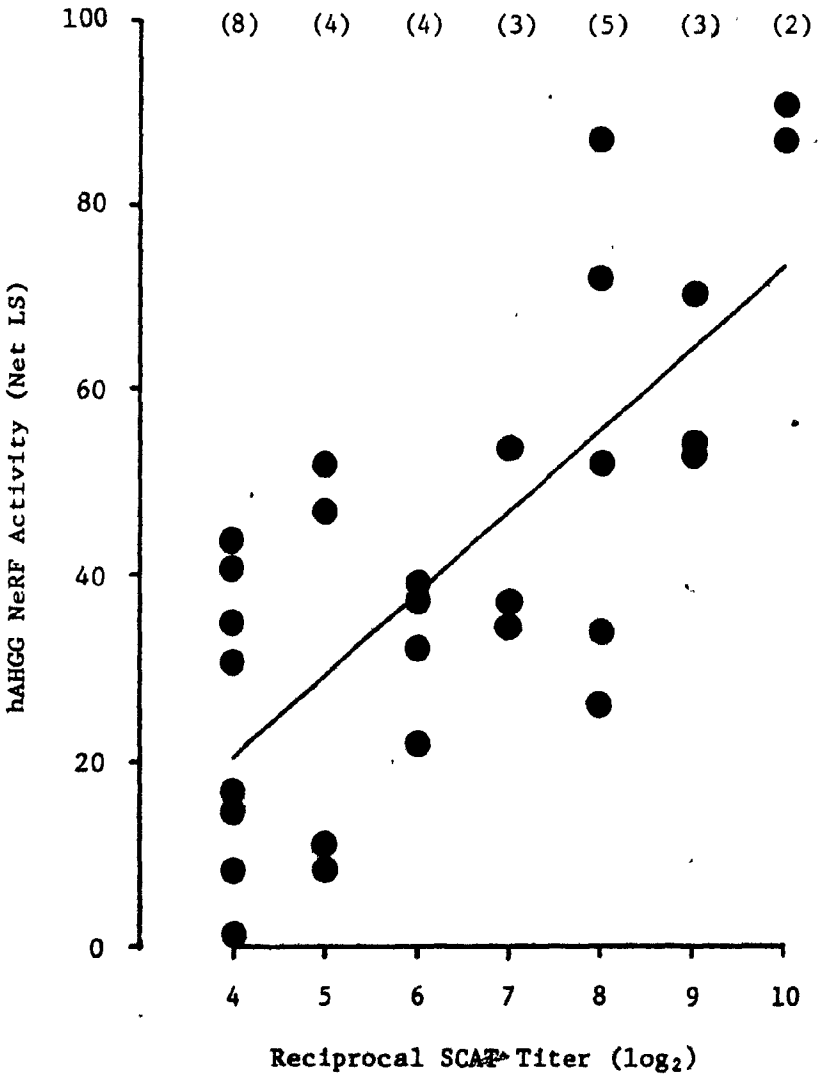


29 heat-decomplemented sera were tested for RF activity with the SCAT assay and with the NeRF assay using glut-aggregated rabbit IgG. Log_2 values for the SCAT titers and the corresponding Net LS values were compared by linear regression analysis. Data obtained were: regression line equation, $Y = 9.8 \log_2 X - 26.6$; $r = 0.86$; $p < 0.001$. Figures in parentheses refer to number of sera tested.

FIGURE 4.17

COMPARISON OF hAHGG NeRF ACTIVITY

WITH SCAT TITER

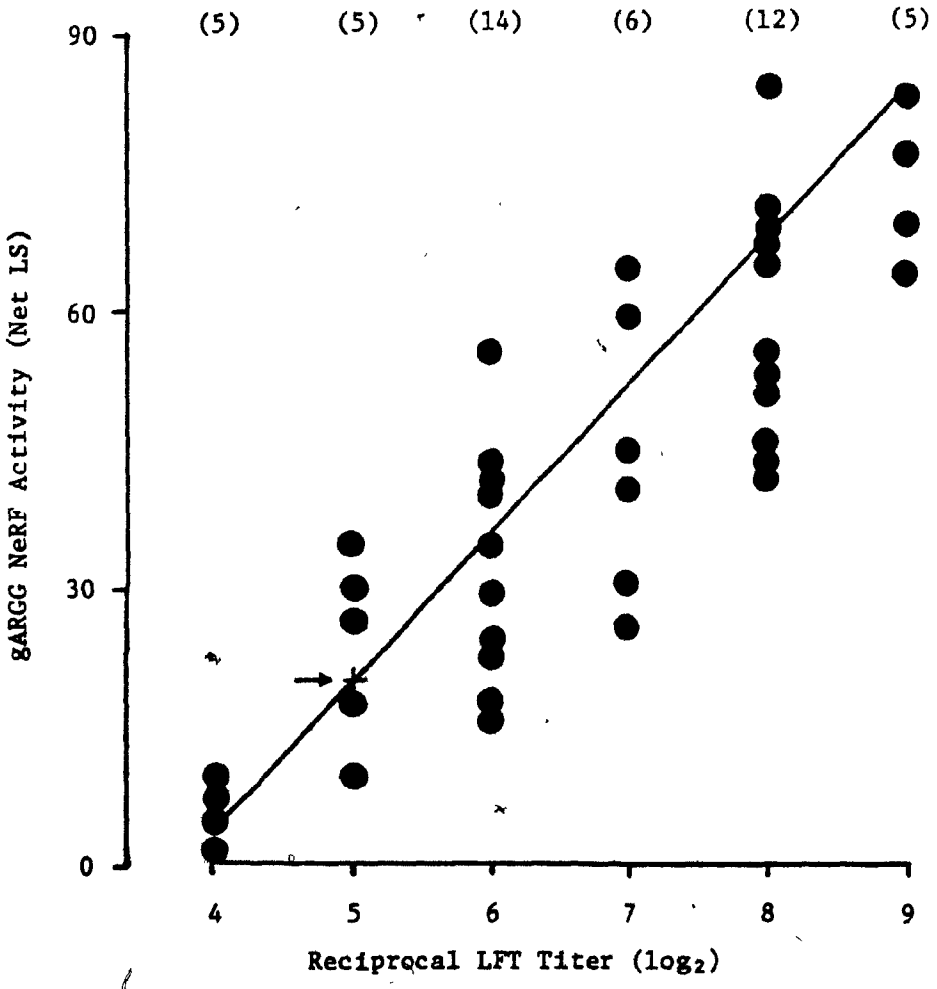


29 heat-decomplemented sera were tested for RF activity with the SCAT assay and with the NeRF assay using heat-aggreagted human IgG. Log_2 values for the SCAT titers and the corresponding Net LS values were compared by linear regression analysis. Data obtained were: regression line equation, $Y = 8.8 \log_2 X - 14.6$; $r = 0.74$; $p < 0.001$. Figures in parentheses refer to number of sera tested.

FIGURE 4.18

COMPARISON OF gARGG NeRF ACTIVITY

WITH LFT TITER

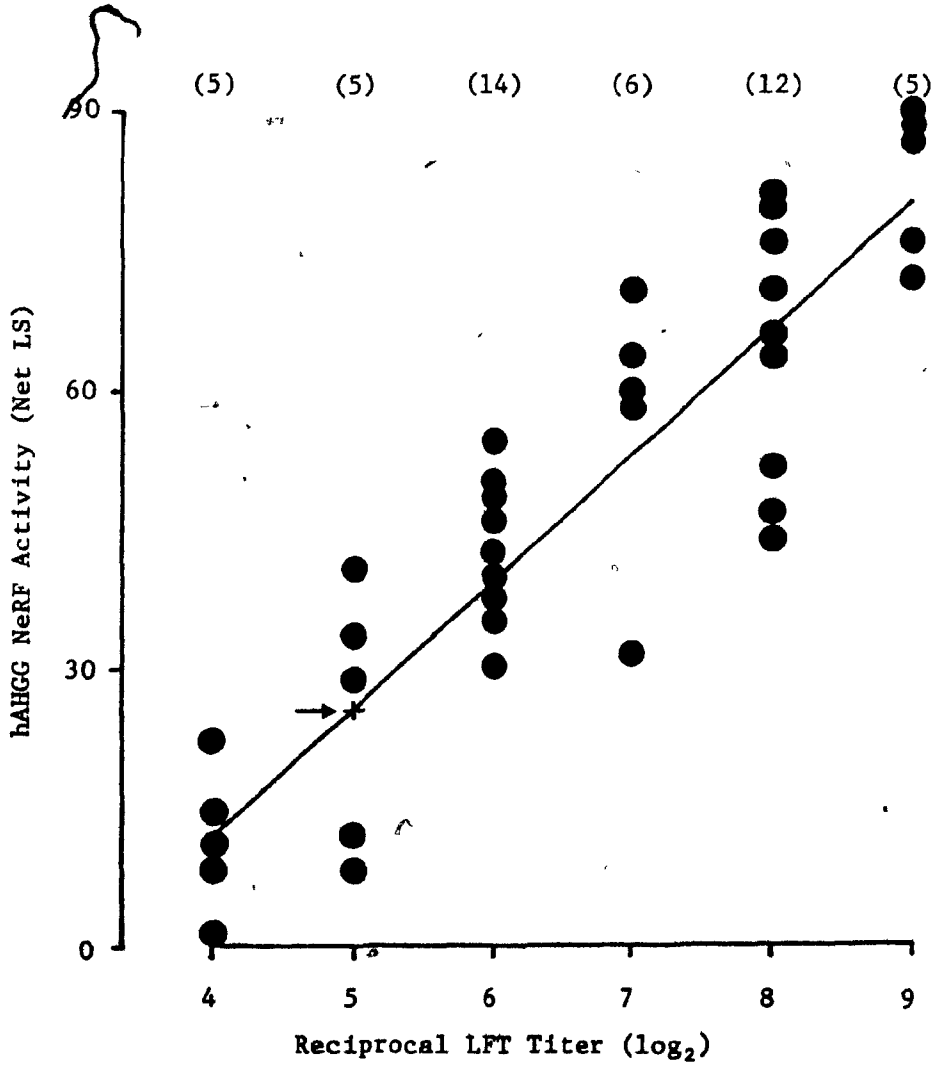


47 heat-decomplemented sera were tested for RF activity with the LFT assay and with the NeRF assay using glut-aggregated rabbit IgG. Log_2 values for the LFT titers and the corresponding Net LS values were compared by linear regression analysis. Data obtained were: regression line equation, $Y = 13.5 \log_2 X - 49.7$; $r = 0.84$, $p < 0.001$. Figures in parentheses refer to number of sera tested. Arrow designates seronegative value for gARGG in this comparison (Net LS = 20)

FIGURE 4.19

COMPARISON OF hAHGG NeRF ACTIVITY

WITH LFT TITER



47 heat-decomplemented sera were tested for RF-activity with the LFT assay and with the NeRF assay using heat aggregated human IgG. Log_2 values for the LFT titers and the corresponding Net LS values were compared by linear regression analysis. Data obtained were: regression line equation, $Y = 13.6 \log_2 X - 41.5$; $r = 0.89$; $p < 0.001$. Figures in parentheses refer to number of sera tested. Arrow designates seronegative value for hAHGG in this comparison (Net LS = 26).

2.6 gARGG vs hAHGG

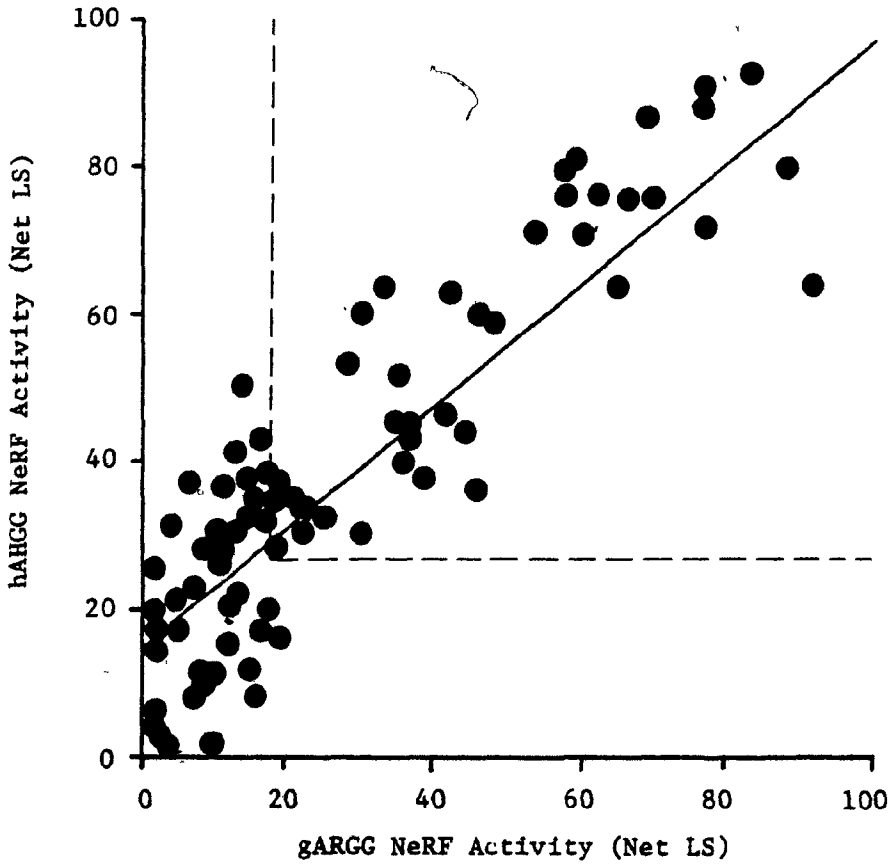
The results of nephelometric rheumatoid factor assays comparing rabbit and human antigen reactivity with sera from 88 patients is shown in Fig. 4.20. Comparison of the Net LS values for 12 normals averaged 1.9 ± 1.5 (range 0.1 - 4.4) and 1.9 ± 1.7 (range 0.5 - 6.1) for the rabbit and human antigens. The regression line for the RA patients as tested by the two antigens intercepts the hAHGG axis at 14.6 Net LS and the slope is 0.82. The r-value as calculated for the linear relationship is 0.91 ($p < 0.001$). Only samples within the seronegative limits deviated appreciably from the regression line. The seronegative limits for the hAHGG antigen was 26 and for gARGG was 20 Net LS.

2.7 hARGG AND hAHGG vs LFT

Results of NeRF assays and LFT titers for 50 RF-sera are shown in Figs. 4.21 and 4.22. A linear relationship was found between the $\log_2$ LFT titres and the NeRF assays with hARGG and with hAHGG. Correlation coefficients of $r = 0.86$ and $r = 0.91$ ($p < 0.001$) were found for the rabbit and human aggregated antigen respectively.

FIGURE 4.20

COMPARISON BETWEEN NeRF ACTIVITY OBTAINED WITH gARGG AND WITH hAHGG

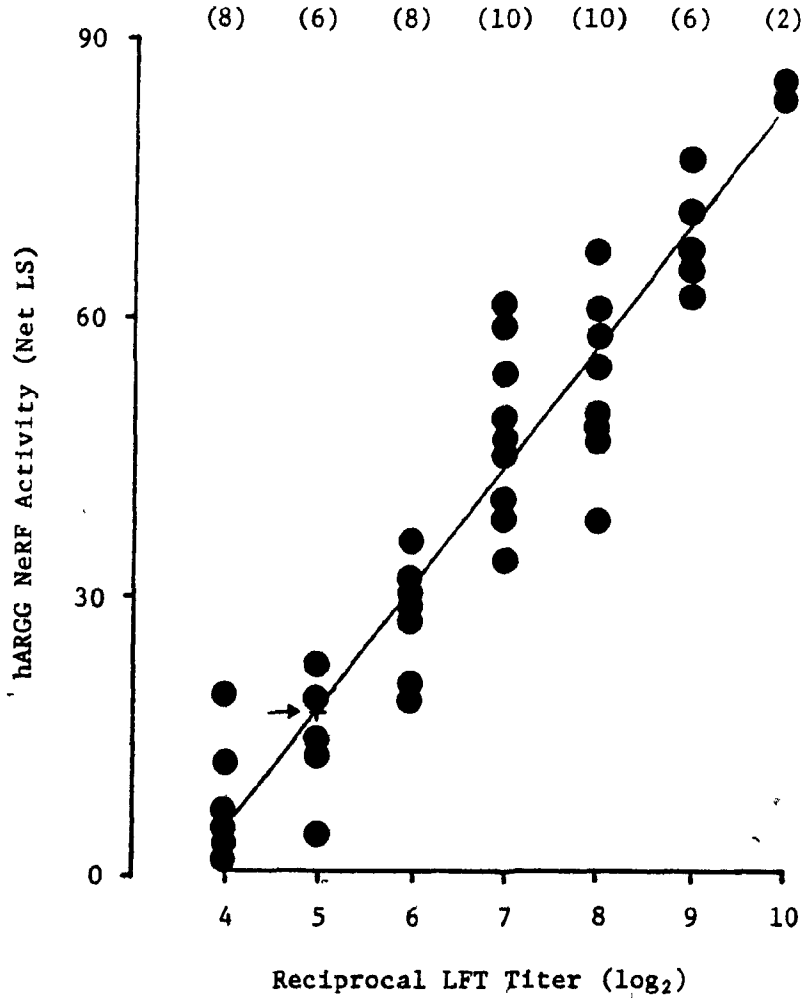


Net LS values for the nephelometric reaction of 88 heat-decomplemented RF-sera with gARGG and with hAHGG were compared by linear regression analysis. Data obtained were: regression line equation, $Y = 0.82X + 14.6$; $r = 0.91$; $p < 0.001$. Hatched lines delineate seronegative limits as determined from Figs. 4.18 and 4.19.

FIGURE 4.21

COMPARISON OF hARGG NeRF ACTIVITY

WITH LFT TITER

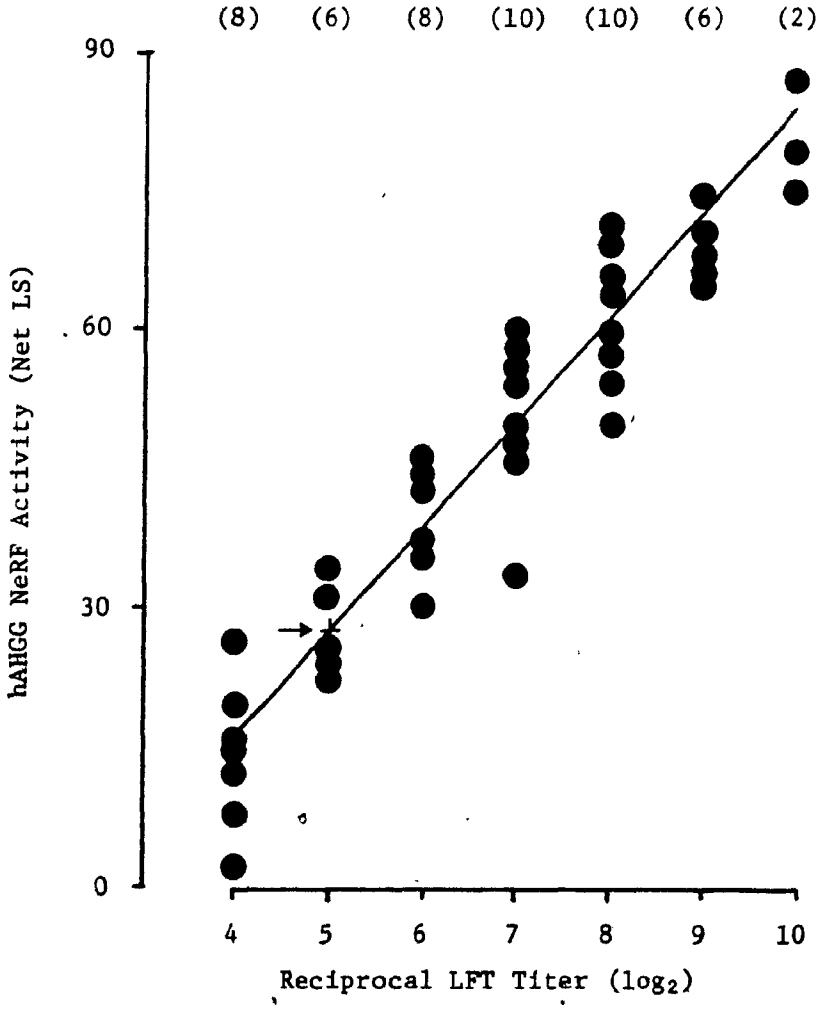


50 heat decomplemented sera were tested for RF-activity with the LFT assay and with the NeRF assay using heat-aggregated rabbit IgG. Log_2 values for the LFT titers and the corresponding Net LS values were compared by linear regression analysis. Data obtained were: regression line equation, $Y = 12.8 \log_2 X - 46.2$; $r = 0.86$; $p < 0.001$; Figures in parentheses refer to number of sera tested. Arrow designates seronegative limit for hARGG in this comparison (Net LS = 18).

FIGURE 4.22

COMPARISON OF hAHGG NeRF ACTIVITY

WITH LFT TITER



50 heat-decomplemented sera were tested for RF activity with the LFT assay and with the NeRF assay using heat-aggregated human IgG. Log_2 values for the LFT titers and the corresponding Net LS values were compared by linear regression analysis. Data obtained were: regression line equation, $Y = 11.0 \log_2 X - 28.6$; $r = 0.91$; $p < 0.001$. Figures in parentheses refer to number of sera tested. Arrow designates seronegative limit for hAHGG in this comparison (Net LS = 27).

2.8 hARGG vs hAHGG

The NeRF assay results for the two antigens, hARGG and hAHGG, are compared in Fig. 4.23. The reactivity of 96 serum samples from RA patients showed a linear relationship with a correlation coefficient of 0.93, a slope of 0.82 and intercepted the hAHGG axis at 16.7 Net LS. There are no sera which are strongly reactive with one antigen that are not also reactive with the other except for sera with low Net LS values, within the seronegative limits. The seronegative limit for the hAHGG is 27 and for hARGG is 18. Net LS values for a similarly tested normal population of 15 sera gave average Net LS values of 1.4 ± 1.1 (range 0.3 - 4.5) for the hARGG and 1.9 ± 1.8 (range 0.5 - 6.0) for the hAHGG

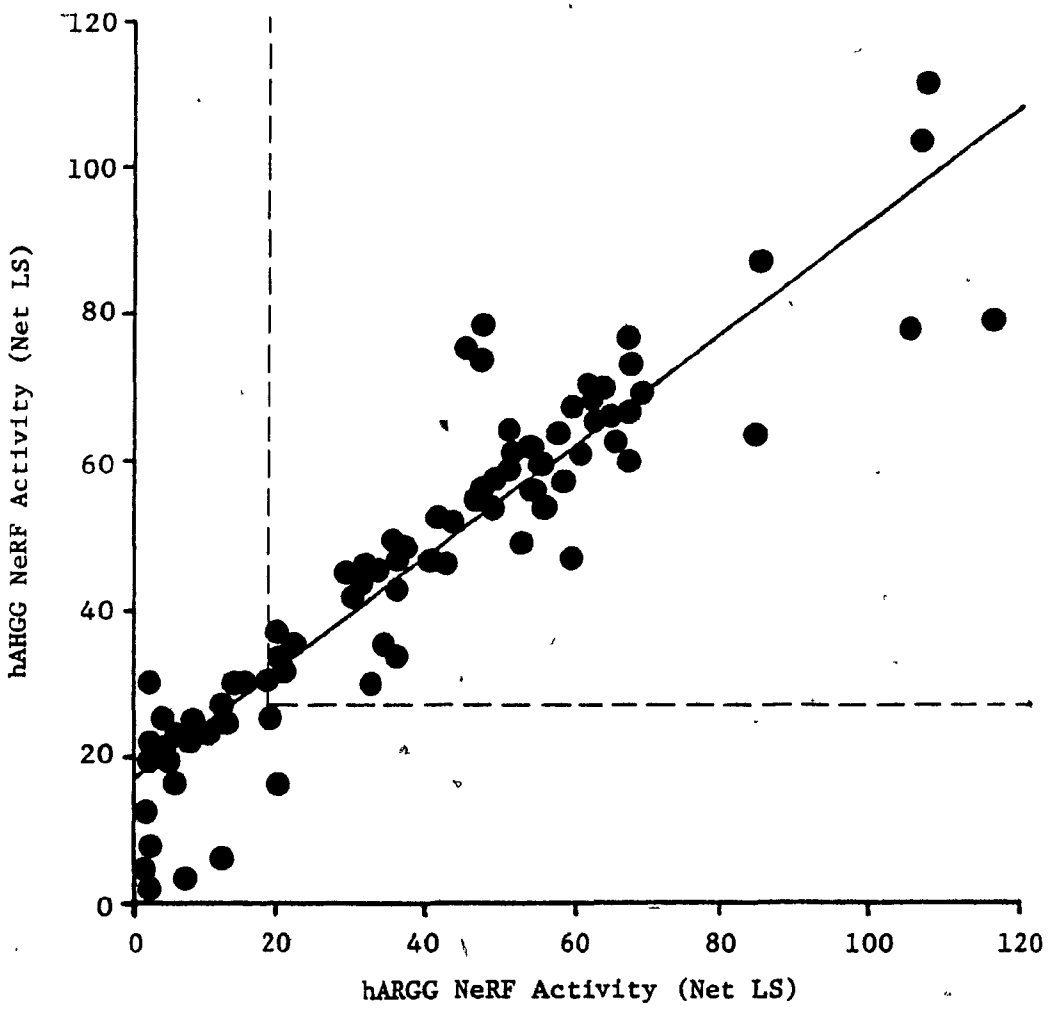
3 CHARACTERISTICS OF RF SERA

3.1 REDUCTION AND ALKYLATION OF RF-SERUM

In order to establish the immunoglobulin class responsible for NeRF activity, reduction and alkylation of serum with cysteine and iodoacetamide was performed on several serum samples. The results of these experiments are summarized in Table 4.6. When serum was incubated with

FIGURE 4.23

COMPARISON BETWEEN NeRF ACTIVITY OBTAINED WITH hARGG AND WITH hAHGG



Net LS values for the nephelometric reaction of 96 heat-decomplemented RF-sera with hARGG and with hAHGG were compared by linear regression analysis. Data obtained were: regression line equation, $Y = 0.82X + 16.7$; $r = 0.93$; $p < 0.001$. Hatched lines delineate seronegative limits as determined from Figs. 4.21 and 4.22.

TABLE 4.6

EFFECT OF REDUCTION AND ALKYLATION OF RF-SERUM ON NeRF ACTIVITY

Cysteine Added (M)	Iodoacetamide Added (M)	%NeRF Activity (untreated =100%)	
		hARGG	hAHGG
-	-	100%	100%
0.025	0.05	87%	92%
0.050	0.10	40%	83%
0.10	0.2	10%	25%
0.25	0.5	1%	1%
0.10	-	41%	56%
-	0.2	99%	102%

Following reduction and alkylation of RF-serum with cysteine and iodoacetamide (final concentrations as noted), samples were dialyzed extensively against Tris-NaCl buffer. Treated samples were then assayed for NeRF activity.

0.1 M cysteine, the NeRF activity was diminished by 90 and 75 percent with the rabbit and human antigens respectively. Although reduction alone decreased activity, reduction followed by alkylation produced a greater decrease in activity than did reduction without subsequent alkylation. Such an effect is consistent with alkylation preventing reformation of di-sulphide bonds. Similar results were observed with 2-Mercaptoethanol treatment of serum.

3.2 EUGLOBULIN PRECIPITATION OF RF-SERUM

The effect of dialysis of RF-containing sera against distilled water is summarized in Table 4.7. The

TABLE 4.7

EFFECT OF EUGLOBULIN PRECIPITATION OF RF-SERA ON NeRF ACTIVITY

Fraction	% NeRF Activity (unfractionated = 100%)	
	hARGG	hAHGG
Unfractionated	100%	100%
Pseudoglobulin	1%	3%
Euglobulin	83%	73%

Following dialysis of RF-serum against distilled water, the euglobulin pellet was resuspended in Tris-NaCl buffer and clarified by centrifugation. Unfractionated, pseudoglobulin (supernatant) and euglobulin fractions were then assayed for NeRF activity.

majority of the nephelometric activity was recovered in the euglobulin fraction with an average of less than 5% of the unfractionated activity remaining in the supernatant. It should be noted that there was a loss of activity in these precipitation experiments which was 17% and 27% for the rabbit and human antigens respectively, and presumably reflects incomplete resolubilization of the euglobulin pellet.

4 NEPHELOMETRIC RHEUMATOID FACTOR ACTIVITY OF PURIFIED POLYCLONAL IgM RHEUMATOID FACTOR (pRF)

4.1 FRACTIONATION OF POLYCLONAL RHEUMATOID FACTOR

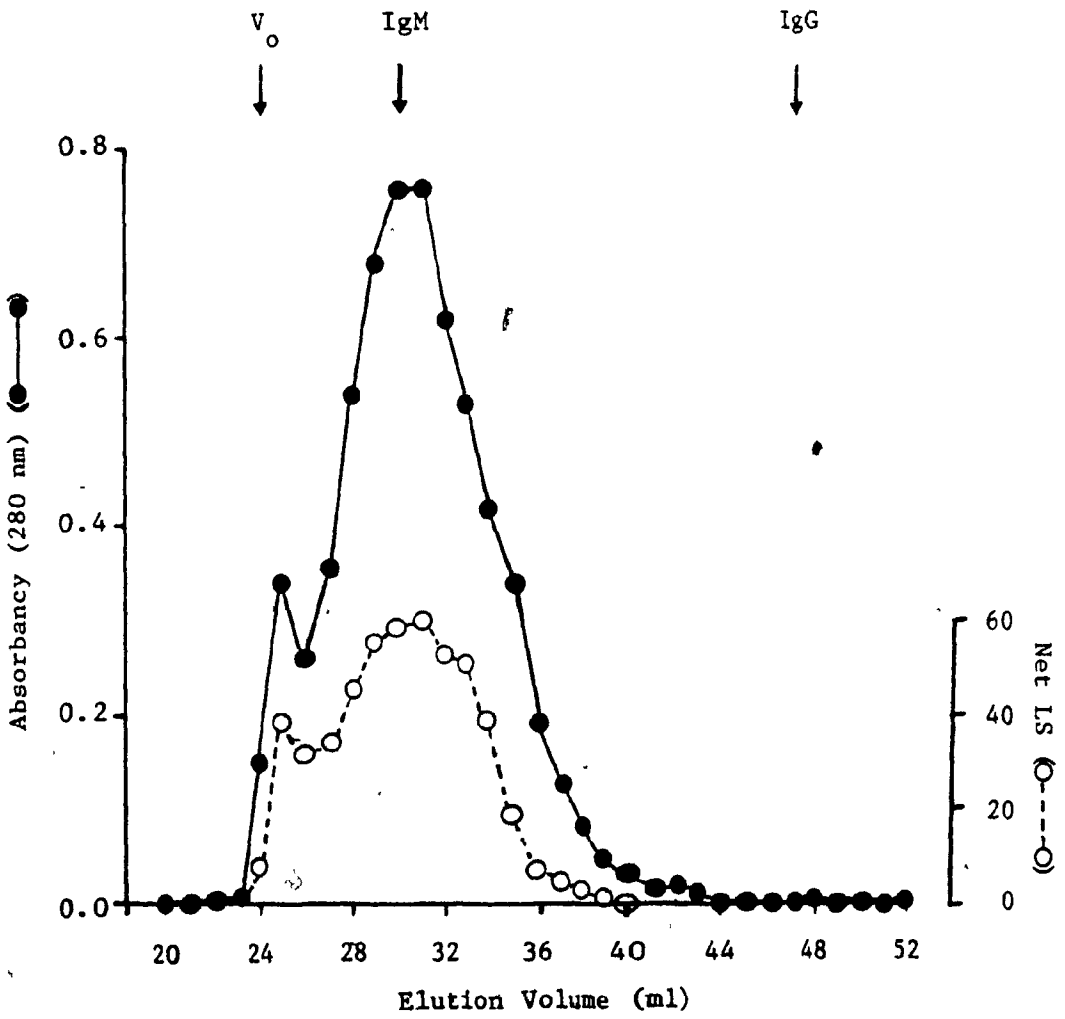
Fractionation of pRF was carried out on BioGel A5m, Fig. 4.24. The majority of the pRF fractionated as a single peak corresponding to the molecular weight of 19S IgM. A small amount of material was recovered in the void volume fractions of the column. When fractions were tested for activity with heat-aggregated human IgG, the reactivity corresponded to the elution profile.

4.2 TIME COURSE OF THE REACTION OF pRF WITH hARGG AND WITH hAHGG

The time course for the reaction between 50 µg. of the purified polyclonal IgM rheumatoid factor and various concentrations of either the rabbit or human aggregates is shown in Fig. 4.25. With both antigens, the light scatter increased with the incubation time, plateauing at 60 minutes and remaining stable for 60 minutes thereafter. Increasing the concentration of hAHGG or hARGG resulted in an increase in the Net LS. The reactions of pRF with hARGG and with hAHGG was similar to the reactions obtained

FIGURE 4.24

GEL FILTRATION CHROMATOGRAPHY OF pRF

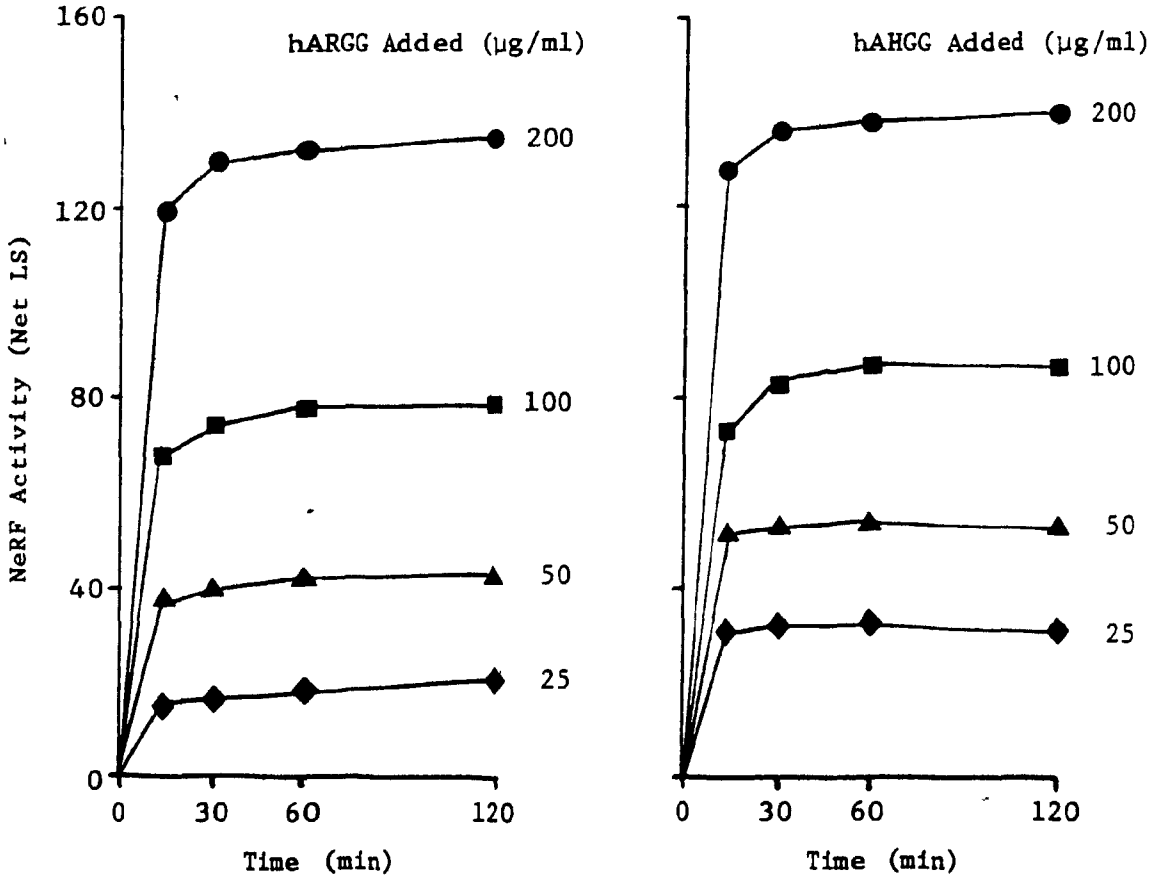


1.0 ml sample of isolated polyclonal rheumatoid factor (pRF) was chromatographed on Bio-Gel A-5m (0.9 x 90 cm) and 1.0 ml fractions collected. Absorbancy (280 nm) and NeRF activity (Net LS) of each fraction are illustrated. V_o, void volume; IgM, elution peak of IgM; and IgG, elution peak of IgG chromatographed separately.

FIGURE 4.25

TIME COURSE OF THE REACTION OF pRF

WITH hARGG AND WITH hAHGG



pRF (final concentration 50 µg/ml) was reacted with various concentrations of hARGG and hAHGG (as indicated) in Tris-NaCl buffer containing no PEG. Net LS was measured after the incubation times as indicated.

with RF-containing serum and these two antigens (Sect. 1.1.17; Fig. 4.8).

4.3 hARGG vs hAHGG

The Net LS values obtained after 60 minutes of incubation for the reaction of pRF with hARGG and with hAHGG are summarized in Table 4.8. The similarity in the

TABLE 4.8

REACTION OF pRF WITH hARGG AND WITH hAHGG

Aggregate Concentration (µg/ml)	NeRF Activity (Net LS)	
	hARGG	hAHGG
50	22	34
100	69	80
200	165	162
400	320	310
800	540	525
1600	760	720

pRF at a final concentration of 50 µg/ml was reacted with various concentrations of rabbit or human antigen in Tris-NaCl buffer. Net LS values were determined after 60 minutes of incubation.

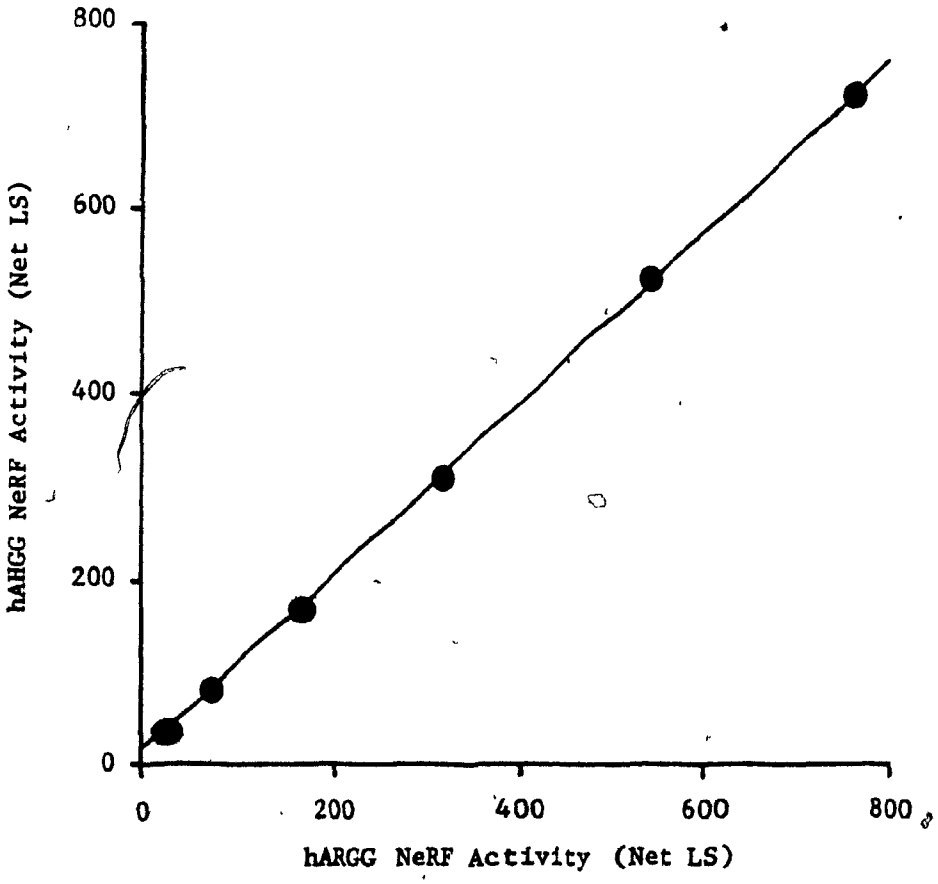
reaction curves with these two antigens is apparent, and when the values for each concentration of antigen are graphed against each other, a linear relationship is seen, Fig. 4.26. When linear regression analysis of this comparison was done, a slope of 0.93, an intercept of 16 on the hAHGG axis and a correlation coefficient of $r = 0.999$ ($p < 0.001$) were obtained. Likewise, the reactions of various amounts of pRF with 50 µg. of either hARGG or hAHGG are presented in Table 4.9. When these data, obtained with the two antigens, are plotted against each other a linear relationship was evident with a slope of 0.74, an intercept of 14 Net LS on the hAHGG axis, and a correlation coefficient of $r = 0.998$ ($p < 0.001$).

4.4 EFFECT OF POLYETHYLENE GLYCOL (PEG)

The previous experiments in this section were done in Tris-NaCl buffer which did not contain polyethylene glycol. The data in Table 4.9 show the effects of adding PEG to the reaction of various concentrations of pRF with a constant amount of either rabbit or human aggregates. At higher pRF concentrations, the Net LS values obtained are similar to those found in the absence of PEG. However, as the concentration of pRF used was decreased, there was an augmentation of the Net LS measured.

FIGURE 4.26

COMPARISON BETWEEN THE NEPHELOMETRIC REACTIONS OF pRF WITH hARGG AND WITH hAHGG



Net LS values (see Table 4.8) for the nephelometric reaction of pRF (final concentration, 50 µg/ml) with various concentrations of hARGG and hAHGG were compared by linear regression analysis. Data obtained were: regression line equation $Y = 0.93X + 16.4$; $r = 0.999$; $p < 0.001$.

TABLE 4.9

EFFECT OF PEG ON THE NEPHELOMETRIC REACTION OF pRF WITH hARGG AND WITH hAHGG

pRF Concentration (µg/ml)	NeRF Activity (Net LS)				% Increase With 1% PEG	
	No PEG		1% PEG			
	hARGG	hAHGG	hARGG	hAHGG	hARGG	hAHGG
12.5	2.4	10.7	23.7	35.5	988	332
25.0	8.1	21.3	52.7	47.5	651	223
50.0	22.3	34.4	71.4	63.4	320	184
100.0	52.3	54.5	92.5	81.0	177	149
200.0	84.9	78.0	114.0	94.2	130	121
400.0	110.6	94.8	133.2	110.0	120	116
800.0	126.0	106.9	150.1	122.6	119	115

pRF (final concentrations as indicated) was incubated with hARGG and with hAHGG at final concentrations of 50 µg/ml in Tris-NaCl buffers containing no PEG or 1.0% PEG (w/v). NeRF activity was determined after 60 minutes of incubation.

4.5 NEPHELOMETRIC REACTION OF pRF ADDED TO NORMAL HUMAN SERUM WITH hARGG AND WITH hAHGG

When various amounts of purified pRF were added to normal human serum (NHS) and subsequently assayed for reactivity with hARGG and with hAHGG, the following results were obtained, Table 4.10. There was a negligible increase in the serum LS when pRF was added to NHS, however, when

TABLE 4.10

REACTION OF hARGG AND OF hAHGG
WITH pRF ADDED TO NORMAL SERUM

Serum pRF Concentration (µg/ml)	Change in Serum LS Following Addition of pRF	NeRF Activity (Net LS)	
		hARGG	hAHGG
125	0.2	10.8	22.2
250	0.5	26.6	39.6
500	1.1	49.7	54.8

pRF was added to serum at the concentrations indicated and incubated for 18 hours at 4°C. prior to assay. LS of serum after addition of pRF and Net LS of serum reacted with hARGG and with hAHGG (50 µg/ml final reaction concentration) were determined after 60 min. incubation.

these samples were reacted with each of the antigens in the NeRF assay, Net LS increased with increasing pRF concentrations added.

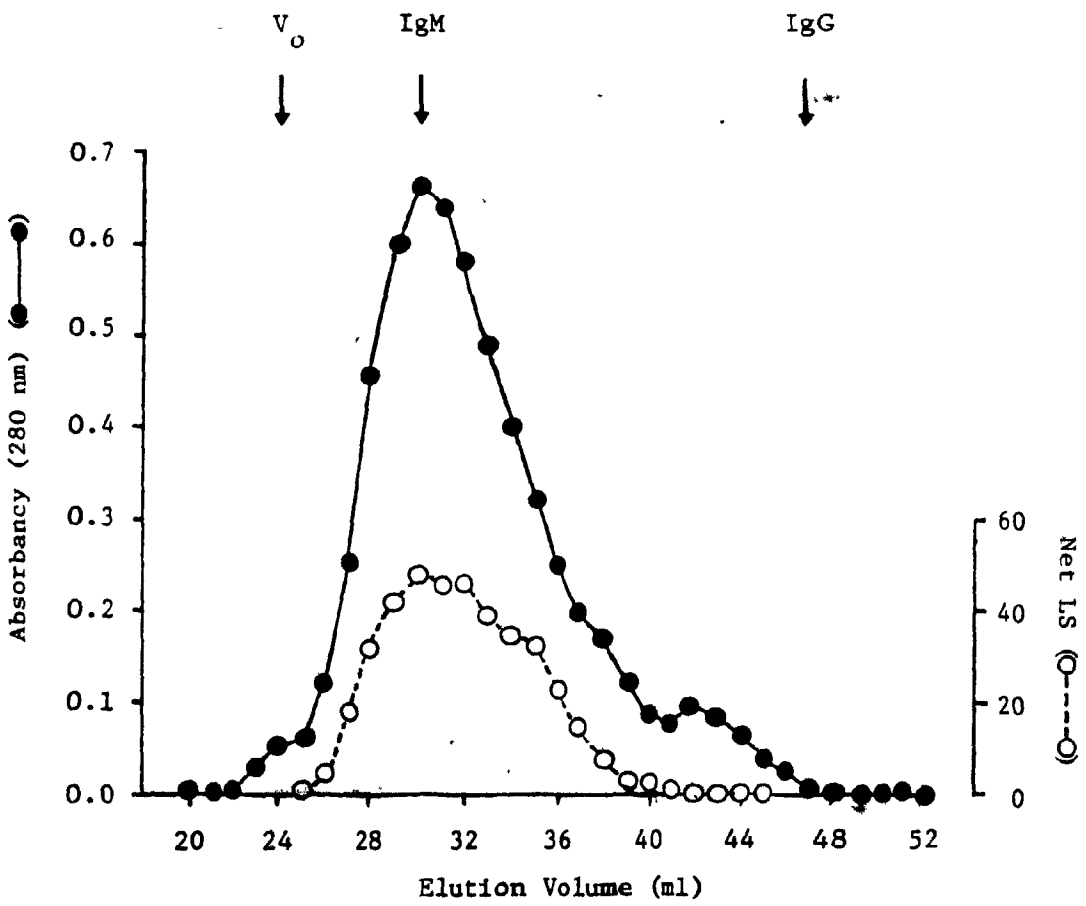
5 NEPHELOMETRIC RHEUMATOID FACTOR ACTIVITY OF PURIFIED MONOCLONAL IgM RHEUMATOID FACTOR (mRF_{og})

5.1 FRACTIONATION OF mRF_{og}

The elution profile of mRF_{og} on BioGel A5m showed a single peak which chromatographed at the same location as 19S IgM and pRF,—Fig. 4.27. When fractions were tested

FIGURE 4.27

GEL FILTRATION CHROMATOGRAPHY OF mRF_{og}



1.0 ml sample of isolated monoclonal rheumatoid factor (mRF_{og}) was chromatographed on Bio-Gel A-5m (0.9 x 90 cm) and 1.0 ml fractions collected. Absorbancy (280 nm) and NeRF activity (Net LS) of each fraction are illustrated. V_o, void volume; IgM, elution peak of IgM; and IgG, elution peak of IgG chromatographed separately.

for nephelometric reactivity with human aggregates, the activity was confined to the 19S material.

5.2 TIME COURSE OF THE REACTION OF mRF_{og} WITH hARGG AND WITH hAHGG

The time course study for the reaction between 50 micrograms of the purified monoclonal IgM-RF and various concentrations of the rabbit and human aggregates is shown in Fig. 4.28. There was an increase in Net LS for both antigens tested over time. Although the reaction of the monoclonal RF_{og} with the human aggregates tended to exceed the Net LS produced with the rabbit aggregates, the curves were otherwise similar.

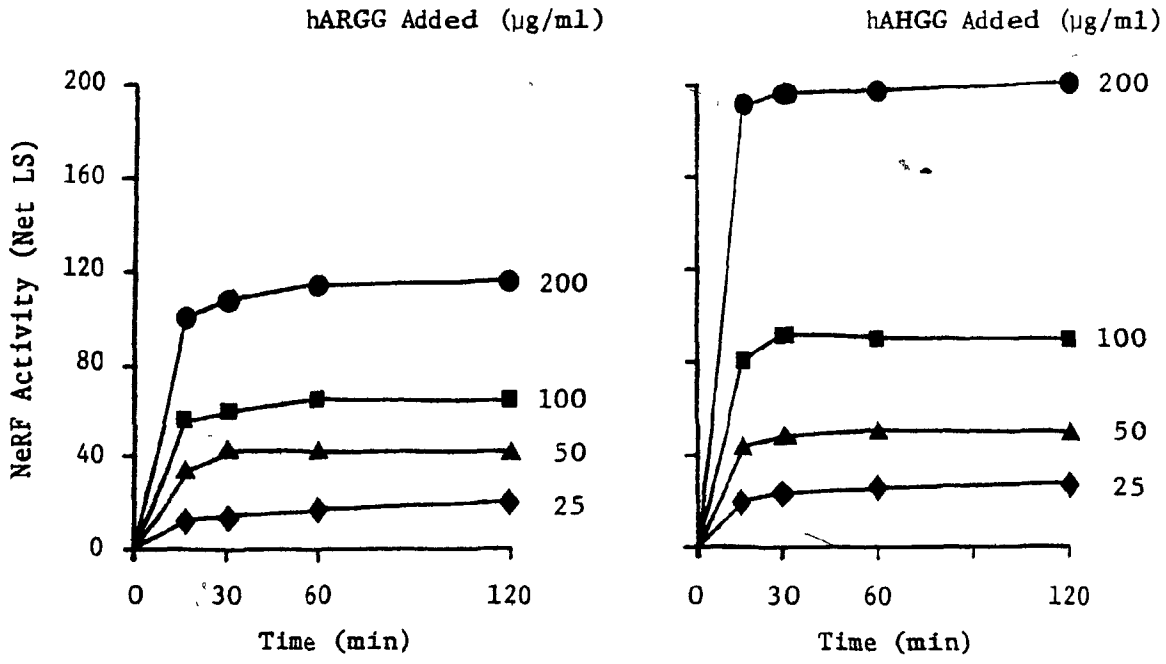
5.3 hARGG vs hAHGG

The Net LS values obtained after 60 minutes of incubation of mRF_{og} and various concentrations of hARGG or hAHGG is summarized in Table 4.11. In contrast to the data that was obtained in studies with RF-serum and purified pRF, the monoclonal RF_{og} preparation showed dissimilar reactivity with the two antigens. It was found that the mRF reaction with the human antigen consistently exceeded the reaction with the rabbit antigen. This was most

FIGURE 4.28

TIME COURSE OF THE REACTION OF mRF_{og}

WITH hARGG AND WITH hAHGG



mRF (final concentration 50 µg/ml) was reacted with various concentrations of hARGG and hAHGG (as indicated) in Tris-NaCl buffer containing no PEG. Net LS was measured after the incubation times as indicated.

TABLE 4.11

REACTION OF mRF_{og} WITH hARGG AND WITH hAHGG

Aggregate Concentration (µg/ml)	NeRF Activity (Net LS)	
	hARGG	hAHGG
25	15	25
50	35	42
100	73	90
200	125	210
400	161	340
800	182	540

mRF at a final concentration of 50 µg/ml was reacted with various concentrations of rabbit or human antigen in Tris-NaCl buffer. Net LS values were determined after 60 minutes of incubation.

apparent at high aggregate concentrations.

6 THE REACTION OF RHEUMATOID FACTOR WITH NATIVE IgG

It has been suggested that one of the biological activities of RF is its ability to bind complement after combination with antigen, presumably IgG. It would appear that if RF and native IgG formed a complex in vitro, then

the reaction could be quantitated by C1q in a nephelometric immune complex assay. The following experiments demonstrate that purified polyclonal IgM-RF can bind with native (7S) IgG and that the complex thus formed is reactive with C1q in the nephelometer.

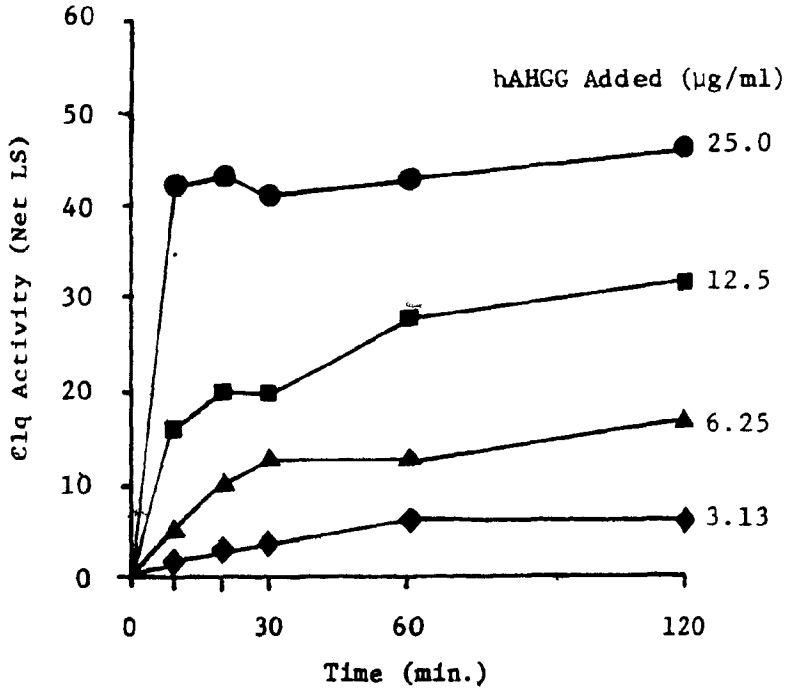
6.1 TIME COURSE OF THE REACTION OF C1q AND hAHGG

Since C1q is known to react with hAHGG, as well as with immune complexes, the capacity for the nephelometer to detect such reactions was investigated. The reaction of 25 µg of purified C1q with various concentrations of heat-aggregated human gamma globulin is shown in Fig. 4.29. There is an increase in Net LS which is dependent on both the aggregate concentration and the incubation time. It was necessary to carry out the reaction in Tris-NaCl buffer containing 2.0% polyethylene glycol (w/v) since this reaction was negligible in buffers containing less than 2.0% PEG. Since both RF and C1q bind aggregates, it was not feasible to use aggregates to assess the reaction of C1q with RF. As a result, purified human native IgG (NHGG) was examined as an antigen, reacting with RF, to analyze the RF-C1q reaction.

FIGURE 4.29

TIME COURSE OF THE REACTION

OF C1q WITH hAHGG



C1q (final concentration, 25 µg/ml) was reacted with various concentrations of hAHGG (as indicated) in 1 ml Tris-NaCl buffer containing 2.0% PEG. Net LS was measured after the incubation times as indicated.

6.2 THE REACTION OF pRF WITH NHGG AND OF pRF-NHGG COMPLEXES WITH C1q

Polyclonal IgM-RF in the presence of 2.0% PEG, reacted with NHGG (Table 4.12) and this reaction was dependent upon the concentration of NHGG employed. Similarly, the Net LS of pRF reacting with NHGG increased with increasing RF concentrations. Unlike the reaction of C1q with aggregated IgG, C1q failed to react with either pRF or human native IgG alone when incubated in 2.0% PEG. When NHGG and pRF were reacted together, and C1q then added to this complex, Net LS increased. This increase was dependent upon the concentration of complexes tested (Table 4.12).

6.3 REACTIONS OF pRF WITH IgG AND OF C1q WITH pRF-IgG COMPLEXES IN NORMAL AND AGAMMAGLOBULINEMIC HUMAN SERA

It was demonstrated (Sect. 6.2) that the reaction between pRF and purified human native IgG could be quantitated in the nephelometer. This raised the possibility that such a reaction might occur between native IgG and RF in serum. As a result, pRF was added to normal and agammaglobulinemic sera which did not contain RF, to determine if

TABLE 4.12

REACTION OF pRF WITH NHGG AND OF
C1q WITH pRF-NHGG COMPLEXES

Activity with Constant pRF Concentration			
NHGG Added (µg/ml)	Net LS	Effect of C1q Addition Net LS	% Increase
0	0	0	-
50	27	39	44%
100	53	65	23%
200	102	127	25%
400	207	249	20%
800	330	474	44%
Activity with Constant NHGG Concentration			
pRF Added (µg/ml)			
0	0	0	-
12.5	38	63	66%
25.0	92	125	36%
50.0	170	257	51%
100.0	328	558	70%
200.0	575	768	34%

pRF was reacted with NHGG in 1.0 ml Tris-NaCl buffer containing 2.0% PEG. Net LS for the RF-IgG reaction was determined after 30 min. of incubation. C1q was subsequently added and the Net LS for the C1q-RF-IgG reaction was measured after an additional 30 min. incubation with subtraction of appropriate controls. Constant concentrations used were: RF, 50 µg/ml; IgG, 250 µg/ml; C1q 25 µg/ml.

RF would react with native IgG in serum.

The reactions of 50 µg of pRF with these sera (heat-decomplemented) in Tris-NaCl buffer containing 2.0% PEG is shown in Table 4.13. In the presence of 2.0% PEG, an increase in the Net LS was observed with the addition of pRF to NHS. Likewise, there was also an increase in the Net LS observed when pRF was added to agammaglobulinemic sera containing very low concentrations of IgG. The net light scattering obtained was dependent upon the concen-

TABLE 4.13

REACTIONS OF RF WITH IgG AND OF C1q WITH RF-IgG COMPLEXES IN NORMAL AND AGAMMAGLOBULINEMIC SERUM

Serum	Serum IgG (µg/ml)	Effect of pRF Addition (Net LS)	Effect of Subsequent Addition of C1q (Net LS)	% Increase
Normal	1005	95.2	160.3	60%
Aγ1	193	24.6	38.5	58%
Aγ2	276	31.0	44.9	45%

pRF (final concentration 50 µg/ml) was reacted with 200 µl of heat-decomplemented serum in 1.0 ml Tris-NaCl buffer containing 2.0% PEG. Net LS for the RF-IgG reaction was determined after 30 min. of incubation. C1q was subsequently added and the Net LS for the C1q-RF-IgG reaction was measured after an additional 30 minute incubation with subtraction of appropriate controls.

tration of IgG present in the serum samples. Addition of Clq to heat-decomplemented normal or agammaglobulinemic sera in the presence of 2.0% (w/v) PEG, but in the absence of added pRF, produced scarcely any increase in Net LS. However, when pRF was added to these sera and subsequently, Clq added, there was an additional increase in net light scatter due to the additon of Clq (Table 4.13).

The demonstration that RF reacted with native IgG in buffer and in serum, and that this RF-IgG complex bound complement, provided a new approach to study the RF-IgG reaction. As a result, the reaction of isolated RF with rabbit and human native IgG (NRGG and NHGG) has been investigated extensively.

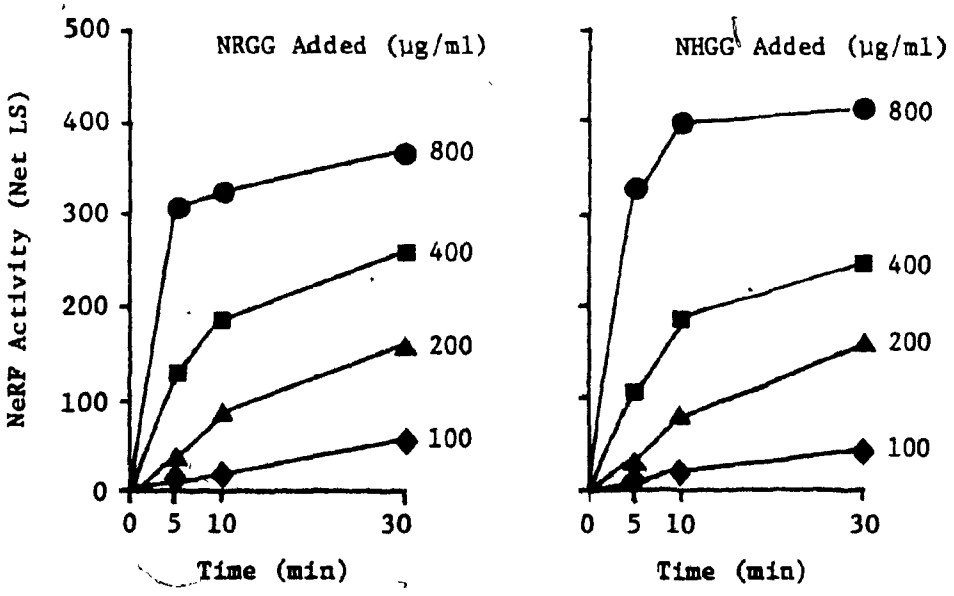
6.4 TIME COURSE OF THE REACTION OF pRF WITH NHGG AND WITH NRGG

The time course study for the reaction of 50 µg. of pRF with various concentrations of either 7S rabbit native IgG (NRGG) or 7S human native IgG (NHGG) is shown in Fig. 4.30. The reactions were similar for the two antigens with pRF in Tris-NaCl buffer containing 2.0% PEG. Similar results were also obtained when 800 µg. of either NRGG or NHGG were reacted with various concentrations of pRF.

FIGURE 4.30

TIME COURSE OF THE REACTION OF pRF

WITH NRGG AND WITH NHGG



pRF (final concentration 50 µg/ml) was reacted with various concentrations of NRGG and NHGG (as indicated) in Tris-NaCl buffer containing 2.0% PEG. Net LS was measured after the incubation times as indicated.

6.5 THE EFFECT OF PEG ON THE REACTION OF pRF WITH NRGG AND WITH NHGG

The previous experiments on the reaction of pRF with native IgG had been carried out in Tris-NaCl buffer containing 2.0% polyethylene glycol. Both antigens reacted similarly with pRF in the presence of 2.0% PEG. When 1.0% PEG was employed in the reaction buffer, there was a decrease in the Net LS values for the reactions with each antigen. However, the reaction of pRF with rabbit native IgG decreased more than with the human native IgG. In the absence of PEG, the reaction of pRF with NRGG was very small in contrast to the decreased, but easily discernable, reaction with NHGG. This data is summarized in Table 4.14.

The Net LS values obtained for each concentration of rabbit and human native IgG were compared at the various concentrations of PEG in the reactions buffer. Linear regression analysis showed a correlation between the two antigens when 1.0% PEG ($r = 0.996$; $p < 0.001$) or 2% PEG ($r = 0.999$; $p < 0.001$) was used in the reaction buffer but no significant correlation was found when PEG was not added. Comparisons were also made between the Net LS values obtained with a given antigen and two different concentrations of PEG. These are summarized in Table 4.15.

TABLE 4.14

THE EFFECT OF PEG ON THE REACTION OF pRF WITH NRGG AND WITH NHGG

Native IgG Concentration (µg/ml)	NeRF Activity (Net LS)					
	NRGG			NHGG		
	PEG Concentration (w/v)			PEG Concentration (w/v)		
	0%	1%	2%	0%	1%	2%
100	1	3	60	3	9	45
200	3	12	163	8	28	135
400	6	40	264	20	75	243
800	5	85	391	41	125	367
1600	4	115	526	53	163	496

pRF at a final concentration of 50 µg/ml was incubated with either NRGG or NHGG (final concentrations as indicated) in Tris-NaCl buffer containing no PEG or PEG, 1.0% or 2.0% (w/v) as indicated. Net LS was measured after 30 minutes of incubation.

TABLE 4.15

COMPARISON OF PEG CONCENTRATIONS ON THE REACTION OF pRF WITH NRGG AND WITH NHGG

Antigen	Comparison of PEG Concentrations (w/v)			Regression Analysis	
	X	vs	Y	r	p
NHGG	0%		1%	0.997	<0.001
"	0%		2%	0.992	<0.001
"	1%		2%	0.994	<0.001
NRGG *	1%		2%	0.990	<0.001

* With 0% PEG, NRGG failed to show a substantial reaction with pRF.

6.6 THE REACTION OF mRF_{og} WITH NRGG AND WITH NHGG

The reaction between monoclonal rheumatoid factor and NRGG and NHGG in the presence of 2.0% PEG is summarized in Table 4.16. mRF_{og} was found to react only with human IgG. Increasing concentrations of NHGG produced higher net light scatter values. The reaction of mRF_{og} with NRGG was small in contrast to the reaction of pRF with this antigen in previous experiments (see Table 4.14).

6.7 THE EFFECT OF PEG ON THE REACTION OF mRF_{og} with NHGG

The effect of various concentrations of PEG on the reaction between mRF_{og} and human IgG is also shown in Table 4.16. There was an enhancement in the Net LS produced by the addition of increasing amounts of polyethylene glycol to the reaction buffer. The enhancement was small with the addition of 1.0% PEG, however, with the addition of 2.0% PEG the enhancement was readily apparent.

TABLE 4.16

THE EFFECT OF PEG ON THE REACTION OF mRF_{og} WITH NRGG AND WITH NHGG

Native IgG Concentration (µg/ml)	NeRF Activity (Net LS)			
	NRGG	NHGG		
	PEG Concentration (w/v)			
	2%	0%	1%	2%
100	3.8	2.9	3.5	15.8
200	7.9	6.5	12.0	58.8
400	16.7	12.4	29.9	115.8
800	24.6	14.4	58.1	182.6
1600	27.1	11.3	41.7	227.3

mRF at a final concentration of 50 g/ml was incubated with either NRGG or NHGG (final concentrations as indicated) in Tris-NaCl buffer containing no PEG or PEG, 1.0% or 2.0% as indicated. Net LS was measured after 30 minutes of incubation. mRF_{og} failed to react with NRGG in the absence of PEG or in buffer containing 1% PEG.

7 KINETICS OF THE REACTION OF RF WITH IgG

7.1 KINETICS OF THE REACTION OF RF WITH AGGREGATED IgG

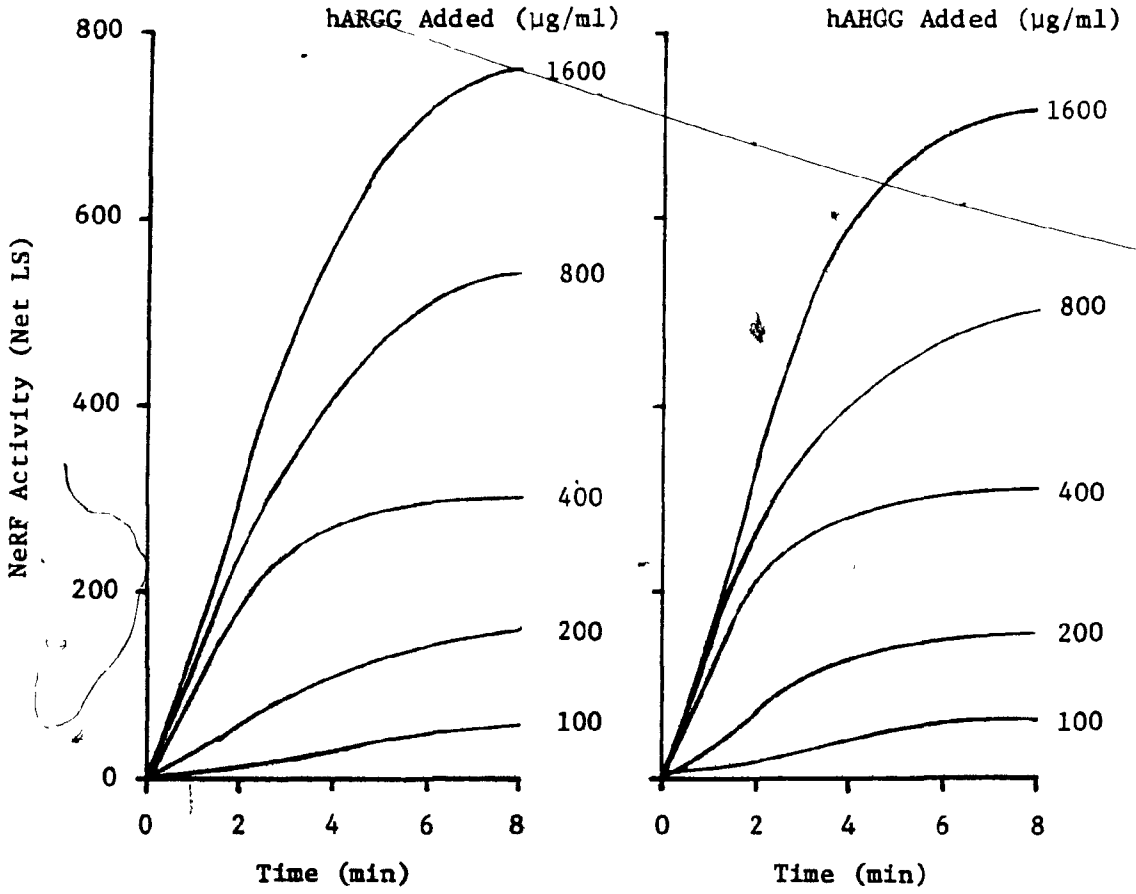
The time curves for the first 8 minutes of the reaction of pRF with various concentrations of hARGG and of hAHGG are illustrated in Fig. 4.31. It is evident that within the first 8 minutes, the reactions of these two antigens with pRF do not differ appreciable. Measurement of peak rates for individual reactions, as well as V_{max} and K_m values for each antigen or RF renders differences in the kinetics of the RF reaction more apparent. This is illustrated in Table 4.17 in which the data from pen recorder tracings (Fig. 4.31) are converted into numerical values. This Table emphasizes the similarity in the kinetics of the reaction of pRF with rabbit and with human aggregates. Differences in such kinetics are illustrated in this Table as well. When the monoclonal RF (mRF_{og}) was reacted with rabbit and with human aggregates and peak rates determined, striking differences were obtained. Earlier results using an end-point analysis showed mRF_{og} reacted poorly with rabbit aggregated IgG (Section 5.3).

Data in Table 4.17 confirm this earlier observation, showing peak rates, V_{max} and K_m are all lower with

FIGURE 4.31

TIME CURVES FOR THE REACTION OF pRF

WITH hARGG AND WITH hAHGG



pRF (final concentration 50 µg/ml) was mixed rapidly with various concentrations of hARGG and hAHGG (as indicated) in 1 ml Tris-NaCl buffer containing no PEG. Net LS was recorded continuosly with a linear pen recorder and time curves redrawn from pen recorder tracings. Peak rate values derived from these data are shown in Table 4.17.

hARGG than with hAHGG. Futhermore, this Table shows there are only slight differences in the kinetics of the reaction of mRF_{og} with hAHGG when compared with the pRF-hAHGG reaction.

TABLE 4.17

KINETICS OF THE REACTION OF RHEUMATOID FACTOR WITH AGGREGATED IgG

Aggregate Concentration (µg/ml)	Peak Reaction Rate (Net LS/min.)			
	pRF		mRF_{og}	
	hARGG	hAHGG	hARGG	hAHGG
50	2	4	2	5
100	10	12	9	16
200	34	48	22	66
400	103	160	34	212
800	173	195	35	240
1600	186	180	33	225
V_{max}	190	198	35	240
K_m	380	300	160	280

Peak rate and V_{max}, expressed in Net LS/min.; K_m, expressed in µg./ml.

7.2 KINETICS OF THE REACTION OF RF WITH NATIVE IgG

The peak rates for the reaction of RF with various concentrations of rabbit and of human native IgG were measured, using both pRF and mRF_{og}. Since polyethylene glycol has a definite effect on the reaction of RF with native IgG, experiments were designed to provide kinetic data on the effect of PEG on this reaction. The results of these experiments are shown in Table 4.18. Here one can see that the peak rate of reaction is increased by PEG and that the concentration of native IgG eliciting the fastest rate (V_{max}) is increased with increased concentration of PEG. Like the similar reactions of pRF with rabbit and with human aggregates, the data show that the reaction of pRF with rabbit and with human native antigens are essentially indistinguishable. This is in contrast to the rates of the mRF_{og} reaction with NHGG. The reaction rates of mRF_{og} with NRGG at all concentrations of rabbit IgG and PEG are less than 1 Net LS/min. and are not included in Table 4.18.

7.3 COMPARISON OF THE KINETICS OF THE REACTION OF RF WITH NATIVE AND WITH AGGREGATED IgG

The time curves for the first 8 minutes of the reactions of pRF, mRF_{og} and mRF_{mi} with NHGG and with hAHGG

TABLE 4.18

KINETICS OF THE REACTION OF RHEUMATOID FACTOR WITH NATIVE IgG

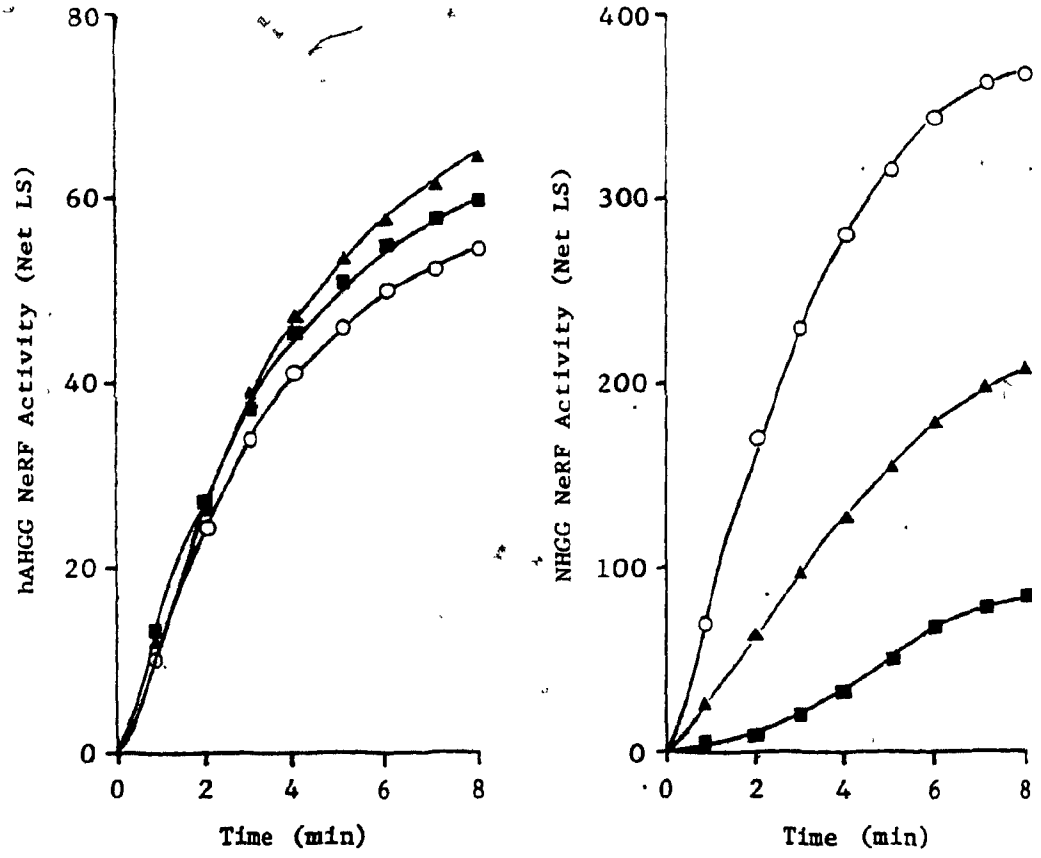
Native IgG Concentration (µg/ml)	Peak Reaction Rates (Net LS/min)								
	pRF						mRF_{og}		
	NRGG			NHGG			NHGG		
	PEG (w/v)			PEG (w/v)			PEG (w/v)		
	0%	1%	2%	0%	1%	2%	0%	1%	2%
100	<1	<1	7	<1	1	5	-	<1	3
200	<1	1	25	1	3	21	-	1	10
400	1	6	77	3	12	71	-	4	21
800	<1	25	197	6	36	185	-	6	46
1600	<1	33	435	6	46	410	-	4	69
2400	nd	33	595	7	50	590	nd	3	83
3200	"	23	720	4	55	690	"	1	68
4800	"	19	965	1	47	960	"	nd	50
6000	"	nd	1050	nd	37	1050	"	"	33
7200	"	"	870	"	nd	825	"	"	nd
V_{max}	≈1*	34	1050	8	56	1050	-	6	84
K_m	≈200*	650	2010	500	600	2020	-	250	750

Peak rate and V_{max}, expressed in Net LS/min.; K_m, expressed in µg/ml.
* Approximate V_{max} and K_m due to very low peak rates. nd, not determined.

are shown in Fig. 4.32. The curves are similar for the reactions of the various RF preparations with aggregated IgG, however, differences are noted with the reaction of these isolated rheumatoid factors with native IgG. pRF is the

FIGURE 4.32

THE REACTION OF THREE ISOLATED RF PREPARATIONS WITH hAHGG AND WITH NHGG



Three isoalted RF preparations (final concentrations 50 µg/ml) were each rapidly mixed with either hAHGG (50 µg/ml; left) or NHGG (800 µg/ml; right) and Net LS measured continuously with a linear pen recorder. Incubation mixtures containing hAHGG did not contain PEG whereas mixtures containing NHGG, contained 2% PEG. (○) pRF; (■) mRF_{og}; (▲) mRF_{mi}.

reactive with NHGG as evidenced by a higher peak rate (190 Net LS/min) with 800 µg of NHGG, whereas the peak rates for mRF_{mi} (89 Net LS/min) and mRF_{og} (43 Net LS/min) are much lower. It is unfortunate that the limited amount of mRF_{mi} did not allow for a more thorough examination of the reaction of this monoclonal RF with IgG.

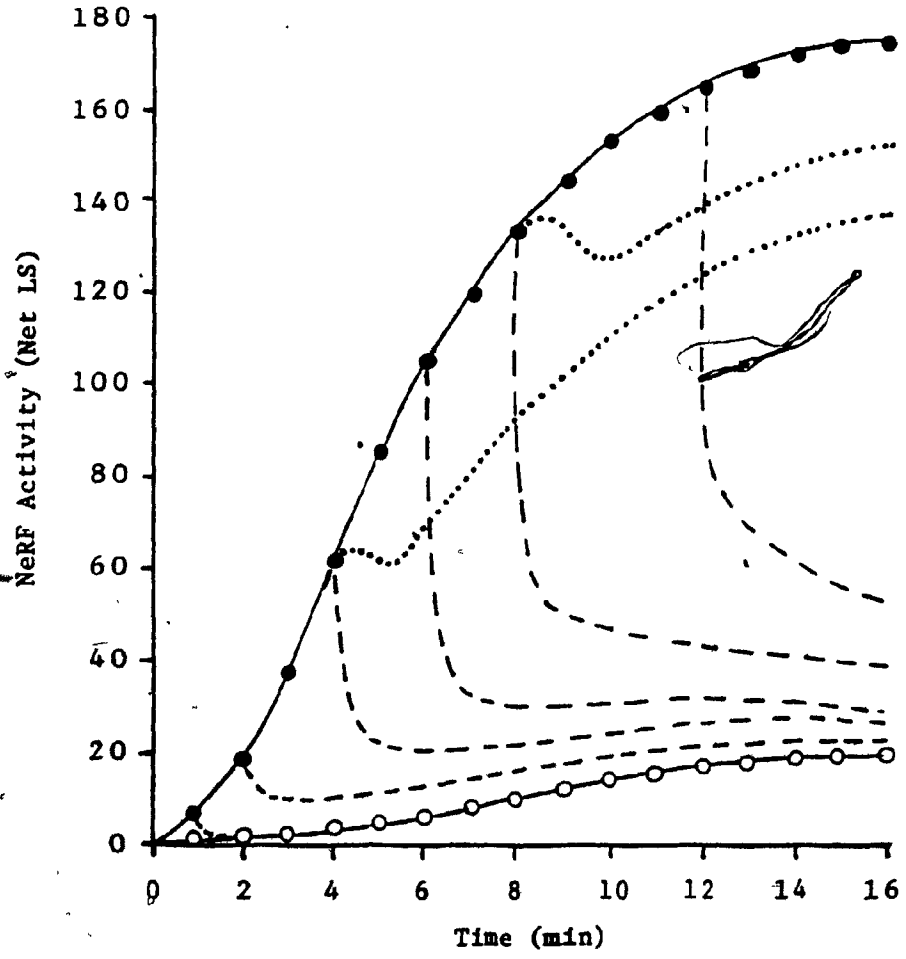
7.4 EFFECT OF PEG ON THE KINETICS OF THE REACTION OF RF WITH NATIVE IgG

PEG has been found to cause a large enhancement in the kinetics of the reaction of RF with native IgG. This enhancement was investigated by diluting the reaction of pRF with NHGG in Tris-NaCl buffer containing 2% PEG as shown in Fig. 4.33. Dilution of this reaction by the addition of 1.0 ml Tris-NaCl buffer containing no PEG diminished the time curve to parallel the time curve for the reaction between pRF and NHGG in Tris-NaCl buffer containing 1% PEG.

FIGURE 4.33

THE EFFECT OF PEG ON THE KINETICS

OF THE REACTION OF pRF WITH NHGG



pRF (50 µg/ml) was rapidly mixed with NHGG (400 µg/ml) in 1.0 ml Tris-NaCl buffer, containing 2% PEG, and Net LS determined continuously with a linear recorder (●). At the times indicated, an equal volume of Tris-NaCl buffer containing no PEG (hatched lines) or Tris-NaCl buffer containing 2% PEG (dotted lines) was added to replicate incubation mixtures and Net LS recorded. The effects of dilution on the pRF-NHGG reaction were estimated by incubating pRF (25 µg/ml) with NHGG (200 µg/ml) in Tris-NaCl buffer, containing 1% PEG and determining Net LS (○).

8 ABSORPTION OF RF ACTIVITY BY IgG

8.1 ABSORPTION OF RF ACTIVITY BY AGGREGATED IgG

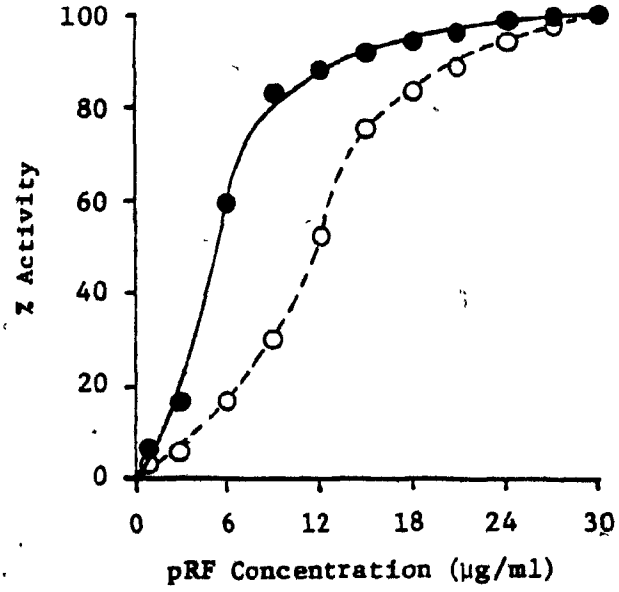
In an attempt to define the reaction of RF with aggregated IgG and with native IgG in greater detail, absorption experiments were done using heat-aggregated IgG, which had been insolubilized to facilitate removal of absorbed RF. In these experiments RF was incubated with insoluble aggregates of rabbit or human IgG and RF activity, after sedimentation of the aggregates by centrifugation, was quantitated with hARGG and with hAHGG. Table 4.19 shows that insoluble human IgG virtually completely removed pRF activity with either antigen, whereas insoluble rabbit IgG removed only part of the pRF activity with the two antigens. Although the decreases in pRF activity following absorption with the insoluble rabbit IgG appeared small, the actual decrease in pRF concentration was approximately 50%. Insoluble human IgG also virtually completely removed mRF_{og} activity, whether this activity was assayed with rabbit or human aggregates after absorption. Again, insoluble rabbit IgG only partially absorbed mRF_{og} activity. The discrepancy between the decrease in RF activity and RF concentration remaining is explained by the activity-concentration curves for RF with aggregated IgG, an example of which is shown in Fig. 4.34.

FIGURE 4.34

ACTIVITY-CONCENTRATION CURVES

FOR THE REACTION OF pRF

WITH hARGG AND WITH hAHGG



pRF at final concentrations indicated was reacted with hARGG (○) and hAHGG (●) in Tris-NaCl buffer containing no PEG. Net LS was measured after 60 minutes of incubation and these values were converted to % Activity using the reaction of 30 µg/ml of pRF with 25 µg/ml of either antigen = 100% Activity.

TABLE 4.19

ABSORPTION OF RF-ACTIVITY BY AGGREGATED IgG

NeRF Antigen	NeRF Reaction Concentrations (µg/ml)		% Activity Remaining (unabsorped RF = 100%)			
			pRF		mRF_{og}	
			Absorbent		Absorbent	
	Antigen	RF	Human	Rabbit	Human	Rabbit
hAHGG	25	30.0	0% (<1)	91% (15)	28% (4)	88% (14)
"	50	"	2% (nd)	96% (nd)	11% (nd)	83% (nd)
"	50	7.5	2% (<1)	76% (4)	0% (<1)	51% (2)
hARGG	25	30.0	1% (<1)	78% (16)	5% (3)	71% (15)
"	50	"	3% (nd)	82% (nd)	0% (nd)	67% (nd)
"	50	7.5	3% (<1)	67% (4)	0% (<1)	4% (<1)

pRF and mRF were absorped with insoluble human or rabbit IgG. Concentrations in the absorption mixture were: RF, 75 or 300µg/ml; absorbent, 1 mg/ml. Following incubation, mixtures were centrifuged (90,000 x g; 30 min; 4°C) and 100 µl of the supernatants assayed for NeRF activity. Figures in parentheses refer to the RF concentration (µg/ml), estimated by standard activity-concentration curves (Fig. 4.34); nd = not determined.

8.2 ABSORPTION OF RF-ACTIVITY BY NATIVE IgG

Insoluble rabbit IgG was not an effective absorbent, since it removed only part of the RF incubated with it, and it was not clear if this failure to completely absorb RF was a property of the absorbent or of the RF itself. As a result, absorption experiments were done using native IgG as the absorbent. In these experiments RF was incubated with native rabbit or human IgG in the presence of 3.5% PEG and the subsequent precipitate removed by centrifugation. In the absence of IgG, pRF or mRF_{og} was not precipitated by PEG concentrations up to 3.5%. When incubated with either human or rabbit native IgG, pRF and mRF_{og} were removed from solution, as shown in Table 4.20. It can be seen that this effect was dependent upon the concentration of IgG incubated with RF. These results show there was little difference in RF activity remaining in the supernatant when RF activity was assayed with hARGG and with hAHGG.

Although native IgG incubated with RF absorbed RF-activity, it was possible that this phenomenon was due to interference by native IgG with the subsequent determination of RF-activity with aggregates. However, this possibility was minimized by assaying the mixtures of RF and IgG before centrifugation to remove complexes and

by analyzing the precipitated RF by resuspension. The results of these experiments are shown in Table 4.21. The lack of diminished RF-activity in uncentrifuged samples containing RF and native IgG indicated that the concentrations of native IgG present in these solutions were insufficient to interfere with the assay of RF-activity with aggregated IgG (hARGG and hAHGG). In addition, RF-activity was recoverable in the pellets emphasizing that RF was absorbed by native IgG.

TABLE 4.20

ABSORPTION OF RF-ACTIVITY BY NATIVE IgG

Isolated RF Concentration (µg/ml)	Absorbent Concentration (µg/ml)	% Activity Remaining (unabsorped RF = 100%) hAHGG	hARGG
pRF (150)	NHGG (50)	98% (105)	72% (105)
	" (150)	48% (45)	26% (48)
	" (450)	4% (18)	2% (<10)
	NRGG (150)	4% (18)	5% (15)
	" (450)	3% (15)	3% (<10)
mRF_{og} (150)	NHGG (150)	61% (53)	31% (50)
	" (450)	25% (30)	14% (27)
	" (750)	4% (16)	3% (<10)
	NRGG (450)	19% (27)	11% (25)
	" (750)	3% (12)	1% (<10)

pRF and mRF were absorped with human or with rabbit native IgG in Tris-NaCl buffer containing 3.5% PEG. Concentrations in the absorption mixture were: RF, 150 µg/ml; IgG, 50-750 µg/ml. Following incubation, mixtures were centrifuged and 100 µl of the supernatants assayed for NeRF activity. Figures in parentheses refer to RF concentration (µg/ml), estimated by standard activity-concentration curves.

TABLE 4.21

ABSORPTION OF RF-ACTIVITY BY NATIVE IgG AND RECOVERY OF RF-ACTIVITY FROM RF-IgG COMPLEXES

Absorbent	Fraction	% Activity Recovered (unabsorped RF = 100%)	
		hAHGG	hARGG
NHGG	Uncentrifuged	116% (150)	99% (150)
"	Supernatant	2% (<10)	1% (<10)
"	Pellet	92% (108)	61% (102)
NRGG	Uncentrifuged	113% (150)	99% (150)
"	Supernatant	0% (<10)	1% (<10)
"	Pellet	99% (143)	85% (135)

pRF was absorped with human or with rabbit native IgG in Tris-NaCl buffer containing 3.5% PEG. Concentrations in the absorption mixture were: RF, 150 µg/ml; IgG, 450 µg/ml. Following incubation, mixtures were centrifuged and the pellets resuspended in Tris-NaCl buffer containing no PEG. 100 µl of uncentrifuged absorption mixtures, supernatants, and resuspended pellets were assayed for NeRF activity. Figures in parentheses refer to RF concentration (µg/ml), estimated by activity-concentration curves.

CHAPTER V

DISCUSSION

Of the three types of specificity exhibited by rheumatoid factors (Chapter II), two have been examined here in detail: species specificity and conformational specificity. Rheumatoid factors have been shown by numerous researchers to react with human and rabbit IgG but not with chicken IgG (Butler & Vaughn, 1964; Normansell & Stanworth, 1968; Stone, 1973). Differences in the assays used to analyze the reaction of RF with each of these antigens have not allowed for exact comparisons to be made between the reaction of RF with human and with rabbit IgG. It is, however, generally accepted that rheumatoid factors are reactive with both human and rabbit IgG and that they exhibit little or no specificity for these two antigens (Normansell, 1972; Stewart et al, 1975). The results presented here confirm that there is no qualitative difference and only small quantitative differences in the reactivity of RF with human and with rabbit antigens.

The assays used here, including the SCAT and LFT assays, have indicated that RF readily reacts with either human or rabbit, native or aggregated IgG, but not with

chicken IgG. Data obtained with the NeRF assay uniformly correlated with the SCAT and LFT titers, emphasizing the "validity" of the NeRF assay but in addition, it provided far more data than could be obtained with agglutination assays, and employed a single methodology to study the reaction of RF with IgG of different species. One of the obvious advantages of the NeRF methodology has been the opportunity to study the reaction of RF with native IgG. In reviewing our results, data obtained with aggregated antigens will be discussed first. This will allow comparisons between our results and results which have been obtained by most of the established RF assays, which employ aggregated, complexed or denatured IgG as antigen.

The reaction of RF with aggregated IgG in the nephelometer is consistent with a reaction due to IgM-RF, as evidenced by the close correlation between the results of the NeRF assay and the results of the standard agglutination assays (SCAT and LFT). Further evidence for IgM rheumatoid factor being responsible for the NeRF reaction was the euglobulin precipitation of RF activity, the loss of RF activity following reduction and alkylation, and the similarity of the reaction of isolated IgM-RF and of RF in a large population of sera with the NeRF antigens.

RF-sera were found to react in a qualitatively similar manner with aggregated rabbit and human IgG anti-

gens in the nephelometric rheumatoid factor assay. This similarity was most evident in the time course studies. In these experiments, the time course of the reaction of heat-decomplemented RF-sera was similar for two of the aggregated rabbit IgG antigens (gARGG and hARGG). Furthermore, these reactions were indistinguishable from the reactions with heat-aggregated human IgG (hAHGG). No obvious differences were noted over the time course studied (10-120 minutes, nor was there any difference in results with regards to the concentration of antigen used.

The reaction of sARGG with RF-sera differed from the time course of the reaction of the other aggregated antigens. After 30 minutes, Net LS decreased when RF-serum was reacted with sARGG, unlike the stable Net LS obtained with the other antigens. Although this fall-off in Net LS may represent the formation of large complexes so large as to be edited out of the LS computation by the nephelometer, it remains unexplained. However, two other types of rabbit aggregated IgG (gARGG and hARGG), which were found to be more stable and useful nephelometrically, did not exhibit this fall-off, indicating it was a feature of the particular aggregation method and not inherent in the rabbit IgG itself. The fall-off phenomenon and the instability of the spontaneous aggregates upon storage, rendered them of very limited value in the NeRF assay. The

higher concentration of sARGG required to elicit reactions of the same Net LS as produced by gARGG or hAHGG, is explainable by a lower concentration of aggregates in the sARGG. The removal of non-aggregated material from sARGG by gel filtration, produced an sARGG preparation (Fraction I) which could be used at the same concentration as the other aggregated antigens. However, Fraction I also exhibited the fall-off phenomenon, like unfractionated sARGG. This suggests that the fall-off was not due to the presence of non-aggregated IgG. Other than this unexplained fall-off, the reactions of sARGG with RF-serum were similar to those obtained with gARGG, hARGG and hAHGG.

Neither the time course of the NeRF reaction, nor the antigen concentrations used in the NeRF assays were able to effectively identify a RF species preference. Similarly, comparisons between the NeRF assay results obtained with the reactions of over 250 sera with the two antigens failed to identify any obvious species specificity which would have been manifested by quantitative differences in reactivity with the different antigens used. In addition, comparisons of these assays with the classical SCAT or LFT, failed to show any significant discrepancy in RF-reactivity with either human or rabbit antigens. When one excludes the sera which can be considered sero-negative (SCAT $\leq$ 1:32; LFT $\leq$ 1:32), then there is no evidence that there is any

preference for either rabbit or human antigen in sero-positive sera. Within the sero-negative range, however, slight preferences for one or the other antigen may be evident but this can be explained in terms of the sensitivity of the various assays for detecting such slight RF-reactivity.

Isolated polyclonal rheumatoid factor (pRF) also reacted with each of the rabbit and human aggregated IgG antigens in the nephelometer. This reaction, like that of RF in serum, showed a time course which was similar for the rabbit and human aggregates and furthermore, the same concentrations of each antigen produced similar results. Thus, isolated polyclonal RF failed to exhibit any species preference.

The reactions of pRF with either human or rabbit aggregated IgG were indistinguishable for 10-120 minutes incubation. In addition, the reaction of pRF within the first 10 minutes was similar when pRF was reacted with either hARGG or hAHGG, emphasizing that the species specificity was the same. This was evident with peak rate V_{max} and K_m measurements, and indicates the lack of quantitative difference in this reaction, using hARGG and hAHGG. Both rabbit and human aggregated IgG absorbed pRF, but only the human insoluble antigen completely removed pRF activity. The aggregated rabbit antigen removed only 9-33% of the RF-

activity, representing 50% of the pRF concentration. This difference, however, appears to reflect the inefficiency of the insoluble rabbit antigen to absorb RF rather than to suggest a species specificity of pRF. This is shown by the ability of rabbit native IgG to completely absorb pRF, like the human native or aggregated IgG.

From these experiments, it was apparent that RF in serum or in purified form did not show species specificity for rabbit or human IgG which had been aggregated. However, it did show specificity which excluded it from reacting with chicken IgG in the nephelometer. These results confirm earlier work done with RF assays which have employed aggregated, complexed or denatured IgG as antigen. Table 2.2 lists the assays which have been used to assay RF. It is apparent that the antigens used in these assays have been aggregated (eg. nephelometric or precipitation assays); complexed (eg. SRBC-anti-SRBC antibodies of the SCAT); or denatured by immobilization or labelling (eg. LFT or other solid phase assays). These methods, therefore, have in common the use of non-native IgG as antigen (Maiolini et al., 1978; Gripenberg et al., 1979). Reviewing Table 2.2 shows that rabbit and human IgG are widely employed as antigens in these assays, emphasizing that RF can react with either antigen. However, direct comparisons of RF reactivity with rabbit and human antigens

are few. Bartfeld (1969b), Waller (1969), Cathcart et al. (1975), and Stankaitiene and co-workers (1978) have examined the reactivity of RF with rabbit and human antigen. Significant differences in reactivity were not observed with aggregated, complexed or denatured antigens. Only Stankaitiene and co-workers used the same methodology to compare the reactions of RF with rabbit IgG and with human IgG, and failed to demonstrate any differences in reactivity. Our results, using a single methodology, as well as SCAT and LFT assays, have also shown there is no apparent RF specificity for rabbit or human IgG which has been aggregated, complexed or denatured.

One of the advantages of the NeRF methodology has been its use to show that RF reacts with native IgG. In the past, the analysis of the reaction of RF with native IgG has been confined to ultracentrifugation (Christian, 1959; Normansell, 1970; 1971) and equilibrium binding methods (Normansell, 1971; Dissanayake et al., 1977) and to inhibition studies (Grubb, 1956; Henney and Stanworth, 1965; Allen and Kunkel, 1966; Johnston and Abraham, 1979). The development of the NeRF assay provided a method to study this phenomenon directly and to compare the reactions of RF with rabbit and human native IgG as well as with aggregated antigens.

The first consideration in discussing the reaction

between RF and native IgG is to establish that native IgG preparations are truly "native". Although it is probably not possible to definitely demonstrate that a preparation of native IgG is totally native, the "nativeness" of IgG used here can be inferred from some of our data. One of the most important observations in this regard was the reaction of C1q with aggregated IgG and its non-reactivity with native IgG. Like other C1q binding assays (Zubler et al., 1976), C1q in the nephelometer was capable of detecting as little as 3 µg/ml of aggregated IgG. Thus, the native preparations used here had to contain less than 3 µg/ml of aggregated IgG, since C1q failed to react with them. The NeRF reaction of pRF with native IgG (800 µg/ml) was equivalent to the reaction elicited by 50 µg/ml of aggregated IgG, which contained >99% aggregated IgG (Results 1.1.1). As a result, the reaction of RF with native IgG cannot be attributed to aggregated IgG present in the native IgG preparations.

There are additional data to support the "nativeness" of our native IgG: gel filtration which showed that the native IgG eluted as a single symmetrical peak of absorbancy; ultracentrifugation (90,000 x g; 75 min) which removed insoluble materials; and filtration through 0.22 µm filters. The effects of polyethylene glycol (PEG) on the reaction of RF with native IgG also suggested that non-

native IgG was absent from our native IgG preparation. Increasing concentrations of PEG enhanced the reaction of RF with native IgG whereas PEG concentrations in excess of 1% diminished the reaction of RF with aggregated IgG. Finally in an attempt to avoid potential denaturation of IgG associated with isolation procedures, pRF was added to serum. The reaction of pRF when added to normal serum and the smaller reaction when added to agammaglobulinemic serum were quantitatively similar to the results obtained with isolated native IgG. These results again suggest RF reacts with IgG which is native.

In 1% and 2% PEG, the reaction of pRF with native IgG was similar for human and for rabbit native IgG, when analyzed by the time course of the NeRF reaction over 5-60 minutes of incubation. Antigen concentrations from 100-800 µg/ml failed to reveal any differences in reactivity. Kinetic analysis also showed that the peak rate, V_{max} and K_m were remarkably similar for rabbit and human antigens. Furthermore, the effects of different concentrations of PEG also support the conclusion that pRF reacts similarly with rabbit and human native IgG. In the absence of PEG, the reaction of pRF with NRGG was much less than the pRF reaction with NHGG (Results, Table 4.14). This was reflected in lower peak rate, V_{max} and K_m values. The lower V_{max} of the reaction of pRF with NRGG, which is accelerated

by the addition of PEG to reaction mixtures, would account for the decreased reactivity of pRF with NRGG, when the assay is read at a fixed time interval (eg. 30 min).

PEG exerts profound effects upon the reaction of RF with IgG, in that it accelerates the rate of reaction and increases the Net LS of the reaction of RF with a given concentration of antigen. The mechanism whereby effects occur is unknown despite its widespread use in a variety of immunological assays. It is unlikely that PEG enhances the RF-IgG reaction by aggregating the IgG antigen, since the LS of native IgG alone is not increased by PEG in contrast to aggregated IgG. Also, at high concentrations of PEG, the Net LS of RF-aggregated IgG reactions is diminished unlike with RF-native IgG reactions which are enhanced. Furthermore, the PEG effect is reversible and does not alter the subsequent reaction of RF with IgG (Results, Fig. 4.33). The well recognized enhancement of antigen-antibody reactions, which do not involve denaturation or aggregation of antigen, also suggests PEG has an effect on the RF-IgG reaction which is not due to the denaturation or aggregation of IgG.

Monoclonal rheumatoid factor (mRF_{og}) reacts with both rabbit and human native IgG but the reaction with rabbit is extremely small when compared with the mRF_{og}-human IgG reaction or the pRF-native IgG reactions. Aggregation

of IgG increased the reactions of both rabbit and human antigens, making the quantitation of the mRF_{og}-rabbit IgG reaction possible. Thus, one observes a great difference in reactivity between rabbit and human IgG with this monoclonal protein. However, mRF_{og} still exhibited weak reactivity with the rabbit antigen, presumably reflecting low affinity for this antigen. This has been found with other monoclonal IgM-RF preparations (Stone, 1973) and also for mRF_{og} by an ELISA RF assay (Karsh, 1981).

The aggregated IgG used here was a large, polydisperse macromolecular complex in contrast to the uniform native IgG. Although the absolute differences in light scattering between the reactions of RF with aggregated and with native IgG could be due to differences in antigen size, the similar reactivity of aggregated IgG with two preparations of monoclonal RF (mRF_{og} and mRF_{mi}) and with polyclonal RF makes possible comparisons independent of antigenic size. Each of these rheumatoid factors reacted differently with native IgG, indicating there had to be a change in reactivity by at least two of these RF preparations to produce similar aggregate reactions (Fig. 4.32). It is not possible to conclude if this change in reactivity was due to: 1. a change in the affinity of rheumatoid factors for aggregated IgG; 2. multivalency; or 3. unmasking of new antigenic sites. However, it is unlikely

that unmasking of antigenic sites accounts for these changes, since monoclonal RF (mRF_{og}) was completely absorbed by native IgG; implying that the same antigens had to be present on native and aggregated IgG. The nephelometer provides quantitative data on the rates of reaction of RF with IgG, by the relationship of these kinetic measurements to measurements fo RF affinity remains to be elucidated. The use of a monodisperse preparation of immune complexes (prepared in antigen excess) would avoid the problems inherent in the polydisperse aggregates, and could provide a reagent whereby such measurements might be made. In addition, it would then be possible to confirm that a change in reactivity of RF with aggregated IgG was due to multivalency, a view favored by most investigators (Normansell, 1971; Eisenberg, 1976; Dissanayake et al., 1977; Hunneyball and Stanworth, 1979).

Laser nephelometry has been shown to be useful for the assay of numerous serum proteins including RF. Our results indicate that the nephelometric RF assay is a valid and versatile assay. In analyzing the nephelometric reaction of RF with IgG, several methods, in addition to the NeRF assay, have been developed. These include:

1. New methods for the aggregation of rabbit IgG, using glutaraldehyde and heating;

2. An assay for the reaction of RF with native IgG with its obvious advantages in being a fluid-phase reaction;
3. Techniques for the kinetic analysis of RF with aggregated and native IgG, which is a model system for other immune reactions;
4. The feasibility of nephelometrically assaying immune complexes by their capacity to bind C1q;
5. A method for absorbing RF with native IgG, providing a simple and gentle method of isolating RF which does not require harsh treatment to recover the RF from the immunosorbent;
6. A simple but useful method for delipidating biological fluids by Freon-MF treatment.

In addition to these methodological developments our work has defined the reaction of RF with IgG, showing that human rheumatoid factor reacts with native IgG as well as with aggregated IgG. Differences in the reaction of RF with human and rabbit IgG could not be demonstrated, despite extensive nephelometric analysis which included kinetics, different methods of antigen preparation, and the survey of over 250 sera. Although aggregated IgG is the reference antigen in most RF assays, results here show that native IgG is also an antigen for RF. RF reacted with native IgG either isolated or in serum. This fluid-phase

reaction exhibited biological activity, demonstrated by the binding of C1q to RF-IgG complexes. Furthermore, both rabbit and human native IgG were able to absorb RF, emphasizing the lack of species specificity exhibited by human rheumatoid factor.

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