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IMMUNOLOGICAL PROPERTIES OF ISOLATED IgG AND IgM ANTI-GAMMA-GLOBULINS (RHEUMATOID FACTORS)*

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SUMMARY

Anti-gamma-globulins of the IgG and IgM classes have been isolated from sera of normal individuals and from patients with osteoarthritis, rheumatoid arthritis and juvenile rheumatoid arthritis. All of the isolated antibodies gave precipitin curves with heat-aggregated, reduced and alkylated gamma-globulin. IgM anti-gamma-globulins gave a positive latex fixation test at 4°C and 37°C while IgG anti-gamma-globulins generally gave a positive test only at 4°C. Anti-gamma-globulins from normals did not fix complement but IgG and IgM anti-gamma-globulins from rheumatoids fixed complement to a similar degree. This *in vitro* complement fixation could account at least in part for the diminished complement levels seen in many rheumatoid synovial effusions.

INTRODUCTION

Rheumatoid arthritis is characterized immunologically by increased levels of serum and synovial fluid anti-gamma-globulins (rheumatoid factors) (Hall, 1961; Panush, Bianco & Schur, 1971; Plotz & Singer, 1956; Ziff, 1957), as well as by depressed synovial fluid complement levels (Hedberg, 1967; Pekin & Zvaifler, 1964; Ruddy *et al.*, 1969). These low levels of complement and complement components are thought to result from immunological consumption and activation of the complement system rather than through other mechanisms (Ruddy *et al.*, 1969; Ruddy *et al.*, 1971). Although complement-fixing antigen–antibody complexes have not been demonstrated in synovial fluids, a number of systems have been described which may contribute to complement depletion within the joint space. Gamma-globulin which has been aggregated *in vitro* will fix complement (Ishizaka & Ishizaka, 1959).

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Aggregates of IgG have been recognized in some rheumatoid synovial fluids on the basis of a precipitation reaction with IgM rheumatoid factor (Hannestad, 1967). In addition, other complexes have been detected both by precipitation with IgM rheumatoid factor or C1q, followed by acid dissociation and identification of the predominant ingredients as IgG and IgG rheumatoid factors (Winchester, Agnello & Kunkel, 1970). Complement-fixing cryoproteins, consisting of IgG, IgM, bound complement components, fibrinogen, DNA and rheumatoid factor have also been noted in rheumatoid effusion (Marcus & Townes, 1971). The intra-articular depletion of complement activity has been noted to be proportional to the titre of rheumatoid factor—that is, IgM anti-gamma-globulins as well as IgG anti-gamma-globulins (Panush *et al.*, 1971; Hedberg, 1967; Winchester *et al.*, 1970). These studies strongly suggest that the complement depletion within synovial fluids may be due at least in part to anti-gamma-globulins reacting with gamma-globulin.

The purpose of our study was, therefore, to isolate, characterize and compare the IgG and IgM anti-gamma-globulins from normals and from patients with osteoarthritis and rheumatoid arthritis, as regards precipitation, latex agglutination and complement fixation

MATERIALS AND METHODS

Sera

Sera were obtained from normal individuals and from patients with osteoarthritis (OA), latex-positive and latex-negative juvenile rheumatoid arthritis (JRA) and latex-positive and latex-negative rheumatoid arthritis (RA).

Isolation of rheumatoid factors

Human Cohn Fraction II (Lederle Labs, Pearl River, New York), 10 mg/ml, was heat-aggregated at 63°C for 30 min and was coupled to cyanogen bromide-activated Sepharose 6B as described by Goetzl & Metzger (1970). The immunoadsorbent (IA) was washed with 0·01 M phosphate-buffered saline (PBS), pH 7·2, until the OD_{280} of the supernatant was zero.

Fifteen to 150 ml of serum were heat-inactivated for 30 min at 56°C and were incubated with the IA at 37°C for 1 hr and at 4°C overnight on a gentle rocker shaker. On the following day, a 2·5 × 20 cm column was packed with the serum–IA mixture and washed with PBS until the OD_{280} was zero. Supernatants from latex-positive JRA or RA samples were saved and concentrated down to the original serum volume and tested for latex agglutinating activity. Elution of serum proteins from the IA was then instituted with 0·1 M sodium acetate buffer, pH 4·5, and the elution monitored by OD_{280} measurements.

Eluates were neutralized to pH 7·4 and concentrated to 1–2 ml by negative pressure dialysis and dialysed versus PBS. Immunoglobulin content was analysed by radial immunodiffusion (Mancini, Carbonara & Heremans, 1965) against monospecific antisera to IgG, IgA and IgM. The presence of other proteins was determined by immunoelectrophoresis (Scheidegger, 1955) utilizing antiserum to whole human serum.

Selected eluates containing a mixture of IgG and IgM anti-gamma-globulins were separated by gel filtration chromatography on Sephadex G-200 using 0·1 M sodium acetate buffer, pH 4·5 (Schronhenloher, Kunkel & Tomasi, 1964). Fractions containing either IgG or IgM were concentrated by negative pressure dialysis to 2 ml, dialysed versus PBS and analysed by radial immunodiffusion.

Latex fixation test

Latex fixation tests were performed at 37°C (Hall, Mednis & Bayles, 1958) or at 4°C for 18 hr.

Quantitation of immunoglobulins

IgG, IgA and IgM concentrations were determined in eluates by radial immunodiffusion as previously described (Panush *et al.*, 1971). Gamma-G subclass concentrations were determined in the serum of patients as previously described (Schur *et al.*, 1970). Gamma-G subclass concentrations were measured in the eluates by a modified Rowe (Rowe, 1969) radioimmunoassay (Dobkin *et al.*, 1974). Specific antiserum was made in rabbits or monkeys to the IgG subclasses (Schur, 1972; Schur *et al.*, 1970). The immunoplates were then overlaid with either radioactively labelled monkey antiserum to rabbit gamma-globulin or rabbit antiserum to monkey gamma-globulin.

Precipitin reactions

IgG, at 10 mg/ml was first reduced with 2-mercaptoethanol and then alkylated with iodoacetamide (Zvaifler & Schur, 1968). This treated IgG was then heat-aggregated for 30 min at 63°C (to be referred to hereafter as 2 ME-γ-glob). An amount of 0·01 ml of isolated IgG or IgM RF was incubated with increasing amounts of 2 ME-γ-glob in a final total volume of 2·57 ml, for 1 hr at 37°C and 18 hr at 4°C. The precipitates were washed with cold PBS, the protein content was measured by the Folin method (Lowry *et al.*, 1951) and the equivalence points were determined (Kabat & Mayer, 1961). Controls included 2 ME-γ-glob and IgG or IgM RF.

Complement fixation

The ability of isolated IgG and IgM RF to fix complement with 2 ME-γ-glob was determined as described previously (Zvaifler & Schur, 1968). Pooled normal human serum, stored at −70°C, was diluted so as to contain 70 CH50 units per ml. Isolated IgG or IgM RF at a protein concentration ranging from 0·002 to 0·112 mg/ml was incubated with 2 ME-γ-glob at equivalence ratios (as determined from the precipitin curves) and in two to eight times RF excess. The mixture of antigen (2 ME-γ-glob)–antibody (RF), complement and buffer (total volume 1·0 ml) was incubated for 90 min at 37°C. Buffer, isolated RF and 2 ME-γ-glob were used as individual controls. The percentage of complement fixed was calculated as described previously (Schur & Becker, 1963). When the isolated IgG or IgM RF or 2 ME-γ-glob exhibited anti-complementary activity, the percentage of total complement fixed by the mixture (RF and 2 ME-γ-glob) was calculated as described previously (Schur & Becker, 1963).

RESULTS

Immunoadsorbent—experimental conditions

Ninety to 95% of the heat-aggregated human Fraction II bound to the cyanogen bromide-activated Sepharose 6B. This IA was stable in 0·1 M, pH 4·5 sodium acetate buffer without releasing coupled IgG from the column. The use of a highly acidic buffer, 25% acetic acid, pH 2·1, or a high molarity buffer, 3 M potassium iodide, resulted in the release of small amounts of IgG from the IA.

Following elution of the RF from the IA with 0·1 M, pH 4·5 acetate buffer, a single peak of protein was detected by OD_{280}. Further treatment of the IA with 0·1 M, pH 2·8 glycine buffer yielded no additional protein.

The supernatants of sera from patients with latex-positive JRA or RA following reaction with IA, in most instances, failed to agglutinate the latex particles.

TABLE 1. Anti-gamma-globulin eluates: immunoglobulin concentration, latex agglutination activity and complement fixing ability

Patient	Diagnosis (Latex)	Eluates (mg/ml)			Latex fixation		Maximum percentage complement fixed	
		IgG	IgA	IgM	37°C	4°C	Equivalence	RF excess
LN	RA (+)	<0·01	0	1·2	1:640	1:320	58	48
HJ	RA (+)							
FX I*		0	0	0·15	1:640	1:320	32	44
FX II*		0·6	0	0	0	1:80	29	43
RG	RA (+)							
FX I		0	0	0·75	1:640	1:640	0	25
FX II		0·25	0	0	1:320	1:640	20	40
DG	RA (+)							
FX I		0	0	0·64	1:640	1:640	39	36
RN	RA (−)	1·4	0	0	0	1:80	66	27
DW	RA (−)	0·03	0	0·56	1:1280	1:1280	n.d.†	n.d.
MC	RA (−)	0·042	0	0·47	1:640	1:640	n.d.	n.d.
DH	JRA (+)							
FX I		0	0	2·0	1:2560	1:2560	57	59
FX II		0·28	0	0	0	1:160	40	37
IG	JRA (+)	0·11	0	0·85	1:1280	1:1280	n.d.	n.d.
MS	JRA (−)	0·54	0	0	0	1:80	33	39
RK	JRA (−)	1·4	0	0	0	1:160	46	68
AD	OA	0·1	0	0	0	1:80	25	35
BE	OA	0·25	0	0	0	1:40	5	0
NB	Normal	0·22	0	0	0	1:80	0	0
LD	Normal	0·4	0	0	0	1:80	0	0
KD	Normal	1·5	0	0	0	1:40	0	0

* FX I and FX II refer to Sephadex G-200 chromatographic fractions.
† N.D. = not determined.

Analysis of the eluates and chromatographic fractions

Protein content. Immunoelectrophoresis with a polyvalent antiserum to human serum revealed only immunoglobulins in the eluates. When the eluates were quantitatively evaluated by radial immunodiffusion, only IgG or IgM or both were found (Table 1). IgM was detected in eluates from all latex-positive sera, in two of three latex-negative RA sera, but not in eluates from latex-negative JRA or OA sera or from normal sera. IgG was detected in all eluates.

When eluates containing both IgG and IgM were separated by Sephadex G-200 chromatography using 0·1 M, pH 4·5 acetate buffer, the first peaks, Fractions I, contained only IgM and the second peaks, Fractions II, contained only IgG (Table 1).

Latex fixation test. The results of the latex fixation tests are shown in Table 1. Eluates and chromatographic fractions from patients with JRA or RA containing IgM gave a positive latex test at both 37° and at 4°C with similar titres. Eluates and chromatographic fractions containing only IgG from normal individuals, patients with OA and patients with latex-negative or -positive JRA or RA gave a positive latex test at 4°C, while no reaction was observed at 37°C, with the exception of one IgG Sephadex G-200 fraction obtained from a patient with latex-positive RA (Table 1).

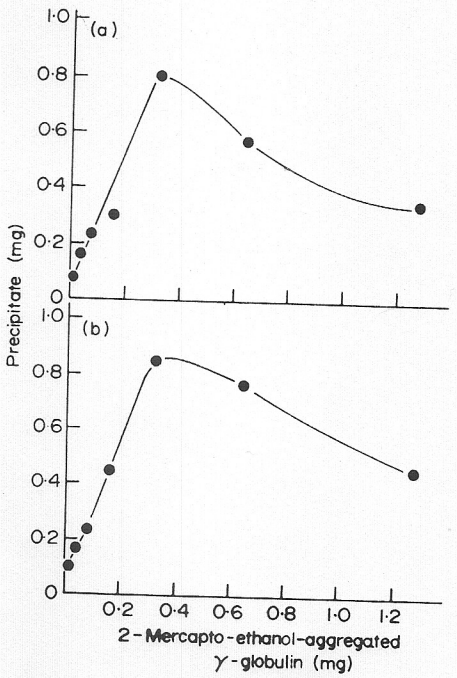

FIG. 1. Precipitin curves of (a) isolated IgG rheumatoid factor and (b) IgM rheumatoid factor anti-gamma-globulins derived from a patient with latex-positive rheumatoid arthritis.

Precipitin reactions. Typical precipitin curves with equivalence zones were detected for all isolated IgG and IgM RF. The equivalence zone varied from sample to sample in no discernible pattern. An example of a precipitin curve given by isolated IgG and IgM RF with nearly identical equivalence zones is given in Fig. 1. The antigen to antibody ratio at the equivalence point was calculated and the results are given in Table 2. The Ag/Ab ratio varied considerably for both IgG and IgM RF.

IgG subclasses. Anti-gamma-globulin eluates containing IgG (see Table 1) were analysed for IgG subclasses. The results are given in Table 3. All of the eluates contained IgG1 and IgG2 and most of them contained IgG3 and some IgG4. The predominant IgG subclass was IgG1. The percentage of anti-gamma-globulin in each IgG subclass did not differ consistently between any of the clinical categories investigated. As a whole, the distribution of the eluates among the IgG subclasses was similar to that of the serum IgG, although there was considerable individual variation.

TABLE 2. Anti-gamma-globulins: equivalence points and complement-fixing efficiencies

IG RF	Ag/Ab ratio*	Units of complement fixed/μg protein	IgM RF	Ag/Ab ratio*	Units of complement fixed/μg protein
HJ RA (+)	107	1·5	HJ RA (+)	320	4·3
RG RA (+)	128	1·4	RG RA (+)	43	0·13
DH JRA (+)	114	2·5	DH JRA (+)	32	0·55
RN RA (−)	23	0·34	LN RA (+)	11	1·6
MS JRA (−)	59	1·7	DG RA (+)	50	1·4
RK JRA (−)	46	2·2	Mean	91	
AD OA	32	1·9			
NB normal	145				
LD normal	320				
KD normal	85				
Mean	106				

$$* \text{ Ag/Ab ratio} = \frac{\text{mg of 2 ME-}\gamma\text{-glob}}{\text{mg of RF}} \text{ at equivalence.}$$

TABLE 3. Gamma-G subclass concentrations in serum and anti-gamma-globulins

Patient	Diagnosis	IgG1 (%)	IgG2 (%)	IgG3 (%)	IgG4 (%)
Serum data					
HJ	RA (+)	89	11	<0·1	<0·1
RG	RA (+)	85	10	3	2
RN	RA (−)	77	5	8	10
DH	JRA (+)	65	23	10	2
RK	JRA (−)	76	15	2	7
MS	JRA (−)	67	14	13	6
AD	OA	77	6	17	<0·1
NB	Normal	73	22	4	1
KD	Normal	58	24	2	16
LD	Normal	64	22	5	9
Eluate data					
HJ (FX II)	RA (+)	84	5	11	n.d.
RG (FX II)	RA (+)	59	8	33	n.d.
RN	RA (−)	82	8	2	8
DH	JRA (+)	70	10	20	n.d.
RK	JRA (−)	63	11	1	25
MS	JRA (−)	76	16	7	1
AD	OA	51	34	16	n.d.
NB	Normal	81	19	n.d.	n.d.
KD	Normal	72	7	1	20
LD	Normal	71	9	16	4

* n.d. = not detectable.

Complement fixation studies. A summary of the results for complement fixation by RF reacting with 2 ME-γ-glob is given in Tables 1 and 2. Even at concentrations of 10 mg/ml, 2 ME-γ-glob by itself fixed very little, if any, complement. In addition, IgG and IgM RF by themselves at concentrations up to 0·24 mg/ml fixed very little, if any, complement. All of the eluates containing IgM RF fixed complement while reacting with 2 ME-γ-glob. Up to 59% of the complement added was fixed by 80 μg of RF and 0·64 mg of 2 ME-γ-glob. There were some differences between the maximum amount of complement fixation at equivalence ratios and in RF excess. IgG RF from latex-positive or latex-negative JRA and RA sera and from two OA sera-fixed complement while reacting with 2 ME-γ-glob, from 5–69% of the added complement. Adding excess RF did not significantly increase complement fixation in this reaction. Three IgG RF (over a wide range of protein concentrations) from normal sera did not fix complement while reacting with 2 ME-γ-glob.

When IgG and IgM RF were compared microgram for microgram, it appeared that they could fix similar amounts of complement when reacting with 2 ME-γ-glob, although there were moderate individual variations. Table 2 gives the number of CH50 units fixed per microgram of either IgG or IgM RF. However, when they were compared on a molar basis, IgM RF was approximately ten times as efficient as IgG RF in fixing equivalent amounts of complement. Maximum complement fixation was given by approximately 10^{-11} M IgM RF and by 10^{-10} M IgG RF.

DISCUSSION

Rheumatoid factors are 19S IgM (Edelman, Kunkel & Franklin, 1958) antibodies to gamma-G globulin which were first noted in the majority of patients with rheumatoid arthritis. Rheumatoid factors have also been detected in other diseases and occasionally IgA and/or IgG antibodies to gamma-G globulins were recognized in some patients with RA (Chodirker & Tomasi, 1963; Heimer & Levin, 1966; Kunkel *et al.*, 1961; Schrohenloher, 1966). Through the use of sensitive solid IgG immunoadsorbent techniques it appears that most, if not all, normal individuals have IgG antibodies to gamma-G globulins (Panush *et al.*, 1971; Torrigiani & Roitt, 1967). IgM anti-gamma-globulins were found only in patients with latex-positive RA or JRA (Bianco *et al.*, 1971; Panush *et al.*, 1971) but levels of IgG and IgA anti-gamma-globulins were higher in the sera of patients with RA and JRA than in normals (Bianco *et al.*, 1971; Panush *et al.*, 1971; Torrigiani & Roitt, 1967).

IgM rheumatoid factors have been shown to precipitate with aggregated gamma-globulin and with immune complexes and form broad precipitin curves. In the present study, precipitin curves with well defined equivalence points were observed with both isolated IgG and IgM anti-gamma-globulins obtained from rheumatoids and normals. There was no apparent difference in the shape of the curves or the equivalence points when anti-gamma-globulins from normals or rheumatoids were used. The IgG and IgM anti-gamma-globulins did not have the same equivalence points.

IgM rheumatoid factors characteristically agglutinate particles sensitized with gamma-G globulin (Hall, 1961; Plotz & Singer, 1956; Ziff, 1957). The agglutination techniques, however, provide only a semiquantitative estimate of the amount of antibody to gamma-globulin present. There was little, if any, correlation in individual sera between the latex fixation titre and the concentration of IgG, IgA or IgM anti-gamma-globulin (Panush *et al.*, 1971). Although this agglutination has been shown to be due primarily to IgM rheumatoid

factors (Franklin *et al.*, 1957), 7S gamma-globulins (Chodirker & Tomasi, 1963) as well as IgG (Heimer & Levin, 1966; Schrohenloher, 1966) and IgA (Heimer & Levin, 1966) rheumatoid factors have on occasion been noted to also give a positive latex-fixation test. In addition, when sera are not heat-inactivated normal sera will also agglutinate gamma-G globulin-sensitized latex particles through reaction with C1q (Ewald & Schubart, 1966). A standard temperature, at which rheumatoid factor tests should be performed, has not been determined. The sensitized sheep cell agglutination test is performed at 4°C (Heller *et al.*, 1955), the Rh agglutination test at room temperature (Waller & Vaughan, 1956) and latex fixation test at 37°C (Cathcart, 1967), although the original description cites use of 56°C (Plotz & Singer, 1956). Capra *et al.*, 1969) have noted an unusual form of rheumatoid factors in patients with infectious mononucleosis and reticulum cell sarcoma which were temperature-dependent and gave agglutination reactions at 4°C. Stimulated by these results, we examined sera from patients with RA and JRA that had negative latex-fixation tests at 37°C. Surprisingly, a significant number of sera from JRA patients gave a positive latex titre at 4°C (Bianco *et al.*, 1971). Many of these JRA patients had elevated levels of IgG anti-gamma-globulins (Bianco *et al.*, 1971). We, therefore, studied IgG anti-gamma-globulins in a latex agglutination test performed at 4°C. Isolated IgG anti-gamma-globulins, whether from normals or from patients with OA, RA or JRA gave a positive latex-agglutination reaction at 4°C and none at 37°C, with one exception (Table 1). By contrast, isolated IgM anti-gamma-globulins, from patients with RA or JRA, gave positive and equal titres at both 4°C and 37°C. It would, therefore, appear that when the standard latex fixation test is performed at 37°C (Cathcart, 1967) positive tests primarily reflect the presence of IgM anti-gamma-globulins. These results are similar to the temperature-dependence of IgG and IgM agglutinating anti-A antibodies (Hummell & Götze, 1966) and rabbit IgG and IgM complement-fixing antibodies (Cunniff & Stollar, 1968).

Early accounts showed that rheumatoid factors did not fix complement and, in fact, appeared to inhibit complement fixation. Later studies showed that IgM rheumatoid factors can fix complement in their reaction with IgG, but only under certain well defined conditions (Zvaifler & Schur, 1968). In the present study, it was demonstrated that both isolated IgG and IgM anti-gamma-globulins from patients with RA, JRA and the IgG from one patient with OA-fixed human complement at 37°C. By contrast, the IgG anti-gamma-globulins from normal individuals and from one patient with OA did not fix complement. IgM RF was more efficient than IgG RF in fixing complement. The difference in complement-fixing ability between anti-gamma-globulins from normal individuals and from patients with rheumatoid arthritis could reflect a difference in the binding constants, conformation, avidity, secondary aggregation or in the IgG subclass of these IgG anti-gamma-globulins. IgG1, IgG2 and IgG3 fix complement while IgG4 does not (Ishizaka *et al.*, 1967). However, there was no apparent difference in the IgG subclass distribution in the anti-gamma-globulins isolated from the different individuals.

Low levels of complement and complement components in rheumatoid synovial fluids correlate not only with elevated levels of IgM and IgG anti-gamma-globulins (Panush *et al.*, 1971; Hannestad, 1967) but also with an unremitting course in rheumatoid arthritis, the presence of subcutaneous nodules, periarticular demineralization, joint space narrowing, cortical impaction, more extensive joint involvement and, on synovial membrane biopsies, more surface synoviocytic giant cells and more subsurface lymphocytic infiltration (Britton *et al.*, 1972). The ability of an antibody to fix complement may, therefore, help to mediate

the inflammatory reactions seen in rheumatoid synovitis. A number of immune complexes could account for the complement fixation within the joint space, including an antigen–antibody complex, aggregated gamma-globulin or rheumatoid factors reacting with either of these two. The first possibility has not been discovered; aggregates of IgG–anti-IgG which precipitate with C1q have been noted (Hannestad, 1967) and rheumatoid factor will fix complement with gamma-G globulin (Zvaifler & Schur, 1968). In the present studies we have demonstrated that both IgG and IgM anti-gamma-globulins can fix complement.

What portion of the depressed synovial fluid complement levels could be accounted for by rheumatoid factor complement fixation with IgG? The maximal number of units of haemolytic complement (C) fixed by each of the (complement-fixing) rheumatoid factor preparations (expressed as units of C fixed per microgram of anti-gamma-globulin protein) is given in Table 2. There was a considerable range of complement-fixing efficiency for both the IgG and IgM anti-gamma-globulins, ranging from 0·13–4·3 units of C fixed per microgram of protein. The mean complement-fixing efficiency for those IgG and IgM anti-gamma-globulins that fixed complement was virtually identical, 1·65 and 1·60 CH50 units per microgram of anti-gamma-globulin, respectively. The mean level of these anti-gamma-globulins in synovial fluids from latex-positive patients with rheumatoid arthritis has been shown previously to be 163 μg/ml of IgG and 99 μg/ml of IgM anti-gamma-globulins (Panush *et al.*, 1971). Therefore, these anti-gamma-globulins could theoretically fix approximately 259 and 160 CH50 units of complement, respectively, after reacting with aggregates of gamma-globulin. The mean complement level in latex-positive rheumatoid synovial fluid was 45 CH50 units/ml and in serum was 240 CH50 units/ml (Britton *et al.*, 1972). In degenerative joint disease the synovial fluid complement levels are approximately one-third of the serum levels in the same patients (Britton *et al.*, 1972). Therefore, if synovial fluid complement levels are less than one-third of the corresponding serum levels, this decrease could represent local complement fixation. Thus, the difference between one-third of 240 CH50 units (mean rheumatoid serum level) and 45 CH50 units (mean rheumatoid synovial fluid level) or 35 CH50 units represents the theoretical amount of complement that could be fixed in each millilitre of latex positive rheumatoid synovial fluid. This value is less than the theoretical complement fixing ability of IgG and IgM rheumatoid factor as determined in the present study. These data suggest that the degree of *in vitro* complement fixation between IgM and/or IgG anti-gamma-globulins and 2 ME-aggregated γ-glob theoretically could account at least in part for the *in vivo* complement depletion in rheumatoid synovial fluids. However, these data and calculations representing static measurements, do not take into account the constant turnover of complement within the joint space. Nor does it exclude complement fixation or depletion by some other mechanism or complexes.

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