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Naturally occurring human antibodies to the F(ab′)$_2$ portion of IgG

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SUMMARY

Antibodies to the F(ab′)$_2$ portion of IgG were detected in the sera of patients and normals using a heat-aggregated F(ab′)$_2$ fragment in a liquid-phase radioimmunoassay. The antibodies were found in 72% of 82 rheumatoid arthritis patients, in less than 20% of patients with other diseases and in a few normal subjects. Anti-F(ab′)$_2$ antibodies were distinct from previously described anti-Fc antibodies and 'serum agglutinators'. They reacted with intact IgG, F(ab′)$_2$ and IgM in binding and inhibition assays and they appeared to have specificity for the Fd portion of IgG molecules, possibly to the V_H region. The anti-F(ab′)$_2$ antibodies were of both 7S IgG and 19S IgM classes and were found in sera as components of intermediate size (between 7S and 19S) and large size (greater than 19S) immune complexes. The possible contribution of autologous anti-F(ab′)$_2$ to abnormal immunoregulation is discussed.

INTRODUCTION

Heterologous anti-immunoglobulins have been shown to have a mitogenic effect on lymphocytes and to modulate immunoglobulin synthesis *in vitro* (Greaves, 1970; Gausset *et al.*, 1976; Möller, 1978). Naturally occurring anti-idiotypic antibodies may have similar immunoregulatory effects *in vivo* as suggested by Jerne (1974). Such anti-idiotypic antibodies would be expected to react with the variable region of the F(ab′)$_2$ portion of antibody molecules and thus might also react with lymphocyte receptors (Cosenza & Kohler, 1972a, 1972b; Kohler *et al.*, 1977). Patients with rheumatoid arthritis (RA) and other diseases often have anti-immunoglobulin antibodies. The specificities of such naturally occurring antibodies other than rheumatoid factors (anti-Fc antibodies) have not been well studied. This paper describes our initial studies of naturally occurring anti-F(ab′)$_2$ antibodies. It is necessary to characterize the various subpopulations of anti-immunoglobulins before examining their likely role as immunoregulatory factors.

A radioimmunoassay for the detection of antibodies with specificity for the F(ab′)$_2$ portion of IgG molecules (anti-Fab antibodies) was developed by modifying our anti-IgG radioimmunoassay (Yamagata *et al.*, 1979). Our results show that anti-Fab antibodies could be detected in many patients with RA, in some patients with other rheumatological disorders and in a few normal subjects as well. Anti-Fab antibodies were inhibited by intact IgG and so must react with antigenic determinants that are exposed on native IgG molecules unlike those previously described (Osterland, Harboe & Kunkel, 1963; Harboe, Rau & Aho, 1965).

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MATERIALS AND METHODS

Collection of sera. Blood samples were obtained by venipuncture from informed patients at the UCLA Center for the Health Sciences and from normal volunteers. After clotting at room temperature, sera were separated and stored at −20°C until use. Patients with rheumatoid arthritis (RA) fulfilled the American Rheumatism Association criteria for definite or classical RA (Ropes *et al.*, 1958), and patients with systemic lupus erythematosus (SLE) fulfilled similar criteria for SLE (Cohen *et al.*, 1971). All other rheumatological diagnoses were those assigned to the patient by the primary care physician but confirmed by a faculty rheumatologist at UCLA.

Isolation of IgG, F(ab')₂ fragments and Fc fragments. Human IgG was purified from Cohn's Fraction II (Miles Labs, Elkhart, Indiana) by DEAE column chromatography. F(ab')₂ fragments were prepared by pepsin digestion of IgG (Nisonoff *et al.*, 1960). The F(ab')₂ was separated by collecting the excluded peak from a Sephadex G-75 column and subjecting this to two successive purifications using Sephadex G-200 to remove small amounts of undigested IgG, Fab and other small fragments. Purified F(ab')₂ gave a single peak of 5S when analysed by sucrose density-gradient ultracentrifugation and produced no visible line when tested at concentrations of up to 24 mg/ml in Ouchterlony immunodiffusion against specific rabbit anti-Fc antiserà (Behring Diagnostics, Somerville, New Jersey).

Fc fragments were prepared by papain digestion of IgG (Porter, 1959). Fc fragments were then isolated by CM and DEAE column chromatography (Franklin, 1960), followed by Sephadex G-100 and Sepharose 4B anti-Fab column chromatography. No precipitin line was observed when tested against a specific rabbit anti-Fab antiserum (Behring Diagnostics, Somerville, New Jersey) at concentrations of approximately 5 mg/ml by the Ouchterlony method.

Preparation of ^{125}I-labelled aggregates of IgG and F(ab')₂. Preparation of radiolabelled stable aggregates of IgG of different sizes has been described previously (Knutson *et al.*, 1979; Yamagata *et al.*, 1979), and preparation of similar aggregates of F(ab')₂ was found to be feasible using a similar protocol. IgG and F(ab')₂ were labelled with ^{125}I (New England Nuclear, Boston, Massachusetts) to a specific activity of 0·1 mCi/mg by the chloramine T method (Hunter & Greenwood, 1962). Aggregations were performed by heating 10 mg/ml of labelled proteins for 30 min at 63°C for IgG and 60°C for 20 min for F(ab')₂. Aggregates of IgG (A-IgG) and F(ab')₂ (A-Fab) with molecular weights of approximately 10×10^6 daltons were then separated by sucrose density-gradient ultracentrifugation. Aggregates were kept in phosphate-buffered saline (PBS) containing 0·5% bovine serum albumin (BSA) to stabilize the aggregates. Isolated A-IgG and A-Fab both produced single narrow peaks in the identical fraction from which they were harvested upon re-ultracentrifugation as we have previously reported for A-IgG (Knutson *et al.*, 1979).

Radioimmunoassays for anti-IgG and anti-Fab antibodies. Our radioimmunoassay method for anti-IgG antibodies (Yamagata *et al.*, 1979) was slightly modified and then adapted to measure anti-Fab antibodies as well as anti-IgG antibodies. Briefly, each 50-μl serum sample to be tested was serially diluted with 0·01 M phosphate-buffered saline, pH 7·4 (PBS), containing 0·05% NaN_3 and 0·5% BSA, using a microdilutor and flexible microtitration plates (Dynatech, Alexandria, Virginia). The plates were then heated to 56°C for 30 min to inactivate the diluted serum. It was important for the assay that inactivation be done *after* dilution of the serum samples.

A-IgG or A-Fab (0·25 μg in 25 μl) were then added to each well and mixed by agitation of the microtitre plate. Seventy-five microlitres of a solution containing polyethylene glycol (2% PEG) (mol. wt 6,000–7,000 daltons), 0·02 M EDTA and approximately 10,000 c.p.m. of ^{22}Na (New England Nuclear) were added to each well and the plates were again agitated and incubated at room temperature for 16 hr. The plates were centrifuged at 3,000 r.p.m. for 40 min, the supernatants were aspirated and the pellets counted for ^{125}I and ^{22}Na activity in a dual-channel automated gamma counter (Beckman, model 7000). After correcting the ^{125}I counts for volume of unseparated supernatant (using ^{22}Na as the volume marker) (Steward, Olsen & Barnett, 1977), the data were plotted as per cent antigen precipitated versus the logarithm of the reciprocal of the serum dilution. The titre of anti-IgG or anti-Fab antibodies for each serum was defined as that dilution which precipitated 33% of the antigen and this was read directly from the semi-log plots.

Purification of anti-Fab and anti-Fc antibodies. Anti-Fab and anti-Fc antibodies were purified from the sera of RA patients by affinity column chromatography using IgG or $F(ab')_2$ coupled to Sepharose-4B (Cuatrecasas, 1970). Antibodies were eluted from the column by lowering the pH of the starting buffer (0·1 M glycine, 0·1 M NaCl) from 8·2 to 3·5.

Anti-Fab antibodies were purified from sera known to contain a high titre of anti-Fab activity by two successive immunoabsorptions with a Sepharose-$F(ab')_2$ column. Anti-Fc antibodies were obtained from the effluent of a Sepharose-$F(ab')_2$ column followed by a Sepharose-IgG column. These were termed 'anti-Fc antibodies', since they reacted with IgG but not with $F(ab')_2$.

Binding of radiolabelled antibodies to Sepharose 4B-coupled ligands. Purified anti-Fab and anti-Fc antibodies were iodinated by the chloramine T method (Hunter & Greenwood, 1962). These labelled antibodies were then tested for their ability to bind various proteins that were coupled to Sepharose 4B beads. Five milligrams of IgG, $F(ab')_2$, Fc and HSA were coupled to each ml of Sepharose 4B gel. Beads were reacted with radiolabelled purified antibodies for 2 hr at room temperature and washed immediately with 0·1 M glycine saline buffer, pH 8·0, containing 0·5% BSA. The per cent ^{125}I bound to the Sepharose was calculated.

Inhibition of anti-Fab antibody activity. The RIA was slightly modified for the inhibition studies as follows: 50 μl of diluted purified anti-Fab antibodies were mixed with 25 μl of inhibitors, 25 μl of A-Fab and 50 μl of 3% PEG solution was then added giving a final PEG concentration of 1%. A dilution of the antibodies that would precipitate 60 to 80% of A-Fab was used for the inhibition studies.

The inhibitors used in these experiments included IgG purified from Cohn's FII, $F(ab')_2$, Fc fragments, heavy (i.e. gamma) and light chains obtained after reduction and alkylation of the IgG by the method of Fleischman, Pain & Porter (1961). Autologous IgG which was free of anti-Fc and anti-Fab activity was prepared by four successive passages over $F(ab')_2$ and IgG Sepharose affinity columns followed by 50% ammonium sulphate precipitation and DEAE chromatography. IgM was purified from Cohn's FII by DEAE and Sephadex G-200 column chromatography.

RESULTS

Purity of A-Fab

The purity of the A-Fab was studied by another approach in addition to the Ouchterlony analyses described in the Materials and Methods section. A-Fab and A-IgG were reacted in the RIA with specific goat anti-Fab antiserum (Miles) and goat anti-Fc antiserum (Kallstad, Chaska, Minnesota). As expected, anti-Fab antiserum reacted with both A-Fab and A-IgG. The A-IgG titre was always slightly higher than the anti-Fab titre. The anti-Fc antiserum had, by chance, a similarly high titre for A-IgG as the anti-Fab antiserum, but failed to react with A-Fab.

Anti-Fab and anti-IgG titres in diseases

Anti-IgG and anti-Fab titres were determined for normal sera ($n = 35$) and for sera from patients with a number of diseases (Fig. 1a, b). An abnormal or 'positive' test was defined as a titre higher than 2 standard deviations above the mean for normals (broken lines in Fig. 1a, b). The upper limit of normal was a titre of 8·6 for the anti-Fab assay and a titre of 26·4 for the anti-IgG assay.

RA patients had the highest prevalence of abnormal titres of both anti-IgG and anti-Fab antibodies (92·7 and 72·0% respectively). Smaller proportions of patients with other rheumatological disorders were positive in the two assays and the titres tended to be lower. The per cent positive sera in these other diseases is included in Fig. 1a and b. Only the group of sera from patients with RA were found to be significantly different from normal by the χ^2 test.

Characterization of anti-Fab antibodies

Anti-Fab and anti-Fc antibodies were purified from patients' sera with rheumatoid arthritis by affinity column chromatography and tested in the A-IgG and the A-Fab assays. Eluted antibodies had lower titres than their respective sera in the A-IgG and A-Fab assays, probably due for the most part to loss of activities during purification. Eluted antibodies were, therefore, greatly

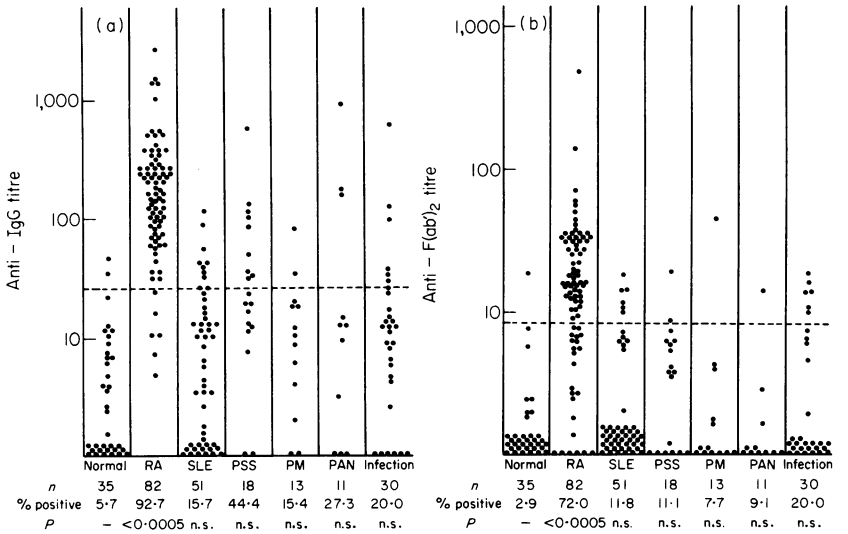

Fig. 1. Serum anti-IgG titres (a) and anti-F(ab')₂ titres (b) were determined in patient groups of rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), scleroderma (PSS), polymyositis (PM), polyarteritis nodosa (PAN) and several infectious diseases (Infection). The titre for each serum was defined as the dilution which precipitated 33% of the antigen. The dotted line shows the mean ±2 s.d. of 35 normal sera. Any value above it was considered abnormal. *P* values were calculated by the χ^2 test. n.s.=not significant.

concentrated before being assayed. Sera from these patients reacted with both A-IgG and A-Fab as already shown. The purified anti-Fab antibodies also reacted with both A-IgG and A-Fab (Table 1). However, anti-Fc antibodies failed to react with A-Fab but reacted with A-IgG as expected. These results show that the anti-Fab antibodies reacted with the F(ab')₂ portion of intact, undigested IgG molecules as well as with A-Fab. This experiment further confirms the specificity of anti-Fab antibodies under study.

In other experiments, purified anti-Fab and anti-Fc antibodies were radiolabelled with ^{125}I and reacted with various proteins bound to Sepharose 4B (Table 2). Anti-Fab antibodies reacted strongly with IgG and F(ab')₂ but only weakly with Fc fragments or with human serum albumin (HSA). Conversely, anti-Fc antibodies reacted strongly with IgG and Fc fragments, but more weakly with F(ab')₂ and HSA. Thus, anti-Fab and anti-Fc antibodies prepared by our immunoabsorption methods were specific and did not cross-react inappropriately with each other's natural ligands.

Four RA sera were tested for their serum agglutinator and pepsin agglutinator activity in the laboratory of Dr Marion Waller. These activities were determined using erythrocytes precoated with Ripley anti-Rh antibody digested by pepsin, papain, subtilisin, trypsin or chymotrypsin (Waller & Blaylock, 1966; Waller, Richard & Mallory, 1969). A test for serum agglutinators is

Table 1. Anti-IgG and anti-Fab activities of antibodies purified from RA sera (titres)

	Serum		Anti-Fab antibody		Anti-Fc antibody	
Patient	A-IgG	A-Fab	A-IgG	A-Fab	A-IgG	A-Fab
D.G.	980	84	34	11	440	< 3
A.P.	636	18	167	34	640	< 3
R.I.	3,200	52	270	64	6,500	< 3
B.S.	1,050	30	18	13	1,200	< 3

Table 2. Per cent binding of anti-F(ab')$_2$ and anti-Fc antibodies to Sepharose-coupled ligands

Antibodies	Patient	Ligands (% binding) IgG	F(ab')$_2$	Fc	HSA
Anti-Fab antibody ^{125}I	R.I.	48·0	43·4	6·9	8·7
	A.P.	48·6	30·1	2·4	n.d.
Anti-Fc antibody ^{125}I	R.I.	69·6	6·3	23·5	11·3

n.d. = Not done.

considered positive if any three of the papain, subtilisin, trypsin or chymotrypsin tests are positive with titre of 1 : 160 or above (Waller *et al.*, 1971). The four RA sera tested were all clearly in the normal range since the highest titre observed for all of the serum agglutinator tests was 1 : 10.

The pepsin agglutinator and our A-Fab radioimmunoassay titres for sera and purified anti-Fab antibodies are shown in Table 3. All of the four RA sera had low titres in the pepsin agglutinator tests. Furthermore, there was an almost total dissociation of the results comparing serum and purified antibodies in the two assays. Two purified antibody preparations (R.I. and G.C.) had lower pepsin agglutination titres but higher anti-Fab titres than the parent serum. Conversely, purified antibodies from B.S. had a higher (though still low) pepsin agglutination titre and a lower titre in the RIA. The 'negative' agglutinator titres and the dissociation of the results for purified antibodies and serum support the view that our assay detects a population of antibodies distinct from those detected by haemagglutination tests.

Inhibition of anti-Fab antibodies by various immunoglobulins and fragments

IgG, IgM, F(ab')$_2$, Fc, γ-chains, light chains or autologous IgG were used as inhibitors in the A-Fab assay using purified anti-Fab antibodies from RA patients. The concentration of inhibitors required to reduce the binding of anti-Fab antibodies to A-Fab by 50% was estimated. The results of the inhibition studies from three RA patients are shown in Table 4. IgG, IgM and F(ab')$_2$ from Cohn's FII were the best inhibitors, while Fc and light chains were poor inhibitors. The γ-chains showed slight inhibition of the reaction. Autologous IgG that was depleted of anti-Fab and anti-Fc activity by immunoabsorption was also a poor inhibitor. A large excess of inhibitor protein was used in each of these studies because A-Fab has a much higher avidity for anti-Fab antibodies than monomeric F(ab')$_2$ due to a higher functional valence for A-Fab.

Immunoglobulin classes of purified anti-Fab antibodies and their size distribution in serum

Anti-Fab antibodies from seven RA patients' sera were purified by immunoabsorption. These antibodies were fractionated by sucrose gradient ultracentrifugation and then assessed for anti-Fab activities in the RIA. Activities sedimented at 19S and 7S in all preparations of purified anti F(ab')$_2$ antibodies tested as shown in Fig. 2. IgG was detected by Ouchterlony analysis in the 7S fractions. Because the protein concentration in the 19S fractions was low, these fractions were

Table 3. Pepsin agglutinator and anti-Fab titres for serum and purified anti-Fab antibodies from RA sera

Patient	Serum RIA	Serum PA	Anti-Fab antibody RIA	Anti-Fab antibody PA
R.I.	52	1 : 10	64	1 : 2
G.C.	21	1 : 10	>192	1 : 2
A.P.	18	<1 : 10	34	1 : 2
B.S.	30	<1 : 10	13	1 : 20

RIA = A-Fab radioimmunoassay, PA = Pepsin agglutinator.

Table 4. Inhibition of anti-Fab antibody activity in three patients with rheumatoid arthritis

Inhibitors	Concentration required to give 50% of inhibition		
	R.I.(1 : 25)	A.P.(1 : 15)	G.C.(1 : 150)
IgG from Cohn's FII	13·0*	11·3	12·3
$F(ab')_2$	13·4	25·0	1·9
Fc	>130·0	>130·0	>130·0
γ-chain	110·0	56·0	120·0
Light chain	>340·0	>340·0	>340·0
IgM	11·2	8·8	22·0
Autologous IgG (free of anti-Fab and anti-Fc activity)	249·0	>250·0	195·0

* Results expressed as μg/0·15 ml.

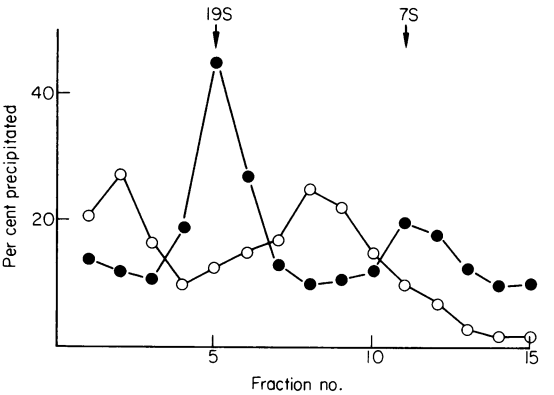

Fig. 2. Size distribution of anti-Fab activities by sucrose density-gradient (10–30%) ultracentrifugation (38,000 r.p.m., 4°C, 18 hr). In purified anti-Fab antibodies (●——●) the activities were found in 19S and 7S fractions. In the serum (○——○) the activities were found in greater than 19S and between 19S and 7S fractions.

passed through an affinity column containing Sepharose-linked goat anti-human IgM. The excluded fraction was devoid of anti-Fab activities whereas the eluate contained more than half of the activity applied. The activities remained in the excluded fraction when these same 19S fractions were passed through a goat anti-human IgG affinity column. Thus, anti-Fab antibodies were both 19S IgM and 7S IgG.

Sera from five RA patients known to be positive in A-Fab assays were ultracentrifuged on sucrose gradients and the various fractions were then assayed for A-Fab activity. The per cent of ^{125}I precipitated by the various fractions from one of the sera is shown in Fig. 2. Sera from the other four patients showed very similar patterns. Anti-Fab activity was mainly detected in greater than 19S fractions and between 19S and 7S fractions, suggesting that the anti-Fab antibodies were circulating in serum as soluble immune complexes.

DISCUSSION

This paper describes the characterization of naturally occurring antibodies in humans that react with the $F(ab')_2$ portion of intact IgG molecules. The radioimmunoassay for detection of anti-Fab antibodies in this paper was similar to that described for our anti-IgG radioimmunoassay

(Yamagata *et al.*, 1979), except that A-Fab was used as the antigen instead of A-IgG. Anti-Fab antibodies were present in the serum of patients with various rheumatological disorders, notably rheumatoid arthritis, and could be detected in patients with chronic infections and in some normal subjects as well. The presence of anti-Fab antibodies could be dissociated from the presence of anti-Fc antibodies.

Inhibition studies were performed to gain insight into the specificity of the anti-Fab antibodies. IgG and $F(ab')_2$ from Cohn's FII were good inhibitors for the reaction between anti-Fab antibodies and A-Fab, whereas Fc fragments were poor inhibitors. Hence, the antigenic determinants are located on the $F(ab')_2$ portion of native intact IgG molecules. Gamma chains were better inhibitors than light chains suggesting that the antigenic determinants are primarily expressed on the Fd portion of heavy chains. Although γ-chains were good inhibitors, they were not as effective as intact IgG. It is therefore possible that full expression of the antigenic sites requires a steric configuration that depends on the presence of light chains in the IgG molecule.

The Fd portion of IgG consists of the variable region (V_H) and part of the constant domain (CH1) of the heavy chain. Several lines of evidence suggest that anti-Fab antibodies are directed to determinants on the V_H region and are thus anti-idiotypic antibodies.

First, there are only two known allotypic determinants in the CH1 domain, Gm1(f) and Gm1(z), but antibodies against these allotypic determinants are rare in RA patients (Grubb, Kronvall & Martensson, 1965; Gold *et al.*, 1965; Brazier *et al.*, 1971). Secondly, IgM was as effective an inhibitor as IgG. The V_H region of γ- and μ-chains from myeloma proteins is known to have more homology than the constant region and there is also data to suggest that the V_H region of both IgG and IgM is coded by the same gene (Capra & Kehoe, 1975). Thirdly, autologous IgG was not as effective an inhibitor as pooled IgG. This suggests that not all autologous IgG molecules express the determinants against which anti-Fab antibodies are directed. Therefore, we believe that anti-Fab antibodies represent anti-idiotypes against a repertoire of idiotypes that are commonly included in pooled IgG. However, we cannot exclude the possibility that anti-Fab antibodies react with unknown allotypic determinants that are present on both IgG and IgM.

The fact that autologous IgG was a poor inhibitor of anti-Fab antibodies might be due to partial *in vivo* suppression of cells producing this repertoire of IgG molecules by anti-Fab antibodies. Thus, the autologous IgG would have less of this repertoire of IgG molecules than normal pooled IgG. Alternatively, idiotype–anti-idiotype complexes formed in the circulation might have been removed by the RES, thus reducing the level of these idiotypes in the serum. Peaks of 7–19S and > 19S anti-Fab activity were detected in sera. This suggests that at least some antigen was initially present in the form of immune complexes. Finally, it is quite possible that autologous IgG expressing the antigenic determinants in question may have been lost during the purification steps done to deplete anti-Fab and anti-Fc activity from autologous IgG before it was used as an inhibitor. These steps included immunoabsorption with both IgG and $F(ab')_2$ Sepharose columns.

Several reports have described antibodies to fragments of IgG obtained by digestion of anti-Rh antibodies with a variety of enzymes (Osterland *et al.*, 1963; Harboe *et al.*, 1965; Natvig, 1966; Waller & Blaylock, 1966; Kormeier, Ing & Mandy, 1968; Waller *et al.*, 1971). These antibodies have been detected by their ability to agglutinate red blood cells coated with such IgG fragments and so have been termed 'serum agglutinators'. The best studied example of these is the 'pepsin agglutinator' which appears to react only with pepsin digests of anti-Rh antibodies but not with intact IgG or other digests of IgG. We feel that our assay detects a population of antibodies that is distinct from the various agglutinators described. First, anti-Fab antibodies were inhibited by intact IgG. Second, the four sera that were examined were negative or had low titres of agglutinator activity and third, the low pepsin agglutinator activity present could be dissociated from the anti-Fab activity. Our purified antibodies probably did contain some agglutinator antibodies because immunoabsorption was performed with $F(ab')_2$ obtained by pepsin digestion of IgG. Thus the anti-Fab antibodies had been demonstrated to be distinct but might be slightly contaminated by the above-mentioned antibodies.

Anti-Fab antibodies were shown to consist of both IgM (19S) and IgG (7S) classes, and they

seemed to circulate predominantly as intermediate complexes (between 7S and 19S) and large complexes (greater than 19S).

Others have shown that heterologous anti-immunoglobulins including anti-F(ab')₂ antibodies can activate human lymphocytes (Greaves, 1970; Gausset *et al.*, 1976, Möller, 1978). Heterologous anti-idiotypes may also modulate immunoglobulin synthesis (Cosenza & Kohler, 1972a, b; Kohler *et al.*, 1977). It is possible that autologous anti-Fab antibodies play an immunoregulatory role *in vivo*. Such regulatory activity might be mediated either through direct reaction of anti-Fab antibodies with certain lymphocyte receptors or through the formation of immune complexes. The increased prevalence of this antibody in RA may account for some of the abnormal regulatory immune responses seen in RA (Silverman *et al.*, 1976; Horwitz & Garrett, 1977).

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