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Research Article

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Anti-Fab Antibodies in Humans
Predominance of Minor Immunoglobulin G Subclasses in Rheumatoid Arthritis

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Abstract

Isoelectric focusing analyses of sera from patients with rheumatoid arthritis (RA) demonstrate two populations of antibodies directed against the Fab portion of pooled human IgG. One population is composed of polyclonal alkaline anti-Fab antibodies (αFABA) and the other, acidic αFABA which are more clonally restricted. In this study we have identified the immunoglobulin classes and subclasses of these antibodies in RA sera.

Enzyme-linked immunosorbent assays (ELISA) demonstrated αFABA in RA sera to be predominantly IgG. A large portion of IgG αFABA existed as immune complexes, inasmuch as dialysis of RA sera against 6 M urea before ELISA analysis was necessary for maximal detection of αFABA activity. Chromatofocusing of RA sera isolated αFABA of different charges and revealed the acidic clonally restricted αFABA to be IgG4 and IgG3, whereas the polyclonal alkaline group contained IgG1, IgG2, and IgG3. Overall, acidic IgG3 and IgG4 comprised 70% of IgG αFABA, and high levels of IgG4 were seen in most RA sera. When αFABA were elevated in normal sera, they were primarily of the IgG4 subclass, and also existed as immune complexes. Serum anti-Fab activity was removed by adsorption of sera with Fab fragments. Anti-Fab antibodies of both κ and λ light-chain types were present in RA sera, and $F(ab')_2$ fragments of RA serum immunoglobulin were found to possess anti-Fab activity.

These studies indicate that αFABA in RA sera are limited to the IgG class, and that most of these antibodies exist as immune complexes and display clonal and minor IgG subclass restriction.

Introduction

Rheumatoid arthritis (RA)[1] is a systemic chronic inflammatory disease, whose pathogenesis is felt to be immunologically mediated (1). Although synovial inflammation predominates clinically, extra-articular manifestations are often found, sometimes in association with a vasculitis (2). Autoantibodies directed against IgG are a characteristic finding in the sera and

1. *Abbreviations used in this paper:* CF, chromatofocusing; ELISA, enzyme-linked immunosorbent assay; αFABA, anti-Fab antibodies; IEF, isoelectric focusing; OA, ovalbumin; RA, rheumatoid arthritis; RF, rheumatoid factor.

synovium of RA patients, and can form circulating immune complexes, high levels of which have been associated with extra-articular activity (3–6).

Both anti-IgG antibodies directed against the Fc portion of IgG, known as rheumatoid factors (RF) and antibodies directed against the $F(ab')_2$ region of IgG have been found in RA sera (7). In the past, studies of anti-IgG antibodies have generally concentrated on RF, which have been well characterized (8, 9). Anti-Fab antibodies (αFABA) in RA were first described by Osterland et al. (10), who reported the presence of "pepsin agglutinators" in serum, antibodies reactive with IgG $F(ab')_2$ fragments but not native IgG (10). Since then αFABA have been shown to have varying specificities for different portions of the Fab and $F(ab')_2$ molecule and to circulate as immune complexes. Additionally, they may also be present in other autoimmune diseases, chronic infections, after immunization, or in some normal individuals (11–15).

We have recently demonstrated that a portion of αFABA in RA and in some normal individuals are a unique group of immunoglobulins which are clonally restricted and have acidic isoelectric points (16). These αFABA require dissociating conditions with urea for their optimal detection by isoelectric focusing (IEF) analysis, confirming their existence in serum as complexes, and are generally in higher quantity in RA sera.

Because only a minority of immunoglobulins are acidic molecules (17), we undertook this present study to isolate RA serum αFABA and to define their immunoglobulin isotypes and subclasses. We have found that the clonally restricted αFABA are primarily restricted to the IgG3 and IgG4 subclasses of IgG, although some individuals may possess αFABA of the IgG1 subclass.

Methods

Sera collection and preparation. Sera from patients with RA who fulfilled the criteria of the American Rheumatism Association for definite or classical RA (18) and sera from normal individuals were studied. All sera were frozen at -20°C until needed. Serum samples to be treated with urea were dialyzed against 6 M de-ionized urea for at least 48 h before analysis by enzyme-linked immunosorbent assay (ELISA) or IEF analysis.

Iodination of proteins. 100 μg of each relevant protein in 0.2 M sodium phosphate buffer, pH 7.3, was iodinated with 1 mCi of $Na^{125}I$ (Amersham Corp., Arlington Heights, IL) to a specific activity of 2,000–3,000 cpm/ng by the chloramine-T method (19). Iodinated proteins were suspended in 1% ovalbumin (OA) to a concentration of 1.5–3.0 μg/ml.

IEF. Agarose IEF was performed as previously described (20, 21). Briefly, 10-μl serum samples were applied to 10% sorbitol–2% agarose gels, containing 6 M urea and 0.5% (vol/vol) ampholines, pH 3.5–9.5 (LKB Produkter, Stockholm, Sweden). Gels were focused at constant power (6.25 W/gel) for 70 min and then incubated 1 h in 37°C saturated sodium sulfate (Na_2SO_4) to precipitate proteins, followed by 1 h in 0.43% dimethyl suberimidate in Tris-buffered, saturated Na_2SO_4, pH 8.0, to cross-link proteins, and 30 min in 0.5% glycine in Tris

buffer, pH 8.0, to block any remaining reactive groups. After incubation in 1% OA to block nonspecific protein-binding sites, gels were overlaid with 30 ml of the iodinated antigen solution (1.5–3.0 μg/ml in 1% OA). Gels were washed overnight in 15 liters of water, fixed with 10% trichloracetic acid, dried, and autoradiographed on Kodak X-R Omat film (Eastman Kodak Co., Rochester, NY).

Chromatofocusing (CF). Standard CF techniques were used to fractionate RA serum proteins (22, 23). 10 ml of each RA serum was precipitated and washed twice with saturated ammonium sulfate (50% vol/vol), resuspended in 6 M urea, and dialyzed against starting buffer. Each prepared serum was chromatofocused over a pH range of 9–6 on a 20-ml polybuffer exchanger (PBE 94, Pharmacia Fine Chemicals, Uppsala, Sweden) column previously equilibrated with the starting buffer, 0.025 M ethanolamine acetic acid, pH 9.4, containing 6 M urea. Serum fractions were eluted with a 1:10 dilution of Polybuffer 96 (Pharmacia), pH 4.0, also containing 6 M urea. 6-ml fractions were collected and the pH of each fraction was determined. All buffers were degassed before use and adjusted to their correct pH.

Purity of IgG Fab fragments. Fab fragments of IgG (Cappel Laboratories, Cochranville, PA) used in these studies were found to contain a single band after sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The Fab fragments were reactive with anti-human antiserum but not with an anti-human Fc (γ chain-specific) antiserum.

ELISA for isotype identification of αFABA. 300 μl of a 30-μg/ml coating solution of Fab fragments of polyclonal human IgG (Cappel Laboratories) in 0.1 M phosphate-buffered saline, pH 7.1 (PBS), was added to wells of a 96-well microtiter plate (Linbro plates, Flow Laboratories, McLean, VA) and incubated overnight at room temperature. The next day the coating solution was aspirated, the plate was washed three times with 1% OA, and 300 μl of 1% bovine serum albumin (BSA) added to each well for 1 h to block unbound protein sites. The plate was washed twice with 1% OA, and either serum samples at multiple dilutions (0.02–0.1 μl), or 5–50-μl aliquots of CF fractions were added to the plate. The volume of each well was brought up to 300 μl with 1% BSA. After an overnight incubation, the samples were removed, the plate was washed three times with 1% OA, and 300 μl of a 1:750 dilution of goat anti-human IgG, IgM, or IgA antibody (Sigma Chemical Co., St. Louis, MO) in 1% BSA was added and incubated for 2 h. After removal of second antibody, the plates were washed twice with 1% OA and six times with distilled water, and then 300 μl of p-nitrophenyl phosphate disodium substrate (Sigma Chemical Co.) was added to each well. Optical densities (OD) were measured with a multichannel spectrophotometer, and converted to nanograms of IgG, IgM, or IgA based upon a standard curve generated by coating wells on a section of each plate with goat anti-human IgG, IgM, or IgA (Tago, Inc., Burlingame, CA) diluted 1:8 in PBS, to which various dilutions of IgG, IgM, or IgA standards were added. Only values determined to be on the linear portion of αFABA titration curves were used for final αFABA determinations.

Specificities of monoclonal anti-human IgG subclass antibodies. All anti-human IgG subclass monoclonal antibodies (Miles-Yeda Ltd., Rehovot, Israel) used in these studies have been found to be specific by ELISA against at least seven purified myelomas of each IgG subclass. These monoclonal antibody have also been tested in the World Health Organization/International Union of Immunological Societies Immunoglobulin Subcommittee's Ig subclass antisera study, and all lots of monoclonals used in our studies have been shown to be IgG subclass-specific without isoallotype reactivity, except possibly for the IgG2 monoclonal antibody, which may have some isoallotype cross-reactivity (personal communication, Miles Scientific Div., Miles Laboratories Inc., Naperville, IL).

ELISA for identification of IgG αFABA subclass. The coating of wells and addition of samples were identical to the ELISA for detection of αFABA isotypes. After removal of samples, 200 μl of mouse monoclonal anti-human IgG1, IgG2, IgG3, or IgG4 (Miles-Yeda Ltd.) antibody diluted with 1% BSA to 1:1000, 1:5,000, 1:120, and 1:5,000, respectively, was added to each well and incubated at room temperature for 4 h. These dilutions represented an excess of antibody in order to

overcome differences in the binding affinities of the monoclonals for their target subclasses. After washing, 200 μl of a 1:650 dilution of goat anti-mouse IgG antibody (American Qualex, Inc., La Mirada, CA), conjugated with alkaline phosphatase (Sigma Chemical Co.) (24), was added to each well and incubated 2 h. Plates were then washed with 1% OA twice and six times with distilled water, after which 200 μl of substrate was added to each well. To standardize development times, a panel of positive control sera and CF fractions was analyzed on each plate, and sample OD were recorded for the plate once the controls reached their predetermined OD values. Comparison of the relative quantities of the different subclasses of IgG was possible as all monoclonal antisera were mouse IgG1, and the same conjugated third antibody was used for all subclass determinations. The percentage of the contribution of each subclass to the total αFABA activity was determined by integration of curve areas.

Effect of urea treatment of serum on IgG and IgG4 antidiphtheria toxoid antibody activity. To determine whether antibodies to other antigens are affected by urea treatment of serum, IgG antidiphtheria toxoid antibody levels were determined in a panel of six sera obtained from diphtheria toxoid booster-immunized volunteers. As seen in Table I, IgG antidiphtheria antibody levels were not significantly different in urea treated or untreated samples $(P > 0.05)^2$. Prior analyses of five of these sera demonstrated that only two of the sera (sera 2 and 3) had acidic antibody bands, which bound the fragment A portion of diphtheria toxoid. These sera were also shown to be IgG4 immunoglobulin (25). When these five urea-dialyzed sera were analyzed for IgG4 antidiphtheria antibodies, only serum 2 and, particularly, serum 3 were found to have IgG4 antidiphtheria antibodies (Table I). Thus, urea treatment of sera did not significantly alter the reactivity of IgG antidiphtheria antibodies with diphtheria antigen. Furthermore, urea treatment of serum did not promote the detection of IgG4 antidiphtheria antibodies, as the only sera found to have IgG4 antidiphtheria antibodies after urea dialyzation were also the only sera shown to possess acidic spectrotypes.

Preparation of purified κ and λ light-chain Fab fragments. An anti-human λ light-chain affinity column was prepared by coupling 300 μg of mouse IgG1 monoclonal anti-human λ light-chain antibody (Bethesda Research Laboratories Molecular Diagnostics, Gaithersburg, MD) in 0.1 M sodium bicarbonate buffer, pH 8.0, to N-hydroxysuccinimide ester agarose (Affi-Gel 10, Bio-Rad Laboratories, Richmond, CA). 500 μg of pooled human polyclonal Fab fragments (Cappel Laboratories) in PBS was circulated through the anti-λ column five times. The column was extensively washed with PBS, and the final wash was determined to be free of all protein measured at OD 280 nm. Purified λ light-chain Fab fragments were eluted from the column with 0.2 M glycine, pH 2.2, and neutralized with Tris salt to pH 8.0. Purified κ light-chain Fab fragments were obtained by passing 75 μg of Fab fragments enriched for κ light chains (the initial Fab solution which had been passed through the column five times) over the regenerated anti-λ column twice, stripping the column with acid and regenerating it before the second passage. The purified λ and κ Fab fragments were diluted to 10 μg/ml, and used to coat ELISA plates. λ Fab fragments were determined by ELISA to be 94% pure, and κ Fab fragments 89% pure, with respect to their light-chain types.

Anti-Fab antibody light-chain analysis. Serum acidic chromatofocused fractions that contained IgG3 and IgG4 αFABA subclasses were selected from six RA patients, and 10 μl of each fraction was reacted in two ELISA plates, one coated with κ Fab fragments and the other coated with λ Fab fragments. After overnight incubation, 200 μl of an IgG1 mouse monoclonal anti-human κ light-chain antibody were added to the λ Fab-coated plate, and 200 μl of a monoclonal anti-human λ light-chain antibody (Bethesda Research Laboratories Molecular Diagnostics) were added to the κ Fab-coated plate. After 2 h of incubation, 200 μl of alkaline phosphatase-conjugated affinity-purified goat anti-mouse IgG (American Qualex, Inc.) was added to both plates. After another 2-h incubation, 200 μl of alkaline phosphatase substrate

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2. Paired *t* test.

Table I. Effect of Urea Treatment of Serum on Antidiphtheria Toxoid Antibody Activity

	Serum sample	1	2	3	4	5	6
*Total IgG antidiphtheria antibody level (OD 405 nm)	Untreated	0.211	0.083	0.435	0.233	0.089	0.233
	Urea-treated	0.079	0.170	0.275	0.066	0.122	0.103
*IgG4 antidiphtheria antibody level (OD 405 nm)	Untreated	0.380	0.749	>2.00	0.609	0.129	—
	Urea-treated	0	0.132	1.392	0.044	0.024	—
‡Presence of acidic clonotypes on IEF analysis		−	+	+	−	−	

* OD values for total IgG and IgG4 antidiphtheria toxoid antibody levels cannot be directly compared with each other in that different developing antibodies with different specific activities were used in the IgG and IgG4 ELISA assays for antidiphtheria toxoid antibody activity.   ‡ Antibody bands with pI > 7 which bind ^{125}I-labeled diphtheria toxoid fragment A as shown below:

Approximate pH
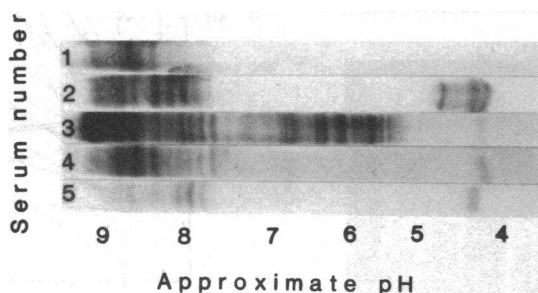
(Sigma Chemical Co.) was added to each plate and the OD were then determined.

Preparation of F(ab')₂ fragments of RA serum immunoglobulin. F(ab')₂ fragments of serum immunoglobulin of four individual RA sera were prepared and tested for αFABA activity in ELISA. Each serum was twice precipitated with saturated ammonium sulfate to enrich for immunoglobulin, and the immunoglobulin fraction was treated with pepsin (Sigma Chemical Co.) according to the procedure of Nisonoff (26). After pepsinization, each serum was extensively dialyzed against PBS to remove small peptides. Analysis of the sera by SDS-PAGE revealed single bands with the same mobility as an F(ab')₂ control; comparison with an IgG control showed no undigested IgG to be present in any of the five sera. The presence of αFABA in the pepsinized serum fractions was determined by incubating each in an ELISA plate coated with purified λ light-chain Fab fragments, followed by sequential addition of mouse monoclonal anti-human κ light-chain antibodies (Bethesda Research Laboratories Molecular Diagnostics), goat anti-mouse IgG (American Qualex, Inc.), and substrate.

Results

Effect of urea on the IEF resolution of αFABA. Previous studies have demonstrated that some αFABA in RA sera exist as immune complexes (12, 27); therefore, to assess fully the clonal heterogeneity of these serum autoantibodies, it was necessary to study those present in immune complexes as well as those circulating as free antibody. When untreated sera were analyzed on a 6 M agarose gel, resolvable αFABA spectrotype patterns were seen (Fig. 1 *a*). Dialysis of the same sera against urea in conjunction with analysis on an agarose gel containing urea resulted in the appearance of additional Fab binding proteins (Fig. 1 *b*). These proteins, which we have previously identified as immunoglobulins (16), were most prominent in the acidic region of the gel, although diffuse uptake was often noted in the alkaline region. A comparison of the total protein pattern with the autoradiographs indicated

that a high proportion of the proteins in the acidic region of the gel had anti-Fab properties (Fig. 1 *c*).

Class distribution of anti-Fab antibodies. Because the majority of the acidic αFABA in RA sera seemed by IEF analysis to exist as immune complexes, we wished to determine whether urea dissociation of these complexes was needed for optimum detection by ELISA. We postulated that, if serum complexes were first dissociated by dialyzing sera against 6 M urea and then diluted 1,000-fold before ELISA, such complexes would be less likely to reassociate with their original partner molecules than to bind to the excess Fab fragments on the ELISA plate. Analysis of 30 RA and 16 normal sera revealed that dialysis of sera against 6 M urea before ELISA analysis resulted in significantly higher levels of IgG αFABA in both the RA (Fig. 2 *a*, $P < 0.001)^2$ and normal (Fig. 2 *b*, $P < 0.001$) groups. The levels of αFABA were significantly higher in RA compared to normal sera for both untreated ($P < 0.0125)^3$ and urea-dialyzed ($P < 0.0025$) samples. IgM and IgA αFABA were either absent or extremely low (<6 µg/ml) in RA sera both before and after dialysis against urea (data not shown).

Specificity of the anti-Fab ELISA. To confirm the specificity of αFABA for Fab fragments, we wished to show that αFABA activity could be decreased by prior adsorption of sera with Fab fragments. As seen in Table II, when sera were preincubated in Fab-coated plates, ~50% of the αFABA activity was removed in four of the five sera examined when compared with matched samples preincubated in BSA-coated plates.

Determination of the IgG subclasses of αFABA among acidic RA proteins fractionated by CF. The above experiments indicated that αFABA were primarily IgG. However, in that the majority of αFABA showed fast mobilities on IEF, they seemed likely to be IgG molecules with acidic pI, i.e., IgG4 (17). To determine the subclass distribution of the αFABA in
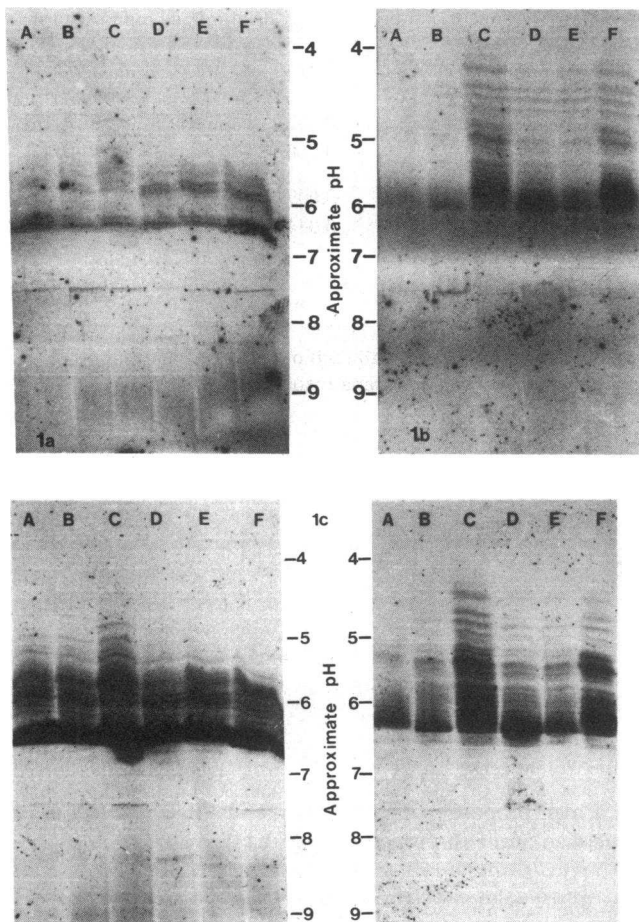
3. Student's *t* test.

Figure 1. Dialysis of serum against 6 M urea enhances the resolution of anti-Fab antibody spectrotypes. (*a*) Untreated sera analyzed on a 6 M urea-agarose gel; (*b*) the same sera dialyzed against 6 M urea before analysis on a 6 M urea-agarose gel; (*c*) total protein stain of sera analyzed in *a* and *b*. Sera *B–E* are RA sera; serum *A* is from a normal individual.
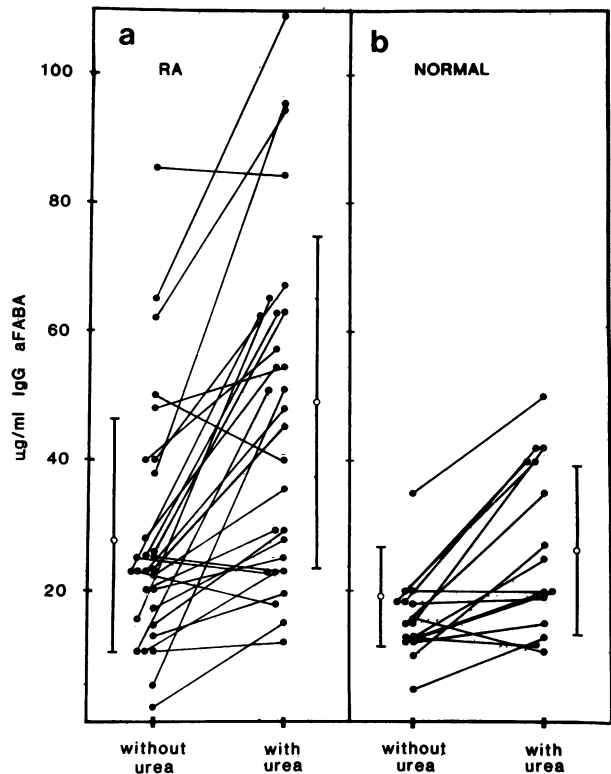

Figure 2. IgG anti-Fab antibody levels in RA (*a*) and normal (*b*) sera determined by ELISA. Dialysis of sera against 6 M urea before analysis significantly increased IgG αFABA levels in both RA ($P < 0.001$) and normal ($P < 0.001$) sera, indicating a large portion of αFABA circulate as immune complexes. Range bar indicates mean value± 1 SD.

relation to their charge, 16 RA sera were preparatively isolated using CF, which separates proteins according to their pI in a manner similar to IEF. The chromatofocused RA sera were fractionated in the presence of 6 M urea into 15 fractions, each of which was assayed by ELISA for each of the four IgG subclasses of αFABA. Examples of CF analyses are shown in Fig. 3. Overall, acidic IgG3 and IgG4 predominated, with some sera also demonstrating elevated levels of alkaline IgG1 (Table III). CF analyses of 10 normal sera for IgG αFABA subclass distributions revealed significantly lower levels of IgG3 ($P < 0.001$) and IgG4 ($P < 0.001$) αFABA, indicating that the elevated IgG3 and IgG4 αFABA levels in RA sera were not an artifact of urea treatment of serum.

Immunoglobulin light-chain analysis of αFABA. Earlier IEF analyses suggested a restricted clonal nature of the acidic αFABA. To further assess this restriction, the light-chain types of the αFABA sera were assessed by a modified ELISA assay. Plates were coated with κ- or λ-containing Fab molecules (isolated by light-chain affinity chromatography), incubated with αFABA, and the binding of αFABA was detected by the complementary anti-human light-chain antisera, i.e., anti-κ on the λ Fab plates and anti-λ on the κ Fab plates. Individual

acidic CF fractions from six RA sera containing only IgG3 and IgG4 αFABA—one with equivalent levels of IgG3 and IgG4 αFABA, two with mostly IgG3 αFABA, and three with mostly IgG4 αFABA—were analyzed for αFABA light-chain

Table II. Removal of αFABA Activity after Adsorption of RA Sera to Fab Fragments

RA patient	αFABA in serum adsorbed to BSA (OD 405 nm)	αFABA in serum adsorbed to Fab fragments (OD 405 nm)	Decrease
			%
W.H.	0.309	0.160	48.2
A.M.	0.355	0.206	42.0
L.I.	0.150	0.084	44.0
L.O.	0.078	0.022	71.8
T.A.	0.143	0.135	5.6

Matched 0.05-μl samples of each serum were preincubated in two plates, one coated with 1% BSA and the other coated with Fab fragments of polyclonal human IgG and blocked with 1% BSA. The samples from both plates were transferred to Fab-coated plates the next day and assayed for αFABA using anti-human IgG antibodies. The percent decrease in αFABA was calculated from the ratio of the decrease in OD found for the samples preincubated in the first Fab plate to the OD found for the samples preincubated in the BSA-coated plate.
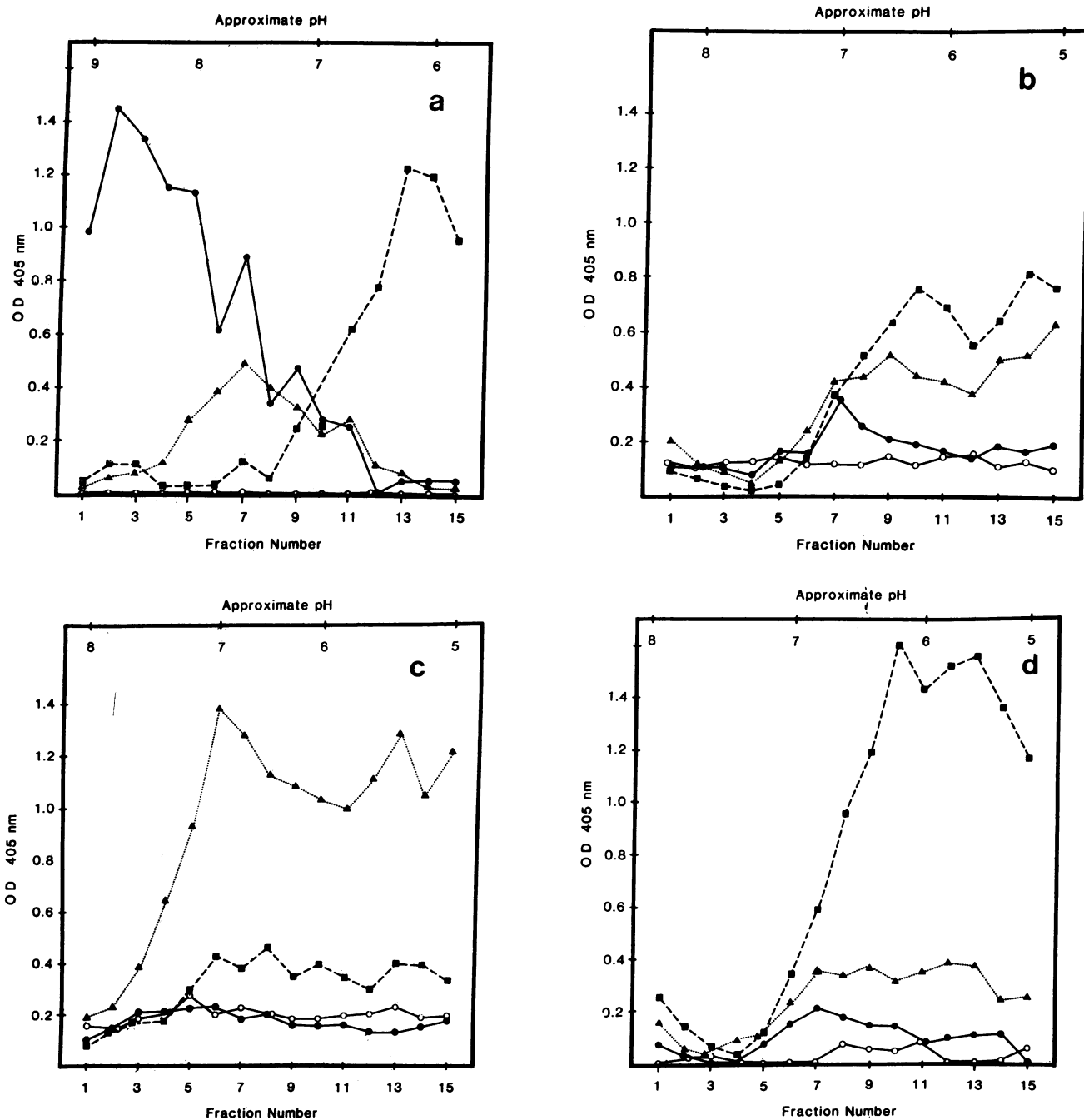
Figure 3. (Continued on next page). Distribution of IgG anti-Fab subclasses in chromatofocused fractions of RA serum *A*. ●——●, IgG1 αFABA; o——o, IgG2 αFABA; ▲·····▲, IgG3 αFABA; ■-----■, IgG4 αFABA. (*A*) Pattern I: elevated IgG1 and IgG4 αFABA; (*b*) pattern II: elevated IgG3 and IgG4 αFABA; (*c*) pattern III: elevated IgG3 αFABA; (*d*) pattern IV: elevated IgG4 αFABA; (*e*) pattern V: low-level αFABA without subclass predominance.

types. All the CF fractions had both κ and λ light-chain αFABA with κ to λ light-chain ratios of 1.1 to 1.5 (Table IV).

Anti-Fab antibody activity in F(ab')₂ fragments of RA serum immunoglobulin. To confirm that the αFABA present in RA sera reacted via Fab–Fab interactions, F(ab')₂ fragments of serum immunoglobulin of four RA patients were prepared and tested in a modified ELISA for αFABA activity presented in the previous section. The RA F(ab')₂ fragments were added to an ELISA plate coated with purified λ Fab fragments, and anti-κ antibodies were used to detect αFABA in the pepsinized RA sera, as the previous set of studies indicated that αFABA of both light-chain types were present. Table V shows αFABA were present in three of the four pepsinized sera studied.

Discussion

Our studies have shown that a group of immunoglobulins with specificities directed against the Fab portion of IgG can be found in elevated levels in the sera of most RA patients. A portion of these antibodies display restricted clonality when analyzed by IEF, exist predominantly as immune complexes, and demonstrate isotype and subclass restriction as well.

This study has shown that the αFABA are primarily IgG, which is consistent with the findings of others (11, 14, 15). Our findings also indicate that αFABA of all four IgG subclasses can exist in RA sera although IgG3 and IgG4 αFABA predominate and comprise ~70% of αFABA isolated from RA sera.
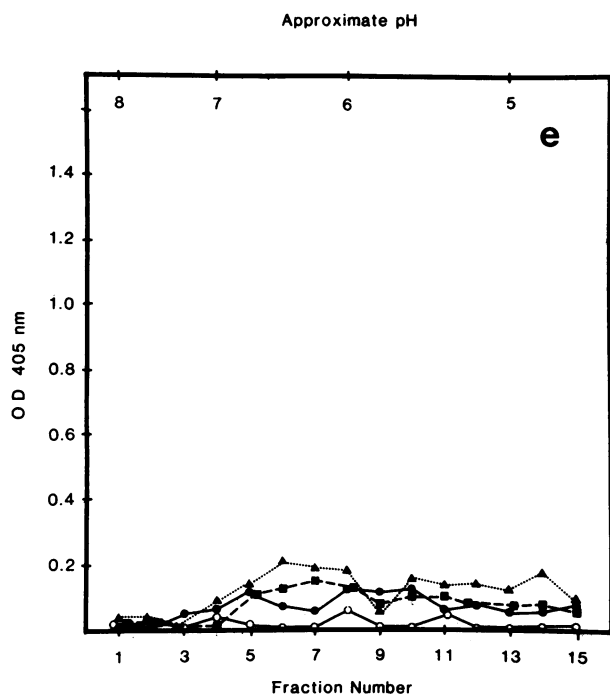
Figure 3.

Approximate pH

(14) have demonstrated that αFABA circulate with anti-keyhole limpet hemocyanin antibodies after immunization of humans with keyhole limpet hemocyanin. Their studies might explain our finding higher IgG4 antidiphtheria antibody levels in untreated sera compared to those found in matched samples dialyzed against urea. Indeed, the only two sera shown to have IgG4 antidiphtheria antibodies after urea dialysis were also the only two sera that had been shown by prior studies to have acidic antidiphtheria IEF clonotypes, which were subsequently found to be IgG4 immunoglobulins (25).

Several findings indicate that our assay systems are specifically detecting anti-Fab antibodies. First, a significant portion of αFABA activity, ~50%, could be removed by adsorption of sera with Fab fragments. Second, 10 normal sera, which were dialyzed against urea, chromatofocused in the presence of urea, and analyzed by ELISA showed low levels of IgG3 and IgG4 αFABA, indicating that the high levels of IgG4 and IgG3 αFABA seen in RA sera were not an artifact resulting from the urea treatment of sera. Lastly, we were able to show that F(ab')₂ fragments of RA serum immunoglobulin also had αFABA activity using light-chain-specific antisera, strongly indicating the antibody nature of serum anti-Fab reactivity.

Although IEF analysis of RA sera indicate that much of the acidic αFABA are clonally restricted, we were unable to demonstrate light-chain restriction of αFABA in CF fractions that were enriched in IgG3 or IgG4 αFABA. Both κ and λ light-chain αFABA were found in the CF fractions in a κ to λ ratio of 1 to 2, similar to that of serum immunoglobulin in general (28). However, due to the constraints of the modified ELISA methods, we would not have detected populations of κ αFABA directed against κ-Fab fragments or λ αFABA directed against λ-Fab fragments, either of which could conceivably comprise a large clonally restricted population of αFABA.

Even though we were able to detect αFABA in normal sera, several findings differentiate αFABA in normals from those found in RA patients. First, reduced quantities of total IgG αFABA, as well as IgG3 and IgG4 αFABA, are found in normal sera as detected by ELISA. Secondly, fewer acidic αFABA antibody bands are found by IEF analysis of normal sera, when compared with RA serum.

In contrast to RA sera, very little IgG3 αFABA is present in normal sera, and when elevated levels of αFABA are present, they are mostly of the IgG4 subclass. The IgG4 αFABA found in normal sera also seem to exist as immune

IgG1, IgG2, and IgG3 αFABA were all found in alkaline CF fractions of RA sera, whereas IgG4 αFABA were found almost exclusively in the acidic CF fractions, all consistent with the known electrophoretic mobilities of the IgG subclasses (17). Of interest, however, was the fact that many of the IgG3 αFABA were acidic immunoglobulins, even though a majority of human serum IgG3 has an alkaline charge.

Dissociation of complexes with urea was necessary for optimum detection of αFABA in RA sera. Furthermore, treatment of sera with urea before analysis was obligatory for detection of the IgG4 subclass of antibodies, either by ELISA or by IEF (16). Our studies therefore indicate that, unless studies of serum αFABA incorporate dissociating conditions during the procedure, αFABA levels might be underestimated, and certain subclasses of αFABA that circulate primarily as complexes may possibly go undetected. Such αFABA may be complexed with serum immunoglobulin. Birdsall and Rossen

Table III. IgG Subclass Distribution of αFABA in RA and Normal Sera

| | αFABA | | | | |
	Total IgG‡	IgG1	IgG2	IgG3	IgG4
RA	12.776±5.406§	2.106±1.840 (16.5%)ll	1.676±1.067 (13.2%)	4.381±3.561 (34.3%)	4.612±2.503 (36.1%)
Normals	3.357±1.546	0.641±0.422 (19.1%)	0.256±0.154 (7.6%)	0.831±0.354 (24.8%)	1.629±1.293 (48.5%)
P value*	<0.001	<0.001	<0.001	<0.001	<0.001

* Student's t test. ‡ Derived from the sum of the optical densities obtained for each IgG subclass of αFABA. § Mean αFABA level±1 SD (OD 405 nm). ll Percent contribution to total αFABA activity.

Table IV. Light-chain Type Analyses of αFABA in RA

Patient	%IgG3 αFABA* in CF fraction	%Ig4 αFABA* in CF fraction	κ αFABA‡	λ αFABA‡	κ/λ
			O.D. 405 nm		
B.E.	47	53	0.191	0.135	1.4
L.I.	84	16	0.257	0.174	1.5
R.O.	80	20	0.275	0.183	1.5
S.A.	22	78	0.130	0.117	1.1
L.O.	0	100	0.177	0.152	1.2
B.A.	21	79	0.118	0.094	1.3

* Acidic CF fractions that possessed only IgG3 and/or IgG4 αFABA were selected.
‡ Matched samples were added to ELISA plates, one coated with λ light-chain Fab fragments and the other coated with κ light-chain Fab fragments. κ αFABA in each sample were determined by adding monoclonal anti-human κ-chain antibodies to the λ Fab-coated plate, and λ αFABA were determined by adding monoclonal anti-human λ-chain antibodies to the κ-coated plate.

complexes because dissociating conditions were necessary for their detection by IEF as well as by ELISA. Thus, although the production of αFABA may be a normal process, the selective differential amplification of a particular subclass may be associated with immune pathologies. Whether increased levels of αFABA, and particularly IgG3 and IgG4 αFABA, are a feature peculiar to RA, or whether they are also amplified in other disorders characterized by immune complexes remains to be determined.

Although at present we can only speculate on the function IgG3 and IgG4 αFABA may have, some may be anti-idiotypic antibodies with immunomodulatory properties (29–33). We have recently found that IgG3 and IgG4 αFABA show differential reactivity against different populations of IgG Fab fragments which have been separated by charge from polyclonal Fab fragments, indicating that not all αFABA recognize a similar determinant on the Fab molecule. In addition, some αFABA may play a direct pathogenic role in RA as a result of deposition of αFABA complexes in synovial tissues. Plasma cells producing αFABA have been demonstrated in RA synovial tissues and antibody complexes with anti-F(ab')₂ activity detected in synovial fluid (34, 35). The poor binding of these

Table V. αFABA Reactivity of RA Serum Immunoglobulin F(ab')₂ Fragments

RA patient	Fab-coated plate*	BSA-coated plate
	OD 405 nm	OD 405 nm
T.A.	0.039	0
B.A.	0.552	0.001
D.E.	0.348	0.018
B.L.	0.211	0.027

* Matched samples of F(ab')₂ fragments of each RA serum were added to two ELISA plates, one of which was coated with λ light-chain Fab fragments of polyclonal human IgG and blocked with 1% BSA, and the other which was coated with 1% BSA. αFABA activity was assayed with monoclonal anti-human κ light-chain antibodies.

complexes with complement (35) is consistent with IgG4, which does not bind complement (36).

Our studies provide new insight into the nature of αFABA inasmuch as it now can be appreciated that multiple families of these antibodies are demonstrated as capable of being differentiated by subclass as well as by the clonality of the response.

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