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Characterization of anti-Fab′ antibodies in human sera: identification of soluble immune complexes that contain hidden anti-KLH and blocking anti-immunoglobulins following immunization with keyhole limpet haemocyanin*

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SUMMARY

After immunization with keyhole limpet haemocyanin (KLH), increased concentrations of anti-KLH and anti-Fab′ antibodies (Abs) were demonstrated in sera from 18 of 20 volunteers. In many cases, post-immunization sera contained soluble immune complexes that incorporated both anti-Fab′ and 'hidden' or 'blocked' anti-KLH antibodies. The complexes containing hidden anti-KLH and blocking anti-Fab′ Abs were not found in pre-immunization sera. The hidden Abs to KLH were revealed by demonstrating increases in anti-KLH activity in sera incubated previously with Fab′ fragments, immobilized on plastic microtitre tray wells. Incubation with insoluble Fab′ did not influence the quantity of anti-tetanus toxoid (TT) that was detected in these sera. Addition of affinity purified anti-Fab′ Abs to samples, previously 'unblocked' by adsorption with immobilized Fab′, depressed their anti-KLH activity to levels present before adsorption, but did not change the quantity of Abs to Dermatophytin, Trychophyton, or TT therein. These results suggest that some autoantibodies generically recognized as 'Fab′ specific' have properties that are usually considered to be characteristic of autoanti-idiotypes.

Keywords autoanti-idiotypes anti-immunoglobulins anti-Fab′ antibodies immune complexes blocking antibodies

INTRODUCTION

Some anti-immunoglobulins, like rheumatoid factors, are commonly associated with collagen-vascular (Mellors *et al.*, 1959; Johnson & Faulk, 1976) or chronic infectious diseases (Bartfeld, 1969; Waller & Duma, 1972). Others have been found in normal donor sera (Bartfeld, 1969). Anti-immunoglobulins may be classified according to their antigenic specificity (Harboe, Rau & Aho, 1965). Certain of these react specifically with determinants in the framework or antigen combining site of the variable region (Kunkel, Mannik & Williams, 1963; Oudin & Michel, 1963). Increased serum levels of these and other anti-immunoglobulins have been demonstrated following specific antigenic stimulation (Brown & Rodkey, 1979; Bokisch, Bernstein & Krause, 1972; Herd, 1973; Cunningham-Rundles, 1982; Geha, 1982; Bankert & Pressman, 1976; Sy *et al.*, 1980;

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Kluskens & Kohler, 1974; Kelsoe, Isaak & Cerny, 1980; Cowdery & Steinberg, 1981; Schecter *et al.*, 1982; Schrater *et al.*, 1979).

Anti-immunoglobulins are also prominent among the antibodies (Abs) released by peripheral blood lymphocytes cultured *in vitro* (Birdsall & Rossen, 1982; Koopman & Schrohenloher, 1980; Tsoukas *et al.*, 1980). Some of the anti-Fab′ Abs released by these cells *in vitro* become incorporated within soluble immune complexes (Birdsall & Rossen, 1982), indicating, perhaps, the presence of idiotype–anti-idiotype or other self associating immunoglobulin–anti-immunoglobulin interactions (Nemazee & Sato, 1982).

The present investigations were undertaken to evaluate whether circulating immune complexes containing anti-Fab′ Abs could also be demonstrated during antigen driven immune responses *in vivo*.

MATERIALS AND METHODS

Human subjects. Twenty adult male volunteers were immunized intradermally with 100 μg of keyhole limpet haemocyanin (KLH). None had positive delayed type hypersensitivity (DTH) responses to the 1st dose of KLH. Thirty days later, they were re-challenged with 100 μg of KLH; all had positive DTH responses to the second injection. Pre-immunization sera and sera drawn 21 days after the last antigen injection were stored at −70°C until tested.

Reagents. IgG was isolated from Cohn Fraction II (CF II) (Sigma, St Louis, Missouri, USA) by ion exchange chromatography on DEAE–Sephacel (Pharmacia, Piscataway, New Jersey, USA). Fab′ was prepared by digesting IgG with pepsin, followed by affinity chromatography over protein A–Sepharose (Pharmacia) to remove intact IgG, and reduction and alkylation to its monomeric form (Birdsall & Rossen, 1982; Cerottini, 1968). IgM was isolated from serum by the method of Masseyeff, Gombert & Josselin (1965) substituting heparin and $MnCl_2$ to remove β-lipoproteins (Mann *et al.*, 1967). Class specific anti-IgG and anti-IgM were absorbed with Fab′–Sepharose to remove L-chain specific Abs and then affinity purified on IgG or IgM Sepharose, respectively. Eluted Abs were digested to their $F(ab')_2$ fragments (Birdsall & Rossen, 1982; Cerottini, 1968) and radiolabelled by the chloramine-T method (Greenwood, Hunter & Glover, 1963). IgG, IgM, Fab′ and KLH Sepharoses were prepared by means of CNBr–Sepharose (Pharmacia). KLH was kindly donated by Dr E.M. Hersh, Dept. Clinical Immunology, M.D. Anderson Hospital, Houston, Texas). Tetanus toxoid (Ultrafined®) and diphtheria toxoid were obtained from Wyeth Lab., Marietta, Pennsylvania, USA; Dermatophytin was obtained from Cutter Lab., Berkeley, California, USA) and Trychophyton from Bioproducts, Tempe, Arizona, USA.

Affinity chromatography. Anti-Fab′ Abs were isolated by affinity chromatography on Fab′–Sepharose. Bound Ab was eluted with 1 M acetic acid. Sources of Fab′ included CF II, pooled pre-immunization sera and pooled post-immunization sera.

Solid phase radioimmunoassay (RIA). Polyvinyl microtitre wells were coated with 100 μl of antigen at 20 μg/ml in 0·1 M, pH 9·6 carbonate. Twenty micrograms of carbodi-imide (Sigma cat. No. C1011), 1 mg/ml in carbonate, was added to crosslink proteins to the plastic. After incubation overnight at 10°C, the plate was washed twice with phosphate-buffered saline with Tween (PBS-Tween), (0·15M NaCl, buffered at pH 7·4 with 0·01M sodium phosphate, containing 0·05% Tween-20). One molar NH_4Cl was added for 30 min to block excess carbodi-imide. The plate was washed twice with PBS-Tween; the wells were filled with 1% bovine serum albumin (BSA) in PBS and the plates were incubated for 30 min. After three additional washes with PBS-Tween, 100 μl of sample was added and incubated overnight at 10°C. Unbound Ab was removed by three washes with PBS-Tween, and bound Ab was detected by addition of ^{125}I-anti-IgG or ^{125}I-anti-IgM in 1% BSA in PBS. After 2 h at 10°C, the plates were washed five times with PBS-Tween; the individual wells were cut out and counted for ^{125}I. To measure the quantity of IgG or IgM detected by this RIA and to prepare standard curves for the assay, microtitre plates were first coated with unlabelled, affinity purified anti-IgG or anti-IgM. Between 0·1 and 100 ng/ml of IgG or IgM were then added to the wells of the microtitre trays. After extensive washing with PBS-Tween, the quantity of ^{125}I-anti-IgG or ^{125}I-anti-IgM that bound to these antigens was measured. Using this data, standard curves were developed that related the quantitity of ^{125}I-anti-immunoglobulin bound to the

quantity of immunoglobulin originally added to that well. This method was also used to measure the total IgG and IgM in unknown samples. In all RIAs non-specific binding was measured by counting the amount of ^{125}I-anti-IgG or -anti-IgM that bound to samples that had been added to microtitre trays coated only with 1% BSA in PBS. The counts of ^{125}I found in wells coated only with BSA were subtracted from counts obtained when the samples were incubated in antigen coated wells, in order to estimate Ag specific binding. These assays were able to consistently measure $<0{\cdot}1$ ng of specific antibody per 100 μl of sample, added to the microtitre well; all assays were done at least in triplicate; results are presented as mean $\pm$ 1 s.d.

Adsorption of blocking Abs. Sera were diluted in PBS containing 1% BSA and incubated overnight at 10°C in microtitre wells coated with Fab′ or BSA. The Ab activity present in the sample incubated with BSA was measured to estimate losses due to non-specific binding.

Measurement of blocking Abs. Blocking was defined as interference with the binding of KLH specific Abs in the solid phase RIA. Fifty microlitres of dilute serum, previously incubated on insolubilized Fab′ fragments, as above, was mixed directly in the antigen coated well with 50 μl of anti-Fab′ Abs (the putative blocking Ab) or 50 μl of 1% BSA in PBS (the control). The RIA was carried out exactly as above except that the sample incubation time was shortened to 4 h at 10°C.

Statistical evaluations. The *t*-test was used to evaluate the significance of change between paired observations. Rank correlations were determined by the Spearman test.

RESULTS

Changes in serum Ab activity following immunization with KLH

IgM anti-KLH Abs increased from an average of 7·7 (range 3·0–13·0) to 14·2 μg/ml (range 5·4–29·0) ($P<0{\cdot}01$, paired *t*-test); IgG anti-KLH increased from an average of 12·6 (range 7·2–19·0) to 72·1 μg/ml (range 11·3–155·0) ($P<0{\cdot}005$, *t*-test). Both IgG and IgM anti-Fab′ Abs increased in 15 of the 20 donors; either IgG or IgM anti-Fab′ Ab increased in all but two (Fig. 1). Increments in IgM anti-Fab′ were correlated with increments in IgG anti-Fab′ Abs ($r_s=0{\cdot}48$, $0{\cdot}01<P<0{\cdot}05$, Spearman rank correlation test).

Identification of immune complexes containing both anti-KLH and anti-Fab′ Abs

To evaluate the specificity of the anti-Fab′ Abs that appeared after imunization with KLH, we attempted to isolate these by affinity chromatography on insolubilized antigen. The quantities of anti-Fab′, anti-KLH, anti-tetanus toxoid (TT) and anti-diphtheria toxoid (DT) Abs, also present in these sera, were determined in order to evaluate how Abs specific for unrelated antigens partitioned on these immunosorbents. The serum applied to the column contained 3,440 ng IgG anti-Fab′, 68,000 ng IgG anti-KLH, 80,000 ng IgG anti-TT and 38,000 ng IgG anti-DT. The eluate from Fab′–Sepharose contained 640 ng of IgG anti-Fab′, 161 ng of IgG anti-KLH, 31 ng of IgG anti-DT

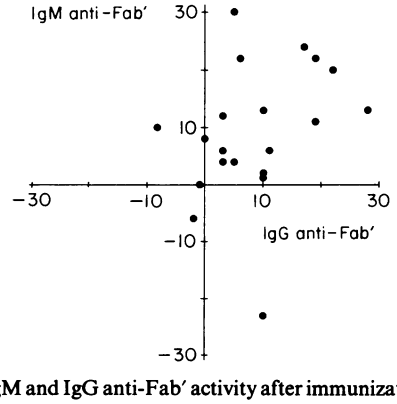

Fig. 1. The net change in serum IgM and IgG anti-Fab′ activity after immunization with KLH (in ng/ml of serum diluted 1:100) is shown for each donor.

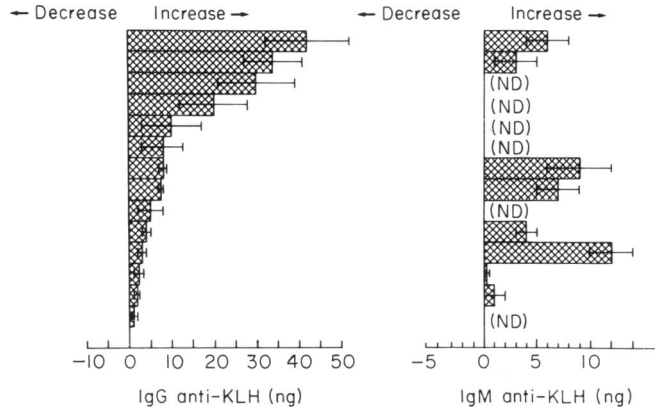

Fig. 2. Sera from 14 donors were adsorbed with immobilized Fab′ fragments or BSA. Bars represent the difference, in ng, in anti-KLH activity, i.e. the Fab′ absorbed sample minus the BSA absorbed sample. Each sample was assayed in triplicate. The error bars indicate ± (1 s.e. of the mean of the Fab′ adsorbed sample + 1 s.e. of the mean of the BSA adsorbed sample). ND = not done.

and 56 ng of IgG anti-TT. This represented recovery, respectively, of 18·6, 0·24, 0·08 and 0·07 % of the specific Abs applied to the column. Similarly after passage over KLH–Sepharose, the eluate contained 97 ng IgG anti-Fab′, 7,110 ng IgG anti-KLH, 97 ng IgG anti-TT and 175 ng IgG anti-DT. These were, respectively, 2·8, 10·5, 0·12 and 0·46% of the specific Abs applied to the KLH–immunoadsorbent. The fact that some anti-KLH Abs co-purified with anti-Fab′ Abs, and vice versa, regardless of the adsorbent used for their isolation, suggested either that some of the anti-KLH Abs in these sera were incorporated in soluble immune complexes that also contain Abs that can bind to Fab′ fragments of IgG, or KLH and Fab′ may display cross-reactive epitopes.

To evaluate these alternatives, IgG anti-KLH was measured before and after the sera were incubated with immobilized Fab′ fragments. If KLH and Fab′ expressed cross-reactive epitopes, incubation with insoluble Fab′ should remove the cross-reacting Abs, decreasing the apparent quantity of anti-KLH Ab in these samples. If, on the other hand, anti-KLH and anti-Fab′ Abs co-purified because they were incorporated in immune complexes, then incubation with insoluble Fab′ fragments might preferentially remove Abs reactive with Fab′. This should leave behind relatively more of the anti-KLH Abs, especially if the postulated immune complexes were products of low affinity antibody–antibody interactions. Consequently, the quantity of anti-KLH Ab should increase. To estimate how much Ab was lost by non-specific absorption, we measured the Ab remaining in aliquots of the same sera, incubated under the same conditions with BSA. We found significantly more IgG and IgM anti-KLH in aliquots of sera incubated with immobilized Fab′, as compared to samples incubated only with BSA ($P < 0{\cdot}005$ for IgG Abs, and $P = 0{\cdot}025$ for IgM Abs, paired t-test) (Fig. 2).

Isolated anti-Fab′ from post-immunization sera block anti-KLH Abs

Anti-Fab′ were isolated from four sera by affinity chromatography on immobilized Fab′ prepared from CF II IgG. When affinity purified anti-Fab′ Abs were added back to the Fab′ adsorbed samples, their IgG anti-KLH activity dropped to that measured prior to incubation with the Fab′ fragments (Table 1). The blocking was not donor specific; anti-Fab′ Abs prepared from any one donor's serum were equally effective in blocking anti-KLH Abs in autologous and allogeneic sera (data not shown).

Specificity of blocking Abs

Incubation of pre-immunization sera with immobilized Fab′ fragments did not change the quantity of IgG anti-KLH detected therein ($P > 0{\cdot}30$), (data not shown). Although the IgG anti-KLH activity of post-immunization sera increased after incubation on Fab′ fragments, there was no

Table 1. Unblocking of IgG anti-KLH activity by preincubation on immobilized Fab′ fragments and subsequent blocking of anti-KLH activity by re-addition of anti-Fab′ Abs

Serum pre-incubated on:	BSA	Fab′	Fab′
Serum then mixed with:	BSA	BSA	anti-Fab′ Abs
Donor			
A	92±1	109±2	96±1
B	56±1	76±1	62±2
C	66±1	86±2	70±3
D	32±1	46±2	40±2

Individual donor sera were diluted in 1% BSA and pre-incubated on either immobilized Fab′ fragments, prepared from CF II, or BSA. These were then combined in equal volumes with either 1% BSA or autologous anti-Fab′ Abs. The anti-Fab′ Abs had been isolated by affinity chromatography on Fab′–Sepharose and were diluted to the same protein concentration before combining with the sera. The samples were then assayed for IgG anti-KLH by the solid phase RIA. Results presented are ng Ab/100 μl; mean ± 1 s.e. of three replicates.

change in the amount of IgG anti-TT Ab in these samples ($P > 0{\cdot}3$) (Fig. 3). IgM anti-TT also remained unchanged after this treatment ($P > 0{\cdot}3$), (data not shown). Moreover, addition of anti-Fab′ Abs isolated from post-immunization sera did not interfere with the binding of Abs specific for Dermatophytin 'O', Trychophyton, or TT even as they decreased the amount of detectable Ab to KLH (Fig. 4).

To evaluate why blocking antibodies specific for the hidden anti-KLH Abs in post-immunization sera could be isolated on Fab′ immunoadsorbents made from Cohn fraction II IgG, we made immunosorbents that contained equal quantities of Fab′ isolated from Cohn F II IgG, from pooled pre-immunization sera or from pooled post-immunization sera. If anti-Fab′ Abs from post-immunization samples were relatively specific for the hidden anti-KLH Abs in these sera, Fab′

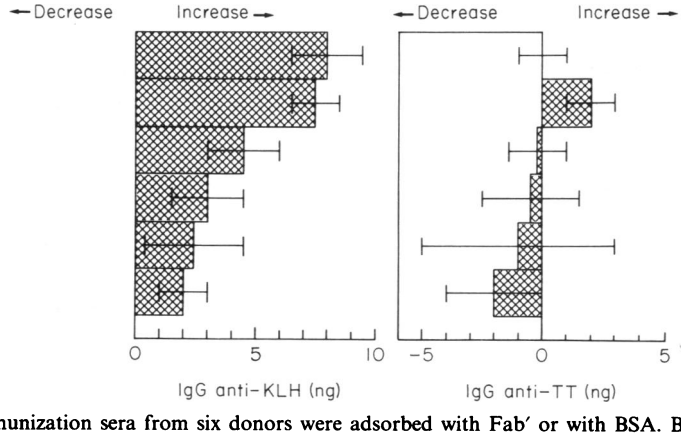

Fig. 3. Post-immunization sera from six donors were adsorbed with Fab′ or with BSA. Bars represent the difference, in ng, in Ab activity, i.e. the Fab′ absorbed sample minus the BSA absorbed sample. Each sample was assayed in triplicate. The error bars indicate ± (1 s.e. of the mean of the Fab′ adsorbed sample + 1 s.e. of the mean of the BSA adsorbed sample).

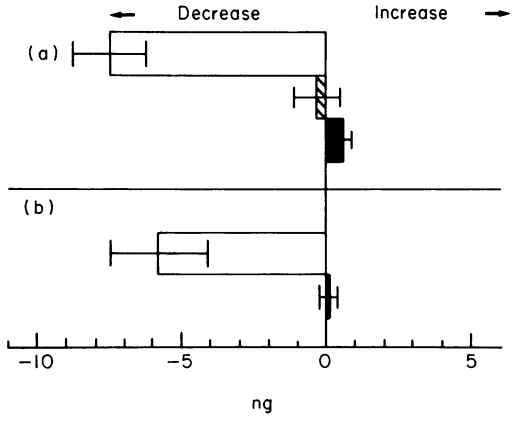

Fig. 4. Change in specific Ab activity after add-back of isolated anti-Fab′ Abs: post-immunization sera from two donors were adsorbed with Fab′ fragments to deplete anti-Fab′ activity. Subsequently, affinity purified Abs specific for Fab′ were added back; control samples received 1% BSA in PBS. Results of assays: for IgG anti-KLH, (□), for anti-Trychophyton (■) and for anti-Dermatophytin (▨). Bars represent the difference, in ng, in the quantity of Ab measured; i.e., (adsorbed serum + anti-Fab′ Abs) minus (adsorbed serum + BSA). Each sample was assayed in triplicate. The error bars indicate ± (1 s.e. of the mean of the samples + anti-Fab′) + (1 s.e. of the mean of the BSA adsorbed sample).

fragments from post-immunization sera should also provide the most effective adsorbent for these blocking Abs. Indeed, anti-Fab′ Abs isolated on Fab′–Sepharose columns made from post-immunization IgG, were seven times more effective, on a weight basis, at blocking anti-KLH activity than anti-Fab′ isolated from the same serum pool using Fab′–Sepharose columns made with Fab′ obtained from Cohn FII or pre-immunization IgG (data not shown).

DISCUSSION

In 18 of 20 donors, IgM or IgG anti-Fab′ Abs increased after immunization with KLH. Anti-Fab′ Abs and anti-KLH Abs tended to co-purify, apparently as immune complexes, during affinity chromatography experiments with these sera, on immunadsorbents that contained immobilized KLH or Fab′ fragments. In post-immunization sera, these complexes contained 'blocked' or hidden antibodies to KLH as well as anti-Fab′ Abs. Hidden anti-KLH Abs were revealed by adsorbing the 'blocking' Abs on Fab′ fragments immobilized on plastic surfaces. Fab′ from pooled post-immunization sera was especially effective at adsorbing these blocking anti-Fab′, suggesting that the blocking anti-Fab′ Abs preferentially bound to Fab′ of immunoglobulins that had appeared following immunization with KLH.

Why blocking Abs preferentially adsorbed to Fab′ immunosorbents is not known. Conceivably, Fab′ present a large array or dense display of cross-reacting determinants that favour adsorption of the blocking Abs, but not the anti-KLH Abs. Some anti-KLH Abs copurified with anti-Fab′ Abs when these were isolated by affinity chromatography on Fab′–Sepharose, indicating that the partitioning of complexes into their anti-KLH and anti-Fab′ Ab components was not complete.

Abs that 'block' anti-KLH could also be isolated on Fab′ fragments made from CF II, albeit with reduced efficiency. The ability of Fab′ from CF II IgG to perform this function probably reflects the fact that this commercially available source of pooled human IgG contains an extraordinarily large diversity of human Abs. Batches of CF II are rarely made from plasma pools of fewer than 2,000 units (personal communication, Dr F. Manuzzi, Miles Laboratories).

The anti-Fab′ Abs isolated from post-immunization sera were incapable of blocking the reactions of anti-Trichophyton, anti-Dermatophytin 'O' or anti-TT Abs with their respective antigens. The specificity for anti-KLH suggests that the blocking anti-Fab′ must react in or near the

antigen combining site. Thus, they have some of the characteristics of anti-idiotypes. Other types of anti-Fab', including those that react with buried or hidden antigenic determinants within the hinge region, may also have been present in these sera. They could also have been detected in the assays used to quantitate anti-Fab' activity but would not be expected to interfere selectively with the antigen specific binding of the hidden anti-KLH, revealed when these samples were incubated with insoluble Fab' (Kormeier, Ing & Mandy, 1968; Osterland, Harboe & Kunkel, 1963).

The complexes containing anti-Fab' and anti-KLH Abs in the post-immunization sera had certain of the characteristics of other naturally occurring immune complexes found in normal donor sera (Morgan, Rossen & Twomey, 1979; Morgan *et al.*, 1982). The anti-immunoglobulin component of these complexes could also be removed by incubating the sera with Fab'–Sepharose. This treatment revealed otherwise hidden, complement-dependent, cytotoxic Abs that can kill a diverse array of cultured human tumor cells (Morgan *et al.*, 1982). The anti-Fab' Abs in these complexes differed from those described in the present study in one important respect: they only blocked tumour cytotoxins isolated from the same donor's serum.

One can marshal evidence both for and against the notion that the bifunctional complexes, described in the present work, are idiotype–anti-idiotype complexes. The most persuasive evidence in favor of this idea is that adsorption of the anti-Fab' Abs selectively increased the anti-KLH activity of the sera. Adding back the isolated anti-Fab' specifically suppressed the anti-KLH activity of adsorbed sera. Against the hypothesis are: (1) unblocking of hidden anti-KLH can be achieved by adsorption with Fab' from CF II and (2) anti-Fab' Abs from one donor's serum also block anti-KLH Abs in other donors' sera.

These objections are not overriding however, when one considers that anti-Fab' Abs, adsorbed to and eluted from Fab' fragments obtained from pooled post-immunization sera, were seven times more effective at blocking IgG anti-KLH Abs than anti-Fab' Abs prepared with Fab' from pooled pre-immunization sera and that KLH is a large, complex Ag which is likely to trigger Ab production by a large number of clones. Thus anti-KLH Abs produced by one donor may cross-react with anti-KLH Abs produced by others. Cunningham-Rundles (1982), found both cross-reactive and individual specific determinants on anti-casein Abs isolated from two IgA deficient individuals. Cross-reactive or common idiotypic determinants have also been recognized among monoclonal rheumatoid factors and among anti-Rh(D) Abs isolated from unrelated donors (Kunkel *et al.*, 1973; Wiliams, Kunkel & Capra, 1968; Forre, Natvig & Michaelsen, 1977).

Because the anti-Fab' in these experiments were isolated by affinity chromatography on Fab' from pooled IgG, it is likely that isolation of Abs reactive with common, cross-reactive epitopes was favored. It might have been possible to demonstrate autologous donor specificity if single donor anti-Fab' Abs had been isolated on Fab'–Sepharose prepared with autologous IgG. Unfortunately, the amount of serum available precluded that experiment.

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