

NATURALLY OCCURRING AUTOLOGOUS ANTI-IDIOTYPIC ANTIBODIES

Participation in Immune Complex Formation in Selective IgA Deficiency*

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About 50% of selective IgA-deficient individuals have very high antibody titers to bovine serum proteins, presumably because of the continuous excessive absorption of dietary antigens from the gastrointestinal tract into the blood stream (1, 2). Most of these individuals also have high levels of circulating immune complexes in their sera, and we have shown (2-4) that these complexes contain bovine proteins. In experimental systems, immunization with antigen-antibody complexes is a very effective means of producing anti-idiotypic antibodies (5). From the network theory of immunoglobulin regulation (6) one might expect that such autoantibodies to the variable regions of antbovine protein antibodies would be found in these human sera. It would also seem probable that these autoantibodies would be important components of circulating immune complexes. This report describes the isolation of auto-anti-idiotypic antibodies to the $F(ab')_2$ of anticasein purified from the sera of two IgA deficient subjects and these experiments demonstrate by antigen blocking that most of these autoantibodies bind anticasein in the region of the casein binding site. In addition, these studies suggest that anti-idiotypic antibodies do participate in *in vivo* immune complex formation, competing with dietary casein antigen for binding sites on the anticasein antibody.

Materials and Methods

Sera and Immunoglobulin Fractions. The sera of two healthy IgA-deficient subjects, A and B, were used in these studies; both had previously been identified as having large amounts of IgG anti-casein antibodies by an enzyme-linked immunosorbent assay (ELISA)¹ (3). Two myeloma proteins, IgG₁ (HA) and IgG₁ (GU) and the IgG fraction of pooled human gamma globulin ("Gamastan", Cutter Laboratories, Berkeley, CA) were purified by passage over DEAE cellulose (Bio-Rad Laboratories, Richmond, CA) equilibrated in 0.01 M Na phosphate buffer pH 7.1. Aliquots of both myeloma proteins and the pooled gamma globulin were pepsin digested (7) for 20 h at room temperature and the $F(ab')_2$ of each isolated by gel filtration on Sephadex G150 (Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Piscataway, NJ).

Affinity Chromatography. Anticasein antibodies were isolated from 75 ml of serum from

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¹ *Abbreviations used in this paper:* AP, alkaline phosphatase; CNBr, cyanogen bromide; ELISA, enzyme-linked immunosorbent assay; NPP, *p*-nitrophenyl phosphate; NS, normal saline; PBS, phosphate-buffered saline; RF, rheumatoid factor; SA, *Staphylococcus aureus*; SDS, sodium dodecyl sulfate.

subject A and 85 ml from subject B by passage of a well-dialyzed, 50%-saturated $(\text{NH}_4)_2\text{SO}_4$ precipitated IgG-containing fraction of serum on Sepharose 4B (Pharmacia Fine Chemicals) to which casein (ICN Nutritional Biochemicals, Cleveland, OH) had been coupled using cyanogen bromide (CNBr) (8). After removal of the nonadherent fractions by passage of 5 column vol of phosphate-buffered saline (PBS), the adherent fractions were eluted by 0.1 M glycine HCl buffer, pH 2.7. These fractions, containing ~ 1.5 mg IgG/ml were passed over DEAE cellulose equilibrated in 0.01 M Na phosphate buffer pH 7.1 to purify IgG and eliminate possible traces of casein, which adheres to cellulose under these conditions (9). In some cases the anticasein fractions were also passed over Sephadex G150 to collect fractions eluting the void volume. 1 mg of the resulting anticasein fractions of each subject were then coupled to 2 ml of CNBr-activated Sepharose 4B (8). IgG fractions of serum A and B that had not adhered to casein-Sepharose, were passed on their respective anticasein columns and potential anti-anti-casein antibodies were eluted by the glycine buffer. These anti-anticasein antibodies were further purified by passage over DEAE cellulose, and then G150 Sephadex to collect fractions eluting in the void volume.

Preparation of Pepsin-digested Alkaline Phosphatase-labeled Anticasein. An aliquot of anticasein antibodies of each donor were pepsin digested (7), and the F(ab')_2 isolated by gel filtration on Sephadex G150. Antigen-specific F(ab')_2 of each were then isolated by repassage and elution from casein-Sepharose. Part of the pepsin-treated, affinity-repurified anticasein of serum A and B (containing about 150 μg IgG/ml) was then coupled to alkaline phosphatase (AP) at an enzyme/antibody ratio of 1:2 (3, 10). The enzyme-antibody conjugates used were eluted in the void volume of a 6×0.2 cm column of Ultragel ACA-22 (LKB-Produkter Brumma, Sweden). The preparations were made 5% in chicken ovalbumin (three-times crystallized; Sigma Chemical Co., St. Louis, MO) to block residual glutaraldehyde and eliminate nonspecific reactions. Approximately 35% of the enzyme-antibody activity of these final preparations could be bound specifically to Sepharose casein as determined in a separate elution study.

Preparation of Alkaline Phosphatase-labeled Casein. Purified sodium caseinate (10) was coupled to AP at an enzyme/protein ratio of 1:2 and then filtered on Ultragel ACA-22 to collect the aggregates eluting in the void volume. The conjugate was made 5% in chicken ovalbumin and used at a dilution of 1:100 in PBS-0.02% Tween-20 (Sigma Chemical Co.).

Assays for Anti-Anticaseins

USING STAPHYLOCOCCUS AUREUS. In separate tubes, increasing amounts (from 25 to 1,600 ng) of F(ab')_2 anticasein of serum A or B were added to 600 ng of anti-anticasein of serum A or B, and 200 ng of AP labeled F(ab')_2 anticasein isolated from the same sera. In other tubes containing both anti-anticasein and AP-anticasein, possible inhibitors of the enzyme-labeled anticasein and anti-anticasein interaction were 10^{-3} M casein, 1 μg of the F(ab')_2 of myeloma proteins HA and GU, or 1 μg of F(ab')_2 of pooled human gamma globulin. In addition, 1 μg of pepsin-digested anticasein of subject B was tested with the anti-anticasein of subject A, and 1.6 μg of pepsin-digested anti-casein of subject A was tested with the anti-anticasein of subject B. In each tube, total volumes of 200 μl were achieved by additions of normal saline (NS). After incubating these reactants overnight at 4°C , 100 μl of a 10% suspension of heat-killed, formalin-fixed *Staphylococcus aureus* (SA) (11) in PBS was added to each sample. After again incubating at 4°C for 1 h, SA pellets were washed three times in PBS containing 0.25% Tween-20 and 1 ml of a 1 mg/ml solution of *p*-nitrophenyl phosphate (NPP) (Technicon Instruments Corp., Tarrytown, NY) was added (3, 11). After 3 h at 25°C , SA pellets were removed by 10-min centrifugation (730 g, GLC-2, Sorvall, Ivan Sorvall, Norwalk, CT), and the OD 400 nm of the supernatant was read (Beckman Acta C iii spectrophotometer, Beckman Instruments, Inc., Fullerton, CA). Control samples contained no anticasein, no anti-anticasein, no enzyme-labeled anticasein, or SA alone.

USING ANTICASEIN BOUND TO POLYSTYRENE. In a further test of the interactions between anti-anticasein, anticasein, and casein, anticaseins (pepsin digested or intact) of subjects A and B were used to coat polystyrene tubes (NUNC, Copenhagen, Denmark), using 0.5 ml of a 1 μg IgG/ml solution in 0.1 M Na carbonate buffer, pH 9.6 per tube, and incubating in a water bath at 37°C for 3 h (8). After washing three times with NS-Tween-20 (0.25%), increasing amounts (1–100 ng) of anti-anticasein of subject A or B in PBS-0.02% Tween were incubated in these tubes for 5 h at room temperature. After washing again, 0.5 ml of the diluted AP

conjugated casein in PBS was added and the incubation continued for 16 h. After rewashing, 1 ml of a 1 mg/ml solution of NPP was added (3, 10), and the resulting yellow color read in a spectrophotometer. Control samples contained no anticasein, or no anti-anticasein. The dilution of the casein conjugate used was previously determined in a preliminary experiment by incubating various dilutions of this conjugate in the anticasein coated tubes. After washing, NPP was added. The dilution of conjugate producing a satisfactory yellow color in a period of one hour was chosen for these tests. All tests were performed in duplicate.

Trace Iodination of Anti-Anticasein. An aliquot of anti-anticasein of subject B was trace iodinated by ^{125}I (New England Nuclear, Boston, MA) using chloramine T (12) and then extensively dialysed against PBS to remove unbound ^{125}I . The final specific activity was calculated to be 5.0 $\mu\text{Ci}/\text{mg}$.

SDS-Polyacrylamide Gel Electrophoresis. Aliquots of the anticasein antibodies of subjects A and B and the ^{125}I anti-anticasein of subject B were reduced and subjected to electrophoresis in 12-cm 5% polyacrylamide gels containing 0.1% sodium dodecyl sulfate (SDS) (13). The resulting gels were either stained with 0.05% Coomassie blue or sliced into 2-mm slices and counted in a Packard Autogamma counter (Packard Instrument Co., Downers Grove, IL).

IgG Quantitation. Radial immunodiffusion (14) was used to determine IgG concentrations for levels ≥ 2 mg, and an ELISA method (to be described separately) for IgG concentrations falling between 10 ng/ml and 2 mg/ml.

Results

The anticasein antibody preparations isolated from the serum of the two individuals contained ~ 1.5 mg of IgG, and the molecular weights of reduced samples analysed on SDS polyacrylamide gel electrophoresis were consistent with the molecular weight of H and L chains of IgG (53,000 and 23,000). The final anticasein preparations had 20 times the anticasein activity per mg IgG (as determined by ELISA) when compared with whole starting serum, and $\sim 40\%$ of the protein of these preparations adhered to casein Sepharose as determined in a separate elution experiment.

The anticasein antibodies of subjects A and B were used to prepare two second-affinity columns for the isolation of anti-anticaseins. An iodinated, further-reduced, purified preparation of subject B's anti-anticasein was found to have a molecular weight profile of H and L chains of IgG on SDS-polyacrylamide gel electrophoresis. 30–35% of the IgG in these anti-anticasein preparations of subject A and B was found to bind to the anticasein of the same donor.

To test the binding of the presumptive auto-anti-idiotypic antibody to anticasein, two kinds of experiments were performed. In the first, increasing amounts of nonlabeled anticasein were introduced to increasingly prevent a limited amount of enzyme-labeled anticasein from binding to anti-anticasein attached to SA. Fig. 1 shows that the AP-conjugated $\text{F}(\text{ab}')_2$ anticaseins of both IgA-deficient patients A and B (panel A and B) are progressively inhibited from binding to their respective anti-antibodies by increasing amounts of unlabeled $\text{F}(\text{ab}')_2$ anticasein. Pepsin-treated pooled human gamma globulin or two IgG myeloma proteins did not inhibit the binding of anticaseins to their respective anticasein antibodies. Casein (10^{-3} M) produced an 80% inhibition of idotype binding for subject A and 95% inhibition for subject B, demonstrating that most, although not all, of the idiotypic antibodies bind in or near the antigen binding site. The anticasein of subject B inhibited by 43% the binding of subject A's anticasein to anti-anticasein; and the anticasein of subject A inhibited the same interaction of subject B by 34%.

In the second assay, a three-party reaction was established to further analyse the capacity of anti-anticasein to block the adherence of casein to anticasein. In Fig. 2, it

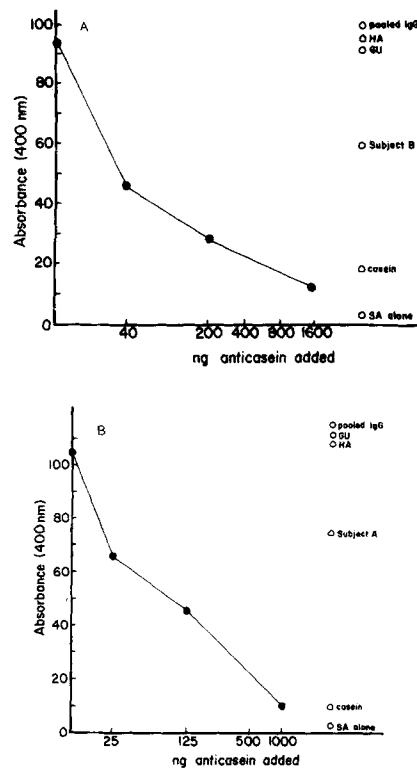


FIG. 1. An ELISA SA assay demonstrates that increasing amounts of anticaseins of subject A (panel A) and subject B (panel B) produce increasing inhibition of the binding of their respective anti-anticaseins to the alkaline phosphatase-labeled anticasein of each. The absorbance 400 nm [abscissa] indicates the relative binding of the enzyme-labeled anticasein. Pooled human gamma globulin and two myeloma proteins produced no inhibition but casein itself produced profound inhibition. Moderate inhibition appears to be present when the anticasein of subject A is tested with the anti-anticasein of subject B (panel B), or vice versa (panel A). The control, SA alone, is indicated.

is seen that as increasing amounts of intact anti-anticasein are added to polystyrene tubes coated with anticasein, progressively less AP-labeled casein is able to bind. (Anti-anticasein and anticasein interactions of subjects A and B are shown in panels A and B, respectively.) The results obtained in these experiments did not differ whether pepsin-digested or intact anticaseins were used to coat the polystyrene tubes.

Discussion

The antigenic individuality of human Ig, a property of the V_H region, was first described for humans by Kunkel et al. (15) and for rabbits by Oudin and Michael (16). That an individual may find regions of his own IG antigenic and produce autoantibodies (anti-idiotypes) is the basis of the Jerne network hypothesis (6), which proposes that all normal in vivo antibody responses are balanced by the reciprocal production of idiotypes and autologous anti-idiotypes. Current experimental evidence in murine models strongly favors cellular and humoral control by idiotypic-anti-idiotypic interactions (17-19), but very few instances of these activities in human systems have yet been described. In one, a T cell antibody with a specific suppressive action on the one-way mixed lymphocyte culture reaction was identified in the serum

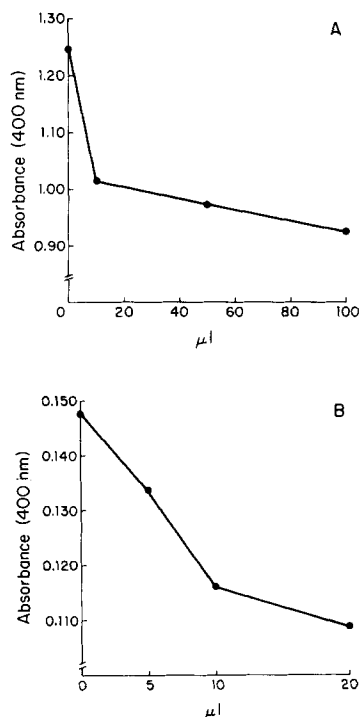


FIG. 2. As increasing volumes of an anti-anticasein preparation of subject A or B (panel A or B) are added to polystyrene tubes coated with the anticasein of the same donor, progressively less binding of alkaline phosphatase-labeled casein is permitted to occur. The relative binding is given by the absorbance at 400 nm, abscissa.

of a long-surviving renal transplant patient (20); and in another, immune complexes containing a tumor cytotoxin complex and an antiglobulin were detected in several normal sera (21). Because, in this latter study, pooled human IgG also bound the tumor-antibody complex, nonspecific antibody interactions are difficult to exclude. Possible anti-idiotypic activity has also been found in the IgM fractions of mixed cryoglobulins (22).

The experiments of the present report show that an autologous anti-anticasein antibody can be isolated from the sera of IgA-deficient individuals. Because these anti-anticaseins could bind to either pepsin treated or intact anti-casein, the observed binding of idiotypic and anti-idiotypic is not due to the release of "hidden determinants" due to pepsin treatment (23). Binding of this nature must be excluded in experiments utilizing pepsin-treated immunoglobulins. To insure that the final preparations were free of casein and/or casein-anticasein complexes that might behave similarly in the assay systems used for anti-anticasein, the potential anti-idiotypic antibodies were passed over Sepharose-casein, DEAE cellulose, and then G150 Sephadex. The [125 I]anti-anticasein of serum B, analyzed on polyacrylamide gel, showed molecular weight bands characteristic of heavy and light chains.

In murine studies, anti-idiotypic antibodies induced by hyperimmunization with antibody can be nearly 100% inhibited from binding to idiotypic by antigen, whereas similar antibodies raised by immunization with antigen antibody complexes are ~75% blocked by antigen (5, 24), indicating that the degree of saturation of antibody with

antigen probably dictates the ultimate production of "site directed" vs. "non-site directed" anti-idiotypic (5, 24). Because IgA-deficient humans have an intermittent saturation of anticasein antibody with ingested antigen (3), it is most interesting that the degree of antigen inhibition found in subjects A and B (80, 95%) falls between the levels found in murine studies for immunization with antibody alone (100%) and immunization with antigen-antibody complexes (75%).

The interactions between anticasein, anti-anticasein, and casein were tested in two systems, one using anti-anticasein bound to SA and another using anticasein bound to polystyrene. The anticasein of subject B could inhibit by 43% the binding of subject A's anticasein to anti-anticasein; in an inverse experiment, subject A's anticasein could inhibit the same reaction of subject B by 34%. Whereas these data may suggest that some of the anticaseins of these individuals could share certain idiotypic determinants, such cross reactivities are not held conclusive unless $\geq 50\%$ inhibition can be demonstrated (25). Although unusual except in families (26), cross-idiotypic reactivities have previously been detected between monoclonal IgG and IgM rheumatoid factors (RF) (27), polyclonal IgM RF (27), cold agglutinins (28), and anti-Rh antibodies of unrelated individuals (29). However, it is possible that IgA-deficient subjects, who already share a particular defect of heavy chain immunoglobulin synthesis, could also share V-region restrictions that limit idiotypic expression, in that the expression of V region and immunoglobulin regions appears to be closely linked genetically.

These experiments suggest that the binding of anti-idiotypic into the circulating immune complexes of an IgA-deficient individual could be dependent upon the content of antigen in these complexes. Because IgA-deficient individuals often have markedly increased antibody titers to bovine gamma globulin, α -lactalbumin, β -lactoglobulin and ovalbumin, in addition to casein (3, 30), it is probable that anti-idiotypic antibodies corresponding to each of these antibodies are produced, and one might expect that the circulating complexes so frequently present in the sera of these individuals could contain continuously fluctuating amounts of antigen and anti-idiotypic, depending on diet. A possible model scheme of these interactions is shown in Fig. 3. This model satisfies our previous observations that IgA-deficient subjects can have high levels of complexes even after an overnight fast, which often falls sharply after ingesting bovine milk (3). Although these could be complexes containing

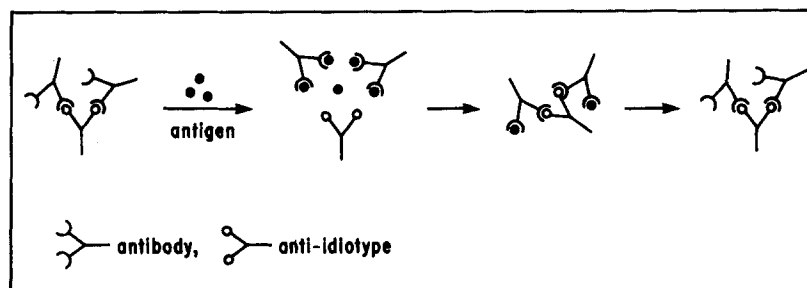


FIG. 3. When fasting, the immune complexes of an IgA-deficient subject are predominantly composed of idiotype and anti-idiotypic. As dietary antigen enters the blood stream, anti-idiotypic is largely displaced by antigen, but as antigen concentrations are reduced, the immune complexes contain both idiotype and antigen. Eventually, as antigen disappears, the original balance of idiotype-anti-idiotypic complex is restored.

long-lived dietary antigens, it is equally probable, in view of the experiments reported here, that these are antibody-anti-idiotypic complexes. In either case, the quantitative level of immune complex would be expected to be diminished by the addition of increasing amounts of a relevant antigen since smaller molecular weight complexes are less effectively bound to Raji cells (31).

The ultimate immunologic effect produced in these individuals by the continuous production of idiotype and anti-idiotypic is a critical issue. In murine studies, anti-idiotypes have been found to be capable of suppressing the expression of idiotypes (32, 33) and may, in other systems, replace antigen as a trigger to idiotype production (34). In addition, both idiotype (35) and anti-idiotypic (36) have been found to generate suppressor T cell activity in murine studies. It has, however, been uncertain to what degree murine experiments could be expected to elucidate human mechanisms. The experiments described here provide evidence of a naturally existing and unique model that may be used to explore these questions in human studies.

Summary

50% of individuals with selective IgA deficiency have high serum titers of antibody to bovine proteins, and high levels of circulating immune complexes that contain bovine antigens. Because in animal studies, immunization with antigen-antibody complexes is a very effective means of producing anti-idiotypic antibodies, we sought such autoantibodies in two sera known to have large amounts of anticasein. After IgG isolation and two-stage affinity chromatography, IgG-like material (molecular weights of H and L chains on SDS-PAGE), with binding activity for the F(ab')₂ of anticasein were isolated from both sera. Pooled human gamma globulin or IgG myeloma proteins did not inhibit binding of specific anti-anticaseins to the corresponding anticasein, but sodium caseinate did block this binding (by 80 and 95%) indicating that most of these autoantibodies have affinity for the casein-binding site. Naturally occurring anti-idiotypic antibodies have been difficult to conclusively demonstrate in human sera; consequently, these experiments provide evidence of a unique model which may be used to explore the network theory of immunoglobulin regulation in humans.

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