A STUDY OF AUTO-ANTI-IDIOTYPES TO BSA

By

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Abstract

In order to study the idiotypic relationships between the antibody populations produced in different species during normal immune responses to ordinary protein antigens, we raised immune sera in mice and chickens using three protein antigens: Bovine serum albumin (BSA), keyhole limpet hemocyanin (KLH) and diphtheria toxoid (DT). An avidin-biotin ELISA was used to measure idiotypic binding between antibody populations from these sera. We found that the chicken sera contained auto-anti-idiotypes (AAI) against antigen specific antibodies which were present in the same serum and which co-purified with those antibodies on antigen-sepharose columns. These AAI were present in secondary response chicken anti-BSA serum at levels comparable to that of the anti-BSA antibody. The chicken AAI also react specifically with Ids in mouse anti-BSA serum. Mouse anti-BSA serum completely inhibited the binding between the chicken Id and AAI. This similarity between the idiotypes of whole populations of antibodies produced in two distantly related species, in the absence of any manipulation with idiotypic or anti-idiotypic reagents, suggests that the AAI detected in this way are internal image antibodies. It indicates there is positive selection for such auto-anti-idiotypes to be internal images.
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<tr>
<td>A 405 nm</td>
<td>absorbance at 405 nm.</td>
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<tr>
<td>AAI</td>
<td>auto-anti-idiotype(s).</td>
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<tr>
<td>B6</td>
<td>C57BL/6 mouse strain.</td>
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<tr>
<td>Bis</td>
<td>N,N'-methylene-bis-acrylamide.</td>
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<td>BSA</td>
<td>bovine serum albumin.</td>
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<tr>
<td>CFA</td>
<td>complete Freund's adjuvant.</td>
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<td>DBA</td>
<td>DBA/2 mouse strain.</td>
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<tr>
<td>DT</td>
<td>diphtheria toxoid.</td>
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<tr>
<td>EDTA</td>
<td>ethylenediamine-tetraacetic acid.</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme linked immunosorbent assay.</td>
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<tr>
<td>h</td>
<td>hour(s).</td>
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<tr>
<td>Id(s)</td>
<td>idiotype(s).</td>
</tr>
<tr>
<td>IFA</td>
<td>incomplete Freund's adjuvant.</td>
</tr>
<tr>
<td>Ig</td>
<td>immunoglobulin.</td>
</tr>
<tr>
<td>KD</td>
<td>kilodalton.</td>
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<tr>
<td>KLH</td>
<td>keyhole limpet hemocyanin.</td>
</tr>
<tr>
<td>Lf</td>
<td>limit of flocculation unit.</td>
</tr>
<tr>
<td>PAGE</td>
<td>polyacrylamide gel electrophoresis.</td>
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<td>PBS</td>
<td>phosphate buffered saline.</td>
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<tr>
<td>SAS</td>
<td>saturated ammonium sulphate.</td>
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<tr>
<td>SDS</td>
<td>sodium dodecyl-sulphate.</td>
</tr>
<tr>
<td>TNP</td>
<td>trinitrophenyl.</td>
</tr>
<tr>
<td>Tris</td>
<td>tris(hydroxymethyl)aminomethane.</td>
</tr>
<tr>
<td>w/v</td>
<td>weight to volume.</td>
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Acknowledgements

I owe a debt of gratitude to you my instructors; Drs. Geoffrey Hoffmann, Julia Levy and Hung-Sia Teh; who have illuminated the doorway before me and introduced me to a world of mysteries. I would especially like to thank Dr Geoffrey Hoffmann for giving me the opportunity to explore. Thank you for your patience and your passionate excitement for simple essence behind intricacies. I hope that my work may honor all of you. Here is my drop, a libation to the sea.

"I devoted myself to study and to explore by wisdom all that is done under heaven. What a heavy burden God has laid on men!" Proverbs 1:13
Introduction

The overall behaviour of the immune system is, in principle, relatively simple. Antigen induces the expression of specific effector functions which remove the antigen. Answering the questions of why and how the immune system manages to produce this desired effect is far more complicated, and so far no widely accepted model of overall immune regulation has been formulated. One popular line of investigation which interests us is the study of idiotypic interactions between specific receptor molecules and between immune cells via their receptor molecules.

The usual approach to studying idiotypes has been to use anti-idiotypic antibodies that have been produced by direct immunization with Ig bearing the idiotype (Id) of interest. This often involves the use of simple antigens (haptens) to produce monoclonals bearing dominant idiotypes. Though this approach has yielded much information about idiotypic interactions (1, 2), it has been suggested that these idiotypic phenomena are artificial and unimportant to overall regulation (3). Since immune responses to complex antigens contain many different Ids, we took a different approach. We immunized two species (mice and chickens) with conventional antigens: BSA, KLH, or DT (diphtheria toxoid) and studied polyclonal auto-anti-idiotypic antibodies in the resulting immune sera.

Auto-anti-idiotypes (AAI) are so called because they are produced by the same individual as the idiotype. In many systems they are produced as a natural part of the immune response to antigens (4) and there is evidence that they are involved in B cell
tolerance. AAI can be detected bound to spleen cells from immunized mice. These antibodies inhibit some of the idiotype positive B-cells from secreting Ig in a plaque assay (5). In humans AAI are produced in normal immune responses (6,7) but they may be involved in the pathology of some immunological disorders such as acquired C1 inhibitor deficiency (8). There is evidence that AAI play a role in preventing auto-immune disease. Mice of strains prone to auto-immunity are deficient in their production of AAI to trinitrophenyl-Ficoll (9). In NZB strain mice the F1 of crosses with normal strains have milder symptoms. These F1 mice all produce AAI directed against the auto-antibody (10). Furthermore, in humans AAI directed against the auto-antibody (anti-DNA) of systemic lupus erythematosus are present at increased levels during remission (11,12) and are normally present in healthy individuals (13). Thus auto-anti-idiotypic antibodies are proving to be very interesting whether they are generally involved in mediating regulation themselves or are by-products of the regulatory process. More needs to be known about their properties and their regulatory roles in the immune system.

In view of the symmetry in idiotypic interactions one might imagine that when an immune response occurs, there could be a "battle" between Ids and anti-Ids specific for the antigen. We reasoned that in such a battle it is quite likely that there should be some survivors on "both sides" (14). In order for a clone to survive complementary clones should have been more or less eliminated or suppressed. Ids that are produced in large amounts would
effectively eliminate their complementary anti-Ids. Anti-idiotypic clones that managed to overcome any complementary Ids would also survive. In different species we would expect different Ids to be survivors since different species should have different repertoires. However, in both species we would expect that the anti-Ids present would bear internal images of epitopes on the antigen (15) because they were all selected by stimulation to be complementary to Ids of binding sites for those epitopes. It follows that surviving anti-idiotypic antibodies to a particular antigen in chicken antisera might bind to Ids in mouse antisera raised against the same antigen. It was this expectation that we set out to test.

In this study we found chicken AAI against antibodies present in the same serum. These chicken antibodies (in animals immunized with BSA) also bind to Id produced by mice. Antigen inhibits both forms of idiotypic binding and mouse anti-BSA serum specifically inhibits the binding between chicken Id and AAI. Thus specific AAI from one species recognize the idiotypes from another species, though neither has been immunized with the other's antibodies. We interpret this cross species recognition of Ids as being the result of selective pressures favouring anti-Ids which are internal images, in preference to anti-Ids which merely recognize one or other private Id.
Materials and Methods

Animals. Inbred DBA/2 and C57BL/6 strain mice were obtained from Jackson Laboratories (Bar Harbour, ME) or raised in our breeding colony from stocks obtained from Jackson and used between 8 and 12 weeks of age. Dekalb breed chickens were obtained from the Poultry Division of the Department of Animal Science at the University of British Columbia.

Preparation of Immune Sera. Chickens were immunized with BSA (Sigma Chemical Company, St. Louis, MO., 25 mg per injection) or KLH (Calbiochem, La Jolla, CA., 5 mg per injection): week 1 in a 50% (v/v) emulsion with CFA s.c., week 2 in PBS i.v., week 3 in IFA s.c., and week 8 in CFA s.c. Serum was obtained 12 days after the last injection. Another group of chickens were injected s.c. at three week intervals with diphtheria toxoid (DT) (Connaught Labs Toronto, Canada). Injections 1 and 2 were 20 Lf and 10 Lf respectively in aluminum hydroxide gel (precipitated form Y, see ref 16) containing 20 Lf/ml and 1 mg/ml Aluminum (as Aluminum Chloride). The third injection was 5 Lf in PBS. Serum was obtained 11 days after the last injection. In addition small samples of serum were obtained from all of the chickens at different times during the immunization schedule. In all cases chicken blood was clotted 2h at 37° C in glass tubes and then refrigerated overnight at 4° C to allow for clot retraction.

Groups of 5 DBA/2 and C57BL/6 mice were immunized intraperitoneally with BSA (7 mg per injection) or KLH (100 µg per injection). The mice were injected with antigen in 1:1 CFA on days 0
and 14, rested for 1 month, injected with antigen in PBS, rested for 3 months, injected with antigen in 1:1 CFA and again 14 days later with antigen in PBS. Serum was obtained 7-10 days after each injection of antigen in saline. The pooled mouse sera obtained after the second soluble boost were used for all experiments unless otherwise stated. In all cases mouse blood was clotted 1h at 37° C and refrigerated overnight at 4° C.

Affinity purification of Chicken Antibodies. Affinity columns were made using antigen coupled to CNBr activated (17) sepharose CL-4B (5 mg BSA, 5 mg KLH or 5000 Lf DT per ml of beads). In each case 70-90% of the protein was coupled and residual reactive sites on the beads were blocked with 0.16 M ethanolamine. Immune serum diluted 1 in 3 with PBS and adjusted to 25 mM in EDTA was then passed one or more times over the appropriate antigen column at 4° C. The column was then washed with PBS-EDTA followed by PBS and eluted with 0.1 N HCl in 0.15 M NaCl. The column was then washed and neutralized and the depleted serum was passed over the column a second time. The column was washed and eluted as before. The fractions were monitored by absorbance at 280 nm (DMS 90 spectrophotometer, Varian). The peak fractions of eluted material were neutralized with 1 M Tris Base. The eluted material from both runs was pooled, concentrated by dialysis on a bed of polyethylene glycol, dialysed against PBS, adjusted to pH 5-6 with 1 N HCl and stored at -20° C.
Adsorption of mouse serum antibodies on antigen columns. Samples (100 μl) of biotinylated serum diluted 1 in 20 were passed over 0.5 ml columns of BSA or KLH sepharose. The samples were washed through over 1 h at 4° C with PBS (10 ml) and eluted with 0.1 N HCl in 0.15 M NaCl (8 ml) while collecting 2 ml fractions. The acid fractions were neutralized with 1 M Tris-Base and all fractions were adjusted to 2.5 ml with PBS.

Preparation of gamma globulin. Chicken sera were precipitated twice (18) with 45% saturated ammonium sulphate (SAS), resuspending with 0.85% (w/v) NaCl. Mouse sera were precipitated with 50% SAS. The final precipitates were washed with 45% SAS, resuspended and dialysed against PBS.

Protein determination. The concentration of protein was estimated where necessary by measuring U.V. absorbance [assuming (18) that 1 A 278 nm = 0.89 mg/ml KLH, 1 A 279 nm = 1.5 mg/ml BSA, and 1 A 280 nm = 0.74 mg/ml fowl γ-globulin or 0.70 mg/ml mouse γ-globulin (as IgG)] or by using the bicinchoninic acid protein assay at 60° for 15 minutes (kit BCA-1 Sigma)

Biotinylation of Proteins and Antibodies. Protein solutions in PBS were biotinylated (19) by adding 0.1 volume of biotinylation reagent [5 mM biotinamidocaproyl N-hydroxysuccinimide ester (Sigma) in dimethyl-formamide] and incubating at room temperature. Serum was diluted 1 in 10 (protein concentration of 10-20 mg/ml) and biotinylated for 4 hours. Antigen and purified antibodies were diluted to 0.2 mg/ml and biotinylated; the reaction was stopped after 2 hours by adding 0.4 volume of 100 mM glycine in 25 mM succinate (pH 5.0). The dimethyl-formamide and unused
reagent were then removed by dialysis against PBS.

Avidin-biotin ELISA for binding to specific antigen or anti-idiotypes. The ELISA assay used was a modification of the standard micro ELISA using the reagents as described by Voller et al (20). For convenience the PBS-Tween was prepared from a 5 fold concentrated stock which contained 0.4 g/l NaN₃ as a preservative. Microtitre plates (Immulon II, Dynatech) were coated with affinity purified antibodies, whole γ-globulin (prepared by ammonium sulphate fractionation) or the protein antigens (100 μl at 10 μg/ml unless otherwise stated) in carbonate buffer pH 9.6 for 3 hours at 37° C. The plates were washed with PBS-Tween, blocked with 5% casein (w/v in PBS-Tween) for 1 hour at 37° C and washed. The biotinylated primary reagent (diluted in 5% casein) was added and allowed to bind for 2 hours at 37° C. The plates were washed and avidin-alkaline phosphatase conjugate (Sigma, 1/400 dilution of a 0.5 mg/ml stock) in 5% casein was added for 1 hour at 37° C. The plates were washed and substrate (Sigma 104: 1 mg/ml p-nitrophenylphosphate in 10% diethanolamine pH 9.8) was added. The plates were incubated at 37° C until sufficient colour development had occurred. Absorbances at 405 nm (A 405 nm) were measured using an ELISA plate reader (Titertec Multiskan).

Inhibition of binding in ELISA. Competitive inhibitors (antigen or serum antibodies) diluted in 5% casein were mixed with an equal volume of the primary reagent (biotinylated antibody or antigen) at twice the desired final concentration. The final concentrations of the biotinylated reagents were: BSA at 50 ng/ml, affinity purified chicken anti-BSA at 200 ng/ml, chicken anti-BSA
serum at a 1/4,000 dilution and mouse anti-BSA serum at 1/30,000. The mixtures were then used in the ELISA assay. The inhibition was calculated from the A 405 nm values according to the equation:

\[
\frac{\text{A}_I - \text{Mean background}}{\text{A}_0 - \text{Mean background}} \times 100
\]

\(A_I\) is the A 405 nm for samples with inhibitor and \(A_0\) is the A 405 nm for the samples without inhibitor. The background is the A 405 nm with uncoated wells (blocked with 5% casein).

Non reducing polyacrylamide gel electrophoresis: separation, preparative fractionation of antibodies. Samples were heated to 60° C for 10 minutes in non-reducing sample buffer (0.0625 M Tris HCl (pH 6.8), 2% SDS and 10% glycerol) and subjected to PAGE (21,22) on linear gradients (23) of 3% to 10% or 3% to 20% T acrylamide (4% C) with stacking gels containing 3% T (5% C) acrylamide [(24) % T is the total % (w/v) of monomer (acrylamide and Bis) and % C is the % of the total monomer contributed by the crosslinking monomer (Bis)]. The gels were then cut into pieces and some sections were stained with Coomassie blue. Unstained sections containing the chicken anti-BSA antibodies to be isolated were sliced into horizontal strips which were individually chopped into 5 mm squares, placed in polypropylene tubes with about 1 gel volume of 0.05 M ammonium bicarbonate and rolled end over end for 3 days at 4° C. The buffer was changed and the gel slices were rolled for an additional 3 days with fresh buffer. The two buffer samples from each slice were pooled and this eluted material was stored at -20°C.
Results

Auto-anti-idiotypic antibodies in affinity purified chicken antibody populations. Affinity purified chicken antibody populations specific for BSA, KLH or DT were biotinylated and an avidin-biotin ELISA was used to measure their binding to plates coated with each of the same affinity purified antibody preparations in non-biotinylated form. The antibodies in each affinity purified preparation react preferentially with antibodies within the same preparation. That is, chicken anti-BSA reacts specifically with chicken anti-BSA, anti-KLH reacts with anti-KLH and anti-DT reacts with anti-DT (Fig. 1). Antibodies in each preparation react only weakly with antibodies specific for other antigens. This specificity indicates that the binding is mediated by the V-region. That is, the purified preparations contain auto-anti-ids (AAI) against the antigen specific antibodies. Since the chicken anti-BSA preparation gave the strongest effect, it was used to further characterize the phenomenon.

Characterization of the antibodies involved in binding of chicken anti-BSA to chicken anti-BSA. We did experiments to show that the specific binding of chicken anti-BSA to chicken anti-BSA is not due to residual antigen in the purified antibodies. Chicken anti-BSA was separated by non-reducing SDS PAGE. The major bands in the chicken anti-BSA migrate at the same positions as a purified yolk IgG standard (Fig. 2). Although the gel was intentionally overloaded no albumin band nor lower molecular weight (degraded albumin) bands could be detected in the chicken anti-BSA, either
Fig. 1. Specific self binding properties of affinity purified chicken antibodies. The results represent the binding in an ELISA of biotin labeled, purified chicken antibodies (□, anti-KLH. ◆, anti-BSA. ■, anti-DT) to plates coated with purified chicken anti-KLH (a), anti-BSA (b) and anti-DT (c). The error bar is the standard deviation of duplicates. In panel (b) the two control curves (those for anti-KLH and anti-DT) overlap, and in some cases the error bar is smaller than the symbol.
Biotinylated antibody (μg/mL)
Fig. 2. Purity of the affinity purified chicken anti-BSA. Samples of proteins were separated by non reducing SDS polyacrylamide gel electrophoresis on a linear 3 to 20% gradient gel and stained with Coomassie Blue. Lane 3 is purified chicken anti-BSA, approximately 30 μg and lane 4 is BSA, approximately 0.3 μg. Lane 2 is chicken egg yolk IgG (25) as a standard. Lane 1 is molecular weight standards (Sigma SDS-6H: myosin (band not visible) β-galactosidase, phosphorylase b, bovine serum albumin, ovalbumin and carbonic anhydrase) with molecular weights as indicated.
Molecular weight (KD)
visually or by scanning densitometer. A BSA standard equivalent to as little as 1% BSA contamination is clearly visible (Fig. 2, 3a). The minimum BSA contamination that would have been needed to account for the ELISA signal was estimated from mixing experiments to be about 5%. Varying amounts of BSA were mixed with irrelevant chicken antibody (anti-KLH) so as to maintain a constant protein concentration and the mixtures were used to coat ELISA plates. The binding of chicken anti BSA to these plates increases linearly up to about 10% BSA. Plates coated at mixtures containing 5% of the protein as BSA bind the same amount of chicken anti-BSA as plates coated with purified chicken anti-BSA. The assay is even less sensitive to the effect of BSA contamination of the biotinylated reagent. The binding to plates coated with chicken anti-BSA of varying quantities of biotinylated BSA or biotinylated chicken anti-BSA was compared. In the approximately linear portion of the binding curves 0.06 \( \mu \text{g/ml} \) of biotinylated BSA produces a signal equal to that of about 0.4 \( \mu \text{g/ml} \) of biotinylated chicken anti-BSA. Thus the biotinylated chicken anti-BSA would have to contain 15% contamination with BSA to account for the binding observed.

Fractionation with preparative gel electrophoresis was used to demonstrate that the specific binding is due to molecules the size of chicken IgG. The affinity purified chicken anti-BSA was eluted with 0.05M ammonium bicarbonate from transverse slices of the unstained portion of the non-reducing SDS PAGE gel. Each eluted fraction was used to coat wells of an ELISA plate to yield a profile of the gel from top to bottom. Biotinylated chicken anti-BSA bound in the ELISA (Fig. 3b) mainly to material from gel slices corresponding
Fig. 3. Molecular size of the chicken auto-anti-idiotypic antibodies. Affinity purified chicken anti-BSA was separated by non reducing SDS polyacrylamide gel electrophoresis and eluted from gel slices as described in the Materials and Methods section. Separate sets of wells on ELISA plates were coated with the material from each of these gel fractions diluted 1/5 with carbonate buffer. The top panel (a) shows densitometer scans of lanes 3 (chicken anti-BSA) and 4 (BSA) of the gel (which is shown in Fig. 2). The bottom panels are ELISA binding of biotinylated affinity purified chicken anti-BSA (b) and mouse anti-(chicken γ globulin) (c) to each gel fraction. The arrows indicate the approximate positions of the pre-stained molecular weight markers (84 KD, fructose-6-phosphate kinase Sigma F-0387; 27 KD, triosphosphate isomerase Sigma T-9400). The asterisks in panel (c) indicate the fractions which were biotinylated and assayed (Fig.4) to determine which biotinylated Ig species bind to the chicken IgG on the ELISA plate.
to the position (Fig. 3a - lane 3) of the main protein band. There was no binding to any material from the slices corresponding to the position of the BSA band (Fig. 3a - lane 4). There was, however, some binding to higher molecular weight material which may be IgM or dimeric IgA (23) since mouse anti-serum against whole chicken γ-globulin binds to some higher molecular weight material in that region of the profile (Fig. 3c).

Material from the gel fractions corresponding to the peaks marked with asterisks in Fig. 3c was biotinylated and binding to chicken anti-BSA was measured (Fig. 4). These fractions represent the higher molecular weight (IgM or IgA) material and the main IgG peak. Binding of material in the peak corresponding to IgG accounts for the vast majority of the signal. Thus the binding between members of the affinity purified chicken anti-BSA population is mainly due to binding among members of the IgG populations rather than binding between IgG and some minor component such as IgM or IgA.

*Idiotype complementarity between antibodies from mouse and chicken immune responses to BSA.* Biotinylated mouse anti-BSA sera bind in the ELISA to affinity purified chicken anti-BSA antibodies but not to chicken anti-KLH antibodies (Fig 5d-i). Furthermore neither normal mouse serum nor hyperimmune mouse anti-KLH sera bind to chicken anti-BSA even though the latter have high titers of anti-KLH antibody (Fig. 5a- c).
Fig. 4. Idiotypic binding is due mainly to interactions between IgG species. Samples (200 μl) of the Ig containing gel fraction extracts marked with asterisks in Fig. 3c were dialyzed against PBS and biotinylated by adding 20 μl of biotinylation reagent, incubated 2 hours and dialyzed against PBS. The biotinylated samples were adjusted to 300 μl and used at 1/50. Binding to plates coated with purified chicken anti-BSA was measured. The error bar is the standard deviation of duplicates. Slices 8 and 9 give the strongest signal and are IgG (compare Fig. 3c).
Relative binding
(A 405 nm)
Fig. 5. Specific binding of mouse serum antibodies to affinity purified chicken anti-BSA measured in an ELISA assay. The results show the binding of various biotin labeled normal or immune mouse sera to plates coated with affinity purified chicken anti-BSA (●), affinity purified chicken anti-KLH (○), BSA (■), and KLH (□). The sera used in the various panels were obtained from: unimmunized mice (a); groups of mice after the second boost with soluble antigen (b, c, f, and i); two individual mice after the first boost with soluble antigen (d and e); a group of mice seven days and ten days respectively after the first boost with soluble antigen (g and h). The data points are means of duplicates.
Biotinylated mouse serum (log of dilution factor)

Relative antibody binding (A 405 nm)

Binding to:
- BSA
- CαBSA
- KLH
- CαKLH

DBA NMS

B6 anti KLH

DBA anti KLH

B6 anti BSA(1)

B6 anti BSA(2)

B6 anti BSA(3)

DBA anti BSA(1)

DBA anti BSA(2)

DBA anti BSA(3)

Biotinylated mouse serum (log of dilution factor)
In pooled sera of both DBA/2 and B6 mice and in the sera of individual mice (Fig 5d,e) a large proportion of the mouse antibody is able to bind the chicken anti-Id. In all except one (Fig. 5f) of the mouse anti-BSA sera tested the titration curves for binding to excess BSA and excess chicken anti-BSA are separated by about a two fold dilution or less. Thus, assuming that equal signals are produced by equal amounts of bound antibody, the sera contain idiotype binding antibody at approximately half or more of the level of anti-BSA antibody.

To rule out antigen as the explanation for this specific binding as well, biotinylated mouse anti-BSA serum was fractionated using preparative gel electrophoresis (Fig 6). All of the material which binds to chicken anti-BSA antibodies (top panels) migrates at the same position as the antibodies which bind to BSA (centre panels) and the main peak of the immunoglobulins as detected by binding to rabbit anti-mouse Ig antibodies (bottom panels). This peak is well resolved from the position of the BSA band (66 KD) where no binding was observed.

Biotinylated mouse anti-BSA sera were also fractionated into antigen binding and non-binding fractions by adsorbing with BSA sepharose columns. More than 90% of the mouse antibodies which bind to chicken anti-BSA are removed by adsorption with BSA sepharose but not KLH sepharose (Fig 7). Antibodies which bind to chicken anti-BSA were recovered in the BSA binding fraction eluted from the BSA column. The simplest explanation is that mouse antibodies against BSA bind to the chicken AAI contained in the purified chicken anti-BSA antibodies.
Fig. 6. Molecular size of the mouse antibodies which bind to the chicken auto-anti-idiotyp.

The protein components of whole biotinylated mouse antisera (B6 anti-BSA on the left, DBA anti-BSA in the centre and DBA anti-KLH on the right) were separated by non-reducing SDS polyacrylamide gel electrophoresis and eluted from gel slices as described in the Materials and Methods section. Each of these fractions was assayed in the ELISA for binding to a plate coated with purified chicken anti-BSA (○), purified chicken anti-KLH (■), BSA (●), and the γ globulin fraction of rabbit antisera against mouse Ig (□). The arrows indicate the approximate positions in the stained portion of the gel of the molecular weight markers (97 KD, phosphorylase b Sigma SDS–6H; 66 KD, bovine serum albumin and 14 KD, α–lactalbumin Sigma SDS–7).
Migration in PAGE (gel slice number)
Fig. 7. The effect of removing anti-BSA antibody from mouse anti-BSA serum on binding to chicken anti-BSA auto-anti-idiotypic. Samples of biotinylated DBA (panel a) and B6 (panel b) mouse anti-BSA sera were passed over small columns of KLH or BSA sepharose. The data shown represents the binding in the ELISA of the antibodies in the first fraction (~2 ml) of the runthrough and of the eluate. The error bar is the standard deviation of duplicates and is negligible in some cases. Practically all of the anti-idiotypic activity is adsorbed to the BSA column.
Figure a: Relative binding (A 405 nm) for KLH column and BSA column in Run-through and Eluate.

Figure b: Relative binding (A 405 nm) with error bars for KLH column and BSA column in Run-through and Eluate.

Column fraction
The time course of the level of anti-BSA and AAI in chickens after immunization with BSA. Chicken γ-globulin fractions were prepared by ammonium sulphate precipitation of small samples of serum obtained throughout the immunization period. The γ-globulin samples were used to coat ELISA plates and the binding of biotinylated BSA, mouse anti-BSA and chicken anti-BSA was measured (Fig. 8). The conditions were adjusted so that each biotinylated reagent was in excess and the signal varied linearly with the proportion of specific antibody in the γ-globulin used to coat the plate. Some BSA binding but very little Id binding antibody is present by day 7 in the primary response after the first immunization. The main peak of both anti-BSA and anti-Id activity is produced during the secondary response. An estimate was made for this assay of the ratio of molar signals for binding of biotinylated antibody as compared to BSA. For the biotinylated proteins coated directly onto ELISA plates biotinylated chicken anti-BSA antibody produces 1.3 ± 0.2 (mean ± SE) times the signal obtained with an equal molar quantity of biotinylated BSA. Since the peak signals (Fig. 8a and c) are roughly the same for binding of biotinylated BSA and for binding of biotinylated affinity purified chicken anti-BSA, this ratio indicates that the concentration of idiotype binding antibodies is about 50-100% of that of the anti-BSA antibodies at this point in the time course.
Fig. 8. Time course of induction of anti-idiotype and anti-BSA antibody in the immune response to BSA. Gamma globulin was prepared from small samples of chicken serum taken at various times through the course of immunization with KLH (□) or BSA (■) and used to coat separate wells of ELISA plates. The data represent the binding of excess biotinylated BSA (a), mouse anti-BSA serum (b), and affinity purified chicken anti-BSA (c) to antibodies in the gamma globulin fraction at each time during the immune response. The error bar (in most cases smaller than the symbol) is the standard deviation of duplicates. For BSA the values were obtained using sera from two chickens. Antigen injections were given at the times indicated by the arrows (in complete (CFA) or incomplete (IFA) Freund's adjuvant or as a solution (Sol) in saline).
Relative binding (A 405 nm units per hour)

- CFA
- Sol
- IFA

Time (weeks)
Antigen (BSA) as an inhibitor of idiotypic binding to chicken anti-BSA. BSA was included as an inhibitor at various concentrations with constant amounts of various biotinylated reagents binding in the ELISA to plates coated with affinity purified chicken antibodies. The binding of both biotinylated mouse anti-BSA serum and affinity purified chicken anti-BSA to affinity purified chicken anti-BSA is completely inhibited by high concentrations of BSA (Fig. 9). This inhibition is specific since KLH does not block the binding of any of the reagents. Higher concentrations of BSA are needed to block the binding of the various anti-BSA antibodies than to block the binding of biotinylated BSA to the purified chicken anti-BSA. This may reflect a greater intrinsic affinity of the Ids and anti-Ids for each other than for BSA or a greater effective affinity due to the multivalence of antibodies.

Specific antiserum as an inhibitor of idiotypic binding to chicken anti-BSA. Specific antisera were tested as inhibitors in the same assay as for inhibition by BSA. The binding of biotinylated affinity purified chicken anti-BSA to affinity purified chicken anti-BSA can be completely inhibited by high concentrations of the chicken anti-BSA serum from either the same chicken or another immune chicken (Fig. 10a). Normal chicken serum and immune serum against another antigen (KLH) give much less inhibition. More importantly, binding of Ids and anti-Ids in the affinity purified chicken anti-BSA can be completely blocked by mouse anti-BSA serum or γ-globulin (Fig. 10b and c). This indicates that a large proportion of the chicken anti-BSA antibodies involved in auto-anti-
idiotypic binding have Ids similar to the mouse anti-BSA antibodies.

This inhibition by mouse serum is not due to mouse serum albumin since non immune and irrelevantly immune mouse sera are more than 10 fold less effective as inhibitors. Furthermore, γ-globulin from mouse anti-BSA serum gives 50% inhibition at 10-20 μg/ml whereas about 1000 μg/ml of BSA is required for 50% inhibition (Fig. 9).
Fig. 9. Inhibition by antigen of idiotypic binding to affinity purified chicken antibodies. BSA (solid symbols) was added at various concentrations to block binding of biotinylated reagents (■: BSA, ●: chicken anti-BSA serum, ▲: mouse anti-BSA serum and ◆: affinity purified chicken anti-BSA) to affinity purified chicken anti-BSA on ELISA plates. As a control, KLH (hollow symbols) was added to the same biotinylated reagents (□, ○, △, ◊). The error bar is the standard deviation of four replicates and is plotted where it is larger than 2.5% inhibition.
Fig. 10. Inhibition of binding between chicken idiotype and auto-anti-idiotype using mouse and chicken anti-BSA serum antibodies. Normal (□), anti-KLH (○) or anti-BSA (●) antibodies (as chicken sera in panel a, as mouse sera in panel b and as γ globulin from mouse sera in panel c) were added at various dilutions to biotinylated affinity purified chicken anti-BSA antibodies in an ELISA with chicken anti-BSA on the plate. The error bar is the standard deviation of four replicates and is plotted where it is larger than 2.5% inhibition. In panel a the two curves [both marked (●)] for blocking by chicken anti-BSA serum are for two sera from individual chickens, one of which is the source of the purified chicken anti-BSA.
Discussion

We find that Id and AAI are present simultaneously in the sera of chickens immunized with conventional protein antigens. In the secondary response to one antigen (BSA) the AAI appears to be present at a concentration of the order of 50-100% of the concentration of the antibodies against the antigen. This indicates that it may be common for AAI to coexist at high levels with the corresponding Ids in immune responses to protein antigens. Furthermore, binding and inhibition experiments show that the chicken AAI also recognize antibodies produced in mice immunized with the same antigen. Nearly all of the chicken AAI appears to bear internal images of the antigen. This confirms for a normal immune response the validity of the conclusion (15) that antibodies which are complementary to an antigen bear "negative images" of epitopes on that antigen. Furthermore, it indicates that the anti-Ids were selected to recognize these negative images (that is, to be internal images).

The presence of AAI in chicken antisera is not very surprising since their production during other normal immune responses is well documented (4). If the anti-Ids are induced by Id they must both be present together at some time during the response. However, the presence of large quantities of these anti-Ids in chicken serum and their ability to copurify with the antigen specific antibodies is curious. They could perhaps be present in sera together with antigen specific antibodies as soluble immune complexes. Such
complexes could bind to an antigen column via the antigen specific antibodies and both components would elute from the column together. Others have reported that Id and AAI can coexist and participate in soluble immune complex formation (26), albeit as part of a pathological condition (selective IgA deficiency).

A second possible explanation for the presence of anti-idiotypic antibodies in the column purified material is that these antibodies may have dual specificity; they may bind both to the antigen and to Ids on antigen specific antibodies. Clones that are both antigen-specific and anti-idiotypic could be expected to have a selective advantage in the immune response. They would receive stimulation from both antigen and antigen-specific idiotypes, and they could continue to be stimulated by idiotypes even after the antigen is eliminated.

The idea of these being dual specific antibodies may not be too far-fetched, since multispecific reactivity between antibodies is common (27,28). Furthermore, Bona et al. (29) have shown that certain anti-idiotypic antibodies raised in A/J mice against the V regions of IgM K_{III}G1 (a human monoclonal rheumatoid factor specific for IgG) also bind to IgG Fc fragments. They gave the name "epibody" to any antibody which binds both to an antigen and to the V regions of other antibodies against the same antigen. Related observations have been made by Kang and Kohler (30) who reported a monoclonal which binds to itself. They raised hybridoma antibodies against T15 (a myeloma which bears the dominant Id of the Balb/c anti-phosphorylcholine (PC) response). One hybridoma was found to react with both PC and T15. This monoclonal binds to
its own V-regions and so they called it an "autobody". Subsequently a \( V_H \) region peptide involved in the binding has been identified (31). Consistent with a dual specificity interpretation of our results is the fact that, to within the time resolution of the study, BSA binding and idiotype binding activity occur in the chicken immune response with identical kinetics (Fig. 8).

If the binding between antibodies in these purified chicken antibody preparations is indeed due to the presence of "epibodies" or "autobodies," this observation could begin to address the important question posed by Kang et al. (31) as to whether autobodies are biological relevant. From this perspective it would be worthwhile to explore more closely the Id and AAI clones in the immune response to BSA. Panels of anti-BSA monoclonal antibodies could be raised and screened for binding to one another and could be used to screen for the AAI producing clones.

If the population of anti-BSA antibodies is in fact selected with time to be self-recognizing, this population may become largely self-regulating. This would then be a relatively simple system of idiotypic regulation, that could be more readily amenable to quantitative analysis than models in which two or more levels of idiotypes are involved.

In contrast to our results it has been reported that AAI produced in human responses to tetanus toxoid do not bind to idiotypes present at that same point in the immune response (6). This is probably because in that report the assay required that the serum be carefully absorbed to remove any antibodies against the antigen. The absorption would also remove any AAI of the kind
described here and could seriously affect the results. Others have reported simultaneous presence of Id and AAI in a rabbit immunized with bacterial polysaccharide (32) and in patients with systemic lupus erythematosus (11,12) selective IgA deficiency (26) and acquired C-1 inhibitor deficiency (8).

We see idiotypic binding of antibodies in chicken serum to the affinity purified chicken antibodies, and we also see mouse anti-idiotypes binding specifically to the chicken idiotypes. The specific interaction between chicken antibodies can be understood in terms of direct stimulation of auto-anti-idiotypic antibodies (4) by Ids present during the immune response. Mouse serum could also contain AAI against mouse Ids. However, this alone would not explain the ability of immune mouse serum to bind to these chicken antibodies and to inhibit the binding of chicken Id to AAI. Any anti-Ids in the mouse serum were not stimulated by the chicken Ids which they recognize and vice versa. The simplest explanation is that a selection process leads to the production in chickens of AAI, the majority of which bear internal images of antigen epitopes that are recognized by mouse antibodies against those epitopes. This is remarkable in that most anti-Ids described in the literature (which are mostly not auto-anti-Ids) are not internal images; see the discussion by Pontillon et al. (33). However, the auto-anti-Ids formed in human responses to house dust mite allergens (7) are also internal image anti-Ids. This suggests that auto-anti-idiotypes in general may have a tendency to bear internal images.

The sharing of idiotypic specificity by antibodies from two different species is in contrast to the behaviour of Ids detected by
anti-Ids raised against purified antibodies. In their classic pioneering studies, Oudin and Michel (34,35) produced anti-idiotypic antibodies by immunizing rabbits with bacterial cells agglutinated with antiserum from individual rabbits. The resulting anti-idiotypic sera precipitated specific antibodies from the original serum but not from the sera of other rabbits immunized with the same antigen. Thus the idiotypic specificities detected by Oudin's anti-idiotypic reagents are not shared even between members of the same species. In general immunization with an antibody leads to production of a range of anti-idiotypic antibodies. Some of these are against Ids which are shared with other (genetically distinct) individuals. A fraction of those which recognize shared Ids do so because they are internal images of epitopes on the antigen and as such could be recognized by any antibody against those epitopes (15,36). Some can even mimic some of the effects of biologically active antigens such as hormones (37). In chickens immunized with BSA almost all (rather than only a few) of the AAI are internal image. This observation suggests that the AAI selected in the course of an immune response to antigen are qualitatively different anti-Ids from those selected by active immunization with Id.

One plausible speculation as to why AAI might tend to be internal image is suggested by the observation that anti-idiotypic T-cell clones can be activated by antibody (38). In vitro cultured human anti-idiotypic T cell lines have been produced which are activated by immune serum Ig when presented in the context of self class II (HLA-DQ). If we speculate that B-cells which present antigen to T-cells get help (lymphokines for example) from those T-
cells then any anti-idiotypic B-cell clone could get help by specifically picking up and presenting antibody molecules to anti-idiotypic T-cells in the same way that antigen specific B-cells pick up antigen at very low concentrations, accumulate it and present it to antigen specific T-cells (39). In this situation B cells producing an internal image would get more stimulation since they would recognize a broad range of idiotypes and thus be able to interact with more anti-idiotypic T-cells. Conversely B cells against antigen epitopes could possibly be stimulated by picking up internal image antibody and presenting it to T-cells. Thus the antigen recognizing clones and the internal image clones could serve to stabilize each other.
Bibliography


