EVOLUTION OF AN IDIOTYPIC DETERMINANT:
ANTI-VAL*

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The occurrence of idiotypic cross-reactivity between individuals of the same species has been demonstrated previously. This phenomenon was first observed with homogeneous human macroglobulins with cold agglutinin activity (1). Using antibodies of the same specificity, cross-idiotypic specificity has been observed in highly inbred strains of mice (2-7) and inbred rabbits (8). This phenomenon also exists for human proteins with anti-y-globulin activity (9). Interstrain idiotypic cross-reactions have also been demonstrated in mice (10, 11). The occurrence of shared idiotypic specificity has been reported in three outbred goats, all of which produce specific antibodies that distinguish human sickle cell hemoglobin (HbS) from normal human hemoglobin (HbA) (12). Genetic observations indicate that the idiotype is inherited as a gene product (6, 7, 13). These results prompted the examination of the idiotypotypes present on relatively homogeneous antibodies of identical specificity produced in closely related species, to determine if a similar idiotype can be produced in two such species.

The species used in this comparison study were goat and sheep; both producing antibodies that distinguish HbS from HbA. A distantly related species, guinea pig, was also examined in this regard. The guinea pigs also produced specific antibody which reacted only with HbS and not HbA, but it lacked the idiotypic cross-specificity present in the sheep and goat antibodies. Anti-idiotypic sera were produced against the goat and sheep antibodies in rabbits. The extent of idiotypic cross-reactivity was measured by immunodiffusion and radioimmunoassay. Strong cross-reactivity was found between the goat and sheep antibodies.

Evidence linking the idiotype with the binding site of the antibody molecule has been described (5, 13, 14). To demonstrate that the idiotypic cross-reactivity between the sheep and goat antibodies also involved the antigen-binding site of the molecules, experiments were done to block antigen binding with different anti-idiotypic sera. Antigen binding to guinea pig antibody was not affected, but binding to sheep and goat antibodies was blocked.

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Abbreviations used in this paper: HbA, normal human hemoglobin; HbS, human sickle cell hemoglobin.
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Materials and Methods

Hemoglobin. HbS was obtained from patients who were heterozygous for this mutation. The HbS is separated from the HbA, on a DEAE-cellulose column, equilibrated with 0.05 M Tris, pH 8.15. HbA; and HbS eluted separately, and HbA, is eluted by making the wash buffer 0.1 M in NaCl.

Anti-HbS Sera. Goats and sheep were immunized with HbS as previously described by Noble et al. (16). Guinea pigs were injected with 5.0 mg of HbS in complete Freund’s adjuvant in the toe pads. 14 days later they were given 0.1 mg of HbS intradermally. Heart punctures were performed on days 21, 23, and 25. The antisera, goat, sheep, or guinea pig, were then prepared as described by Tan-Wilson et al. (17).

Isolation of 7S Anti-Val. HbA, or HbS was conjugated to CNBr-activated Sepharose 4B (Pharmacia Fine Chemicals, Inc, Piscataway, N. J.) as instructed by the manufacturer. The purified antibody populations were isolated as described by Schreiber and Reichlin (12) with some modifications. The anti-HbS antiserum, after dialysis to pH 7.5 (0.05 M phosphate, 0.5 M sodium chloride, 0.01 M sodium azide) was passed through a column of HbA, Sepharose at a flow rate of 70 ml/h. The eluate from this column, which contained all of the antibodies which would not bind HbA, was collected and concentrated to the original volume. It was then passed through a column of HbS-Sepharose. The column was washed with starting buffer until the optical density of the eluate at 280 nm had fallen to less than 0.02 OD units. The buffer was changed to 1.0 M acetic acid, 0.5 M sodium chloride, and the antibodies were eluted off in a sharp peak, as determined by the OD at 280 nm. These antibody-containing fractions were immediately pooled and neutralized with solid trizma base (Tris) and then dialyzed against starting buffer. These antibodies bind HbS and not HbA and are termed anti-Val antibodies. Their specificity was confirmed by fluorescence titration as described by Noble et al. (16).

Anti-Idiotype Antiserum. Antisera to goat 6 anti-Val and sheep 26 anti-Val Fab’ fragments were raised in white New Zealand rabbits as described by Schreiber and Reichlin (12). The rabbit anti-goat 6 anti-Val anti-idiotype serum used in this study was the same as that used and described by Schreiber and Reichlin. It was obtained by absorbing the rabbit anti-goat 6 anti-Val serum with a goat anti-HbA serum. Rabbit anti-sheep 26 anti-Val anti-idiotype serum was obtained by absorbing the anti-sheep 26 anti-Val serum with normal sheep γ-globulin (Schwarz/Mann Div., Becton, Dickinson & Co., Orangeburg, N. Y.).

Measurement of Anti-Idiotype Reaction by Radioimmunoassay. The procedure of Schreiber and Reichlin (12) was used with some modifications. Homologous anti-Val (7S) antibodies were trace labeled with 131I by means of the chloramine-T method (18). Free 131I was separated from labeled anti-Val by gel filtration through a 0.9 × 15 cm Bio-Gel P-4 column equilibrated with 0.1 M phosphate buffer pH 7.0.

131I-anti-Val was diluted so that 50 μl of the solution contained approximately 3,000 cpm. The same dilution was used for each experiment. The concentration of anti-Val in this solution has been calculated to be about 10 ng/ml. 50 μl of diluted 131I-labeled anti-Val were mixed with 100 μl of a dilution of rabbit anti-idiotype serum such that about 50% of the total counts added were bound. The anti-idiotype serum was diluted with normal rabbit serum to provide carrier γ-globulin for precipitation. The reaction was allowed to incubate for 18-24 h at 4°C, after which time an excess of sheep antiserum to rabbit γ-globulin was added to precipitate all rabbit immunoglobulin in the reaction tube. Immunoprecipitation was allowed to proceed for 24 h at 4°C, after which time the precipitates were washed three times in a neutral wash buffer containing 1% albumin and then counted in a Nuclear Chicago gamma-ray spectrometer (Nuclear-Chicago Corp., Des Plaines, Ill.). Inhibition experiments were performed using the above method, except that 50 μl of inhibitor solution were mixed with 50 μl of 131I-labeled anti-Val and then 100 μl of the anti-idiotype serum added. This same procedure was also used to measure the inhibition of the anti-idiotype reaction by hemoglobin. In those experiments, hemoglobin was used as the inhibitor, and the above method was followed.

Measurement of Inhibition by Anti-Idiotype Sera of the Reaction of HbS with Anti-Val Antibodies. HbS was trace labeled with 131I using the chloramine-T method (18). Free 131I was separated from 131I-labeled HbS by gel filtration through a 1.4 × 16 cm Sephadex G-25 column equilibrated with 0.1 M phosphate buffer, pH 7.0. The trace-labeled HbS was diluted so that 50 μl contained about 3,000 cpm.
Results

The Demonstration of Interspecies Idiotypic Cross-Reactivity by the Ouchterlony Technique. The specificities of the absorbed anti-idiotypic sera were first examined by gel diffusion according to the method of Ouchterlony. The anti-idiotypic serum directed toward goat 6 anti-Val formed no precipitin line when diffused against normal goat γ-globulin.

As shown in Fig. 1, no precipitin line was formed upon diffusion of this anti-idiotypic serum against a goat anti-cytochrome c serum. In contrast, precipitin lines formed upon reaction with goat 6 anti-HbS serum and sheep 26 anti-HbS serum. The two lines interact to indicate a reaction of identity between these two antigens. No reaction was observed with guinea pig anti-HbS serum. In a separate experiment, this anti-idiotypic serum was also found to form an observable precipitin line with a second sheep anti-HbS serum, sheep 27.

The specificity of the anti-idiotypic serum directed toward sheep 26 anti-Val was virtually the same. It failed to react with normal sheep γ-globulin and with guinea pig anti-HbS serum but gave clear precipitin lines with sheep 26 anti-HbS serum, sheep 27 anti-HbS serum, and with goat 6 anti-HbS serum. The identity reaction obtained with goat 6 anti-HbS and sheep 26 anti-HbS sera can be seen in Fig. 2. This is a clear demonstration of interspecies idiotypic cross-reactivity.

Direct Binding of $^{131}$I-Labeled Anti-Val to Anti-Idiotypic Serum. An indirect precipitation method was used to measure the direct binding of $^{131}$I-labeled anti-Val antibodies to the anti-idiotypic serum directed toward goat 6 anti-Val.

50 µl of an appropriate dilution of anti-idiotypic serum were mixed with 100 µl of a dilution of anti-Val antibodies. The dilution of anti-Val, in carrier γ-globulin, is such that there is an excess of antigen ($^{131}$I-labeled HbS). The mixture of anti-idiotypic serum and anti-Val is incubated for 10 min at 4°C, after which time 50 µl of the $^{131}$I-HbS are added. After an 18- to 24-h incubation period, the appropriate serum is added to precipitate all the reacting immunoglobulin. Immunoprecipitation was allowed to proceed for 24 h at 4°C, after which time the precipitates were washed three times in a neutral buffer and counted in a Nuclear-Chicago gamma-ray spectrometer.
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**Fig. 2.** Ouchterlony experiment showing reaction of identity between sheep and goat anti-HbS sera. Center well contains Ra 585 anti-idiotypic serum which is specific for sheep 26 anti-Val.

**Fig. 3.** Radioimmunoassay titer in which Ra 326 serum is diluted and the concentrations of the $^{125}$I-labeled goat 6 anti-Val (○—○) and sheep 26 anti-Val (○—○) remain constant.

$^{125}$I-labeled goat 6 anti-Val or sheep 26 anti-Val was mixed with serial dilutions of this anti-idiotypic serum. After an appropriate incubation time, immunoprecipitation was accomplished by addition of excess sheep antisera to rabbit $\gamma$-globulin. The results of one such titration are shown in Fig. 3. The homologous antibody, goat 6 anti-Val, bound to the anti-idiotypic serum as was expected. There was also binding of the heterologous sheep 26 anti-Val, although the level of binding was much lower than that of the homologous anti-Val population. Again interspecies idiotypic cross-reactivity is demonstrated, but the identity reactions seen in Figs. 1 and 2 must be only qualitative since a clear quantitative binding difference is demonstrable.

**Inhibition of the Anti-Idiotypic Reaction by Purified Antibody Populations.** The ability of different antibody populations to inhibit the reaction of the anti-idiotypic sera with radiolabeled homologous anti-Val antibodies offered a more quantitative measure of the specificity of these anti-idiotypic sera and the idiotypic cross-reactions. The procedure used for these measurements is described in the Materials and Methods.

In Fig. 4 the inhibition of the reaction of the anti-idiotypic serum directed toward goat 6 anti-Val with radiolabeled goat 6 anti-Val by cold goat 6 anti-Val antibodies, sheep 26 antibodies, and normal sheep $\gamma$-globulin are compared.
Inhibition of this anti-idiotypic reaction occurs not only with the homologous antibody, but also with the heterologous sheep 26 anti-Val populations. The level of inhibition by the sheep 26 anti-Val was much lower than that by the homologous goat 6 anti-Val, again emphasizing that they are not identical. There is no inhibition by normal sheep gamma globulin.

Guinea pig anti-Val was isolated in the same manner as goat and sheep anti-Val and used in these inhibition studies. The results of one such experiment are shown in Fig. 5. Guinea pig anti-Val demonstrates a very weak, if any, cross-reaction with the Ra 326 serum, even though it has the same specificity as the goat and sheep anti-Val (i.e., reacts with HbS and not HbA). This anti-Val, from a distantly related species, does not contain a demonstrable cross-reactive idiotype.

Reciprocal experiments using rabbits immunized with sheep 26 anti-Val were also performed. The results of one such experiment using anti-idiotypic serum from Ra 585, specific for sheep 26 anti-Val, are shown in Fig. 6. Inhibition experiments were performed using purified antibodies to inhibit the binding of $^{131}$I-labeled sheep 26 anti-Val to the Ra 585 anti-idiotypic serum. As expected, the homologous sheep anti-Val inhibits the reaction quite strongly. A heterologous sheep anti-Val from another sheep, sheep 27, also inhibits this anti-

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FIG. 4. Inhibition of the binding of $^{131}$I-labeled goat 6 anti-Val to Ra 326 anti-idiotypic serum by: (●—●), goat 6 anti-Val; (□—□), sheep 26 anti-Val; and (○—○), normal sheep gamma globulin.

FIG. 5. Inhibition of $^{131}$I-labeled goat 6 anti-Val binding to Ra 326 serum by: (○—○ and ●—●), goat 6 anti-Val; (■—■), guinea pig anti-Val; and (□—□), normal goat gamma globulin.
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**FIG. 6.** Inhibition of $^{131}$I-labeled sheep 26 anti-Val binding to Ra 585 serum by: (○—○), sheep 26 anti-Val; (□—□), sheep 27; (△—△), goat 6 anti-Val; and (▲—▲), guinea pig anti-Val. (●—●), normal sheep gamma globulin.

The idiotypic reaction quite strongly but not to the same extent as the homologous sheep 26 anti-Val. It has previously been shown that the anti-Val from different individual goats does not show identical inhibition patterns in experiments such as these (11). The other antibody population that strongly inhibits this reaction is goat 6 anti-Val, demonstrating once again the idiotypic cross-reactivity of relatively homogeneous antibodies with the same specificity from two closely related species. Guinea pig anti-Val was also tested and did not inhibit this anti-idiotype reaction.

**Inhibition of the Anti-Idiotype Reaction by Hemoglobin.** HbS and HbA1 were used as inhibitors of the anti-idiotype reaction as is described in the Materials and Methods. The results of the inhibition of the reaction between HbS and goat 6 anti-Val by the anti-idiotype serum from Ra 326 which is specific for goat 6 anti-Val were reported by Schreiber and Reichlin (12). The antigen, HbS, could only block 40% of the anti-idiotype reaction. We have found that the anti-idiotype reaction between Ra 585 serum and sheep 26 anti-Val is inhibited 26% by HbS. HbA1 did not inhibit this reaction. These results indicate that the idiotype is defined to some extent by antigenic sites on anti-Val which lie outside the HbS-binding region.

**Inhibition of the HbS Anti-Val Reaction by Anti-Idiotype Sera.** Since HbS can partially block the reaction of an anti-Val population with its anti-idiotype serum a portion of the idiotypic determinants must include regions within the HbS-binding site on anti-Val. Therefore, the anti-Val HbS reaction should be inhabitable by anti-idiotype. Furthermore, a comparison of cross-inhibitions offers a means of assessing the similarities of the antigen-binding sites on the anti-Val antibodies from different species.

The anti-Val being studied was mixed with various dilutions of an anti-idiotype serum and incubated at 4°C for 10 min. The mixtures were then tested for their ability to bind $^{131}$I-labeled HbS (see Materials and Methods). The results are shown in Fig. 7. To demonstrate that the system was truly idiotypic, antisera directed against antigens other than hemoglobin were used. The two antisera were goat 4 anti-human cytochrome c and sheep 193 anti-human myoglobin. The anti-idiotype sera were unable to significantly inhibit antigen binding to these antisera. The inhibition levels noted in these experiments were attributed to nonspecific binding. The homologous anti-idiotype antibody, Ra...
326, inhibits antigen binding to goat 6 anti-Val very strongly, to nearly 100% inhibition. The heterologous anti-idiotypic serum, Ra 585, also inhibits the antigen binding of goat 6 anti-Val quite strongly. Normal rabbit serum does not significantly inhibit this binding reaction. Similar results were obtained using sheep 26 anti-Val. The homologous anti-idiotypic antibody, in this case Ra 585, inhibited the binding of antigen to sheep anti-Val quite strongly. The Ra 326 antiserum also inhibits the antigen binding to this anti-Val. However, the effect seems to dilute out much more quickly. Anti-Val produced in guinea pigs was also used in these experiments. Neither Ra 326 nor Ra 585 anti-idiotypic sera could inhibit the binding of 131I-labeled HbS to the guinea pig anti-Val (Fig. 7).

Discussion

The results which have been presented, clearly establish the occurrence of interspecies idiotypic cross-reactivity between antibody populations of identical specificity, anti-Val, from both sheep and goats. This cross-reactivity is dramatically observed in double-diffusion Ouchterlony experiments in which sheep and goat antisera containing anti-Val antibodies form precipitin lines of identity with the anti-idiotypic sera directed towards either sheep or goat anti-Val populations.

Quantitative studies of this cross-reactivity, using the radioimmunoassay, reveal that this identity is only apparent. In every case the inhibition of the binding of radiolabeled homologous idiotype required much less cold homologous idiotype than heterologous idiotype. This difference in inhibitory effectiveness was approximately 100-fold. There are two ways in which such a difference can be explained. It might be that the cross-reacting idiotype represents a very small fraction of the heterologous anti-Val population. Alternatively, the heterologous anti-Val might bind to the anti-idiotypic antibodies with a much lower affinity than does the homologous anti-Val. The results presented in Fig. 7 strongly favor the latter explanation. The reaction of goat 6 anti-Val with HbS can be more than half inhibited by rabbit anti-sheep anti-Val. Likewise, well over half of the reaction of HbS with sheep 26 anti-Val can be inhibited by rabbit
anti-goat anti-Val. In addition, the occurrence of clear precipitin lines by the reaction of the anti-idiotypic sera with their heterologous idiotypes in our Ouchterlony experiments is inconsistent with there being a 100-fold reduction in the effective idiotype concentration in these sera. Thus we must conclude that these anti-Val populations are not identical but that they cross-react idiotypically only with substantially reduced affinities for the anti-idiotypic sera.

It is important to note that the observed idiotyp and idiotypic cross-reactivity involves the major fraction of not only the anti-Val, but also the anti-idiotypic antibodies. Both of the anti-idiotypic sera react with virtually all of the antibodies which make up the anti-Val population against which they were elicited. Even when we limit our consideration to the anti-idiotypic antibodies which block the antigen-binding sites on the anti-Val antibodies, we find that these anti-idiotypic antibodies can block the reaction of HbS with about 90% of their homologous anti-Val populations. In addition, for both anti-idiotypic sera the majority of the reaction with the labeled homologous anti-Val population can be inhibited by the heterologous anti-Val preparation. Therefore, for almost all anti-Val antibodies from goat 6 there appear to exist idiotically cross-reacting antibodies in the anti-Val population from the sheep and vice versa.

One can envision a trivial explanation for idiotypic cross-reactivity if one imagines an antigen-binding site to be a template of the antigenic site to which it is directed and to bind by virtue of having a complementary three-dimensional structure and charge profile. If this complementarity were precise then any two antibodies directed toward the same antigenic determinant would have binding sites with very similar surface morphologies even though these might be produced by very different amino acid residues and protein-folding interactions. Since it is the surface structures with which the anti-idiotypic antibodies interact directly, idiotypic cross-reactivity might indicate only similarity in specificity rather than similarity in protein structure. There are two arguments against this proposal. The first is the failure of the guinea pig anti-Val antibodies to cross-react with our idiotypic sera. Here a similar specificity fails to produce idiotypic relatedness. The second, is the ability of cross-reacting idiotypes to inhibit a larger percentage of the anti-idiotypic reaction than can be inhibited by the antigen itself. Sheep 26 anti-Val can inhibit 80% of the reaction between Ra 326 antiserum and goat 6 anti-Val while HbS inhibits only 40% of this reaction. This requires that roughly half of the idiotypic cross-reactivity results from regions on the surface of goat 6 anti-Val antibodies that are outside the HbS-binding site.

The data presented here clearly demonstrate the existence of idiotypic cross-reactivity between antibodies from two closely related species. This suggests that the genes coding for the variable regions of these antibodies may have evolved along with other genes in these species. Evidence exists which demonstrates that the variable regions of mouse anti-phosphorylcholine antibodies are specified by germ-line genes (7). The results of mating experiments, using a rabbit population with monoclonal-like antibodies to streptococcal group C carbohydrate, suggest that the idiotype is inherited as a gene product (8). These observations support the idea that genes coding for an idiotype could evolve and produce such protein structures in two closely related species.
Summary

An antibody population which reacts only with human sickle cell hemoglobin (HbS) and not with normal human hemoglobin, has been isolated from goat, sheep, and guinea pig antisera. These antibody populations termed anti-Val (Val), isolated from an individual goat (no. 6) and sheep (no. 26) have been used to elicit anti-idiotypic responses in rabbits. These anti-idiotypic sera were used to study the idiotypic cross-reactions between the goat and sheep anti-Val. Strong cross-reactions were present using either Ra anti-goat anti-Val or Ra anti-sheep anti-Val. Guinea pig anti-Val did not cross-react with these anti-idiotypic sera. Binding of HbS to the anti-Val of the goat and sheep could be blocked by the anti-idiotypic sera, but the binding of HbS to the guinea pig anti-Val could not. These data demonstrate idiotypic cross-reactivity between two closely related species.

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References

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