THE HUMAN NATURAL ANTI-Gal IgG

III. The Subtlety of Immune Tolerance in Man as Demonstrated by Crossreactivity Between Natural Anti-Gal and Anti-B Antibodies

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Immune tolerance to self antigens is an important characteristic of the immune system. This is the mechanism by which the immune system distinguishes between self and foreign antigens. Immune tolerance, according to Burnet's clonal selection theory, results from the deletion of lymphoid clones that produce antibodies that can interact with self antigens (1, 2). Elimination of these autoreactive clones occurs during an embryonic stage by an unknown mechanism. This hypothesis has been challenged by studies (3) that have shown that human sera contain natural antibodies against various proteins that are normally present in man. Guilbert, Avrameas, and their colleagues (4, 5) have shown, by the use of affinity chromatography purification methods, that normal human sera contain natural antibodies to evolutionarily conserved proteins including cytochrome c, actin, tubulin, fetuin, collagen, and even human albumin. Using the same approach, Lutz et al. (6) demonstrated antibodies in human sera to the band 3 molecule of human red cells. The occurrence of these autoantibodies suggest that self-reactive clones are not eliminated by the tolerance mechanism as proposed by Burnet (1) and Jerne (2).

Due to the low concentration of these natural antibodies in human serum (<0.5 μg/ml), and their considerable degree of crossreactivity (4, 5), these antipeptide antibodies may not serve as an appropriate system for the study of immune tolerance to self antigens. Analysis of the interaction between naturally occurring anti-carbohydrate antibodies and chemically defined carbohydrate antigens should provide a more precise system to study the effect of immune tolerance on the interaction between natural antibodies and self antigens. Binding of antibodies to glycosidic epitopes does not involve electrostatic bonds. Thus, weak crossreactivities observed with anti-protein antibodies due to partial similarities in charge arrays on various protein epitopes are avoided. Binding of the glycosidic epitope occurs within the antibody molecule rather than on the surface, providing a firm attachment of the uncharged epitope, in which Van der Walls forces and hydrogen bonding constitute the dominant binding force (7). This

This study was supported by National Institutes of Health grants AG-06299 to U. Galili, AM-25094 and AM-16095 to S. B. Shohet, and CA-32826 to B. A. Macher. Address correspondence to Dr. Uri Galili, Cancer Research Institute, M-1282, University of California, San Francisco, CA 94143-0128.

J. EXP. MED. © The Rockefeller University Press - 0022-1007/87/03/0693/12 $1.00

Volume 165 March 1987 693-704
type of interaction requires a high degree of structural fitting between the antigen and the combining site on the antibody molecule.

Human anti-carbohydrate antibodies are, like other antibodies, polyclonal in nature. The combining sites of the different clones vary in the extent of the area complementary to the antigenic epitope (8). Nevertheless, natural anti-carbohydrate antibodies display a high degree of specificity. For example, the difference in the binding specificities of anti-blood group A and B antibodies is related to the presence or absence of a N-acetyl residue on the terminal galactosyl of the A and B blood group determinant (7, 9; Table I).

We have recently described (10) another natural anti-carbohydrate antibody, anti-Gal, that also displays this same sort of narrowly defined specificity. Anti-Gal is a polyclonal antibody present in every individual and constitutes as much as 1% of the circulating IgG. Anti-Gal isolated from AB sera by affinity chromatography was found to display a distinct specificity for glycosphingolipids with a Galα1→3Gal glycosidic epitope. However, it did not bind to closely related glycosphingolipids with a Galα1→3(Fucα1→2)Gal epitope, which is the blood group B determinant (11; see illustration in Fig. 1). It can be hypothesized that the inability of anti-Gal from AB individuals to interact with the B antigen results from an effective immune tolerance mechanism. Thus, in individuals with B or AB blood type, tolerance prevents the production of anti-Gal clones capable of interacting with the fucosylated Galα1→3Gal epitope (i.e., the B antigen). If this assumption is correct, then this restrictive effect of immune tolerance should not occur in individuals lacking the blood group B antigen. Therefore, in individuals of blood type A or O (Table I) the anti-Gal reactivity should include antibody clones that can interact with both Galα1→3Gal and Galα1→3(Fucα1→2)Gal epitopes as illustrated in Fig. 1. In the present study, we examine the extent by which immune tolerance affects the spectrum of anti-Gal specificities produced in individuals of different blood groups.

Materials and Methods

Isolation of Anti-Gal from Normal Human Serum. Anti-Gal was purified from normal human serum by affinity chromatography according to a modification of the method described previously (10, 11). Heat-inactivated plasma was loaded on a column containing the immunoadsorbent Synsorb 90 (Chemiomed, Edmonton, Canada) with the glycosidic epitope Galα1→3Galβ1→4Glc-R. After extensive washing with PBS, the antibodies retained on the column were eluted by incubation with 0.5 M melibiose (α-galactosyl glucoside) for 4 h at 37°C. The carbohydrate was removed by repeated dialysis against
large volumes of PBS. The anti-Gal preparation was brought to a concentration of 100 μg/ml in PBS. This concentration corresponds to the anti-Gal concentration of normal human serum (10).

Isolation of Anti-A and Anti-B Antibodies. Sera containing anti-A or anti-B blood group antibodies were incubated for 1 h at 24°C with equal volumes of washed packed A type or B type red cells, respectively. The red cells were washed five times in 10 volumes of PBS and lysed by hypotonic shock. After three additional washes, the antibodies adsorbed on the red cell membranes were eluted with glycine-HCl buffer (pH 2.6) and brought immediately to pH 7.4 by addition of 1 N NaOH. The eluates were dialyzed against PBS and the anti-blood group reactivity was assessed by hemagglutination with the relevant red cells.

Glycosphingolipids. Globoside (Gb₄) was purchased from Supelco, Inc. (Bellefonte, PA). Human P₁ glycolipid was a gift from Drs. H. Clausen and S. Hakomori (Fred Hutchinson Cancer Research Center, Seattle, WA). B₂ glycolipid antigen was prepared from human B red cells by Folch partitioning (12) and HPLC on an Iatrobead column using a gradient of isopropanol/hexane/water (13). All other glycosphingolipids were prepared in our laboratory as previously reported (11, 14–16).

Immunostaining of Glycosphingolipids on Thin-Layer Plates. Glycosphingolipids were chromatographed on aluminum-backed Silica Gel 60 high-performance thin-layer chromatography (HPTLC) plates (E. M. Laboratories, Inc., Cincinnati, OH) in CHCl₃/MeOH/H₂O 60/35/8 vol/vol. Immunostaining was done according to a modification (17) of the method of Magnani et al. (18). The plate was dried and dipped (60 s) in a solution of 0.1% polyisobutyl methylacrylate (Polysciences, Inc., Warrington, PA) in hexane, and was air dried. The plate was soaked overnight at 5°C in PBS with 5% BSA.

1 Abbreviation used in this paper: HPTLC, high-performance thin-layer chromatography.
for 2 h in anti-Gal or purified anti-B (5 μg/ml), 1 h in biotinylated goat anti-human IgG (Vector Laboratories, Inc., Burlingame, CA), and thereafter in the Vector ABC reagent containing avidin-alkaline phosphatase, and finally in an alkaline phosphatase substrate solution (Vector Laboratories, Inc., Burlingame, CA). The plate was rinsed for 10 min in PBS between each incubation step.

Defucosylation of the B, Antigen. B, (10 μg) was treated with 0.1 M TCA for 1 h at 80°C to remove the fucose residue (19). After cooling, equal volumes of methanol and water were added to the hydrolysate and the mixture was taken to dryness. The defucosylated B, was resuspended in CHCl₃/MeOH, 1/1 (vol/vol) and analyzed by TLC immunostaining with anti-Gal.

Hemagglutination Assay. Indirect hemagglutination activity of the isolated antibodies was titrated by mixing 50 μl of twofold serial dilutions of the antibody with an equal volume of a 1% red cell suspension in V-shaped wells of a microtiter plate. After a 30-min incubation at room temperature, the red cells were washed three times by spinning the plates for 3 min at 800 g, decantation of the supernatant, and resuspension of the pelleted red cells. Thereafter, the red cells were resuspended in 50 μl rabbit anti-human IgG antibodies (Dako, Copenhagen, Denmark). Agglutination was evaluated after the red cells had settled at room temperature for 2 h. Titers were expressed as the greatest dilution of antibody solution that caused complete agglutination.

Removal of Anti-Gal Reactivity from Human Serum. The proportion of anti-B antibodies that have a crossreacting activity with anti-Gal was determined by analyzing anti-B activity in sera depleted of anti-Gal. Adsorption of anti-Gal was performed by two methods: (a) Adsorption of human A and O sera on rabbit red cells. These red cells lack the B antigen, as indicated by the capacity of rabbits to produce anti-B antibodies (20), but express an abundance of Galα1→3Gal epitopes (21). Normal heat-inactivated A and O type sera were mixed with an equal volume of packed rabbit red cells and incubated for 30 min at room temperature. The red cells were removed by centrifugation and the procedure was repeated three times. Thereafter, the absorbed sera were assayed for anti-B reactivity by hemagglutination assay with human B red cells. (b) Adsorption of human A and O sera on a Galα1→3Galβ1→4Glc-R immunoadsorbent column was used to remove anti-Gal (Synsorb 90, ChemibioMed, Edmonton, Canada). This procedure was repeated three times. The anti-B reactivity remaining in the effluent after this procedure was assessed by the hemagglutination assay.

Results

Demonstration of the Specificity of the Natural Anti-Gal Antibodies Isolated from AB Sera. In our previous study (10), we showed that anti-Gal isolated from normal AB sera displays a distinct specificity for the Galα1→3Gal residue of the rabbit CPH molecule, and not for the Galα1→4Gal residue of the rabbit CTH molecule. To further demonstrate the restricted specificity of this natural antibody a number of reference glycosphingolipids, listed in Table II, were studied for anti-Gal binding. The interaction of anti-Gal with these compounds was analyzed by immunostaining on TLC plates. In addition to the interaction with rabbit CPH (Fig. 2, lane 2), anti-Gal bound to bovine CPH and CHH (Table II), all of which have previously been shown to have terminal Galα1→3Gal residues (21–23). Removal of the terminal galactosyl units by treatment with α-galactosidase abolished the interaction with anti-Gal (not shown). Glycosphingolipids with terminal Galα1→4Gal residues such as rabbit CTH and human P₃ antigen, or glycosphingolipids with terminal Galβ1→4GlcNAc, or nongalactosyl terminal carbohydrate residues, were not immunostained by anti-Gal (Table II). Furthermore, the presence of a branching fucose on the penultimate galactose residue of Galα1→3Gal (i.e., B₄ antigen) prevents anti-Gal binding (Fig. 2, lane 5). The
TABLE II
Glycosphingolipids Tested for Anti-Gal Binding

| Name                  | Source     | Structure                                      | Anti-Gal binding |
|-----------------------|------------|------------------------------------------------|------------------|
| CPH (ceramide pentahexoside) | Rabbit     | Galα1→3Galβ1→4GlcNAcβ1→4Galβ1→4Glc1→1Cer      | +                |
| CHH (ceramide heptahexoside) | Cow        | Galα1→3Galβ1→4GlcNAcβ1→4Galβ1→4Glc1→1Cer      | +                |
| Glucosyl ceramide     | Man        | Glc1→1Cer                                     | -                |
| Lacotyl ceramide      | Man        | Galβ1→4Glc1→1Cer                               | -                |
| CTH (ceramide trihexoside) | Rabbit    | Galα1→4Galβ1→4Glc1→1Cer                        | -                |
| Globoside             | Man/pig    | GalNAcβ1→4Galα1→4Galβ1→4Glc1→1Cer             | -                |
| Paragloboside         | Man        | Galβ1→4GlcNAcβ1→5Galβ1→4Glc1→1Cer             | -                |
| Forsman               | Sheep      | GalNAcα1→3Galα1→4Galβ1→4Glc1→1Cer             | -                |
| P1                    | Man        | Galα1→4Galβ1→4GlcNAcβ1→5Galβ1→4Glc1→1Cer      | -                |
| B1                    | Man        | Galα1→3(Fucα1→2)Galβ1→4GlcNAcβ1→5Galβ1→4Glc1→1Cer | -            |
| GM3                   | Man        | NeuAca2→8Galβ1→4Glc1→1Cer                      | -                |
| Sialylparagloboside   | Man        | NeuAca2→3Galβ1→4GlcNAcβ1→5Galβ1→4Glc1→1Cer    | -                |

B antigenicity of this glycosphingolipid was demonstrated by immunostaining with isolated human anti-B antibody (Fig. 2, lane 4). Removal of the branching fucosyl by mild acid treatment resulted in the comigration of the B1 antigen derivative with CPH (Fig. 2, lane 6), and the immunostaining of this derivative by anti-Gal (Fig. 2, lane 7).

Differences in the Specificity of anti-Gal Isolated from the Serum of Individuals of Various Blood Types. As shown in the previous section, the ability of anti-Gal from AB serum to distinguish between the Galα1→3Gal epitope and the B epitope was not surprising, since AB individuals lack any antibodies capable of interacting with the B antigen as a result of immune tolerance (Table I). However, we would expect A and O blood type individuals who lack the B antigen to have anti-Gal clones interacting exclusively with the Galα1→3Gal structure, and in addition, anti-Gal clones interacting with the Galα1→3Gal structure regardless
Table III

Hemagglutinating Reactivity* of anti-Gal Isolated from Individuals of Various Blood Types$

| Serum from blood type | Hemagglutination titer |
|-----------------------|------------------------|
|                       | Rabbit RBC             | A type RBC§ | B type RBC§ |
| A                     | 1:1,024                | -           | 1:16        |
| B                     | 1:512                  | -           | -           |
| AB                    | 1:1,024                | -           | -           |
| O                     | 1:512                  | 1:4         | 1:32        |

* Indirect hemagglutination using rabbit anti-human IgG as a second antibody.

§ Representative data of experiments performed with three to four individuals of each blood type.

of whether a branching fucose is present or not (see illustration, Fig. 1). The latter anti-Gal clones would thus immunostain both CPH and B antigen. To test this hypothesis, anti-Gal was isolated from the serum of A type individuals by the same method used for purifying anti-Gal from AB type donors (i.e., Galα1→3Gal immunoabsorbent affinity chromatography). Like anti-Gal from AB serum (Fig. 2, lane 2), anti-Gal from A serum readily bound to rabbit CPH and to rabbit glycosphingolipids with longer carbohydrate chains near the origin of the TLC plate (Fig. 2, lane 8). These are neutral glycosphingolipids with long complex carbohydrate chains (15–25 carbohydrate units) terminating in Galα1→3Gal residues (24). However, unlike anti-Gal from AB serum, the anti-Gal from A serum readily bound to the B antigen (Fig. 2, lane 9). These findings imply that anti-Gal in A serum has a broader spectrum of specificity than anti-Gal in AB serum. The anti-Gal in A serum consists of antibodies that recognize not only Galα1→3Gal, but also Galα1→3(Fucα1→2)Gal epitopes (see Fig. 1).

This conclusion was further supported by hemagglutination studies. Hemagglutination of B type red cells, which express Galα1→3(Fucα1→2)Gal, and rabbit red cells with Galα1→3Gal epitopes, was studied using anti-Gal prepared from individuals of various blood types (Table III). It should be stressed that rabbit red cells do not express the B antigenic epitope. As previously observed (10), anti-Gal from all sera agglutinated rabbit red cells at a titer of 1:512 to 1:1,024. However, anti-Gal from A and O serum also agglutinated B type red cells, whereas anti-Gal from B or AB serum had no such reactivity. The A type red cells, used as control, were not agglutinated by anti-Gal from A, B, or AB sera. A low hemagglutination titer of A red cells was observed with anti-Gal from O serum. This finding suggests that anti-Gal produced in individuals lacking both the A and B antigenic epitopes has a broader spectrum of specificity than anti-Gal produced in individuals of other blood types. Thus, anti-Gal from O type serum, which bound primarily to the Galα1→3Gal epitope on the immunoabsorbent, contained antibody clones capable of interacting with the A and B antigenic epitopes.

Anti-Gal Reactivity within Anti-B Antibodies. The results presented in the previous section suggest that the anti-Gal antibodies of A and O blood type individuals contain anti-B activity. To further demonstrate the overlap between
anti-Gal and anti-B reactivities in these individuals, purified anti-B was analyzed for anti-Gal activity. Anti-B antibodies were purified from A serum by adsorption on and elution from B-type red cells. These anti-B antibodies were assayed for anti-Gal activity by immunostaining glycosphingolipids with Ga\(\alpha\)1→3Gal epitopes. In addition to their reactivity with the B\(_1\) antigen (Fig. 3, lane 3), these antibodies readily bound to bovine and rabbit CPH (Fig. 3, lanes 1 and 2), and to bovine CHH (Fig. 3, lane 1), all of which have terminal Ga\(\alpha\)1→3Gal residue (21–23). The presence of anti-Gal reactivity within the purified anti-B antibodies was also demonstrated in hemagglutination studies (Table IV). The anti-B eluates agglutinated both B type red cells and rabbit red cells. Control experiments with anti-A antibodies eluted from A red cells demonstrated a lack of agglutinating reactivity with rabbit red cells. These results demonstrate that purified anti-B antibodies also contain anti-Gal reactivity.

It was of interest to determine the proportion of anti-B antibodies with anti-Gal reactivity versus that of pure anti-B [i.e., reacting exclusively with the Ga\(\alpha\)1→3(Fuc\(\alpha\)1→2)Gal epitope]. For this purpose, sera of A and O type individuals that contain anti-B reactivity (Table I) were adsorbed three times on equal volumes of rabbit red cells to remove anti-Gal antibodies, then assayed by
TABLE V
Decrease in Anti-B Reactivity After Adsorption of Anti-Gal

Discussion
The results presented in the current study suggest that the spectrum of specificities of a natural antibody produced against a given antigen is strictly determined by the tolerance mechanism. This mechanism allows for the production of antibody clones with specificities toward a variety of structures other than self antigens. Anti-Gal is produced in every individual and is defined as an antibody that interacts with Galα1→3Gal epitopes. However, the spectrum of anti-Gal specificity is dependent on the individual's blood type. Thus, B and AB type individuals produce anti-Gal antibodies that only bind to Galα1→3Gal epitopes; whereas A or O type individuals who lack the B epitope on their red cells have clones of anti-Gal that bind to both Galα1→3Gal and the B antigen. This was shown by immunochemical and hemagglutination analysis. Moreover, adsorption experiments to remove anti-Gal indicated that the majority of the antibodies designated anti-B are in fact anti-Gal clones that can also interact with the Galα1→3(Fucα1→2)Gal epitope. Only a small proportion of anti-B antibodies exclusively recognize the Galα1→3(Fucα1→2)Gal epitope (pure anti-B illustrated in Fig. 1). Thus, in contrast to the general view that anti-B antibodies are immunoglobulin molecules recognizing the Galα1→3(Fucα1→2)Gal epitope, our data indicate that anti-B is a heterogeneous antibody population that consists mostly of antibodies recognizing both the B antigenic determinant and the Galα1→3Gal epitope, and of a relatively small proportion of antibodies that
exclusively bind to the Galα1→3(Fucα1→2)Gal epitope. The observation that anti-B sera contain antibodies that interact with a structure that is distinct from the B antigen but closely resembles it (B-like), was reported 60 yr ago by Landsteiner and Philip-Miller (20, 25), and more recently by Owen (26). These investigators made two apparently contradictory observations: (a) Rabbit red cells can adsorb a large proportion, but not all, of the anti-B reactivity found in A serum; and (b) that rabbits could produce anti-B antibodies after immunization with B red cells. Thus, the antigen on rabbit red cells interacting with the anti-B was designated a B-like antigen (26). Our data suggest that the B-like antigen is the Galα1→3Gal epitope, which is abundantly expressed on rabbit red cells and interacts with the anti-B antibodies that overlap with anti-Gal.

Another interesting observation by Landsteiner and Philip-Miller (25) was that the B-like antigen is expressed on red cells of New World monkeys, but not of Old World monkeys or apes. This has been confirmed by others (27–29) who showed that New World monkey red cells, like rabbit red cells, express a B-like antigen that can adsorb only part of human anti-B reactivity. Nevertheless, these primates could produce antibodies to human B red cells. In a recent study (30, 31), we found that anti-Gal from AB sera interacts with red cells of New World monkeys, but not with red cells of Old World monkeys or apes. Furthermore, by immunostaining with anti-Gal, we demonstrated that New World monkey red cells contain CPH with a Galα1→3Gal epitope, similar to that found in rabbit and bovine red cells. These findings suggest that the Galα1→3Gal epitope is the B-like antigenic determinant found in various mammals, including New World monkeys.

The finding that anti-Gal and anti-B have overlapping reactivities suggests that various clones of anti-Gal display different degrees of fitting to the Galα1→3Gal structure (Fig. 1). This observation is in line with the elegant studies of Kabat (8), who demonstrated the occurrence of clones of human anti-dextran antibodies with combining sites of various sizes. Our study shows that a proportion of anti-Gal antibody clones crossreact with the B antigenic determinant. These clones only exist in individuals in whom the B antigen is absent. In an individual expressing the B antigen, such overlapping clones are absent due to immune tolerance. The complete absence of anti-Gal clones capable of reacting with self blood group B antigen suggests that the immune tolerance mechanism exerts a powerful effect, preventing the generation of antibodies to self antigens. Related to this study are our recent observations on the contribution of anti-Gal to the immune-mediated destruction of senescent and pathologic human red cells (10, 32–34). These studies have suggested that Galα1→3Gal epitopes are present on human red cells in small amounts, and in a cryptic form. During the aging of red cells in circulation, these epitopes become exposed de novo, which results in anti-Gal binding to the red cells and their subsequent destruction. The occurrence of large amounts of anti-Gal, and the presence of its corresponding antigen in a cryptic form suggests that immune tolerance is only effective with noncryptic self antigens. Thus, the demonstration of natural antibodies to antigenic structures that are normally compartmentalized and not exposed to the immune system (i.e., intracellular antigens or antigens on brain tissues) may not contradict the hypothesis that differentiation between self and nonself antigens is a result
of immune tolerance. Elucidation of the mode by which this tolerance mechanism functions, using precise immunochemical approaches such as described in the present study, will greatly enhance the understanding of the basic processes in the ontogeny of the immune system.

Summary

A well-defined antigen/antibody system was used to evaluate the effect of immune tolerance on the spectrum of specificities of natural antibodies. The antibody used in this study, anti-Gal, is a naturally occurring, polyclonal IgG that constitutes 1% of the circulating IgG in humans. We have previously shown that anti-Gal, purified from AB sera, specifically interacts with glycosphingolipids bearing a \( \text{Gal}a1\rightarrow3\text{Gal} \) epitope, but not with the closely related B antigen in which the penultimate galactose of the \( \text{Gal}a1\rightarrow3\text{Gal} \) epitope is fucosylated \( \text{Gal}a1\rightarrow3(\text{Fuc}a1\rightarrow2)\text{Gal} \). This narrow specificity was assumed to be the result of an effective immune tolerance mechanism that prevents the expression of antibody clones that can recognize both the \( \text{Gal}a1\rightarrow3\text{Gal} \) and the self B epitopes. If the assumption that immune tolerance determines the range of anti-Gal specificity is correct, then anti-Gal from individuals lacking the B antigen (A and O blood types) would be expected to interact with both \( \text{Gal}a1\rightarrow3\text{Gal} \) and \( \text{Gal}a1\rightarrow3(\text{Fuc}a1\rightarrow2)\text{Gal} \) epitopes. In this study, anti-Gal from the serum of individuals of various blood types was purified by affinity chromatography on \( \text{Gal}a1\rightarrow3\text{Gal} \) adsorbent and tested for its reaction with the B antigen. Whereas anti-Gal from AB and B individuals only reacted with \( \text{Gal}a1\rightarrow3\text{Gal} \) epitopes, anti-Gal from A and O individuals reacted with both \( \text{Gal}a1\rightarrow3\text{Gal} \) and B epitopes. Furthermore, it was determined that the majority of anti-B reactivity in A and O individuals is in fact anti-Gal antibodies capable of recognizing both \( \text{Gal}a1\rightarrow3\text{Gal} \) and B epitopes. It can be concluded from these results that immune tolerance accurately controls the spectrum of natural antibody specificities by preventing the production of antibody clones that can interact with self antigens.

Received for publication 13 November 1986.

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