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Association of Bacterial Carbohydrate-Specific Cold Agglutinin Antibody Production with Immunization by Group C, Group B Type III, and *Streptococcus pneumoniae* Type XIV Streptococcal Vaccines

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Rabbits immunized with group B type III, group C, and *Streptococcus pneumoniae* type XIV streptococcal vaccines developed autoantibodies reactive with autologous and isologous erythrocytes and human O-positive erythrocytes at reduced temperatures. The cold agglutinin antibodies were present in both the immunoglobulin M (IgM) and IgG fractions of group C streptococcal antiserum and in the IgM fraction of group B type III and *S. pneumoniae* type XIV antisera. BALB/c, CF1, and local strains of mice immunized with group B type III and *S. pneumoniae* type XIV streptococcal vaccines also produced a cold agglutinin antibody reactive with rabbit and human erythrocytes. The cold agglutinin antibodies were reactive with saccharide compounds representative of the determinants present on the individual bacterial carbohydrate structures, individual vaccine preparations, and isolated polysaccharides. The group C antibodies in rabbits were reactive with sugar ligands in the following order: *N*-acetylgalactosamine > melibiose > lactose > galactose > glucose. Group B type III and *S. pneumoniae* type XIV cold agglutinin antibodies in rabbit antisera, however, displayed reactivities different from group C antibodies and from each other. Group B type III antibodies reacted with galactose > lactose > *N*-acetylgalactosamine > glucose > rhamnose; *S. pneumoniae* type XIV antibodies reacted with lactose > melibiose > galactose > glucose > *N*-acetylgalactosamine. The same relative ligand specificity was observed for the cold agglutinin antibodies in *S. pneumoniae* type XIV mouse antisera. The cold agglutinin antibodies in group B type III and *S. pneumoniae* type XIV antiserum reacted with erythrocytes at higher temperatures (up to 31°C) than did group C antibodies (up to 14°C). In addition, *S. pneumoniae* type XIV antibodies did not discriminate between I- or i-bearing human erythrocytes to a significant extent. The results obtained provide substantial evidence that autoreactive cold agglutinin antibodies produced by immunization with these vaccines represent subpopulations of bacterial carbohydrate-specific antibodies that cross-react with mammalian carbohydrate structures.

The association of autoreactive erythrocyte antibodies with infectious disease (13) and animal immunization (7, 12) has been of interest for many years. The majority of these antibodies react with human and rabbit erythrocytes at temperatures below 37°C and are consequently classified under the general category of cold agglutinin antibodies. The characteristic erythrocyte reactivity of these antibodies correlates with the reactivity of certain human monoclonal (usually Waldenström) immunoglobulins, and therefore is thought to be associated with Ii region blood group carbohydrate structures (15). The i antigenic determinants recognized by human anti-i antibodies represent structures present on a linear oligosaccharide with repeating galactose (β-1-4) *N*-acetylglucosamine (β-1-3) units; human anti-I antibodies react, depending upon their individual specificity, with regions of a branched oligosaccharide structure formed by addition of galactose (β-1-4) *N*-acetylglucosamine (β-1-6) branches to the i-active region (4). Blood group-bearing glycoproteins, in addition to the core oligosaccharide structures comprising the Ii regions, may also present serine or threonine residues (or both) substituted with *N*-

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acetylgalactosamine (GalNAc) via O-serine or O-threonine glycosidic linkages (or both).

The appearance of these antibodies during infection or immunization (or both) may be a result of formation of an immunogenic erythrocyte, a nonspecific polyclonal response due to infection or immunization conditions, or a cross-reactive antibody population generated in direct response to the infectious or immunizing agent. Recently, it was shown that rabbits hyperimmunized with group C streptococcal vaccine produced cold agglutinin antibodies (6). The apparent ligand specificity of these antibodies, GalNAc, strongly suggested a bacterial cell wall carbohydrate structure stimulus which led to production of an erythrocyte cross-reactive antibody population. Given the development of bacterial vaccines for human use, the potential for immunization to lead to autoreactivity of this type is of obvious concern.

The present study was designed to investigate the relationship between the appearance of erythrocyte-autoreactive antibodies and the vaccine used for immunization. The rationale involved utilized the carbohydrate structural characteristics of the individual vaccines and correlation of these determinants with the relative sugar ligand specificity demonstrated for any cold agglutinin antibodies that appeared as a result of immunization of both rabbits and mice. The following vaccines were used: group C, B, and *Streptococcus pneumoniae* type XIV streptococcal heat-killed cells. The results of this study revealed that group B-, C-, and *S. pneumoniae* type XIV-immune rabbits each produced cold agglutinin antibodies of different relative ligand specificity. Further, mice immunized with *S. pneumoniae* type XIV vaccine produced cold agglutinin antibodies of similar ligand specificity as those produced in rabbits. The data support the contention that certain antibodies produced as a consequence of bacterial challenge can cross-react with mammalian erythrocyte determinants. Such reactivity therefore could result in an autoimmune response.

MATERIALS AND METHODS

Preparation of vaccines and immunization protocol. Group C strain C-74 vaccine was prepared as previously described (6). Group B type III (typing of the group B organisms was kindly performed by C. J. Baker, Baylor College of Medicine, Houston, Tex.) and *S. pneumoniae* type XIV vaccines were prepared by growing the bacteria in Todd-Hewitt broth at 37°C for 18 to 24 h. The cells were pelleted by centrifugation and washed three times with sterile saline. The cells were suspended in 30 ml of sterile saline and heat killed by incubation at 70°C for 30 min. After the killed vaccine was washed four times with sterile saline, the cells were suspended to an optical density of 11.5 at 450 nm and then frozen until use. New Zealand White rabbits were injected intravenously three times per week over a 4- to 6-week period, rested for 2 to 3 months, and injected as before. The vaccine dosage was increased by 0.25-ml increments beginning with 0.25 ml the first week to 1.0 ml the last week of immunization. Preinoculation and weekly bleedings were taken via the central ear artery. The serum was routinely prepared at 37°C and tested immediately. The serum samples were stored frozen at −20°C until further use. BALB/c, CF-1, and local outbred agouti-colored mice were immunized with *S. pneumoniae* type XIV streptococcal vaccine intravenously via a tail vein. The immunization protocol followed that given the rabbits. The bleeding schedule followed that of the rabbits and was performed via the retroorbital plexus.

Antibacterial response. Vaccine antibody responses were measured by vaccine agglutination as described by Herd and Spragg (8).

Erythrocyte preparations. Rabbits were bled from the central ear artery into Alsever solution. Sheep and bovine (ox) erythrocytes were obtained from Colorado Serum Co., Denver, Colo. Whole human O-positive blood was provided by Lawrence Memorial Hospital as outdated preparations. In addition, a blood typing panel was provided by Lawrence Memorial Hospital. Each erythrocyte sample was washed three times and suspended to 1% (vol/vol) in 0.02 M phosphate–0.15 M NaCl buffer (pH 7.2).

Hemagglutination assays. Agglutination activity in rabbit immune sera against autologous and isologous rabbit erythrocytes was assayed by adding an equal volume of erythrocytes (0.3%, vol/vol) to twofold 50-μl dilutions of antisera in microtiter plate wells and incubating the reaction mixtures at 4°C or 37°C (or both). The cold agglutinin antibody titer was recorded as the reciprocal of the highest dilution of antiserum that yielded a positive reaction. No significant difference in agglutination was observed when immune sera were tested against various rabbit isologous erythrocyte preparations. Heterologous human (types A, B, OI, and Oi) sheep, and bovine (ox) erythrocytes were examined in the presence of whole serum and purified cold agglutinin antibody, either as described above or in direct slide agglutination assay. Mouse immune sera were similarly tested against rabbit, human, bovine (ox), and mouse erythrocytes.

Cold hemagglutination inhibition assays. Antiserum preparations (25 μl) were diluted twofold in V-bottom microtiter plate wells. To these wells was added 50 μl of a 0.3% (vol/vol) suspension of rabbit erythrocytes and 25 μl of 0.02 M Tris HCl–0.15 M NaCl–0.1% (wt/vol) gelatin buffer, pH 8.0 (Tris-gelatin buffer). After incubation for 4 h at 4°C, hemagglutination was assessed on a scale of 4+ (highest) to + (lowest). The antiserum dilution which yielded a 2+ reaction was subsequently used in inhibition assays. Individual carbohydrate solutions were prepared at 2 mg/ml in Tris-gelatin buffer. A 25-μl (representing 50 μg) sample of carbohydrate solution was diluted twofold, and 25 μl of the appropriately diluted antiserum was added to each well. The microtiter plates were incubated 1 h at room temperature, and 50 μl of a 0.3% (vol/vol) suspension of rabbit erythrocytes was added. After incubation at 4°C for 4 h, hemagglutination was measured. The lowest amount of inhibitor that completely

inhibited agglutination of the erythrocytes was recorded.

Cold hemagglutinin antibody purification. Antibodies reactive with erythrocytes at reduced temperature were purified as previously described (6). Cold agglutinin antibodies were purified from group C and *S. pneumoniae* type XIV antisera; however, recovery of these antibodies from group B antisera was poor. Consequently, subsequent examination of antibody activity in group B antisera utilized the exclusion volume (void volume fraction) of Sephacryl S-200 fractionated whole group B antisera. Group C and *S. pneumoniae* type XIV antisera were prepared at 37°C. After the addition of antisera to an equal volume of packed rabbit erythrocytes at 37°C, the suspension was incubated for 1 h at 4°C and centrifuged at 800 × *g* for 15 min. After centrifugation, the supernatant volume was removed, and an equal volume of 0.02 M phosphate–0.15 M NaCl buffer (pH 7.2) was added. The suspension was incubated at 37°C for 1 h and at 0°C for 1 h and centrifuged at 4°C, and the supernatant volume was discarded. These procedures were repeated three times. The material that eluted after the final 37°C incubation step was concentrated by ultrafiltration to the original serum volume and fractionated on a 2.4- by 100-cm Sephacryl S-200 column equilibrated and eluted in the presence of 0.02 M Tris-hydrochloride–0.5 M NaCl–0.02% (wt/vol) sodium azide buffer (pH 8.0). Individual fractions were assayed for cold agglutinin antibody activity, pooled, and concentrated. Pooled fractions were examined by sodium dodecyl sulfate-agarose-polyacrylamide gel electrophoresis and by immunodiffusion analysis against goat anti-rabbit μ chain- and goat anti-rabbit γ chain-specific antisera. By these criteria, group C antisera yielded purified cold agglutinin antibody activity in both the immunoglobulin M (IgM) and IgG immunoglobulin classes. No IgG antibody was detected in the IgM fraction, and no IgM monomers were detected in the IgG fraction. Only IgM immunoglobulin and cold agglutinin antibody activity was detected in the material purified from *S. pneumoniae* type XIV antisera. Attempts to purify these antibodies from group B antisera were unsuccessful. In further studies, only the purified IgM cold agglutinin antibodies, or the IgM-containing serum fractions were used unless otherwise noted.

Temperature dependence of erythrocyte reactivity. The precise temperature requirements for cold agglutinin antibody interaction with rabbit erythrocytes were evaluated by analyzing twofold serial dilutions of whole group C and group B type III and *S. pneumoniae* type XIV streptococcal antisera for cold agglutinin antibody activity at 4°C. The last dilution of each antiserum which gave a 4+ agglutination reaction was used in subsequent assays. Samples (50 μl) of the antiserum dilution from each of the respective antisera were placed in duplicate microtiter wells in seven different microtiter plates. After the addition of 50 μl of 1% (vol/vol) rabbit erythrocytes to each well, one plate was incubated at each of the following temperatures; 5, 10, 15, 20, 25, 30, and 37°C for 90 min. The wells were scored for their agglutination reaction from 0 to 4+.

Hemolysis assays. Cold agglutinin antibody-dependent, complement-mediated hemolysis was examined in a biphasic temperature hemolysis assay similar to that of Lau and Rosse (11), with purified antibodies and whole or fractionated antisera. Before the assay, all purified antibody preparations were equilibrated by dialysis against 0.02 M Tris-hydrochloride–0.128 M NaCl–0.5 mM Mg^{2+}–0.15 mM Ca^{2+}–0.1% (wt/vol) bovine serum albumin (μ = 0.15, pH 7.4). The material was diluted twofold, and excess guinea pig complement was added, followed by the addition of rabbit erythrocytes to a final 0.25% (vol/vol) suspension. Solutions were incubated for 1 h at 0°C and 1 h at 37°C. Fourfold the lowest amount of test agent required for 100% hemolysis was subsequently determined and used for erythrocyte sensitization in complement titration assays. Guinea pig complement was titrated under these conditions to provide a 50% lysis endpoint. From these data, the amount of complement required to provide 4 50% lysis endpoint units was calculated. Subsequently, purified antibody or whole or fractionated antisera were diluted twofold, 4 50% lysis endpoint complement units was added in the presence of a 0.25% (vol/vol) suspension of rabbit erythrocytes, and a 50% lysis endpoint was determined. In hemolytic inhibition assays, the amount of antibody or whole or fractionated antisera that yielded 50% hemolysis was used for erythrocyte sensitization. The extent of hemolysis was determined by absorbance measurements at 413 nm of supernatant volumes.

Hemolysis inhibition studies. Solutions of individual saccharide compounds were prepared at initial concentrations at 50 mM and diluted twofold. Carbohydrate solutions were prepared at initial concentrations of 100 μg/ml; these solutions were diluted twofold to approximately 1.0 ng/ml. To inhibitor solutions were added an amount of purified antibody or whole or fractionated antiserum, complement, and erythrocytes to a final volume of 1.5 ml. After incubation at 0°C for 1 h and 37°C for 1 h, the extent of hemolysis was determined. Control solutions included those prepared in the absence of inhibitor, the absence of complement, and the absence of antibody or whole or fractionated antiserum. In addition to these controls, the microcomplement fixation test (14), which utilized either sheep or bovine (ox) erythrocytes, was used to determine the extent of anti-complementarity associated with solutions of inhibitor or antibody.

Radioactively labeled antibody studies. To assess the specificity of purified cold agglutinin antibodies isolated from *S. pneumoniae* type XIV antisera, these antibodies were trace labeled by using the Enzymobead reagent (Bio-Rad Laboratories, Richmond, Calif.) to a specific activity of 100 cpm/ng. These antibodies were subsequently reacted with *S. pneumoniae* type XIV vaccine to assess specific binding and group C and micrococcal vaccines to assess nonspecific reactions. Approximately 110 ng of ^{125}I-labeled antibody was added to bovine serum albumin-coated tubes containing 200 μl of a 1:10 dilution of vaccine (absorbance at 450 nm, 1.15). After incubation for 2 h at room temperature, the suspensions were centrifuged, the supernatant volume was discarded, and the pellet was suspended and washed once with 0.02 M phosphate–0.15 M NaCl buffer made 2% (wt/vol) in bovine serum albumin (pH 7.2). After centrifugation, the supernatant volume was discarded, and the pellet was counted in a Packard crystal scintillation gamma counter. Binding inhibition studies were performed in the presence of individual solutions containing 50 mM

lactose or glucose. Antibody labeled at higher specific activities, i.e., 3,000 and 6,000 cpm/ng, were inactive with respect to agglutination of erythrocytes at 4°C.

RESULTS

Cold agglutinin antibody response and erythrocyte reactivity. Shown in Fig. 1 are the polysaccharide structures associated with group B type III, group C, and *S. pneumoniae* type XIV streptococci. Earlier experiments that utilized anti-group C streptococcal serum had revealed cold agglutinin antibodies that were preferentially reactive with GalNAc (2, 6). These data suggested the autoreactive antibodies observed were, in fact, bacterial carbohydrate specific, but cross-reactive with erythrocyte determinants. To address this hypothesis, rabbits were individually immunized in the present study with vaccine prepared from group C, group B type III, and *S. pneumoniae* type XIV streptococci. Sera were examined at 4°C for the ability to agglutinate rabbit erythrocytes. All rabbits immunized produced vaccine and erythrocyte-reactive antibodies during week 1 of immunization. The vaccine agglutination titers rose during week 1 and plateaued at week 4. The highest vaccine titers attained were 1:1,048; 1:512; and 1:4,096 for group B, *S. pneumoniae* type XIV, and group C antisera, respectively. The hemagglutination titer of these antibodies in group C and *S. pneumoniae* type XIV antisera rose during week 2, usually plateaued during week 3, and thereafter remained constant. Group B cold agglutinin antibody titers did not rise after week 1 and in some instances declined. Among the rabbits examined, peak cold agglutinin antibody titers varied from 1:128 to 1:256 in group C-immune animals, 1:80 to 1:160 in *S. pneumoniae* type XIV-immune animals, and 1:20 in group B-immune animals. All preinoculation sera were negative with respect to cold agglutinin antibody, but in some instances showed a 1:20 to 1:40 group C vaccine titer.

Cold agglutinin antibody activity could be detected in only the IgM fractions of whole group C antisera fractionated by S-200 Sephacryl gel chromatography. Further, only the void volume fractions of group B and *S. pneumoniae* type XIV antisera similarly fractionated revealed cold agglutinin antibody activity. Cold agglutinin antibodies purified from group C and *S. pneumoniae* type XIV antisera by elution from rabbit erythrocytes were subsequently subjected to fractionation by S-200 Sephacryl gel chromatography, and 4-ml fractions were individually assayed for activity. Only the IgM peak of erythrocyte-purified *S. pneumoniae* type XIV material (as judged with reaction with goat anti-rabbit μ chain-specific antisera) and the exclusion volume of group B antisera contained cold agglutinin antibody activity. In contrast, both IgM and IgG fractions of erythrocyte-purified cold agglutinin antibodies isolated from group C antisera were active. Further examination of these antisera utilized either purified IgM cold agglutinin antibody or the exclusion volume of S-200 Sephacryl chromatographed antisera.

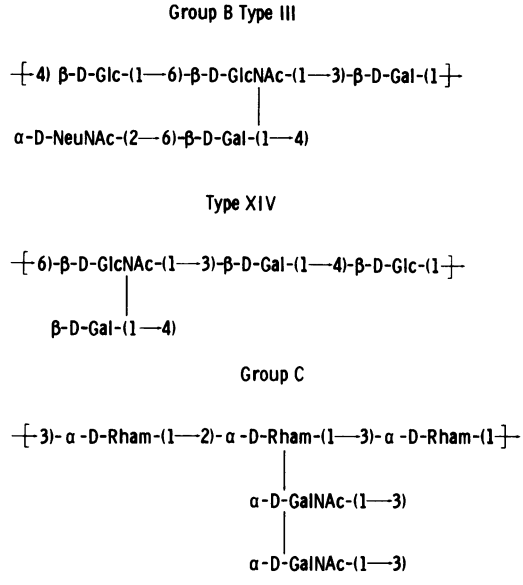

FIG. 1. Structures of polysaccharides assigned to group B type III, *S. pneumoniae* type XIV, and group C streptococci.

Cold agglutinin erythrocyte reactivity. Reactivity with erythrocytes was further examined using rabbit, bovine (ox), human type OI, human type Oi, and human type A, human type B, and sheep erythrocytes. Purified IgM cold agglutinin antibody preparations isolated from group C and *S. pneumoniae* type XIV antisera reacted with rabbit erythrocytes at 4°C, and these antibodies also reacted with human type O erythrocytes at 4°C. Furthermore, when tested by direct slide agglutination assay, group C IgM cold agglutinin antibodies strongly agglutinated sheep and human type A erythrocytes at 37°C. In addition, both group B fractionated serum and purified *S. pneumoniae* type XIV IgM cold agglutinin antibodies strongly agglutinated human type B erythrocytes at 37°C. In contrast, when either whole serum or purified material was examined, neither preparation agglutinated human O or bovine (ox) erythrocytes at 37°C nor bovine (ox) erythrocytes at 4°C. *S. pneumoniae* type XIV whole antisera and purified IgM antibodies were further examined for the ability to agglutinate human type OI and human type Oi erythrocytes. Essentially no difference in reactivity was de-

tected, since a titer of 1:80 was observed when antisera were tested against human type OI erythrocytes and a titer of 1:160 was observed when *S. pneumoniae* type XIV antisera were tested against human type Oi erythrocytes.

When the 19S fraction of group B, group C, and *S. pneumoniae* type XIV antisera were tested in the biphasic temperature hemolytic assay, all were capable of initiating hemolysis of rabbit erythrocytes. Further, the antibodies were efficient hemolysins, since usually less than 2 μg of purified material was active in producing 50% lysis when IgM cold agglutinin antibodies purified from group C and *S. pneumoniae* type XIV antisera were examined.

Temperature requirements of erythrocyte-cold agglutinin antibody interaction. During the course of the present investigation, group B and *S. pneumoniae* type XIV rabbit antisera were observed to cause significant agglutination of rabbit erythrocytes at room temperature, whereas most group C antisera failed to agglutinate erythrocytes until much lower temperatures were achieved. To evaluate the precise temperature requirements of cold agglutinin antibody-erythrocyte interaction, group C, group B, and *S. pneumoniae* type XIV whole antisera were compared for their ability to effect agglutination of rabbit erythrocytes at various temperatures. When a 2+ agglutination reaction was used as an arbitrary reference point, group C antisera yielded such a reaction at 14°C and below, whereas both group B and *S. pneumoniae* type XIV antisera were reactive at 31°C and below.

Vaccine and carbohydrate reactivity. The parallel appearance of cold agglutinin antibody activity and bacterial antibody activity during immunization with each of the vaccine preparations suggested an association between the antibody population observed and the vaccines that elicited the response. Absorption studies were performed in an attempt to differentiate between erythrocyte-reactive and bacteria-reactive antibody. Similar to data obtained in previous studies (6), when the void volume fraction of group C antisera was absorbed only once with group C vaccine, cold agglutinin antibody titers dropped from 1:256 to 1:16. When purified group C IgM cold agglutinin antibody (at approximately 1 mg/ml) was absorbed once with group C vaccine, the hemagglutination titer decreased from 1:5,000 to 1:40. Absorption of the void volume fraction of group B and *S. pneumoniae* type XIV antisera with their respective vaccines yielded similar results. The cold agglutinin antibody titer decreased from 1:320 to 1:32 and 1:640 to 1:8, respectively. The relationship between *S. pneumoniae* type XIV cold agglutinin antibodies, specificity for individual vaccines, and the dissimilarity between these antibodies and the antibodies elicited in group C antisera was further examined using purified ^{125}I-labeled antibodies. When approximately 110 ng of antibody was added to *S. pneumoniae* type XIV, group C, and micrococcal vaccines, 97, 2, and 2 ng of antibody, respectively, were bound. When binding was examined in the presence of 50 mM lactose and 50 mM glucose, binding to *S. pneumoniae* type XIV vaccine was reduced by 63% in the presence of lactose and uninhibited in the presence of glucose. Further, when the cold hemagglutinin activity present in whole serum was inhibited in the presence of purified carbohydrate preparations, rabbit erythrocyte agglutination in the presence of group B antiserum was completely inhibited in the presence of approximately 750 ng of group B carbohydrate; erythrocyte agglutination in the presence of *S. pneumoniae* type XIV antiserum was completely inhibited in the presence of 300 ng of *S. pneumoniae* type XIV polysaccharide. These data clearly revealed specific cold agglutinin antibody reactivity associated with specific bacterial vaccine reactivity.

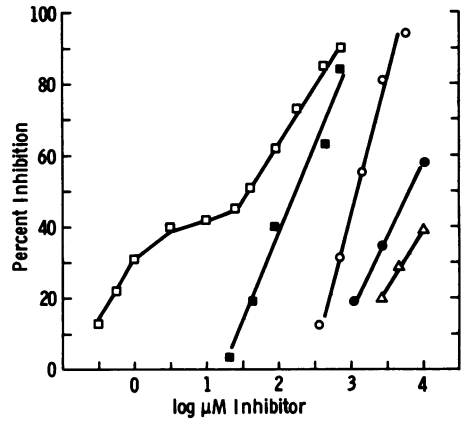

FIG. 2. Effect of lactose (□), melibiose (■), galactose (○), glucose (●), and GalNAc (△) on *S. pneumoniae* type XIV rabbit cold agglutinin antibody-sensitized rabbit erythrocyte hemolysis.

Ligand reactivity. To more thoroughly investigate the ligand binding characteristics of these various cold agglutinin antibody preparations, biphasic temperature hemolysis inhibition studies were initiated. Shown in Fig. 2 are data representative of purified antibody isolated from *S. pneumoniae* type XIV rabbit antisera. As shown, lactose was approximately fivefold more reactive than galactose and manyfold more reactive than GalNAc. In contrast (Table 1), cold agglutinin antibody purified from group C immune sera was preferentially inhibited in the presence of GalNAc. Further, the data in Table 1 demonstrate that galactose was significantly

TABLE 1. Inhibition of rabbit cold agglutinin antibody hemolytic activity in biphasic temperature hemolytic inhibition assays

Cold agglutinin antibody source	Amt of saccharide or polysaccharide required for 50% inhibition							
	Saccharide (mM)					Polysaccharide (ng)		
	Lactose	Melibiose	Galactose	GalNAc	Glucose	Type XIV	Group B	Group C
Group C	12	3	25	1	>50			7.5
Group B[a]	8		2	13	>50		50	
Type XIV	0.04	0.2	1.3	16	6.3	15		

[a] Sephacryl S-200 void volume fraction.

more inhibitory of cold agglutinin antibody hemolytic activity in the void volume fraction of group B immune sera when compared to lactose and GalNAc. These data revealed a clear difference among the cold agglutinin antibodies' ligand reactivity in association with the different antipolysaccharide responses in the individually immunized animals. Also, group B cold agglutinin antibody activity was inhibited less than 50% by 20 mM rhamnose. In addition, individual hemolytic responses were inhibited in the presence of nanogram quantities of purified polysaccharides (Table 1). To further examine the association of cold agglutinin antibody ligand specificity with the antipolysaccharide immune response, mice were immunized with *S. pneumoniae* type XIV vaccine.

The data in Fig. 3 represent saccharide inhibition studies of cold agglutinin antibody hemolytic activity (toward rabbit erythrocytes) present in the void volume Sephacryl S-200 fractions of pooled BALB/c *S. pneumoniae* type XIV antisera. As shown, the relative ligand specificity observed was identical to the data obtained when rabbit *S. pneumoniae* type XIV antisera or purified material was examined. As was observed when rabbit *S. pneumoniae* type XIV antisera were tested, lactose was a substantially more effective inhibitor of hemolysis when compared with galactose or GalNAc. From the data shown in Tables 2 and 3, the strain of mouse examined did not alter the relative ligand inhibition observed. However, when *S. pneumoniae* type XIV polysaccharide was used as antigen in complete Freund adjuvant, the preferred ligand was melibiose. Mice immunized with complete Freund adjuvant only did produce a very weak cold agglutinin antibody response inhibitable by melibiose. Therefore, it is possible that the presence of adjuvant containing mycobacteria participated in the response. Importantly, however, cold agglutinin reactivity with GalNAc remained extremely low (Table 2). Interestingly, BALB/c and CF1 group B antisera cold agglutinin antibody reacted with rabbit erythrocytes at room temperature and, in some cases, at 37°C, although hemagglutination titers were significantly lower than those observed at 4°C (data not shown).

DISCUSSION

Cold agglutinin antibodies of the IgM class have been observed in the sera of rabbits immunized with *Mycoplasma pneumoniae*, *Listeria monocytogenes* and *Streptococcus* MG vaccines (7, 12). These antibodies could be effectively removed from sera by absorption with the vaccine used for immunization. Further, these antibodies were reactive with saccharide ligands that contained galactoside moieties. It was difficult in these studies, however, to determine whether the autoreactive antibodies were erythrocyte specific, but cross-reactive, with the bacteria, or vice versa. The present investigation approached this problem by utilizing the recognized differences and similarities among the carbohydrate structures associated with three different microorganisms.

The carbohydrate structures associated with group C, group B type III, and *S. pneumoniae* type XIV streptococcal bacteria are known. Consequently, the reactivity of cold agglutinin antibodies could be more easily defined. Where-

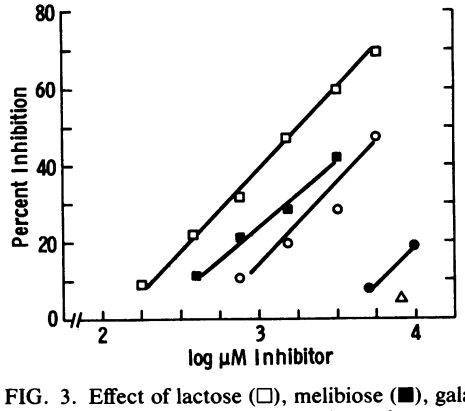

FIG. 3. Effect of lactose (□), melibiose (■), galactose (○), GalNAc (●), and glucose (△) on *S. pneumoniae* type XIV BALB/c mouse cold agglutinin antibody-sensitized rabbit erythrocyte hemolysis.

TABLE 2. Saccharide inhibition of BALB/c cold agglutinin antibody activity in biphasic hemolytic inhibition assay[a]

Immunizing agent	Amt of saccharide required for 50% inhibition (mM)				
	Lactose	Melibiose	Galactose	GalNAc	Glu
Type XIV polysaccharide	1.1	0.6	1.9	40% at 10 mM	None at 10 mM
Type XIV vaccine	1.7	3.5	5.3	32[b]	50[b]

[a] Antisera were from mice immunized with type XIV polysaccharide and vaccine.
[b] Extrapolated to obtain 50% endpoint.

as the immunodominant structural features of the group C carbohydrate antigen are the α-1, 3-linked GalNAc disaccharide residues, both group B type III and *S. pneumoniae* type XIV determinants are devoid of GalNAc determinants. Further, *S. pneumoniae* type XIV and group B type III carbohydrate antigens are essentially identical except for a terminal *N*-acetylneuraminic acid residue associated with the core structure of the group B type III polysaccharide (10).

In the biphasic temperature hemolysis inhibition assay, GalNAc was the most effective inhibitor of group C cold agglutinin antibody activity. These data substantiated previously obtained data on this particular system (6). In contrast, the cold agglutinin antibodies induced by rabbit immunization with either group B type III or *S. pneumoniae* type XIV vaccines were most reactive with galactose or lactose, respectively. Furthermore, the 50% inhibition endpoints obtained in the presence of several other mono- and disaccharides clearly distinguished group C cold agglutinin antibodies from both group B and type III and *S. pneumoniae* type XIV streptococcal cold agglutinin antibodies. In addition, the relative inhibition profiles observed for group B type III and *S. pneumoniae* type XIV cold agglutinin antibodies were similar. It is clear, therefore, that immunization with bacteria per se does not result in the appearance of "nonspecific" cold agglutinin antibodies. Indeed, these antibodies are unique and reflect ligand specificities which are predicted on the basis of bacterial carbohydrate determinants.

Baker and Kasper (1) have recently shown that the terminal sialic acid moiety of the group B type III carbohydrate antigen is exceedingly acid labile and is readily hydrolyzed during culture of the microorganisms in Todd-Hewitt broth, due to accumulation of acid metabolites. The pH of the cultures in the present investigation after 24 h of incubation had dropped to 5.0. Thus, the culture conditions employed very likely resulted in partial desialation of the type III carbohydrate antigen. Such a loss of sialic acid would render the type III antigen structurally identical to the *S. pneumoniae* type XIV antigen. Consequently, the similarity between cold agglutinin antibody ligand reactivity in both group B type III and *S. pneumoniae* type XIV antisera would predictably be similar if the cold agglutinin antibodies produced had, indeed, arisen in direct response to the bacterial carbohydrate structures. By similar reasoning, the clear differences in the ligand specificity of group C cold agglutinin antibodies in comparison to the antibodies generated by immunization with the other two vaccines reflects a specificity of these antibodies for the carbohydrate structures associated with the bacterial antigens.

The cold agglutinin antibodies in group B rabbit antisera reactive with galactose and lactose probably represent a type III carbohydrate-specific subpopulation. There is substantial evidence that group B specific antibodies are predominantly reactive with rhamnose determinants (3). Although this particular sugar in the present study was inhibitory of the group B type III cold agglutinin antibodies, a high (approximately 20 mM) concentration was required to inhibit at the 50% level. It has also been demonstrated that galactose was an effective inhibitor of purified type III polysaccharide interaction with type-specific antisera, although the majority of the reactivity with the polysaccharide appeared directed toward sialic acid determinants (3). Thus, although it appears that galactosyl-containing structures are involved in the cold agglutinin activities observed in the current

TABLE 3. Saccharide inhibition of cold agglutinin antibody activity in biphasic temperature hemolytic assay[a]

Mouse strain	Amt of saccharide required for 50% inhibition (mM)				
	Lactose	Melibiose	Galactose	GalNAc	Glucose
Local	0.8	5.6	2.2	20% at 10 mM	20% at 10 mM
CF1	0.3	0.6	1.4	20% at 10 mM	20% at 10 mM

[a] Antisera were from randomly bred, agouti-colored mice and CF1 mice immunized with type XIV vaccine.

study, there is not an absolute requirement that the antibodies be strictly galactose specific to observe erythrocyte reactivity. When mice were immunized in the present study, it was clear that the cold agglutinin antibodies that appeared were strikingly similar to those produced in rabbits. It appears highly unlikely that three different strains of mice would produce antibodies in a nonspecific manner that reflected such similar carbohydrate specificity and, in turn, reflected the same characteristics as the cold agglutinin antibodies produced in rabbits. Interestingly, when the mice were immunized with group C streptococcal vaccine, no cold agglutinin antibodies were detected. A recent study by Coligan et al. (5) provided data that clearly point to the unlikely possibility of an immune response in mice to group C streptococcal carbohydrate GalNAc determinants, since such determinants are identical to the glycan region of the Forssman glycolipid, which mice express. Thus, hyperimmunization per se and other nonspecific events cannot easily explain the appearance of these autoreactive antibodies. When anti-*S. pneumoniae* type XIV rabbit and mouse antisera were compared, it was interesting to note that the inhibition of rabbit cold agglutinin antibody isolated from pooled antisera exhibited a biphasic inhibition response in the presence of lactose. Therefore, there appeared to be two relatively distinct lactose-reactive rabbit cold agglutinin antibody populations, one of lower affinity and one of higher affinity. This type of inhibition was not observed when mouse antisera were examined.

The association of cold agglutinin antibodies with overt pathological symptoms is dependent upon the temperature required for erythrocyte-antibody interaction (13). Even the amount of such antibodies can become a secondary consideration when antibody binding to erythrocytes at near-physiological temperatures is a characteristic of the response. In the present study, both group B type III and *S. pneumoniae* type XIV cold agglutinin antibodies reacted with erythrocytes at significantly higher temperatures in comparison with the group C antibodies. It is possible that the structural similarities between *S. pneumoniae* type XIV structures and Ii oligosaccharides is the explanation for the relatively better agglutination activity exhibited by both rabbit and mouse antisera for rabbit erythrocytes. However, the ability of these antibodies to react with erythrocytes at temperatures as high as 31°C could have been due to the degree of exposure on the erythrocyte surface of determinants with which the antibodies were reactive, or a generally higher affinity for saccharide determinants may have been present.

The relative ease with which cold agglutinin antibodies were produced in the present study suggests that careful attention be paid to human immunization schedules that utilize bacterial carbohydrates. Clearly, the potential for production of autoreactive antibodies exists and should be carefully evaluated.

Indeed, the recent demonstration that certain suppressor cell-reactive antibodies are inhibitable in the presence of lactose (9) suggests a potential for perturbation of normal immune regulatory mechanisms if a sufficient quantity of galactose-binding cold agglutinin-like antibodies are induced by immunization.

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