CLINICAL AND DIAGNOSTIC LABORATORY IMMUNOLOGY, Mar. 1995, p. 125–131 Vol. 2, No. 2
1071-412X/95/$04.00+0

Anti-α-Galactosyl Immunoglobulin A (IgA), IgG, and IgM in Human Secretions†

RANDA M. HAMADEH,[1]* URI GALILI,[2] PING ZHOU,[1] AND J. M. GRIFFISS[1,3]

Centre for Immunochemistry and Department of Laboratory Medicine,[1] and Department of Medicine,[3] University of California San Francisco, San Francisco, California 94143, and Department of Microbiology and Immunology, Medical College of Pennsylvania, Philadelphia, Pennsylvania 19129[2]

Received 16 May 1994/Returned for modification 12 August 1994/Accepted 11 November 1994

Anti-α-galactosyl (anti-Gal) is a natural human serum antibody that binds to the carbohydrate Galα1,3Galβ1,4GlcNAc-R (α-galactosyl epitope) and is synthesized by 1% of circulating B lymphocytes in response to immune stimulation by enteric bacteria. We were able to purify secretory anti-Gal from human colostrum and bile by affinity chromatography on silica-linked Galα1,3Galβ1,4GlcNAc. We found similar secretory anti-Gal antibodies in human milk, saliva, and vaginal washings. Secretory anti-Gal from milk and saliva was exclusively immunoglobulin A (IgA); that from colostrum and bile also contained IgG and IgM isotypes. Serum was also found to contain anti-Gal IgM and IgA in addition to the previously reported IgG. Anti-Gal IgA purified from colostrum and bile had both IgA1 and IgA2. Secretory anti-Gal from saliva, milk, colostrum, and bile agglutinated rabbit erythrocytes (RRBC) and bound to bovine thyroglobulin, both of which have abundant α-galactosyl epitopes. The RRBC-hemagglutinating capacity of human saliva, milk, bile, and serum was specifically adsorbed by immobilized Galα1,3Galβ1,4GlcNAc but not by Galα1,4Galβ1,4GlcNAc, Galβ1,3GalNAc, Galβ1,4GlcNAc, Galβ1,4GlcNAcα1,2Man, or Fucα1,2Galβ1,4GlcNAc. No RRBC-hemagglutinating activity could be detected in rat milk, rat bile, cow milk, or rabbit bile, suggesting a restricted species distribution for secretory anti-Gal similar to that found for serum anti-Gal. Colostral anti-Gal IgA bound strongly to a sample of gram-negative bacteria isolated from the throats and stools of well children as well as to an *Escherichia coli* K-1 blood isolate. Colostral anti-Gal IgA inhibited the binding of a *Neisseria meningitidis* strain to human buccal epithelial cells, suggesting that this antibody may play a protective role at the mucosal surface.

Anti-α-galactosyl (anti-Gal) antibodies are natural human polyclonal antibodies that bind specifically to the mammalian carbohydrate structure Galα1,3Galβ1,4GlcNAc-R (α-galactosyl epitope) on glycoproteins and glycolipids (13, 15). Anti-Gal has been associated with autoimmune thyroid disorders (9), Henoch-Shoenlein purpura and immunoglobulin A (IgA) nephropathy (7), scleroderma (11), inner ear disease (23), Chagas' disease (1, 30), and malaria (26). Old World monkeys, apes, and humans are unique among mammals in that they produce large amounts of anti-Gal (1% of circulating IgG) but do not synthesize detectable α-galactosyl epitopes on their cell surfaces (14, 17, 18). In contrast, nonprimate mammals, prosimians, and New World monkeys lack anti-Gal but produce an abundance of α-galactosyl epitopes (more than 10^6 α-galactosyl epitopes per cell) (14, 18).

The terminal Galα1,3Gal structure which is part of the α-galactosyl epitope is also present within the lipopolysaccharides (LPS) and capsular polysaccharides (K) of a number of gram-negative bacteria, many of which normally colonize the human gastrointestinal tract (2, 6, 21). Anti-Gal is thought to be produced as a result of constant stimulation by these bacteria (16) in a way that is analogous to the synthesis of blood group antibodies in response to normal enteric bacterial antigens (32). Many enteric organisms that make Galα1,3Gal structures have been associated with autoimmune conditions, notably in *Klebsiella* and *Yersinia* infections (20). Occasionally, these bacteria can survive within the bloodstream to cause sepsis. Many more gram-negative bacteria that cause sepsis bind anti-Gal than do bacteria of the same species that are isolated from the stools of healthy individuals (19). This may be related to the ability of anti-Gal IgG to block the alternative pathway of complement-mediated lysis of bacteria subsequent to binding to α-galactosyl-like epitopes on their LPS, thereby promoting their survival in the nonimmune host (19).

Since many bacteria capable of interacting with anti-Gal are part of the normal gut flora (16, 19), it was of interest to determine whether anti-Gal may be present in a secretory form as an IgA isotype. Secretory anti-Gal IgA in the throat or gut could promote host defense against invasive diseases caused by bacteria that surface express α1,3-galactosyl residues by binding to them and preventing their attachment to mucosal epithelial cells. On the other hand, the association of high levels of serum anti-Gal IgA with nephropathy and purpura (7) and of anti-Gal IgG with thyroiditis (9) suggests that these antibodies may also promote autoimmune diatheses. Detailed analyses comparing anti-Gal under normal and pathologic conditions have not been done.

In this study, we describe anti-Gal in human secretions and compare its isotypes with those of anti-Gal in normal human serum. We found that human saliva, colostrum, milk, bile, and vaginal washings contain anti-Gal antibodies mostly of the IgA isotype, whereas normal human serum contains anti-Gal of isotypes IgG, IgM, and IgA. The anti-Gal IgA in human colostrum bound to commensal gram-negative bacteria and pre-

* Corresponding author. Present address: Veterans Administration Medical Center/111W1, 4150 Clement St., San Francisco, CA 94121. Phone: (415) 221-4810, ext. 3788.

† Paper no. 66 from the Center for Immunochemistry of the University of California at San Francisco.

vented the adhesion to human buccal cells of a representative strain, suggesting that this antibody may play a protective role at the mucosal surface.

MATERIALS AND METHODS

Human and animal secretory fluids. We obtained serum samples from two laboratory volunteers (blood types A and B). We obtained colostrum from a nursing laboratory colleague and milk samples from the same colleague, one of us, and four anonymous healthy donors (3). Vaginal washes from three anonymous healthy donors were the kind gift of G. Brooks, University of California San Francisco. A human bile sample was collected intraoperatively from a patient undergoing a wedge resection of the liver for carcinoma and an incidental cholecystectomy. This patient had no gallstones and a normal gallbladder pathology. This bile sample was a kind gift from L. Way, Veterans Administration Medical Center, San Francisco, Calif. We collected saliva from two laboratory volunteers. We bought pooled human colostral IgA from Sigma Chemical Co. (St. Louis, Mo.).

Milk samples from lactating rats and cows were a kind gift from A. Budayr, and rat and rabbit bile samples were from B. Aagard, both of whom are at the Veterans Administration Medical Center, San Francisco, Calif.

Bacterial strains. We cultured stool samples obtained from 10 healthy infants and toddlers on Mueller-Hinton agar plates and saved all aerobic gram-negative organisms that grew. Neisserial strains were obtained from a collection of such strains in our laboratory. *Escherichia coli* K-1 was cultured from the blood of a child with gram-negative sepsis.

Agglutination of RRBC. The α-galactosyl epitope is the most abundant carbohydrate structure on rabbit erythrocytes (RRBC) (8, 10, 28). Therefore, these RRBC have been used routinely to screen for the presence of anti-Gal and to determine its titer (9, 14, 15, 17).

We studied the hemagglutinating activity of the following samples: six human milk samples (individually and pooled), two human saliva samples (individually and pooled), three human vaginal washes (individually and pooled), two human serum samples (individually and pooled), one human colostral sample, pooled colostral IgA (Sigma) at a starting concentration of 1 mg/ml, one human bile sample, two rat milk samples (pooled), three cow milk samples (pooled), and two rabbit bile samples (pooled).

We mixed twofold serial dilutions of each sample with an equal volume of a 0.5% RRBC suspension in V-shaped wells of a microtiter plate. The diluent was phosphate-buffered saline (PBS) of pH 7.4. Agglutination (i.e., "carpet" formation by RRBC) was evaluated after the samples had settled for 1 h at room temperature. Titers were expressed as the reciprocal of the highest dilution that caused complete agglutination.

Demonstration of α-galactosyl specificity of secretory and serum anti-Gal. Silica-linked synthetic oligosaccharides SYNSORB columns (Chembiomed, Edmonton, Alberta, Canada) were tested for their ability to adsorb the RRBC hemagglutinating activity (i.e., anti-Gal activity) of saliva, milk, and serum obtained from one individual. The following oligosaccharides on SYNSORB columns were used: Galα1,3Galβ1,4GlcNAc, Galα1,4Galβ1,4GlcNAc (blood group P), Galβ1,3GalNAc (T antigen), Galβ1,4GlcNAc (*N*-acetyllactosamine), Galβ1,4GlcNAcα1,2Man, and Fucα1,2Galβ1,4GlcNAc (blood group H) or silica alone (no carbohydrate) as control for nonspecific adsorption. Saliva, milk, or serum samples were diluted twofold in PBS and applied to the columns (1 ml of adsorbent per column) listed above. The effluents were collected and stored. The material bound to the columns was then eluted with 0.1 M glycine HCl buffer (pH 2.6) and immediately neutralized with 0.1 N NaOH. Both the effluent and eluate of each sample (saliva, milk, or serum) obtained after affinity chromatography on each of the columns described above were tested for their ability to agglutinate RRBC.

Determination of the anti-Gal isotypes by ELISA with a solid-phase α-galactosyl epitope. To examine the specificity and the isotypes of anti-Gal in whole human serum, saliva, milk, colostrum, and purified pooled human colostral IgA, we used a highly sensitive enzyme-linked immunosorbent assay (ELISA) with bovine thyroglobulin enriched for α-galactosyl epitopes (30 epitopes per molecule) (29). This α-galactosyl-enriched (BS^+) bovine thyroglobulin was obtained by affinity chromatography on a *Bandeiraea simplicifolia*-Sepharose column (Vector Laboratories, Burlingame, Calif.) as described previously (29) and was diluted to a concentration of 20 μg/ml in carbonate buffer (pH 9.5) to coat microtiter wells. To prevent nonspecific binding, 1% bovine serum albumin (BSA) in carbonate buffer was added to the wells, and the plates were incubated for 1 h at room temperature. Serial twofold dilutions in PBS of the following samples were then added in triplicate to the wells of the microtiter plates, and the plates were allowed to incubate for 1 h at room temperature: (i) a 1:10 solution in PBS of serum, saliva, or milk obtained from one individual (blood type A^-) and of a whole colostrum sample obtained from another (blood type O^-); and (ii) a 1:2 solution in PBS of pooled human colostral IgA (1 mg/ml; Sigma). The wells were then washed five times with 0.05% Tween-PBS, and horseradish peroxidase-linked rabbit anti-human IgG, IgA, or IgM (Dako) diluted 1:1,000 in PBS was added to the wells and incubated for 1 h at room temperature. The wells were then washed again with 0.05% Tween-PBS, and peroxidase substrate (Sigma ODP-10 reagent, 0.5 mg/ml) was added. The A_{450} of the solution in the plates was read after 30 min of incubation with the substrate with a Bio-Rad ELISA reader.

Purification of anti-Gal. We used our reported method (16) to isolate anti-Gal from blood type B normal human serum, pooled human colostral IgA, and a human bile sample.

(i) Purification of anti-Gal from serum. To examine the relative proportions of high- and low-avidity serum anti-Gal for the ligand Galα1,3Galβ1,4GlcNAc, we loaded 20 ml of blood type B serum onto a 5-ml affinity chromatography column of silica beads to which the oligosaccharide epitope Galα1,3Galβ1,4GlcNAc-R had been conjugated (SYNSORB 115; Chembiomed). We washed the column with PBS and then serially eluted the column-bound antibodies, first with 0.5 M NaCl (termed the NaCl anti-Gal fraction), then with 0.4 M melibiose (α-galactosyl glucoside; termed the melibiose anti-Gal fraction), and lastly with 0.1 M glycine HCl (pH 2.6; termed the glycine HCl anti-Gal fraction). Glycine-eluted fractions were neutralized immediately with 0.1 N NaOH. Columns with silica alone (with no conjugated carbohydrate) were used as described above to control for any nonspecific binding of antibodies to silica.

We dialyzed all of the eluates against PBS and determined the concentrations of anti-Gal IgG, IgA, and IgM in these samples by radioimmunodiffusion (RID; Behring Diagnostics Inc., Somerville, N.J.). We confirmed the values obtained by RID by repeating these measurements by rate nephelometry (Beckman Instruments, Inc., Brea, Calif.). The latter determinations were done by the staff of the Chemistry Laboratory at the Veterans Administration Hospital in San Francisco. The concentrations of total IgG, IgA, and IgM in the original serum sample were measured simultaneously by RID.

To further examine the specificity of the serum anti-Gal isotypes for Galα1,3Galβ1,4GlcNAc, we measured the binding by ELISA of the NaCl, melibiose, and glycine HCl anti-Gal eluates (obtained from both the SYNSORB 115 and the control silica columns) to solid-phase bovine thyroglobulin as described above. The secondary anti-isotype antibodies were alkaline phosphatase-linked goat anti-human IgG, IgA, or IgM (Sigma) diluted 1:500 in PBS, and the substrate was 75 μl of a disodium *p*-nitrophenyl phosphate (2 mg/ml in 100 mM sodium bicarbonate–10 mM $MgCl_2$ [pH 9.5]; Sigma). The absorbance of the plates was read after 20 min of incubation with the substrate with a Bio-Rad 2550 ELISA reader and a 405-nm filter. This experiment was repeated twice.

(ii) Purification of anti-Gal from human bile. We diluted 4 ml of human bile into 4 ml of PBS and loaded it onto the SYNSORB 115 column or the control silica column. We used 0.1 M glycine HCl in PBS (pH 2.6) to elute column-bound antibodies. The concentrations of anti-Gal IgG, IgA, and IgM in the eluates were determined by RID and rate nephelometry as outlined above for serum anti-Gal. The concentrations of total IgG, IgA, and IgM in the original bile sample were measured simultaneously by RID.

We confirmed the α-galactosyl specificity of bile anti-Gal and examined its isotypes by assessing its binding to bovine thyroglobulin, as detailed above, and by assessing its ability to hemagglutinate RRBC.

(iii) Purification of anti-Gal from human colostral IgA. We loaded 5 mg of pooled human colostral IgA diluted in PBS onto the SYNSORB 115 column or onto the control silica column and then eluted the column-bound antibodies with 0.1 M glycine HCl in PBS (pH 2.6). We determined the concentration of anti-Gal IgA in these samples by RID.

We confirmed the α-galactosyl specificity of colostral anti-Gal IgA by assessing its binding to bovine thyroglobulin in an ELISA (as detailed above for serum anti-Gal) and by assessing its ability to hemagglutinate RRBC.

Determination of bile and colostral anti-Gal IgA1 and IgA2 by ELISA. We used an ELISA to determine the subclass of anti-Gal IgA in colostral IgA and in bile. Briefly, we used 75 μl of a 20-μg/ml solution in PBS of colostral anti-Gal IgA or 75 μl of a 20-μg/ml solution in PBS of bile anti-Gal post-SYNSORB 115 treatment to coat the wells of a microtiter plate. Nonspecific binding was blocked by 0.5% BSA in PBS for 30 min. Wells were then washed with PBS and incubated for 1 h with a 1:500 solution in PBS of monoclonal anti-human IgA1 or monoclonal anti-human IgA2 bought from Zymed Laboratories, South San Francisco, Calif. After washing the wells, we added 75 μl of alkaline phosphatase-linked goat anti-mouse IgG (Sigma) diluted 1:500 in PBS to each well and incubated the plates for 1 h. After extensive washing of the plates with PBS, we added the phosphatase substrate and read the absorbances of the solutions in the plates as described above. Samples were run in triplicate.

Binding of colostral anti-Gal IgA to members of the family *Enterobacteriaceae* by a dot blot assay. This assay was performed as described previously (22). Gram-negative bacteria isolated from stool and blood cultures were plated onto Mueller-Hinton agar and incubated at 37°C for 18 h. We then dotted each organism onto a piece of nitrocellulose paper, which was air dried and incubated in a 1% casein solution for 1 h to block nonspecific binding sites. We then reacted the paper for 3 h at room temperature with a 2-μg/ml solution in 1% casein of colostral anti-Gal IgA. The blot was then washed with PBS, incubated for 1 h in a 1:500 solution in 1% casein of an alkaline phosphatase-conjugated goat anti-human IgA, and developed with a naphthol substrate and fast red solution as described above. Control blots that had on them only bacteria with secondary antibody but no primary antibody were included. We recorded the results as positive when a red color was clearly visible as compared with controls. At least two independent observers recorded the results.

Effect of colostral anti-Gal IgA on the adhesion of *Neisseria meningitidis* to human buccal cells. We used a group C *N. meningitidis* strain that bound

TABLE 1. Anti-Gal reactivity in human and animal specimens

Source of specimen	Titer for:						
	Colostrum	Colostral IgA	Milk	Saliva	Bile	Vaginal wash	Serum
Human	1:2,058	1:64	1:256	1:128	1:16	1:4	1:128
Rat			None		None		
Cow			None				
Rabbit					None		

colostral anti-Gal IgA by dot blot (see above) to study the effect of anti-Gal IgA on bacterial adhesion. Human buccal cells were isolated by scraping the buccal mucosa of a laboratory volunteer three times with a sterile wooden blade, suspending the scrapings in 0.05 M HEPES-minimal essential medium buffer (pH 7.45; HEPES buffer), and washing the cells three times by pelleting at 170 $\times$ *g* and resuspending them in HEPES buffer (HEPES is *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid). A buccal cell suspension of 2×10^5 cells per ml was prepared in HEPES buffer, and a hemocytometer was used to count the cells. The *Neisseria* strain was grown on gonococcal agar (Difco) and resuspended in HEPES buffer to a final dilution of 2×10^7 CFU/ml of buffer. Two hundred and fifty microliters (5×10^6 CFU) of the bacterial suspension was then either preincubated or not with 100 μg of anti-Gal IgA in HEPES buffer for 30 min at 37°C and subsequently incubated with 250 μl of the buccal cell suspension (5×10^4 cells) for 30 min at 37°C. The mixture was then centrifuged at 170 $\times$ *g* for 3 min, the pellet was resuspended in HEPES buffer, and the cells were washed with HEPES. Twenty-microliter samples were then placed on glass slides, fixed with methanol, stained with Gram's stain, and examined by light microscopy at ×1,000. The number of organisms attached per buccal cell was then counted. Thirty buccal cells were examined for each reaction mixture, and the mean and standard deviation of the number of bacteria attached per buccal cell were calculated for each reaction mixture. Buccal cells alone (without added bacteria) were examined to control for any preexisting adherent bacteria.

RESULTS

Analysis of anti-Gal reactivity in human and animal specimens. We studied human secretory fluids, including colostrum (one sample), milk (six samples), saliva (two samples), bile (one sample), vaginal washings (three samples), and human serum (two samples) for anti-Gal activity as indicated by RRBC agglutination. We assayed each sample individually as well as pooled when more than one sample was available for each secretory fluid. All of the human fluid samples agglutinated RRBC at various titers. Human milk agglutinated RRBC at titers ranging from 1:64 to 1:512, depending on the milk sample. Human saliva agglutinated RRBC at titers ranging from 1:64 to 1:256. Human serum agglutinated RRBC at titers ranging from 1:128 to 1:256.

The hemagglutination titers for pooled samples when more than one sample was available for each category are shown in Table 1. Human colostrum had very high RRBC-hemagglutinating activity. Note that the pooled human colostral IgA (purchased from Sigma) had a much lower hemagglutination titer (starting concentration, 1 mg/ml) than the whole human colostrum sample, indicating either the added hemagglutinating activity of other anti-Gal isotypes (IgM or IgG) in whole colostrum, loss of activity of commercial colostral IgA after purification, or person-to-person variability in anti-Gal IgA titers in colostrum. Pooled human milk and pooled human saliva had hemagglutinating titers comparable to that of serum, whereas bile had a lower titer. The hemagglutinating titer of the pooled vaginal washings was low, probably as a result of the diluting effect of the wash solution.

These data suggest that anti-Gal antibodies are present in human colostrum, milk, saliva, bile, and vaginal washings. The actual α-galactosyl epitope specificity of these RRBC-agglutinating secretory antibodies is described below (Table 2). No anti-Gal reactivity was detected in pooled rat milk, rat bile, cow milk, or rabbit bile, implying that secretory antiGal, like serum anti-Gal, is species restricted and not produced in nonprimate mammals.

TABLE 2. Specificity of the RRBC-hemagglutinating activity of human saliva, milk, and serum

Carbohydrate or control	Hemagglutination titer for:					
	Saliva		Milk		Serum	
	Effl[a]	Elu[a]	Effl	Elu	Effl	Elu
Galα1,3Galβ1,4GlcNAc	1:2	1:32	1:16	1:64	1:8	1:64
Galα1,4Galβ1,4GlcNAc	1:64	0	1:64	1:16	1:64	1:4
Galβ1,3GalNAc	1:64	0	1:256	1:2	1:128	0
Galβ1,4GlcNAc	1:64	0	1:256	0	1:64	0
Galβ1,4GlcNAcα1,2Man	1:64	0	1:256	0	1:64	0
Fucα1,2Galβ1,4GlcNAc	1:64	0	1:256	0	1:64	0
Control (silica)	1:64	0	1:256	0	1:128	0

[a] Effl, effluent; Elu, eluate.

All assays were run in triplicate and were reproducible within one hemagglutinating titer (not shown).

Specificity of the RRBC-hemagglutinating activity of saliva, milk, and serum. We tested the abilities of various synthetic carbohydrates linked to silica bead columns to absorb the RRBC-agglutinating activity from saliva, milk, and serum obtained from one individual. The results are shown in Table 2. The agglutinating activity of serum was specifically adsorbed onto Galα1,3Galβ1,4GlcNAc-R adsorbent, as published previously (13, 15). As in serum, the RRBC-agglutinating activities of saliva and milk were removed by chromatography on synthetic Galα1,3Galβ1,4GlcNAc-R. Low-pH eluates from Galα1,3Galβ1,4GlcNAc-R columns contained significant RRBC-hemagglutinating activity; for milk and serum, eluates from Galα1,4Galβ1,4GlcNAc also had some hemagglutinating activity, albeit to much lower levels. These results indicate the presence of anti-Gal in saliva and milk as well as in serum.

Binding of human saliva, milk, colostrum, and serum to solid-phase α-galactosyl epitopes (BS^+ thyroglobulin). We screened for the presence of anti-Gal and determined the anti-Gal isotype in human saliva, milk, whole colostrum, commercially available colostral IgA, and human serum by examining the binding of these samples to BS^+ bovine thyroglobulin in an ELISA. BS^+ bovine thyroglobulin, like RRBC, has abundant Galα1,3Galβ1,4GlcNAc structures (i.e., 30 epitopes per molecule [29]). The results are shown in Fig. 1. The anti-Gal isotype was determined by the different secondary antibodies used in this assay.

All samples contained anti-Gal. The predominant anti-Gal isotype in saliva and milk was IgA; anti-Gal IgA also predominated in whole colostrum, although IgG and IgM were also detected, albeit at lower titers. Serum had all three anti-Gal isotypes. The binding of commercially available colostral IgA (Sigma) to BS^+ thyroglobulin could be adsorbed completely by chromatography over Galα1,3Galβ1,4GlcNAc (SYNSORB 115), confirming that colostral anti-Gal IgA was specific for this carbohydrate structure.

Concentrations of anti-Gal IgG, IgA, and IgM in human serum and bile and of anti-Gal IgA in human pooled colostral IgA: affinities of serum anti-Gal isotypes for Galα1,3Galβ1, 4GlcNAc. To determine the relative avidity distribution of the isotypes of serum anti-Gal for Galα1,3Galβ1,4GlcNAc, we measured the concentrations of IgG, IgA, and IgM in sequential eluates of low-avidity (NaCl anti-Gal), higher-avidity (melibiose anti-Gal), and highest-avidity (glycine anti-Gal) fractions by RID (Table 3). Rate nephelometry gave very similar values to those obtained by RID (not shown). A silica

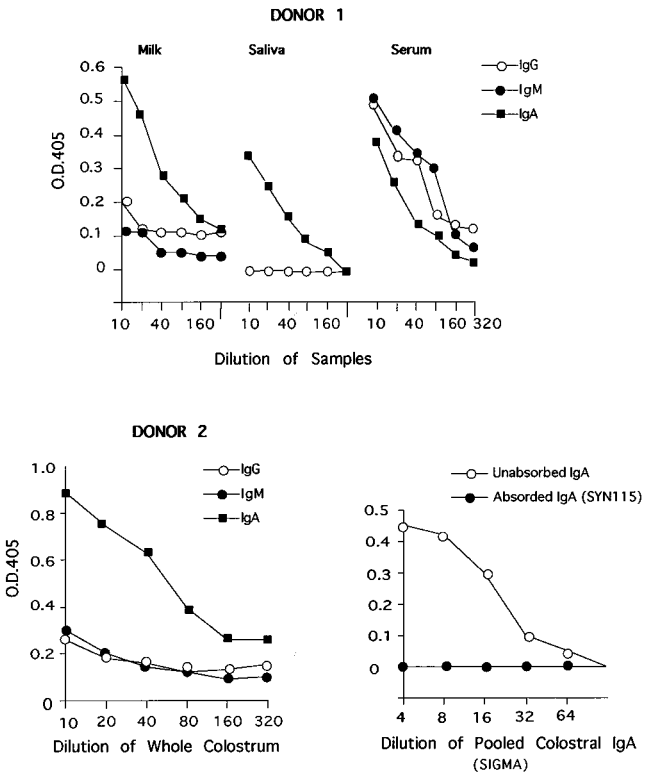

FIG. 1. Immunoglobulin isotype binding to BS^+ bovine thyroglobulin in an ELISA. Immunoglobulin isotype binding was detected by use of different secondary antibodies (IgG, IgM, and IgA). Human colostral IgA was immunodepleted of its BS^+ binding activity by passage over silica-linked Galα1,3Galβ1,4GlcNAc (SYNSORB 115). O.D., optical density.

column was used to control for nonspecific binding of antibodies to silica.

Serum anti-Gal contained all three isotypes, i.e., IgG, IgA, and IgM. Each of the anti-Gal isotypes had low-avidity (NaCl eluate), intermediate-avidity (melibiose eluate), and high-avidity (glycine HCl eluate) antibody populations that bound to Galα1,3Galβ1,4GlcNAc-silica. However, the proportions of these populations differed for each anti-Gal isotype. Most of the anti-Gal IgG was represented in the intermediate-avidity pool, whereas the anti-Gal IgM and IgA were represented mostly by higher-avidity antibodies against this structure.

TABLE 3. Concentration of immunoglobulin isotypes eluted from immobilized Galα1,3Gal and native silica[a]

Specimen type and elution	Concn (μg/ml)					
	IgG		IgA		IgM	
	Syn 115	Silica	Syn 115	Silica	Syn 115	Silica
Serum (sequential elutions)						
1. NaCl (0.5 M)	2.01	3.58	0.10	0.36	0.24	0.20
2. Melibiose (0.4 M)	26.20	0.43	3.53	0.22	14.30	0.98
3. Glycine HCl (0.1 M)	4.84	3.42	6.10	0.21	30.00	4.54
2. + 3. Melibiose + glycine HCl	31.04	3.85	9.63	0.43	44.30	5.52
Bile: glycine elution only	11.80	0.06	10.70	<0.06	0.00	0.00
Colostral IgA (Sigma): glycine elution only			20.20	1.00		

[a] The concentrations of immunoglobulin in whole serum or bile, determined by RID, were as follows. For serum, IgG, IgA, and IgM concentrations were 11,000, 1,950, and 975 μg/ml, respectively. For bile, these concentrations were 3,000, 103, and 240 μg/ml, respectively. The colostral IgA concentration was 5 mg/ml.

The concentrations of immunoglobulins eluted with 0.5 M NaCl from Galα1,3Galβ1,4GlcNAc-silica and from native silica were similar. In contrast, only small amounts of antibody could be eluted from the silica-alone column with melibiose or glycine HCl after NaCl, with one notable exception: nearly equal amounts of the IgG isotype were eluted with glycine from both the SYNSORB and silica columns.

To examine and compare the specificities of these eluted antibodies for Galα1,3Galβ1,4GlcNAc, we studied the binding of these antibodies to bovine thyroglobulin in an ELISA (Fig. 2). The NaCl eluates from both SYNSORB 115 and silica-alone columns had no specificity for this epitope on bovine thyroglobulin (results not shown). However, both melibiose and glycine fractions from the SYNSORB 115 column, but not those from the silica-alone column, bound bovine thyroglobulin. This strongly suggested that the glycine-eluted IgG antibody population that bound to the silica column was very different from that bound to the SYNSORB 115 column, even though their concentrations were similar by RID.

Of interest was that the eluate with the highest avidity for bovine thyroglobulin was the melibiose eluate for all three anti-Gal isotypes (Fig. 2). The avidity of the melibiose eluates, therefore, was different for their cognate epitope on the SYNSORB column, i.e., Galα1,3Galβ1,4GlcNAc-silica (trisaccharide), compared with that on bovine thyroglobulin, where this structure is part of a longer saccharide.

Table 3 also shows the concentrations of anti-Gal IgG, IgA, and IgM in bile and of anti-Gal IgA in pooled colostral IgA by RID. Rate nephelometry gave values similar to those of RID (not shown). It should be noted again that anti-Gal IgA and IgG isotypes were present in bile and that bile anti-Gal IgA represented around 10% of total bile IgA in this bile sample. No anti-Gal IgM was found in this sample, although another bile sample did show large amounts of anti-Gal IgM in addition to amounts of anti-Gal IgA and IgG similar to those found in the sample represented in Table 3 (results not shown). Only a small amount of bile anti-Gal bound nonspecifically to silica. Bile anti-Gal also bound bovine thyroglobulin (not shown).

Anti-Gal IgA could be purified from pooled colostral IgA and represented around 0.5% of total colostral IgA. Only a small amount (around 0.5% of total anti-Gal IgA) bound nonspecifically to silica. The RRBC-agglutinating activity of colostral anti-Gal IgA was 1:16 (starting concentration, 100 μg/ml). Colostral anti-Gal IgA bound bovine thyroglobulin (not shown), as did the whole colostral IgA sample (Fig. 1).

Subclass of human colostral and bile anti-Gal IgA. The subclasses of secretory anti-Gal IgA were studied by ELISA using monoclonal antibodies against IgA1 and IgA2. As shown in Fig. 3, anti-Gal isolated from colostral IgA and from bile contains both IgA1 and IgA2.

Binding of colostral anti-Gal IgA to enteric gram-negative bacteria by dot blot. Colostral anti-Gal IgA bound to all of the bacteria we tested (results not shown). These were mostly isolated from throat cultures (*N. meningitidis*) and normal stool cultures (*Escherichia coli*, *Enterobacter cloacae*, and *Klebsiella pneumoniae*). Of interest also was that anti-Gal IgA bound an *E. coli* K-1 blood isolate (results not shown).

Effect of colostral anti-Gal IgA on the adhesion of *N. meningitidis* 15515 to human buccal cells. We examined the effect of colostral anti-Gal IgA on the adhesion of *N. meningitidis*

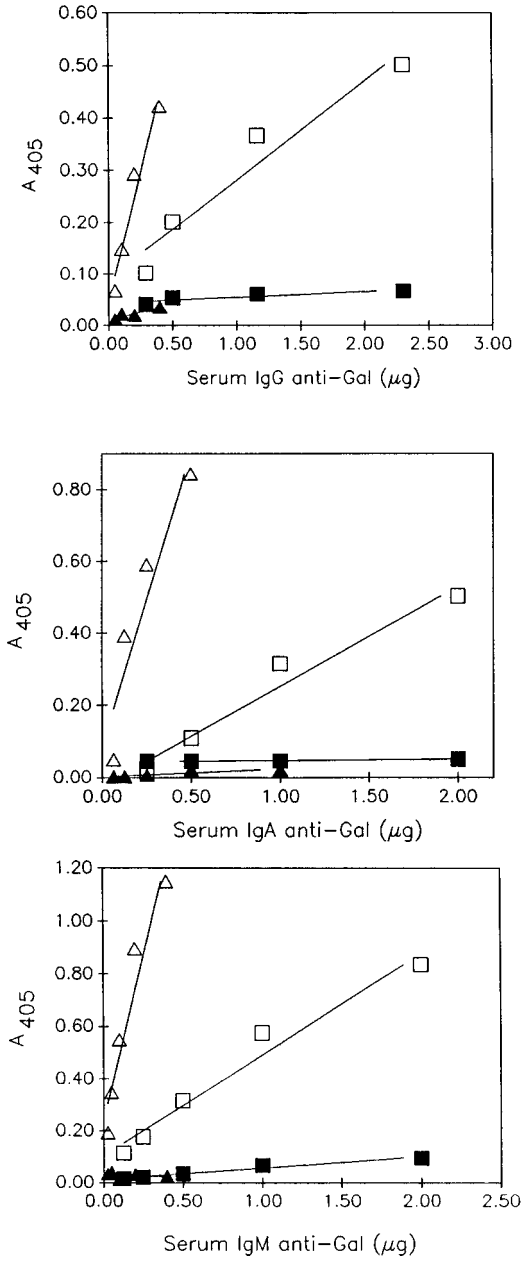

FIG. 2. Binding of melibiose and glycine HCl eluates from SYNSORB 115 and silica columns to bovine thyroglobulin in an ELISA. Symbols: △, melibiose eluates from SYNSORB 115 ▲, melibiose eluates from silica; □, glycine HCl eluates from SYNSORB 115; ■, glycine HCl eluates from silica. Sodium chloride eluates (see Table 3) from SYNSORB 115 and silica columns had no binding to bovine thyroglobulin and are not represented in these graphs. Immunoglobulin isotype binding was detected by the use of different secondary antibodies. (The equation for the melibiose eluate serum anti-Gal IgG is $y = 0.05 + 0.99x$; that for the glycine eluate anti-Gal IgG is $y = 0.08 + 0.19x$, indicating a higher avidity of the melibiose eluate for bovine thyroglobulin compared with that of the glycine eluate. The equation for the melibiose eluate anti-Gal IgM is $y = 0.24 + 2.48x$, and that for the glycine eluate anti-Gal IgM is $y = 0.1 + 0.39x$; the equation for melibiose eluate anti-Gal IgA is $y = 0.09 + 1.62x$, and that for glycine eluate anti-Gal IgA is $y = 0.03 + 0.28x$, supporting similar conclusions.)

15515 to human buccal cells. In the absence of anti-Gal, 34 bacteria on average bound to each buccal cell, whereas only 3 bacteria on average bound each cell in the presence of this antibody ($P < 0.001$) (Fig. 4).

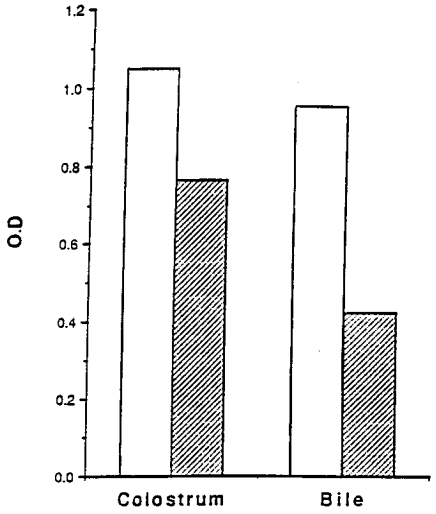

FIG. 3. Subclasses of colostral and bile anti-Gal IgA. Symbols: open bars, IgA1; hatched bars, IgA2. OD, optical density.

There was one bacteria on average bound to each buccal cell in control reaction mixtures that contained buccal cells alone without added *N. meningitidis* 15515 (results not shown).

DISCUSSION

This study describes for the first time the presence of α-galactosyl antibodies in human secretions (saliva, milk, colostrum, bile, and vaginal washings) that have the same specificity as that of human serum anti-Gal. Galili et al. have shown that anti-Gal is abundantly present in all normal human serum (1% of all circulating IgG) and appears to have a relatively restricted specificity for terminal Galα1,3Galβ1,4GlcNAc-R structures (15, 17). We purified secretory antibodies of the same specificities from pooled human colostral IgA and from human bile, using affinity chromatography on silica-linked Galα1,3Galβ1,4GlcNAc, a method described previously for the isolation of serum anti-Gal (17). In addition, we screened for the presence of such antibodies in whole human colostrum, human milk, saliva, and vaginal washings by examining the ability of these samples to agglutinate RRBC and to bind bovine thyroglobulin, both of which express an abundance of

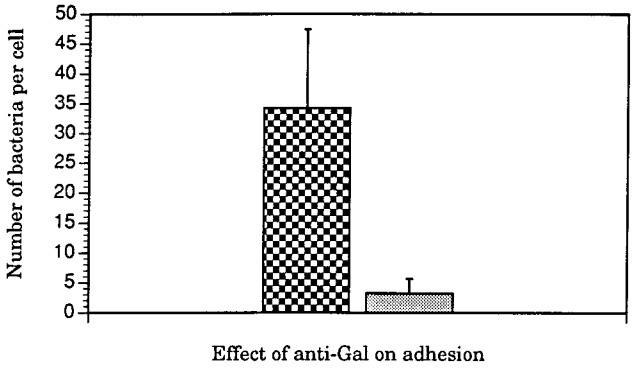

FIG. 4. Effect of colostral anti-Gal IgA on the adhesion of *N. meningitidis* 15515 to human buccal cells. Symbols: ▩, adhesion of *N. meningitidis* to buccal cells in the absence of anti-Gal; ▨, adhesion of *N. meningitidis* to buccal cells in the presence of anti-Gal ($P < 0.001$).

Galα1,3Galβ1,4GlcNAc structures (15). Secretory anti-Gal was found in these samples and appeared to be predominantly IgA in saliva, milk, and bile, although IgG and IgM also were present in human colostrum and IgG was present in human bile. Colostral IgA with anti-Gal specificity represented around 0.5% of pooled colostral IgA and contained both IgA1 and IgA2 subclasses. Similarly, bile anti-Gal contained abundant amounts of anti-Gal IgA of both IgA1 and IgA2 subclasses. These results indicate that anti-Gal appears to be abundantly present in human secretions and accounts for a significant proportion of the total secretory immunoglobulin pool.

Considerable diversity in the recognition of α-linked galactose epitopes by human natural serum antibodies has been reported previously: α1,3, α1,2, and α1,4 antibody specificities all have been described (33). The highest avidity for serum anti-Gal was for galactose α1,3-galactose, with a reduction in avidity noted when the position of the α-linkage was modified, in decreasing order from α1,2 and then α1,6 to the almost-inactive α1,4 bond (33). These studies, however, did not examine the individual clonal affinities of anti-Gal nor did they look into differences in affinities among anti-Gal isotypes.

On the basis of elution studies with melibiose, anti-Gal had been thought to be predominantly IgG (13). Recently, however, Sandrin et al. (27) reported that human natural anti-pig IgM antibodies bound Galα1,3Gal structures, providing evidence that IgM anti-Gal also circulates in humans. We have confirmed the presence of circulating IgM anti-Gal and described for the first time the presence of high titers of anti-Gal IgA in human serum. In addition, we found that the relative avidities for silica-linked Galα1,3Galβ1,4GlcNAc differed among the three isotypes. Anti-Gal IgG was represented mostly in the melibiose-eluted (intermediate-avidity) antibody pool. In contrast, anti-Gal IgA and IgM were represented mostly in high-avidity glycine HCl eluates. In the case of anti-Gal IgM, this may be due to the pentameric nature of the molecule and the higher number of Fab fragments that can bind to Galα1,3Galβ1,4GlcNAc.

Interestingly, when the specificity of these antibodies for Galα1,3Gal on bovine thyroglobulin was examined, we found that the melibiose eluates of all three isotypes had higher avidity for bovine thyroglobulin than the glycine eluates. This may be because antibody that has been eluted by low pH may have become partially denatured, but all glycine eluates were immediately neutralized to avoid this effect. Another explanation is that the Galα1,3Gal cognate epitope on the silica-linked trisaccharide had a different conformation than that on bovine thyroglobulin, where it is part of a much longer, and occasionally branched, polyglycosylceramide. Another finding in our study was the description in human serum and milk of antibodies recognizing Galβ1,3GalNAc and Galα1,4Galβ1,4Glc NAc.

Studies on the distribution of serum anti-Gal and the Galα1,3Gal epitope among primates have revealed a reciprocal evolutionary pattern (14, 18). Old World monkeys, apes, and humans have suppressed expression of the Galα1,3Galβ1, 4GlcNAc structure concomitant with the production of serum anti-Gal. Nonprimate mammals, prosimians, and New World monkeys express this epitope on cell surface glycoproteins and glycolipids but do not synthesize anti-Gal. This study also shows that secretory anti-Gal follows a similar evolutionary distribution in that it is found uniquely in human secretions and does not appear to be present in bile or milk samples from other mammals. That serum anti-Gal is restricted to humans and Old World monkeys and is produced by 1% of B lymphocytes (12) implies some evolutionary advantage to synthesizing this antibody. The reason for the excessive production of circulating and secretory anti-Gal in humans is not clear as yet.

Serum anti-Gal binds to a large number of enteric gram-negative bacteria (19). We had postulated that such enteric organisms may be responsible for constantly stimulating the synthesis of serum anti-Gal in humans (16). It is also likely that these same bacteria stimulate the local production of secretory anti-Gal at the intestinal level. It is further possible that anti-Gal plays an important role in the defense against such bacteria.

Hypothetically, such a protective role of secretory anti-Gal IgA may be mediated by binding to organisms both in the throat and the gut and preventing their attachment to mucosal surfaces. Such a mechanism may be related to the well-established findings that breast-feeding effectively protects the newborn against gastrointestinal, respiratory, and ear infections (5, 24) and may prevent neonatal sepsis (25, 35).

Our observation that bile contains anti-Gal IgA and IgG is particularly intriguing in light of our recently published observation that serum anti-Gal binds to gram-negative and gram-positive bacteria isolated from pigmented gallstones (31). In the present study, we found that human colostral anti-Gal IgA binds to enteric gram-negative organisms isolated from the stools of normal infants and toddlers and to a blood isolate of *E. coli* K-1, the serotype that causes neonatal sepsis (4).

The reported associations of high levels of serum anti-Gal IgA with purpura and nephropathy (7) and of anti-Gal IgG with autoimmune thyroid disorders (34), however, leaves open the question of whether anti-Gal may actually promote autoimmune diatheses. We have found that anti-Gal binds more often to gram-negative organisms isolated from the bloodstreams of patients with sepsis than to similar bacteria cultured from normal stools (19). For *E. coli* and *Citrobacter freundii*, expression of α-galactosyl structures is a necessary but insufficient attribute for survival within the bloodstream (19). The ability of serum anti-Gal to block the alternative complement pathway-mediated killing of certain gram-negative bacteria may promote their intravascular survival (19).

Many organisms that cause sepsis or are associated with autoimmune disorders originate in the gut (20). These bacteria then have to survive in the bloodstream to cause disease or establish metastatic infections (20). In view of the present study, it is possible that whereas with some organisms expression of α-galactosyl surface structures that bind anti-Gal may be used to evade the host's circulatory immune defense, with others this natural antibody may contribute to the prevention of their invasion into the body.

ACKNOWLEDGMENTS

We would like to thank Gary A. Jarvis for his critical review of the manuscript.

This work was supported in part by the Veterans Administration and Public Health Service grants AI 31461 (R. Hamadeh), AI 21171 (J. M. Griffiss), and AG 06299 (U. Galili) and by the University of California San Francisco Research Evaluation and Allocation Committee grant 36517 (R. Hamadeh).

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