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Reaction of Human Colostral and Early Milk Antibodies with Oral Streptococci

F. MICHAEL EGGERT[1]* AND B. W. GURNER[2]

Department of Stomatology, Faculty of Dentistry, University of Alberta, Edmonton, Alberta, Canada T6G 2N8,[1] and Division of Immunology, Department of Pathology, University of Cambridge, Cambridge CB2 2QQ, England[2]

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Colostrum or early breast milk or both from each of 16 healthy women contained agglutinating antibodies for all normal streptococcal inhabitants of the human oral cavity (*S. mutans*, *S. sanguis*, *S. mitis*, and *S. salivarius*), including those which colonize the neonatal oral cavity in significant numbers. Agglutination correlated with the amount of immunoglobulin A (IgA) binding to bacterial surfaces as measured by mixed reverse passive antiglobulin hemagglutination. Surprisingly, colostral IgA agglutinated our control organism, *Brucella abortus*. Low levels of colostral or milk IgM and IgG antibodies also reacted with all of the test bacteria. Absorption studies with an enzyme-linked immunosorbent assay showed that a proportion of antibodies in colostrum and early milk is specific for each of the different oral streptococci. Fractionation on Sepharose 4B indicated that 11S secretory IgA is the predominant form of colostral and milk antibody for all of the test bacteria, including *B. abortus*. No evidence was found that reactions other than antigen-antibody reactions resulted in binding of colostral immunoglobulins by any of the test bacteria.

The spectrum of antibacterial antibodies in human colostrum and early milk is of interest in understanding the role of secretory antibodies both during immediate postnatal development and in subsequent development and control of a normal gastrointestinal tract flora. This role of secretory antibodies might be readily investigated in the human oral cavity because of its accessibility. Sterile at birth, within hours the human oral cavity acquires a microbial flora (22). Colostral immunoglobulin A (IgA) antibodies react with common members of the gastrointestinal tract flora (1, 5, 13, 23). Studies of human colostral antibodies reacting with oral organisms have focused exclusively on *Streptococcus mutans* (5, 13). Recent studies have investigated effects of secretory antibodies and other glycoproteins on adherence of intact oral bacteria to simulated tooth surfaces (19, 21, 25), but none have investigated maternal colostral antibodies against those bacterial species that colonize the epithelia of the neonatal oral cavity.

We set out to (i) demonstrate the occurrence of maternal colostral milk antibodies against those oral streptococcal species that colonize the neonatal oral cavity, (ii) measure the antibody class and the levels of these secretory antibodies, (iii) distinguish the antibodies from non-immunoglobulin glycoproteins also capable of reacting with bacteria, and (iv) determine whether the colostral antibodies exhibited specificity for individual species of oral streptococci.

MATERIALS AND METHODS

Human colostrum and early breast milk collected during the first 5 days postpartum from 15 healthy women at Mill Road Maternity Hospital (Cambridge, England) and from 1 woman at The London Hospital (London, England) were cleared and stored at −30°C (12). Immunoglobulin concentrations were measured by using rocket immunoelectrophoresis by R. J. Thompson (Department of Clinical Biochemistry, Addenbrooke's Hospital, Cambridge, England) with serum IgA standards (DAKO) and correction for secretory IgA (2, 28).

Oral streptococci. *S. sanguis* B-12, *S. mutans* Ingbritt IB, *S. mitis* B-121, *S. salivarius* B-215, and *Brucella abortus* Weybridge were obtained and grown as described previously (11, 13). Confirmation of streptococcal identities was performed by J. Hardie and R. A. Whiley (The London Hospital Medical College, Dental School, London, England). All of the bacteria were negative for protein A-like reactivity (15).

Direct agglutination and mixed reverse passive antiglobulin hemagglutination (MRPAH) using the bacterial dilution procedure, each with appropriate controls, were performed as previously described (11, 13). Class-specific antiglobulins were provided by R. R. A. Coombs (University of Cambridge, Cambridge, England). Reverse passive antiglobulin hemagglutination (RPAH) with the same indicator cells as MRPAH is as sensitive as radioimmunoassay (27) and more easily undertaken with simple equipment. MRPAH permits rapid and sensitive quantitation of antibacterial antibody classes in large numbers of samples without specialized equipment. Phosphate-buffered saline (PBS) (2 volumes of PBS:1 volume of water) used during bacterial sensitization and washing gave one to two dilution steps higher antibody titers with colostrum than isotonic PBS and no evidence of false-positive reactions. There was no detectable nonspecific immunoglobulin uptake by *B. abortus* using a human chronic brucellosis serum previously absorbed three times with *B. abortus*. Isotonic PBS was used during the hemagglutination assay and chromatography. Calcium dependence of reactions was tested using PBS containing 0.05 M EDTA (12, 20).

Bacteria used for enzyme-linked immunosorbent assay (ELISA) were sensitized and washed as for MRPAH. Sensitized bacterial suspensions were reacted with an equal volume of 1/50 dilutions of peroxidase-labeled anti-human alpha-chain specific antiglobulin (DAKO) for 30 min at 20°C. After washing, bacterial suspensions which had reacted with the antiglobulin were titrated in doubling dilutions and tested at 492 nm for peroxidase (31) with a Titertek Multiskan (Flow Laboratories). A baseline control was provided by measuring serial dilutions of unsensitized bacterial suspensions which had been reacted with peroxidase-labeled antiglobulin.

For cross-absorptions, each 0.1 ml of colostrum was

* Corresponding author.

TABLE 1. Colostral anti-streptococcal antibodies measured by direct agglutination[a]

Organism	Titer				Spec act IgA[b]
	Mean	SEM	Range	Median	
S. sanguis B-12	5.8	0.7	1–10	6	0.232 ± 0.060
S. mutans IB	5.4	0.4	3–9	6	0.221 ± 0.050
S. mitis B-121	6.7	0.5	3–10	6	0.128 ± 0.029
S. salivarius B-215	4.3	0.4	1–6	4	0.575 ± 0.125
B. abortus	3.8	0.6	1–10	3	1.369 ± 0.327

[a] Titers are $\log_2$. The control organism was *B. abortus*. $n = 16$.
[b] Specific agglutinating activity of IgA: concentration of colostral IgA (milligrams per milliliter) containing one agglutinating unit ± SEM.

allowed to react for 30 min at 20°C with bacteria from 0.1 ml of a stock suspension (2 to 4 μl of packed bacteria per absorption step). This procedure was repeated three times. Colostral specimens were selected for cross-absorption from those demonstrating appreciable antibody titers by MRPAH; therefore, this regimen did not produce total antibody absorption.

Chromatography on a Sepharose 4B column (Pharmacia GB; 1.6 by 80 cm) used upward flow of isotonic PBS at 15 ml/h. Fractions (7.5 ml) were collected with flow monitoring at 279 nm (LKB Uvicord S). Cleared 2-ml samples (colostra code names, SHE, McG, and CAL; milk code name, SAN) were chromatographed, purified immunoglobulins in fractions were identified and quantitated by RPAH, and then the fractions were reconcentrated to 2 ml by ultrafiltration (Amicon CF 25 Centriflo cones) and tested for antibodies by MRPAH.

Statistical methods included linear regression, correlation analysis, and one-way analysis of variance (4).

RESULTS

Concentrations of colostral IgA and IgM. Concentrations of colostral IgA and IgM agreed with previously published values (3, 7): IgA, 10.1 mg/ml ± 2.2 standard error of the mean (SEM), range, 1.1 to 35.9 mg/ml, $n = 16$; IgM, 0.8 mg/ml ± 0.4 SEM, range, 0.1 to 7.0 mg/ml, $n = 16$. Colostral IgA and IgM concentrations were strongly correlated: IgM = 0.090 + 0.032 IgA; $n = 15$; $r = 0.69$; $P < 0.01 > 0.001$.

Direct agglutination. All test bacteria, including *B. abortus*, were agglutinated by all colostra (Table 1). Specific agglutinating activities of IgA in 16 colostra showed that *B. abortus* was less strongly agglutinated than the oral streptococci (Table 1). All agglutinations were EDTA insensitive.

MRPAH. All test bacteria reacted with colostral IgA in all specimens. IgM and IgG antibodies were frequently present (Table 2). Use of 0.05 M EDTA during all stages had no measurable effect on uptake of any antibody class onto any of the test bacteria. *B. abortus* is more readily agglutinated than the test streptococci, which are similar to each other in agglutinability. An MRPAH titer for antibodies to *B. abortus* represents less immunoglobulin bound to the bacterial surface than an equal titer of antibody to the streptococci.

Agglutination versus immunoglobulin uptake. Direct agglutination and the uptake of secretory IgA antibodies by bacteria were significantly correlated (Table 3). There was no significant correlation between direct agglutination and either IgM or IgG uptake except for reactions of IgM antibodies with *B. abortus* and IgG antibodies with *S. salivarius* (Table 3).

Chromatography. In all specimens secretory IgA eluted between colostral 19S IgM and 7S IgG (Fig. 1). The bulk of colostral IgA is 11S secretory IgA (29). Because the anti-gamma reagent was more sensitive than the others, RPAH was only used to accurately locate the elution positions of the three immunoglobulin classes. In all four specimens the elution position of colostral 11S IgA on Sepharose 4B coincided with the elution position of direct agglutinating activity for *S. mutans* and *S. salivarius* (Fig. 1). In all specimens the elution position of IgA antibodies reacting with streptococci also coincided with that of 11S IgA (Fig. 1). The slight skewing of secretory IgA antibodies measured by MRPAH toward the 19S fraction is in agreement with previous findings of higher-molecular-weight polymers of secretory IgA (29). IgM and IgG antibodies were detected after fractionation (not shown).

Antibodies to the control organism, *B. abortus*, produced

TABLE 2. Reaction of colostral antibodies with oral streptococci measured by MRPAH[a]

Organism	Anti-globulin	Titer			
		Mean	SEM	Range	Median
S. sanguis B-12	G	1.0	0.3	0–4	0
	M	1.3	0.5	0–8	0.5
	A	5.2	0.6	1–10	5.0
S. mutans IB	G	1.9	0.3	0–5	2.0
	M	2.3	0.5	0–7	2.0
	A	5.5	0.6	1–10	6.0
S. mitis B-121	G	2.8	0.3	1–5	3.0
	M	1.6	0.4	0–6	1.0
	A	5.9	0.6	1–10	6.0
S. salivarius B-215	G	2.6	0.5	0–5	2.5
	M	2.4	0.5	0–7	2.0
	A	6.4	0.6	1–10	6.5
B. abortus	G	0.9	0.5	0–9	0
	M	1.7	0.4	0–5	1.5
	A	5.8	0.7	1–10	5.5

[a] Titers are $\log_2$. $0 = \log_2 < 1$. Control organism, *B. abortus*. $n = 16$.

TABLE 3. Correlation between direct agglutination (DA) titers and binding of immunoglobulins measured by MRPAH

Organism	DA versus IgA uptake ($n = 16$)		DA versus IgM uptake ($n = 16$)		DA versus IgG uptake ($n = 16$)	
	r	P	r	P	r	P
S. sanguis B-12	0.66	<0.01 >0.001	0.15	NS[a]	0.02	NS
S. mutans IB	0.55	<0.05 >0.01	0.49	NS	0.41	NS
S. mitis B-121	0.52	<0.05 >0.01	0.47	NS	0.09	NS
S. salivarius B-215	0.62	<0.05 >0.01	0.43	NS	0.54	<0.05 >0.01
B. abortus	0.56	<0.05 >0.01	0.64	<0.01 >0.001	0.35	NS

[a] NS, Not significant.

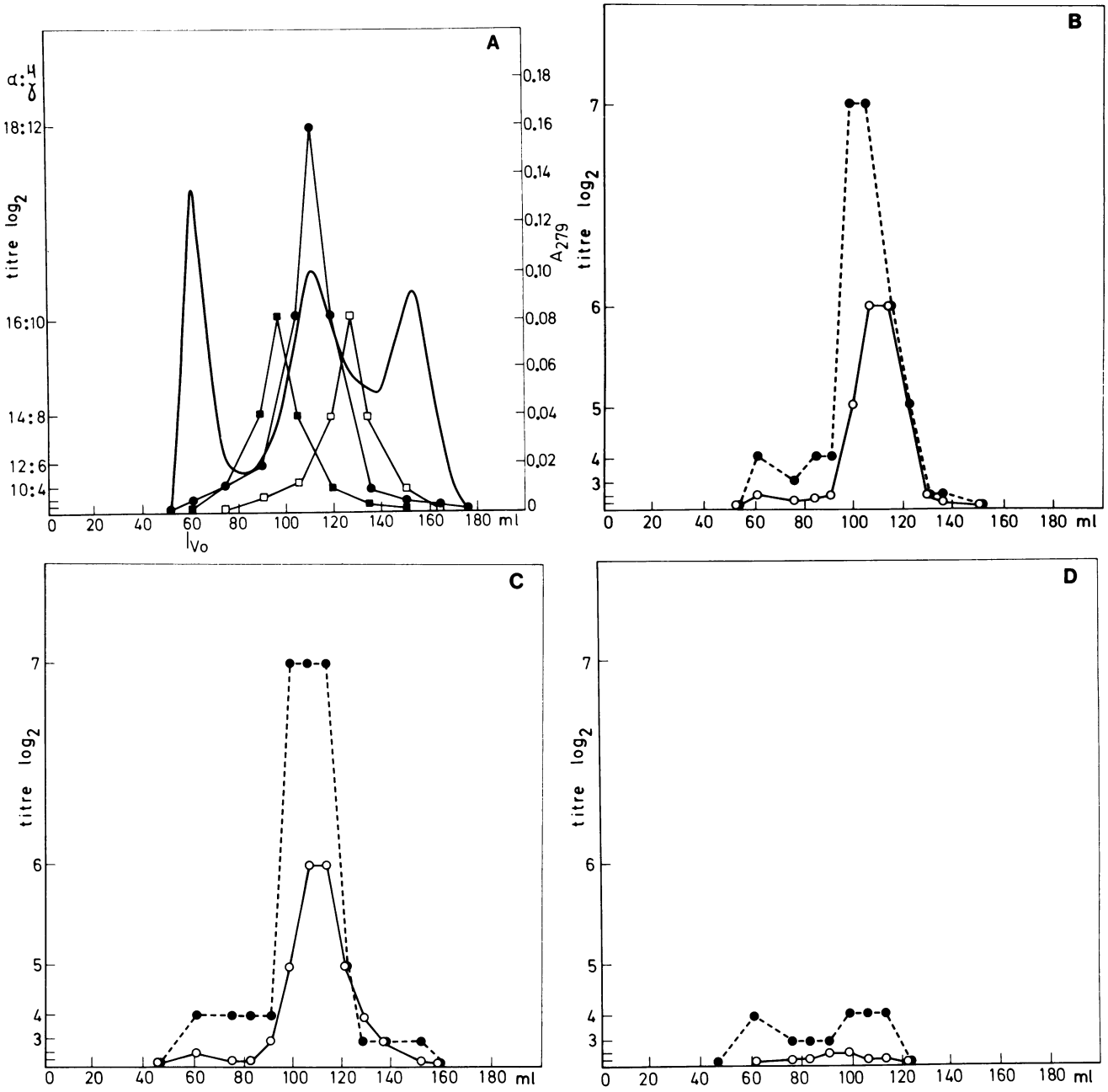

FIG. 1. Gel filtration of human colostral antibodies on Sepharose 4B. The column was 1.6 by 80 cm, the sample was McG (2 ml), the flow was 15 ml/hr, and the eluent was PBS. (A) Elution of IgA, IgM, IgG, and other proteins by RPAH. ●, IgA; ■, IgM; □, IgG. A_{279}, Absorbance at 279 nm. (B) Elution of IgA antibodies to *S. mutans* IB. (C) Elution of IgA antibodies to *S. salivarius* B-215. (D) Elution of IgA antibodies to *B. abortus*. Symbols: ○, direct agglutination; ●, IgA (MRPAH) (IgM, and IgG not shown).

much lower peaks than antibodies to streptococci (Fig. 1) in both colostra tested for this antibody. In the specimen of milk, antibodies to oral streptococci were readily detected after chromatography but the initially very-low-titer antibody to *B. abortus* was lost.

Void volume was revealed by opalescence of fractions containing very-high-molecular-weight complexes not fractionated at all on Sepharose 4B (Fig. 1), unlike blue dextran (Pharmacia), most of whose smaller molecules enter Sepharose 4B. Low titers of IgA in void volume fractions reacting with test bacteria were associated with minimal direct agglutination (Fig. 1), nor could this IgA uptake be inhibited with EDTA.

Cross-absorption and ELISA. Only reactions with the homologous absorbing organisms showed consistent and marked lowering of antibody levels in three colostra (Table 4). Analysis of variance of the three experiments indicated that statistically significant differences exceeded ±13 to ±17% at the 98% confidence level (Table 4). Values within these limits reflect experimental variation. There was an indication of a weak partial cross-reaction between *S. mutans* IB and *S. salivarius* B-215 with *S. mutans*, but not with *S. salivarius* as absorbing agent. The basis for this partial cross-reaction was not further investigated. Colostrum Lee had low-affinity antibodies that were difficult to absorb (Table 4).

TABLE 4. Specificity of colostral anti-bacterial antibodies determined by cross-absorption and ELISA

Organism	Antibodies (% of unabsorbed control)[a]										
	Den colostrum			Row colostrum				Lee colostrum			
	IB	Bru	215	IB	Bru	215	121	IB	Bru	215	121
S. mutans IB	24	70	86	36	123	92		60	89	94	
B. abortus	105	38	78	112	40	102		97	76	106	
S. salivarius B-215	48	84	12	82	66	43		62	80	52	
S. mitis B-121	60	89	63				37				55
S. sanguis B-12	95	85	114								

[a] After absorption three times with *S. mutans* Igbritt (IB), *B. abortus* (Bru), *S. salivarius* B-215 (215), and *S. mitis* B-121 (121). The 98% confidence limits were Den, ± 16%; Row, ± 13%; and Lee, ± 17%. Significant values ($P < 0.02$) exceeded 100% ± confidence limit. One-way analysis of variance: Den ($F = 40.5$; df 14, 254; $P < 0.005$); Row ($F = 60.5$; df 9, 171; $P < 0.005$); Lee ($F = 15.8$; df 9, 80; $P < 0.005$). Underlining emphasizes homologous absorptions in contrast with heterologous absorptions.

DISCUSSION

We have illustrated two aspects of secretory antibodies which occur naturally in human colostrum. First, the 11S IgA antibodies reacted with the test streptococci (Fig. 1). The non-immunoglobulin glycoproteins of gastrointestinal tract secretions that react with both bacteria and immunoglobulins (11, 12, 20) were not detected. Second, because antibodies in all normal colostra reacted with all test bacteria, we had to show that at least a proportion of each observed reaction was specific. Cross-absorption followed by ELISA demonstrated that a proportion of antibodies in colostra were specific for surface components of each of the test bacteria, including the unexpected reaction with *B. abortus* (Table 4). This is the first demonstration of specific human colostral antibodies against such a wide range of oral streptococcal species, including those that colonize the neonatal oral cavity in significant numbers (22).

Intact bacteria provide the most realistic simulation of the interaction of secretory antibodies with bacteria in vivo (30) and their effects on bacterial adherence to oral surfaces (19, 21, 25). For all bacteria direct agglutination was significantly correlated with IgA uptake (Table 3). The bulk of colostral and milk agglutinating activity resided in the secretory 11S IgA fraction eluting between IgM and IgG, in agreement with our previous results (10). Colostral IgM and IgG antibodies were demonstrated by us previously (13).

Secretory IgA antibodies measured with antiglobulins can be clearly separated both from other immunoglobulin classes and from high-molecular-weight glycoproteins producing bacterial aggregation and causing nonspecific immunoglobulin binding by bacteria (10, 12, 17, 20). There was no evidence of such glycoprotein activity in our samples. In none of the chromatographic fractions were the reactions of colostral immunoglobulins (IgA, IgM, and IgG) EDTA sensitive, unlike the reactions of the high-molecular-weight glycoproteins (11, 12, 17, 20). IgA in void volume fractions that reacted with bacteria most likely represents small amounts of aggregated IgA. Secretory component is not involved in nonspecific reactions of 11S IgA with streptococci (S. Suzuki and R. R. A. Coombs, unpublished data), and nonspecific IgA binding is uncommon with oral streptococci (14). There was so little nonspecific binding of secretory IgA to the test bacteria that we were able to demonstrate specific binding of small amounts of colostral antibodies to *B. abortus* against a background of relatively high total IgA concentrations.

Highly sensitive methods such as MRPAH and ELISA increase the chance of observing unusual antibody reactions, since even trace amounts of cross-reacting antibody can be demonstrated. The sensitivity of these methods is such that colostral IgM and IgG antibodies can be demonstrated directly (13). Specific activities of colostral antibodies against oral streptococci were more than 50% higher than those against *B. abortus*. The low-titer colostral antibody reacting with *B. abortus* may have been induced by lipopolysaccharide from an unrelated gram-negative organism in a manner analogous to that of serum and salivary antibodies which cross-react with lipoteichoic acid from different gram-positive bacteria (8, 26). The low affinity of such cross-reacting antibodies to *B. abortus* is reflected by the difficulty of achieving total absorption during cross-absorption studies. A significant proportion of our naturally occurring colostral antibodies were specific for each species of oral streptococcus. The nature of the bacterial surface antigens with which the colostral antibodies react specifically is under investigation.

The finding that all normal human colostra we tested contained secretory IgA antibodies against a broad range of the normal adult oral flora (Tables 1 and 2) might at first seem unremarkable. However, it is of biological interest that the mother provides such high titers of antibodies against common oral streptococci. Our current knowledge suggests that maternal secretory antibodies are involved in the development of a normal human infant gastrointestinal tract flora (16, 24, 30). A mother provides her infant with these secretory antibodies at a time when normal bacteria, originating from her (6, 9, 18), begin to populate the infant gastrointestinal tract. Reactions of human colostral antibodies with noncariogenic oral streptococci are as strong as those of colostral antibodies against *S. mutans*, suggesting an equivalent biological importance to the neonate. The secretory immune system of a healthy human mother does not appear to be engaged in the selective provision of colostral antibodies against highly pathogenic bacteria. Any biological effect of colostrum from a healthy mother appears to involve provision of the neonate with high titers of secretory antibodies against the entire range of oral streptococci, as shown here, and also against a wide range of other normal inhabitants of the adult gastrointestinal tract (1, 23) in combination with any antibodies against pathogens.

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