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Selective concentration of IgD class-specific antibodies in human milk

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SUMMARY

The participation of human IgD class antibody in local immune responses of breast tissue was studied by analysing the sera-to-milk ratios of total IgD, IgM, IgA, IgG isotypes and albumin found in matched samples, and by analysing the sera-to-milk (S/M) ratios of IgD, IgM, IgA, IgG antibodies against *Haemophilus influenzae* capsular polysaccharide (PRP), phosphorylcholine, tetanus and in some cases diphtheria antigens. The study group consisted of eight women immunized during pregnancy with PRP, and control, unimmunized women. Albumin, and total IgG showed high S/M ratios. IgA had a low S/M ratio as expected, consistent with reports that IgA is locally concentrated. Total IgD and IgM isotype ratio values were intermediate between IgG and IgA suggesting they were selectively concentrated in breast fluids due to local production or transport mechanisms, or both. Ratios for specific antibodies of IgA and IgM isotypes and for total IgA and IgM isotype showed parallel data. Among the IgD antibodies, those specific for PRP and phosphorylcholine suggested a higher degree of selective concentration as compared with tetanus antigen. In the group of unimmunized women, although selective concentration of total IgD was observed, specific antibody studies were inconclusive due to the low milk IgD antibody levels encountered. The results indicate that IgD (and also IgM) may participate in local immune responses of human breast tissues and fluids; possibly influenced by the nature of the antigen, the state of immunization and the hormonal environment (pregnancy).

Keywords IgD and milk

INTRODUCTION

Mucosal immunity is mediated by secretory IgA which is the major immunoglobulin isotype of human secretions. It is thought that enteric sensitization by antigen is followed by the dissemination of committed IgA-precursor B cells to various peripheral secretory organs (Hanson, 1982; Mestecky, 1987). Among the non-gastrointestinal sites of mucosal or local immunity, the mammary gland has special features. The lactating breast shows an influx of IgA-producing lymphoid cells which may be tissue specific. A mammary gland autotransplant into the anterior chamber of the eyes of mice treated with lactogenic hormones demonstrated IgA-producing-cell infiltration (Franklin, Prendergast & Silverstein, 1978). Women immunized with the bacterial capsular polysaccharide of *Haemophilus influenza* (PRP) during pregnancy, in contrast to women immunized prior to becoming pregnant, show evidence of increased specific antibody production in breast milk (Insel, Amstey & Pichichero, 1985). Up to 3×10^6 white blood cells/ml are found in human colostrum. These cells are predominantly macrophages, but include B and T lymphocytes (Parmely, Beer & Billingham, 1976). Breast feeding has been reported to protect the human newborns against gastrointestinal, respiratory and possibly allergic problems; the mechanism is unclear and the observations remain controversial.

In addition to IgA, other Ig classes, including IgD, may be involved in local immunity of the breast. In support of IgD involvement, Steel & Leslie (1985) reported high levels of locally produced IgD in rats. In humans, Bahna, Keller & Heiner (1982) showed that six out of 14 women had high colostrum/plasma antibody ratios of IgD and IgE 'allergy-related' antibodies and concluded there was evidence for local concentration of antibodies in breast tissue secretions.

The last studies in particular were similar in design to the present efforts. Both measure human total immunoglobulin classes and specific immunoglobulin class antibodies in sera and milk of mothers. The current efforts differ in that they tested for a different panel of non-allergy-related specific antibodies, several of which have been shown to involve IgD class antibodies. In addition to confirming observations on selective concentration of IgD specific antibodies in breast secretions, the

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experimental results suggest the possibility of differences among antigens in isotype specific responses.

MATERIALS AND METHODS

Sources of sera and milk

As part of a study of parenteral immunization, eight 34–36-weeks pregnant women were immunized with the type b *H. influenzae* capsular polysaccharide (PRP); sera and milk samples were collected 1 month post-partum as described (Insel *et al.*, 1985; Amstey *et al.*, 1985). Controls were eight unimmunized pregnant women of comparable ages.

Micro-ELISA

A sandwich-type micro-ELISA was used to measure IgD, IgM, IgG, and IgA in 96-well polyvinylchloride plates (Dynatech, Alexandria, VA). Plates were coated with anti-class-specific immunoglobulin antibody diluted in bicarbonate buffer overnight at 5°C, and then were blocked with 0·5% bovine serum albumin (BSA) for 2 h. Test sample or standards were next added for 2 h. A third step involved incubation with a biotin anti-human immunoglobulin specific for the assayed human immunoglobulin class, for 3 h. Lastly, an avidin horseradish peroxidase conjugate and OPD substrate was added to develop a colorimetric reaction (Litwin & Zehr, 1987a). The assay was performed at room temperature. Between steps, plates were sequentially washed with deionized water and phosphate-buffered saline (PBS) containing 0·05% Tween 20. Human immunoglobulin serum standards were employed (Calbiochem-Behring Diagnostics, San Diego, CA) to construct standard curves. Goat affinity-purified reagents were used throughout. For IgG the antibody used to coat the plate was obtained from Southern Biotech., 2040-01 (Birmingham, AL), and the upper antigamma layer was from Sigma, B1140 (St Louis, MO). For IgM the coating antibody was obtained from Tago, 4102 (Burlingame, CA) and the upper layer antibody from Sigma, B1265. For IgA the coating layer was from Southern Biotech., 2050-01, and the upper layer from Sigma, B1015. For IgD the first antibody was from Tago, 4105 and the upper layer antibody anti-delta from Sigma, B2140. The last or uppermost layer in all cases was avidin-peroxidase from Sigma, A3151. To confirm the specificity of the micro-ELISA assays, inhibition studies were undertaken. IgD specificity was analysed by adding purified monoclonal immunoglobulin proteins at the same time as the anti-class antibody; inhibitor concentrations ranged from 0·01 to 10 μg/ml. Chromatographically purified monoclonal IgM was obtained from Cooper Biomedical (Malvern, PA), 21039; monoclonal IgA was from Behring (San Diego, CA), 401100; monoclonal IgG was purified from a myeloma sera by salt precipitation followed by protein A chromatography; monoclonal IgD (Gow) was purified by anti-delta-Sepharose immunoaffinity chromatography and showed a characteristic delta band on SDS–PAGE run under reducing conditions. Different anti-delta antibodies were employed in the micro-ELISA and in the anti-delta immunoaffinity column.

MicroELISA was adapted to detect specific antibodies of IgD, IgM, IgG, and IgA isotypes. The following antigens were used to coat 96-well microwell plates: phosphorylcholine (PC)–BSA provided by Dr B. Pollok, Guthrie Foundation for Medical Research, Sayre, PA; PRP (Insel & Anderson, 1986a); and diphtheria and tetanus toxoids (Mass Biol Lab, Boston, MA). Plates were coated at predetermined optimum dilutions for 90 min at 37°C in PBS for PRP, and 3 h at room temperature in a carbonate–bicarbonate coating buffer for all other antigens, then blocked with 0·5% BSA–PBS Tween for 2 h at room temperature. The test sample was added for 2 h at room temperature or overnight at 4°C. A biotinylated antiglobulin was next added. The same biotinylated agents were employed as used in the 'sandwich' micro-ELISA. The reaction was developed with avidin-horseradish peroxidase followed by the appropriate substrate and read at 60 min. Between steps, plates were washed. For the PC antibody assay, polyvinylchloride plates (Dynatech, 001-010-2801) were used. For all other assays polystyrene plates were obtained from Flow Laboratories, 76-381-04 (McLean, VA). Spectrophotometric readings were converted to arbitrary units taken from standard curves constructed for each individual specific antibody ELISA by assigning 100

Table 1. Sera/milk (S/M) ratios of IgD class-specific antibodies in immunized mothers

Patient no.	Albumin (μg/ml)	Total IgD (AU)	IgD Antibodies* Anti-PRP	Anti-PC	Anti-tetanus
1					
S	29000	3·4	47	15	40
M	>310	0·05	33	11	0·60
S/M	<94	68	1·4	1·4	67
2					
S	13000	140	4·5	13	40
M	NT	4·3	16	100	NT
S/M	NT	33	0·28	0·13	NT
3					
S	17000	48	100	82	100
M	NT	0·23	10	6·2	NT
S/M	NT	210	10	13	NT
4					
S	17000	12	18	24	31
M	200	0·06	7·5	<15	<0·60
S/M	85	200	2·4	>1·6	>52
5					
S	NT	32	>290	28	90
M	200	0·13	63	2·8	<0·60
S/M	NT	250	>4·6	10	>150
6					
S	21000	5·8	>290	26	75
M	220	<0·063	57	1·5	<0·60
S/M	92	92	>5·1	17	>130
7					
S	29000	54	100	22	68
M	240	0·36	25	2·4	<0·60
S/M	120	150	4·0	9·2	>110
9					
S	17000	2·0	50	22	55
M	>310	<0·005	12	2·4	<0·60
S/M	<55	>400	4·2	9·2	>92

* Anti-diphtheria antibodies of IgD isotype were tested in six subjects. The ratios of S/M were >34, >36, >64, >84, >84, >84.

AU, arbitrary units; NT, not tested.

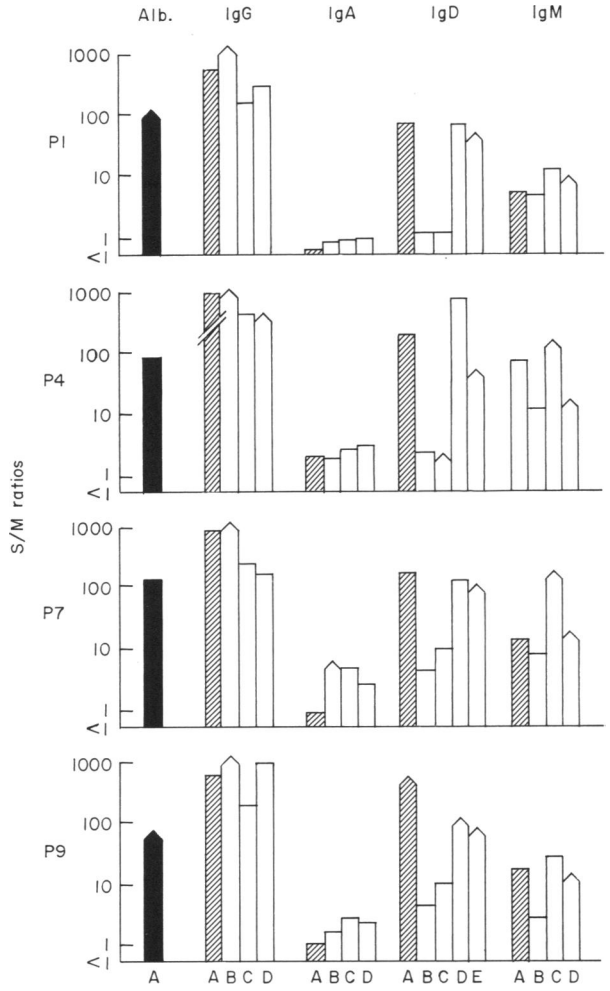

Fig. 1. Serum-to-milk ratios (S/M) in four immunized mothers. Alb., albumin. A, total isotype values; B, anti-PRP antibody; C, anti-PC antibody; D, anti-tetanus antibody; and E, anti-diphtheria antibody. □, total isotype values. The units for the original data (sera and milk values) are in μg/ml for all A bars and are arbitrary units for B, C, D and E bars. Pointed bars are > values.

arbitrary units to the sample which resulted in a high optical density but still could be diluted for a standard curve. All samples, serum or breast fluids from an individual were assayed simultaneously for antigen or isotype.

Albumin determinations

Albumin was measured by using radial immunodiffusion plates with standards from Behring Diagnostics (LC-Partigen Albumin Kit).

RESULTS

Immunoglobulin classes and albumin

Consistent with published studies (Keller *et al.*, 1985), neither albumin nor IgG showed evidence of local concentration in breast milk. This is indicated by the high serum/milk (S/M) ratios in individual samples (Table 1, Fig. 1) or median S/M ratios in Table 2. In sharp contrast to IgG and albumin and consistent with prior observations, IgA values were similar in milk and sera in most samples; S/M ratios were unity or less. IgD and IgM showed intermediate patterns; total IgD ratios ranged from 33 to >400 and total IgM ratios from 5·7 to 190. The IgD ratios were less than the IgG ratios in all instances. These findings raised the issue of local production of IgD and IgM antibodies.

Isotype-specific antibody (Tables 2, 3).

Specific antibodies of each class of the *H. influenzae* type b PRP, PC, tetanus and in some cases diphtheria antigens were quantified. The choice of antibody system was based on the availability of purified antigens and a reliable assay, the desire to test both polysaccharide and protein antigens, and the fact that an immunized group of mothers was available (Insel *et al.*, 1985). There was no evidence for local concentration of IgG antibody in milk: S/M ratios for specific IgG antibodies were consistently high. In contrast, IgA-specific antibodies showed low S/M ratios for all antigens consistent with local concentration of IgA in milk. IgD anti-PRP and IgD anti-PC ratios in PRP-immunized mothers were lower than total IgD S/M ratios. However, IgD anti-tetanus S/M ratios were similar to total IgD S/M ratios. IgM showed similar total and specific IgM antibody S/M ratios: IgM ratios of anti-PC and anti-PRP did not differ from anti-tetanus antibodies. The data are interpreted as indicating local IgM and IgD concentration in breast tissue or milk which differs sharply in the pattern of antibody responses among the antigens tested.

Comparison of PRP immunized and unimmunized mothers

PRP-immunized mothers (patients 1–9) were compared with eight unimmunized pregnant control women (patients 10–17) to determine the role of recent antigen contact. Results showed selective concentrations of total IgD in certain sera. Specific antibodies were too low for analysis in unimmunized individuals.

DISCUSSION

The observation of IgD antibodies in breast milk focuses attention on the role of IgD in local immunity. Earlier published observations documented the presence of IgD-secreting cells in breast tissues (Brandtzaeg, 1983). The current report suggests that these cells are producing local antibody. Interpretation of the IgD data is facilitated by the clearcut findings for the IgG and IgA isotypes, which, as predicted from past findings, show respectively a lack of, and strong evidence for selective concentration of specific antibodies. IgG and IgA can thus serve as negative and positive 'controls.'

Several explanations for the phenomenon of local concentration of IgD can be considered. IgA-producing cells make initial antigenic contact in the gastrointestinal submucosa and then migrate to other local sites of immunity. This model may be valid for IgD with modifications. IgD-producing cells are normally absent from the gastrointestinal tract but they are frequently encountered in the tissues of the upper aerodigestive tree (Brandtzaeg, Surjan & Berdal 1978; Korsrud & Brandtzaeg, 1980; Litwin & Zehr, 1987a, 1987b) where they could theoretically initiate antigenic contact and then migrate to breast tissue. Since there is no secretory component associated with IgD, it would be difficult to explain how migrating submucosal cells could secrete IgD into the luminal structures of the breast. The possibility of intra-epithelial migration of lymphoid cells in the breast was raised by Seelig & Beer (1981). It is also possible that

Table 2. Summary of sera/milk (S/M) median ratios in PRP-immunized and unimmunized mothers

	Median* (Range) [Number of samples]				
	IgD	IgM	IgA	IgG	Albumin
Immunized					
Total	175 (33–>400) [8]	15 (5·7–190) [6]	0·98 (0·13–2·2) [6]	940 (440–2700) [6]	<94 (<55–120) [5]
Anti-PRP	4·1 (0·28–10) [8]	5·9 (1·8–11) [8]	2·8 (0·48–>4·8) [8]	>9600 (all >1300) [7]	
Anti-PC	9·2 (0·13–>17) [8]	>68 (10–>130) [4]	2·6 (0·82–7·2) [6]	200 (130–400) [4]	
Anti-Tetanus	>100 (all >52) [6]	>11 (all >7·0) [4]	2·3 (0·84–2·9) [4]	>310 (140–1000) [4]	
Unimmunized					
Total	>50 (22–>2800) [8]				
Anti-PRP	>7·5 (all>6·3) [7]				
Anti-PC	>18 (3.1–>24) [8]				

* Total antibody and albumin in μg/ml; specific antibody in arbitrary units.

the substantial number of immune cells observed in colostrum and milk (Parmely *et al.*, 1976) produce IgD specific antibody.

The present results can be critically compared with those of Keller *et al.* (1985) who used colostrum rather than milk, plasma rather than sera and a colostrum/plasma ratio which is the reciprocal of the S/M ratio employed in the current study. The experimental approaches applied in both investigations are similar and a comparison of respective data, after recalculation for the different ratio, indicates that in general, the same basic findings are present; there is selective concentration of IgA, IgM and IgD in human breast fluids in certain subjects; further, there is selective concentration of specific antibody. Certain disparities may be of importance. Keller *et al.*, (1985) found more pronounced selective concentration of total IgD than we did, and marked individual differences between antibody ratios. Keller *et al.* (1985) employed an entirely different panel of antigens including lactoglobulin, bovine serum albumin, Bermuda grass and alpha-gliadin in subjects who were presumptively exposed to the antigens naturally.

In the present work, the differences noted between elicitation of IgD antibodies to PRP, PC and tetanus antigens would be consistent with differencs between IgD mucosal immune responses to different classes of antigens. Different IgD isotype responses to carbohydrate *versus* protein antigens have been previously speculated. The reasoning is that IgD antibody is primarily elicited by capsular polysaccharides of bacteria in the upper aerodigestive tree (Litwin & Zehr, 1987a, 1987b) where IgD-producing cells are present in relatively large numbers (Brandtzaeg *et al.*, 1978; Korsrud & Brandtzaeg, 1980; Litwin & Zehr, 1987a). The supporting experimental evidence for the above hypothesis, includes the existence of easily detectable IgD class anti-PRP and anti-PC antibodies in human sera (unpublished data from our laboratory). Alternatively, the data may reflect the PRP immunization of the experimental subjects. The present experimental evidence cannot distinguish between the above possibilities.

Pregnancy is known to influence immune responses. PRP-immunized pregnant women showed >20-fold greater titres of *H. influenzae* capsular antibody as compared with unimmunized pregnant women, suggesting that the mammary gland preferentially attracted and trapped activated lymphocytes during pregnancy (Insel *et al.*, 1985). The selective concentration of IgD anti-PC may also be related to 'stimulation' during immunization with PRP of spontaneous-secreting IgD anti-PC-producing cells circulating in low numbers in healthy persons (unpublished data). The ubiquitous nature of PC antigen would facilitate continuous antigenic exposure and consequent immune stimulation. The lack of selective concentration of IgD anti-tetanus and anti-diphtheria could thus reflect lack of frequent mucosal stimulation as opposed to a distinction as to how immune responses view carbohydrate *versus* protein antigens.

The major focus of this study has been IgD, in keeping with studies from this laboratory which have been concerned with the regulation and role of IgD-producing cells (Litwin & Zehr,

1987a, 1987b, 1987c) and IgD-specific antibody. It should be noted that these data also demonstrate selective concentration of IgM in breast fluids. This phenomenon is not widely appreciated. Other investigators have emphasized the presence of specific IgE antibody in breast fluids (Bahna *et al.*, 1982). Current information, although incomplete, suggests that the complexity of local immune mechanisms may involve immunoglobulin class and subclass differences.

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