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Antibacterial and Antitoxin Responses in the Serum and Milk of Cholera Patients

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Antibacterial and antitoxin responses in the acute and convalescent (7 to 10 days) sera of 14 cholera patients were determined by various serological techniques. Similar studies were also carried out with corresponding milk samples of six of these patients who were lactating women. A significant rise in antibacterial titers was observed in all convalescent serum and milk samples. A similar rise in antitoxin titers was observable in all serum and four milk samples. Specificity of the antibacterial titers was further evaluated by the indirect hemagglutination test using lipopolysaccharide antigen, and close correlations were noted between these titers and vibrio agglutination ($P < 0.001$) and vibriocidal ($P < 0.001$) titers of sera. Serum and milk convalescent cholera patients could effectively neutralize cholera toxin action in vivo, although the neutralizing activity of serum was higher than that of milk. Determination of antibody titers by the enzyme-linked immunosorbent assay demonstrated that anti-lipopolysaccharide activity in sera belonged predominantly to immunoglobulin M (IgM) and, to a lesser extent, to IgG and IgA, whereas such activity in milk was mostly contributed by secretory IgA, although some IgM antibodies also could be detected. On the other hand, antitoxic activity in convalescent sera primarily belonged to IgG, whereas such activity in milk was almost exclusively contributed by secretory IgA. These results demonstrate that an antibody response in the mammary gland was stimulated due to the antigen exposure in the gut and are consistent with the idea of a common homing pattern of immunocytes within the secretory immune system. Moreover, some differences in the antibody production mechanism between the systemic and secretory immune systems are indicated.

Cholera is a gut-associated disease in which pathogenesis is caused by a toxin (enterotoxin) secreted by the multiplying vibrios in the small intestine. Immunity to cholera may result from either antibacterial or antitoxic, or both, immune responses (20). However, the precise mechanism of protective immunity is not clearly understood. Currently available data indicate that protection afforded by the conventional cholera vaccine is incomplete and, therefore, of very limited public health value (22). On the other hand, field studies (49) as well as recent studies with human volunteers (6, 26) have suggested that clinical cholera provides immunity, at least for some period of time, and that reinfection is more likely to occur with organisms of heterologous serotype. Therefore, it is quite logical that studies on the immunological mechanism in cholera infection should be focused on analyses of various types of immune responses in convalescent cholera patients and evaluation of their protective role. Unfortunately, most studies reported so far on human cholera have dealt with either the antibacterial or antitoxic form of systemic immunity, and only limited information is available on the role of secretory antibodies. There are three principal reasons for this: (i) the difficulty in collecting small intestinal secretions from patients; (ii) the instability of the intestinal antibodies due to rapid enzymatic degradation; and (iii) the lack of a suitable technique to detect small amounts of specific antibodies present in these secretions. Recently, several groups of workers (1, 2, 16, 19, 32, 40) have shown that the stimulation of antibody response in the gut may be disseminated to other distant secretory sites involving bronchial, mammary, and salivary glands. It is likely that measurement of the specific immunoglobulin A (IgA) response in saliva, milk, and colostrum may provide indirect information on the intestinal IgA response. Moreover, a simple yet sensitive enzyme-linked immunosorbent assay (ELISA) method (10, 44) is now available which can measure small quantities of specific antibody present in these secretions.

All of these developments prompted us to reinvestigate both antibacterial and antitoxin

responses in the sera of acute- and convalescent-phase cholera patients by various in vitro and in vivo methods. The secretory antibody response was also investigated in milk samples of patients who were lactating women, and results from corresponding sera were compared. Antibody heterogeneity in both systemic and secretory responses was analyzed by determining class-specific titers against purified lipopolysaccharide (LPS) and toxin antigens by the ELISA technique. These studies were aimed at detailed analyses of various types of antibody responses in human cholera.

MATERIALS AND METHODS

Collection of serum and milk. Sera were collected from bacteriologically confirmed cholera patients (12 female and 2 male) admitted to the Infectious Diseases Hospital, Calcutta. All strains of *Vibrio cholerae* isolated from the stool culture of these patients were of El Tor biotype and Inaba serotype. Both acute phase (within 24 h of admission) and convalescent-phase (7 to 10 days later) sera were collected. Of these 14 patients, 6 were lactating women from whom milk samples were also collected simultaneously. Milk was processed by centrifuging at 20,000 × *g* for about 30 min at 4°C. The clear middle layer (between the fat layer at the top and pellet at the bottom) was carefully withdrawn and stored frozen.

Estimation of serum and milk immunoglobulins. IgM, IgG, and IgA levels were determined in serum and milk by single radial immunodiffusion (28). Heavy-chain-specific goat anti-human IgM, IgG, and IgA antisera (Hyland Laboratories, Inc., Costa Mesa, Calif.), respectively, were used for this purpose. Specificities of these antisera were checked by immunodiffusion tests. A World Health Organization reference preparation (67/97) was used as a standard for IgM, IgG, and IgA estimations, and results were expressed as milligrams per milliliter of serum (21).

Vibriocidal antibody titer. Serum vibriocidal tests were carried out by following the plating method of Finkelstein (13), *V. cholerae* 569B Inaba was used as the target organism.

Anti-LPS IHA titer. Crude LPS was prepared from *V. cholerae* 569B Inaba by the phenol-water extraction method (48). This crude preparation was treated with cetyltrimethylammonium bromide to remove nucleic acid material. The protein content of the final LPS preparation varied between 3 and 5% as determined by the modified Folin method (27).

The indirect hemagglutination (IHA) test was carried out in microtiter plates, using serially double-diluted test samples and human group O erythrocytes sensitized with LPS (200 μg of LPS per ml of 2% erythrocyte suspension) (38). All samples were pretreated with heat inactivation at 56°C for 30 min.

Anti-LPS ELISA titer. IgM, IgG, IgA, and secretory IgA (SIgA) anti-LPS antibody titers of serum and milk were determined by the micro-ELISA technique as described by Voller et al. (45) with minor modifications. Alkaline phosphatase (type VII; Sigma Chemical Co., St Louis, Mo.) was used as the enzyme and was conjugated with immunoglobulin fractions of sheep anti-human IgG (γ-chain specific), IgM (μ-chain specific), IgA (α-chain specific), and SIgA (secretory-chain specific) antisera by the glutaraldehyde conjugation method (46).

Micro-ELISA plates (Dynatech Laboratories, Inc., Alexandria, Va.) were sensitized by the addition of 20 μg of LPS in 100 μl of coating buffer in each well followed by overnight incubation at 4°C. Wells were washed with 0.15 M phosphate-buffered saline containing 0.05% Tween 20 (PBS-Tween). Next, the wells were filled with 100 μl of test samples serially double diluted with PBS-Tween containing 1% fetal calf serum. The initial dilution of the test sample in the first well was 1:25 in all cases. Plates were incubated at 37°C for 1 h and washed, and enzyme-labeled anti-human IgM-IgG-IgA (diluted to 1:400 in PBS-Tween containing 1% fetal calf serum) or SIgA (diluted to 1:50) was added. After further incubation for 1 h at 37°C, the wells were washed and 100 μl of *p*-nitrophenyl phosphate solution (in 0.1 M diethanolamine buffer, pH 9.8) was added to each. The color that developed was recorded after 30 min of incubation at 37°C. Titers were expressed as the highest sample dilution giving a definite yellow color that matched visually the color of a *p*-nitrophenol solution of about 0.6 optical density unit at 400 nm.

Antitoxin IHA titer. Antitoxin IHA titers were determined by using diluted test samples and tanned human erythrocytes sensitized with purified cholera toxin (Schwarz/Mann, Orangeburg, N.Y.) (14). Usually, 100 μg of toxin was used per ml of 1.5% tanned erythrocyte suspension. IHA titers were expressed as the reciprocal of the highest dilution of the sample giving a positive reaction. Occasionally, these titers were also expressed in antitoxin units (AU) by comparison with the titer of a reference equine antitoxin preparation (Swiss Serum and Vaccine Institute, Berne, Switzerland) which contained 24,000 AU per ml of serum.

Antitoxin ELISA titer. Immunoglobulin class-specific antitoxin titers of serum and milk were determined by the micro-ELISA method. Each well of the micro-ELISA plate was coated with 2 μg of purified cholera toxin, and all subsequent steps were identical to those described earlier for anti-LPS ELISA.

Antitoxin titer by toxin neutralization tests. Antitoxin titers of serum and milk were also determined by toxin neutralization methods. The rabbit ileal loop test (9) and skin permeability factor (PF) assay (8) were adopted for this purpose.

The ileal loop test was performed in rabbits (about 1.5 kg) fasted for 24 h before the experiment. Usually, 12 to 15 ligated loops (each about 5 cm in length) were prepared in the ileum portion of the small intestine. For each set of experiments, toxin-antitoxin mixtures were prepared by mixing a constant amount of purified cholera toxin with at least five different dilutions of the test sample as well as of the reference antitoxin preparation. Then 0.5 ml of each of these mixtures (containing 0.2 μg of toxin) was injected into different loops of the same rabbit which also contained positive (toxin only) and negative (saline only) control loops. After about 20 h, the rabbit was sacrificed and fluid accumulation in each loop was measured separately.

The fluid volume per centimeter of loop length was calculated for each loop and plotted against the dilution of the test sample and reference antiserum in a semilogarithmic scale. The dilution of the sample showing 50% reduction in the volume/length value was taken as its neutralization titer, which was then expressed in antitoxin units per milliliter.

Toxin-antitoxin mixtures for the PF assay were prepared with five different dilutions of the test as well as reference samples. A 0.1-ml sample of each mixture (containing 0.1 μg of toxin) was injected intradermally at different sites in the depilated skin of a rabbit. The same rabbit was also injected with 0.1 ml of toxin (0.1 μg) and saline at two other sites, which served as positive and negative controls, respectively. After 18 to 20 h, the rabbit was given 1 ml of 5% Evans blue intravenously. Bluing zone diameters were measured after 2 h and plotted against antitoxin dilution in a semilog scale. The highest dilution of a sample that reduced the bluing zone diameter to 4 mm was taken as its titer, which was then expressed in antitoxin units per milliliter.

RESULTS

Immunoglobulin changes in serum and milk. IgM, IgG, and IgA levels were determined in the serum and milk of cholera patients (Table 1). The mean serum IgA level was slightly increased in convalescent patients (2.4 ± 0.7 mg/ml), although this increase was not statistically significant ($P > 0.1$) compared with the acute-phase level (1.9 ± 0.9 mg/ml). No such increase was noted in the serum IgG and IgM levels.

Immunoglobulin estimation for milk samples showed that IgA was the predominant class of immunoglobulin. The mean IgA level in milk increased significantly ($0.05 > P > 0.01$) during the convalescent period. A similar increase ($0.05 > P > 0.01$) was noted in the mean IgM level. Low levels of IgG were detected in milk; these levels remained essentially unchanged.

Antibacterial antibody response in serum. A significant increase (4- to 16-fold) in the agglutinating antibody titers against *V. cholerae* (Inaba) was observed in the sera of all convalescent cholera patients.

Serum vibriocidal titers were quite variable in the acute phase and ranged between 1 and 4.85 ($-\log_{10}$ value). A definite rise (10- to 1,000-fold) in the vibriocidal titers, however, was noticed in all convalescent sera, with titers ranging between 3 and 7.

A significant rise (fourfold or more) in anti-LPS IHA titers was observed in all but one serum. Reciprocal titers of acute-phase sera were ranged between 4 and 32, whereas those of convalescent-phase sera were between 32 and 256. A comparison between vibrio agglutination and anti-LPS IHA titers showed a highly significant correlation ($r = 0.88$, $P < 0.001$) between these two values. Similarly, anti-LPS IHA titers were found to correlate well ($r = 0.81$, $P < 0.001$) with the corresponding vibriocidal titers.

It is evident from micro-ELISA results (Table 2) that anti-LPS activity belonged primarily to antibodies of the IgM class, although some activity was also exhibited by IgG and IgA. A significant (4- to 32-fold) increase in IgM ELISA titers was noted in all convalescent-phase sera. Similarly, serum IgG and IgA ELISA titers were also significantly (≥4-fold) raised in eight and seven patients, respectively. Micro-ELISA did demonstrate the presence of anti-LPS antibodies of the IgM class in all acute-phase sera, whereas the IgG and IgA classes of anti-LPS antibodies were demonstrable in five and seven acute-phase sera, respectively.

Antitoxin antibody response in serum. A significant rise (eight- to 16-fold) in the serum antitoxin IHA titers was noted in all patients studied. Antitoxin IHA titers in convalescent-phase sera ranged between 32 and 512, whereas they were between 4 and 32 in 11 of 14 acute-phase sera. No antitoxic activity was detected in the remaining three acute sera.

Micro-ELISA results (Table 2) showed that serum antitoxin antibodies belonged primarily to the IgG class, the titers of which were elevated significantly (4- to 16-fold) in all cases. Antitoxin IgG ELISA titers of convalescent-phase sera ranged between 100 and 1,600, and the same was

TABLE 1. *Concentration of immunoglobulin in serum and milk samples of acute- and convalescent-phase cholera patients*

Sample	No.	Immunoglobulin concn (mg/ml)[a]		
		IgM	IgG	IgA
Acute serum	14	1.5 ± 0.6	19.1 ± 4.6	1.9 ± 0.9
Convalescent serum	14	1.6 ± 0.4	17.9 ± 2.8	2.4 ± 0.7
P[b]		>0.1	>0.1	>0.1
Acute milk	6	0.26 ± 0.05	0.17 ± 0.03	1.2 ± 0.2
Convalescent milk	6	0.44 ± 0.14	0.16 ± 0.04	1.6 ± 0.3
P[b]		$0.05 > P > 0.01$	>0.1	$0.05 > P > 0.01$

[a] Expressed as mean ± standard deviation.

[b] When compared with corresponding acute-phase values.

TABLE 2. *Immunoglobulin class-specific anti-LPS and antitoxin ELISA titers of sera from cholera patients*

Patient no.	Anti-LPS ELISA titer (reciprocal)						Antitoxin ELISA titer (reciprocal)					
	IgM		IgG		IgA		IgM		IgG		IgA	
	Act[a]	Con[b]	Act	Con	Act	Con	Act	Con	Act	Con	Act	Con
1[c]	200	3,200	25	50	50	400	200	100	100	1,600	25	50
2[c]	100	800	<25	<25	50	50	25	50	<25	100	<25	<25
3[c]	100	800	25	100	25	50	100	100	25	400	<25	50
4[c]	25	200	<25	50	<25	50	50	50	25	400	<25	<25
5[c]	25	200	<25	25	<25	25	50	100	<25	200	<25	25
6[c]	25	100	<25	25	<25	50	25	25	<25	100	<25	<25
7	25	100	<25	100	50	200	25	25	50	800	<25	25
8	200	3,200	25	400	50	400	50	25	200	1,600	25	50
9	50	800	25	200	50	400	100	100	25	400	<25	<25
10	200	3,200	<25	25	25	50	100	50	<25	200	<25	<25
11	25	800	25	200	<25	25	50	50	25	400	<25	25
12	50	800	<25	200	<25	25	50	100	25	400	<25	25
13	25	200	<25	25	<25	<25	25	25	25	100	<25	<25
14	100	800	<25	50	<25	50	100	100	<25	200	<25	25

[a] Act, Acute phase.
[b] Con, Convalescent phase.
[c] Lactating woman.

also demonstrable (≥25) in nine acute sera. Interestingly, little change in the antitoxin IgM ELISA titer was noted during the convalescent period. A significant rise in the IgA antitoxin titer was noted in one case only (Table 2).

Toxin-neutralizing activities of paired sera from five cholera patients were determined by both rabbit ileal loop and PF assay methods (Table 3). These samples were chosen on the basis of their high antitoxin IHA titers, which are also presented in Table 3 for comparison. It is apparent that antitoxin titers determined by all the three methods agreed reasonably well.

Antibacterial and antitoxin responses in milk. Antibacterial and antitoxin responses in milk samples were determined by the IHA method (Table 4). Similar results obtained with corresponding sera are presented for comparison. A definite rise in the anti-LPS antibody level was observable in the milk samples of all six patients, their convalescent titers ranging between 8 and 256. Anti-LPS IHA titers could be demonstrated in only three acute-phase samples. Similarly, only two of six patients had antitoxin IHA titers in their acute-phase samples, although a significant rise in these titers was noted in four patients. Two patients (no. 1 and 3), showed particularly high anti-LPS as well as antitoxin IHA titers in their convalescent-phase milk and serum samples (Table 4).

Micro-ELISA results (Table 5) indicated that anti-LPS activity in milk belonged primarily to the SIgA class of antibodies, the levels of which rose significantly during the convalescent period. The same was true for the antitoxic activity in milk, where a definite rise in SIgA titers was noted in convalescent-phase samples. Both anti-LPS and antitoxin antibodies of the SIgA class were present in the acute-phase samples of two patients (no. 2 and 3) only. Milk samples also contained some anti-LPS IgM ELISA titers,

TABLE 3. *Serum antitoxin titers of cholera patients as determined by various methods*

Patient no.	Reciprocal antitoxin titer (AU/ml)					
	IHA		Rabbit ileal loop assay		PF assay	
	Act[a]	Con[b]	Act	Con	Act	Con
1[c]	80	600	40	620	40	710
3[c]	40	600	<30	270	<40	290
7	80	1,200	70	600	70	670
8	80	1,200	100	710	110	900
12	80	600	80	790	110	900

[a-c] See Table 2.

TABLE 4. *Anti-LPS and antitoxin IHA titers of serum and milk samples of cholera patients*

Patient no.[a]	Anti-LPS IHA titer (reciprocal)				Antitoxin IHA titer (reciprocal)			
	Serum		Milk		Serum		Milk	
	Act[b]	Con[c]	Act	Con	Act	Con	Act	Con
1	32	256	32	256	32	256	16	256
2	8	128	<4	16	<4	32	<4	8
3	16	128	8	64	16	256	8	128
4	8	64	<4	16	8	128	<4	4
5	4	32	<4	8	<4	32	<4	8
6	4	32	4	16	8	128	<4	4

[a] Lactating women.
[b] Act, Acute phase.
[c] Con, Convalescent phase.

TABLE 5. *Immunoglobulin class-specific anti-LPS and antitoxin ELISA titers in milk samples of cholera patients*

Patient no.[a]	Anti-LPS ELISA titer (reciprocal)						Antitoxin ELISA titer (reciprocal)					
	IgM		IgG		SIgA		IgM		IgG		SIgA	
	Act[b]	Con[c]	Act	Con	Act	Con	Act	Con	Act	Con	Act	Con
1	50	400	<25	<25	100	800	<25	50	<25	25	100	800
2	<25	100	<25	<25	<25	50	<25	<25	<25	<25	<25	50
3	<25	100	<25	<25	50	400	<25	<25	<25	<25	25	400
4	25	100	<25	<25	<25	200	<25	<25	<25	<25	<25	100
5	<25	25	<25	<25	<25	100	<25	<25	<25	<25	<25	100
6	<25	<25	<25	<25	<25	50	<25	<25	<25	<25	<25	100

[a-c] See Table 4.

which increased significantly in four of six cases. IgG antibodies could be detected in only one of these samples.

Paired milk samples from two patients (no. 1 and 3) were further tested by toxin neutralization assays. Convalescent-phase samples of these patients did exhibit toxin-neutralizing activities by both ileal loop and PF assay methods. However, neutralization titers, although agreeing reasonably well between each other, were somewhat lower than those obtained by the IHA method (Table 6).

TABLE 6. *Antitoxin titers in milk samples of cholera patients as determined by various methods*

Patient no.[a]	Reciprocal antitoxin titer (AU/ml)					
	IHA test		Rabbit ileal loop assay		PF assay	
	Act[b]	Con[c]	Act	Con	Act	Con
1	40	600	<40	110	<40	120
3	20	300	<30	80	<40	90

[a-c] See Table 4.

DISCUSSION

A significant increase in both antibacterial and antitoxin titers was noted in the sera of convalescent-phase cholera patients. On the other hand, serum immunoglobulin levels did not show a parallel increase. In fact, IgG and IgM levels remained more or less unchanged during the convalescent period, whereas the IgA level showed a moderate, albeit insignificant, rise (Table 1). This result was probably due to the effect of hemoconcentration (caused by diarrhea) in the acute-phase sera, a conclusion that was supported by their higher IgM, IgG, and IgA values compared with those obtained with normal individuals living in the area (15).

Agglutination and vibriocidal titers determined in this study were in reasonable agreement with those obtained by earlier workers (12, 30, 34, 39, 47). So far, little information has been available on the anti-LPS antibody response in human cholera, although its protective role has been shown in field trials (22) as well as in experimental animals (41). Data presented here clearly establish close correlations between serum anti-LPS titers and corresponding vibrio agglutination ($P < 0.001$) and vibriocidal ($P < 0.001$) titers. This, in turn, suggests that the later antibodies were probably directed mainly against the somatic LPS antigen. the alternate possibility, of course, is that antibacterial responses were directed against both LPS and non-LPS antigens, in which case their relative contributions remained essentially unchanged. Such non-LPS antigens might include the cell wall protein (33) and flagellar antigens (11), both of which were found to be protective in experimental animals. It is difficult to exclude completely the possibility that some such antigen was present in our LPS preparation, which also contained about 3 to 5% protein.

The demonstration of the rise in serum antitoxin titers in patients convalescing from cholera was in general agreement with earlier observations (3, 8, 14, 23, 36). It is interesting to note that our antitoxin titers, determined by three methods, agreed reasonably well among each other when expressed in antitoxin units (Table 3). This investigation provides us with valuable information on the antigen specificity as well as the antibody heterogeneity of the systemic immune response in cholera. Thus, simultaneous determination of serum anti-LPS and antitoxin IHA titers and a reasonable correlation ($r = 0.55$, $0.01 > P > 0.001$) between these two values suggest that antibody production against the LPS antigen did not interfere appreciably with that against the toxin antigen, and vice versa. An interesting difference, however, was noted when serum antibody responses against these antigens were analyzed with respect to immunoglobulin classes. Anti-LPS activity in conva-

lescent-phase serum was found to be associated mainly with IgM and, to a lesser extent, with IgG and IgA antibodies (Table 2). On the other hand, antitoxin antibodies belonged primarily to the IgG class. Interpretation of these results is difficult, although it is tempting to speculate that differences in the biochemical and immunological properties between the T-cell-independent LPS and T-cell-dependent toxin antigens (24) might have been responsible.

A definite increase in both antibacterial and antitoxin titers was evident in convalescent-phase milk samples. The antigen specificity of these antibacterial titers was further established by the demonstration of anti-LPS antibodies (Table 4). Antitoxin antibodies in milk could effectively neutralize cholera toxin action in vivo, although titers determined by neutralization methods were somewhat lower than the corresponding IHA titers (Table 6). Two of these patients (No. 1 and 3) showed particularly high anti-LPS and antitoxin titers compared with those of the other four patients (Table 4). Moreover, such antibodies were also demonstrable in their acute-phase milk samples. Antibody titers in milk correlated well with the corresponding serum antibody titers. However, a major difference between serum and milk antibody responses was clearly evident from the ELISA results (Tables 2 and 5). Thus, milk antibodies directed against the toxin antigen were predominantly of the SIgA class, which was in contrast to the IgG antitoxin response in serum. In the case of LPS antigen, mainly SIgA and some IgM antibodies were detectable in convalescent-phase milk samples. These findings were in general agreement with the observation that a significant rise in milk IgA and IgM (but not IgG) levels could be detected during the convalescent period (Table 1).

Demonstration of specific antibodies in the milk of cholera patients poses an interesting question regarding their origin and biosynthetic mechanism. It has been established (18, 43) that SIgA antibodies are synthesized locally, and the same is probably true for IgM antibodies in secretions (5, 37). Analysis of the present data on the basis of the above information excludes the possibility that most of the milk antibodies were derived by transudation from serum. Rather, these results are consistent with the idea of a common homing pattern of immunoctyes within the secretory immune system (7, 17, 35). Specific SIgA antibodies have been demonstrated in the colostrum of pregnant women infected with *Salmonella typhimurium* (2). In another report, IgA-producing cells were demonstrated in the milk of women orally immunized with *Escherichia coli* (16). Further evidence in favor of this hypothesis is accumulating (4, 25, 29, 31, 32, 40, 42).

The results presented here clearly demonstrate the stimulation of various types of antibacterial and antitoxin responses in the serum and milk of cholera patients against antigenic stimuli which were essentially restricted within the gut. However, the relationship between the antibody response in the gut and in the mammary tissue could not be established. It is evident that, unlike the serum antibody response, the secretory response followed a similar pattern in the cases of IPS and toxin antigens with regard to the production of SIgA antibodies. These observations suggest some differences in the antibody production mechanism between the systemic and secretory immune systems.

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