INFECTION AND IMMUNITY, Aug. 1980, p. 545-550
0019-9567/80/08-0545/06$02.00/0

Vol. 29, No. 2

Neutralizing Activity Against *Clostridium difficile* Toxin in the Supernatants of Cultured Colostral Cells

NAOKI WADA,[1]* NAOMI NISHIDA,[1] SUSUMU IWAKI,[1] HITOSHI OHI,[1] TOSHIO MIYAWAKI,[1] NOBORU TANIGUCHI,[1] AND SHUNSUKE MIGITA[2]

Department of Pediatrics, School of Medicine,[1] and Department of Molecular Immunology, Cancer Research Institute,[2] Kanazawa University, 13-1 Takaramachi, Kanazawa 920, Japan

Human colostral specimens were obtained from 60 Japanese postpartum women within the first 3 days after delivery. Neutralizing activity against *Clostridium difficile* toxin was evaluated with Y1 adrenal cells in miniculture. When Y1 adrenal cells were exposed briefly to the toxin, they showed a rounding response in culture, resembling that effected by *Escherichia coli* enterotoxin; however, preincubation of the toxin with aqueous phase of colostrum significantly reduced its cytopathic effect on Y1 adrenal cells. Of 60 colostral specimens, 17 samples had neutralizing activity against the toxin. Cell-free supernatants of colostral cells cultured for 7 days without mitogens contained significant amounts of both immunoglobulin A (IgA) and IgM, but very small amounts of IgG. Neutralizing activity of cell-free supernatants of cultured colostral cells was evaluated as described above. Neutralizing activity against the toxin was identified in five samples of culture supernatants out of 60 colostral cell specimens. In all five cases, the aqueous phase of colostrum also had a neutralizing effect against *C. difficile* toxin. Neutralizing activity against the toxin found in five supernatants of cultured colostral cells was completely abolished only by anti-human IgA antibody as assessed by immune precipitation.

Culture supernatants of human colostral cells contain immunoglobulins, mainly of the immunoglobulin A (IgA) class, which seem to be largely secreted or released from colostral macrophages (17). Colostral macrophages have been suggested as a possible vehicle for the storage and transport of preformed immunoglobulins in the breast milk. However, little information is available regarding the contribution of colostral macrophages, the principal cells in colostrum, to the immune defense of the neonatal intestine. Necrotizing enterocolitis has been increasingly seen in low-birth-weight infants, but is relatively uncommon in breast-fed infants (8). Barlow et al. developed a rat model of necrotizing enterocolitis of the neonate, in which freeze-thawed rat milk was not protective, but unprocessed rat milk or the formula supplemented with viable rat milk cells exerted effective protection (2, 16). They speculated that some of the milk cells ingested remained viable in the upper gastrointestinal tract of the rat for up to 1 week and continued to exert active protection against necrotizing enterocolitis.

Now, there is growing evidence that toxin-producing *Clostridium* species might be a positive cause and the final step in the pathogenesis of necrotizing enterocolitis (8). Recently, *Clostridium difficile* was also incriminated as a cause of antibiotic-associated colitis (6, 10). *C. difficile* elaborates a toxin which is heat-labile and cytopathic for cells in tissue culture (20), and produces vascular permeability factors in rabbit skin (18). In this work, neutralizing activity against *C. difficile* toxin of 7-day-culture supernatants of human colostral cells was assessed by the use of Y1 adrenal cell assay. Neutralizing activity against *C. difficile* toxin was found in five culture supernatants of colostral cell specimens, and neutralizing activity appeared to reside largely in the IgA fraction of culture supernatants as assessed by immune precipitation.

MATERIALS AND METHODS

Preparation of colostral cells. Colostral specimens were collected from 60 Japanese postpartum women during the first 3 days after delivery. All samples of colostrum were diluted 1:2 with phosphate-buffered saline (PBS, pH 7.2) and then defatted by centrifugation for 10 min at 500 × *g* at room temperature. Fluid phase was again centrifuged for 20 min at 12,000 × *g*, and this aqueous phase was used in the neutralizing assay for *C. difficile* toxin. The sedimented cells were washed twice with PBS and suspended in RPMI 1640 medium (GIBCO Laboratories, Grand Island, N.Y.) containing 20% heat-inactivated fetal bovine serum (GIBCO). These cell suspensions were overlaid on a mixture of sodium metrizoate and Ficoll (Lymphoprep, Nyegaard Co., Oslo, Norway) and

centrifuged for 20 min at 400 × *g* at room temperature. The cells isolated from the interface of the gradient were washed three times with PBS and suspended to 2.0×10^6 cells per ml in RPMI 1640 containing 20% fetal bovine serum. The cells obtained in this way were more than 90% viable as judged by trypan blue dye exclusion and consisted of macrophages (33 to 78%), as determined by phagocytosis of yeast particles (13) or nonspecific esterase staining (11), lymphocytes (10 to 28%), and neutrophils (4 to 30%), as judged by stained morphology. In the present work, these cells were used as colostral cells.

Cell cultures. Colostral cells were cultured at a concentration of 2.0×10^6 cells per ml in RPMI 1640 supplemented with L-glutamine (0.3 mg/ml), penicillin (200 U/ml), amphotericin B (5 μg/ml), gentamicin (10 μg/ml), and 20% fetal bovine serum. All cultures were grown in a final volume of 1.0 to 3.0 ml of culture medium in plastic culture tubes (no. 2027, Falcon Plastics, Oxnard, Calif.). The tubes were incubated in a humidified environment of 5% CO_2 in air at 37°C for 7 days. After incubation, the culture supernatants were separated from the cellular pellet by centrifugation for 15 min at 400 × *g* and then stored at −20°C.

Immunoglobulin determination. The amounts of IgA, IgM, or IgG in the culture supernatants were measured by a photometric immunoassay of latex agglutination with near infrared turbidimetry devised by M. Sawai et al. (22). Briefly, 0.6 ml of appropriately diluted culture supernatant or control immunoglobulin (19 to 2,000 ng) was added to 1.0 ml of a suspension of latex particles (about 0.2 μm in diameter) coated with anti-IgA, -IgM, or -IgG, and then changes in the turbidity of latex were read by near infrared turbidimetry (Mitubishi Chemical Industries, Ltd., Tokyo, Japan).

***C. difficile* toxin and cytopathic effect on cultured cell lines.** Y1 adrenal, HeLa, and FL (amnion) cells were maintained in Ham-F12 media (Nissui Seiyaku Co., Ltd., Tokyo) supplemented with 12.5% horse serum (GIBCO), 2.5% fetal bovine serum, and kanamycin (100 μg/ml). These cell lines were subcultured into a 96-well microplate (no. 3042; Falcon Plastics) at 37°C in a humidified environment of 5% CO_2 in air. The tissue culture was available for assay when a monolayer was formed, usually after 1 or 2 days.

A strain of *C. difficile* (ATCC 17859, kindly supplied by S. Nishida, Department of Microbiology, Kanazawa University) was grown in brain heart infusion broth (BBL Microbiology Systems, Cockeysville, Md.) for 48 h at 37°C under anaerobic conditions (80% N_2, 10% CO_2, 10% H_2). The culture was then centrifuged at 12,000 × *g* for 10 min to remove bacteria. The supernatant was filtered through a 0.45-μm-pore size membrane filter (Millipore Corp., Bedford, Mass.), and used as the source of toxin. Toxin titration was carried out by making serial twofold dilutions in PBS. Evaluation of the cytopathic effect of toxin on cell lines was performed by a modification of the method of Sack and Sack (21). Each 12-μl sample was added to cell monolayers grown on a microplate. After 5 min of exposure, the culture medium containing the toxin was removed with a micropipette, the cultured cells were washed with PBS, and fresh medium was added. The cells were observed for morphological changes under a phase-contrast inverted microscope after 18 to 24 h of incubation.

Neutralizing activity of the aqueous phase of colostrum and culture supernatants. Neutralizing activity against *C. difficile* toxin in the 7-day-culture supernatants of colostral cells was analyzed by Y1 adrenal cell assay. Each supernatant was tested on two occasions in duplicate, together with *C. difficile* toxin in a final concentration of 50% tissue culture infective dose ($TCID_{50}$). Before application to the cell layers, culture supernatants and toxin were incubated together at 37°C for 60 min. Furthermore, the aqueous phase of colostrum was examined for neutralizing activity to *C. difficile* toxin by this method.

Classes of immunoglobulins responsible for neutralizing activity against *C. difficile* toxin in the culture supernatants of colostral cells were assessed by immune precipitation. Immune precipitation was performed by a modification of the method of George and Cohen (5). Samples of the supernatants were incubated with human serum as a carrier and rabbit anti-human monospecific antibodies against IgA, IgM, or IgG (Behringwerke, A.G., Marburg, Federal Republic of Germany) at antigen-antibody equivalence for precipitation. The human serum was selected from one healthy donor, whose serum did not neutralize the toxin in the dose used.

Precipitation was carried out for 1 h at 37°C and then overnight at 4°C. After incubation, the supernatant was separated from immune precipitates by centrifugation at 10,000 × *g* for 15 min and used in the neutralization assay.

RESULTS

Immunoglobulins in culture supernatants of colostral cells. In 7-day cultures of colostral cells (2×10^6 cells per ml), mean concentrations of IgA and IgM were 3,826 ng/ml (range, 318 to 15,010 ng/ml) and 1,063 ng/ml (range, 405 to 2,604 ng/ml), respectively (Fig. 1).

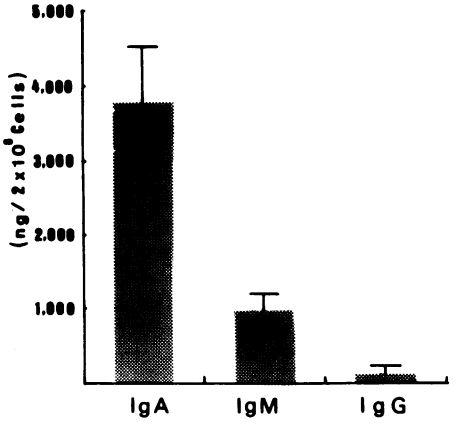

FIG. 1. *Immunoglobulins in culture supernatants of colostral cells. Colostral cells were cultured in a density of 2.0×10^6 cells per ml for 7 days. Vertical bars represent standard errors of the means of results from 60 specimens.*

Concentration of IgG in the culture supernatants was markedly lower (mean, 154 ng/ml; range, 51 to 815 ng/ml) than those of IgA and IgM.

Y1 adrenal cell assay for *C. difficile* toxin. When Y1 adrenal cells were exposed briefly to an appropriate dilution of culture filtrates of *C. difficile*, characteristic changes in cell morphology were observed in ongoing culture. These morphological changes were discernible by 4 to 6 h after the exposure of the toxin to the cell, with maximal rounding response occurring 18 to 24 h later. Changes in cell morphology appeared to be irreversible up to 48 h of culture and to be similar to those caused by exposure to *Escherichia coli* enterotoxin. Since *C. difficile* toxin has now been demonstrated to be cytopathic to HeLa and FL cells in tissue culture (3, 18, 20), the sensitivity of Y1 adrenal cells to the toxin was compared to that of HeLa or FL cells. As shown in Table 1, Y1 adrenal cells were as sensitive as HeLa or FL cells to graded doses of *C. difficile* toxin. Cell responses to the toxin, morphological changes, and time course of cytopathic effects were similar regardless of the cell type. On heating at 56°C for 30 min, culture filtrate of *C. difficile* exerted no cytopathic effect on any of the three cell lines.

Neutralization of *C. difficile* toxin by the aqueous phase of colostrum and culture supernatants of colostral cells. The aqueous phase of colostrum and the 7-day-culture supernatants of colostral cells were examined for the neutralizing activity against *C. difficile* toxin by means of Y1 adrenal cell assay.

Culture supernatants were preincubated with 1 $TCID_{50}$ of *C. difficile* toxin and then applied to cells. Reduction of the cytopathic effects to less 10% was chosen as evidence of neutralization. By this criterion, 5 (8.6%) of 60 culture supernatants had neutralizing activity. Since neutralizing activity of cell-free supernatants was relatively weak, a precise evaluation of the grade of neutralizing activity by serial dilutions was difficult to perform. Cell-free supernatants showing no neutralizing activity did not reveal any activity even at severalfold concentrations.

The aqueous phase of colostrum was preincubated with 12 μl of 3 $TCID_{50}$ of *C. difficile* toxin, and 17 (28.3%) of 60 specimens had neutralizing activity (see below). In all five cases of culture supernatants, the aqueous phase of colostrum also had neutralizing activity against *C. difficile* toxin.

Immunoglobulin class responsible for neutralizing activity. As shown in Table 2,

TABLE 2. *Identification of immunoglobin class of toxin neutralizing antibodies in culture supernatants of colostral cells*[a]

Colostral cell samples (50 μl)	Rounding response			
	Rabbit serum[b] (10 μl)	Anti-IgA (10 μl)	Anti-IgM (5 μl)	Anti-IgG (8 μl)
Medium[c]	+	+	+	+
A	−	+	−	−
B	−	+	−	−
C	−	+	−	−
D	−	±	−	−
E	−	+	−	−

[a] The culture supernatants of colostral cells were incubated with 1 μl of human serum as carrier and the appropriate volume of rabbit anti-human IgA, IgM, or IgG antibodies at antigen-antibody equivalence for precipitation. After separation from immune precipitates by centrifugation, the supernatants were preincubated with 12 μl of *C. difficile* toxin (1 $TCID_{50}$) for 60 min at 37°C. Then, the mixture of culture supernatants and the toxin was added to Y1 adrenal cells in microcultures. Rounding response of Y1-adrenal cells was determined 18 to 24 h after incubation: +, >50%; ±, 10 to 50%; −, <10% rounded cells.

[b] Nonimmune rabbit serum.

[c] RPMI 1640 with 20% fetal bovine serum.

TABLE 1. *Effect of C. difficile toxin on Y1 adrenal, HeLa, and FL cell lines*

Cell line[a]	Morphological change[b]										$TCID_{50}$[c]
	In toxin concn:									In PBS	
	2^0	2^1	2^2	2^4	2^6	2^8	2^9	2^{10}	2^{11}		
Y1-adrenal	+	+	+	+	+	+	−	−	−	−	2^8
HeLa	+	+	+	+	+	+	+	−	−	−	2^9
FL	+	+	+	+	+	+	−	−	−	−	2^8

[a] Cells were subcultured onto 96-well microplates. Monolayers were obtained by seeding 10^4 cells per well in growth medium for 24 h of incubation at 37°C in a humidified environment of 5% CO_2 in air. Then, each 12-μl sample was applied into these monolayer cells in the microplate.

[b] Titration was carried out by serial twofold dilutions in PBS. +, >50% cytopathic effect; −, <50% cytopathic effect.

[c] Cells were observed for morphological changes under a phase-contrast inverted microscope after 18 to 24 h of application of the toxin for 5 min. The titer was reciprocal and is expressed as *C. difficile* toxin in a final concentration of 1 $TCID_{50}$.

the neutralizing activity in the culture supernatants of colostral cells was completely eliminated by precipitation with rabbit anti-human IgA antibody in four of five neutralizing activity-positive supernatants. In one supernatant (D), weak neutralizing activity against the toxin was still observed after the immune precipitation by anti-IgA antibody. Further addition of anti-IgA serum showed no appreciable effect on the results. These results suggest that the neutralizing activity against *C. difficile* toxin resides largely in IgA in the culture supernatants of colostral cells.

Concentrations of IgA in the culture supernatants of five positive and the remaining negative samples were 2,928 ± 1,111 (standard error) and 4,040 ± 857 ng/ml, respectively (Fig. 2). This difference in IgA concentration was not statistically significant. This result suggests that the concentration of IgA from cultured colostral cells is not directly related to the neutralizing activity against *C. difficile* toxin.

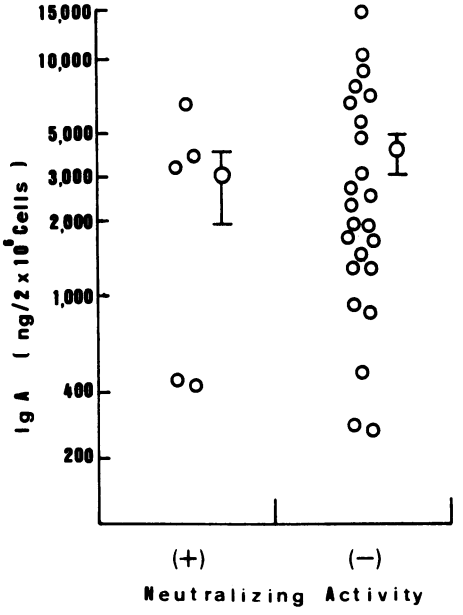

FIG. 2. *The relationship between IgA concentrations and neutralizing activity against C. difficile toxin in culture supernatants of colostral cells. The samples which reduced the 50% cytopathic effect of toxin on Y1 adrenal cells to less than 10% were considered to have neutralizing activity against the toxin. Circles with bars on the right side of individual plotted results correspond to the geometric mean values. Vertical bars represent standard errors of the means. The difference of IgA concentrations between two groups was not statistically significant, as evaluated by Student's t test (P > 0.5).*

DISCUSSION

Several lines of evidence suggest that human colostrum may provide a passive protection for the neonatal gastrointestinal surface against microorganisms during the period before the active development of secretory immunity (9, 12, 19). The predominant class of immunoglobulin in human colostrum is secretory IgA (14, 15), a form of antibody structurally suited to function in the external environment of the intestine. It has been suggested that the specificity of these IgA-associated antibodies reflects the spectrum of antigens to which lymphoid cells in the gastrointestinal tract of the mother have been exposed (1). Holmgren et al. reported that secretory IgA antibodies against toxins of enterotoxigenic *E. coli* and *Vibrio cholerae* were demonstrated in breast milk of Pakistani women, but not in the milk of Swedish women (7). They speculated that these different results between two groups of lactating women may be attributable to varying opportunities for previous enteric exposure to these microbes. Besides soluble factors such as immunoglobulins, human colostrum contains virtually all types of immunologically active cells, of which 70 to 80% are macrophages, about 10% are lymphocytes, and the rest are polymorphonuclear leukocytes. Although little is known regarding the contribution of milk cells to the defense mechanism of the neonate, colostral cells, principally macrophages, may play an important role in protecting the newborn from necrotizing enterocolitis.

Crago et al. demonstrated by immunofluorescence techniques that colostral macrophages contained immunoglobulins of the IgA and IgM classes, secretory component, and lactalbumin (4). The coincidental appearance of these proteins in single macrophages but not in lymphoid cells indicated that colostral macrophages acquired these proteins by ingestion from the environment. Pittard et al. reported that colostral cells, even when cultured without mitogens, released immunoglobulins, mainly IgA class, in the culture supernatants (17). They proposed that this immunoglobulin originated largely from colostral macrophages which served as possible vehicles for storage and transport of preformed immunoglobulins. Active synthesis of appreciable amounts of IgA by lymphoid cells was not observed. In the present work, the 7-day-culture supernatants contained IgA and IgM: mean concentration of IgA (3,826 ng/2 × 10^6 cells) was about three times that of IgM (1,063 ng/2 × 10^6 cells). Only negligible amounts of IgG (mean value, 154 ng/2 × 10^6 cells) were present in the culture supernatants. These results agreed well

with those of Pittard et al. (17), except for a relatively high concentration of IgM.

Recently, several species of toxin-producing clostridia have been increasingly incriminated as a possible cause and the final step in the pathogenesis of necrotizing enterocolitis of the neonate (8). Although *C. difficile* has not been incriminated as a cause of necrotizing enterocolitis (23), for convenience, a toxin produced by *C. difficile* which was isolated from a patient with antibiotic-associated colitis was used as a model of clostridium toxin in this work. This toxin was heat labile and cytopathic for cells (20) such as FL (3, 20) or HeLa (18) in tissue culture. As shown in Table 1, the Y1 adrenal cell line also was as sensitive to *C. difficile* toxin as were the FL and HeLa cell lines, resulting in a similar morphological change upon exposure to the toxin. Neutralizing activity against *C. difficile* toxin could be assessed by inhibition of toxin-inducible morphological change in cultured monolayers of Y1 adrenal cells with the addition of aqueous phase of colostrum or cultured supernatants of colostral cells. Samples (25 μl) of serial twofold dilutions of colostral specimens in PBS were incubated with 12 μl of 3 $TCID_{50}$ of *C. difficile* toxin. Neutralizing activities of colostral specimens, expressed as the highest twofold dilution of the colostral specimen abolishing the cytopathic effect of the toxin, were 3, 8, 3, 1, 2, and 0 for toxin concentrations of 2^0, 2^1, 2^2, 2^3, 2^4, and 2^5, respectively. Of 60 colostrum specimens from lactating Japanese women, 17 showed neutralizing activity against *C. difficile* toxin. If the assumption of Holmgren et al. (7) is correct, about one-fourth of Japanese women might have experienced enteral exposure to *C. difficile*. Among the 17 cases having colostral antibody against *C. difficile* toxin, only five colostral cell specimens released neutralizing antibody in the culture supernatants. From the results of immune-precipitation analysis, the neutralizing antibodies in the culture supernatants mainly belonged to the IgA class of immunoglobulin. Any cellular specimen from colostrum having no neutralizing activity did not release antibody for *C. difficile* toxin. However, total concentration of IgA in the culture supernatants showed no significant difference whether or not culture supernatants of colostral cells contained neutralizing antibody.

The reason why only one-third of colostrum specimens having neutralizing activity against *C. difficile* toxin contained cells, principally colostral macrophages, capable of releasing IgA antibody against the toxin and the biological significance of such colostral macrophages remain to be elucidated. However, since colostral macrophages are capable of a slow release of immunoglobulins having antibodies for various antigens, exhibit phagocytic function, and can tolerate large variations in the environment, it may be plausible to speculate that these cells may contribute to the defense process taking place in the gastrointestinal tract of the newborn and may play an important role in protecting the newborn against certain forms of necrotizing enterocolitis.

ACKNOWLEDGMENTS

We thank Motoichi Hatano, Cancer Research Institute, Kanazawa University, for his technical advice on performance of neutralization assay by cultured cell lines, and Shoki Nishida and Shin-ichi Nakamura of the Department of Microbiology for their critical review of this manuscript. We also gratefully acknowledge the excellent technical assistance of Naoki Moriya.

This work was supported in part by a grant of Morinaga Hooshi-kai.

LITERATURE CITED

1. **Ahlstedt, S., B. Carlsson, L. Å. Hanson, and R. M. Goldblum.** 1975. Antibody production by human colostral cells. I. Immunoglobulin class, specificity, and quantity. Scand. J. Immunol. **4**:535–539.
2. **Barlow, B., T. V. Santulli, W. C. Heird, W. A. Blanc, J. Pitt, and J. N. Schullinger.** 1974. An experimental study of acute neonatal enterocolitis—the importance of breast milk. J. Pediatr. Surg. **9**:587–595.
3. **Chang, T.-W., P.-S. Lin, S. L. Gorbach, and J. G. Bartlett.** 1979. Ultrastructural changes of cultured human amnion cells by *Clostridium difficile* toxin. Infect. Immun. **23**:795–798.
4. **Crago, S. S., S. J. Prince, T. G. Pretlow, J. R. Mcghee, and J. Mestecky.** 1979. Human colostral cells. I. Separation and characterization. Clin. Exp. Immunol. **38**: 585–597.
5. **George, E. R., and H. J. Cohen.** 1979. Kinetics of immunoglobulin synthesis and secretion by resting and pokeweed mitogen-transformed human lymphocytes. Clin. Immunol. Immunopathol. **12**:94–104.
6. **George, R. H., J. M. Symonds, F. Dimock, J. D. Brown, Y. Arabi, N. Shigva, M. R. B. Keighley, J. A. Williams, and D. W. Burdon.** 1978. Identification of *Clostridium difficile* as a cause of pseudomembranous colitis. Br. Med. J. **1**:695.
7. **Holmgren, J., L. Å. Hanson, B. Carlson, B. S. Lindblad, and J. Rahimtoola.** 1976. Neutralizing antibodies against *Escherichia coli* and *Vibrio cholerae* enterotoxins in human milk from a developing country. Scand. J. Immunol. **5**:867–871.
8. **Kliegman, R. M.** 1979. Neonatal necrotizing enterocolitis. Implications for an infectious disease. Pediatr. Clin. North Am. **26**:327–344.
9. **Larsen, S. A., Jr., and D. R. Homer.** 1978. Relation of breast versus bottle feeding to hospitalization for gastroenteritis in a middle-class U.S. population. J. Pediatr. **3**:417–418.
10. **Larson, H. E., A. B. Price, and P. Honour.** 1978. *Clostridium difficile* and the etiology of pseudomembranous colitis. Lancet **i**:1063–1066.
11. **Li, C. Y., K. W. Lam, and L. T. Yam.** 1973. Esterases in human leukocytes. J. Histochem. Cytochem. **21**:1–12.
12. **Michael, J. G., R. Ringenback, and S. Hottenstein.** 1971. The antimicrobial activity of human colostral antibody in the newborn. J. Infec. Dis. **124**:445–448.

13. **Miller, M. E.** 1969. Phagocytosis in the newborn infant. Humoral and cellular factors. J. Pediatr. **74**:255–259.
14. **Murillo, G. J.** 1971. Synthesis of secretory IgA by human colostral cells. South. Med. J. **64**:1333–1337.
15. **Ogra, S. S., and P. L. Ogra.** 1978. Immunologic aspects of human colostrum and milk. I. Distribution characteristics and concentrations of immunoglobulins at different times after the onset of lactation. J. Pediatr. **92**: 546–549.
16. **Pitt, J., B. Barlow, and W. C. Heird.** 1977. Protection against experimental necrotizing enterocolitis by maternal milk. I. Role of milk leukocytes. Pediatr. Res. **11**: 906–909.
17. **Pittard, W. B., III, S. H. Polmar, and A. A. Fanaroff.** 1977. The breast milk macrophage. A potential vehicle for immunoglobulin transport. RES J. Reticuloendothel. Soc. **22**:597–603.
18. **Rifkin, G. D., F. R. Fekety, and J. Silva, Jr.** 1977. Antibiotic-induced colitis. Implication of a toxin neutralised by *Clostridium sordellii* antitoxin. Lancet **ii**: 1103–1106.
19. **Robinson, J. E., B. A. M. Harvey, and J. F. Soothill.** 1978. Phagocytosis and killing of bacteria and yeast by human milk cells after opsonisation in aqueous phase of milk. Br. Med. J. **3**:1443–1445.
20. **Rolfe, R. D., and S. M. Finegold.** 1979. Purification and characterization of *Clostridium difficile* toxin. Infect. Immun. **25**:191–201.
21. **Sack, D. A., and R. B. Sack.** 1975. Test for enterotoxigenic *Escherichia coli* using Y1 adrenal cells in miniculture. Infect. Immun. **11**:334–336.
22. **Sawai, M., S. Morita, S. Sato, H. Nishida, N. Moriya, and S. Migita.** 1979. Latex agglutination by near infrared turbidimetry, its application for immunoglobulin assay of culture fluid of lymphocytes. Proc. Jpn. Soc. Immunol. **9**:114–115.
23. **Stoll, B. J., A. J. Nahmias, C. Wickliffe, A. W. Brann, Jr., V. R. Dowell, Jr., and D. N. Whaley.** 1980. Bacterial toxin and neonatal necrotizing enterocolitis. J. Pediatr. **96**:114–115.