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Immunoglobulins and antibodies in colostrum and milk of Guatemalan mayan women[1]

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SUMMARY

Colostrum and milk from a population exposed to a high risk of malnutrition and infection were evaluated for immunoglobulins and antibodies against selected bacterial and viral pathogens. IgA levels were high in colostrum (333 mg/100 ml) as well as later in the protracted weaning period (242 mg/100 ml) (242 mg/100 ml). IgM was detectable in colostrum (36 mg/100 ml) but not after 4 weeks of lactation. Antibody titers against bacterial and viral pathogens were detected in colostrum, and less frequently in milk. A correlation was observed between levels and frequency of antibody titers with endemicity of pathogens in the population. The importance of these findings is discussed in regard to early acquired local immunity and intestinal viral and bacterial colonization, and with reference to the importance of breast-feeding in pre-industrial societies.

INTRODUCTION

Breast feeding and its relationship with resistance to infection have been subjects of continuing interest and investigation. With the advent of further knowledge regarding the secretory immunologic system, it has been suggested that con-

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ferring local passive immunity may be an important function of secretory IgA in colostrum and breast milk (1-3). Recent reviews in the literature summarize in detail the current state of knowledge on the secretory immunologic system (4, 5).

A lower incidence of disease and mortality in breast-fed infants has been reported from various parts of the world (6). This applies to a wide range of illnesses including gastrointestinal disorders, measles, whooping cough, otitis media and respiratory infections. Due to the high incidence of gastrointestinal disease, especially in developing countries, the study of contributory factors has been explored. These include (a) the high risk of contamination through artificial feeding of infants; (b) the poor environmental sanitation affecting preparation of food or providing a continuous source of infection by direct or indirect contact; (c) the lack of a protective effect of the intestinal microflora in artificially fed children, and (d) the lack of other specific and non-specific components of maternal milk. Regarding the latter, immunoglobulins, lysozyme, immune cells, and interferon have been investigated (7-11).

Of these factors, immunoglobulins have attracted most attention. An effect of human colostral antibodies on polioviruses and *Escherichia coli* has been demonstrated. Several investigators showed that antipoliovirus antibody in colostrum and milk interferes with intestinal viral replication. Inhibition was found to be directly correlated with antibody titers (12-14). Recently Akao *et al.* (15) demonstrated an identical mode of neutralization of poliovirus 2 by both serum IgG and secretory IgA, whether derived from maternal milk or stool extracts of immunized children. This study supports the concept that secretory IgA of colostrum or milk effectively neutralizes enteroviruses in the intestinal lumen and functions as a protective factor against infection.

There is evidence for correlation between immunoglobulin levels in colostrum and a reduction in titers of coliform bacteria in stools of breast-fed neonates (16). It was shown that, as the levels of immunoglobulin decreased, hemagglutinating and bacterial activity of colostrum and stool extracts also decreased. More importantly, when colostral immunoglobulins were high, coliform titers were lower than those in bottle-fed neonates not receiving colostrum. As immunoglobulins decreased, coliform counts rose in breast-fed neonates approa-

ching the counts observed in bottle-fed infants. This work suggests a direct effect of colostral immunoglobulins on intestinal flora (16).

Since evidence points to an effect of colostral antibodies on infectious agents, and considering the importance of breast-feeding, especially in developing countries, a study was initiated in 1968 in Santa María Cauqué. This is a semi isolated Guatemalan Mayan village with a population of 1,300, where studies of nutrition and infection are underway (17). Immunoglobulin levels and antibody titers against a variety of pathogens were measured in colostrum and milk. The prevailing socio-economic conditions in the village result in deficient enviromental sanitation and personal hygiene, chronic malnutrition and crowding. Favorable conditions exist for the spread and endemicity of pathogenic agents.

The pattern of breast-feeding and weaning have been recorded. It is customary for newborns to be nursed by one to three foster mothers immediately after birth; only 20% of mothers nurse during the first day after delivery. Infants are almost exclusively breast-fed during the first three months; sugar or rice water is supplied occasionaly in the first few days. Weaning follows a protracted course with solid foods (corn, bread, noodles, beans) introduced by the end of the first year of life. The mode of complete weaning is 28 months (17, 18).

MATERIALS AND METHODS

Collection of Samples

All samples of human colostrum and milk were collected under field conditions in the mothers' homes. The established rapport of INCAP workers was invaluable in obtaining the cooperation of the mothers. Multiple samples were collected from 43 women between parturition and 4 weeks thereafter. Colostrum was collected within three days after delivery; later milk specimens were obtained at weekly intervals allowing a variation of two days. Additional samples were collected from 24 women in later stages of lactation. In total, 248 samples were obtained from 71 mothers. An attempt was made to obtain at least 2 ml of colostrum or milk on each occasion. The first samples were collected with sterile breast pumps;

later the mothers were able to express adequate samples manually into sterile containers. The specimens were studied bacteriologically (19) and then were frozen for further study.

Processing of Samples

Two ml of each specimen were centrifuged at 2,000 rpm's (1,000 × g) at 4°C for 90 minutes to separate the fat and sediments from the relatively clear supernates. These were transferred with Pasteur pipettes to sterile one-dram vials for storage at −20°C.

Quantitation of Immunoglobulins

IgA and IgM levels were measured in supernates using the radial immunodiffusion technique (2) with commercial plates (Hyland); 11-S standards were used to quantitate initial samples through the courtesy of Dr. T. B. Tomasi. These specimens then served as standards for later determinations. To assure further removal of lipid material, the samples were centrifuged again prior to testing at 1,500 rpm at 5°C for 30 minutes. Centrifugation was carrier out in hematocrit tubes from which the supernates could be transferred directly to the wells of immunodiffusion plates without disrupting the fat layer.

Investigation of Bacterial Antibodies

Passive hemagglutinating antibodies against the "0" polysaccharide of *Shigella dysenteriae* 2, *S. flexneri* 1a, *S. flexneri* 6, *S. sonnei*, *Salmonella panama*, and *Escherichia coli* 0111: B4, were investigated using an adaptation (21) of the microtiter technique (22). Supernates of colostrum and milk were inactivated at 56°C for 30 minutes. Two-fold dilutions were made (1:2 to 1:1024) in triethanolamine buffer saline. A volume of 0.05 ml of sample was required to test for antibodies against each antigen.

Quantitation of Bacterial Inhibitory Activity

To test for direct *in vitro* inhibitory activity, supernates from centrifuged unheated specimens of colostrum and milk were diluted (1:5, 1:10, 1:20) in phosphate buffer saline (PBS) containing 0.5 per cent lactalbumin hydrolysate. Dilutions were made in sterile transparent plastic trays (96 wells).

A 10^{-8} dilution of a 17-hour culture of *E. coli* 0111: B4 in trypticase soy broth (BBL) was made in PBS. Volumes of 0.2 ml of the dilution of bacterial culture were added to 0.2 ml volumes of colostrum or milk (and their dilutions) and were mixed in the wells by gentle shaking for one minute. Trays were covered and incubated at 37°C for 3 hours. After incubation, wells were filled with tergitol 7 agar (BBL) with 0.004% triphenyltetrazolium chloride, melted and cooled at 45°C; the contents were mixed using sterile wooden applicator sticks. The trays were sealed and incubated at 37°C for 18 hours. Colonies were counted under low power magnification; end points were read when there was 50 per cent inhibition of bacterial growth in comparison to control wells containing bacterial dilution without colostrum or milk.

Investigation of Viral Antibodies

Viral neutralization tests were done using poliovirus 1 and coxsackievirus B5. Samples were heated at 56°C for 30 minutes before testing. Initially all samples were screened at a 1:10 dilution. Positive specimens were diluted (4-fold) in PBS. A volume of 0.2 ml containing 400 $TCID_{50}$ of virus was mixed with an equal volume of each dilution. After incubation at 37°C for one hour, 0.1 ml of each dilution was inoculated into each of two culture tubes of HEp-2 cells fed with Melnick's medium. Cell cultures were incubated at 37°C and held for two weeks.

RESULTS

Immunoglobulins

IgA was quantitated in 133 samples and IgM in 34. The mean level of IgA in colostrum was 333 mg/100 ml; mean values for 1st, 2nd, and 3rd day colostrum were 433, 322, and 326 mg/100 ml, respectively. Levels fell consistently during the first four weeks of lactation (Figure 1), and remained low until late in lactation when the mean level rose to 242 mg/100 ml. Statistical analysis of mean IgA values, using the multiple F test of Duncan (23), is summarized in Table I. The distribution of observed concentrations of IgM is shown in Figure 2. IgM was consistently detected only in colostrum, and showed a mean value of 36 mg/100 ml; after 4 weeks it was no longer detectable.

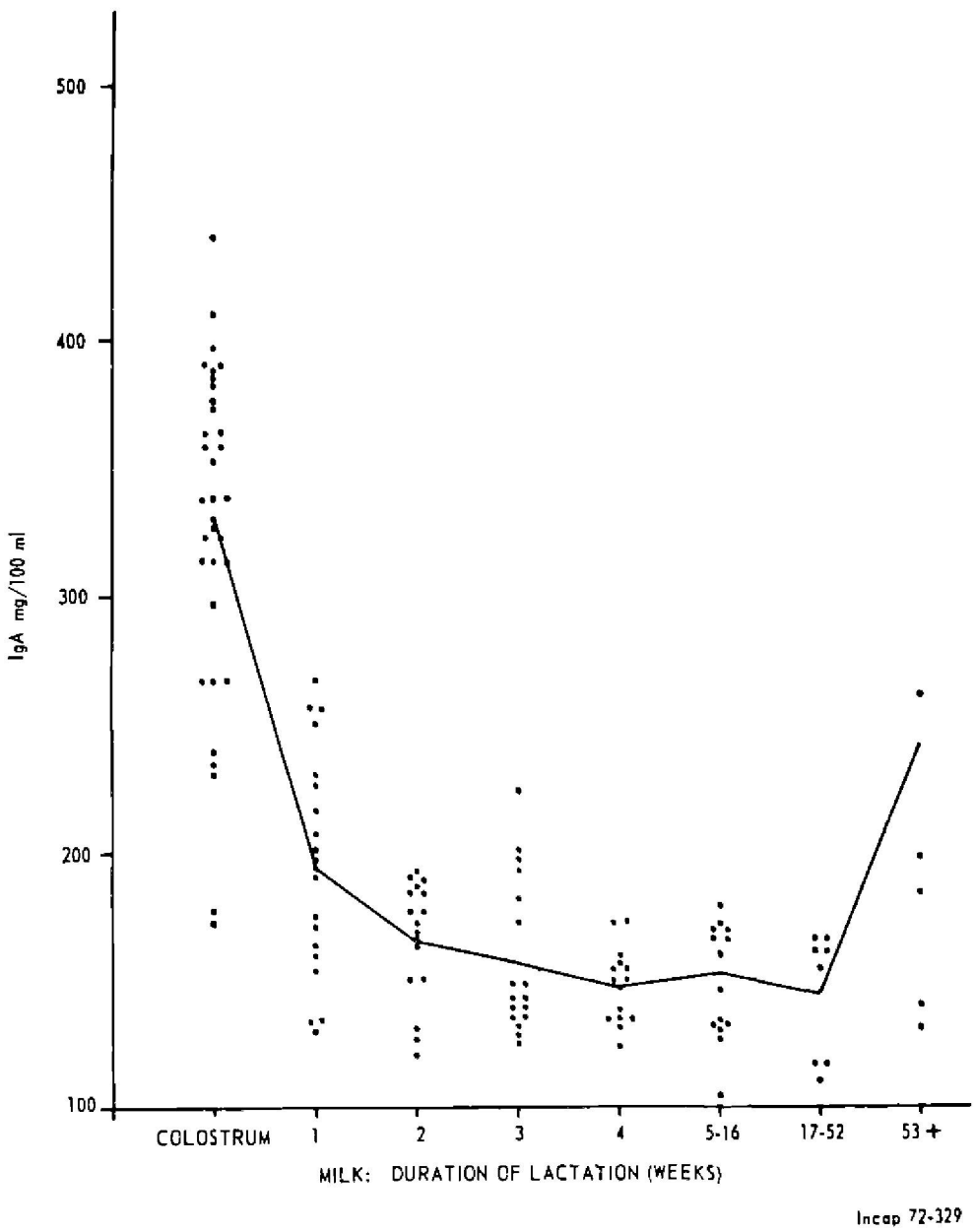

Figure 1.—Distribution of IgA concentrations in colostrum and milk of Guatemalan Mayan women, Santa María Cauqué, 1968. Line indicates mean values.

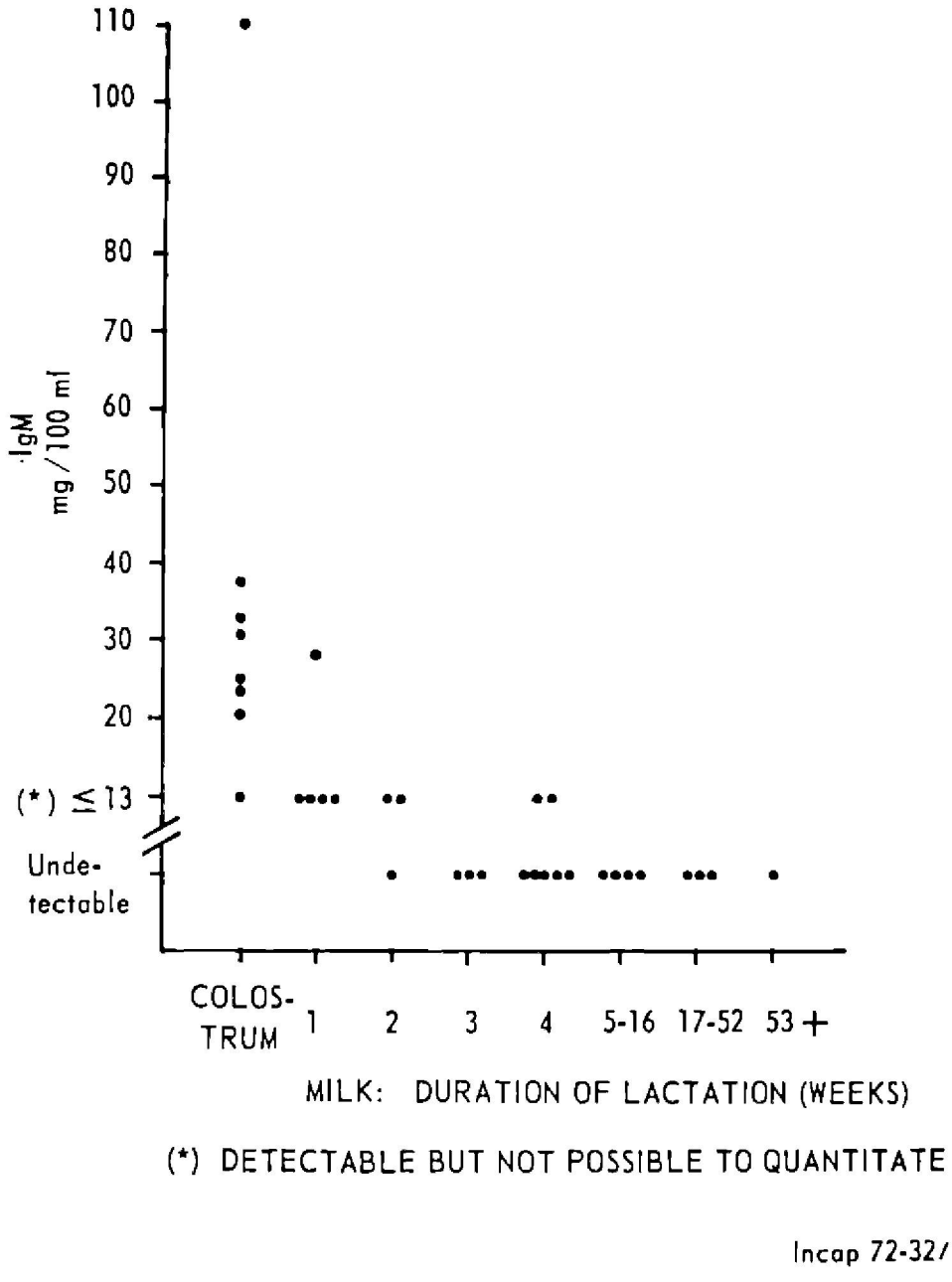

Figure 2.—Distribution of IgM concentrations in colostrum and milk of Guatemalan Mayan women, Santa María Cauqué, 1968.

TABLE Nº 1
STATISTICAL ANALYSIS OF IgA CONCENTRATIONS IN COLOSTRUM AND MILK

Duration of lactation	n	Mean	S. D.
Colostra (3 days)	35	333.2*	71.3
Milk (weeks)			
1	20	195.7*	42.9
2	17	166.3	23.0
3	17	158.3	30.7
4	15	147.8	15.0
5 - 16	14	152.1	21.4
17 - 52	8	144.4	24.9
> 52	7	241.7*	111.7

* Values are different ($p \leqslant 0.05$) from all other mean values using multiple F test of Duncan.

Passive Hemagglutinating Antibodies

Table II presents the geometric means of reciprocal titers of supernates of all samples tested. Figure 3 indicates the percentage of specimens with titers (1:2 or greater) during the course of lactation. The percentage of titers against *S. flexneri* (serotypes 1a and 6) persisted throughout lactation, while the number of titers against *S. dysenteriae* 2, *S. sonnei, Salmonella panama* and *E. coli* 0111:B4 fell during lactation. Where titers existed the levels fell significantly only with *S. flexneri* (serotype 1a and 6) and *S. dysenteriae* 2, as shown by the multiple F test of Duncan ($p \leqslant 0.05$).

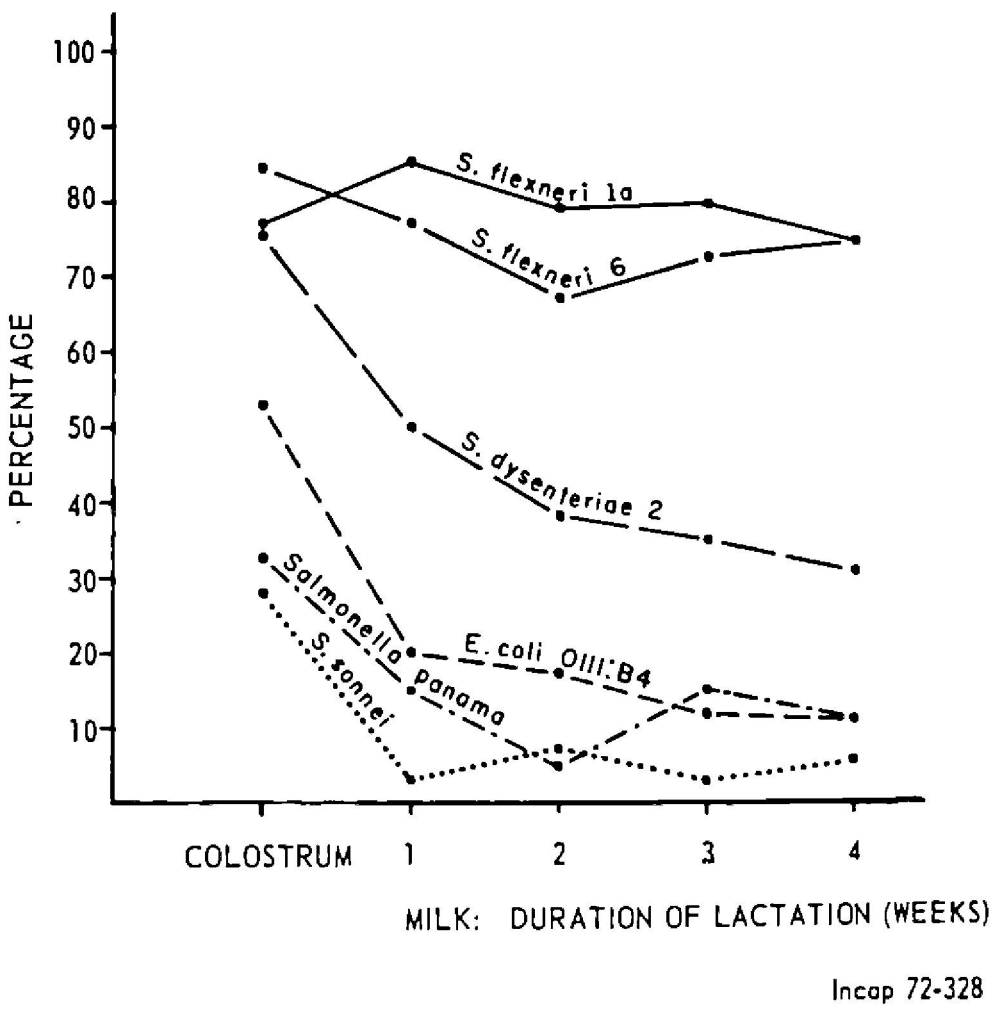

Figure 3.—Percent of samples tested containing passive hemagglutinating bacterial antibodies to "0" polysaccharide.

TABLE Nº 2

PASSIVE HEMAGGLUTINATING ANTIBODY IN COLOSTRUM AND MILK

Duration of lactation	E. coli 0111:B4	Salmonella panama	S. dysenteriae 2	S. flexneri 1a	S. flexneri 6	S. sonnei
Colostrum (1-3 days)	6.4(16/30)*	4.6(10/30)	18.8(22/29)	23.3(24/31)	11.0(26/31)	2.6(8/29)
Milk (weeks)						
1	5.2(8/40)	5.0(6/40)	8.3(20/40)	7.7(33/39)	6.1(30/39)	(1/40)
2	5.4(7/42)	8.0(2/42)	10.4(16/42)	7.8(33/42)	6.3(28/42)	5.0(3/42)
3	5.3(5/41)	6.4(6/41)	9.3(14/40)	7.8(33/41)	5.0(30/41)	(1/40)
4	3.4(4/36)	5.7(4/36)	10.3(11/35)	6.7(27/36)	4.7(27/36)	4.0(2/35)
5 - 16	(0/14)	(0/14)		11.0(13/14)	6.0(12/14)	
17- 52	(0/8)	(0/8)		8.0(7/8)	8.0(6/8)	
52	(1/5)	(1/5)		8.0(5/5)	5.3 (5/5)	

* Geometric mean of the reciprocal titers and, in parentheses, numbers of specimens with antibody in total examined.

Bacterial Inhibitory Activity

Twenty-five samples, including 7 colostra, were tested for *in vitro* capacity to inhibit *E. coli* 0111:B4. Inhibitory activity was found in 5 of the 7 colostra; none of the 18 milk specimens showed activity. Figure 4 shows the appearance of inhibition in the test system. A direct correlation was observed btween inhibitory activity and hemagglutinating antibody titers, Table III.

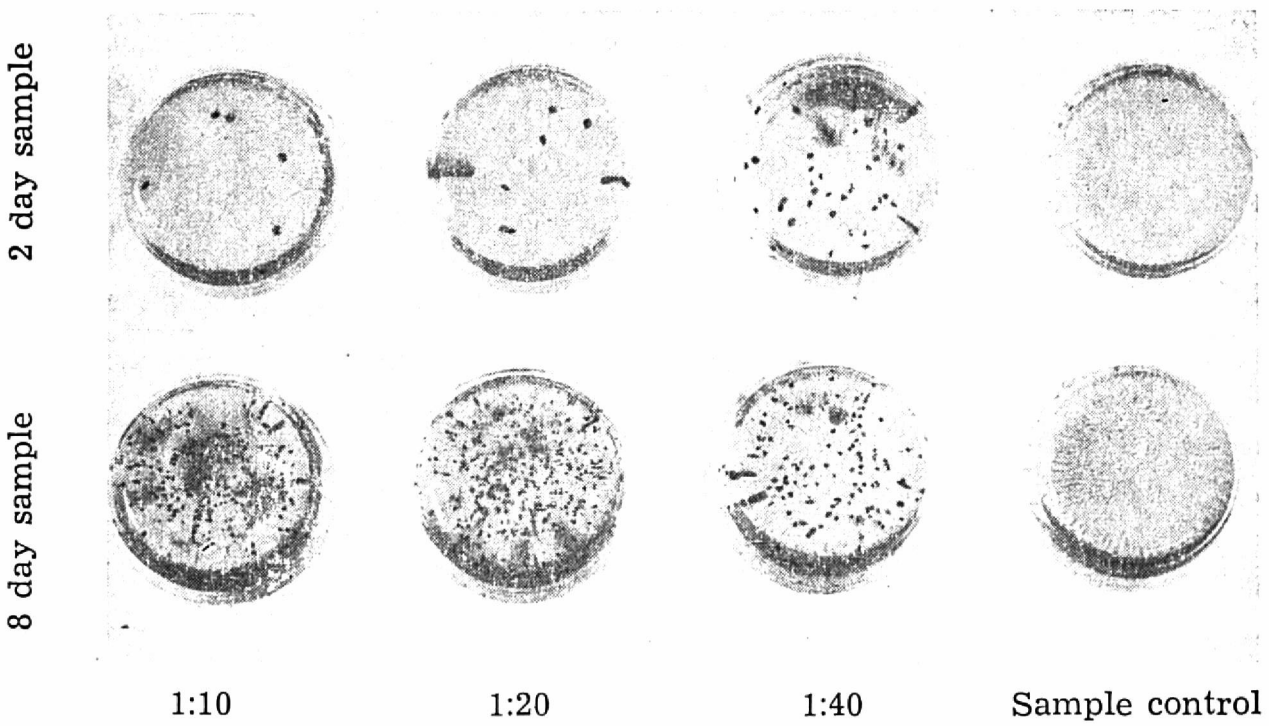

Figure 4.—Samples of colostrum and milk tested for bacterial inhibitory activity using E. coli 0111: B4. 0.2 ml of 10^{-8} dilution of E. coli 0111: B4 added to each sample dilution.

Viral Neutralization

Forty-nine samples, including 9 colostra, were tested for viral neutralizing antibody. Eight of the colostra had titers against poliovirus 1, ranging from 1:10 to 1:160; six of the colostra had titers against coxsackie B5 ranging from 1:10 to 1:640. Later in lactation titers were less frequent and lower, as shown in Table IV.

TABLE Nº 3

PASSIVE HEMAGGLUTINATING ANTIBODY AND BACTERIAL INHIBITORY ACTIVITY AGAINST E. coli 0111:B4

Subject	Duration of lactation (days)	Bacterial Inhibition titer	Hemagglutination titer
1	2	1:20	1:4
	7	0	0
2	2	1:40	1:4
	8	0	0
3	3	1:20	1:4
	8	0	0
4	3	1:10	trace
	8	0	0
5	3	1:10	*
	6	0	0

* Nonspecific hemagglutination.

TABLE Nº 4

VIRAL NEUTRALIZING ANTIBODY IN COLOSTRUM AND MILK

Duration of lactation	Poliovirus 1	Coxsackievirus B5
Colostrum (1-3 days)	0-160* (8/9)	0-640 (6/9)
4-28 days	0-10 (1/15)	0-10 (1/15)
29 days - 52 weeks	0-10 (1/20)	0 (0/20)
> 52 weeks	0-10 (2/5)	0 (0/5)

* Range of reciprocal determinations; number of positive samples in total tested shown in parentheses.

DISCUSSION

Prolonged breast-feeding is part of the cultural pattern throughout pre-industrial societies. This is important not only in terms of its nutritive value and its being relatively protected from contamination (24), but because it has been found to contribute protective mechanisms against infection. This acquires greater significance owing to the high prevalence of infection in developing societies. Industrialization is rapidly expanding to urban areas of developing countries and affecting the pattern of breast-feeding, even in rural areas. There is a public health concern that abrupt weaning in the very early months of life will not only carry a nutritional risk, but will remove defense mechanisms against infection. The present study deals with some of the protective factors that are present in milk of women in an ecosystem where malnutrition and infection are highly prevalent.

Reported values of IgA in colostrum vary widely; a rapid downward trend in the first three days of lactation has been described, which may explain some of the variation. The values obtained in this study were, however, similar to others from different areas of the world. The mean IgA concentration for 2nd day colostrum was 322 mg/100 ml compared with 693 mg/100 ml (25); 750 mg/100 ml (7); 260 mg/100 ml (16); and approximately 500 mg/100 ml (26).

Data are available on the volume of breast milk, ingested per day during the first two years of life, in a region ecologically similar to the study area (27). Considering this information and the average IgA content in milk, excluding colostrum, a child may receive between 700-1300 mg of IgA per day. Since secretory IgA is sufficiently resistant to proteolysis (28), these quantities probably reach the intestinal lumen.

The persistance of hemagglutinating antibody titers against *S. flexneri* (serotypes 1a and 6) may be related to the higher prevalence of the *flexneri* group in the village as compared to other serotypes (29). It should be noted that *Shigella* infection is very rare prior to weaning, thus suggesting that IgA exerts a protective influence, although other factors such as lysozyme may play a role as indicated before.

Passive hemaglutinating antibody titers were correlated with inhibitory bacterial activity as previously shown by

others (16). Although hemagglutinating antibody cannot be assigned a bactericidal role, this correlative observation, however, suggests the value of examining such titers.

Patterns of antibody activity in colostrum and milk reflect the endemicity of bacterial and viral pathogens in the population studied, as determined by continuous isolation technique. Such antibody activity may play a significant role during intestinal colonization of the infant by averting infection with enteric pathogens, particularly in a period where other host defense mechanisms have not developed or are in the process of being established.

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RESUMEN

Inmunoglobulinas y anticuerpos en calostro y leche de mujeres Mayas de Guatemala

Se determinaron inmunoglobulinas y anticuerpos contra ciertos virus y bacterias en calostro y leche, en una población expuesta a un alto riesgo de desnutrición e infección. Los niveles de IgA fueron altos en calostro (333 mg por 100 ml) así como más tardíamente durante el destete que es tardío en la región (242 mg por 100 ml). El calostro contenía IgM (36 mg por 100 ml), más no así la leche a las 4 semanas de lactancia. Se demostraron títulos de anticuerpos contra bacterias y virus en calostro y menos frecuentemente en leche. Se observó una correlación entre los niveles y frecuencia de títulos de anticuerpos y la endemicidad de ciertos patógenos en la población. La importancia de estos hallazgos se discute en relación a la inmunidad local adquirida tempranamente, a la colonización e infección intestinal por bacterias y virus, y finalmente con referencia a la importancia de la alimentación al seno en sociedades pre-industrializadas.

BIBLIOGRAPHY

(1) Kenny, J. F., M. I. Boesman & R. H. Michaels. Bacterial and viral coproantibodies in breast-fed infants. Pediatrics, 39: 202-213, 1967.

(2) Bellanti, J. A. Role of local gamma-A-immunoglobulins in immunity. Amer. J. Dis. Child., 115: 239-246, 1968.

(3) Heremans, J. F., P. A. Crabbe & P. L. Masson. Biological significance of exocrine gamma-A-immunoglobulin. **Acta Med. Scand.**, 179 (Suppl. 445): 84-88, 1966.

(4) Tomasi, T. B. Human immunoglobulin A. **New Engl. J. Med.**, 279: 1327-1330, 1968.

(5) Dayton, D. H., P. A. Small, R. M. Chanock, H. E. Kaufman & T. B. Tomasi (Eds): **The Secretory Immunologic System**, USDHEW, PHS, National Institutes of Health, Bethesda, Maryland, 1969.

(6) Harfouche, J. K. The importance of breast-feeding. **J. Trop. Pediat.**, 16: 133-175, 1970.

(7) Bazzi, C., R. Cattaneo, V. Mignone & M. V. Farina. Further observations on immunoglobulins of external secretions. **Progr. Immunobiol. Standard.**, 4: 333-335, 1970.

(8) Jolles, P. & J. Jolles. Lysozyme from human milk. **Nature**, 192: 1187-1188, 1961.

(9) Smith, C. W. & A. S. Goldman. The cells of human colostrum. I. **In-vitro** studies of morphology and functions. **Pediat. Res.**, 2: 103-109, 1968.

(10) Murillo, G. J. & A. S. Goldman. The cells of human colostrum. II. Synthesis of IgA and lC. **Pediat. Res.**, 4: 71-75, 1970.

(11) Schafer, T. W., Lieberman & P. E. Came. Studies on the kinetics of interferon. Appearance of milk and its effect on resistance of suckling mice to virus challenge. Presented at International Colloquium on Interferon and Interferon Inducers, Leuven, Belgium, Sept., 1971.

(12) Lepow, M. L., R. J. Warren, N. Gray, V. G. Ingram & F. C. Robbins. Effect of Sabin type 1 poliomyelitis vaccine administered by mouth to newborn infants. **New Engl. J. Med.**, 264: 1017-1078, 1961.

(13) Plotkin, S. A., M. Katz, R. E. Brown & J. S. Pagano. Oral poliovirus vaccination in newborn African infants. **Am. J. Dis. Child.**, 11: 27-30, 1966.

(14) Katz, M. & S. A. Plotkin. Oral polio immunization of the newborn infant; a possible method for overcoming interference by ingested antibodies. **J. Pediat.**, 73: 267-270, 1968.

(15) Akao, Y., A. Sasagawa, S. Shiga & R. Kono. Comparative studies on the mode of neutralization reaction of poliovirus type 2 with serum IgG and secretory IgA from mothers's milk and fecal extract. **Japan J. Med. Sci. Biol.**, 24: 135-152, 1971.

(16) Michael, J. G., R. Ringenback & S. Hottenstein. The antimicrobial activity of human colostral antibody in the newborn. **J. Inf. Dis.**, 124: 445-448, 1971.

(17) Mata, L. J., J. J. Urrutia & B. García. Effect of infection and diet on child growth: Experience in a Guatemalan village. **Ciba Foundation Study Group** N^{o} 31, **Nutrition and Infection**, Boston (Little, Brown and Company), 1967, pp. 112-134.

(18) Mata, L. J. & J. J. Urrutia. Intestinal colonization of breast-fed children in a rural area of low socioeconomic level. **Ann. N. Y. Acad Sci.**, 176: 93-109, 1971.

(19) Wyatt, R. G. & L. J. Mata. Bacteria in colostrum and milk of Guatemalan Indian women. **J. Trop. Pediat.**, 15: 159-162, 1969.

(20) Mancini, G., A. O. Carbonara & J. F. Heremans. Immunochemical quantitation of antigens by single radial immunodiffusion. **Int. J. Immunochem.**, 2: 235-254, 1965.

(21) Cáceres, A. & L. J. Mata. Hemaglutinación indirecta para la investigación de anticuerpos a enterobacteriáceas. **Rev. lat-amer. Microbiol.**, 12: 137-144, 1970.

(22) Lee, M. R., N. S. Ikari, W. C. Branche Jr. & V. M. Young. Microtiter bacterial hemagglutination technique for detection of **Shigella** antibodies **J. Bact.**, 91: 463, 1966.

(23) Duncan, D. B. Multiple range and multiple F tests. **Biometrics, 11:** 1-42, 1955.

(24) Jelliffe, D. B. & E. F. P. Jelliffe. The uniqueness of human milk - An Overview. **Am. J. Clin. Nutr.**, 24: 1013-1024, 1971.

(25) Ansaldi, N., G. Cirioti & J. Liberatori. Le immunoglobuline nel latte e nel colostro di donna. **Minerva Pediatrica, 20:** 1969-1981, 1968.

(26) Ammann, A. J. & E. R. Stiehm. Immune globuline levels in colostrum and breast milk, and serum from formula-and breast-fed newborns. **Proc. Soc. Exptl. Biol. Med.**, 122: 1098-1100, 1966.

(27) Martínez, C. & A. Chávez. Nutrition and development in infants of poor rural areas. I. Consumption of mother's milk by infants. **Nutr. Rep. International**, 4: 139-149, 1971.

(28) Tomasi, T. B. Jr. Structure and functions of mucosal antibodies. **Ann. Rev. Med.**, 21: 281-298, 1970.

(29) Mata, L. J., R. Fernández & J. J. Urrutia. Infección del intestino por bacterias enteropatógenas en niños de una aldea de Guatemala, durante los tres primeros años de vida. **Rev. lat-amer. Microbiol. Parasitol.**, 11: 102-109, 1969.