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Serum Hemolytic and Bactericidal Activity in Breast and Formula-Fed Infants

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Whether formula or breast feeding influences the functional activity of the complement system from birth to three months of age has been studied. The classical pathway was evaluated by assessing hemolytic activity, based on the capacity of the intact complement system to lyse sheep erythrocytes when coated with specific antibodies. The bactericidal activity of the serum against *Staphylococcus aureus* and *Escherichia coli* was used to evaluate the alternative complement pathway. Sera were obtained from neonates (40 ± 2 weeks of gestation), and one-month or three-month old infants, fed either breast or formula. Control serum was obtained from healthy adults between 22 and 30 years of age. The hemolytic capacity of serum from breast-fed infants of one month and three months of age was significantly greater than that of the serum from infants which had been fed formula milk.

Key words: Complement, Formula milk, Mother's milk, Serum.

The incidence and mortality due to infection in man is greater in the early months of life than at all other periods with *Staphylococcus aureus* and *Escherichia coli* being amongst the more common infectious agents (15). Numerous studies have demonstrated that breast fed infants are less susceptible than formula fed infants to these various infections (2-4, 8, 9, 14, 16), and human colostrum and milk has been shown to possess a wide variety of host-resistance factors (13).

The complement system plays a key role in the host defence against microorganisms and its importance is clearly seen in those infants with complement deficiencies who have an increased risk of developing severe and recurrent microbial infections (5). No transport system exists

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for maternal complement proteins to cross the placenta. For this reason, the fetus and neonate are completely dependent upon the synthesis of these proteins by their own cells.

Since recent studies have suggested a mutual relationship between nutrition and optimum immune responses (1), the aim of this study was to investigate whether formula or breast feeding influences the functional activity of the infants complement system from birth until three months of age. Measurement of the serum hemolytic capacity in the presence of red-blood-cell antibodies was used as an indicative measure of activation of the classical pathway, and bactericidal activity against *S. aureus* and *E. coli* used to evaluate the alternative complement pathway.

Materials and Methods

Groups.– Newborn infants: 80 neonates (40 ± 2 weeks of gestation) who were born consecutively in the Hospital Materno-Infantil in Badajoz, were selected from healthy mothers between 26 ± 4 years of age previously informed of the protocol involved. All infants or mothers who developed problems postnatally were eliminated from the study. The mothers were chosen on the basis of a normal pregnancy and past medical history.

One-month-old infants: At the moment of birth, feeding of the neonates was separated randomly divided into two groups: half of the infants were breast-fed and the other half formula-fed (Nidina I). At one month of age, serum samples were taken. Three-month-old infants: Similar groups as above were established. However, serum samples were not taken until the infants were three months of age.

This study was approved by the ethical committee of the University of Extremadura.

Serum.– Blood samples were taken from the umbilical cord in neonates and by venipuncture from one-month and three-month old infants. After allowing the blood to clot at room temperature, samples were kept overnight at 4 °C. Subsequently, the blood was centrifuged for 15 min at 300 x *g* and the serum separated and stored at -70 °C in small aliquots until required for assay. A pool of serum obtained from eleven healthy young men aged from between 22 and 30 years was used for control values. Parallel determinations were made with heat inactivated serum (56 °C, 30 min) in order to ensure that any possible modifications in the presence of non-decomplemented serum were due to the complement.

For the study of the hemolytic activity of complement, the serum was diluted in Isogever to 1/200, 1/300, 1/400 and 1/500 dilutions. For the study of serum bactericidal activity, the sera were diluted in MHB (Mueller-Hinton Broth) to a dilution of 1/16.

Bacteria.– The bacteria used were *Staphylococcus aureus* (ATCC 9144) and *Escherichia coli* (ATCC 35218) obtained from the Departamento de Microbiología de la Facultad de Medicina, (Badajoz, Spain).

Isogever preparation.– Gelatine (10 %) (Sigma) plus 0.425 g sodium azide (Sigma) were dissolved in 100 ml of distilled water heated to 56 °C until dissolved. The Isogever was prepared by mixing 1 ml gelatin solution and 100 ml dissolved Complement Fixation Test (CFT, Flow). The mixture could be kept at 4 °C, protected from light and was viable for two weeks.

Hemolysin (Cerdarlane), a specific antibody for sheep erythrocytes, was diluted 1:1000 in Isogever.

Sheep red blood cell (SRBC) treatment.– SRBC's (Biomedics) were first

washed twice (10 min, 300 x g) with PBS (phosphate buffered saline solution) and then once with Isogever, followed by incubation for 30 min at 37 °C with hemolysin. After this time, the cells were adjusted to a concentration in distilled water that gave 100 % absorbance at 415 nm (total hemolytic absorbance).

Serum hemolytic activity.– Serum hemolytic activity was evaluated by using a modification of the CH-5C (Capacity hemolytic) technique of GAITHER and FRANK (7). Thus, 100 µl SRBC were incubated for 30 min at 37 °C with the various dilutions of serum at a final volume of 500 µl. Following this 1.5 ml Isogever was added to each sample and centrifuged for 2 min at 500 x g. The supernatants from each sample were read spectrophotometrically at 415 nm, using Isogever as a blank. Finally, the values obtained were transformed into a Probit index using the formula:

$$\text{Probit} = Y/100 - Y$$

where

$$Y = \frac{\text{Sample absorb.} - \text{Blank absorb.}}{\text{Total hemolysis absorb.}} \; 100$$

The CH-50 is obtained when the Probit index is equal to 1.

Serum bactericidal activity.– The serum was diluted in MHB to a titer of 1/16 with a final tube volume of 100 µl. To this, 10 µl of 1 x 10^5 CFU (colony forming unity) of *S. aureus* or *E. coli* per ml was added. In addition, there was a tube with MHB and bacteria inoculum but without serum. Content tubes were seeded onto Mueller-Hinton agar plates and incubated at 37 °C, 100 % humidity, and 5 % CO_2. After 24 h, CFUs were determined and the results expressed as a growth percentage (CFU %) by means of the expression: CFU (%) = CFU samples with serum/CFU samples without serum x 1, where 100 % (maximum growth) represents the number of CFU surviving in a control sample and where the bacteria had been incubated in the absence of serum.

Statistical analysis.– The data are expressed as mean ± standard deviation of the number of experiments performed. They were subjected to one-way analysis of variance using Kruskal-Wallis ranks, followed by Mann-Whitney two-tailed U test, $P < 0.05$ being taken as the minimum significance level.

Results

The influence of formula feeding and breast feeding upon the infant's serum hemolytic capacity is shown in table I. The results are expressed in CH-50 units, which represent the capacity of serum complement to lyse 50 % of SRBC in the presence of a specific antibody. At birth, complement hemolytic capacity was undetectable by means of the CH-50 technique. Both one month breast fed and formula fed infants had serum with a lower CH-50 value when compared to the adult controls, although this decrease was statistically significant ($p < 0.05$) only in the formula fed group. However, serum

Table I. *Hemolytic activity of serum (CH-50 units) from breast-fed and formula-fed infants.*
Each value represents the mean ± S.D. of 40 determinations performed in duplicate. Controls are obtained from adult's serum.

	One month old infants	Three month old infants
Control	179 ± 33	179 ± 33
Breast-fed	163 ± 55	184 ± 21
Formula-fed	126 ± 15*°	160 ± 21°

* < 0.05 with respect to control values.
° < 0.05 with respect to breast-fed values of the same age.

from three month old formula-fed and breast-fed infants both possessed values which were similar to those of the control. There was a significant increase in the number of CH-50 units in the serum of three month old breast-fed children compared to that of three month old infants being formula fed.

The bactericidal mechanism of serum is mediated principally by the alternative complement pathway. In fig. 1 the results of either formula feeding or breast feeding from birth upon one and three month old infant's serum bactericidal activity against *E. coli* and *S. aureus*, respectively, are shown. Minimum bactericidal activity (which correlates with an increase in the CFU %) was obtained in serum samples taken immediately after birth. No differences were obtained between the bactericidal activity of control samples compared with that of one-month or three-month old sera from either formula fed of breast fed infants. The bactericidal activity in serum samples from one month and three month old infants, independently of the form of feeding was greater than that in the serum of neonates.

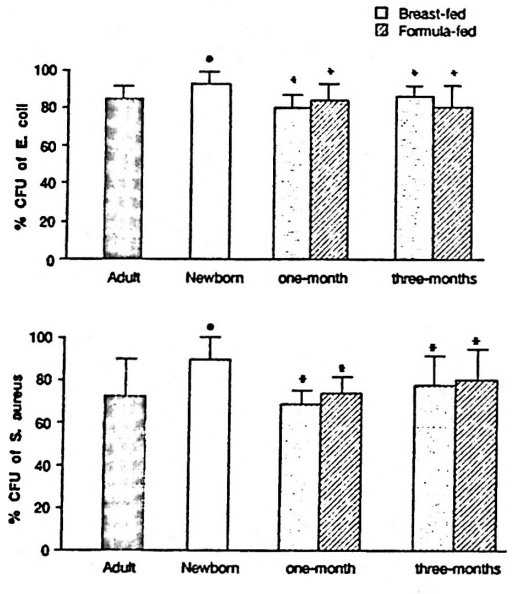

Fig. 1. *Bactericidal activity against* Escherichia coli *and* Staphylococcus aureus *of serum from newborn, one and three month old either breast-fed or formula-fed infants.*
The values are expressed in CFU % (colony forming unity) of *E. coli* or *S. aureus* in presence of samples. Each value represents the mean ± SD of 40 determinations performed in duplicate. Controls were obtained from adult serum. • $p < 0.05$ with respect to control values; * $p < 0.05$ with respect to newborn infants' values.

Discussion

Human maternal milk is the natural food for the newborn in the first months of their life, presenting many advantages since it is always ready to use, is inexpensive and ensures a more rapid growth in the children. Taking into account the results observed recently by our research group, human maternal milk can also be said to induce immunological advantages. Thus, FORNER *et al.* (6), have found that serum from breast-fed infants: 1) Presents a greater chemotactic activity than serum from formula-fed infants and 2) Induces a greater attachment and ingestion capacity of *Candida albicans* by neutrophils than incubation with serum from formula fed infants.

The complement system constitutes a crucial mechanism in the development of the immune system, since it serves as a vital link in the action of antibodies against a variety of antigens, as well as the recruitment and activation of inflammatory cells and lytic destruction of certain foreign cell-membranes. The complement system in the neonate demonstrates deficiencies when compared with adults and no transport system exists for maternal complement proteins to cross the placenta (15). Considering that human milk possesses a wide variety of host-resistant fac-

tors in addition to T and B lymphocytes, macrophages, mononuclear cells, humoral components, lysozyme and lactoferrin (10), the influence of breast-feeding and formula feeding upon the development and/or activation of the complement system from birth to three months of age has been evaluated in this study.

Measurement of the hemolytic activity of complement, based on the capacity of the intact complement system to lyse sheep erythrocytes when coated by specific antibodies, was used to assess the classical pathway. Complement activity in the serum of one month old breast-fed infants was greater than that of infants fed artificial milk (table I). This observation may be due to the presence in human milk of a high concentration of macrophages which are able to synthesise complement factors (2); alternatively human milk also contains some proteins of the complement system, although in very small amounts (11). At three months of age, however, the efficiency of the classical. complement pathway was similar in both groups of infants. By this time of life, the activity and/or development of the complement system classical pathway may have matured.

Bactericidal activity against *E. coli* and *S. aureus* (which correlates with an increase in the CFU %) was lowest in newborn infant serum (fig. 1). Similar results were observed by McCRACKEN and SHINEFIELD (11) and MILLER (12). This fact may be the reason for the higher incidence of bacterial infection suffered by neonates. However, no significant differences in bactericidal activity were observed in serum from one month or three month old infants irrespective of the type of milk they were fed as compared to control values (serum from adults). For this reason, it is postulated that the alternative complement pathway fully matures one month after birth.

In conclusion, breast feeding does appear to have a positive role in the activation and/or development of the classical complement pathway during the first month of extrauterine life.

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C. BARRIGA, I. POMBERO, J. DURÁN, A. FORNER, J. CARDESA y A. B. RODRÍGUEZ. *Actividad hemolítica y bactericida del suero en niños alimentados con leche artificial o materna*. Rev. esp. Fisiol. (J. Physiol. Biochem.), 51 (4), 213-218, 1995.

Se valora si durante los primeros meses de vida, –desde el momento del nacimiento hasta los tres meses–, la alimentación natural o artificial influye sobre la actividad funcional del sistema del complemento de vida. La vía clásica ha sido evaluada a través de la actividad hemolítica, basada en la capacidad del sistema del complemento en lisar hematíes recubiertos con anticuerpos específicos. La actividad bactericida del suero frente a *Staphylococcus aureus* y *Escherichia coli* se ha utilizado para valorar la ruta alternativa del complemento. Los sueros han sido obtenidos de recién nacidos (40 ± 2 semanas de gestación), de un mes y de tres meses de vida alimentados con leche artificial o materna. Los sueros controles proceden de adultos sanos entre 22 y 33 años. La capacidad hemolítica de los sueros obtenidos de niños alimentados al pecho, al mes y a los tres meses de vida, es significativamente superior al que presentan los sueros de niños que han sido alimentados con leches artificiales.

Palabras claves: Complento, Leche materna, Leche artificial, Suero.

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