Deficient Induction of the Immune Response to Oral Immunization with Cholera Toxin in Malnourished Rats during Suckling

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Malnourished rats during suckling were orally immunized with cholera toxin (CT) after different periods of refeeding. Intestinal fluids, sera, and supernatant fluids from cultured mesenteric lymph node (MLN) cells were obtained after rats were given three doses of CT and analyzed by enzyme-linked immunosorbent assay (ELISA) to evaluate the specific antibody response. Serum-specific immunoglobulin G (IgG), IgA, and IgM were severely diminished in malnourished rats immunized with three doses of CT after 1 week of refeeding when compared with those of controls. Also, a decreased IgA ELISA titer of the intestinal fluids and abrogation of the capacity to neutralize the CT in the intestinal ligated loop test were found. When a booster was given at 113 days of age, the immune response continued to be affected in the serum and the intestinal fluid. The results from the analysis of the supernatant fluids from cultured MLN cells were coincident with those mentioned above. When one dose of CT was administered into Peyer's patches (PP) after 1 week of refeeding, an impaired immune response was found in the intestinal fluid of malnourished rats during suckling compared with that of controls. This result together with the analysis of supernatant from MLN and PP cell cultures suggests that antigen triggering in the PP was affected. When the refeeding period was extended to 30 days and then the first dose of CT was administered, the antibody immune responses in intestinal fluid serum and supernatant fluid approached control values. These observations reinforce the fact that the gut-associated lymphoid tissue immaturity of the rats when they received the first CT dose (at 28 days old) was the main reason for the decreased immune response observed in the experimental group.

It is well known that malnutrition provokes severe alterations in the thymus (36) and in the T-cell-dependent areas of lymphoid organs (2, 7). These alterations lead to a decrease in the capacity to produce antibodies to T-cell-dependent antigens (4, 8) and alter cell-mediated immunity (2, 15) as well as cytokine synthesis (28). The mucosal immunity is also affected and may result at least in part, in defective immunoglobulin A (IgA) antibody responses on mucosal surfaces (5). In addition, different studies have shown a decreased number of intraepithelial lymphocytes and IgA-positive B cells in the lamina propria (6). It has been reported that malnutrition during suckling provokes a delay in the cell cycle in bone marrow (3, 29). In previous work, we have reported that rats malnourished during suckling present, after 30 days of refeeding with a stock diet, a delay in B-cell terminal differentiation as well as decreased percentages of $CD5^+$, $CD4^+$, and $CD8^+$ T cells in gut-associated lymphoid tissue (GALT). The last alteration remained even after 90 days of refeeding (11). The GALT is the principal zone of induction in the mucosal immune system; therefore, it is of interest to clarify whether the alterations mentioned above have functional consequences in some steps of the development of the mucosal immune response. For this purpose, cholera toxin (CT), which is known to be a potent mucosal immunogen (10, 21, 22, 32, 34), was administered to rats by the oral route after different periods of refeeding.

MATERIALS AND METHODS

Animals. Wistar rats kept at a constant temperature of 18 to 21°C and under a 12-h day-night cycle were used. One-day-old rats were randomly assigned to either an experimental or a control group. To achieve undernutrition, one mother nursed 15 to 16 pups (malnourished rats [MNR]) (38). Well-nourished rats (WNR) suckled in litters of six to eight pups were used simultaneously as controls. At weaning, the MNR and the WNR were fed a stock diet (24% protein) ad libitum. To define their nutritional status, the rats were weighed and the serum protein concentrations were measured by the methods of Lowry et al. (18a).

Immunizations and sample collection. The immunization schedules used were as follows. CT (Sigma Chemical Co., St. Louis, Mo.) dissolved in phosphate-buffered saline (PBS) with 3% sodium bicarbonate was administered intragastrically by means of a neonate cannula under light anesthesia. One group of MNR and WNR (group 1) received three doses of CT at intervals of 1 week (10, 7.5, and 7.5 µg) (31). Immunization started when the rats were 28 days old, i.e., when germinal centers begin to mature (35). Seven days after the third dose, the rats were bled under ether anesthesia and the sera were stored at −20°C. To obtain intestinal secretions, the rats were killed, the small intestine was dissected out and washed to remove any associated blood, and the intestinal secretions flushed out with 4 ml of EDTA (50 mM) plus 0.1 mg of soybean trypsin inhibitor (Sigma) per ml were collected in conical tubes (9). After adding 20 µl of 100 mM of phenylmethylsulfonyl fluoride (Sigma) in ethanol, the tubes were then centrifuged. The supernatants were stored at −20°C until used. A second group of MNR and WNR (group 2) that received three doses of CT as described above were monitored until the rats were 113 days of age (92 days of refeeding), after which an intragastric booster of CT was administered (15 µg). Seven days later, samples were collected as described above. A third group of MNR and WNR (group 3) received three doses of CT (15 µg) at 7-day intervals, the first one at 50 days of age (30 days of refeeding). One week after the last dose, the samples were collected.

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To study the induction of the immune response, 28-day-old MNR and WNR were immunized with CT into Peyer's patches (PP) as follows. Rats were anesthetized with ether, and a midline incision was made in the abdominal wall of each. Ten micrograms of CT in 10 μl of PBS was injected into five PP of the middle small intestine. Five days later, samples were collected.

Determination of total IgA and antitoxin antibody levels. Antitoxin antibodies were determined by an enzyme-linked immunosorbent assay (ELISA) with slight modifications to the technique described by Lycke et al. (23). Briefly, polystyrene microtiter plates (Corning Glass Works, Corning, N.Y.) were coated with GM1 ganglioside (Sigma) at a concentration of 2 μg/ml in PBS and incubated overnight at room temperature. After rinsing three times with PBS, purified CT (2 μg/ml in PBS) was added, and the plates were incubated as described above. Residual protein-binding sites were blocked with PBS containing 8% defatted milk. Intestinal fluid samples were analyzed at dilutions of 1/5 and 1/50. Sera were analyzed at dilutions of 1/20 and 1/10,000, and supernatant fluids were analyzed at a dilution of 1/2. Samples were incubated for 1 h at 37°C. Antitoxin antibodies were detected with affinity-purified goat anti-rat immunoglobulin class-specific antisera (Bethyl Laboratories, Inc.), followed by IgG affinity-purified rabbit anti-goat immunoglobulin horseradish peroxidase conjugate (Sigma). The microtiter plates were washed five times with PBS-0.05% Tween between each step. O-Phenylenediamine, 1 mg/ml with 0.025% H_2O_2, was finally added to the wells, the reaction was stopped with $SO_4H_2 \cdot 2N$, and the A_{492} was read. Antitoxin levels in sera and in intestinal fluid were expressed as arbitrary ELISA units obtained from standard curves derived from 40 samples with different levels of anti-CT antibodies with a cutoff of 0.4 above the background. Antitoxin ELISA units in supernatant from cell cultures were obtained from a standard curve with a cutoff of 0.2 above the background. Every time, a reference sample was run simultaneously with the unknown samples.

For determination of whole IgA in intestinal fluid, the plates were coated with affinity-purified goat IgG anti-rat α chain (Bethyl; 6 μg/ml) in 0.05 M carbonate buffer (pH 9.5) and incubated overnight at room temperature. Samples were incubated for 2 h at 37°C. The reaction was developed with a horseradish peroxidase conjugate-specific anti-rat IgA (Bethyl). The optical densities were converted into immunoglobulin concentration (in micrograms per milliliter) by interpolation in the lineal range of a sigmoid standard curve derived from purified IgA (ICN Biomedicals, Inc.). To calculate the total micrograms of IgA, concentrations obtained in this way were adjusted for the volume of recovered intestinal fluid. In 32-day-old rats, since the length of the small intestine of each MNR was approximately one-fourth shorter than that of each WNR, total values of IgA were corrected by an adjustment factor. The same protocol was used to measure the IgA concentration in serum. An identical protocol was used to determine the IgG concentration in serum, except that affinity-purified goat anti-rat IgG (Sigma; 3 μg/ml) was used to coat the plates and affinity-purified goat anti-rat IgG coupled to horseradish peroxidase (Bethyl) was used as the detector antibody. The standard curve was derived from results with a calibrate serum (Bethyl).

Protection in intestinal ligated loop test. The intestinal ligated loop technique was done as described by Lange and Holmgren (17). Briefly, rats were fasted for 24 h prior to the operations. The abdomen of each rat was surgically opened under ether anesthesia, and a 10- to 14-cm loop was ligated in the mid-part of the small intestine. One microgram of CT in

TABLE 1. Body weights and serum protein concentrations of MNR during suckling and of controls (WNR)

Age (days)	Rat group (n)	Body wt (g)	Serum protein concn (mg/ml)
21	WNR (18)	51 ± 5[a]	54 ± 12
	MNR (29)	26 ± 3[b]	56 ± 14
28	WNR (23)	79 ± 9	50 ± 10
	MNR (23)	47 ± 7[b]	42 ± 15
50	WNR (25)	169 ± 32	58 ± 12
	MNR (27)	122 ± 23[c]	62 ± 16
70	WNR (10)	215 ± 35	ND
	MNR (10)	175 ± 25[c]	ND
120	WNR (12)	342 ± 67	ND
	MNR (12)	264 ± 56[c]	ND

[a] Values are means ± standard deviations.
[b] $P < 0.0001$ when compared with WNR value (by Student's t test).
[c] $P < 0.01$ when compared with WNR value (by Student's t test).

0.2 ml of PBS was injected into the loop, and the abdomen was closed. After 4 h, the rats were surgically opened, the loops were surgically removed and weighed, and their lengths were determined. The weight-per-length ratios (in grams per centimeter) of normal, malnourished, and nonimmunized control rats were compared.

MLN and PP cell cultures. Mesenteric lymph nodes (MLN) were removed after the rats were bled. Single-cell suspensions were prepared by forcing the MLN through a stainless-steel mesh and then washing and suspending them in RPMI 1640 containing 10% fetal calf serum, 25 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid), 2 mM L-glutamine, 0.05 mM 2-mercaptoethanol, and antibiotics (100 U of penicillin per ml and 100 μg of streptomycin per ml). To prepare PP cell suspensions, the intestine was removed and flushed with PBS. All visible PP were removed and incubated in citrate buffer (50 mM trisodium citrate in PBS, 100 U of penicillin per ml, 100 μg of streptomycin per ml, 1 mM dithiothreitol) for 3 min at room temperature to remove mucus, after which they were thoroughly rinsed in complete medium. Cells were released from the PP by forcing them through a stainless-steel mesh. Five million cells were placed in individual wells of 24-well flat-bottom plates (Corning) and incubated at 37°C in 5% CO_2–95% humid air for 1 week without replacing the medium. Triplicates were run at the same time. The supernatants were harvested, centrifuged, and stored at −20°C. At the end of the culture period, cell viability was evaluated by trypan blue exclusion.

Statistical analysis. Student's t test was used for comparison between two means. Analysis of variance and the Tukey-Kramer test were used for multiple-group comparisons. The ELISA titers were transformed logarithmically prior to analysis. The normality of samples and homogeneity of variance were confirmed by the Kolmogorov-Smirnov test and Scheffe-Box test, respectively. For the nonparametric samples, the Mann-Whitney U test was used.

RESULTS

Body weight and serum protein concentration. The body weights of the MNR remained low throughout the experimental period. However, we did not find any alteration in the serum protein concentrations of the MNR (Table 1).

Anti-CT antibodies in intestinal fluid, serum, and supernatant fluids from cultured MLN cells. The IgA anti-CT titers in intestinal fluid were significantly diminished in the MNR of group 1 (35 ± 13 ELISA units) compared with those of the
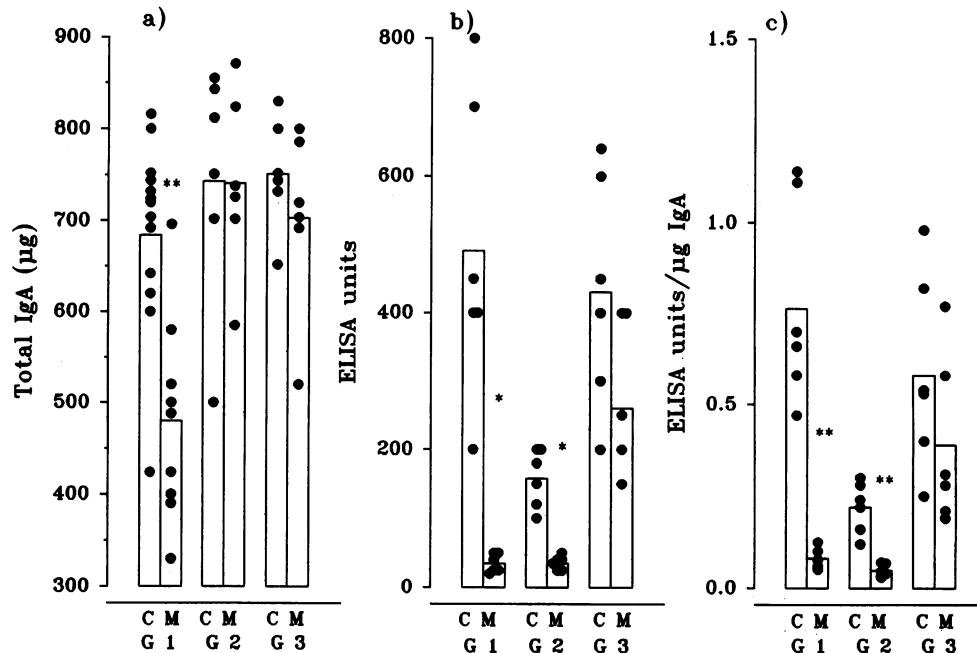
FIG. 1. Total IgA (a), IgA anti-CT antibodies (b), and ratio of IgA anti-CT antibodies to total IgA (c) in intestinal fluid of controls (C) and MNR (M) during suckling. Rats of group 1 (G1) were immunized orally three times with CT beginning at day 28 of age (1 week of refeeding). Rats of group 2 (G2) were immunized like those of group 1 and at 113 days of age (92 days of refeeding) received a booster. Rats of group 3 (G3) were immunized orally three times with CT beginning at 50 days of age (30 days of refeeding). The antibody levels were determined by ELISA and expressed as arbitrary ELISA units. *, $P < 0.0001$; **, $P < 0.001$. Student's t test was used.

WNR (491 ± 220 ELISA units). MNR that received a booster at 113 days of age (group 2) continued to have significantly lower specific IgA levels in the intestinal fluid than the WNR (35 ± 9 ELISA units versus 158 ± 42 ELISA units). However, then the first dose of CT was administered after 30 days of refeeding (group 3), the ability to respond to the antigen was restored in the MNR although a nonsignificant difference persisted (260 ± 115 ELISA units in The MNR versus 430 ± 170 ELISA units in the WNR) (Fig. 1). In the MNR of group 1, the total IgA was significantly diminished when compared with that of the WNR (480 ± 111 μg versus 683 ± 101 μg). IgA in the intestinal fluid of the MNR of groups 2 and 3 did not differ from that of dose- and age-matched controls (Fig. 1). No differences were found in the concentrations of IgG and IgA in serum between the WNR and the experimental rats of the three groups.

The MNR in groups 1 and 2 had a significantly lower ratio of IgA anti-CT ELISA units per microgram of total IgA in the intestinal fluid than WNR (0.08 ± 0.027 versus 0.77 ± 0.28 in group 1 and 0.048 ± 0.017 versus 0.22 ± 0.07 in group 2). In group 3, the ratio of ELISA units per microgram of IgA in the MNR (0.39 ± 0.23) approached control values (0.58 ± 0.26) (Fig. 1).

In the analysis of protection against CT challenge by the ligated intestinal loop test, the WNR in group 1 demonstrated a partial ability to neutralize the CT (0.186 ± 0.055 g/cm), whereas no antitoxin activity was observed in the MNR (0.30 ± 0.027 mg/cm), in which the fluid accumulation in the loops was as high as in the nonimmunized rats (0.32 ± 0.028 mg/cm) (Fig. 2). The low protection ability observed in control rats may be due to the low doses of CT employed in the immunization schedule.

In the MNR of group 1, the levels of IgG, IgM, and IgA anti-CT antibodies in serum were significantly lower (6,489 ±

6,231, 205 ± 195, and 335 ± 331 ELISA units, respectively) than those in the WNR (112,678 ± 125,339, 1,436 ± 1,907, and 1,590 ± 1,427 ELISA units, respectively). Similar results were obtained in group 2 for specific IgG, IgM, and IgA levels when the MNR (1,189 ± 1,762, 73 ± 66, and 74 ± 38 ELISA units, respectively) were compared with the WNR (25,357 ± 14,246, 657 ± 475, and 328 ± 99 ELISA units, respectively). Nonsignificant differences were found between both groups when the
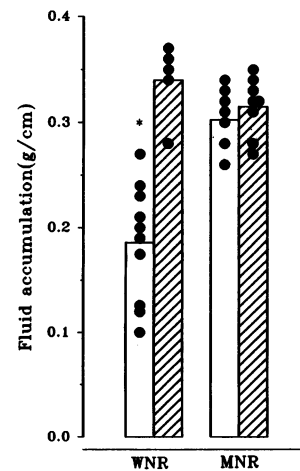

FIG. 2. Protection against CT challenge in the ligated intestinal loop test. WNR and MNR during suckling were immunized orally with three doses of CT at 28, 35, and 42 days of age. Four hours after the intraduodenal challenge, fluid accumulation in the intestinal loop of the immunized (open bars) and nonimmunized rats (hatched bars) was compared. *, $P < 0.001$. Student's t test was used.
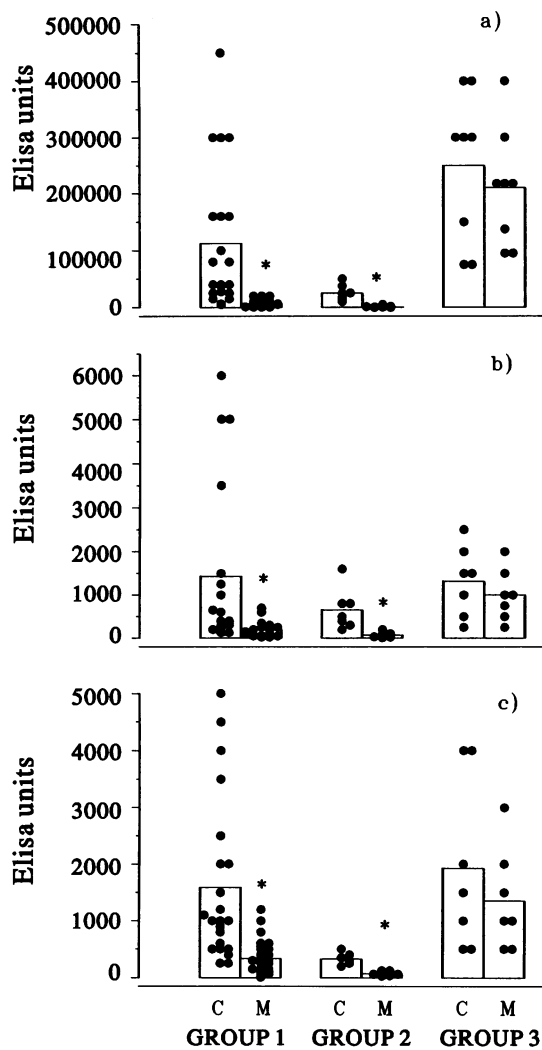
FIG. 3. IgG (a), IgM (b), and IgA (c) anti-CT antibodies in sera of controls (C) and MNR (M) during suckling of groups 1, 2, and 3 (for details, see legend of Fig. 1). *, $P < 0.0001$; **, $P < 0.01$. Student's t test was used.

first dose of CT was administered after 30 days of refeeding (group 3) (Fig. 3).

IgG and IgA anti-CT levels in supernatant fluid from MLN cell cultures of the MNR of group 1 were significantly diminished (2.6 ± 1.3 and 1.4 ± 1.6 ELISA units, respectively) when compared with those of the WNR (54.4 ± 44.7 [$P < 0.0001$] and 13.6 ± 11.2 [$P < 0.05$]). Similar results were found in the MNR of group 2 (0.8 ± 0.8 and 1.4 ± 1.6 ELISA units, respectively, for IgG and IgA) when compared with the results for WNR (27.2 ± 22.3 [$P < 0.0001$] and 7.6 ± 5.4 [$P < 0.0001$]). No differences were found in group 3.

Anti-CT antibodies after immunization into PP. When one dose of CT was administered into PP of rats at 28 days of age, the specific IgA in intestinal fluid was drastically decreased in the MNR (4.0 ± 1.4 ELISA units) when compared with that in WNR (156 ± 151 ELISA units). Also, total IgA was lower in the MNR (65.4 ± 11.2 µg) than in the WNR (156 ± 25.6 µg). Nonetheless, the MNR had a significantly lower ratio of IgA anti-CT ELISA units per microgram of total IgA in the intestinal fluid than the WNR (0.069 ± 0.023 versus 0.944 ±

0.930) (Fig. 4). Supernatants from PP cell cultures of the MNR showed a lower level of specific IgG, IgA, and IgM anti-CT antibodies (1.2 ± 1.7, 0.8 ± 1.1, and 0.86 ± 1.0 ELISA units, respectively) than those from the WNR (8.0 ± 4.9, 9.6 ± 6.0, and 2.8 ± 1.1 ELISA units, respectively). Similar results were found in the supernatants from the MLN cell cultures when these levels in the MNR (1.2 ± 1.8, 0.4 ± 0.9, and 0.8 ± 1.1 ELISA units, respectively) were compared with those in the WNR (12.8 ± 11.8, 5.2 ± 2.7, and 4.8 ± 3.0 ELISA units, respectively) (Fig. 5). The levels of serum-specific IgG, IgA, and IgM were significantly lower in the MNR (72 ± 52, 36 ± 26, and 84 ± 49 ELISA units, respectively) than in the WNR (832 ± 488, 320 ± 192, and 416 ± 214 ELISA units, respectively; $P < 0.001$).

DISCUSSION

In the present study, we show that malnutrition during suckling results in a decreased level of total IgA in the intestinal fluid, in a diminished antibody immune response against CT in intestinal fluid and sera, and in a decreased production of antibodies against CT in MLN cell cultures. Furthermore, an impairment in the neutralization of CT in the ligated loop test was observed. We could further see that the impairment in the ability to mount an antibody response when MNR were fed CT was probably due to a deficient induction of the immune response in the PP, since the administration of CT into PP resulted in very low titers of specific antibodies in intestinal fluid, in supernatant from PP and MLN cell cultures, and in serum. Likewise, the MNR that received three doses of CT were not able to generate a normal immune response when a booster was delivered after 3 months of refeeding.

In this study, we observed no differences in the levels of total IgA in serum between the WNR and the MNR. It has been described that protein-calorie malnutrition in mice and children provokes an increase in IgA concentration in serum (25, 27). This excess of serum IgA may represent an impairment of a mechanism for the removal of IgA from serum. However, the normal values we found in MNR seem to indicate that a deficient production of IgA in mucosal surfaces, perhaps due to a reduced capacity to generate the IgA-committed cells (18), may be the cause of low levels of IgA in intestinal fluid. This is an agreement with the scarce number of cells bearing IgA observed in the PP and MLN of MNR during suckling (11, 12). Nevertheless, the ratio of IgA anti-CT antibodies per microgram of total IgA in the intestinal fluid indicates that the low antibody response to CT is due to a deficiency in the specific immune response rather than a decrease in the levels of the immunoglobulin concentration. Cholera toxin is one of the few nonreplicating antigens that is capable of developing immunological memory when administered by the oral route (20, 22, 30). In the present study, when a booster was administered after 92 days of refeeding (group 2), a very poor antibody immune response was obtained in MNR. These results may be indicating on the one hand that a certain number of antibody-producing cells in the MNR are producing less IgA than the same number of antibody-producing cells in the WNR, thus suggesting an alteration in the terminal B-cell differentiation at the effector site throughout the experimental period. On the other hand, the same results could be explained by a deficient induction of the immune response when the first doses of CT were administered rather than by a deficient long-term memory immune response due to permanent alterations in the gut at the time of booster delivery. However, when the schedule of immunization began after 1 month of refeeding, the specific immune response in the MNR approached control values,
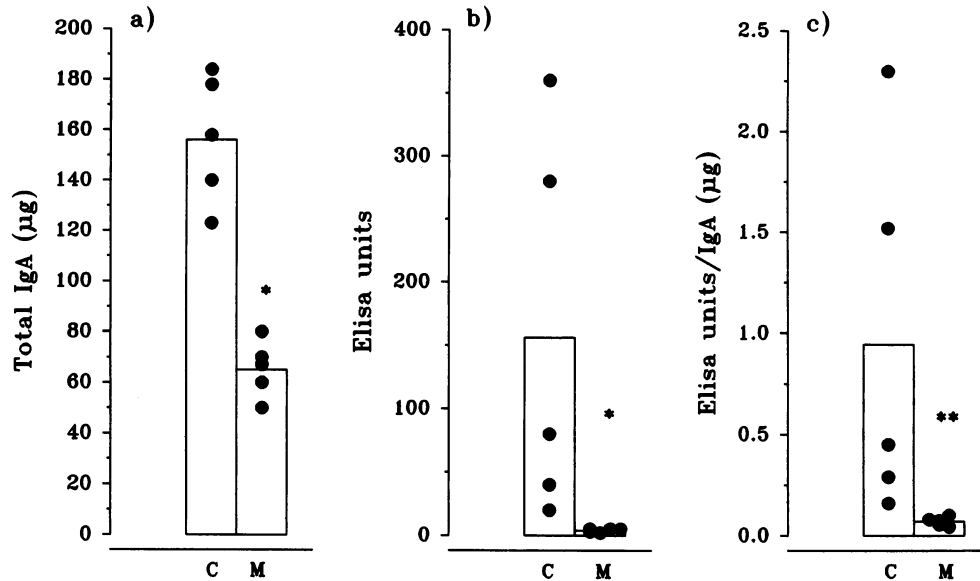
FIG. 4. Total IgA (a), IgA anti-CT antibodies (b), and ratio of IgA anti-CT antibodies to total IgA (c) in intestinal fluid of controls (C) and MNR (M) during suckling. MNR and controls were immunized with 10 μg of CT into PP at 28 days of age. *, $P < 0.001$; **, $P < 0.01$. Student's t test was used.

indicating that at that time, the microenvironment in PP required for optimal induction was present and the terminal differentiation of B cells in the lamina propria had taken place in the MNR. Since in group 2 the booster was given after 3 months of refeeding (i.e., when the MNR are able to mount a normal immune response to CT) and the immune response continued to be affected, we think that a deficient induction of the immune response is the cause of the impaired immune response found in the MNR.

CT is a thymus-dependent antigen (19) which requires functional CD4$^+$ T cells in the gut for the development of a host defense mechanism against it (14). Recent findings show that in mice, the oral route preferentially stimulates the Th2 subset (26, 39, 40). Previously, we found a decrease in the CD4$^+$ subset in GALT together with a high frequency of pre-B cells and immature T cells in the MLN of 28-day-old MNR (11, 12). Therefore, it is possible that the immaturity of the GALT when rats received the first doses of CT (groups 1 and 2) may be the main cause of the impaired immune response in this kind of malnutrition. The absence of a correlation between the level of antibody immune response and the nutritional status evaluated as serum protein concentration (indicating a moderate malnutrition) also supported this point of view. This hypothesis is in agreement with another study (25) which shows that moderate protein malnutrition in mice does not severely affect the IgA immune response to sheep erythrocytes administered orally.

Protein-calorie malnutrition and vitamin A deficiency have been reported to affect the migration and localization of MLN blasts seeding the gut lamina propria (24). It is possible that this event also occurs in our model, but the results presented here seem to indicate that malnutrition during suckling affects earlier steps of the immune response.

There are discrepant results concerning the effect of malnutrition on the immune response to CT administered orally. Previous studies in rats have shown that a low protein diet (4% protein) provokes a decreased number of IgA-producing cells, but this alteration was reversed when the CT was administered
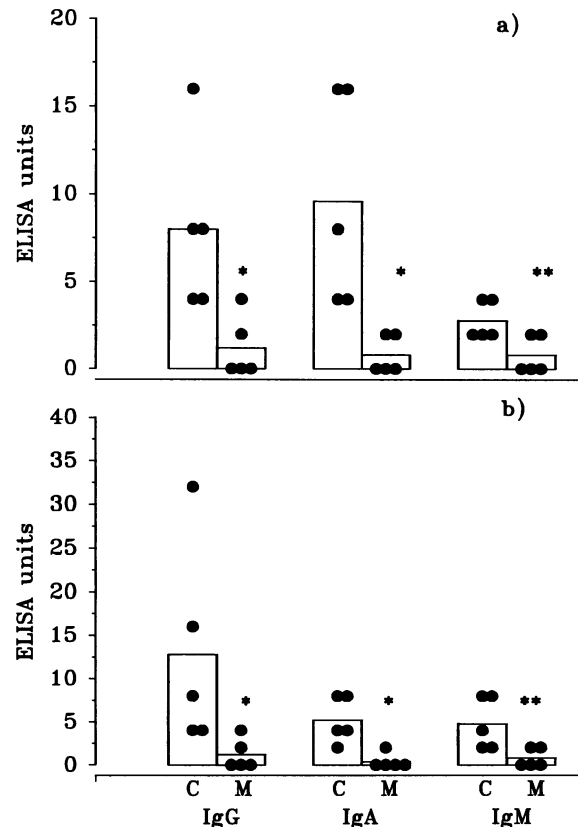

FIG. 5. IgG, IgA, and IgM anti-CT antibodies in supernatant fluids from PP (a) and MLN (b) cell cultures of controls (C) and MNR (M) during suckling. MNR and controls were immunized with 10 μg of CT into PP at 28 days of age. Values are based on triplicate determinations. *, $P < 0.01$; **, $P < 0.05$. The Mann-Whitney U test was used.

with the refeeding (1, 16). Studies in children indicate that no alterations are found in the antibody response to orally administered cholera toxin B subunit if malnutrition is measured as low weight in relation to age (13). Furthermore, in another kind of immune deficiency such as that which can be observed in aging rats, an alteration in the number of CT antibody-containing cells in the gut lamina propia as well as diminished levels in specific IgA in the intestinal fluid has been described (33).

Our results indicate that when rats are malnourished during suckling, it is necessary to allow a long period of refeeding prior to the administration of the CT to restore the immune response. Results described by others indicate that in adult rats, low food intake (50% of that of controls) by itself does not impair the immune response to CT (37). This model of adult malnutrition could be compared with the malnutrition during suckling we used (in both models, malnutrition is due to the low food intake). Our experimental results showing that malnutrition during suckling severely affects the immune response to CT administered orally should be interpreted as a consequence of malnourishment of rats in an early period of life, which leads to the GALT immaturity.

The results presented in this report may be taken into account when vaccination campaigns are carried out in developing countries.

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