

## Rotavirus-Inhibitory Activity in Serial Milk Samples from Mexican Women and Rotavirus Infections in Their Children during Their First Year of Life

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A total of 75 children born in rural Mexico were followed for diarrheal diseases and rotavirus (RV) excretion during the first year of life. For 18 children, an average of 14 serial breast milk samples were obtained between days 2 and 360 after delivery and were tested for RV-inhibitory activity. Of these samples, 70, 62, and 85% showed inhibitory activity against serotype (ST) 1 human RV, ST4 human RV, and ST3 simian RV, respectively; the median titers were 10, 10, and 20, respectively. Some 89% of the milk samples showed RV-specific antibodies in an enzyme-linked immunosorbent assay (median titer, 20). Surprisingly, 98% of the milk samples inhibited ST6 bovine RV. ST6, but not ST1, RV-inhibitory activity survived heat treatment (10 min at 80°C). Of the 18 children tested, 13 children experienced 23 episodes of diarrhea (enterotoxigenic [ $n = 8$ ] and enteropathogenic [ $n = 3$ ] *Escherichia coli*, *Campylobacter jejuni* [ $n = 4$ ], *Shigella flexneri* [ $n = 2$ ], RV [ $n = 1$ ]) and 5 children experienced 6 RV infections. Only one RV infection was associated with diarrhea. The five RV excretors did not differ from the nonexcretors with respect to the RV-inhibitory activity in the breast milk fed to them. The RV-inhibitory titers were too low in the majority of the studied Mexican milk samples to indicate an important effect of breast-feeding on the take rate of oral human, simian, or reassortant RV vaccines. Breast-feeding might, however, inhibit the take rate of a bovine RV vaccine.

Rotaviruses (RVs) are one of the most important causes of acute gastroenteritis in both industrialized and developing countries (18). Active immunization against RV is the subject of intensive research (19). Currently, experimental RV vaccines are given orally as live attenuated viruses. Vaccine viruses undergo a limited replication in the intestines of infants (1). There is thus some concern that antibodies to RV in human breast milk might affect the take rate of these vaccines in breast-fed vaccinees (11, 31). An analysis of several RV vaccine trials has not indicated a strong adverse effect of breast-feeding on seroconversion to RV vaccination (14, 22, 27, 28, 31). Nearly all of those studies were conducted in industrialized countries. Notably, however, some RV vaccines which conferred protection in children from industrialized countries (29, 30) failed to protect children from developing countries (11, 16). No explanation was given for these failures, but it was discussed whether breast milk from women in developing countries contained substances harmful to the vaccine virus (11, 16, 28). Here we report inhibitory activities against current RV vaccine candidates in serial breast milk samples from Mexican women. We found only low inhibitor concentrations against human RV (HRV) and several HRV × animal RV reassortants. In contrast, high inhibitor concentrations against bovine RV (BRV) NCDV were detected. These might influence the take rate of bovine RV-based vaccines.

### MATERIALS AND METHODS

**Study design.** The present study was carried out in the rural village of Lugar Sobre la Tierra Blanca in the state of

Morelos, approximately 180 km southwest of Mexico City (8, 9). A total of 75 children born consecutively between 15 August 1985 and 26 January 1986 were enrolled in the study after oral, informed consent was obtained from both parents. The medical personnel living in the village examined all 75 children during the first 24 h after birth and visited each family every 48 h over the next 12 months in order to record episodes of diarrhea. All except 2 of these 75 children were breast-fed at birth and 40% were still being breast-fed at 1 year of age (9); for 18 women, sufficient amounts and numbers of serial breast milk samples were available to us. Demographically, these 18 women did not differ significantly from those who ceased breast-feeding earlier.

For the purpose of the present study, diarrhea was defined as four or more bowel movements in 24 h with a liquid or semiliquid consistency and/or the presence of blood or mucus in the stools, as detected by the mother or child-minder and confirmed by the examining physician. A prodromic period of 48 h was operationally defined for the study; organisms obtained in cultures taken within 48 h of the initiation of symptoms were considered to be associated with the disease. An episode of diarrhea was considered to be ended when a child showed no symptoms for 72 h and the stools were considered to be normal by the examining physician. After each case of diarrhea, daily home visits were made by both the physician and the social worker in charge of the family.

An average of 14 serial breast milk samples were obtained from 18 Mexican women between days 2 and 360 after delivery. Milk was collected by manual expression into sterile containers after the nipple was cleaned with disinfectant and after the first couple of drops were discarded. The samples were obtained throughout the period of lactation

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and were immediately frozen and maintained at  $-20^{\circ}\text{C}$  until further use. Weaning was defined as the date at which the child stopped receiving breast milk. Serum samples were not obtained.

Once every fortnight from the time of birth, a sample of feces from each child was collected in sterile containers. Within 1 h, the sample was inoculated onto appropriate media for the detection of putative pathogenic bacteria (9). The rest of the sample was maintained under refrigeration and was sent within 24 h to Mexico City for the identification of RV by using a commercial enzyme-linked immunosorbent assay (ELISA; Enzygnost; Behringwerke, Marburg, Federal Republic of Germany). RV-positive samples were confirmed by electron microscopy. A sample of feces was also collected and handled in a similar way whenever a child had diarrhea.

**RV inhibition test.** The milk samples were tested for inhibitory activity against serotype (ST) 6 bovine RV NCDV, a first-generation RV vaccine candidate (19), and ST3 simian RV strain RRV, a second-generation RV vaccine candidate (19). Milk samples were also tested for inhibitory activity against the RRV-based reassortant RV strains (23, 24) D  $\times$  RRV, DS-1  $\times$  RRV, and ST3  $\times$  RRV. These third-generation RV vaccine candidates contain the VP7 protein of human ST1, ST2, and ST4 RVs, respectively, and the VP4 protein of simian RV RRV. In addition, inhibitory activities against reassortant DS-1  $\times$  UK (23), which contains the VP7 protein of human ST2 RV strain DS-1 and the VP4 protein of bovine ST6 RV strain UK, and two authentic HRV strains (ST1:Wa, ST4:Hochi) were measured in these milk samples. RV Hochi and ST3 have different P types (21), but they have the same G type (serotype 4A [13]).

The peroxidase focus reduction test described previously (4) was used. A twofold dilution series starting with a 1:5 dilution of milk in M199 medium (Seromed, Berlin, Federal Republic of Germany) was prepared.

When the number of infected cells was reduced by 50% compared with the number of virus-infected control cells, the sample was said to be inhibiting. Four microscopic fields were evaluated for each well, and each sample was tested in duplicate.

RV-specific antibodies in breast milk were determined by an ELISA described previously (6). Contrary to previous reports, a polyvalent conjugate (Sigma, St. Louis, Mo.) was used to reveal bound milk antibody.

## RESULTS

**Inhibitory activity against ST1 RV.** Similar titers and prevalences were detected against ST1 HRV Wa and against HRV  $\times$  simian reassortant RV D  $\times$  RRV (Table 1). In fact, 189 samples showed similar inhibitory titers (twofold or less titer difference) with ST1 HRV Wa and D  $\times$  RRV. Twenty-two samples showed fourfold or greater higher titers against ST1 HRV Wa than against RV D  $\times$  RRV, and 31 samples showed fourfold or greater higher titers against RV D  $\times$  RRV than against ST1 HRV Wa. Thus, for inhibitory activity directed against ST1 RV, the human or simian origin of VP4 is of minor importance.

Table 2 shows the RV Wa-inhibitory titers in serial breast milk samples from women whose children experienced an episode of diarrhea or RV infection. We observed only two women (e.g., woman 17) with high inhibitory activities in their milk (inhibitory titer,  $\geq 80$  in the majority of the samples). In contrast, seven women (e.g., woman 16) were identified who had consistently low inhibitory activities in

TABLE 1. RV-inhibitory activities in 255 serial breast milk samples from 18 Mexican women

| Rotavirus strain  | Origin                | G sero-type | No. (%) of inhibitory samples <sup>a</sup> | Median titer (range) |
|-------------------|-----------------------|-------------|--|----------------------|
| Wa                | Human                 | 1           | 179 (70)                                   | 10 (<5-160)          |
| D $\times$ RRV    | Human $\times$ simian | 1           | 171 (67)                                   | 10 (<5-320)          |
| DS-1 $\times$ RRV | Human $\times$ simian | 2           | 232 (91)                                   | 20 (<5-160)          |
| RRV               | Simian                | 3           | 216 (85)                                   | 20 (<5-320)          |
| ST3 $\times$ RRV  | Human $\times$ simian | 4           | 253 (99)                                   | 40 (<5-640)          |
| Hochi             | Human                 | 4           | 159 (62)                                   | 10 (<5-320)          |

<sup>a</sup> A sample was defined as inhibitory if it reduced at a 1:5 milk dilution the number of infected cells by 50%.

their milk (no sample showed inhibitory titers of  $\geq 20$ ). In general, no marked fluctuations in the ST1 HRV Wa-inhibitory titers were observed between serial milk samples. Of note, inhibitory titers were not significantly higher in colostrum milk samples than in mature milk.

**Inhibitory activity against ST2, ST3, and ST4 RVs.** Some 91% of the milk samples showed inhibitory activity against the ST2 HRV  $\times$  simian reassortant RV DS-1  $\times$  RRV, and 85% of the milk samples showed inhibitory activity against ST3 simian RV RRV (Table 1).

In our test we could not use authentic ST2 and ST3 HRV strains because their slow replication in MA-104 cells led to overgrowth of the bacteria that originated from the milk samples. The inhibitory activity of the milk which was directed against RRV-derived VP4 became evident when we compared the inhibitory activities against two reassortant RV strains, DS-1  $\times$  RRV and DS-1  $\times$  UK. These two strains with ST2 HRV-derived VP7 protein differed in the origin of their VP4 proteins. Note that BRV UK is a nonhemagglutinating animal RV strain (17). At a 1:20 milk dilution, 51% of the Mexican milk samples inhibited the growth of DS-1  $\times$  RRV reassortant RV, whereas only 31% of the milk samples inhibited growth of DS-1  $\times$  UK reassortant RV with VP4 of RV UK.

Similarly, all but two milk samples inhibited the growth of ST4 HRV  $\times$  simian reassortant RV ST3  $\times$  RRV at the lowest milk dilution, whereas only 62% inhibited the growth of ST4 HRV Hochi (Table 1). Thus, for inhibitory activity against these two ST4 RV strains, the human versus simian origin of VP4 seems to be important.

**ELISA.** Given the limited variations in the virus-inhibitory titers that we observed, we considered it necessary to confirm by another assay system that RV-specific antibodies were indeed being measured in our peroxidase focus reduction test. The focus reduction test detects any inhibitory activity in milk, not only that of antibody. The milk samples were therefore tested for the presence of RV-specific antibody by ELISA; 228 (89%) of the 255 milk samples showed RV-specific antibody in the ELISA (median titer, 20; titer range, <5 to 1,280).

Table 2 shows the RV-specific ELISA antibody titers in serial breast milk samples of 18 Mexican women. Women with the highest RV Wa inhibitory titers (e.g., woman 17) also showed the highest RV antibody titers by ELISA. Of note was the fact that a greater fluctuation in ELISA titers than inhibitory titers was seen between serial milk samples. Heating of the milk samples for 10 min at  $80^{\circ}\text{C}$  destroyed both RV Wa-inhibitory activity and ELISA antibody reactivity.

**Inhibitory activity against BRV NCDV.** Clear-cut evidence

TABLE 2. HRV Wa-inhibitory titers in serial breast milk samples from Mexican women whose children experienced an episode of diarrhea or RV infection

| Woman no. | Titer of inhibitory activity to ST1 HRV Wa in breast milk specimen sampled on the following approximate day after delivery: |                 |    |    |    |                 |                 |                |                 |                 |                 |                  |                  |                 |                |
|-----------|---|-----------------|----|----|----|-----------------|-----------------|----------------|-----------------|-----------------|-----------------|------------------|------------------|-----------------|----------------|
|           | 3   | 6               | 15 | 20 | 60 | 90              | 120             | 150            | 180             | 210             | 240             | 270              | 290              | 330             | 360            |
| 1         | 30  | 20              | <5 | 20 | 5  | 10              | <5              | <5             | 10              | <5              | <5              | <5               | <5               | <5 <sup>a</sup> | — <sup>b</sup> |
| 5         | 20  | 20              | 5  | 20 | <5 | 10              | <5              | <5             | <5              | <5              | 10              | <5               | <5 <sup>a</sup>  | <5              | 5              |
| 6         | <5  | 40              | 20 | 20 | 30 | 5               | 20 <sup>c</sup> | 20             | 20              | 30              | 20              | 20 <sup>a</sup>  | 10               | 30              | 30             |
| 7         | 20  | <5              | 10 | 10 | <5 | <5              | —               | 20             | <5 <sup>c</sup> | 5 <sup>a</sup>  | <5 <sup>a</sup> | <5               | <5               | <5              | —              |
| 9         | 20  | 10              | 5  | 5  | 5  | <5              | <5              | 10             | <5 <sup>c</sup> | <5              | <5              | <5               | <5 <sup>a</sup>  | <5              | —              |
| 10        | 10  | <5              | <5 | 5  | 5  | <5              | <5              | 20             | <5              | 5 <sup>a</sup>  | <5 <sup>a</sup> | 20               | 20               | 20              | 20             |
| 11        | 60  | 5               | 5  | <5 | <5 | <5 <sup>a</sup> | <5              | <5             | <5              | 10              | <5 <sup>a</sup> | <5               | <5               | <5              | 5              |
| 12        | 40  | 60              | 60 | 60 | 10 | 60              | 60              | 40             | 40              | 40 <sup>c</sup> | 20              | 40               | 60               | 40 <sup>c</sup> | —              |
| 13        | 10  | 5               | <5 | 5  | 10 | <5              | <5 <sup>a</sup> | 5 <sup>a</sup> | <5              | <5              | <5              | <5               | 5                | 5               | —              |
| 14        | 20  | 20 <sup>a</sup> | 10 | 10 | <5 | 20              | 10              | 10             | 20 <sup>a</sup> | 40 <sup>a</sup> | 10              | 10               | 5 <sup>a</sup>   | 5               | —              |
| 15        | <5  | 60              | 30 | <5 | 10 | 80              | 40              | 80             | 20 <sup>a</sup> | 10              | 80              | 30               | 10               | 40              | —              |
| 16        | <5  | 5               | <5 | <5 | <5 | —               | 10              | <5             | 5               | <5              | <5              | <5 <sup>a</sup>  | 5 <sup>a,c</sup> | —               | <5             |
| 17        | 80  | 80              | 80 | 20 | 20 | 80              | 80              | 60             | 80              | 60              | 160             | 160 <sup>a</sup> | 80               | 20              | 20             |
| 18        | 60 <sup>a</sup>   | 40              | 60 | 60 | 60 | 40              | 20              | 40             | 60              | 60              | 40              | 60 <sup>c</sup>  | 60 <sup>a</sup>  | 60 <sup>a</sup> | —              |

<sup>a</sup> Diarrhea was observed in the corresponding child at the indicated time after delivery.

<sup>b</sup> —, sample not available.

<sup>c</sup> RV was detected in the corresponding child at the indicated time after delivery.

that inhibitory activity that is not antibody in nature was present in the Mexican breast milk samples came from the observation of inhibitory activity to ST6 BRV NCDV.

At a 1:40 dilution, all but four Mexican milk samples inhibited the growth of BRV NCDV. In 251 samples (98%), heat treatment (10 min at 80°C) had no effect on this inhibitory activity but resulted in the complete loss of RV-specific antibody activity, as assessed by ELISA. At a 1:80 dilution, 167 of 255 (65%) breast milk samples still inhibited the growth of BRV NCDV.

Digestion of 15 selected milk samples with porcine pancreatic trypsin (1 mg/ml; Flow Laboratories) or *Vibrio cholerae* neuraminidase (0.2 U/ml; Behringwerke) did not reduce NCDV-inhibitory activity in 14 and 13 milk samples, respectively. A control experiment (12, 31) showed that neuraminidase treatment of MA-104 cells results in a 10-fold reduction of NCDV-infected cells in comparison with the number observed in untreated control cells, thus demonstrating the activity of the neuraminidase on the cellular receptor. In 9 of 15 selected samples, NCDV-inhibitory activity was quantitatively recovered by 50% ammonium sulfate precipitation.

**Antibody titers in milk, diarrhea, and RV infection during the first year.** During the first year of life, 13 of 18 children who received the breast milk studied experienced at least one episode of diarrhea. Of these, six children experienced one episode, five children had two episodes, one child had three episodes, and another child had four episodes during the first year of follow-up (Table 2). A total of 23 episodes of diarrhea were observed: eight were associated with enterotoxigenic *Escherichia coli*, three with enteropathogenic *E. coli*, four with *Campylobacter jejuni*, two with *Shigella flexneri*, and 1 with RV. Seventeen episodes (74%) occurred in children older than 6 months of age. Five children did not experience an episode of diarrhea. They did not receive milk with higher RV-inhibiting activities or higher ELISA antibody titers (data not shown).

Five of 18 children (28%) excreted RV during the first year of life, and 1 child excreted RV twice. Only one of these six RV infections was associated with diarrhea (Table 2). The five RV excretors did not receive milk with lower RV-inhibitory activities (Table 2) or lower ELISA antibody titers (Table 3).

## DISCUSSION

Inhibitory activities to HRV and HRV × animal reassortant RV strains and RV-specific ELISA antibody were detected in most breast milk samples from Mexican women. Titers were not higher than those previously observed in the colostrums of German women (5). Passive immunization of Chilean children with a bovine milk immunoglobulin preparation showing antibody titers comparable to the inhibitory titers in Mexican breast milk (measured in the same laboratory) failed to prevent RV infection (3). On the basis of the results obtained with HRV or simian RV strains, we conclude that RV-inhibitory titers were too low in the majority of the Mexican women to indicate an important effect of breast-feeding on the take rate of oral RV vaccines (28). This conclusion was supported by the observation that breast-feeding did not prevent RV infection in 5 of the 18 Mexican children studied. Our data thus corroborate the conclusions of several studies which observed no strong adverse effect of breast-feeding on RV vaccine take rate (14, 22, 27, 31). High inhibitory titers against ST6 BRV NCDV were detected in milk from Mexican women. This result was surprising because ST1 RV has been reported to be the most prevalent RV strain in Mexico, whereas ST6 RV has not been isolated from Mexican children (26). However, inhibitory activity against BRV NCDV was not antibody mediated, since it resisted a heat treatment which was shown by ELISA to destroy antibody activity. Heat-resistant anti-RV activity has previously been reported for African and Scandinavian colostrum samples (25). Contrary to the activity in mature Mexican milk, this heat-resistant activity was trypsin sensitive and could not be precipitated by 50% ammonium sulfate. Previous studies have shown that sialic acid glycoproteins, sialic acid oligosaccharides, and mucins also inhibit simian RV SA11 (12, 32). Like BRV NCDV, but unlike HRV, simian RV SA11 is a hemagglutinating RV (12). However, a neuraminidase concentration that apparently destroyed the receptor for BRV NCDV on MA-104 cells did not affect the NCDV-inhibitory activity of the Mexican milk samples. In addition, various fractions of human milk oligosaccharides purified from pooled breast milk (10) failed to inhibit BRV NCDV at physiological (7) concentrations (3a).

TABLE 3. RV-specific antibody titers in serial breast milk samples from Mexican women as assessed by ELISA

| Woman no. | RV-specific antibody titer in breast milk specimen sampled at the following approximate day after delivery assessed by ELISA: |                 |     |     |     |                 |                 |                |                 |                  |                  |                  |                   |                 |                |
|-----------|---|-----------------|-----|-----|-----|-----------------|-----------------|----------------|-----------------|------------------|------------------|------------------|-------------------|-----------------|----------------|
|           | 3   | 6               | 15  | 20  | 60  | 90              | 120             | 150            | 180             | 210              | 240              | 270              | 290               | 330             | 360            |
| 1         | 40  | 80              | 20  | 10  | 20  | 5               | 5               | 10             | 20              | 5                | 40               | 20               | 20                | 10 <sup>a</sup> | — <sup>b</sup> |
| 5         | 40  | 640             | 320 | 640 | 20  | <5              | 10              | 5              | 20              | 40               | <5               | 5                | 10 <sup>a</sup>   | 5               | 40             |
| 6         | <5  | 20              | 10  | 40  | 10  | 5               | 5 <sup>c</sup>  | 5              | 10              | 5                | 5                | 10 <sup>a</sup>  | 5                 | 5               | 5              |
| 7         | 80  | 320             | 10  | 20  | 40  | 320             | —               | 40             | 40 <sup>c</sup> | 10 <sup>a</sup>  | 5 <sup>a</sup>   | <5               | 10                | 40              | —              |
| 9         | 20  | 40              | 40  | 160 | 20  | 40              | 40              | 5              | 20              | <5               | <5               | <5               | <5 <sup>a</sup>   | <5              | —              |
| 10        | —   | 40              | 40  | <5  | 40  | 160             | 320             | 20             | 160             | 320 <sup>a</sup> | 320 <sup>a</sup> | 320              | 160               | 320             | 80             |
| 11        | 160   | 10              | 5   | 5   | <5  | <5 <sup>a</sup> | 20              | 5              | <5              | 5                | 40 <sup>a</sup>  | <5               | <5                | 5               | 5              |
| 12        | 320   | 640             | 40  | 10  | 5   | 10              | 10              | 20             | 20              | <5 <sup>c</sup>  | 10               | 20               | 40                | 10 <sup>c</sup> | —              |
| 13        | 640   | 20              | —   | 10  | 40  | 80              | 40 <sup>a</sup> | 5 <sup>a</sup> | 40              | 80               | <5               | —                | 5                 | 20              | —              |
| 14        | 40  | 80 <sup>a</sup> | 20  | 5   | 40  | <5              | <5              | 10             | 5 <sup>a</sup>  | 10 <sup>a</sup>  | <5               | <5               | 10 <sup>a</sup>   | 20              | —              |
| 15        | 80  | 5               | 10  | 10  | 320 | 10              | 1,280           | 320            | 20 <sup>a</sup> | <5               | <5               | 10               | 10                | 80              | —              |
| 16        | 20  | 10              | <5  | 5   | 5   | 10              | 5               | <5             | 80              | <5               | 10               | <5 <sup>a</sup>  | 10 <sup>a,c</sup> | 20              | <5             |
| 17        | 80  | 80              | 320 | 160 | 160 | 40              | 320             | 20             | 80              | 80               | 160              | 160 <sup>a</sup> | 320               | 80              | 320            |
| 18        | 80 <sup>a</sup>   | 80              | 80  | 80  | 10  | 10              | 5               | 10             | 20              | 10               | 20               | 10 <sup>c</sup>  | 20 <sup>a</sup>   | 10 <sup>c</sup> | —              |

<sup>a</sup> Diarrhea was observed in the corresponding child at the indicated time after delivery.

<sup>b</sup> —, sample not available.

<sup>c</sup> RV was detected in the corresponding child at the indicated time after delivery.

Although not antibody in nature, these inhibitors might interfere with the replication of the BRV vaccine in breast-fed children. A recent meta-analysis of trials with the BRV vaccine did not show a strong negative effect of breast-feeding on the BRV vaccine take rate (14). A similar result was also observed in a field trial conducted in Peru (20). Data on RV-inhibitory titers in breast milk samples are not available from Peru and are scarce for women in other developing countries (15, 28, 33). The poor result of BRV vaccine trials in Gambian and Rwandan children led to speculations that local breast milk contained substances harmful to the vaccine virus (11, 16). We do not yet know whether the non-antibody inhibitor against BRV NCDV is also found in the breast milk of women from other parts of the world (2). In view of the variable results obtained with BRV vaccines in different parts of the world, it might be advisable to test local breast milk for this inhibitor before launching an RV vaccination trial.

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