Virus-Induced Colostral Cell Cytokine Stimulation of Human Leukocyte Natural Killer Cytotoxicity

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Natural killer cytotoxicity is an important antiviral defense mechanism. Human peripheral blood mononuclear cells cultured with herpes simplex virus (HSV)-infected cells produced a cytokine. This substance stimulated adult natural killer cytotoxicity from 53.0 ± 10.5% to 79.8 ± 12.8% (P < 0.01) against HSV-infected target cells. These data resulted in a calculated cytokine-dependent cellular cytotoxicity (CDCC) value of 65.8%. Cytokine production was not stimulated by uninfected cells and was independent of the presence or absence of antibodies to HSV in sera of donors of mononuclear cells. Cells from humancolostrum also produced an HSV-stimulated cytokine which mediated CDCC by using both adult (19.8 ± 3.9%) and neonatal (18.6 ± 3.4%) mononuclear effector cells. Colostral cell cytokine production was also independent of donor HSV serology. Not all colostral cultures produced the cytokine, and in general colostrum-stimulated CDCC was lower than peripheral blood leukocyte-stimulated CDCC. Colostral cell cytokine stimulation of neonatal natural killer cytotoxicity may account in part for the increased nonspecific resistance of breast-fed infants to viral infection.

Classic (9) and recent epidemiological studies (3, 6, 20) have documented the protective effect of breast feeding against various infections in human infants. Recent studies also have revealed that the protective effect is not always related to the serological immune status of the mother or to the presence of breast milk antibody (23, 29, 32). In at least one study, the presence of viable colostral leukocytes was necessary to protect animals against Klebsiella-induced necrotizing enterocolitis (25).

We have previously demonstrated the existence of colostral cell antibody-dependent cellular cytotoxicity to herpes simplex virus (HSV)-infected cells (15). Our more recent studies have revealed significantly lower levels of antibody-dependent cellular cytotoxicity and natural killer cytotoxicity mediated by colostral cells when compared with blood cells (16).

Human neonatal natural killer cytotoxicity to HSV-infected cells is low (2, 13, 17) but can be stimulated by human interferon (13). Human peripheral blood lymphocytes and colostral cells incubated with virus-infected cells produce interferon (7, 10, 21). We therefore sought to determine whether human colostral cells were able to produce a substance (cytokine) when cultured in the presence of virus-infected cells that could stimulate the low neonatal natural killer cytotoxicity to HSV-infected cells. This could serve as a possible mechanism of colostral cell-dependent, nonspecific stimulation of the immune system of neonates.

MATERIALS AND METHODS

Colostral cells. After obtaining informed consent, we collected colostrum from mothers 2 to 4 days postpartum using an Egnell type SMB breast pump (16, 24). Colostral cells were washed four times in Hanks balanced salt solution (GIBCO Laboratories, Grand Island, N.Y.) and diluted to desired concentrations in minimal essential medium (GIBCO) supplemented with 10% inactivated fetal calf serum (Flow Laboratories, Inc., Rockville, Md.), penicillin (50 U/ml), and streptomycin (50 μg/ml) (supplemented minimal essential medium).

Blood leukocytes. Heparinized blood (30 U/ml) was obtained from healthy laboratory workers and postpartum lactating women. Neonatal umbilical cord blood samples were collected from healthy term infants at delivery in heparinized syringes (10 to 30 U of heparin per ml of blood) from the placental side of the ligated umbilical cords. Blood was processed by dextran sedimentation followed by Ficoll-Hypaque (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.) buoyant density centrifugation (13, 16, 17, 22). The separated mononuclear cells (a combination of lymphocytes and monocytes) obtained at the interface were washed four times in Hanks balanced salt solution and suspended in supplemented minimal essential medium at the desired concentrations. All human experimentation included informed consent and was reviewed and approved by the Committee for the Protection of Human Subjects, University of Texas Medical School, Houston, Texas.
**HSV immune status.** We assayed sera for anti-HSV antibody by determining the antibody-dependent cellular cytotoxicity activity (14, 17, 18).

**Cytokine culture preparations.** Leukocytes from blood or coelomus were cultured in supplemented minimal essential medium in sterile polystyrene capped culture tubes (12 by 75 mm; Scientific Products Div., McGaw Park, Ill.). Where indicated, leukocytes were cultured alone (unstimulated) or in the presence of Chang liver cells (2.5 × 10^6/ml) or Chang liver cells infected the previous day with 1 to 2 PFU of HSV type 1 (HE strain, virus stimulated). The final concentrations of leukocytes were utilized to result in Chang liver cell/leukocyte ratios of 1:200 (5 × 10^6 cells per ml) to 1:30 (7.5 × 10^6 cells per ml). Leukocytes were cultured at 37°C in a humidified atmosphere of 95% air and 5% CO_2 for 18 to 42 h. The cultures were then centrifuged (400 × g for 15 min), and the cell-free supernatants were collected and stored at −80°C before use.

**Microcytotoxicity assay.** The assay for cellular cytotoxicity was performed as described previously (13–18). The target cells were 51Cr-labeled Chang liver cells which had been infected 18 h previously with HSV type 1. To each microtiter well was added 100 µl of mononuclear effector cells (1.5 × 10^6 cells per well), 50 µl of target cells (5 × 10^5 cells per well), and 50 µl of cytokine preparation or medium. The covered plates were then incubated at 37°C in a humidified atmosphere of 95% air and 5% CO_2 for 18 h.

For a determination of the amount of 51Cr released from the target cells, 100 µl was aspirated from the top of each well without the cell button being disturbed. We then added 100 µl of 1 M NaOH to each well and aspirated the total volume into a separate container. All samples were counted in a Beckman Biogamma 4000 gamma counter (Beckman Instruments, Inc., Palo Alto, Calif.) for 1 min. Chromium release was calculated according to the following formula: percentage of 51Cr released = [2A(A + B)] × 100, where A equals counts per minute in the top 100 µl, and B equals counts per minute in the bottom 100 µl, to which 1 M NaOH was added.

Natural killer cytotoxicity was defined as: ([(percentage of 51Cr release of target cells + mononuclear effector cells) – (percentage of 51Cr release of target cells)]/[100 – percentage of 51Cr release of target cells)] × 100.

Cytokine-dependent cellular cytotoxicity (percent CDCC) was defined as: [(percentage of 51Cr release of target cells + mononuclear effector cells + virus-stimulated cytokine preparation) – (percentage of 51Cr release of target cells + mononuclear effector cells + unstimulated cytokine preparation)]/[100 – (percentage of 51Cr release of target cells + mononuclear effector cells + unstimulated cytokine preparation)] × 100.

Spontaneous 51Cr release of HSV-infected cells was 18.1 ± 3.0% in 18 h. These values were not affected by virus-stimulated or unstimulated cytokine preparations alone. All assays were performed in triplicate, with standard deviations of less than 5%.

**Statistical method.** Data are expressed as the mean ± standard error of the mean of experiments. The significance of differences of mean values was determined by Student’s paired two-tail t test.

**RESULTS**

Cytokine produced from peripheral blood mononuclear cells enhanced adult mononuclear cell natural killer cytotoxicity. Initial experiments were designed to determine the optimum culture and assay conditions for detecting mononuclear cell-derived cytokines. Mononuclear cells in the presence of HSV-infected Chang liver cells, uninfected Chang liver cells, or medium alone were incubated at various cell ratios for 18 h. The culture supernatants were then assayed for stimulating activity in the natural killer cytotoxicity assay. In the assay, the supernatants were preincubated for 2 h with unrelated mononuclear effector cells. Target cells were then added and further incubated for 18 h.

The natural killer cytotoxicity of mononuclear effector cells incubated with medium only was 45.9 ± 8.9%. There was no significant increase in the natural killer cytotoxicity of mononuclear cells in the presence of cytokine supernatants from leukocytes incubated with medium only (53.0 ± 10.5%). In contrast, the natural killer cytotoxicity of mononuclear effector cells preincubated in supernatants of cultures of leukocytes plus HSV-infected cells mediated significantly higher (P < 0.01) natural killer cytotoxicity (79.0 ± 12.9%). These assays were performed with a final dilution of culture supernatant of 1/4 (mean ± standard error of the mean of four separate experiments). These data resulted in a calculated CDCC of 65.8 ± 14.9%, using the CDCC formula (see above). Similar results were obtained with supernatants from cultures of 100 to 30 leukocytes per virus-infected cell. Thus, cultures of mononuclear cells incubated with HSV-infected cells contained a cytokine that mediated CDCC.

We examined the effect of various culture conditions and manipulations of the leukocytes on cytokine production as measured by CDCC (Table 1). There was no significant difference in cytokine production when cultures of mononuclear cells from four HSV seronegative donors incubated with HSV-infected cells were compared with those from six HSV seropositive donors incubated with HSV-infected cells. Cytokine activity was greater (P < 0.002) in mononuclear cell cultures incubated with HSV-infected cells than in matched mononuclear cell cultures incubated with uninfected cells. The CDCC activity of supernatants of mononuclear cells incubated with uninfected cells was not significantly higher than that of cells incubated only with medium. Thus, whereas HSV-infected cells stimulated mononuclear cells to produce a cytokine which mediated CDCC, uninfected cells did not.

Since it is possible that macrophages have a
TABLE 1. Cytokine production by peripheral blood mononuclear cells

| Leukocyte culture | % CDCC |
|-------------------|--------|
| Mononuclear cells incubated with HSV-infected cells | 37.3 ± 4.2 |
| Mononuclear cells from HSV seronegative donors incubated with HSV-infected cells | 45.3 ± 14.0 |
| Mononuclear cells from HSV-seropositive donors incubated with HSV-infected cells | 50.6 ± 9.2 |
| Mononuclear cells incubated with uninfected cells | 9.2 ± 4.8 |
| Mononuclear cells incubated with HSV-infected cells plus latex | 20.0 ± 6.5 |
| Mononuclear cells incubated with calf serum-free medium | 7.4 ± 3.6 |
| Mononuclear cells incubated with medium alone, HSV-infected cells added immediately before harvesting | 5.4 ± 4.3 |

*Percent CDCC (mean ± standard error of the mean of four to six separate experiments) of mononuclear cells incubated for 2 h with 18-h cytokine-containing culture supernatants, before the addition of target cells in the 18-h cytotoxicity assay. The infected cell/leukocyte ratio in the cytokine cultures was 1:100, and the final dilution of cytokine culture supernatants in the cytotoxicity assay was 1/4.

role in cytokine production (4, 5, 30), and since latex is a macrophage inhibitor (18), we analyzed the effect of latex (diameter, 1.09 μm; Dow Diagnostic, Indianapolis, Ind.) on cytokine production by adding 20 μl of 10% latex to the leukocyte cultures during incubation. Latex was removed by centrifugation during the supernatant harvest and was not present in the cytotoxicity assay. There was a significant (P < 0.05) reduction of CDCC when latex was present in the cytokine cultures for 18 h (Table 1). This indicates that the macrophage played a role in cytokine production.

Since fetal calf serum has been shown to stimulate natural killer cytotoxicity, we examined a series of cultures which contained neither infected cells nor fetal calf serum. There was no significant change in natural killer cytotoxicity when medium without calf serum was used. Therefore, fetal calf serum itself did not contribute to the cytokine production causing CDCC.

For confirmation that the cytokine was a product of the interaction of leukocytes and HSV-infected cells, mononuclear cells and HSV-infected cells were incubated in separate tubes, combined after 18 h, and immediately harvested. There was no significant increase in the CDCC of cells exposed to these supernatants (Table 1). Thus, the cytokine production was a result of the prolonged interaction of leukocytes and virus-infected cells.

There was no significant difference in cytokine production from leukocytes of individuals who were seropositive or seronegative for HSV antibody (Table 1). To further exclude the possibility of in vitro antibody production during culture, which would then mediate antibody-dependent cellular cytotoxicity, we used staphylococcal protein A to absorb immunoglobulin G (IgG) which could have mediated antibody-dependent cellular cytotoxicity (14). Cytokine preparations were absorbed for 1 h with an equal volume of protein A-Sepharose CL-4B (Pharmacia, lot 12630) and then separated by centrifugation (500 × g for 15 min) before being used in the cytotoxicity assay as previously described (14). The CDCC activity of the supernatants after protein A treatment (37.5 ± 4.5%) was similar to that before such treatment (39.0 ± 3.0%) in two experiments. Thus, the cytokine produced was not IgG mediating antibody-dependent cellular cytotoxicity, but was a nonantibody leukocyte product.

Cytokine produced from colostral cells enhanced the CDCC of adult mononuclear cells. Colostral cells were cultured with HSV-infected cells or medium at various HSV-infected cell/colostral cell ratios. After an 18-h incubation, the supernatants of these cultures were tested for CDCC activity with adult human peripheral blood mononuclear cells. Of 10 colostral cell cultures, 4 were active (CDCC > 10%) at infected cell/leukocyte ratios of 100:1, and 6 of 13 were active at ratios of 30:1. Both preparations tested at ratios of 60:1 were active. Table 2 shows the percentage of CDCC mediated by the active colostral cell culture supernatants. Thus, 12 of 25 (48%) colostral cultures stimulated with virus-infected cells produced a cytokine which

| Cell ratio | n (%) | % CDCC |
|------------|-------|--------|
| 1:100      | 4 (40) | 15.5 ± 2.5 |
| 1:60       | 2 (100) | 22.0 ± 0 |
| 1:30       | 6 (46)  | 13.8 ± 0.9 |

*Ratio of infected Chang liver cells to responding colostral cells in cytokine cultures.
* n, Number of active samples with CDCC >10% (percentage of total samples tested).
* CDCC of samples with activity >10%.
increased adult mononuclear cell killing of HSV-infected cells (CDCC). Colostro cell-stimulated CDCC tended to be lower than blood leukocyte-stimulated CDCC (Tables 1 and 2).

The effect of colostrum donor HSV serology on the ability of colostral cells to stimulate CDCC was analyzed. Colostral cell cytokine preparations from four seropositive women yielded CDCC values of 20.9 ± 7.6%, and six samples from seronegative women mediated CDCC with an activity of 18.4 ± 5.8%. Thus, there was no effect of maternal serological status on the ability of their colostral cells to produce cytokines which could mediate CDCC.

**Cytokine-dependent cellular cytotoxicity of neonatal mononuclear cells stimulated by supernatants of colostral cell cultures.** Table 3 shows the results of HSV-stimulated colostral cell culture preparations on CDCC when the cultures were incubated with either adult or neonatal leukocyte effector cells. In this series of experiments, 57.1% (8/14) of adult mononuclear cell—colostral supernatant combinations and 31.2% (5/16) of neonatal mononuclear cell—colostrum supernatant combinations responded with over 10% of CDCC. The magnitude of CDCC of those cells able to respond (>10%) was similar among neonatal cells (18.6 ± 3.4%) and adult cells (19.8 ± 3.9%). Thus, responsive neonatal leukocytes were as sensitive to increased cytotoxicity mediated by colostral cell-produced cytokine as were adult leukocytes.

**DISCUSSION**

We demonstrated that human leukocytes cultured with virus-infected cells secreted a substance that increased natural killer cytotoxicity. This substance was not produced after culture with uninfected cells. Production of the substance was independent of the serological status of the leukocyte donor. The substance was not absorbed by staphylococcal protein A; therefore, it was not an IgG antibody. This confirmed that increased cytotoxicity was not due to in vitro antibody production with subsequent antibody-dependent cellular cytotoxicity, as has been shown to occur in other systems (2, 8). Using cytokine as broadly defined to mean a substance produced by a stimulated leukocyte, we demonstrated CDCC.

Interferon is known to stimulate natural killer cytotoxicity (1, 5, 13, 19, 28, 30) and is probably among the cytokines responsible for CDCC. We have documented interferon titers of 87.8 ± 29.7 U/ml in 18-h HSV-stimulated leukocyte cultures (S. Kohl, S. B. Greenberg, and M. Harmon, manuscript in preparation). There are other cytokines, such as interleukin-2, (which has also been shown to increase natural killer cytotoxicity [11]), that have not yet been assayed in our system. More specific identification of the cytokine and the cell responsible for its production in blood and colostrum is under way.

Colostral cells cultured with virus-infected cells also produced a cytokine which was independent of the serological status of the cell donors and which also mediated CDCC. It is known that colostral cells may release IgA (27). The fact that colostral cells from both seropositive and seronegative women mediated CDCC negates the role of specific colostral IgA-mediated cytotoxicity. Indeed, high concentrations of IgA-rich, lipid-free colostrum are inhibitory to cellular cytotoxicity (16). We have shown that colostral cell cytokine preparations can often increase the normally low neonatal natural killer cytotoxicity activity (2, 13, 17) by the mediation of CDCC. As we have previously shown with purified human interferon, the leukocytes from responsive neonates were as sensitive to this cytokine as were the leukocytes from adults, although fewer cells of neonates were responsive (13). Although these results were anticipated, recent work has shown that stimulated human leukocytes may produce substances that either increase (interferons) or decrease (prostaglandin E2) natural killer cytotoxicity (19). Since stimulated macrophages produce prostaglandin E2 (19), and since colostrum is rich in macrophages compared with blood (26, 31), the lower CDCC activity of colostral supernatants may be due to low interferon or interleukin production, high prostaglandin production, or other, unidentified factors.

Colostral leukocytes themselves have been shown to be poor effector cells in cytotoxicity and bactericidal assays when compared with matched adult blood cells (16, 24). Nevertheless, there are data to suggest that the cellular component of colostrum is crucial in the protection of the suckling infant from infection (25). Colostral cells have been previously shown to produce interferon (7, 21) and a lymphocyte-derived che-

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**TABLE 3.** Comparison of CDCC of neonatal and adult mononuclear cells stimulated by colostral cell cultures supernatants

| Mononuclear cell source | % Responding cells<sup>a</sup> | % CDCC<sup>b</sup> |
|------------------------|-------------------------------|------------------|
| Adult                  | 57.1 (8/14)                  | 19.8 ± 3.9 (n = 8)|
| Neonatal              | 31.2 (5/16)                  | 18.6 ± 3.4 (n = 5)|

<sup>a</sup> Percentage of mononuclear cells with CDCC over 10%. (Number responding/number tested.)

<sup>b</sup> Mean ± standard error of the mean of three experiments involving three different adult and neonatal mononuclear cells with 10 different cytokine supernatants. Only the values for responding samples (n) are reported (not all 10 cytokine supernatants were available in each experiment).
motacitic factor when stimulated by mitogens (12) and to transport immunoglobulins (27). Thus, while being poor effector cells, colostral cells may have a strong immunological secretory function and may produce or transport soluble factors capable of affecting the immune competence of the effector cells of infants in vitro. These factors, among others, may explain the nonspecific protection afforded suckling animals and humans from a variety of infectious diseases (26, 31). Further studies of animal models and humans will be necessary to determine whether the soluble immune substances can be active in vivo as well as in vitro.

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