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Human colostrum contains an activity that inhibits the production of IL-2

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SUMMARY

The effect of human colostrum on T cell immune function was investigated. Colostrum inhibited the proliferation of human T cells activated by allogeneic, concanavalin A (Con A) or phytohaemagglutinin (PHA) stimulation. Colostrum also inhibited the production of IL-2 by Con A-activated human peripheral blood T cells and by Con A-activated Jurkat cells, a human T lymphoma line. Similarly, human colostrum inhibited the production of IL-2 by EL4 cells, a murine thymoma line, when stimulated with phorbol myristate acetate. The inhibitory activity was not cytotoxic and could not be neutralized by antibody to transforming human growth factor β.

Keywords colostrum T lymphocytes interleukin-2 inhibition

INTRODUCTION

We have shown that breast feeding, when compared with formula, significantly enhanced the T cell-mediated response to BCG vaccine given within 1 month after birth, and enhanced B cell immunity to polyribose phosphate of *Hemophilus influenzae b* conjugate vaccine (Pabst *et al.*, 1989; Pabst & Spady, 1990). These observations suggested that there might be an immune-enhancing activity in early milk or colostrum. A mitogenic activity in milk that acts on fibroblasts (Tapper, Klagsbrun & Neumann, 1979) and a proline rich protein that stimulates the proliferation of B lymphocytes (Julius, Janusz & Lisowski, 1988) have been described. Mincheva-Nilsson *et al.* (1990) have reported activities in human milk that both enhance and suppress T lymphocyte proliferation, the former activity being associated with lactoferrin.

Here we confirm that human colostrum inhibits T cell proliferation in assays where a T cell response is stimulated. We also show that this inhibition can be explained in part by an activity in human colostrum that inhibits the production of IL-2.

MATERIALS AND METHODS

Materials

Cells were cultured in RHFM medium consisting of RPMI 1640 (GIBCO, Grand Island, NY), 10 mM HEPES (Sigma), 10^{-4} M 2-mercaptoethanol and 10% fetal bovine serum (FBS) (GIBCO).

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For human peripheral blood mononuclear cells (PBMC), 10% AB serum was substituted for FBS. Recombinant human IL-2 was secreted from yeast cells transformed with an expression vector containing a synthetic human IL-2 cDNA (Barr *et al.*, 1984). Human transforming growth factor β (TGFβ1) and neutralizing rabbit antibody to TGFβ were obtained from R&D Systems (Minneapolis, MN). Concanavalin A (Con A), phytohaemagglutinin (PHA-P) and phorbol 12-myristate 13-acetate (PMA) were supplied by Sigma Chemical Company (St Louis, MO).

Colostrum

Colostrum samples were collected within one day of birth from healthy women who delivered their infants at term at the University of Albert Hospital. Samples were centrifuged (1500 *g*; 15 min, room temperature). The supernatant fluid was retained, avoiding pelleted cells and the overlaying fat pellicle. Samples were pooled (groups of six), sterilized by gamma irradiation (^{137}Cs source, 5000 rad) and stored at -70°C.

Cells

Peripheral blood mononuclear cells, obtained from healthy volunteers, were prepared by centrifugation of blood over Ficoll-Paque (Pharmacia, Uppsala, Sweden). Jurkat (human T helper lymphoma), RPMI 7666 (human EB-transformed B lymphocyte), EL4 (murine T helper thymoma) and MTL2.8.2 (murine IL-2-dependent CTL) cell lines were passaged *in vitro* in the laboratory.

Proliferation assays

Cells (10^5) were cultured in triplicate for 24 h in a 0·2 ml final volume in 96-well flat-bottomed microtitre trays (Costar). 0·25 μCi/well [^{3}H]-TdR (2 Ci/mmol, NEN, Boston, MA) was added for the final 6 h. Cultures were harvested on a Skatron harvester (Lier, Norway) and the radioactivity incorporated was determined by β-scintillation counting. For the mixed lymphocyte reaction (MLR) 10^5 responder cells were mixed with 10^5 irradiated (1500 rad) stimulator cells. Cultures were incubated for 5 days and pulsed with [^{3}H]-TdR as described above.

Assay for IL-2

This was performed as described previously (Hooton, Gibbs & Paetkau, 1985) using the IL-2-dependent murine cell line MTL2.8.2 and monitoring the reduction of the tetrazolium dye [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (Mosmann, 1983). IL-2 activity is expressed relative to a reference standard from Biological Resources Branch, NCI-FCRF (Frederick, MD).

RESULTS

Inhibition of lectin- and alloantigen-induced T lymphocyte proliferation

PBMC from normal human volunteers were incubated with Con A (3 μg/ml) or PHA (4 μg/ml) in the presence or absence of 10% colostrum for 3 days. Figure 1a shows a representative experiment. Both lectins stimulate T cell proliferation only and in both cases colostrum inhibited that proliferation. A similar inhibition of proliferation was observed with one- and two-way MLRs set up with human PBMC (Fig. 1b).

Colostrum at 10% was not toxic to any of the assay cells as determined by vital dye staining (eosin) at the end of the culture. In addition, colostrum added to either a human T cell (Jurkat) or human B cell (RPMI 8666) tumour cell line had no effect on proliferation as measured by [^{3}H]-TdR incorporation (Fig. 2a). 10% colostrum had no significant effect on the IL-2-dependent proliferation of the murine MTL2.8.2 cell line (Fig. 2b). This observation indicates that colostrum has no direct effect on the IL-2 molecule and that it does not affect the way in which IL-2 interacts with the IL-2 receptor on the MTL2.8.2 cells.

In contrast to the lack of toxicity of colostrum, we have found that human milk taken 2 weeks or more post-partum was toxic to the EL4 test cells (with or without PMA). This toxicity was not due to complement-mediated lysis (data not shown).

Inhibition of production of IL-2

The fact that colostrum inhibited the proliferation of T cells in immune or immunomimetic responses, but did not inhibit the proliferation of autonomously growing cells, suggested to us that an intermediate process in the immune response was being inhibited. An obvious candidate was the production of IL-2, a lymphokine necessary for the growth of T cells.

A significant problem in measuring IL-2 in the medium during an *in vitro* immune response is that the IL-2 produced is also consumed. However in a limiting dilution assay, where the number of T lymphocytes is limiting, IL-2 produced from a single cell can be detected (Vie & Miller, 1986). We have adapted a limiting dilution assay by adding a constant number of PBMC to each culture so that each well receives IL-2-producing cells

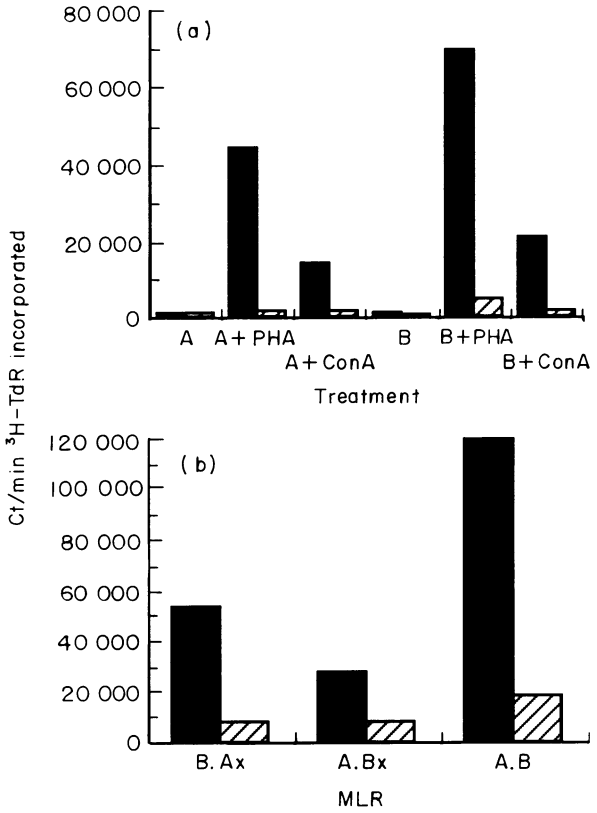

Fig. 1. Effect of colostrum on the proliferation of stimulated PBMC. (a) Lectin stimulation: 10^5 PBMC from two healthy adult volunteers (A or B) were incubated for 3 days in the presence of medium only, 4 μg/ml PHA or 3 μg/ml Con A with (■) or without (■) 10% pooled colostrum. (b) Mixed lymphocyte reaction: 10^5 PBMC were incubated with (■) or without (■) 10% pooled colostrum for 5 days as described in Materials and Methods. Ax and Bx refer to irradiated stimulator cells. Proliferation was measured by [^{3}H]-TdR incorporation as described in Materials and Methods.

but not so many cells that a significant amount of IL-2 is consumed. Five hundred PBMC were incubated with Con A (3 μg/ml) and 2500 irradiated (5000 rad) EBV-transformed human lymphocytes as feeder cells, together with graded doses of colostrum in a microtitre plate. After 3 days in culture the plate was irradiated (5000 rad) to kill the cells. Five thousand MTL2.8.2 cells were added to each well and the cultures incubated for a further 24 h. The cultures were pulsed for 6 h with [^{3}H]-TdR and harvested as described in Materials and Methods. The uptake of the tritiated label was taken as a measure of MTL2.8.2 proliferation, and therefore as a measure of IL-2 present in the culture medium. Figure 3 shows that colostrum inhibited the proliferation of MTL2.8.2 in a dose-dependent manner suggesting that colostrum was inhibiting the amount of available IL-2 in the medium. Colostrum from different donors showed similar activities, being active down to 1% by volume.

To confirm in a more direct way that IL-2 production was inhibited, cloned Jurkat cells were incubated with Con A (15 μg/ml) and PMA (5 ng/ml) in the presence or absence of 10% colostrum. After 24 h culture, the supernatant medium was removed and tested for IL-2 as described in Materials and

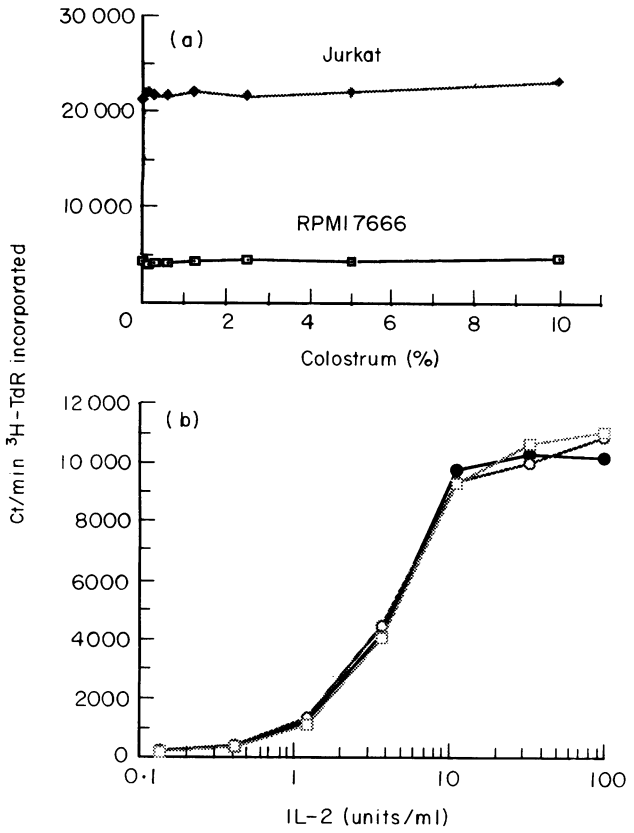

Fig. 2. Effect of colostrum on the proliferation of lymphocyte cell lines. (a) 10^5 human T cells (Jurkat) and B cells (RPMI 7666) were incubated with graded doses of pooled colostrum. (b) Murine IL-2-dependent MTL2.8.2 cells were incubated at 10^4 cells (5×10^4 cells/ml) per well with graded doses of recombinant IL-2 in the presence (◇) and absence (●) of 10% pooled colostrum or (□) 5 ng/ml TGFβ in a final volume of 0·2 ml RHFM. The cells were incubated for 24 h. [^{3}H]-TdR incorporation was measured as described in Materials and Methods.

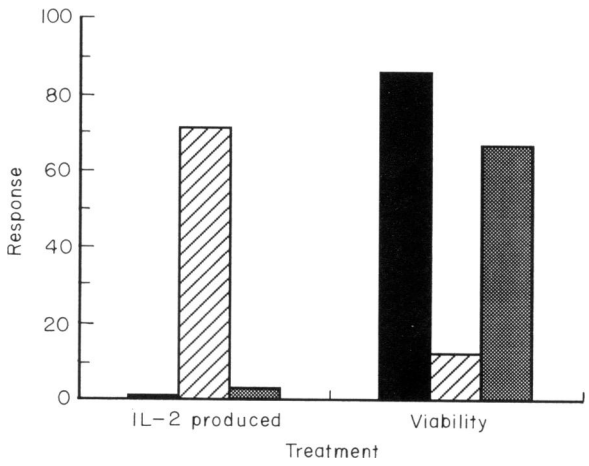

Fig. 4. Effect of colostrum on the viability of, and the production of IL-2 by, Jurkat cells. 10^5 Jurkat cells were incubated for 24 h with 5 μg/ml PMA, 15 μg/ml Con A and 10% pooled colostrum in a final volume of 0·2 ml RHFM. 100 μl supernatant medium was removed and tested for IL-2 as described in the Materials and Methods section. ■, Medium; ▨, PMA + Con A; ▩, PMA + Con A + colostrum.

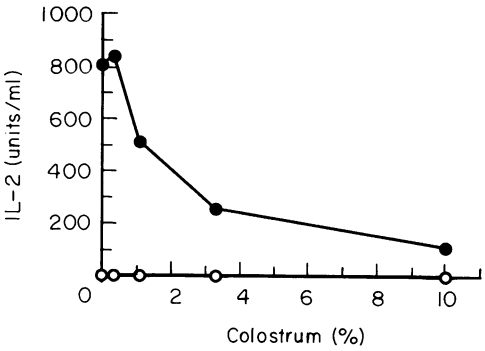

Fig. 5. Effect of colostrum on the production of IL-2 by EL4 cells. 10^5 EL4 cells were incubated for 24 h with 10 ng/ml PMA and graded doses of pooled colostrum in a final volume of 0·2 ml RHFM. IL-2 was measured as described for Fig. 4. ●, with PMA; ○, without PMA.

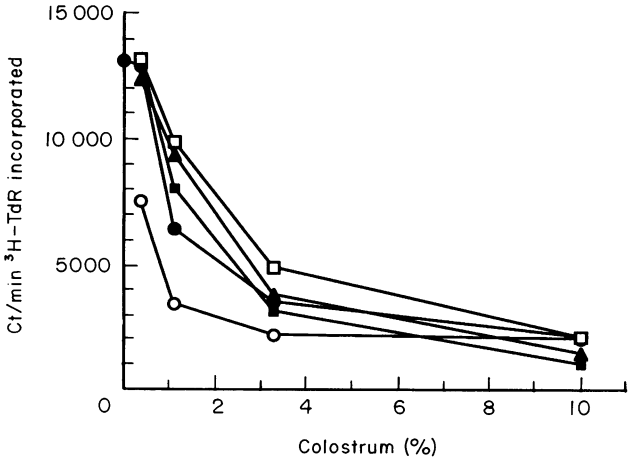

Fig. 3. Effect of colostrum on the production of IL-2 by Con A-stimulated PBMC. See text for details. d1–d5 refer to colostrum samples from individual donors. ●, d1; ○, d2; ■, d3; □, d4; ▲, d5.

Methods. Colostrum inhibited the production of IL-2 by this cloned cell line and also prevented the cell death that normally accompanies the stimulation (Fig. 4).

One argument against the use of Con A as a stimulator is that a glycoprotein in colostrum might bind Con A and prevent its action. The proliferation inhibitory activity in colostrum has been shown to be a Con A-binding protein (Mincheva-Nilsson *et al.*, 1990). To avoid this problem we used the murine T helper cell line, EL4, which produces IL-2 when stimulated with PMA. The cell line has the added advantage that it does not possess glucocorticoid receptors. Colostrum inhibited the production of IL-2 by PMA-stimulated EL4 in a dose-dependent manner (Fig. 5).

Recently there has been a report of an activity similar to TGFβ in bovine milk (Stoeck *et al.*, 1989) that is immunosuppressive. We found that human TGFβ inhibited the production of IL-2 by PMA-stimulated EL4, the activity saturating at about 1 ng/ml (Fig. 6a). Human TGFβ had no effect on the IL-2-dependent proliferation of MTL2.8.2 (see Fig. 2b). Anti-

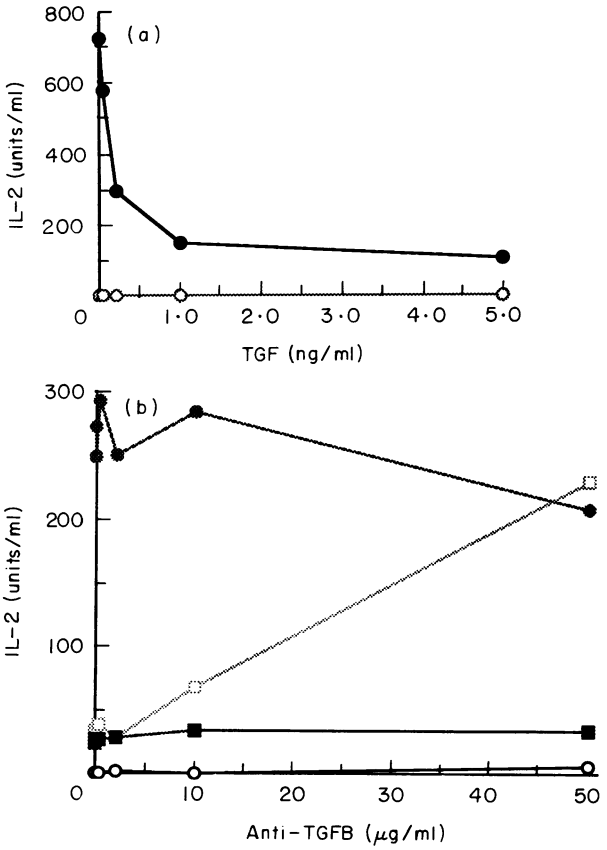

Fig. 6. Effect of human TGFβ1 on the production of IL-2 by EL4 cells. 10^5 EL4 cells were incubated for 24 h with the indicated additions in a final volume of 0·2 ml RHFM. 100 μl supernatant was removed and tested for IL-2. (a) EL4 were incubated with (●) and without (○) 10 ng/ml PMA with graded concentrations of TGFβ1. (b) EL4 cells were incubated with medium (○), 10 ng/ml PMA (●), PMA + 1 ng/ml TGFβ1 (□) and PMA + 10% pooled colostrum (■) and graded doses of rabbit anti-human TGFP as indicated.

TGFβ antibody neutralized the TGFβ-mediated inhibition but not the colostrum-mediated inhibition of IL-2 production by these cells (Fig. 6b). This indicates that the IL-2-inhibitory activity in colostrum is not TGFβ.

DISCUSSION

In this report we provide confirmation of the presence of one or more inhibitory activities in human colostrum that inhibit T cell function as measured by proliferation in lectin-stimulated or mixed lymphocyte assays (Mincheva-Nilsson *et al.*, 1990). Colostrum was not toxic to any cells tested and did not inhibit the proliferation of autonomously growing cells. The inhibitory activity is therefore not a general inhibitor of proliferation or DNA synthesis but appears to be restricted to cells that are proliferating as a result of immune activation in the form of antigen or lectin. This result is in contrast to that of Barta, Barta & Crisman (1990) who report the presence of DNA synthesis inhibiting factor(s) (DNA-SIF) in bovine milk whey.

We have shown that colostrum contains a substance or substances whose activity inhibits the production of IL-2 in Con A-activated cells, both human PBMC and a human T lymphoma. It could be argued that a glycoprotein in milk binds Con A (which binds mannose residues) preventing the binding of the lectin to the cell surface and activating it. Mincheva-Nilsson *et al.* (1990) have shown that the proliferation inhibitory activity present in human colostrum and milk binds Con A. However, the inhibition of IL-2 production is not due merely to the competitive binding of Con A by milk glycoprotein since colostrum also inhibited the production of IL-2 by PMA-stimulated EL4 cells. We do not know if this is the same activity that inhibits the proliferation of lectin- and allogeneic-stimulated PBMC. However, colostrum is not a general inhibitor of proliferation, and it seems likely that it inhibits the development of T cells, either in their responsiveness to lymphokine or in the production of lymphokine(s). At any rate, the reduced proliferation of immune-activated cells can be explained by the inhibition of lymphokine production.

Human milk and colostrum contain many lymphokines and growth factors, some of which are inhibitory in immune responses. In particular, bovine milk growth factor (MGF) has been shown to inhibit Con A-induced proliferation of human PBMC and also IL-2- and IL4-dependent proliferation of human T cells (Stoeck *et al.*, 1989). These investigators have also shown that in this respect MGF, TGFβ1 and TGFβ2 have very similar activities. Espevik *et al.* (1990) have reported that TGFβ at 1 ng/ml inhibited the production of IL-2 by IL1β-stimulated EL4-NOB-1 murine thymoma cells. TGF activity has been reported in human colostrum (Noda, Umeda & Ono, 1984). TGFβ1 inhibited the production of IL-2 by PMA-stimulated EL4 (Fig. 6). We have shown that TGFβ1 is not the inhibitory activity in colostrum as anti-TGFβ did not affect this inhibition at concentrations of antibody that neutralized 1 ng/ml TGFβ1.

IL-10 (CSIF, cytokine synthesis inhibitory factor), a cytokine produced by Th2 helper cells in the mouse, inhibits the production of cytokines, including IL-2, in both the mouse and the human (Vieira *et al.*, 1991). We have been unable to test if human IL-10 is the activity in colostrum that inhibits IL-2 production due to lack of available reagents. Vieira *et al.* (1991) also report that IL-10 inhibits the ability of macrophages to stimulate cytokine synthesis. The fact that the inhibitory activity in colostrum works directly on IL-2-producing cells suggests that the activity is not IL-10.

The function of an immune-inhibitory activity in colostrum is not clear. One possible function might be in preventing allogeneic reactions of the graft-*versus*-host variety. There is substantial evidence that breast milk contains immunoactive cells including macrophages, B cells and T cells (Crago *et al.*, 1979; Parmely, Beer & Billingham, 1976). The T lymphocytes are almost exclusively memory cells (Bertotto *et al.*, 1990) and human milk and colostral lymphocytes have been demonstrated to proliferate in response to alloantigens (Parmely *et al.*, 1977). Although there is evidence that milk lymphocytes enter the neonate's tissues in rats (Parmely *et al.*, 1976), mice (Weiler, Hickler & Sprenger, 1983), lambs (Schnorr & Pearson, 1984), and in baboons (Jain *et al.*, 1989) there is no evidence of colostral cells in the circulation of the human neonate.

Mincheva-Nilsson *et al.* (1990) postulate that the inhibitory activity down-regulates the immune response to environmental antigens in neonates until T suppressor activity can develop and thereby prevent atopic reactions to these antigens in later life. The inhibitory activity would be exerted in the gut of the infant inhibiting the response of intestinal wall lymphocytes. This would prevent hyperactive responses there against intestinal

content antigens. This in turn might reduce hypersensitivities.

We have demonstrated that one component of colostrum inhibits the production of IL-2 but not the effect of IL-2. Since IL-2 has a pivotal role in T cell-dependent immune responses, colostrum will have a profound effect on the development of the immune response to any T-dependent antigen. It remains to be determined whether the immunoregulatory activity of colostrum affects primarily the immune cells in colostrum itself or the developing lymphoepithelium in the infant's gut, or both.

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