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Antioxidant Properties of Human Colostrum

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ABSTRACT. Because it has recently been hypothesized that human milk is antiinflammatory, the effects of aqueous human colostrum on human polymorphonuclear leukocyte (PMN) respiratory burst activity and selected enzymatic activities was examined. Aqueous colostrum was found to spontaneously reduce ferricytochrome C in a concentration-dependent manner, prohibiting use of the standard assay to measure superoxide production. It also caused a significant concentration-dependent prolongation of the lagtime from stimulation of PMN with phorbol myristate acetate to the appearance of hydrogen peroxide. Substitution of an enzymatic peroxide-generating system for PMN did not alter the effect of colostrum. Colostrum also suppressed myeloperoxidase activity and lysozyme activity, but not β-glucuronidase activity in PMN lysates. Inclusion of colostrum in an *in vitro* assay of PMN-mediated cell detachment significantly suppressed this PMN-mediated effect. These data demonstrate that aqueous human colostrum significantly interferes with PMN oxygen metabolic and enzymatic activities that are important in the mediation of acute inflammation. (*Pediatr Res* 24: 14–19, 1988)

Abbreviations

PMN, polymorphonuclear leukocytes
PMA, phorbol myristate acetate
H_2O_2, hydrogen peroxide
HBSS, Hanks' balanced salt solution without Ca^{++}, Mg^{++}, and phenol red

The PMN is the acute inflammatory cell of the body, and as such, performs two important roles: microbial killing and propagation/modulation of acute inflammation (1). Proper performance of these roles is dependent on an intact capability to generate oxygen metabolites via the respiratory burst (2, 3). Although the presence of large numbers of PMN in a physiologic fluid other than blood is usually taken as evidence of acute inflammation, human colostrum contains approximately 2×10^6 PMN/ml (4) without evidence of acute inflammation in the breast. The PMN present in colostrum are hypofunctional compared to blood PMN, having decreased oxygen metabolism and bactericidal activity (4) as well as depressed chemotactic responsiveness (5). To examine whether the hypofunction of colostral PMN and the lack of acute inflammation in the newly lactating breast might reflect inherent "antiinflammatory" characteristics of human colostrum, we tested the *in vitro* effects of colostrum on PMN oxygen metabolism at two levels: oxygen metabolite production by the PMN and oxygen metabolite-mediated cellular injury.

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METHODS

Colostrum specimens. Samples of human colostrum were collected by breast pump from mothers 1 to 4 days postpartum. The colostrum was kept on ice until separated by centrifugation (4°C, 81,000 × *g*, 30 min). The time from colostrum collection to separation was typically 30–60 min. After centrifugation, the aqueous fraction of the separated colostrum was harvested by puncturing the centrifuge tube above the cell pellet with a needle and pumping the aqueous fraction out at 2–4 ml/min using a peristaltic pump. Aqueous colostrum was stored in aliquots at −70°C until studied; all studies used either fresh aqueous colostrum (never frozen) or aqueous colostrum thawed once. For experiments that used whole, acellular colostrum, fresh colostrum was centrifuged at 300 × *g* for 10 min at 4° C, the cream layer and aqueous phase decanted, remixed, and then used or frozen in aliquots at −70° C until used.

PMN studies. PMN were purified from heparinized human blood as described (6). PMN oxygen consumption was measured with a Clark oxygen electrode (7) using PMA, 20 ng/ml final concentration, as the stimulus. In these experiments, PMN were used at 2.5×10^6 cells/ml. PMA-stimulated neutrophil hydrogen peroxide production was measured by following horseradish peroxidase mediated oxidation of scopoletin by H_2O_2 (8). In these experiments, PMN were used at 10^6 cells/ml.

For studies examining the effects of aqueous colostrum on PMN enzyme activities, a lysate of purified PMN (approximately 10^7/ml) was used as the source for the enzymes myeloperoxidase, β-glucuronidase, and lysozyme. Separate aliquots of aqueous colostrum and PMN lysate were assayed for each enzyme. The sum of the enzyme activity in colostrum plus the activity in the PMN lysate activity (referred to as the "predicted" activity) was then compared to the activity of identical colostrum and PMN lysate aliquots first mixed together and then assayed (referred to as the "observed" activity). Pairs of predicted and observed values were then plotted against one another, and the regression line through the points was compared to a line with a slope of 1 by *t* test. Myeloperoxidase activity of PMN lysates was measured using *O*-dianisidine as the chromagen (9). Specifically, triplicate 50-μl samples (either PMN lysate + H_2O colostrum + H_2O, or colostrum + lysate) were combined with 200 μl of MPO buffer (made by combining 6 ml of HBSS with Ca^{++} and Mg^{++} without phenol red, containing 1% bovine serum albumin, with 5 ml of 0.1 M phosphate buffer, pH 7.2, with 1 ml of 1.25 mg/ml. *O*-dianisidine in water with 1 ml of 0.05% H_2O_2 in water) and incubated in 96-well microtiter plates for 15 min at room temperature. After 15 min, color formation was read immediately (at 460 nm) on an automated ELISA plate reader. Triplicate determinations were then averaged, and compared as described above. Lysozyme and β-glucuronidase activities were measured by standard methods (10, 11).

The effects of stimulated PMN on HEp-2 cell adherence were studied as follows. HEp-2 cells [a continuous cell line originally derived from a human epidermoid carcinoma (12) and frequently used to examine interactions between pathogenic enteric bacteria

and epithelial cells (13, 14)] were grown to monolayers in 24-well tissue culture plates using RPMI 1640 media with 10% fetal calf serum, 2 mM glutamine, 50 μg/ml streptomycin, and 50 μg/ml penicillin added. Monolayers were washed twice with HBSS, and 5×10^6 purified PMN were added to quadruplicate wells (approximately 10 PMN: 1 HEp-2 cell). PMA (20 ng/ml final concentration), whole, acellular colostrum (10% final concentration), and HBSS were added, and the cell cultures were incubated for 2 h at 37° C. After 2 h the quadruplicate wells were washed twice with HBSS, the cells remaining were solubilized in 6 N NaOH, and the total protein content of each well was determined (15) and the quadruplicates were averaged.

Cytochrome C reduction. One part aqueous colostrum or diluted aqueous colostrum was mixed with 1 part ferricytochrome C (1.78 mg/ml) and kept at room temperature for 10 min. Ferricytochrome C reduction was then quantitated by measuring the OD_{549} of each sample and comparing it to a blank that substituted water for the aqueous colostrum.

Acellular production of hydrogen peroxide. In some experiments, an acellular H_2O_2-generating system was substituted for purified PMN. In these experiments, a stock glucose solution (1 M) was used to bring the final glucose concentration to 10 mM, and glucose oxidase, 0.25 U, was added. At 35° C, pH 5.1, this system produces 250 nmol of H_2O_2/min; under the assay conditions used (room temperature, pH 7), this system produces 65.19 ± 5.76 nmol H_2O_2/min.

Calculation of colostral reducing activity. Cytochrome C-reducing activity of colostrum was calculated by a standard method (16) and is expressed in nmol/ml. H_2O_2-depleting activity was calculated by multiplying the lag time (in min) by the final rate of H_2O_2 production (in nmol/min), divided by the volume of colostrum added.

Electrophoretic concentration of colostrum. Aliquots of aqueous colostrum were diluted 2- to 4-fold with 0.001 M phosphate buffer, pH 8. They were then concentrated using an electrophoretic concentrator (ISCO, Lincoln, NB) with 3.5-kDa dialysis membranes at either end of the concentration cuvette. This device uses an electric field to concentrate charged molecules at the surface of a dialysis membrane with defined mol. wt. exclusion characteristics (17). The charge of the targeted molecule is controlled by the pH of the dilution buffer used. Dilution buffer of pH 8 was used to dilute the colostrum based on preliminary experiments examining both the electrophoretic mobility and the pH stability of the colostrum activities. Concentration was performed for 2 W hours per cuvette at 4° C.

Data shown are the mean ± SEM; unless otherwise noted, statistical significance was determined by paired sample *t* test whenever natural pairing occurred; otherwise, Student's *t* test was used.

RESULTS

Effects of aqueous colostrum on PMN oxygen metabolism and enzymes. Initial experiments performed to examine whether aqueous colostrum had an effect on PMN superoxide production demonstrated that aqueous colostrum reduced ferricytochrome C spontaneously (Fig. 1). The degree of ferricytochrome C reduction was dependent on the amount of colostrum added; time course experiments showed that ferricytochrome C reduction began immediately on mixture, and was complete by 10 min at room temperature. Spontaneous reduction of cytochrome C was not affected by the presence of superoxide dismutase (75 U/ml). In comparison, the antioxidants; methionine (1 mM), mannitol (50 mM), and dimethylsulfoxide (280 mM) did not spontaneously cause ferricytochrome C reduction. Aqueous colostrum was also found to spontaneously reduce 0.1% nitroblue tetrazolium (data not shown). The ferricytochrome C reducing capacity of aqueous colostrum was calculated to be 497 ± 47 nmol/ml (n = 17). Boiling (10 min at 100° C) resulted in retention of 96 ± 11% of this activity (n = 4).

Because PMN superoxide production could not be assessed in the presence of aqueous colostrum, PMA-stimulated hydrogen peroxide production by PMN was examined. Aqueous colostrum caused a dose-dependent prolongation of the lagtime from PMA addition to scopoletin oxidation (H_2O_2 production) (Fig. 2, *left*). This increase in the lag time was not due to delay in the onset of the respiratory burst, as aqueous colostrum had no effect on the time of onset of PMA stimulated oxygen consumption (Fig. 2, *right*). The effect of 10% aqueous colostrum on the rate of H_2O_2

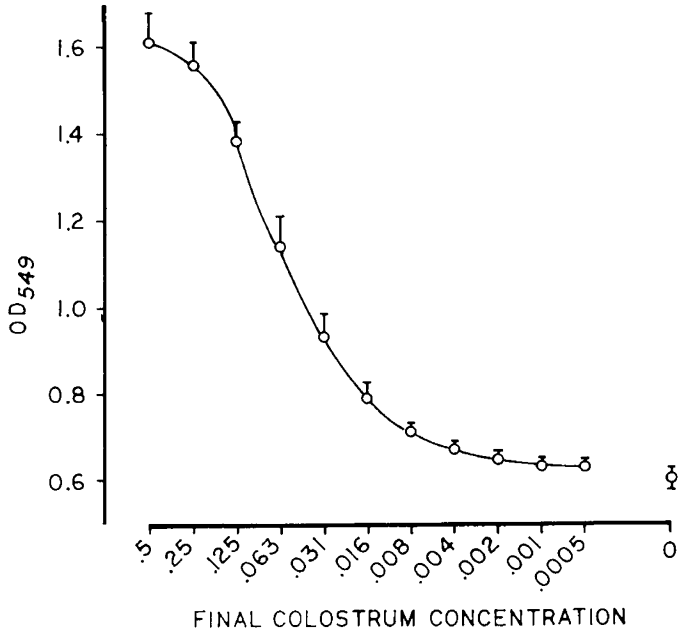

Fig. 1. Effects of combining cytochrome C and aqueous human colostrum. The ordinate shows OD549, absorption maximum for ferrocytochrome C. The *abscissa* shows final aqueous colostrum concentration in assay.

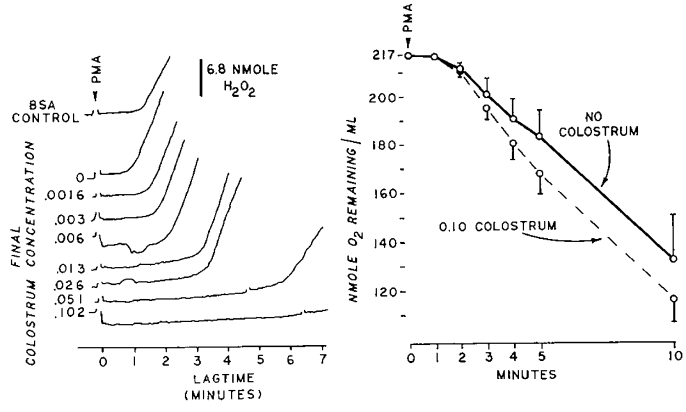

Fig. 2. *Left*, patterns of H_2O_2 production by human PMN in the presence of various concentrations of aqueous colostrum. The final aqueous colostrum concentrations are shown. A bovine serum albumin stock solution (17 mg/ml) was used as a control for protein content. The *upper tracing* shows a 0.10 final concentration of this stock. The lag time (in min) is shown on the *abscissa*. The *arrow* denotes time of cell stimulation with PMA (20 ng/ml final). The *bar* shows 10 fluorescence units, equal to the production of 6.8 nmol of H_2O_2. The *tracings* show scopoletin fluorescence: upward deflection denotes loss of fluorescence due to oxidation by H_2O_2. *Right*, PMA-stimulated PMN oxygen consumption in the presence and absence of aqueous colostrum. The nmol of oxygen remaining are shown on the *ordinate*. Time in min is shown on the *abscissa*. The *arrow* denotes time of stimulation with PMA. Data points are mean ± SEM. n = 5 for no colostrum; n = 8 for 0.10 colostrum.

production by PMN could not be evaluated because of the prolonged lagtimes it caused. Lagtime prolongation was still seen in the presence of 2.5% colostrum, and at that concentration, the rate of PMN H_2O_2 production was not significantly different than control (15.98 ± 2.17 *versus* 15.08 ± 2.39 nmol H_2O_2/min/2.5 × 10^6 PMN, control *versus* 2.5% colostrum, $n = 7$).

Aqueous colostrum had no effect on PMN β-glucuronidase activity, assessed by comparing the activity of the enzyme in the absence (Fig. 3, *predicted*), and presence (Fig. 3, *observed*) of aqueous colostrum. In the presence of 50% aqueous colostrum, β-glucuronidase activity was 105.8 ± 7.0% of control. Aqueous colostrum significantly depressed both PMN myeloperoxidase and lysozyme activities (Fig. 3, *middle* and *bottom*); 49.3 ± 5.9% of predicted myeloperoxidase activity ($p < 0.001$ *versus* predicted) and 86.0 ± 5.4% of predicted lysozyme activity ($p < 0.01$ *versus* predicted) was observed in the presence of 50% aqueous colostrum.

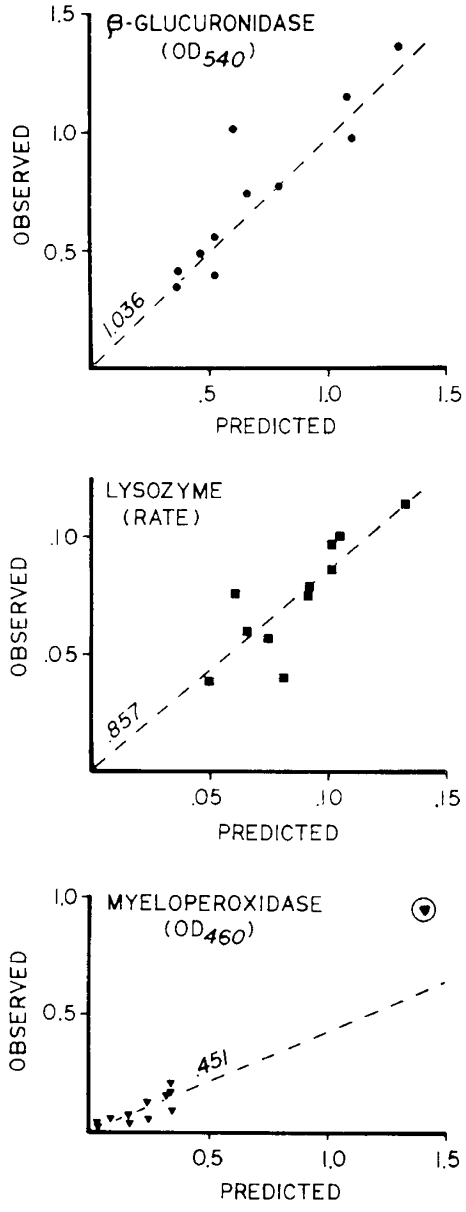

Fig. 3. The effects of aqueous colostrum on β-glucuronidase, lysozyme, and myeloperoxidase activities from human PMN lysates. The *ordinates* show observed values (activity of combined PMN lysate and colostrum). *Abscissa* shows predicted values (activities of PMN lysate and colostrum measured individually, then added). *Dashed lines* show regression slope for the data with intercept fixed at 0. *Numbers* above *dotted lines* are the numerical slopes. The *circled point* in the *lower panel* is an experimental value excluded from the regression calculation because it is an outlier.

Effects of aqueous colostrum on PMN-mediated HEp-2 cell detachment. As summarized in Table 1, HEp2 cells in tissue culture were not detached from the tissue culture substrate by purified PMN, by aqueous colostrum, by PMN with colostrum or by PMA alone. However, in the presence of PMN-stimulated by PMA, 42.0 ± 6.2% of starting cellular protein remained after 2 h. Addition of aqueous colostrum (10%) to this condition partially abrogated the effect of the stimulated PMN, increasing the fraction of starting cellular protein to 73.3 ± 8.5% after 2 h ($p < 0.001$, without colostrum *versus* with colostrum, paired sample *t* test).

Effects of aqueous colostrum on an acellular H_2O_2-producing system. To simplify examination of the effects of aqueous colostrum on PMN H_2O_2 production, an enzymatic H_2O_2-producing system was substituted for PMN in further experiments. In the presence of excess glucose, glucose oxidase produces H_2O_2 at a constant rate, which is dependent on the glucose oxidase concentration. Aqueous colostrum delayed onset of scopoletin oxidation by this H_2O_2 producing system in a dose-dependent fashion, similar to its effect on PMN produced H_2O_2 (Fig. 4). The H_2O_2 depleting activity of colostrum, using this cell-free system, was 918 ± 54 nmol/ml ($n = 18$). Boiling (10 min at 100° C) resulted in retention of 51 ± 11% of activity ($n = 6$). In an additional experiment, the effects of high concentrations of colostrum on

Table 1. *Effects of colostrum on PMN-mediated HEp2 cell detachment*

Condition	(*n*)	% of total protein remaining: Mean	SEM	
HEp2 cells alone	10	100		
HEp2 cells + PMN	9	96.54	3.85	
HEp2 cells + colostrum	9	108.89	7.78	
HEp2 cells + PMN + colostrum	4	87.88	7.39	
HEp2 cells + PMA (20 ng/ml)	4	103.50	5.60	
HEp2 cells + PMA + PMN	10	41.99	6.24	$p < 0.001$
HEp2 cells + PMA + PMN + colostrum	10	73.30	8.51	$p < 0.001$

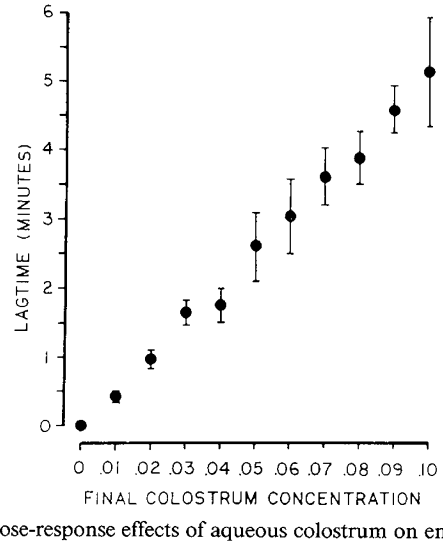

Fig. 4. Dose-response effects of aqueous colostrum on enzymatically produced H_2O_2. Final colostrum concentration is shown on the *abscissa*. Lag time from the addition of glucose oxidase to the appearance of H_2O_2 is shown on the *ordinate*.

H_2O_2 produced by glucose + glucose oxidase were examined (Fig. 5). In two of three specimens tested, colostrum concentrations of 90% prolonged the lagtime to the appearance of H_2O_2 to more than 60 min.

Ten percent aqueous colostrum did not significantly decrease the rate at which H_2O_2 was produced by glucose + glucose oxidase (65.19 ± 5.76 *versus* 54.79 ± 7.86 nmol/min, control *versus* 10% colostrum, n = 10 and 9, respectively). Treatment of colostrum with 50 mM aminotriazole (a catalase inhibitor) for 30 min at 4° C decreased H_2O_2-depleting activity to 94 ± 8% of control (n = 4). In two experiments, treatment of colostrum with 0.05% sodium azide as an inhibitor of catalase (30 min, room temperature) decreased its H_2O_2 depleting activity to 92 and 93% of control. Inclusion of catalase into the cell-free H_2O_2-generating system did not produce a lag time; rather, it decreased the apparent rate of H_2O_2 production. In an experiment not shown, substitution of this H_2O_2-producing system for exogenously supplied H_2O_2 in the myeloperoxidase assay, with continuous monitoring of myeloperoxidase activity, demonstrated that the suppressive effect of colostrum on myeloperoxidase activity was related to its depleting effect on H_2O_2, the substrate for myeloperoxidase. In this system, colostrum caused a delay in the onset of myeloperoxidase activity, but did not affect the rate.

To examine the relationship between the aqueous colostrum element responsible for ferricytochrome C reduction and the element responsible for depleting H_2O_2, the following experiment was performed: colostrum, glucose, and glucose oxidase were combined and the oxidation of scopoletin was monitored fluorometrically. Aliquots of this assay mixture were then removed at different times and examined for ferricytochrome C-reducing activity. Aliquots removed immediately after combining aqueous colostrum, glucose, and glucose oxidase produced initial ferricytochrome C reduction followed by ferricytochrome C reoxidation (Fig. 6*A*). Delay of aliquot removal until after the onset of scopoletin oxidation (*i.e.* H_2O_2-depleting activity was exhausted) resulted in no ferricytochrome C reducing activity (Fig. 6*B*). Substitution of water for glucose oxidase in the H_2O_2 assay resulted in prompt reduction of ferricytochrome C by the aqueous colostrum, which could then be reoxidized by addition of glucose oxidase (Fig. 6, *control*).

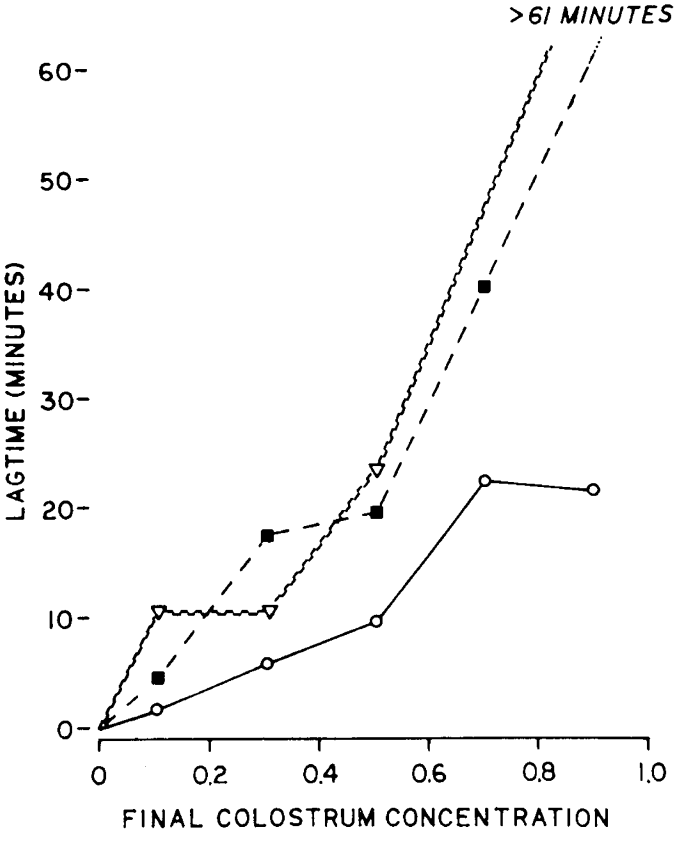

Fig. 5. The effects of increasing human colostrum concentrations on the lag time to the appearance of glucose + glucose oxidase produced H_2O_2. Data from three different human colostrum specimens are shown.

Electrophoretic concentration of colostral activity. Both the cytochrome C-reducing activity and the H_2O_2-depleting activity of colostrum could be electrophoretically concentrated: cytochrome C-reducing activity concentrated 4.2 ± 0.4-fold (n = 7); H_2O_2-depleting activity concentrated 5.3 ± 0.6-fold (n = 6); in three experiments, total colostral protein concentrated 6.9 ± 2.7-fold.

DISCUSSION

It is generally accepted that breast-feeding benefits the newborn infant, providing superior nutrition as well as cellular and humoral elements that might bolster the neonate's host defenses. It has recently been hypothesized that human milk might also be antiinflammatory, based on the paucity of inflammatory mediators it contains, the presence of multiple antiinflammatory components in it, and the depressed functional characteristics of many of the leukocytes it contains (18). Whether this characteristic is real or how it might or might not be beneficial to the infant is currently speculative.

It is known that colostrum, the initial product of lactation, is different from mature milk in its fat composition, protein composition, and the types of leukocytes it contains (19). PMN are the predominant phagocytic cell type in colostrum, whereas in transitional and mature milk, mononuclear phagocytes predominate. The PMN plays a major role in acute inflammation, and the presence of large numbers of PMN in body tissues or fluids (except bone marrow and blood) is synonymous with acute inflammation. The presence of relatively large numbers of PMN in colostrum typically without signs of acute mammary inflammation is somewhat paradoxical. We hypothesized that the PMN present in colostrum are the result of maternal host defense responses to opening up new potential routes of microbial invasion from the environment (*i.e.* initiating lactation), and that the presence of such activated, exudate PMN would be potentially injurious to the naive, neonatal intestinal epithelium unless their inflammation-inducing capabilities were suppressed. Because the PMN propagates/modulates acute inflammation via its production of oxygen metabolites and secretory products (1, 20), we chose to examine the effects of aqueous, acellular colostrum on these aspects of PMN function.

We were unable to examine the effects of colostrum on PMN superoxide production using a standard assay (superoxide dismutase inhibitable ferricytochrome C reduction) because acellular colostrum spontaneously reduced the cytochrome C in a rapid, dose-related manner. Interesting facets of this effect were that the reduction was not mediated by an endogenous superoxide generating system (it was not affected by superoxide dismutase), and the reduction did not appear to be enzymatic (the rate rapidly plateaued before complete reduction of the cytochrome C) (Fig. 6, *control*). We therefore examined the effects of colostrum on PMN production of H_2O_2, the ultimate product of the respiratory burst (21).

Colostrum was found to delay the appearance of PMN-produced H_2O_2. The data show that this effect was not mediated by interference with the onset of the PMN respiratory burst per se (colostrum did not cause a lag before the onset of oxygen consumption). Using an enzymatic, acellular system to produce H_2O_2, the same effect was seen. In both systems, the major effect of colostrum was to delay the appearance of H_2O_2, with little effect on its ultimate rate of appearance (Fig. 2). Such an effect is not likely to be enzymatic, as enzymatic destruction of H_2O_2 causes dose-dependent changes in the rate of H_2O_2 production in this system. Rather, the colostral component(s) is more likely

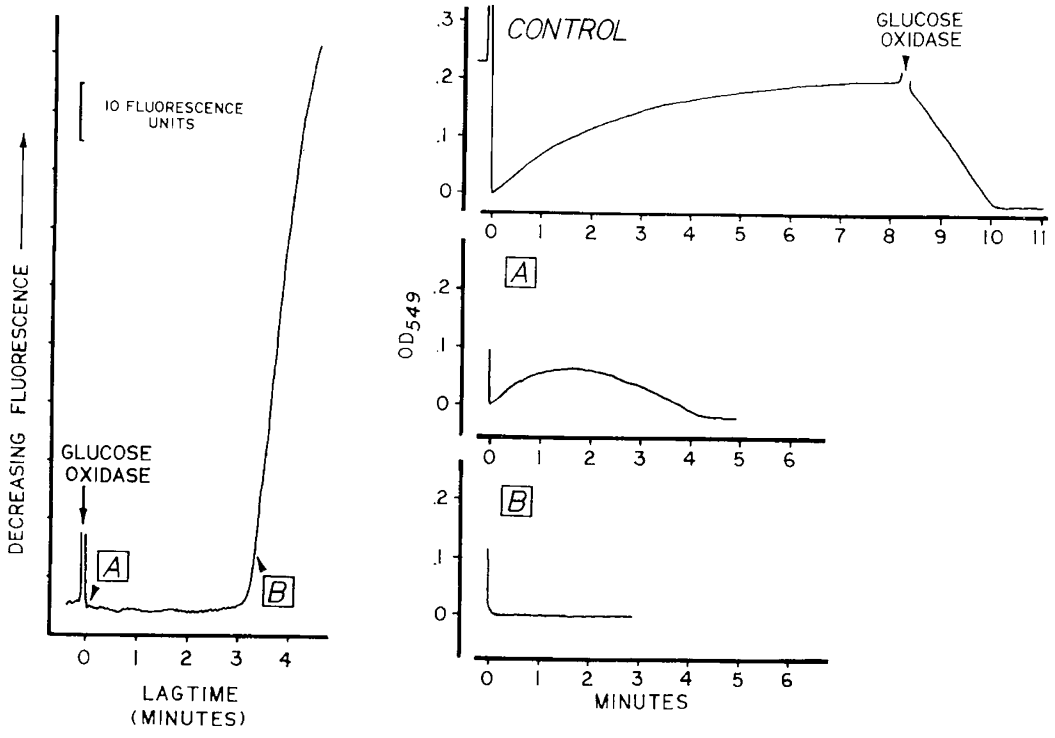

Fig. 6. Effects of exhaustion of colostral H_2O_2 depleting activity on colostral cytochrome C reducing activity. *Left*, tracing of scopoletin oxidation (glucose + glucose oxidase produced H_2O_2) in the presence of 0.10 aqueous colostrum. *A* and *B* show times at which aliquots were removed from parallel experiments and assayed for ferricytochrome C reducing activity. *Right* (*top*), spontaneous ferricytochrome C reducing activity of aqueous colostrum. OD549 is shown on the *ordinate*; time in min is shown on the *abscissa*. *Arrow* shows time of addition of glucose oxidase. (*Middle*), spontaneous ferricytochrome C reduction by an aliquot removed at time point *A* (*bottom*), spontaneous ferricytochrome C reduction by an aliquot removed at time point *B*.

an oxidizable moiety that is present in micromolar concentrations in whole colostrum, which when completely oxidized by H_2O_2, no longer competes with scopoletin for the H_2O_2 produced either by stimulated PMN or glucose + glucose oxidase. The question arises whether the colostral component that becomes oxidized as cytochrome C is reduced is the same component that is oxidized by H_2O_2. Based on the calculated colostral content of each activity (497 and 918 nmol/ml, respectively) they do not appear to be one and the same. However, as shown in Figure 6, exhaustion of H_2O_2-depleting activity in the glucose-glucose oxidase system is temporally related to loss of the cytochrome C-reducing activity.

The observed effects of colostrum on myeloperoxidase activity can possibly be explained by its H_2O_2-depleting activity. Hydrogen peroxide is the substrate for this enzyme; depletion of exogenously added H_2O_2 would result in less apparent enzyme activity, as was observed in the standard assay. By substitution of a continuous source of H_2O_2 (glucose + glucose oxidase) for exogenously added H_2O_2 and following myeloperoxidase activity continuously, it was seen that the suppressive effect of colostrum resulted from a delay in the onset of myeloperoxidase activity, and not a change in the rate of chromogen production.

To examine whether the antioxidant activities of colostrum might have relevance at the cellular level, we tested the effects of colostrum on PMN mediated HEp-2 cell detachment. HEp-2 cells were used because they are an epithelial cell line commonly used to study enteric microorganism/epithelial cell interactions. Because colostrum would exert its effects at the level of the mucosal surface in the intestine, this cell line was considered more appropriate for examining PMN effects than classically used vascular endothelium. As might be anticipated, PMA-stimulated PMN caused significant detachment of HEp-2 cells; 10% whole colostrum significantly ameliorated this PMN-mediated effect, consistent with its antioxidant capabilities. It is tempting to speculate that such a sparing effect might help protect the neonatal intestine from potentially injurious maternal PMN that are present in the colostrum.

These studies do not chemically identify the colostral component(s) that mediate its antioxidant activity. The concentration of the antioxidant activity at the surface of a 3.5-kDa dialysis membrane at pH 8 suggests the component(s) is larger than 3.5 kDa and that it carries a positive charge at pH 8. In a single experiment (data not shown) using pH 5 buffer for electrocentration, the antioxidant activity did not concentrate, suggesting that the component(s) is either uncharged or unstable at that pH. A number of small antioxidant compounds are reported to be present in human colostrum: ascorbate is present in concentrations of 47–104 mg/liter (267–591 nmol/ml) in cellular, whole colostrum (22); cystine and taurine are present in acellular, aqueous colostrum at concentrations of 25 ± 9 and 413 ± 71 nmol/ml (23); α-tocopherol is present in cellular, whole colostrum at a concentration of about 23 nmol/ml (24). Studies examining the possible roles of each of these components in colostral antioxidant activity are currently underway.

These studies are the first to show that human colostrum creates a chemically reducing environment in which PMN-produced H_2O_2 is not available as an oxidant or a substrate for other PMN enzymatic systems. They also show that colostrum can protect against PMN-mediated cellular injury. Taken together, they support the concept that colostrum is antiinflammatory.

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