Antigen-Driven Immunoglobulin Production by Human Colostral Lymphocytes

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ABSTRACT. It has long been recognized that human milk contains substantial quantities of secretory IgA which is elaborated by plasma cells located proximal to the ductal epithelium. The presence of lymphocytes, granulocytes and macrophages in the milk itself raises the question of whether these "shed" milk cells might potentially serve a function with regard to protecting the suckling neonate from infection. The functionality of milk B-lymphocytes has remained particularly controversial. In this report, we describe the use of a novel technique of milk cell separation prior to cell culture and demonstrate the ability of colostral B-lymphocytes to elaborate immunoglobulin in response to antigenic stimulation using vaccine strain polio virus. (*Pediatr Res* 25:295-299, 1989)

Abbreviations

BM, breast milk
PBMC, peripheral blood mononuclear cells
LeuOME, leucine *O*-methyl ester
PWM, pokeweed mitogen

Although it is well established that human milk secretory IgA is produced locally in the lactating mammary gland, the ability of "shed" colostral B-lymphocytes to produce immunoglobulin *in vitro* has remained a topic of debate (1). Various groups of investigators have reported evidence that these cells will produce IgA under appropriate conditions (2-5), but others have failed to demonstrate functionality using similar methodologies (6-8). It might be hypothesized that these B-cells would not be fully functional on the basis of studies showing that colostral T-cells, natural killer cells, macrophages, and polymorphonuclear cells are generally hyporesponsive when studied *in vitro*. Reversing the argument, if the majority of colostral cell types exhibit some degree of functionality, then it is hypothetically probable that colostral B-cells retain activity. One difficulty encountered in the study of these cells is the abundance of IgA found within milk phagocytic cells, which easily obscures the relatively smaller amount of IgA that might be synthesized *de novo*. We have, therefore, used a novel method for the preparation of reasonably pure colostral lymphocytes and have tested the hypothesis of functionality by determining the ability of colostral B-cells to elaborate Ig *in vitro* in response to inactivated polio virus.

Received April 29, 1988; accepted November 7, 1988.

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Supported in part by NIH Biomedical Research Support Grant 2S07-RR05321. HBS is the recipient of a 1987 Burroughs Wellcome Young Investigator Award, National Foundation for Infectious Diseases. SAS is the recipient of Research Center Development Award 1-K04-CA00896 from the National Cancer Institute.

MATERIALS AND METHODS

Collection of blood and milk samples. After we obtained informed consent, we drew blood samples from a peripheral vein and obtained BM by means of an electric breast pump (Egnell Inc., Cary IL). The milk was immediately diluted 1:1 with sterile physiologic saline and brought to the lab in an ice slurry to prevent macrophage clumping.

Isolation of blood mononuclear cells. PBMC were separated from fresh heparinized (20 U/mL) blood by standard density gradient centrifugation over a solution of sodium metrizoate and Ficoll (Lymphoprep, Nygaard, Oslo, Norway) (9). Briefly, after 2:1 dilution with PBS (pH 7.4), sedimentation was carried out at $300 \times g$ for 30 min at room temperature. Bouyant cells were harvested by aspiration, resuspended in PBS and sedimented at $450 \times g$ for 15 min, and subsequently washed twice more with RPMI 1640 culture medium (RPMI, Grand Island Biological Co., Grand Island, NY). Cells were finally resuspended for counting in RPMI supplemented with 10% heat-inactivated FCS, 80 μ g/mL of gentamicin (Schering Pharmaceutical, Kenilworth, NJ) and 2-mM/mL fresh glutamine (hereafter referred to as complete RPMI).

Preparation of colostral cells. Colostral cells were sedimented at $450 \times g$ for 15 min at room temperature. The cell-free supernate (whey) was partially delipidated using sterile cotton-tipped swabs, then carefully aspirated and stored at -70°C for later processing. Complete RPMI containing 5-mM LeuOME was prepared, adjusted to pH 7.4, and filter sterilized (10). A 10-mL plastic syringe was packed with 1 mL sterile nylon wool, 2 mL glass beads, and 7 mL Sephadex G-10 (Pharmacia Fine Chemicals, Piscataway, NJ), then thoroughly equilibrated with 15 mL LeuOME. The sedimented colostral cells were gently resuspended in 1 mL of LeuOME and allowed to run into the G-10. The column was closed and placed in a 37°C incubator (5% CO_2 , 95% air atmosphere) for 45 min. Cells were eluted with stirring using 10 mL LeuOME. The cells were sedimented as above, resuspended in 2 mL complete RPMI, and carefully layered over a discontinuous gradient of Lymphoprep (sp gr, 1.077) and Ficoll adjusted to sp gr 1.060 in RPMI. Centrifugation was carried out as for blood mononuclear cells. The zone above sp gr 1.060 was discarded, and the midzone down to sp gr 1.077 was collected by aspiration (see Table 1). This lymphocyte-enriched midzone was diluted with PBS, and the cells were sedimented as above. The cells were washed twice by sedimentation in complete RPMI and resuspended in a small vol of the same.

Cell counting. Total nucleated cells were counted using a solution of gentian violet and 2% acetic acid (Türk's solution). Identity and viability of these cells was determined by Wright's stain (Camco Quik-stain, American Hospital Supply, Edison, NJ) α -naphthyl butyrate esterase staining (Technicon Instrument Corp., Tarrytown, NJ), and trypan blue exclusion.

Table 1. Results of colostrum cell separation, showing representative purification of colostrum lymphocytes from other colostrum cells by sequential LeuOME, Sephadex G-10, and discontinuous Ficoll gradient treatment*

	Fraction A (%)	Fraction B (%)
MAb staining		
GAM-FITC	1.0	1.0
M-IgG	0.5	1.0
CD2 positive	4.5	70.9
CD20 positive	1.0	10.0
Wright staining		
Granulocytes	50.0	27.0
Lymphocytes	3.0	71.0
Monocytes	47.0	2.0

* Fraction A is taken from the Ficoll gradient above sp gr 1.060; fraction B is taken from the zone between sp gr 1.060 and 1.078. M-IgG, mouse IgG as first antibody; GAM-FITC, fluorescein-conjugated goat-antimouse second antibody alone.

Cell culture. Syngeneic blood macrophages were used as antigen-presenting cells as follows: PBMC were prepared as 2×10^6 viable lymphocytes/ml of complete RPMI. In a 96-well serocluster plate (Costar, Data Packaging Corp., Cambridge, MA), each of 45 wells was filled with 100 μ L PBMC suspension. After 60 min of incubation at 37°C, 30 of the wells were washed three times with complete RPMI (multichannel pipette) to remove nonadherent cells. Three culture conditions were then established: 15 wells of adherent PBMC (syngeneic macrophages) alone in 100 μ L of RPMI; 15 wells of adherent PBMC (syngeneic macrophages) plus 100 μ L of macrophage-depleted colostrum lymphocytes; and 15 wells still containing 100 μ L each of PBMC suspension. Triton X-100 (100 μ L of 1%) was added at time 0 to triplicate wells of adherent PBMC, adherent PBMC plus colostrum cells, and total PBMC (preculture lysis). To triplicate wells of each culture condition, 100 μ L of one of the following was then added: complete RPMI, PWM at 10 μ g/mL in complete RPMI, sham-polio diluted 1×10^{-6} in complete RPMI, or polio antigen diluted identically. The Triton was instilled, followed by repeated vigorous pipetting, and the lysed cells were stored at -20°C. After a 9-d incubation at 37°C in a 95% air, 5% CO₂ atmosphere, 28 μ L of 4% Triton X-100 were added to each well with vigorous pipetting; the triplicate wells were pooled and stored at -20°C.

Preparation of antigens and mitogens. PWM (Grand Island Biological Co.) was reconstituted in complete RPMI to a concentration of 10 μ L/mL. Virus stocks of polio virus type I Mahoney were prepared at 35°C in a closed culture of African green monkey kidney cells (MA-104, M. A. Bioproducts, Walkersville, MD) in 40 mL of Eagle's minimal essential medium with 2% FCS, 200 U/mL penicillin, 0.4 mg/mL streptomycin, 0.03 mg/mL gentamicin, and 5 μ g/mL fungizone. Noninfected (sham) culture supernate was used in control experiments, and was processed in an identical fashion. Cellular debris was removed from 400 mL of pooled culture supernates by sedimentation at $2600 \times g$ for 20 min. Virus was quantitated before inactivation, with 0.1 mL of infected culture supernate diluted 1:100 000 showing 50% lysis of a monkey kidney cell monolayer, giving a TCID₅₀ of 10^{-5} . The virus-containing and sham culture supernates were inactivated by 30-min exposure to UV A irradiation in an open tray. Inactivation was confirmed by lack of cytopathic effect on reinoculation. Virus was then precipitated using NaCl (2.2 g/100 mL) and solid polyethylene glycol 6000 (10 g/100 mL) (11). The precipitate was collected by sedimentation at $3000 \times g$ for 20 min at 4°C, resuspended in STE buffer (Tris-HCl 0.01 M, NaCl 0.10 M, Na₂EDTA 0.001 M, pH = 7.5), and sonicated (MSE Sonicator, 40 W, 1.5 min). After cesium chloride density

centrifugation (0.5 g/mL) ($35\,000 \times g$ for 48 h at 15°C type Sw41Ti rotor), a single visible band was collected and dialyzed extensively against PBS. Final purification of virus by Sephacryl S-300 superfine molecular sieve chromatography yielded 65 mL of a solution containing 84 μ g/mL of protein ($E_{280}^{1\%} = 16.2$). Noninfected pooled culture supernates underwent identical processing.

Micro-ELISA. Micro-ELISA was used to measure Ig levels in culture supernates as previously described (12), except that each incubation step (coating, sample binding, conjugate binding) was accomplished in 1 h using an orbital shaker in a 37°C warm room. Pooled supernates were assayed in triplicate. Sensitivity was determined to be 5 ng/mL.

Plasma cell staining. Cells were cultured as above for 7 d, then harvested and washed three times in FA buffer (Difco Laboratories, Inc., Detroit, MI). Cytospin preparations were fixed with cold acetone $\times 30$ min, washed with PBS and stained with fluorescein-conjugated goat-antihuman IgG/A/M. Cells were examined by epifluorescence and phase contrast microscopy.

Monoclonal antibody staining. Unseparated colostrum cells were analyzed as previously described (13), using a modification of the whole blood technique of Hoffman and Hansen (14). Separated cells from each zone of the final density gradient were washed three times in FA buffer and resuspended at 1×10^6 /mL. A total of 50 μ L of cell suspension was mixed with 50 μ L of reconstituted (1:40) T11/CD2 (pan T-cell marker), B4/CD20 (pan B-cell marker), mouse-antihuman glomerular basement membrane or FA buffer. After 30 min of incubation on ice, the cells were washed twice and similarly reacted with fluorescein conjugated goat-antimouse antibody. Cells were examined by epifluorescence and phase contrast microscopy. Unseparated colostrum cells were similarly stained and examined by flow microfluorometry on a Coulter Epics-V cytometer (Coulter Electronics, Inc., Hialeah, FL).

Statistical analysis. The results of isotype production as measured in ng/mL in replicate (ELISA) were analyzed by paired *T* test. The validity of the test was confirmed by *F* test which showed equivalent variance in the samples being compared. All *p* values shown represent 2-tailed significance.

RESULTS

To determine that appropriate lymphocyte subpopulations were present to support Ig synthesis, colostrum cells were first studied by flow microfluorometry. In 10 samples (collected d 1–5 postpartum), the following composite phenotype was detected: CD20+ 5.3% $\pm$ 2.7; CD2+ 12% $\pm$ 10; CD4+ 8.3% $\pm$ 6.3; and CD8+ 7.1% $\pm$ 3.4. The mean T4/T8 ratio was 1.3 ± 0.7 (2 SD). These frequencies are in agreement with other published reports (15).

As it has been reported that PWM fails to stimulate Ig synthesis by colostrum B-cells, inactivated polio virus was chosen for testing on the assumption that a small proportion of migratory, gut-derived colostrum B-cells would retain immunological memory for this enteric antigen. Polio antigen was prepared as described and added to cultures of normal donor peripheral blood lymphocytes to characterize the response. The responses of six donors to serial dilutions of the antigen are illustrated in Figure 1. There were no differences in Ig production between wells containing RPMI and wells containing sham polio (IgA *p* = 0.6, IgG *p* = 0.6, IgM *p* = 0.9). All donors showed normal boosting of IgG, IgM, and IgA production in response to PWM (data not shown). The responses to polio were variable and mainly limited to the IgG isotype consistent with an antigenic (not mitogenic) response. The results of a seventh donor were removed from the pool because of failure to respond to PWM. It was later learned that this young woman had hyperprolactinemia. Initial colostrum cell culture experiments were performed using unseparated cells, prepared by sedimentation of colostrum at $300 \times g$ over a

Fig. 1. Polio antigen stimulation of PBMC. PBMC from six control (male or nulliparous female) donors were cultured for 9 d in the presence of RPMI alone, or with optimal concentration of PWM, sham-polio (diluted 1×10^{-5}), or polio antigen (shown on X-axis, exponential concentration as diluted serially from stock). Pooled culture supernates were assayed in triplicate by ELISA for concentration of total IgA, IgG, and IgM. Typical polyclonal activation was seen in response to PWM with several 100-fold elevation of IgG and IgM over RPMI alone, with no significant difference between RPMI and sham-polio (S) stimulation in any isotype (data not shown). Paired T test analysis of the response to sham-polio *versus* peak response to polio revealed significant response to polio in IgG ($p = 0.01$) consistent with an anamnestic response.

cushion of FCS to remove debris. This technique was chosen over Ficoll-Hypaque as the latter is associated with poorly controlled separations resulting from the altered buoyant density of lipid-laden colostrous leukocytes. Cells were then cultured for 7 d at 37°C (5% CO₂, 95% humidified air) in RPMI 1640 alone, or with added PWM or polio antigen. No difference in postculture supernatant IgA concentration was seen between PWM-stimulated and RPMI control cultures. However, using 1% vol/vol (final concentration) Triton X-100 to lyse aliquots of cells pre- and postculture, it was seen that postculture supernatant IgA concentrations exceeded preculture concentrations in 13 of 14 cases ($p < 0.001$).

There was a stronger suggestion of *de novo* IgA synthesis in response to polio antigen when lysates of prelysed controls were compared with supernate + lysates of antigen-stimulated cells ($p < 0.001$). However, pre- and postculture group means were not significantly different due to the release of large quantities of preformed IgA. Although the preculture Triton-lysed control allowed for measurement of this parameter, substantial dilution of samples was required to achieve linearity in the absorbance readings by ELISA, leading to unacceptable variance in replicate determinations. To solve this problem, several approaches were tested for the preculture removal of IgA-containing phagocytic cells.

Sephadex G-10 column adsorption alone was inadequate for removal of these cells to any substantial degree, consistent with previous reports (16). Discontinuous Percoll gradients appeared to offer some advantages except in cases where large numbers of lipid-laden granulocytes were present. Therefore, a new technique of cell separation was developed based on the lethal effects of lysosomotropic L-amino acid esters on peripheral blood macrophages (10). Unlike the rapid effects on peripheral blood cells, incubation of washed whole colostrous cell preparations in 5-mM LeuOME for periods of up to 6 h failed to remove macrophages completely as judged by Wright-stained cytocentrifuge preparations. However, by using LeuOME, Sephadex G-10, and a discontinuous Ficoll gradient in sequential steps it was possible to

achieve 60–95% purity of colostrous lymphocytes, with macrophage contamination reduced to <5%. The adequacy of this technique was confirmed by MAb staining of the isolated cell fraction (Table 1).

Colostrous lymphocytes were isolated from 10 postpartum donors by this method. Six of these had adequate numbers of cells, which were prepared at 1×10^6 viable lymphocytes/mL in culture as described in "Materials and Methods." In these cultures, the background signal (preformed IgA released at time 0 by Triton lysis) was reduced by 10- to 50-fold compared with cultures of unseparated milk cells, thus reducing variance in the ELISA. No Ig was detected in the prelysed or postculture syngeneic adherent-cell only wells, confirming that Ig production in the syngeneic adherent cell + colostrous lymphocyte wells was not a result of contaminating peripheral blood lymphocytes. The wells containing postpartum syngeneic PBMC showed constitutive production of IgA, IgG, and IgM that was not enhanced by PWM, but was to a variable extent by polio. The latter did not achieve statistical significance. In five of six cases, milk lymphocytes cultured in RPMI or sham-polio alone produced IgA with a mean increase of 8 859 ng/mL over prelysis background (range, 709–21 465 ng/mL; $p = 0.01$). In four of six cultures with RPMI or sham-polio alone, IgM production was noted with a mean increase over prelysis background of 2 674 ng/mL (range, 27–10 761 ng/mL; $p < 0.05$). No IgG was detected. In four of six cultures, polio antigen stimulation was associated with a further boosting of IgA levels in the postculture supernates as compared to sham-polio stimulation, with a mean increase of 5 605 ng/mL (range, 346 – 11 723 ng/mL; $p < 0.01$). The results of a representative experiment are illustrated in Figure 2.

Wright-Giemsa staining of colostrous cell preparations before culture has revealed an abundance of cells with typical morphologic appearance of plasma cells. Several attempts were made to identify plasma cells in postculture cell preparations after stimulation with polio antigen. Of five samples examined, none contained cells with typical intracytoplasmic staining by fluorescein-labeled anti-Ig.

Fig. 2. Representative results of IgA and IgM production by colostral B-cells from a single donor. Colostral lymphocytes, isolated and depleted of macrophages as described, were cultured in 96-well round-bottom plates precoated with adherent syngeneic PBMC. Pooled supernates from triplicate wells of each condition illustrated (lysis with Triton X-100 at time 0 or after 9 d of culture) were assayed for total IgA, IgM, and IgG by ELISA. Cells lysed at time 0 contained preformed IgA and IgM. Cells cultured with RPMI, sham-polio, or polio antigen produced equivalent amounts of IgM, which significantly exceeded the prelyse control ($p < 0.05$). Cells cultured with RPMI or sham-polio produced significant amounts of IgA compared with the prelyse control ($p = 0.01$). Culture with polio antigen was associated with a significant increase in IgA production compared with culture in RPMI or sham-polio ($p < 0.01$). IgG production could not be detected. Control, adherent cell-only wells produced no Ig. Error bars represent 1 SD.

DISCUSSION

The present communication describes the use of a novel technique for the separation of BM lymphocytes, and the *in vitro* response of milk B-cells to polio antigen. Our findings of IgA and IgM production by cultured milk B-cells support the hypothesis that these cells retain some degree of functional activity after being shed into the milk.

The results are supported by various independent reports. Specifically, human colostral cells cultured in the presence of ^{14}C -labeled amino acids have been noted to elaborate IgA as detected by immunoelectrophoresis and by autoradiography (2). Negative autoradiographic results were obtained in these experiments using cell-free colostrum, frozen and thawed colostrum, and colostral cells cultured with puromycin or actinomycin D. Short term culture of colostral cells has also revealed increasing amounts of IgA, free of secretory component, in culture supernates plus cell lysates assayed at 24, 72, and 120 h (3). Ahlstedt et al. (4) showed 80% inhibition of IgA plaques from colostral cells by addition of 10 $\mu\text{g}/\text{mL}$ puromycin. Finally, stable clones of IgA-producing B-lymphocytes have been developed by EBV transformation of colostral lymphocytes (5).

It has been suggested that they are senescent cells, based on their failure to respond to PWM as a polyclonal B-cell activator (6). This conclusion does not take into consideration the possibility that BM B-cells may be a unique population that is selectively unresponsive to pokeweed (17). In this regard, it has been shown that milk macrophages bind phytohemagglutinin but not PWM (18) and that they express unique surface markers (19). Furthermore, our results indicate that even peripheral blood lymphocytes obtained during the 1st wk postpartum fail to respond to polyclonal B-cell activation by PWM. Taken together with our findings of a single "normal" control with hyperprolactinemia and failure to respond to PWM, this raises the possibility that increased prolactin levels associated with lactation may influence the colostral B-cell PWM response. Indeed, prolactin has been shown to compete with cyclosporine for a common binding site on murine T-lymphocytes, and can reverse cyclosporine-induced immunosuppression (20). Related findings support a role for prolactin in the maintenance of T-cell immunocompetence (21).

In some cases, the absence of demonstrable BM B-cell function may be related to methodologic approach. Separation of lym-

phocytes from other milk cells is a difficult task, and the use of unseparated cells risks the release from accessory cells of pre-formed IgA, and possibly suppressive substances (22), into the culture system. Early work from our lab suggests that BM B-cells may be regulated in a unique fashion by the products they elaborate (23). However, these earlier studies were hindered by the methodologic problem of isolating milk lymphocytes from milk phagocytes which contain large quantities of preformed IgA. This problem has been overcome through the use of LeuOME combined with specifically designed, discontinuous density gradient centrifugation. LeuOME is a lysosomotropic compound that is rapidly metabolized to free leucine after passive diffusion into the lysosomes of phagocytic cells. Leucine, a more polar compound than LeuOME, diffuses only slowly out of the lysosomes, with accumulation leading to osmotic rupture and subsequent death of the cell via autodigestion (10). LeuOME abolishes natural killer cell function and T-cell mitogen responsiveness through selective destruction of macrophages. In general, lymphocytes retain >95% viability after treatment with LeuOME (24).

In preliminary studies using optimal concentrations of LeuOME alone to pretreat milk cells, it was found that only 50% of esterase-positive cells were destroyed even with prolonged (6-h) incubations. Peripheral blood esterase-positive cells were effectively eliminated after 45 min. It is possible that this differential susceptibility is related to a protective effect mediated by the large quantities of lipid in the lysosomes of BM phagocytes. To improve on this technique, LeuOME-treated BM cells were incubated in a Sepharose G-10 column, and the eluted cells subsequently separated by discontinuous Ficoll density gradient centrifugation with sp gr zones designed to isolate lymphocytes (1.060 – 1.078 sp gr). Analysis of the cell distributions in the various layers after centrifugation, using standard Wright-giemsa and fluorescein-labeled MAb staining, revealed 60–90% purification of BM lymphocytes in the density band between 1.060 and 1.078. The pellet with sp gr 1.078 consists of polymorphonuclear neutrophils, G-10 granules, subcellular debris, and occasional lymphocytes.

The choice of appropriate stimuli for colostral B-cells appears to be of importance. BM cells are thought to be mature, having been exposed in gut-associated lymphoid tissue to specific enteric antigens before IgA isotype switching and migration to the breast. Studies in which *E. coli*-derived antigens are ingested by lactating

women demonstrate the subsequent appearance of antigen-specific IgA-class milk antibodies (25). Similar findings have been reported after oral immunization with polio vaccine (26). These studies provided the rationale for our use of vaccine strain polio virus antigen as a stimulus for IgA production by isolated colostrum lymphocytes. The observed synthesis of Ig in RPMI alone and in four of six cases the synthesis of IgA in response to polio antigen suggests that B-cells are shed from the breast at various stages of differentiation. Consistent with this hypothesis are the observations of both plasmablasts and medium-sized lymphocytes staining with CD20 MAb in the preculture samples. The ability of milk B-cells to respond to polio antigen in a 7-d culture implies that these are primed B-cells that undergo terminal differentiation, and further implies the presence of lymphokines such as IL-5 (27) and IL-6 (28) in the culture milieu. Our findings support the hypothesis that *in vitro* synthesis of IgA and IgM does occur in properly designed colostrum cell cultures. The only alternative explanation would be that preformed molecules are reduced to monomeric form during the culture period, and that this degradation is enhanced by polio antigen. This is unlikely, but can readily be tested. Studies are in progress to determine whether the observed response to polio is antigen specific.

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