Clin. exp. Immunol. (1979) 38, 585–597.

Human colostral cells

I. SEPARATION AND CHARACTERIZATION

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(*Accepted for publication* 17 *April* 1979)

SUMMARY

Analyses of the cells present in human colostrum obtained from fifty-four healthy donors during the first four days of lactation revealed that there were $3{\cdot}3 \times 10^6$ (range $1{\cdot}1 \times 10^5$–$1{\cdot}2 \times 10^7$) cells per ml of colostrum. Based on histochemical examinations, it was found that this population consisted of 30–47% macrophages, 40–60% polymorphonuclear leucocytes, 5·2–8·9% lymphocytes, and 1·3–2·8% colostral corpuscles; epithelial cells were rarely encountered. The identity of various cell types was confirmed by Wright's stain and by a series of histochemical techniques which disclosed the presence of non-specific esterase, peroxidase, and lipids. For further characterization, the different types of cells were separated by various methods, such as Ficoll-Hypaque density centrifugation, isokinetic centrifugation on a linear Ficoll gradient, adherence to glass or plastic, and phagocytosis of carbonyl iron. Immunohistochemical staining with FITC- and/or TRITC-labelled reagents to IgA, IgM, IgG, κ- and λ-chains, secretory component, lactoferrin, and α-lactalbumin were applied to unseparated as well as separated colostral cells. Polymorphonuclear leucocytes (staining for peroxidase) as well as macrophages and colostral corpuscles (staining for non-specific esterase) exhibited numerous intracellular vesicles that contained lipids as well as various combinations of milk proteins. Lymphoid cells did not stain with any of these reagents and plasma cells were not detected among the colostral cells. Individual phagocytic cells contained immunoglobulins of the IgA and IgM classes, both κ and λ light chains, secretory component, lactoferrin, and α-lactalbumin. The coincidental appearance of these proteins in single, phagocytic cells but not in lymphoid cells indicate that the cells acquired these proteins by ingestion from the environment. Markers commonly used for the identification of B lymphocytes (surface immunoglobulins) and T lymphocytes (receptors for sheep red blood cells) were unreliable for the analysis of colostral cells (unless accompanied by subsequent morphological characterization) because strong fluorescence was observed on the surface of many non-lymphoid cells and because numerous macrophages and colostral corpuscles formed rosettes with sheep red blood cells (SRBC). Lymphocytes, often found in association with colostral macrophages or corpuscles, were classified as T cells.

INTRODUCTION

Substantial evidence indicates that the ingestion of colostrum and milk may provide passive protection to the infant (Hanson & Winberg, 1972; Goldman & Smith, 1973; Welsh & May, 1979). In addition to humoral defence factors such as immunoglobulins (Ig), lactoferrin and lysozyme, viable cells have been detected in colostrum and milk by several investigators (Smith & Goldman, 1968; Lascelles, Gurner &

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Coombs, 1969; Parmely & Beer, 1977). Among these cells macrophages, colostral corpuscles of Donnè, (probably macrophages laden with fat, which are also termed foamy macrophages), PMN, lymphocytes, and epithelial cells have been detected. Both T and B cells have been identified in the lymphocyte population (Diaz-Jouanen & Williams, 1974; Parmely, Beer & Billingham, 1976; Parmely *et al.*, 1977).

The function of colostral cells is only partly understood. It has been suggested that colostral T lymphocytes or their products may confer cell-mediated immunity to the infant (Mohr, 1973; Parmely *et al.*, 1977; Ogra, Weintraub & Ogra, 1977; Schlesinger & Covelli, 1977; Ogra, Weintraub & Ogra, 1978). B lymphocytes and their progeny have been implicated in the production of IgA which is the predominant immunoglobulin in milk (Murillo & Goldman, 1970). Furthermore, it has been suggested that the specificity of these IgA-associated antibodies reflects the spectrum of antigens to which lymphoid cells in the gastrointestinal and respiratory tracts of the mother have been exposed (Ahlstedt *et al.*, 1975; Goldblum *et al.*, 1975). The detection in colostrum of cells that actively produce IgA varies with the findings of Pittard, Polmar, & Fanaroff (1977). Although these authors confirmed the release of IgA from cells present in colostrum, they propose that this immunoglobulin originates from colostral macrophages which serve as passive vehicles for IgA transport and release. Active synthesis of IgA by lymphoid cells was not observed. This variance may be partly explained by the heterogeneity of colostral cells and by difficulties encountered during the separation and characterization of these cells.

In the studies reported here, we separated colostral cells by modifications of centrifugation on Ficoll gradients and by the ability of colostral cells to adhere to different surfaces. Unseparated and separated cell populations were subsequently characterized by histochemical and immunohistochemical criteria. Particular attention was given to characterization of immunoglobulin-containing cells in colostrum.

MATERIALS AND METHODS

Collection of milk. Milk was collected by manual expression from the first to fourth day post-partum from donors who had no apparent signs of mastitis. Milk or colostrum was expressed into sterile conical tubes and held on ice. Samples were processed within 1 hr of collection.

Milk samples were diluted 1:2 with Dulbecco's PBS and spun at 400 *g* for 10 min. The cell pellet was washed twice in PBS and resuspended either in PBS with 1% BSA or in RPMI 1640 (Gibco Company, Grand Island, New York) with 20% heat inactivated foetal bovine serum (FBS) (Reheis Chemical Company, Kankakee, Illinois), 2 mM L-glutamine (Gibco), 100 μg/ml penicillin and 100 u/ml Streptomycin (Gibco).

Cell separation techniques. The cellular components of milk were separated by one of the following techniques: (1) Ficoll-Hypaque density centrifugation, (2) isokinetic centrifugation, (3) adherence to glass, (4) filtration through glass wool columns or (5) ingestion of carbonyl iron.

(1) Ficoll-Hypaque separations were performed by a modification of Böyum's method (1968). Washed colostral cells ($1{\cdot}0$–$4{\cdot}0 \times 10^7$) were resuspended in PBS and gently layered on 3 ml of Ficoll-Hypaque (density 1·078) in a 15 ml plastic culture tube. These preparations were spun at room temperature at 400 *g* for 40 min. After centrifugation the buffy interface was removed and washed for further processing.

(2) Isokinetic centrifugation was performed according to the method of Pretlow (1971). Colostral cells were adjusted to a concentration of $1{\cdot}0$–$4{\cdot}0 \times 10^7$ cells in 7 ml of PBS with 10% BSA and then gently layered onto a gradient consisting of Ficoll in Joklik's tissue culture medium. The gradient ranged from 2·7% Ficoll at the sample-gradient interface to 5·5% at the gradient-cushion interface. The gradients were centrifuged at 97 *g* (800 r.p.m.) at 2–4°C for 16 min. Four ml fractions were collected from the top by displacement with sucrose, using the tapping cap previously described (Pretlow, Weir & Zettergren, 1975). In order to assure linearity of the gradient, the refractive index of each fraction was checked. Washed cells were resuspended in PBS with 1% BSA and 0·03% azide (PBS/Azide) and cytocentrifuge preparations were made for histochemical or immunochemical characterization.

(3) Removal of adherent cells by their adherence to plastic and glass surfaces. To remove adherent cells, washed colostral cells were resuspended in complete RPMI 1640 and allowed to adhere to glass or petri plates for 2 hr at 37°C in 5% CO_2. The non-adherent cells were carefully decanted and the adherent cells were removed by gentle agitation with a Pasteur pipette. After washing, adherent and non-adherent cells were resuspended in PBS/Azide, and cytocentrifuge slides were made.

(4) Attempts were also made to remove adherent cells by filtering colostral cells through glass wool columns (McGhee *et al.*, 1979). Washed colostral cells ($1{\cdot}0$–$5{\cdot}0 \times 10^7$) were resuspended in Eagle's MEM with 2% FBS and then applied to a well washed 7 ml glass wool column packed into a 12 ml plastic syringe. The non-adherent cells were washed through with 25–50 ml of MEM+2% FBS, washed in PBS, and resuspended in PBS/Azide for cytocentrifuge preparation.

(5) Separation of phagocytic cells. To remove phagocytic cells, washed colostral cells were resuspended in complete RPMI

1640 and incubated with carbonyl iron at 37°C for 15 min with gentle agitation. Cells that ingested iron were removed by three passages over a magnet.

Separated and unseparated preparations were examined histochemically and immunochemically to determine cell purity and to identify cell types.

Histochemical techniques. Differential cell counts were determined on slides stained with Wright's stain. Cells were differentiated as macrophages, colostral corpuscles, epithelial cells, PMN and lymphocytes. The irregular shape of many colostral cells could have resulted in an imprecise or erroneous morphological classification, and therefore additional histochemical markers were used. The presence of peroxidase granules (Wintrobe, 1962) was used to further identify PMN, and non-specific esterase (Yam, Li & Crosby, 1971) was used as a marker for macrophages and colostral corpuscles. Sudan Black and Oil Red O stains were used to detect the presence of lipids, and methyl green-pyronine stains were used to detect plasma cells. The procedures described in the second edition of the *Manual of Histologic and Special Staining Techniques* of the Armed Forces Academy of Pathology (1960) were used. At least 200 cells were counted and results were expressed as percentage of the total.

Reagents. Polyvalent antisera to human IgG+IgM+IgA and monospecific antisera to IgG, IgM and IgA (specific for heavy [H] chain), and to κ chains was purchased from Behring Diagnostics, (Calbiochem Company, San Diego, California). Monospecific anti-IgG, anti-IgA, and anti-IgM reagents were also prepared in this laboratory as described in detail elsewhere (Crago & Mestecky, 1979). Anti-secretory component (SC) reagents were prepared by immunizing rabbits or a goat with (1) SC purified from the milk of a hypogammaglobulinaemic mother, (2) SC chemically cleaved from secretory IgA or, (3) by adsorption of anti-secretory IgA serum with IgA myeloma proteins. Details of these procedures and controls of specificity were described (Crago & Mestecky, 1979). Anti-α-lactalbumin was produced by immunizing rabbits with α-lactalbumin purified from human milk by the method of Brodbeck *et al.* (1967). Methods for the purification of lactoferrin and for the production of anti-lactoferrin antisera have also been described (Cole *et al.*, 1976). Details of the preparation and fluorochrome labelling of γ-globulin fractions and $F(ab')_2$ fragments of these antisera were described by Winchester (1976).

Immunochemical techniques. Colostral cells were examined for the presence of intracellular Ig by staining with fluorescein isothiocyanate (FITC)-labelled antisera of polyvalent Ig or monospecific antisera to IgA, IgM, or IgG. Cell preparations were also stained with tetramethylrhodamine isothiocyanate (TRITC)-labelled anti-IgA or IgM, anti-SC, anti-lactoferrin, or anti-α-lactalbumin. To determine the type of light chain (L chain) present, cells were stained with antisera to κ-(FITC-labelled) or λ-(TRITC-labelled) type L chains. Cell preparations were examined for the coincident appearance of these markers by double staining with various combinations of FITC-labelled anti-Ig, anti-IgG, -IgM, and -IgA or anti-κ chain reagents, and with TRITC-labelled anti-SC, anti-lactoferrin, anti-α-lactalbumin, anti-IgA or anti-λ chain reagents. TRITC-labelled anti-λ chain reagent was a gift from Dr W. Hijmans, TNO, Rijswijk, The Netherlands. Details of the methods for intracellular and surface fluorescence staining have been previously described (Crago & Mestecky, 1979).

To determine the proportion of B and T lymphocytes in human colostrum, cell preparations were incubated with neuraminidase-treated SRBC (N-SRBC) (Hoffman & Kunkel, 1976) for T cell enumeration. A TRITC-labelled $F(ab')_2$ fragment of unabsorbed anti-IgG which also detected L chains on IgA and IgM molecules was used to detect the presence of surface Ig. For T cell determinations, at least 200 white cells were counted, and results were expressed as a percentage of the total number of cells that formed rosettes. A cell which had three or more N-SRBC attached was counted as a rosette. To allow morphological identification of rosetted cells, cytocentrifuge slides of rosette preparations were also stained with Wright's stain.

Fluorescence microscopy. Cells stained with fluorochrome-labelled reagents were examined with a fluorescence microscope (Orthoplan, Leitz Company, Wetzlar, West Germany) equipped with a vertical illuminator (Ploempak 2, Leitz). A super pressure mercury lamp (HBO 100W/2, Osram, West Germany) and a high pressure xenon lamp (XBO 75W/2, Osram, West Germany) were used as light sources for optimal TRITC or FITC excitation, respectively. Photographs were taken with a Leitz camera (Orthomat) on Kodak Ektachrome 400 film developed at ASA 800.

RESULTS

Variability in the numbers and types of colostral cells

Colostrum (day 1 to day 4 post-partum) was collected from fifty-four patients, cells were counted and cell types were determined on the basis of their morphological appearance and histochemical and immunohistochemical staining. Macrophages, PMN, lymphocytes, colostral corpuscles and epithelial cells were detected. Colostral corpuscles were not grouped with macrophages because of their morphological appearance (small, dense and with a centrally located nucleus, surrounded by a large, foamy cytoplasm). Epithelial cells were encountered rarely and were not included in statistical analysis (Table 1). Cell counts were expressed as the number of cells per ml of milk collected. A great variability in the total numbers of cells and distribution of various cell types was observed not only among individuals, but also in single patients studied longitudinally. During the first four days post-partum, colostrum contained $3{\cdot}3 \times 10^6$ cells/ml (a mean value of fifty-four patients for the first four days post-partum) with a range of

TABLE 1. Daily variations in the cells from human colostrum

	Day 1 Post-partum					Day 2 Post-partum					Day 3 Post-partum					Day 4 Post-partum				
Patient	Number of cells $\times 10^6$	*L	M	PMN	CC	Number of cells $\times 10^6$	L	M	PMN	CC	Number of cells $\times 10^6$	L	M	PMN	CC	Number of cells $\times 10^6$	L	M	PMN	C
†1	2·6	‡23·0	43·0	34·0	0·0						1·3	7·5	69·0	22·5	1·0					
2	2·5	15·5	41·5	34·5	8·5	6·7	1·5	24·5	71·0	2·5										
3																1·3	8·0	25·0	64·5	2·5
4						7·7	16·0	37·5	46·5	0·0	1·3	4·5	36·5	48·0	10·5					
5						0·6	24·0	57·5	12·5	6·0	0·11	12·5	59·5	27·0	0·5	0·7	14·0	42·5	43·0	0·0
6	9·4	3·0	15·5	81·5	0·0	5·0	2·0	18·5	79·5	0·0										
7						3·9	0·0	12·0	88·0	0·0	3·5	0·0	3·5	96·5	0·0					
8											2·0	1·0	53·0	37·5	8·0	0·6	4·0	77·0	17·0	7·0
9											1·4	2·0	27·5	70·5	0·0	1·5	3·0	40·5	54·5	2·0
10											4·3	6·0	16·0	75·5	2·5	0·8	0·5	15·0	82·5	4·0
11						11·6	1·0	7·0	89·5	2·5	8·6	1·5	14·5	83·0	1·0					
12	6·9	4·0	34·0	59·5	2·5		4·0	34·0	62·0	0·0	2·4	17·5	59·0	70·0	3·5					
13	1·6	9·5	32·5	58·0	0·0															
§Mean value	3·5	8·9	47·8	40·0	2·8	5·1	6·2	30·7	61·3	1·8	3·1	5·2	32·2	60·6	1·9	1·6	6·7	37·6	54·4	1·3

* L = Lymphocyte, M = macrophage, PMN = polymorphonuclear leucocyte, CC = colostral corpuscle.
† Patients 1–13 are representative of fifty-four patients examined.
‡ Numbers represent percent of 200 cells counted.
§ Values represent mean of fifty-four patients.

$1{\cdot}1\times10^5$–$1{\cdot}2\times10^7$. Statistical analyses were performed to determine whether a significant increase or decrease in total cell numbers occurred during this period within the group of fifty-four patients. Had all patients been followed for the full four days, the analyses would have been that for a complete randomized block design. However, due to the unavailability of milk samples, counts were not obtained for every patient for each day of the study. The resulting imbalance required obtaining appropriate tests by the method of least squares utilizing a standard statistical computer package (Barr *et al.*, 1976). Despite great variability in cell counts, analysis revealed no significant increase or decrease in total number of cells/ml of colostrum when a day-to-day comparison was made ($P = 0{\cdot}3$). However, analysis indicated a slight downward trend in cell counts ($P = 0{\cdot}09$) during the period of study.

Macrophages and PMN were the predominant cell types present (Table 1); the mean value of total cells (days 1–4) scored as macrophages ranged from 30·0–47·0%, for PMN from 40·0–60·0%, for lymphocytes from 5·2–8·9%, and for colostral corpuscles from 1·3–2·8% of the total cells in colostrum. Differential cell counts were subjected to the same statistical analysis used above. There was no significant difference in the number of macrophages, colostral corpuscles or PMN during the first four days postpartum. While there was no significant difference in the number of lymphocytes present from day to day, analysis of the linear trend again showed a slight downward trend ($P = 0{\cdot}1$) in the number of lymphocytes present during day 1–4 of lactation.

Histochemical characterization

Due to the unusual morphological appearance of macrophages and PMN (see below) probably caused by the presence of intracellular lipid, additional histochemical staining was performed to further characterize the cells. Different morphological characteristics were noted between colostral macrophages and PMN, as compared to the same cell types in peripheral blood. Numerous vacuoles seen in colostral cells often distorted the shape of the cell. Therefore, additional histochemical staining procedures were used to further verify cell identity. Oil Red O and Sudan Black stains showed large numbers of lipid-containing vacuoles in macrophages, PMN and colostral corpuscles; lymphoid cells did not react with either of the lipid stains. Many extracellular particles also stained for lipids and these were present in the cell suspensions despite extensive washing. Macrophages and colostral corpuscles contained non-specific esterase and this enzyme was occasionally observed in PMN but never detected in lymphoid cells. Peroxidase-containing granules were detected in PMN. Only rarely were these granules detected in macrophages and colostral corpuscles, but never in lymphoid cells. Lymphocytes appeared morphologically identical to those in peripheral blood. Both small and large lymphocytes were seen with a pronounced preponderance of the former type. We were unable to detect cells that corresponded morphologically to plasma cells when Wright's stain or methyl-green pyronine were used.

Separation techniques

In our initial attempts to separate the cells in colostrum, a Ficoll-Hypaque density gradient analogous to that employed for the separation of mononuclear cells from peripheral blood, was used. Although this procedure depleted the granulocyte population, the lymphocytes were still contaminated with large numbers of macrophages and colostral corpuscles. In attempts to remove adherent cells, washed colostral cells were allowed to adhere to glass or plastic petri plates for 2 hr at 37°C in 5% CO_2, and the non-adherent cells were subjected to the same Ficoll-Hypaque centrifugation. This procedure did not appreciably increase the purity of the cells since many macrophages and colostral corpuscles did not adhere. The removal of phagocytic cells by ingestion of carbonyl iron was equally ineffective because many of these cells did not ingest the iron.

Because it appeared that the intracellular lipid would not allow a separation based on differences in density, we turned to the isokinetic gradient. This gradient is designed so that cells sediment at constant velocities as functions of their diameters and densities (Pretlow, 1971). This gradient separated colostral cells into three main areas (Fig. 1, Table 2). The top of the gradient (fractions 1–6) contained debris and very few intact cells. Lymphocytes were found in fractions 9–14 with a population 60–90% pure. The next zone of the gradient contained macrophages and colostral corpuscles (49–58% pure) with

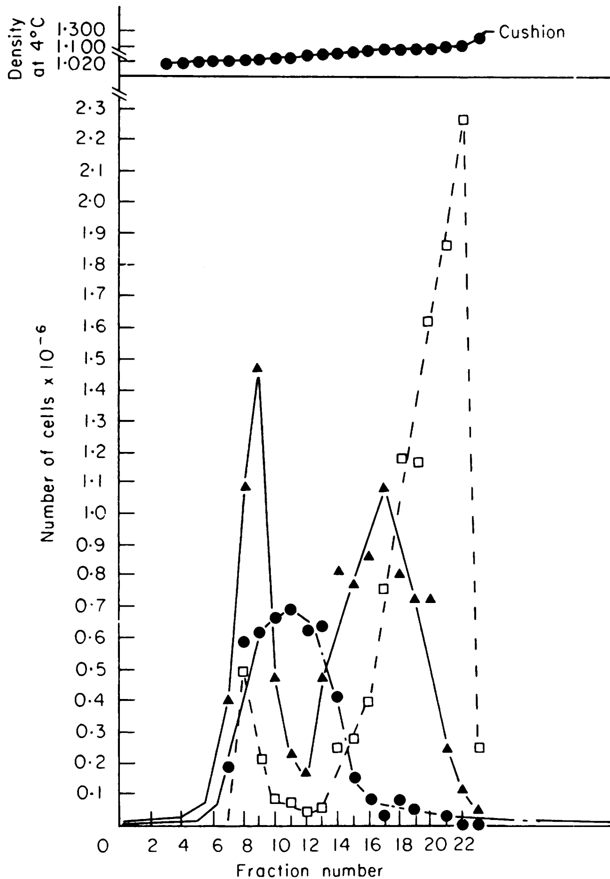


FIG. 1. Distribution of cell types separated from human colostrum by isokinetic centrifugation on a linear Ficoll gradient. Type of cells in each fraction, was determined by histochemical criteria (Wright's stain, non-specific esterase, peroxidase, and lipid stains see Fig. 2b–d and Fig. 3h). The cells were also stained with fluorochrome-labelled reagents to various milk proteins (see results). (●) Lymphocytes, (▲) macrophages, (□) PMN.

a diphasic peak (fractions 7–10 and fractions 14–20). PMN were spread throughout the bottom half of the gradient, but were concentrated in fractions 19–22 (purity ranging from 50%–90%). Photographs of representative fields of cell types purified by isokinetic centrifugations are shown in Fig. 2b–d. The histochemical analyses described above confirmed the identity of cells in various fractions. It was not possible to separate macrophages and colostral corpuscles. Due to the tendency of cells from colostrum to aggregate, many cell clumps were found at the bottom of the gradient and were not included in the total. This method gave a recovery ranging from 60–80%, if aggregated cells were disregarded. Because of the variability in cell numbers and characteristics among individuals examined, the purity of the isolation was not always reproducible.

Isolation of lymphocytes and adherent cells from colostrum was also accomplished by fractionation through glass wool columns in conjunction with adherence to glass or plastic. Washed colostral cells suspended in complete media were first incubated in petri plates for two hr and subsequently the non-adherent cells were carefully decanted. Glass wool columns were used to further deplete the lymphocytes of adherent cells. The recovery of cells from these columns averaged 6·8% of the initial cell number and gave an 8–10 fold increase in purity of lymphocytes. This method had the advantage that cells were easily recovered and remained 90–98% viable.

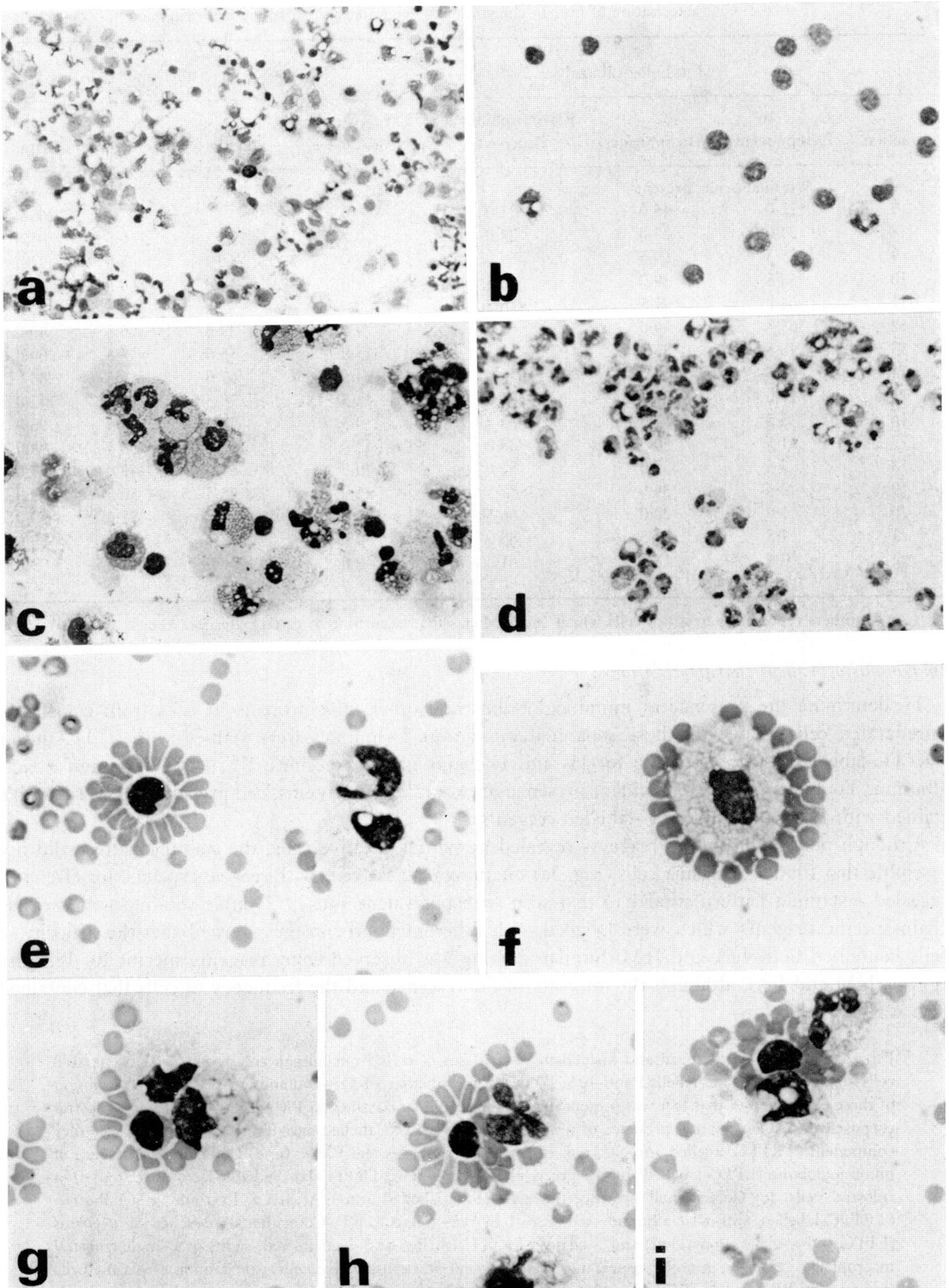


FIG. 2. Cells from human colostrum (stained with Wright's stain): (a) unseparated cells; (b) lymphocyte-enriched fraction from isokinetic gradient (fraction 11, see Fig. 1); (c) macrophage and colostral corpuscle-enriched fractions from isokinetic gradient (fraction 16, see Fig. 1); (d) PMN-enriched fraction from isokinetic gradient (fraction 21, see Fig. 1); (e) colostral lymphocyte forming rosette with N-SRBC; (f) colostral corpuscle forming rosette with N-SRBC; (g, h, i) colostral lymphocytes interaction with macrophages and colostral corpuscles. The lymphocytes were identified as T cells by their ability to form rosettes with N-SRBC.

TABLE 2. Characterization of human colostral cells separated by isokinetic centrifugation

	Morphological analyses				Histochemical analyses		
Fraction	Lymphocytes	Macrophages	Polymorphonuclear leucocytes	Colostral corpuscles	Esterase	Peroxidase	Lipid
1–6	No intact cells present						
7	*22·0	45·0	25·0	8·0			60·0
8	27·0	23·0	23·0	0·0	72·5	16·5	85·0
9	25·5	60·5	9·0	5·0	72·0	7·0	75·0
10	53·5	39·0	6·5	1·0	48·0	6·5	50·0
11	64·0	21·5	6·5	8·0	33·0	4·5	32·0
12	67·5	19·0	4·5	9·0	37·0	6·5	40·0
13	48·5	36·0	4·5	11·0	55·0	5·5	60·0
14	26·5	53·0	16·0	4·5	67·0	16·5	80·0
15	11·0	56·5	21·0	11·5	79·5	11·5	92·0
16	5·5	57·5	26·5	10·5	70·5	25·5	96·0
17	1·5	50·5	35·0	13·0	61·5	38·5	98·0
18	3·5	35·0	51·0	10·5	51·0	49·0	97·0
19	2·5	34·0	57·5	11·0	43·5	56·5	99·0
20	1·0	20·0	66·5	9·5	45·5	52·0	99·0
21	1·5	10·5	88·0	0·0	13·0	86·5	99·0
22	0·5	18·5	81·0	0·0	16·0	83·5	99·0

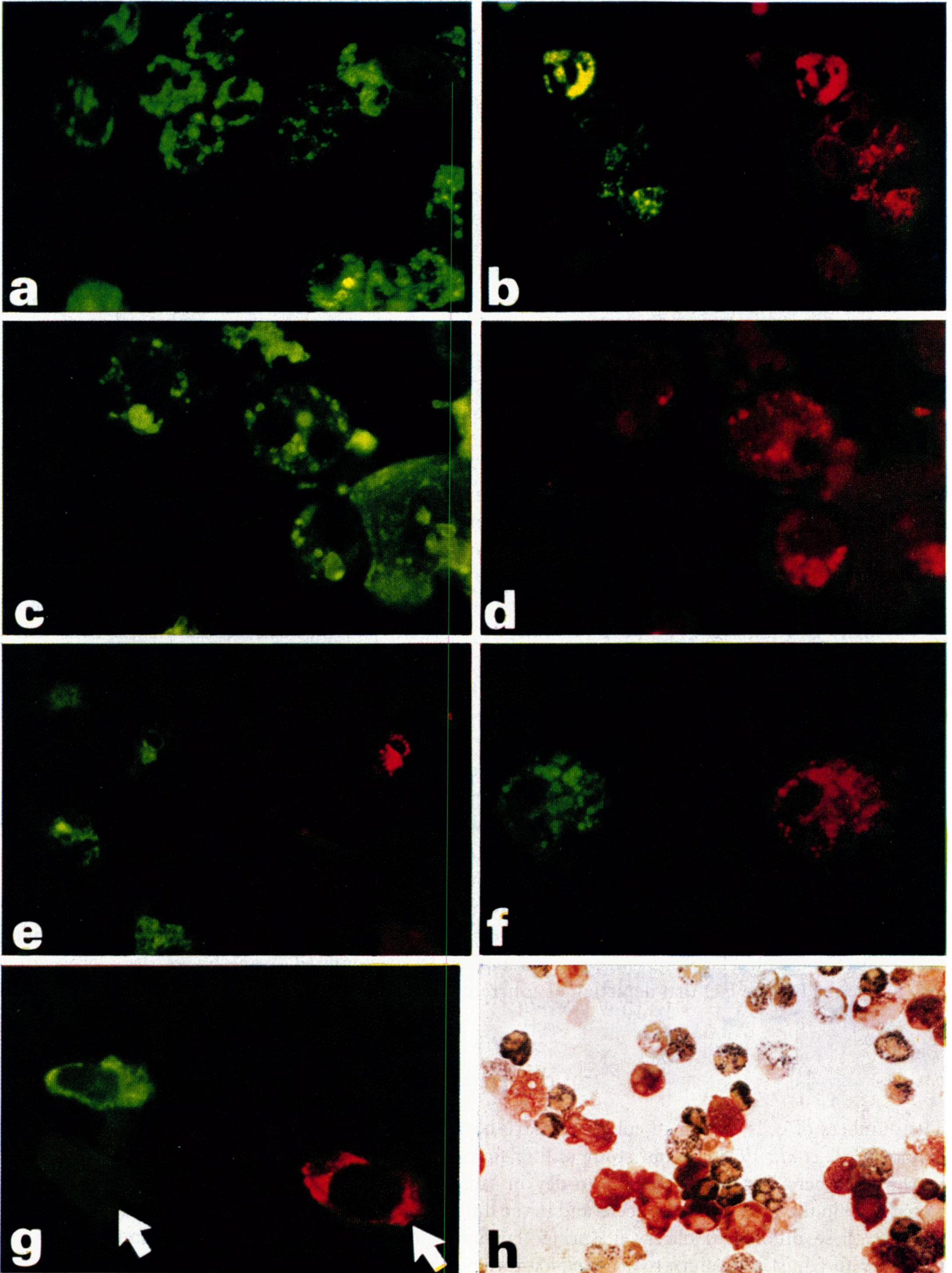
* Numbers represent percent of cells which could be classified according to given criteria (200 cells counted).

Immunohistochemical characterization

To determine the presence of immunoglobulins and other milk proteins in cells from colostrum, unseparated cells as well as those separated by various techniques were stained with FITC-and/or TRITC-labelled reagents specific for H- and L-chains of Ig molecules, SC, lactoferrin, and α-lact-albumin. To detect possible coincident presence of two different proteins, cell preparations were double stained with FITC- and TRITC-labelled reagents.

Although polyvalent anti-Ig reagents revealed numerous positive cells, the staining pattern did not resemble that found in plasma cells (Fig. 3a). Staining of these cells with reagents specific for H-chains revealed a staining pattern similar to that seen with polyvalent anti-Ig. Double staining with two H-chain-specific reagents which were labelled with different fluorochromes showed that the majority of cells contained both IgA and IgM. Similar staining was observed using reagents specific for IgA and IgG or IgM and IgG, although the intensity of fluorescence and the frequency of cells that contained IgG was markedly low.

FIG. 3. Immunohistochemical and histochemical analyses of cells from human colostrum. (a) Unseparated cells stained with FITC-labelled anti-IgA. Note the morphology of IgA-containing cells. Wright's staining of these cells revealed that IgA was present in cells that could be classified as PMN, macrophages, or colostral corpuscles. (b) Coincidental presence of immunoglobulin (FITC-labelled anti-Ig, green, left) and secretory component (TRITC-labelled anti-SC, red, right) in macrophages and PMN. (c, d) Coincidental presence of immunoglobulin (FITC-labelled anti-Ig, green, c) and lactoferrin (TRITC-labelled anti-lactoferrin, red, d) in colostral cells. (e) Coincidental presence of IgA (FITC-labelled anti-IgA, green, left) and α-lactalbumin (TRITC-labelled anti-α-lactalbumin, red, right) in colostral cells. (f) Coincidental presence of κ-chains (FITC anti-κ-chain, green, left) and λ-chains (TRITC-labelled anti-λ-chain, red, right) in a single colostral macrophage. Note the granular appearance of fluorescent areas in positive cells. (g) Staining of plasma cells (induced by PWM stimulation of peripheral blood lymphocytes) with anti-κ-chain, green, left) and anti-λ-chain (TRITC-labelled anti-λ-chain, red, right). The same reagents (f and g) did not stain the same plasma cells for both, κ- or λ-chains. The cell marked with an arrow contained λ-chain (right) but no κ-chain (left). (h) Combined non-specific esterase-peroxidase staining applied to cells from fraction 18 of isokinetic gradient. Cells staining red contain non-specific esterase and were classified as colostral corpuscles or macrophages. Cells staining blue-green contain peroxidase and were identified as PMN.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Further experiments were performed on unfractionated cells or cells fractionated by isokinetic centrifugation or adherence to glass wool columns to determine the type of cells that stained for two Ig classes. Cells were stained with combinations of reagents (FITC- and TRITC-labelled) which would disclose two intracellular proteins, including H- and L-chains, SC, lactoferrin, or α-lactalbumin. Staining with reagents specific for Ig components, (H-chains), in conjunction with reagents specific for a protein not produced by plasma cells (SC, lactoferrin, or α-lactalbumin) indicated that both immunoglobulins and SC or lactoferrin occurred in the same cell (Fig. 3b–d). Few cells contained α-lactalbumin in combination with any of the Ig H-chains (Fig. 3e). In contrast to the mutually exclusive presence of L chain types in plasma cells from bone marrow or PWM-stimulated peripheral blood lymphocytes which express only one L chain type in single cells (Fig. 3g), the majority of individual cells in colostrum contained both κ and λ L-chains (Fig. 3f). Contrary to plasma cells which exhibit a smooth, cytoplasmic pattern of fluorescence, the fluorescence observed in colostral cells was distinctly granular. In addition, the morphology of Ig-containing cells (as revealed by phase-microscopy or subsequent haematological staining) did not resemble that of plasma cells.

Identical double staining procedures were applied to cells separated by the various techniques described above. The most pronounced staining for Ig, as well as for other milk proteins, was observed in cells (obtained from glass adherence or isokinetic centrifugation) that were previously shown by histochemical techniques to correspond to macrophages, colostral corpuscles, or PMN (Fig. 3h). Coincidental presence of Ig with both κ- and λ-chains, SC, lactoferrin and α-lactalbumin in macrophages and PMN, indicated that these proteins were acquired through ingestion from the external environment. Fractions that contained primarily lymphocytes did not exhibit intracellular Ig, SC, lactoferrin, or α-lactalbumin.

Numerous particles that resembled and stained brightly with all fluorochrome-labelled reagents were differentiated by a combination of fluorescence and phase-contrast microscopy.

An attempt was made to enumerate B lymphocytes by staining with a TRITC-labelled $F(ab')_2$ fragment of polyvalent anti-human Ig. However, the majority of cells, as well as extracellular debris, exhibited a strong surface fluorescence, which made accurate counts unreliable. In contrast, the same reagent applied to peripheral blood lymphocytes from twelve individuals revealed surface fluorescence on only 5–16% of cells (Crago & Mestecky, 1979).

Attempts were also made to determine the proportions of T lymphocytes in separated and unseparated colostral cell preparations by allowing the cells to rosette with N-SRBC. Although 60–73% of the lymphocytes formed rosettes (Fig. 2e), numerous cells identified by histochemical criteria as macrophages or colostral corpuscles also formed rosettes (Fig. 2f).

A considerable number of lymphocytes were found in association with macrophages and/or colostral corpuscles in both separated and unseparated cells. This phenomenon was previously observed by Smith & Goldman (1970). To determine whether these lymphocytes could be classified as B or T cells they were analysed for their ability to form rosettes. The majority (90%) of these lymphocytes could be classified as T cells (Fig. 2g–i). Therefore, some loss of lymphocytes encountered during fractionation procedures may be ascribed to the fact that depletion of adherent cells also removed lymphocytes attached to them.

DISCUSSION

The numbers of cells in human colostrum have been reported to vary greatly from day to day among donors (Ogra *et al.*, 1978). In our study of fifty-four patients, we found this variability to exist not only between mothers, but also from day to day in samples collected from the same mother. Differences were found in the total number of cells and in the differential analyses of cell types. In order to determine whether these differences observed during the first four days post-partum were significant, it was necessary to adjust the values to account for variances, and subject these adjusted values to statistical analyses. These analyses revealed that, during the period studied, there was no significant increase or decrease in either the total number of cells or in the numbers of specific cell types. However, there was a downward linear trend in both total cell number and the percentage of lymphocytes during the four

day study period. These results indicate that studies performed on milk samples taken on any of the first four days post-partum may be considered as statistically equivalent.

Differential counts on cells from colostrum were hampered by the unusual morphology of the cells. Often, as a result of the large amount of intracellular lipids, colostral cells appeared quite different from their counterparts in peripheral blood. These morphological variations necessitated the use of a combination of morphological, histochemical, and immunohistochemical criteria to identify cell types.

Standard methods for the separation of different cell types were not applicable to the cells from colostrum. Ficoll-Hypaque gradients or adherence to glass did not result in a significant purification of any of the colostral cell types. Only isokinetic centrifugation or adherence to glass coupled with glass wool fractionation were applicable. However, the overlap of various cell populations caused by irregular separation (most probably due to intracellular lipids present in phagocytic cells) led to low yields of cells with a high degree of purity. In our study, the most practical method for isolation of lymphocytes proved to be the combination of adherence to plastic with subsequent glass wool fractionation.

The analyses of colostral cells are of interest because their characterization may clarify the mechanisms that regulate the secretory immune system and eventually lead to the induction of humoral and cellular protection for the neonate. Several models have been proposed to explain the presence, in external secretions, of IgA-associated antibodies and IgA-containing cells with a specificity for antigens encountered in the gut. It has been postulated that gut- or bronchus-associated lymphoid tissues (GALT or BALT) are the sources of IgA-precursor cells sensitized to ingested or inhaled antigens. These cells emigrate from the GALT or BALT via the thoracic duct, enter the circulation, and eventually seed subepithelial mucosal tissues of the intestinal and respiratory tracts (Craig & Cebra, 1971; Rudzik *et al.*, 1975; Cebra *et al.*, 1976; Husband & Gowans, 1978), mammary glands (Roux *et al.*, 1977), and possibly salivary and lacrymal glands. Indeed, the spectrum of antibody specificities found in secretory IgA of mammary, salivary, and lacrymal glands induced as a consequence of natural or artificial immunizations (Goldblum *et al.*, 1975; Carlsson *et al.*, 1976; Arnold, Mestecky & McGhee, 1976; Mestecky *et al.*, 1978a) supports the concept of a common mucosal secretory IgA system (Cebra *et al.*, 1976; Parmely & Beer, 1977; Bienenstock *et al.*, 1978; Hanson *et al.*, 1978; Mestecky *et al.*, 1978b). The detection, in colostrum and milk, of cells which formed indirect plaques with erythrocytes coated with lipopolysaccharide isolated from a strain of *E. coli* (086), which temporarily colonized the mothers' intestines, would favour the local production of IgA by plasma cells that originated in GALT (Goldblum *et al.*, 1975; Ahlstedt *et al.*, 1975). The contention that these plaque-forming cells represent plasma cells that actively synthesize IgA was supported by the ability of puromycin to inhibit plaque formation. Furthermore, free IgA-associated antibodies to *E. coli* 086 were absent (or present in low titres) in milk but consistently detectable, by plaque assay, in milk cells. However, no morphological examinations of these plaque-forming cells were performed to confirm their identity as plasma cells. Moreover, the proportions of plaque-forming cells to the total number of colostral cells (up to 8%) is uncomfortably high. Since no cells that correspond to plasma cells were detected in colostrum and lymphoid cells comprise slightly over 10% or less of total cells (Parmely *et al.*, 1976; Ogra *et al.*, 1978, this study) these results would indicate that almost the entire population of lymphoid cells would be involved in the formation of antibodies to a single antigen. The observation that plaques could be developed by anti-α-chain as well as anti-SC reagents must be viewed with regard to the established fact that SC is not produced by plasma cells or lymphocytes. These findings could be explained by the presence of preformed secretory IgA (polymeric IgA with SC) in these cells. Coincidental appearance of immunoglobulins with non-immunoglobulin proteins (SC, lactoferrin, and α-lactalbumin) in single cells as observed in our studies, and also discussed by Brandtzaeg (1978), strongly suggests that these proteins were acquired from the environment by phagocytic cells. The strongest evidence for the passive acquisition of immunoglobulins by colostral phagocytic cells was obtained from double-staining with FITC-labelled anti-κ and TRITC-labelled anti-λ chain reagents. In contrast to plasma cells which stain with either anti-κ or anti-λ reagents, Ig-containing cells in colostrum invariably stained with both reagents. The granular pattern of immunofluorescence staining and the histochemical analyses confirmed that macrophages are indeed the principal Ig-containing cells in milk and colostrum. This conclusion is in agreement with the results of Pittard

et al. (1977). These authors demonstrated that breast milk macrophages contain large amounts of IgA, mitogen stimulation of colostral cells does not result in a significant increase in IgA production, and increases in extracellular Ig can be accounted for by decreases in intracellular macrophage-associated Ig.

The origin of colostral macrophages and the site where they acquire intracellular Ig remains unclear. The coincidental presence of IgA and SC as well as other milk proteins in single phagocytic cells would suggest that they acquire these proteins after transport of IgA through epithelial cells. The biological significance of the presence of Ig and milk proteins within phagocytic cells of colostrum remains speculative. Because milk macrophages are capable of a slow release of Ig, exhibit phagocytosis, and can tolerate large variations in the environment, it has been suggested that these cells may contribute to the defense processes taking place in the gastrointestinal tract of the newborn (Pittard *et al.*, 1977).

An interesting observation concerns the interaction of milk macrophages with lymphocytes, previously described by Smith & Goldman (1970). In our investigations, these lymphocytes were almost exclusively classified by a rosetting technique as T cells. This interaction may be of some importance in the expression of the biological function of macrophages (activation by T cell products), T cells (presentation of antigen by macrophages), or B cells (help or suppression).

We are grateful to Ms R. Kulhavy, Ms Annette Pitts and Mr C. Jennings for invaluable technical assistance and Miss C. Sims for editorial advice. We would like to thank Dr E. Bradley, Department of Biostatistics, University of Alabama in Birmingham, for statistical analyses of data.

Supported by US PHS grants GM-07164, AI-10854 and DE-02670.

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