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# Characteristics of milk cells

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Human colostrum and milk represent complex biological liquids containing different immunoactive agents that change during lactation. These include immunocompetent cells like macrophages and lymphocytes with blood origin. Morphological characteristics of human leucocytes were assessed by the use of light microscopy, scanning electron microscopy and transmission electron microscopy. Additional characteristics of milk cells in colostrum were, investigated using membrane markers after lectins, Ag I/II or MBB activation. These examinations proved lymphocytes and macrophages as potential antigen presenting cells.

*Key words:* milk cells, colostrum, mucosal immunity.

## Introduction

Human colostrum and milk represent complex biological liquids containing different immunoactive agents that change during lactation. Among them milk cells exist, in concentration of 1-3x$10^6$ in ml cells. These immunocompetent cells are neutrophils, macrophages and lymphocytes which have blood origin. Their range varies among individuals and time of lactation. T-lymphocytes are more abundant in comparison to B-lymphocytes that could be no more than 6% of total number of lymphocytes.

## Materials and Methods

### Light and electron microscopy

For the characterization of leukocyte population 512 samples of human breast milk from healthy donors were collected. Samples were taken between 2$^{nd}$ and 30$^{th}$ days post partum. Centrifugation at 200g was performed for 20 min to isolate leucocytes, followed by PBS washing. Afterwards cells were processed for light microscopy (LM), transmission electron microscopy (TEM) and scanning electron microscopy (SEM).

Observation under LM was performed on cell smears after the method of Wright-Farbung [1]. Eosin methylene blue was added to cell smears and left for one min, then 1 ml of distilled water was added and left another 3 min. Slides were dried at room temperature.

For SEM, cells were washed three times in PBS and left to adhere on cover slips for 20 min. They were then fixed in 2, 5% glutaraldehyde, dehydrated in graded ethanol solutions and covered with gold. Observation was performed under JEM-35 scanning electron microscope. For TEM observation milk samples were diluted three times with cold PBS and centrifuged three times at 1000g. Then cells were resuspended in 3% agarose in PBS and put on ice and fixed in 2,5% glutaraldehyde and post fixed in 1% $OsO_4$. Then samples were dehydrated in graded ethanol solutions and embedded in resin. Ultra thin sections were cut and observed under transmission electron microscope Opton EM 109.

**Cultivation and Proliferation**

Human milk samples were centrifuged at 200g for 20 min. and washed 4 times in PBS supplemented with 10% fetal calf serum (FCS). ($1 \times 10^6$) cells were incubated in RPMI 1640 medium containing 10% FCS for 24, 48 and 72 hours in presence of 10 μg MBB, 5 μg Con A or 0, 1 μg Ag I/II at 37C, 5% $CO_2$. Control samples contained only cells, cultured in RPMI 1640 medium and 10% FCS. For proliferation assay cells were incubated with 1 μCi $^3$H-Thy. Labeled DNA was harvested onto DEAE filters and triplicate sample readings were obtained by scintillation counting /Beckman/.

**Indirect Peroxidase Technique**

$1 \times 10^6$ cells were stimulated with mitogens or antigens and cultured in presence of the following mAb: mB1, CD-71, CD-3, CD-4, RFD-7 and MA-5. Then cells were washed in PBS and cultured in presence of anti mouse Ig conjugated with peroxidase. 200 cells were counted on each sample and colored after Wright for cytological examination. For DNP and RNP activation of milk cells we performed methylene blue fast green method [2].

## Results and Discussion

Human neutrophils and macrophages from breast milk showed regular morphology, compared to those, obtained from peripheral blood but had more granules, impregnated with lipids and carbohydrates. (see Fig. 1 and Fig. 2). Lymphocytes showed no morphological differences to those found in peripheral blood.

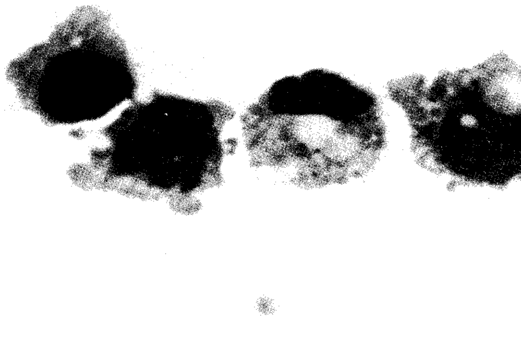

Fig.1. Macrophages from human colostrum, with pinocitotic vesicles or with microclasmatozes. (x 1000)

Fig.2. Leukocyte in human colostrum (x 1000)

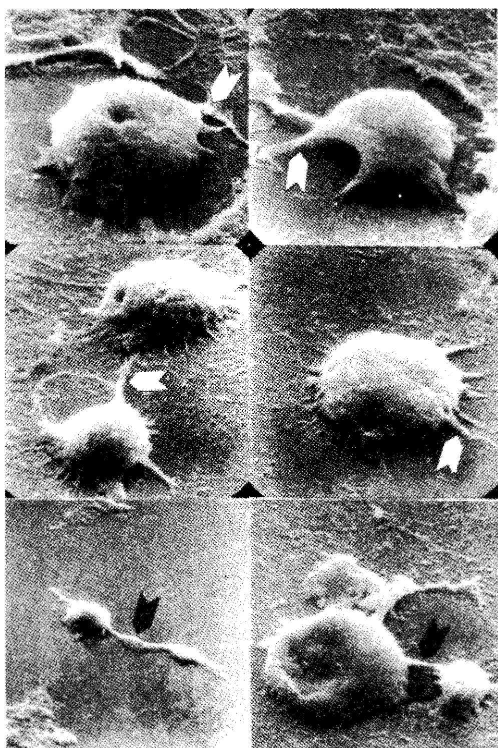

Fig.3.SEM micrograph of milk cells, with protrusions. Originally x 10000.

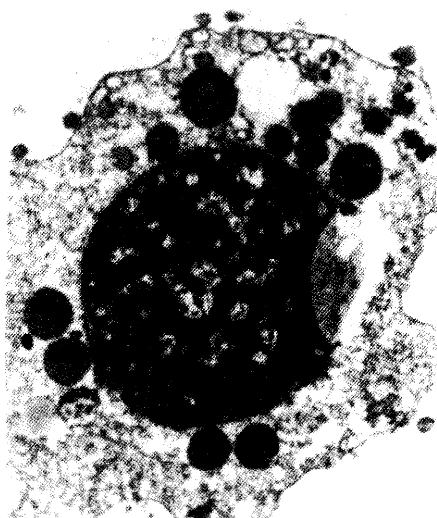

Fig.4. TEM micrograph of milk macrophage. Originally x 12000

Both TEM and SEM confirmed morphological characteristics of peripheral blood cells. Visibly (Fig. 3), cells possessed protrusions enabling their close contact with each other. TEM micrographs (Fig.4) represented typical neutrophils with plenty of lipid and carbohydrate granules and often with activated nuclei.

Activation of milk cells with Con A or MBB was performed and expression of several markers was assessed. The highest expression was found on second and third days of activation as visible in Fig 5.

| ConA/MBB stimulation | DAY 0 to 4 | | | | |
|---|---|---|---|---|---|
| Marker | 0 | 1 | 2 | 3 | 4 |
| HLA-DR | 14/14 | 72+/69+ | 58+/58+ | 43+/44+ | 20/20 |
| CD71 | 2/2 | 21+/27+ | 62+/57+ | 78+/61+ | 40+/36+ |
| CD3 | 12/12 | 50+/59+ | 72+/58+ | 76+/59+ | 47+/33+ |
| CD4 | 10/10 | 28+/26+ | 29+/34+ | 40+/43+ | 25+/20+ |
| 3H-Thy Incorporation | -/- | 4,1++/2,1++ | 7,0++/4,5++ | 6,1/6,6++ | 3,8+/6,2+ |

Fig.5. Expression of markers after ConA or MBB stimulation of milk cell.

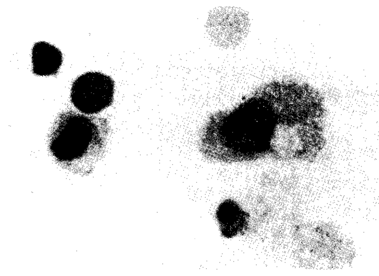

Fig.6. DNP and RNP staining of milk cells after Zvetkova E. et al. (x250).

| mAb | Specificity | Day0 | Day2 | Day4 |
|---|---|---|---|---|
| RFD7 | macrophages | 65±4 | 38±11 | 0±5 |
| MA5 | blood monocytes | 26±3 | 84±8 | 93±6 |
| mB1 | antiHLA-DR | 25±9 | 80±12 | 90±4 |

Fig.7. Expression of macrophages markers after *in vitro* stimulation with AgI/II.

After activation with AgI/II antibody, up regulation of RFD7 expression was observed, mostly in tissue macrophages and MA5 and mB1 were present only in 20% of cells (Fig.7)._Expression of the latter two markers increased with time of cultivation, which means macrophages in human colostrum were capable of inducing *de novo* synthesis and differentiation after activation with mitogens. The observation was confirmed by another cytological method. DNP and RNP staining of milk cells (see Fig. 6) revealed nuclear activation and possibility of *de novo* synthesis in milk macrophages.

Previous studies have proved that milk cells could pass the neonatal intestinal barrier. The precise mechanisms of crossing the intestinal mucosa have not been established yet [3]. After ingestion the maternal cells populate the lymph nodes, and could be often found in other tissues of the newborn. Thus they express their immunomodulating effects on neonates [4, 6]. Major histocompatibility complex (MHC) Class II antigens are fully expressed on newborn human intestinal mucosa [5]. MHC represents membrane glycoproteins, present on the cell surfaces of leucocytes. Class II antigens have limited expression- they are present only on mononuclear phagocytes and B-lymphocytes. Activation of milk cell with Con A, MBB or AgI/II demonstrated that maternal macrophages and lymphocytes expressed MHC Class II antigens *in vitro*, which means they are potential antigen-presenting cells. Activation with other antigens [7] showed similar to our results: colostral lymphocytes cross intestinal mucosa of newborn and remain immunologically active.

In conclusion the neonate intestine represents a unique system, unaffected by any antigens. Newborns receive adult antigen-presenting cells from maternal colostrum and milk. These cells are not recognized as foreign. Thus immune responses in neonates could be modulated in order of their survival.

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