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Immunocytochemical characteristics of milk cells after in vitro activation with *St. mutans* AgI/II

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Human milk cells were in vitro stimulated with St. mutans membrane protein antigen — AgI/II. Using monoclonal antibodies to main lymphocyte and macrophage markers, it is shown that the antgen activates predominantly the helper T lymphocytes and a small fraction of macrophages.

Key words: milk lymphocytes, milk macrophages, membrane marker expression, in vitro activation.

Introduction

Human milk from early in lactation differs from most other external secretions because it contains viable leukocytes. These include macrophages, polymorphonuclear leukocytes, lymphocytes and a few epithelial cells. Human colostral and milk cells could be activated in in vitro cultures by mitogens (PHA, PWM, Con A, LPS) or antigens (PPD, MBP) [2, 5]. They have been shown to differ in a number of membrane properties (resetting, adherence, mobility) and membrane marker expression from the corresponding peripheral blood mononuclear cells [3, 7, 9].

The aim of the present study was to present quantitative kinetic data concerning the main lymphocyte and macrophage marker expression by milk cells after in vitro stimulation with a bacterial protein antigen — *St. mutans* AgI/II [8].

Material and Methods

Cell cultures

Milk samples were collected from 20 healthy donors between the 2nd and 10th day-postpartum. Cells were pelleted at 100× g for 10 min, and washed in PBS. 1×10^6 cells per ml were incubated in medium RPMI 1640 containing 10% FCS and antibiotics for 24h, 48h, 72h and 96h in the presence of 0.1 µg/ml AgI/II, at 37 °C, 5%CO_2. Control cultures contained the same cell number in medium.

Proliferation assay

16h before harvesting 1 μCi ^{3}H-thymidine (UVVV-Prague, Czech Republic) was added to each sample and the incorporation counted in a beta-scintillation counter (Beckman). All samples were done in triplicate.

Indirect immunoperoxidase technique

Cultured cells were washed 3 times in medium and centrifuged for 1 min at 500xg to obtain cytospin speciments. Then the samples were fixed in cold aceton, endogenous peroxidase exhausted in methanol/H_2O_2 solution, and treated for 30 min with medium containing 20% normal bovine serum. The following monoclonal antibodies were used — mB1 (anti-HLA-DR) [4], anti-CD71 (anti-transferrin receptor, OKT9 —ORTO), anti-CD3 (DAKO), anti-CD4 (DAKO), anti-CD8 (DAKO), MA5 (anti-blood macrophage) [6], RFD7 (anti-tissue macrophage, Royal Free Hospital, London). The treatment with each of them was for 1h at 4 °C following washing and incubation with anti-mouse Ig conjugated to peroxidase. The development of the reaction was with diaminobenzidine and H_2O_2. 200 cells were counted in each sample. After the examination of peroxidase reaction the samples were stained after Wright for routine cytological expertise.

Results and Discussion

In preliminary studies we titrated AgI/II concentrations and found the optimal ones, giving maximal proliferative response of 1×10^6 cells to be 0.1μg/ml. This maximum was reached on day 4 of the culture.

A representative experiment for proliferation of milk cells upon activations with AgI/II.

Day	0	1	2	3	4
Mitogenic index	-	4.6	5.8	9.3	11.8

Drastic changes occur during cultivation period among different cell populations in the milk. The polymorphonuclear leukocytes, which were predominantly neutrophils, disappear after 24h of culture. A very interesting behaviour showed the macrophages. On day 0, most of the macrophages were positive for RFD7 monoclonal antibody, a marker for tissue macrophages, and only 25% were positive for MA5, a marker for blood macrophages. The HLA-DR positivity of the macrophages was in parallel to MA5 one. After 2 days of culture the macrophages started distructing, and on day 4 they consisted about 10% from whole milk population (Table 1). The tissue macrophage marker RFD7 decreased to 0% and the activated by AgI/II milk macrophages all bear the blood macrophage marker MA5, and were all HLA-DR positive. We suggest a division of these cells, or at least a part of them, in the culture and de novo synthesis of membrane marker molecules under the antigenic stimuli. This result significantly differs from our previous observation, where the milk macrophages do not divide under Con A and MBP stimuli [5].

The milk lymphocytes were shown to be mostly T-lymphocytes [1]. We confirm this with their reaction with anti-CD3 monoclonal antibody. With the advancing of the culture time and the destruction of the polymorphonuclears first, and the majority of the macrophages second, the T-lymphocytes become the predominant cell type in the culture (Table 2). In the case of AgI/II stimulation they were also about 70%

T a b l e 1. Membrane properties of milk macrophages after AgI/II stimulation (in per cent positive cells)

MAB	Specificity	Day		
		0	2	4
RFD	Tissue	65+4	38+11	0+5
MA5	Blood macrophage	25+3	84+8	93+6
MB1	anti-HLA-DR	25+9	80+12	90+4

T a b l e 2. Time-dependent expression of lymphocyte markers (in per cent positive cells) under AgI/II stimulation

Marker expressed	Day				
	0	1	2	3	4
CD3	12+5	46+7	76+11	78+9	89+3
CD4	8+7	26+9	48+9	52+6	61+5
CD71	12+5	21+8	40+1	62+4	78+9
HLA-DR	14+7	20+9	43+6	68+3	86+9

CD4 positive which means that they are of helper type and this result is different from MBP stimulation where CD4 positive fraction was 40-50%.

As could be expected the expression of CD71, which is the transferring receptor, increases in parallel with the thymidine incorporation. This suggests a metabolic activation of T lymphocytes and supports the possibility for IL-2 synthesis, and further autocrine activation of the committed clones.

The last but not least is the expression of the HLA-DR monomorphic determinants by the activated milk cells. As it is known from numerous reports, upon activation by antigens or mitogens the T-lymphocyte starts class II expression and becomes immunologically committed. The macrophage is constantly class II positive. Therefore in our system the initial expression of HLA-DR on day 0 should be due to macrophages and later on the culture time to the activated under AgI/II T-lymphocytes. The temporal profiles of the AgI/II dependent MHC class II expression appeared very similar to those for the same marker expression on peripheral blood mononuclear cells (our observation).

On the basis of the above data we might conclude that AgI/II is a very potent antigenic stimulus for milk T-cells, activating mostly T-helpers in this population. This rises the possibility of isolating AgI/II specific T-helper clones which could be used to influence the development and maturation of the immune system of infants, when given during the first days postpartum.

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