Bovine whey protein concentrate supplementation modulates maturation of immune system in suckling rats

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During neonatal life, challenges from breast milk and microbial flora promote immune system maturation. Immunonutrition in these stages may become an important way to increase natural defence systems. The aim of this study was to determine the effect of a daily bovine milk whey protein concentrate (WPC) supplement on the intestinal and systemic immune systems in suckling rats. The composition of intraepithelial and lamina propria lymphocytes (IEL and LPL) was analysed by flow cytometry. Systemic and intestinal humoral immune responses were determined by sera Ig levels and Ig-secreting cell quantification by ELISA and ELISPOT, respectively. From birth, suckling Wistar rats were supplemented with WPC or standard infant formula (SIF). The WPC group showed the same proportion of most of the main mucosal cell subsets as the reference animals. However, in the first days of life WPC enhanced the innate immunity by increasing the NK cell proportion in both epithelial and lamina propria (LP) compartments. A rise in intestinal CD8+ IEL was also induced by WPC supplementation. A time-course of sera Ig levels and spontaneous IgA, IgM and IgG production by LPL and mononuclear cells from blood and spleen, in the WPC group, exhibited a similar pattern to those pups fed only by dam’s milk. In summary, the present results show the effects of WPC on enhancing mucosal innate immunity during early life.

Suckling: Rat: Intraepithelial lymphocytes: Lamina propria lymphocytes: Whey: Milk

Material and methods

Animals

Pregnant Wistar (G7) rats were obtained from Harlan (Barcelona, Spain). They were housed in individual cages under controlled temperature and humidity conditions in a
12 h:12 h light:dark cycle, and were fed with a commercial diet (AIN-93G, Harlan) and water ad libitum. Animals were monitored daily and allowed to deliver naturally. The day of birth was identified as day 1 of life. Litters were unified to ten pups per mother, with free access to the nipples and rat diet. Studies were performed in accordance with the institutional guidelines for the care and use of laboratory animals established by the Ethical Committee for Animal Experimentation at the University of Barcelona and Catalonia Government (CEEA-UB Ref. 849/00).

**Dietary supplementation**

Animals were distributed in 3 groups depending on the supplementation diet: the experimental group receiving WPC; the supplemented reference group, receiving standard infant formula (SIF) and the reference group (REF) (20 animals each). Animals from the WPC group were supplemented with 0.2 g/kg/day of a premium ultra grade bovine whey concentrate (CEEA-UB Ref. 849/00).

**Isolation and purification of intraepithelial and lamina propria lymphocytes**

Intestinal IEL and LPL suspensions were obtained by chemical and enzymatic reactions from 2-4 animals depending on their postnatal age following procedures established previously in our laboratory. Both IEL and LPL suspensions were later purified and cell number and viability were determined.

**Immunofluorescence staining and flow-cytometer analysis**

2 × 10⁵ IEL or LPL were stained by a double immunofluorescence technique. The mouse anti-rat monoclonal antibodies conjugated to fluorescein isothiocyanate or phycoerythrin used here included anti-CD3 (1-F4), anti-CD4 (OX-35), anti-CD8a (OX-8), anti-CD25 (OX-39), anti-CD45 (OX-1), anti-TCRαβ (R73), anti-TCRγδ (V65) and anti-NKR-P1A (10/78), all from BD Pharmingen (San Diego, CA, USA); anti-CD2 (OX-34), anti-CD45RA (OX-33) and anti-CD90 (OX-7) from Caltag (Burlingame, CA, USA) and anti-CD8β (3-41) from Serotec (Kidlington, Oxford, UK). Staining was developed following procedures described in previous studies, and results were analysed with an Epics XL flow cytometer (Coulter Corp., FL, Hialeah, USA).

**ELISPOT and ELISA techniques**

A solid-phase enzyme-linked immunosorbent technique (ELISPOT) was used to quantify IgA-, IgG- and IgM-secreting cells (SC) from blood, spleen and lamina propria (LP) following the conditions established in previous studies. Spots were automatically counted by ELISPOT-reader system (AID Diagnostica, Strassberg, Germany) and expressed as the number of Ig-SC per 10⁶ cells. Sera IgG, IgM and IgA concentration was determined using a sandwich ELISA technique standardised previously.

**Statistical analysis**

SPSS 10.0 (SPSS Inc, Chicago, IL, USA) was used for statistical analysis by conventional one-way ANOVA considering the experimental group as an independent variable. When supplementation had a significant effect on the dependent variable, the LSD test was applied. Significant differences were accepted when P<0.05.

**Results**

**Animal growth**

Body weight increase was unaffected by either daily WPC or SIF supplementation during the first half of the suckling period, and showed growth similar to the reference group. However, during late suckling, higher body weight gain was found in those animals supplemented with SIF (P<0.05, data not shown).
IEL composition in small intestine

WPC supplemented animals showed the same percentages in most of the main subsets within the intraepithelial (IE) compartment as the reference group during suckling period: total gated leukocytes (CD45\(^+\), ~90%), IE-T lymphocytes (CD3\(^+\), ~60 to 90% from day 4 to 21, respectively), and CD8\(^+\) IEL (~80-50% from day 4 to 21, respectively). Moreover, in all studied groups, the TCR\(\alpha\beta\)- cell proportion in CD8\(^+\) IEL increased from ~25% (day 4) to ~50% (day 21), and the TCR\(\gamma\delta\)- cell percentage in CD8\(^+\) IEL rose from ~15% (day 4) to ~25% (day 21).

Supplementation with WPC induced an increase in NKR-P1A\(^+\) (a rat NK cell marker) cell proportion in the IE compartment compared to both the reference and SIF groups at the beginning of the suckling period (\(P<0.05\), Fig. 1A). Later, all groups showed a similar development pattern for this subset. Moreover, the CD8\(^+\) cell proportion in NKR-P1A\(^+\) IEL markedly decreased during the entire suckling period in all studied groups (data not shown).

The CD8\(\alpha\alpha\)/CD8\(\alpha\beta\) ratio in IEL, which may represent the relationship between lymphocytes developed in the intestinal environment with respect to cells with typical phenotype from extra-mucosal sites, was modified by WPC supplementation. Thus, the CD8\(\alpha\alpha\)/CD8\(\alpha\beta\) ratio in the WPC group was higher than in the reference and SIF groups, from day 7 and throughout the entire sucking period (\(P<0.05\), Fig. 1B).

LPL composition in small intestine

Total gated leukocytes in LP (CD45\(^+\)) rose from ~65% (day 4) to ~95% (day 21) in all groups. There was a physiological increase of B cell proportion (CD45RA\(^+\) cells) during suckling in LP that was not modified by the supplements during this period. Moreover, the proportion of CD90 molecules in B cell surfaces, a negative marker of maturation, was ~75% in the three experimental groups during suckling (data not shown).

The percentage of CD4\(^+\) LPL ranged between ~10–30% during suckling in all three groups, and the proportion of these cells bearing CD3 (typical T cell marker), CD2 (adhesion molecule), CD25 (T cell activation marker) and CD90, were not modified by the supplements. With respect to CD8\(^+\) LPL, CD8\(\alpha\alpha\)/CD8\(\alpha\beta\) ratio and TCR\(\alpha\beta\) percentages were similar among WPC, SIF and reference groups (data not shown).

At day 4 of life, similar to the IE compartment, the NKR-P1A\(^+\) cell proportion in LPL in WPC supplemented animals (~27%) was higher than those in the SIF (~21%) and reference (~17%) groups (\(P<0.05\), Fig. 1C). However, at the end

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**Fig. 1.** Developmental time-course of intestinal cells during the suckling period under supplementation conditions. (A) NKR-P1A\(^+\) cells with respect to total IEL, (B) CD8\(\alpha\alpha\)/CD8\(\alpha\beta\) ratio in total IEL, (C) NKR-P1A\(^+\) cells with respect to total LPL, and (D) NKR-P1A\(^+\) CD8\(^+\) LPL with respect to NKR-P1A\(^+\) LPL. Each data corresponds to the mean ± SEM (\(N=5-10\) animals). Statistical differences: \(a P<0.05\) WPC vs. Reference, \(b P<0.05\) SIF vs. Reference, \(c P<0.05\) WPC vs. SIF.
of the suckling period the NKR-P1A+ LPL proportion in the WPC group did not differ from that of the other groups. Moreover, the youngest (day 4) WPC supplemented animals showed a higher proportion of NKR-P1A+ LPL bearing CD8+ marker compared to the reference and SIF groups (P<0.05, Fig. 1D). On the other hand, at the end of the suckling period a decrease in this subset in the WPC and SIF groups was observed (P<0.05, Fig. 1D).

Spontaneous Ig production by blood, spleen and LP mononuclear cells

Since B cells represent a predominant subset during suckling3,11, we studied the ability to spontaneously secrete IgG, IgM and IgA antibodies by means of ELISpot in different immune compartments. LP cells showed no IgA-SC or IgG-SC throughout suckling period. On day 21, the number of IgM-SC was similar in all groups (50–115 IgM-SC/10⁶ cells, Table 1). Spleen neonatal cells barely secreted IgG or IgA but did produce IgM. The number of IgM-SC increased in the spleen after the first week of life similar to the WPC and reference groups: (~300 IgM-SC/10⁶ cells during first week of life and ~1000 IgM-SC/10⁶ cells at the end of suckling. Blood Ig-SC numbers were very low for all Ig isotypes in suckling rats ((40 Ig-SC/10⁶ cells, Table 1). Effects of WPC on antibody secretion were not found.

Serum Ig levels

Serum IgG and IgM levels were detected from the first week and increased until day 21 with values ranging between ~0.5–2.5 mg/ml and ~5–150 µg/ml, respectively. IgA levels rose more than 100 times only during last week of suckling (~75–1000 ng/ml). Any serum Ig time-course was not substantially modified by supplementation with WPC in comparison to the reference group. Data from day 21 are shown in Table 1 as a summary of these results.

Discussion

A vast body of literature is focused on the associated effects of bioactive compounds present in milk and colostrum on immune health. However, little is known about the influence of these dairy products on the mucosal and systemic immune system maturation during suckling. In this study, most of the main intestinal lymphocyte subsets, i.e. CD45+ (total leukocytes) (CD3+, TCRαβ+, TCRγδ+ and CD4+ (T cells) and CD45RA+ (B cells), followed the same time-course in WPC supplemented suckling rats as they did in the reference group. However, WPC supplementation from birth increased NKR-P1A+ cell proportion in early life (day 4) in both intestinal compartments consider Ed. NK cells constitute the first line of GALT defence and, as reported previously, they represent a high proportion of rat IE and LP compartments during the first days of life, decreasing with age1,3,11. Our results showed that bioactive compounds present in WPC, by increasing NK cell proportion in GALT, enhanced innate immunity in early life. A similar effect in spleen from adult mice fed with colostrum has been described14. An increase in the NK cell proportion after lactoferrin administration has been found in circulating cells in adult rats15 and in intestinal cells in mice16. Moreover, our data derived from the end of suckling (day 21) suggested that NKR-P1A+ LPL from WPC supplemented animals exhibited a more mature intestinal phenotype (NKR-P1A+ CD8-), which corresponds to that found in adult age, than reference animals did.

Intestinal CD8α+ IEL correspond to a non-conventionally selected lymphocyte population that develops in the gut microenvironment and expresses this particular isoform of CD8 co-receptor17. Our results showed an increase throughout the entire suckling period in the CD8α+ /CD8αβ+ IEL ratio in the WPC group. These data suggest that WPC enhances immune responses developed specifically at mucosal sites by increasing the proportion of gut CD8α+ IEL, perhaps by means of a local effect of WPC compounds. Similar to an NK cell increase derived from lactoferrin consumption, a rise in CD8+ intestinal cells was also found elsewhere16.

The present study shows that WPC promotes the expansion during early age of cell subsets involved in innate and mucosal immune response. This is another mechanism by which they may contribute to the largely studied properties and benefits of colostral and whey proteins in preventing gastrointestinal infections14–18. This effect may be partially attributed to the high concentration of lactoferrin present in the dairy product used in the present study (9.2 mg/g of dried WPC).

The immunoenhancing effect of whey proteins on the production of antibodies against a specific antigen has been well documented19. Our results showed that WPC supplementation failed to enhance the humoral immune response during suckling (blood, LP and spleen) in the absence of specific stimulation. This result could be explained by the low ability of immune cells to produce Ig during this period3,11. However, the dairy supplementation used in this study did not disturb the physiological acquisition of Ig production capacity, as

### Table 1. Effect of WPC and SIF supplementation on humoral immune response in suckling rats

<table>
<thead>
<tr>
<th>Group</th>
<th>IgG (µg/ml)</th>
<th>Mean ± SEM</th>
<th>IgM (µg/ml)</th>
<th>Mean ± SEM</th>
<th>IgA (ng/ml)</th>
<th>Spleen*</th>
<th>Mean ± SEM</th>
<th>Blood*</th>
<th>Mean ± SEM</th>
<th>LP†</th>
<th>Mean ± SEM</th>
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<tr>
<td>WPC</td>
<td>2466.0</td>
<td>111.5</td>
<td>129.5</td>
<td>18.9</td>
<td>1001.1</td>
<td>108.9</td>
<td>856.7</td>
<td>353.3</td>
<td>4.10</td>
<td>2.3</td>
<td>106.0</td>
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<tr>
<td>SIF</td>
<td>2146.3</td>
<td>335.2</td>
<td>127.4</td>
<td>23.6</td>
<td>734.8</td>
<td>71.7</td>
<td>1060.9</td>
<td>141.9</td>
<td>4.10</td>
<td>2.6</td>
<td>52.4</td>
</tr>
<tr>
<td>Reference</td>
<td>2392.3</td>
<td>110.4</td>
<td>147.6</td>
<td>7.2</td>
<td>1012.6</td>
<td>139.7</td>
<td>935.5</td>
<td>222.4</td>
<td>4.88</td>
<td>2.5</td>
<td>114.5</td>
</tr>
</tbody>
</table>

Results correspond to 14 (†) or 21 (*) day-old animals. Number of IgM-secreting cells (SC) are expressed with respect to 10⁶ cells. N = 5–10 animals per experimental group.
evidenced by the similar pattern of development found in all of the experimental groups.

In summary, this study demonstrates that daily supplementation with WPC during suckling improves the development of intestinal and innate cells: CD8αα⁺ IEL and NK cells in IE and LP compartments.

Conflict of interest statement

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References