Cow's IgG1 and its proteolytic digests stimulate immunoglobulin formation in mouse spleen cell cultures

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The aim of this work is to investigate the effect of cow's immunoglobulin G1 (IgG1) and its gastrointestinal proteinase digests on the formation of immunoglobulin, the expression of some cytokine mRNAs, and the ratio of several immunocompetent cells in mouse spleen cell cultures. Immunoglobulin G1 was prepared from Holstein cow's colostrums, digested with pepsin, trypsin, chymotrypsin, or pepsin and pancreatin. Peptide fragments with a molecular mass of less than 20,000 were then recovered from each digest as proteolytic digest for investigation. Intact IgG1 and all its digests (except for peptic) significantly stimulated IgA formation, while intact IgG1 and its digests (especially tryptic) enhanced IgG formation. Intact IgG1, and both tryptic and chymotryptic digests noticeably increased mRNA expressions of cytokines secreted by type 2 helper T (Th2) cells such as IL-4, IL-5, and IL-6. Moreover, intact IgG1 and all its digests significantly increased CD4+ and CD19+ cell ratios. These results suggest that intact IgG1 and its gastrointestinal proteolytic digest may stimulate the production of IgA, a major immunoglobulin in intestinal tracts, via the modulation of cell numbers and/or functions of B and Th2 cells.

Bovines IgG1 and seine proteolytischen Verdauungsaufschluss stimulieren die Immunoglobulinbildung in Mäusen-Splenzellkulturen


18 Immunoglobulin G1 (proteolysis, stimulation of IgA)  18 Immunoglobulin G1 (Proteolyse, Stimmung von IgA)

1. Introduction

Immunoglobulin G (IgG) is the most abundant antibody in bovine colostrum. Cow's IgG consists of 2 subclasses, IgG1 and IgG2. More than 90% is IgG1 in the colostrum, although blood contains almost equal amounts of IgG1 and IgG2 (1). This fact suggests that cow's IgG1 plays a physiologically important role in calf intestinal tracts, in addition to protecting the host against pathogenic organisms.

Kobashi et al. (2) reported that IgA production of Peyer's patch cells was higher in X-irradiated mice given cow's IgG-rich milk than in those given normal milk. Ishida et al. (3) demonstrated that the oral administration of cow's IgG-rich milk significantly augmented some immunological functions of gut-associated lymphoid tissue cells. However, it is unclear how active immunity is regulated by cow's IgG, in particular by the IgG1 subclass.

In a previous paper, Ohnuki and Otani (4) demonstrated that about half of the antigen-binding and 15% of the protein G-binding activities of cow's milk IgG1 ingested in mouse stomach were recovered in feces. This observation confirms that undigested IgG1 and its digests coexist in gastrointestinal tracts in mice that orally ingests cow's IgG1. Thus, our study was designed to investigate the effects of cow's IgG1 and its gastrointestinal proteolytic digests on the formation of IgA and IgG (major antibodies in local and systemic immunities, respectively), expressions of some cytokine mRNAs, and ratios of immunocompetent cells in mouse spleen cell cultures.

2. Materials and methods

2.1 Materials

P Rabbit anti-bovine IgG1 and IgG2 sera were obtained from Nordic Immunological Laboratories (Tilburg, NL). Goat anti-mouse IgG was purchased from Jackson Immuno Research Laboratories (West Grove, PA, USA). Horseradish peroxidase (HR)-labeled sheep anti-mouse IgG, goat anti-mouse IgA, and HR-labeled goat anti-mouse IgA were from Bethyl Laboratories, Inc, Montgomery, TX, USA). BioLegend (San Diego, CA, USA) was the supplier of phycoerythrin (PE)-labeled anti-mouse CD4 monoclonal antibodies (mAb, clone RM4-5), PE-labeled anti-mouse CD49b mAb (clone DX5), biotin-labeled anti-mouse CD8a mAb (clone 53-6.7),
bion-labeled anti-mouse CD19 mAb (clone MB19-1), biotin-labeled anti-mouse CD11b mAb (clone M1/70), and phycocerythrin/cyamine 5 (PE/Cy5) -labeled streptavidin. Pepsin, trypsin, chymotrypsin and pancreatin were purchased from Sigma Chemicals (St. Louis, MO, USA). TRIZol reagent was from Invitrogen Japan (Tokyo). M-MLV reverse transcriptase and Taq DNA polymerase were obtained from Qiagen (Tokyo). Defined fetal bovine serum (FBS) was from HyClone Laboratories (Logan, UT, USA), and RPMI-1640 from Nissui Pharmaceutical Co, Tokyo. Remaining chemicals were of the highest analytic grade commercially available.

2.2 Colostral IgG1

IgG1 was prepared from fresh colostrum secreted by a Holstein cow within 48 h after delivery using a combination of ammonium sulfate fractionation and anion-exchange chromatography. The IgG1 subclass was confirmed by means of immunodiffusion with rabbit anti-bovine IgG1 or IgG2 serum (5).

2.3 Preparation of proteolytic digest

IgG1 (200 mg) was dissolved in 5 ml of 0.01M HCl or 0.01M sodium phosphate buffer (pH 7.5). The IgG1 solution was adjusted to pH 2 (for peptic digestion) or 7.5 (for trypic or chymotryptic digestion) by addition of 1 M HCl or NaOH, adjusted to 10 ml with distilled water, and used as a substrate solution. Digestion was carried out in a test tube at ratios of IgG1 : proteinase = 50:1 (for pepsin) and 100:1 (for trypsin or chymotrypsin) with continuous shaking in a water bath at 45°C for 6 h. The pH of the IgG1 solution reacted with pepsin was immediately adjusted to 7.2 with 1 M NaOH, and centrifuged at 3000 rpm for 30 min at 4°C. On the other hand, in the case of trypsin and chymotrypsin, the same amount of each proteinase was added, and the test tube was kept for an additional 3 h under the same conditions. Then, each digest was immediately adjusted to pH 7.2 with 1 M NaOH or 1 M HCl, and centrifuged at 3000 rpm for 30 min at 4°C. All supernatants obtained by centrifugation were subjected to ultrafiltration at 4°C on an Amicon Ultrafiltration Cell Model 52 (Amicon Corporation, Danvers, MA, USA) with an Advantec UP-20 membrane with a 20-kDa exclusion (Advantec MFS, Tokyo, Japan). The ultrafiltrate was recovered as a peptic, trypic or chymotryptic digest of IgG1. On the other hand, after the IgG1 was digested with pepsin for 6 h as mentioned above, the digest was adjusted to pH 7.5 with 1 M NaOH. Two milligrams of pancreatin were added to the adjusted digest and subjected to continuous shaking in a water bath at 45°C. After 6 h, another 2 mg of pancreatin was added, and the digest was kept for an additional 3 h under the same conditions, after which the ultrafiltrate was prepared from the digest according to the same procedure mentioned above, and used as a peptic-pancreatic digest. The amount of peptide recovered was about 16, 18, 20, and 29% of the IgG1 amount used as a substrate in the peptic, trypic, chymotryptic, and peptic-pancreatic digests, respectively.

2.4 Spleen cell suspensions and cell cultures

Six week-old male C3H/HeN mice were obtained from the Japan SLC (Hamamatsu, Japan). A single-cell suspension was prepared by disrupting spleens in RPMI-1640 medium containing 5% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin as previously reported (8). Final concentrations of the cells, and IgG1 or its proteolytic digests were 5 × 10^6 viable cells/ml and 0 to 100 μg/ml, respectively. The cell suspension mixture was cultured for 12 h (for mRNA expression) or 72 h (for immunoglobulin level and cell ratio) at 37°C in a humidified 5% CO₂-95% air atmosphere.

2.5 Immunoglobulin assay

The sandwich enzyme-linked immunoassay method (ELISA) was used to determine the levels of IgA and IgG in the cell culture supernatant, and principally carried out as previously reported (6). The immunoglobulin level was represented as an immunoglobulin index calculated by the following formula: Immunoglobulin index = ELISA value (A490 nm) for culture with IgG1 or its digest/ ELISA value (A490 nm) for culture without IgG1 or its digest.

2.6 Reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was extracted from spleen cells and used for RT-PCR as previously reported (7). The primer sequences of IL-6 and GAPDH followed those of a previous paper (7) and IL-4 and IL-5 followed those of Yang et al. (8). The primer sequences of IL-2 followed ULETT et al. (9). The primer sequences of the other cytokines were as follows: IL-10, (Sense) 5′-GGTTCCGACCTACAAGGTA-3′, (Antisense) 5′-GCTGGTGGTATACTGAGACAC-3′; INF-γ, (Sense) 5′-CCAATTTGGTGTTGTGG-3′ (Antisense) 5′-GCGGGTCTG-3′; IL-12 p40, (Sense) 5′-CAAGTTCTTGGGTCCTGCTGTC-3′ (Antisense) 5′-GGTCTATACTGACGAGCTG-3′; TNF-α, (Sense) 5′-CAAGTTCTTGGGTCCTGCTGTC-3′ (Antisense) 5′-GGTCTATACTGACGAGCTG-3′; IL-10, (Sense) 5′-GGTTCCGACCTACAAGGTA-3′, (Antisense) 5′-GCTGGTGGTATACTGAGACAC-3′; INF-γ, (Sense) 5′-GCTGGTGGTATACTGAGACAC-3′, (Antisense) 5′-GCGGGTCTG-3′; IL-12 p40, (Sense) 5′-CAAGTTCTTGGGTCCTGCTGTC-3′ (Antisense) 5′-GGTCTATACTGACGAGCTG-3′. The result was represented as an expression index calculated by the following formula: Expression index = (Band strength for a given cytokine in the presence of IgG1 or its digest) / (Band strength of the simultaneously treated GAPDH) (Band strength for a given cytokine in the absence of IgG1 and the digest).

2.7 Cell analysis by personal cell functional analyzer

The mouse spleen cell was incubated with mouse Fc block solution for 15 min at 4°C, and reacted with PE-labeled anti-mouse mAbs specific to CD4 or CD49b, or with biotin-labeled anti-mouse mAbs specific to CD8, CD19 or CD11b for 15 min at 4°C. Cells already reacted with the biotin-labeled antibody were further incubated with PE/Cy5-labeled streptavidin for 15 min at 4°C. Finally, all cells were analyzed using a Guava Personal Cell Function Analyzer (Guava Technologies, Hayward, CA, USA).

2.8 Statistical analysis

Immunoglobulin index and cell ratio were expressed as means ± standard deviation. The significance in the difference between cultures with and those without IgG1 or its digest was determined by Student's t-test.
3. Results and discussion

Figure 1 shows immunoglobulin indexes of supernatants from mouse spleen cells cultured for 72 h with IgGl and its digests. Both IgA and IgG indexes significantly increased in the culture with intact IgGl and tryptic digest. In addition, the former also significantly increased in chymotryptic and peptic-pancreatic digests as did the latter in peptic digest. IgA and IgG are the dominant immunoglobulins in gastrointestinal and systemic immune systems, respectively (1). The present results suggest that cow's IgGl, which is the most abundant immunoglobulin in bovine colostrum, plays a physiological role in the development of an active humoral immune system in addition to conferring passive immunity in newborn calves.

Figure 2 shows the expression index of mRNA for some cytokines of mouse spleen cells cultured for 12 h with IgGl and its digests. Intact IgGl showed more than a 2-fold increment of mRNA expressions for IL-5 and IL-6, and a 1.5- to 2-fold increment of mRNA expressions for IL-4, IFN-γ, IL-12 p40 and TGF-β, whereas little influence on expressions of mRNAs for IL-2 and IL-10. On the other hand, digests noticeably increased expressions of mRNAs for IL-4 and IL-6. In addition, peptic digest increased expressions of mRNAs for IL-2, IFN-γ and TGF-β, while tryptic and peptic-pancreatic digests increased the mRNA expression for IL-5. Peptic and chymotryptic digests increased expressions of mRNAs for TGF-β, whereas peptic-pancreatic digest markedly reduced expressions of mRNAs for IL-12 p40 and IL-10. Type 2 helper T cells produce IL-4, IL-5 and IL-6 (10). IL-4 is a one of B cell growth factors and stimulates IgG formation, while IL-5 promotes the survival of IgA B cells and their maturation into IgA secreting cells (11). IL-6 plays an important role in promoting the terminal differentiation of B cells to IgA secreting cells (12).

These facts provide at the cytokine level that bovine IgGl and its digests stimulate IgA and IgG formations in mouse spleen cell cultures.

Table 1 shows the ratios of CD4+ , CD8+ , CD19+ , CD11b+ and CD49b+ cells in mouse spleen cells cultured for 72 h with IgGl or its digests. Intact IgGl significantly increased the ratios of CD4+ and CD19+ cells, whereas it showed little influence on those of CD8+ , CD11b+ and CD49b+ cells. Tryptic, chymotryptic and peptic-pancreatic digests significantly increased the ratio of CD49b+ cells, in addition to those of CD4+ and CD19+ cells. On the other hand, the ratio of CD8+ cells significantly increased in cultures with tryptic, chymotryptic and peptic and chymotryptic digests, whereas no intact IgGl or its digests had any influence on CD11b+ cells. CD4 and CD19 are typical cell surface antigens of helper T cells and B cells, respectively (13, 14). At the immunocompetent cellular level, these facts support our above finding that intact IgGl and its digests stimulate IgA and IgG formations in mouse spleen cell cultures. On the other hand, CD8, CD11b and CD49b, which are typical cell surface antigens of cytotoxic T cells, monocytes/macrophages, and natural killer cells, respectively, are well known contributors to cellular immunity (15-17). Hence, digestion of cow's colostrum IgGl may produce peptides that regulate cellular as well as humoral immunity.

Antigen-presenting cells such as dendritic cells and macrophages possess some types of IgG receptors (FcγRI) on their cell surface. FcγRI and FcγRII stimulate immunoglobulin formation when IgG binds to the receptor, whereas FcγRIIb inhibits it. Moreover, FcγRII can interact with not only IgG-antigen complex but also with free IgG, with which FcγRIIb hardly interacts at all (15). Thus, the stimulation of IgG and IgA formations observed in the present study may be due to the binding of IgGl to FcγRI on antigen-presenting cells. Further study is in currently progress to clarify this point.

Fig. 1: Effects of IgGl and its digests on IgA (upper) and IgG (lower) formations in mouse spleen cell cultures. The results are represented as immunoglobulin indexes against cell cultures without IgGl and its digest. Values significantly differ from the IgGl and its digest-free culture at *P<0.05, **P<0.01 and ***P<0.001.

Fig. 2: Effects of IgGl and its digests on mRNA expressions of some cytokines in mouse spleen cell cultures. Cells were cultured for 12 h with or without IgGl and its digests. Numbers represent the expression index against the IgGl and its digest-free culture. A: IgGl and its digest-free culture, B: IgGl, C: Peptic digest, D: Tryptic digest, E: Chymotryptic digest, F: Peptic-pancreatic digest.

Table 1 shows the ratios of CD4+, CD8+, CD19+, CD11b+ and CD49b+ cells in mouse spleen cell cultures.
In conclusion, the present study demonstrates that, at the cytokine and immunocompetent cellular levels, IgG1 and its gastrointestinal proteinase digests stimulate the formation of immunoglobulin, particularly IgA, in mouse spleen cell cultures.

Acknowledgment
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5. References
(4) OHNUI, H., OTANI, H.: Milchwissenschaft, in press
(6) ZHANG, F. M., OTANI, H.: Milchwissenschaft 60 (2) 175-179 (2005)

Table 1: Ratios of several immunocompetent cells in the mouse spleen cell cultures with or without IgG1 and its digests

<table>
<thead>
<tr>
<th>Cell</th>
<th>Concentration (μg/ml)</th>
<th>Intact</th>
<th>Peptic</th>
<th>Tryptic</th>
<th>Chymotryptic</th>
<th>Pepsin-pancreatic</th>
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<tr>
<td>CD4⁺</td>
<td>0</td>
<td>17.40 ± 0.53</td>
<td>14.76 ± 0.29</td>
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<td>50</td>
<td>19.10 ± 0.72**</td>
<td>13.60 ± 0.30**</td>
<td>16.90 ± 0.60**</td>
<td>18.93 ± 0.51**</td>
<td>15.06 ± 0.55</td>
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<td>21.80 ± 0.45***</td>
<td>13.73 ± 1.21</td>
<td>14.80 ± 0.56</td>
<td>18.53 ± 0.83*</td>
<td>18.76 ± 0.81**</td>
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<tr>
<td>CD8⁺</td>
<td>0</td>
<td>6.79 ± 0.37</td>
<td>5.07 ± 0.16</td>
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<td>7.38 ± 0.54</td>
<td>5.23 ± 0.40</td>
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<td>100</td>
<td>7.48 ± 0.68</td>
<td>6.03 ± 0.21*</td>
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<td>CD19⁺</td>
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<td>42.90 ± 0.36</td>
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<td>42.90 ± 0.36</td>
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<td>37.23 ± 0.46**</td>
<td>47.93 ± 0.61***</td>
<td>43.96 ± 1.08</td>
<td>48.40 ± 1.01***</td>
<td>45.70 ± 0.56**</td>
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<td>100</td>
<td>40.96 ± 0.69***</td>
<td>54.16 ± 0.91***</td>
<td>44.90 ± 0.81***</td>
<td>56.46 ± 0.76***</td>
<td>45.26 ± 0.93**</td>
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<tr>
<td>CD11b⁺</td>
<td>0</td>
<td>9.23 ± 0.64</td>
<td>8.67 ± 0.81</td>
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<td>50</td>
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<tr>
<td>CD49b⁺</td>
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<td>3.43 ± 0.46</td>
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<td>4.97 ± 0.67*</td>
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<td>4.60 ± 0.46**</td>
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Values significantly differ from IgG1 and its digest-free culture at *P<0.05, **P<0.01 and ***P<0.001.