A humoral immunoregulatory mechanism of bovine milk immunoglobulin G *via* Fcγ receptors in mice

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Abstract

Oral ingestion of bovine milk immunoglobulin (Ig) G had been reported to suppress responses of serum and intestinal antigen-specific IgG and IgA in mice. In contrast, bovine milk IgG significantly stimulated production of IgG and IgA in mouse Peyer's patch cell cultures as well as spleen cell cultures. The milk IgG bound to some cells in the spleen cell cultures while the orally ingested milk IgG bound to some Peyer's patch cells. The binding of milk IgG to the spleen cells was strongly inhibited by anti-mouse FcγRI, whereas that of bacteria and milk IgG complexes prepared from intestinal contents of mice given orally milk IgG to Peyer's patch cells was suppressed noticeably by anti-mouse FcγRII/III. Moreover, a mixture of *Escherichia coli* and its non-specific milk IgG significantly increased ratios of $CD80^+CD11c^+$ and $CD83^+CD11c^+$ cells in mouse Peyer's patch cell cultures whereas a mixture of *Escherichia coli* and its specific milk IgG significantly reduced these ratios.

1. Introduction

Ruminants transfer many kinds of anti-infectious substances to neonates *via* milk. Hence, bovine milk contains a large quantity of immunoglobulin (Ig) G. Because of this, attempts have been made to use bovine milk IgG as a passive

immunomodulatory dietary ingredient. Several investigators have reported that bovine milk IgG specific to intestinal microorganisms protects animals including humans against intestinal infections (1).

The authors demonstrated that bovine milk IgG stimulated antibody responses in mouse spleen cell cultures (2), whereas oral ingestion of bovine milk IgG suppressed the response in mice (3). However, it is unclear why bovine milk IgG has different effects on antibody responses *in vitro* and *in vivo*.

Immunocompetent cells such as dendritic cells and macrophages possess several types of IgG receptors (FcγR) on their surface. FcγR Ⅰ and FcγRⅢ stimulate the formation of immunoglobulin when IgG binds to the receptor, whereas FcγRⅡb inhibits it. Moreover, FcγR Ⅰ may highly interact with monomeric IgG, while FcγRⅡb may little interact with antigen-free IgG (4). Therefore, the different effects of bovine milk IgG on antibody responses *in vitro* and *in vivo* may be due to the difference in Fcγ receptors for milk IgG on immunocompetent cells.

This study was carried out in order to clarify the different effects of bovine milk IgG on mouse antibody responses in spleen cell cultures and following oral ingestion.

2. Materials and methods

This experiment was conducted in accordance with the guidelines for the Regulation of Animal Experimentation at the Faculty of Agriculture, Shinshu University, and according to Law No. 105 and Notification No. 6 of the Japanese government.

2.1 Materials

Anti-bovine IgG1 was obtained from Bethyl Laboratories (Montgomery, TX,

USA). Phycoerytherin/cyanine 5 (PE/Cy5)-labeled streptavidin, PE-labeled anti-mouse CD80, biotin-labeled anti-mouse CD11c (clone N418) and anti-mouse CD32/16 (clone 93) were purchased from BioLegend (San Diego, CA, USA). Anti-mouse CD64 was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-mouse CD83 was from R & D systems (Minneapolis, MN, USA). Iodoacetic acid sodium salt, 2-iminothiolane hydrochloride and PE were purchased from Merck (Hohenbrunn, Germany), MP Biomedicals (Solon, OH、USA) and Far East Bio-Tec (Taiwan, China), respectively. PE-labeled anti-bovine IgG1 was prepared according to the procedure of Vernot *et al*. (5). RPMI-1640 medium was purchased from Nissui pharmaceutical (Tokyo, Japan). Penicillin G potassium and streptomycin sulfate were obtained from Meiji Seika (Tokyo, Japan). IgG-stripped fetal bovine serum (FBS) was purchased from Quest Biomedical (West Midlands, UK). Other chemicals were of the highest analytical grade commercially available.

2.2 Immune milk and milk IgG

Immune milk, which was produced from cows immunized with a killed bacterial mixture containing *Esherichia coli* was kindly provided by Kanematsu Wellness Corporation (Tokyo, Japan). Whey powder was prepared from the immune milk, and milk IgG was prepared from 33%-saturated ammonium sulfate precipitates of the immune milk whey by anion-exchange chromatography (6). Milk IgG was confirmed by immunoblotting analysis to consist of 95.9% IgG1 and 4.1% IgG2 using antibodies specific to bovine IgG and its subclasses after polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate (7).

2.3 Cell cultures

Six-week-old male C3H/HeN mice were obtained from Japan SLC

(Hamamatsu, Japan). Spleen cell suspensions were prepared according to a procedure described previously (2). Peyer's patch cell suspensions were prepared from mice orally given or not given milk IgG. In brief, a mixture of 24 mg of milk IgG and 180 mg of immune milk whey was dissolved in 0.6 ml of distilled water. The solution was injected into mouse stomachs by using an oral sonde needle. After 0, 0.5, 1, 2, 3, 6 and 12 h of the injection, Peyer's patches were recovered from mice sacrificed with an overdose of ether. The Peyer's patch cell suspension was prepared according to the procedure of TAGLIABAUE *et al*. (8), and adjusted to 1×10^6 viable cells/ml. A Peyer's patch cell suspension was also prepared from mice not given the mixture of milk igG and immune milk whey.

The Peyer's patch cells were cultured with milk IgG (0 to 100 μg/ml) at 37 °C in RPMI-1640 medium containing 5% IgG-stripped FBS, penicillin (100 U/ml), and streptomycin (100 μg/ml). After 2 h cultivation, the binding of IgG to cells was assayed with a personal cell functional analyzer, or GUVA PCA (Guava Technologies, Hayward, CA, USA). After 72 h , antibody levels in the culture supernatants were determined by sandwich enzyme-linked immunosorbent assay (ELSA).

2.4 Preparation of bacteria and milk IgG complexes

Mice were orally given the mixture of milk IgG and immune milk whey as described above, and after 2 h, intestinal (duodenum to appendix) contents were recovered. The material was suspended in distilled water, and the suspension was centrifuged at 1300 x g for 3 min. The supernatant was collected and then centrifuged at 5700 x g for 3 min. The precipitate was washed three times with HBSS, and suspended in HBSS. The number of bacteria in the suspension was adjusted to 1×10^7 CFU/ ml. The amount of milk

IgG was determined by Guava PCA using PE-labeled anti-bovine IgG1 which bound about 16.4 % of the bacteria in the suspension.

Alternatively, a mixture of milk IgG (10 mg/ml HBSS) and *Escherichia coli* NBRC 3301 (1×10^8 CFU/ml HBSS) was incubated for 1 h at room temperature, and washed three times with HBSS. The washed mixture was used as a mixture of *E. coli* and *E. coli* -specific milk IgG.

2.5. Preparation of E. coli-non-specific milk IgG

A mixture of milk IgG (10 mg/ml HBSS) and E. coli (1×10^{11} CFU/ml HBSS) was incubated for 1 h at room temperature. After the incubation, the mixture was membrane-filtrated with a disposable syringe filter unit (DISMIC-25cs, Advantec, Tokyo, Japan). The filrate was confirmed not to contain any *E. coli*, and used as *E. coli*-non-specific milk IgG.

2.6 Antibody assay

The sandwich ELISA for determination of IgG and IgA levels in cell culture supernatants was carried out as described previously (9). The immunoglobulin level was represented as an antibody index calculated with the following formula: Antibody index = ELISA value (A 490 nm) for culture with milk IgG/ ELISA value (A 490 nm) for culture without milk IgG.

2.7 Assays for determining the binding of milk IgG to cells and expression of specific molecules (CD11c, CD80 and CD83) on cells

The binding of milk IgG and expression of specific molecules on cells were evaluated by Guava PCA after immunostaining with the appropriate antibodies.

2.8 Statistical analysis

Data were expressed as the means ± standard deviation. The significance of differences was tested with Student's *t*-test.

3. Results

3.1 Effects of milk IgG on the secretion of IgG and IgA by mouse spleen cells and Peyer's patch cells

Spleen cells and Peyer's patch cells from mice which had not been given milk IgG were cultured with the IgG for 72 h, and levels of mouse IgG and IgA in culture supernatants were determined. As shown in Fig. 1, milk IgG significantly stimulated the secretion of IgG and IgA in both the cell cultures. The stimulatory effect of milk IgG was grater for IgA than for IgG. The IgA level in Peyer's patch cell cultures was approximately 2-fold higher in the presence (100 μg/ml) than absence of milk IgG.

Fig. 1:

3.2 Detection of milk IgG on mouse spleen cells and Peyer's patch cells

Spleen cells from mice which had not been given milk IgG were incubated with the IgG, and stained with PE-labeled anti-bovine IgG1. As shown in Fig. 2a, the relative fluorescence intensity of the cells incubated with milk IgG was at least 10 times higher than that in the absence of milk IgG.

Fig. 2:

Similarly, Peyer's patch cells prepared from mice orally given milk IgG were stained with PE-labeled anti-bovine IgG1. As shown in Fig. 2b, the relative fluorescence intensity of some Peyer's patch cells of mice orally given milk IgG was about 10 times higher than that of cells from mice not given milk IgG.

3.3 Effects of anti-mouse CD64 and anti-mouse CD32/16 on the binding of milk IgG to spleen cells or Peyer's patch cells

Spleen cells from mice which had not been given milk IgG were incubated with the IgG in RPMI-1640 medium in the presence of anti-mouse CD64 or anti-mouse CD32/16, and then stained with PE-labeled anti-bovine IgG1. As shown in Fig. 3a, the relative fluorescence intensity of the spleen cells incubated with milk IgG was obviously decreased by the addition of anti-mouse

Fig. 3:

CD64, but little changed by the addition of anti-mouse CD32/16. Similarly, the relative fluorescence intensity of Peyer's patch cells, which had been prepared from mice not given milk IgG, incubated with the IgG clearly decreased on the addition of anti-mouse CD64, but changed little with the addition of anti-mouse CD32/16 (data not shown).

Peyer's patch cells were incubated with bacteria and a milk IgG complex prepared from mouse intestinal contents in the presence of anti-mouse CD64 or anti-mouse CD32/16, and stained with PE-labeled anti-bovine IgG1. As shown in Fig. 3b, the relative fluorescence intensity of the cells incubated with the bacteria and milk IgG complex was noticeably reduced in the presence of anti-mouse CD32/16 but little changed in the presence of anti-mouse CD64.

3.4 Effects of mixtures of E. coli and E. coli-specific or non-specific milk IgG on the expression of CD80 and CD83 on CD11c$^+$ cells

Peyer's patch cells from mice which had not been given milk IgG were incubated with mixtures of *E. coli* and *E. coli*-specific or non-specific milk IgG, and proportions of CD80$^+$CD11c$^+$ or CD83$^+$CD11c$^+$ cells were determined by Guava PCA. As shown in Fig. 4, the mixture of *E. coli* and the specific IgG had significantly reduced ratios of CD 80$^+$CD11c$^+$ and CD83$^+$CD11c$^+$ cells whereas the mixture of *E. coli* and the non-specific IgG had significantly increased ratios of both the cells. In particular, the proportion of CD80$^+$CD11c$^+$ cells increased about 1.3-fold with the addition of *E. coli* (1×10^6 CFU/ml) and the non-specific milk IgG (200 μg/ml), whereas that of CD83$^+$CD11c$^+$ cells was reduced about half by the addition of *E. coli* (1×10^7 CFU/ml) and the non-specific IgG (400 μg/ml).

Fig. 4:

4. Discussion

Previously, the authors demonstrated that milk IgG stimulated production of IgG and IgA in mouse spleen cell cultures, whereas oral ingestion of the IgG suppressed the antibody response in mice (2, 3). Orally ingested proteins are known to affect gut-associated lymphoid tissues (GALT) such as Peyer's patch, intraepithelium, and lamina propria (10). Hence, the different effects of milk IgG on the antibody responses *in vitro* and *in vivo* might be due to the difference in immunocompetent materials used, i.e., the spleen versus Peyer's patches. The secretion of IgG and IgA, however, was significantly stimulated in both the spleen cell culturesand Peyer's patch cell cultures (Fig. 1). Thus, the different effect is concluded not to be attributable to the difference in immunocompetent mterials.

There are several types of IgG receptors, FcγRI, FcγRII, FcγRIII, etc., on immunoconpetent cells (11). These distinct classes of Fcγ receptors include CD64 (FcγRI), CD32 (FcγRII) and CD16 (FcγRIII). CD64 , a 70kDa cell surface glycoprotein with high affinity for monomeric IgG, is expressed constitutionally on some cells including monocytes. The low-affinity receptors, FcγRII and FcγRIII, have a putative role in mediating humoral immune responses (11). In the present study, milk IgG was demonstrated to bind to mouse spleen cells and Peyer's patch cells (Fig. 2). The binding of antigen-free milk IgG to the cells was inhibited by the addition of anti-mouse CD64 (Fig. 3a), while that of bacteria and milk IgG complex to Peyer's patch cells was suppressed by the addition of anti-mouse CD32/16 (Fig. 3a). These results suggest that antigen-free milk IgG predominantly binds to FcγRI whereas bacteria-bound IgG predominantly binds to FcγRII/III. On the other hand, a mixture of *E.coli* and non-specific milk IgG increased the expression of CD80 and CD83 on $CD11c^+$ cells whereas a mixture of *E. coli a*nd *E. coli* -specific milk IgG suppressed the expression (Fig.

4). CD11c is part of the integrin family and expressed on monocytes including dendritic cells. Dendritic cells are professional antigen presenting cells (12). Dhodapkar *et al.* (13) reported that the expression of CD80 and CD83 on dendritic cells was suppressed by the blockage of FcγRIIb. Oki *et al.* (14) demonstrated that CD80 was essential for humoral immune responses. These findings suggest that antigen-free IgG stimulates the production of immunoglobulin by binding to FcγRI while antigen-bound IgG suppresses it by binding to FcγRIIb.

In conclusion, the different effects of milk IgG on mouse antibody responses in spleen cell cultures and by following administration may be attributable to the difference in receptors on accessory cells for milk IgG. In the cell culture, milk IgG is antigen-free and binds to accessory cells *via* FcγRI, and increases immunoglobulin production *via* the stimulation of CD80 expression. In contrast, milk IgG forms antigen-antibody complexes with intestinal bacteria when milk IgG is orally given in mice. The antigen-bound milk IgG binds to accessory cells *via* FcγRIIb, and suppresses the immunoglobulin production *via* the inhibition of CD80 expression.

Acknowledgements

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5. References

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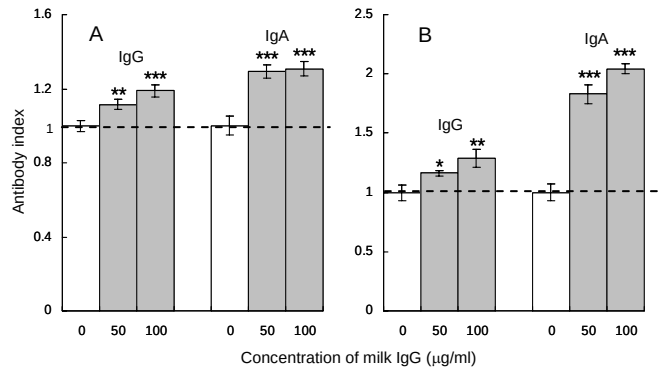

Fig. 1: Effects of milk IgG on production of IgG and IgA in mouse spleen cell (A) and Peyer's patch cell (B) cultures. The spleen cells and Peyer's patch cells were cultured for 72 h. Values are significantly different from those without milk IgG at *P<0.05, **P<0.01 and ***P<0.001.

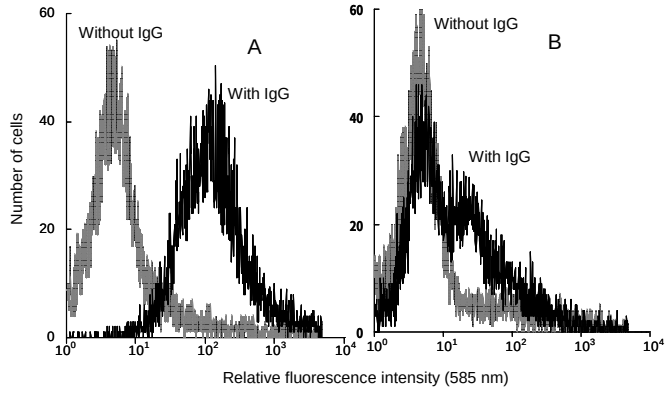

Fig. 2: Relative fluorescence intensity due to milk IgG on mouse spleen cells, which had been prepared from mice not given milk IgG, cultured with the IgG (A) and on Peyer's patch cells, which had been prepared from mice orally given milk IgG (B).

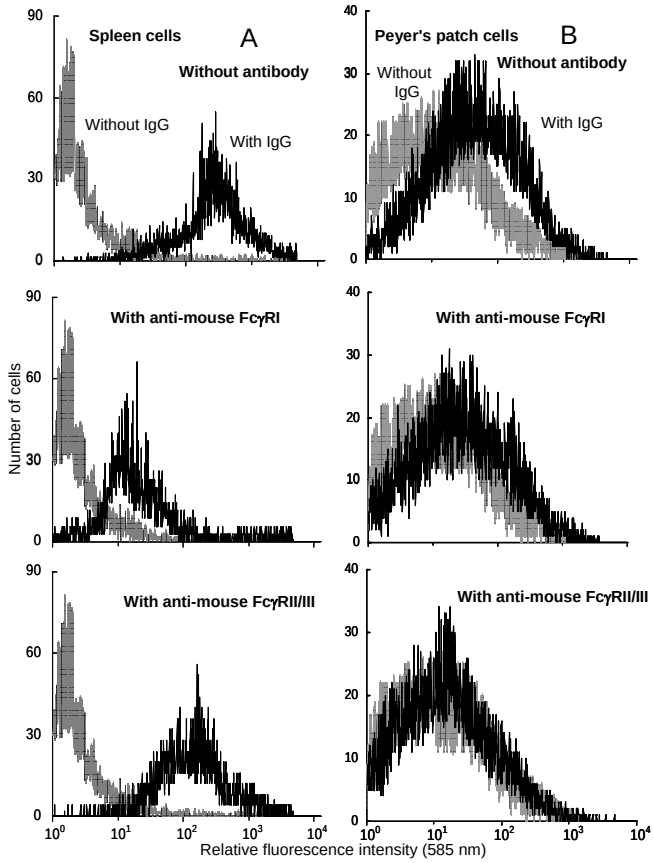

Fig. 3: Effects of anti-mouse Fc receptors on relative fluorescence intensity due to milk IgG on mouse spleen cells cultured with the IgG (A) and on Peyer's patch cells cultured with bacteria and milk IgG complex, which had been prepared from mice orally given milk IgG (B).

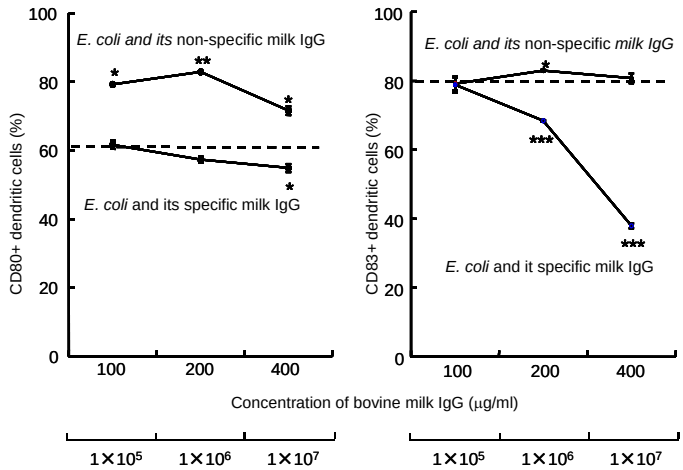

Fig. 4: Effects of *E. coli* and its specific or non-specific milk IgG on ratios of $CD80^+CD11c^+$ and $CD83^+CD11c^+$ cells in mouse Peyer's patches.