The effect of horse milk lactoferrin on mice cellular immune response:
Lymphocyte proliferation and interleukin production

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Abstract

Lactoferrin (Lf) is an iron-binding glycoprotein, has been known as a nonspecific immunomodulator. The aims of this research were to study the effect of horse milk lactoferrin on mice lymphocyte proliferation and interleukin (IL-2, IL-4) production. Female Balb/c mice 6-8 weeks old, fed horse milk lactoferrin 1.0 mg/mouse/day, in assay period of 28 days. On the 7th, 14th, 21st, and 28th day, lymphocyte proliferation was assayed with MTT, IL-2 and IL-4 were analyzed by ELISA. Result indicate, lymphocyte proliferation of mice fed horse milk lactoferrin was higher than the control and bovine milk lactoferrin on the day of 14th, whereas lymphocyte proliferation on the 28th day of mice fed bovine milk lactoferrin was higher than horse milk lactoferrin and the control. Level of IL-2 and IL-4 in lymphocyte culture, and IL-4 in serum of mice fed horse milk lactoferrin, were higher than the control, whereas serum IL-2 was undetectable.

Keywords: Horse milk lactoferrin, lymphocyte proliferation, interleukin production

Introduction

Horse milk and horse milk products have been known as health food. However, the scientific basis to support this practice has not been proofed, particularly the horse milk lactoferrin as an immunomodulator. Lactoferrin (Lf) is an iron-binding 80 kDa glycoprotein, belongs to the transferrin family that is present in leukocytes, and many exocrine secretions (e.g. milk, saliva, tears, mucosal and genital secretions) (Karthikeyan et al., 1999). Lactoferrin is an example of a minor milk protein (Steijns, 2001), has a very broad range of biological functions relating to the host defense system and thus, is a multifunctional protein (Shimazaki et al., 1998). Biological activity of lactoferrin is directly related to its three major structural properties: 1) it is organised in two homologous lobes, with one iron-binding site each, 2) the presence of glycosylation sites, and 3) the highly basic N-terminus mediates the binding to several eukaryotic and prokaryotic structure (Aguila and Brock, 2001). The biological function of lactoferrin e.g. as antimicrobial, antiviral, antioxidant, and an immunomodulator (Brink, 2000).

Domain arrangements in the transferrin proteins to be an important structural feature related to their specific biological functions. Based on the structures of transferrin, it can be stated that the native apoproteins of transferrin family adopt three forms: 1) with both the N and the C lobes closed, as observed in horse apolactoferrin, 2) with the N lobe open and the C lobe closed, as observed in human apolactoferrin, and 3) with both the N and the C lobes open, as found in duck apotransferrin (Sharma et al., 1999). Apolactoferrin and partially saturated N-lobe forms being predominant in vivo (Aguila and Brock, 2001).

The content and composition of lactoferrin glycan greatly varies according to species (Karthikeyan et al., 1999, McAbee and Walsh, 2000), and the glycosylation sites is related to resistance to mucosal proteases (Aguila and Brock, 2001). The amino acid
sequence of lactoferrin that is involved in receptor binding is important in the interaction with its receptor (Schambacher et al., 1993), because immunomodulation function of lactoferrin is mediated by receptor in intestinal brush border, lymphocytes and macrophages (Yuki et al., 1998).

Naturally ingested food components can potentially interact with a variety of lymphoid cells along the gastrointestinal tract. Several factors can influence these interactions, including the solid or liquid form of the food component and the oral modes of exposure. Immunomodulation requires intact molecules or active peptides capable of reaching immunocompetent cells (Steir et al., 2004).

The effect of bovine and human lactoferrin on the immune system have been observed (Yuki et al., 1998; Debbabi et al., 1998). However, the effect of horse milk lactoferrin in immune response have not been studied. Horse milk lactoferrin is expected to be a functional food or nutraceuticals that enhance the immune response.

Materials and Methods

Isolation of horse milk lactoferrin

Milk from local horse in Yogyakarta, was defatted by centrifugation. Horse milk lactoferrin was isolated from horse skin milk on ion exchange chromatography and gel filtration according to Sharma et al. (1999).

Lymphocyte proliferation assay

Mice spleens were removed, suspended in RPMI medium, and crushed by a syringe plunger, tissue debris was removed. The suspension was centrifuged, supernatant was removed. The spleen cells were resuspended with ammonium chloride solution to lysed red blood cells. Spleen cells were washed 2x with RPMI medium, and resuspended with complete medium (RPMI + FBS), and counted to the final concentration of 5 x 10^6/ml. (2.5 x 10^6 / well). Lymphocyte were cultured on microplate 96 well (200 µl/well). PHA mitogen were added to the final concentration of 5 µg/ml or Con-A 40 µg/ml (10 µl/well). Microplate was incubated at 5 % CO2 for 72 hours at 37 °C. Methyl tetrazolium (MTT) solution (5 mg/ml dissolved in PBS) was added 10 µl/well on microplate, and incubate for 4 h at 5 % CO2, incubator. Ten percent SDS in 0.01 M HCl was added on microplate well (100µl/well) and incubate overnight at room temperature. Read the optical density (OD) of microplate culture at 550 nm in microplate reader (Wijayant, 1996). Stimulation index (SI) of lymphocyte was calculated by dividing the average of OD value in mitogen-stimulated cultures by the average OD value in unstimulated cultures (Loa et al., 2001).

IL-2 and IL-4 analysis in lymphocyte culture and serum

Add 50 µl of Plate Reagent to each well which had been coated with Anti-Mouse IL-2 or Anti-Mouse IL-4. Add 50 µl of Standards (Mouse IL-2 or Mouse IL-4) or Samples to each well in duplicate. Cover plate and incubate at 37°C for 2 hours in humidified incubator. Wash plate 5x with Washing buffer. Add 100 µl of prepared Conjugate Reagent (Anti IL-2/ Anti IL-4- HRP Conjugate Reagent) to each well. Cover plate and incubate at 37°C for 1 hour in humidified incubator. Wash plate 5x with washing buffer. Add 100 µl Premixed TMB (tetramethyl benzidine) Substrate Solution to each well. Develop plate in the dark at room temperature (20-25°C) for 30 minutes. Stop reaction by adding 100 µl of Stop Solution to each well. Measure absorbance on a plate reader at 450 – 500 nm. Calculate the results using graph paper or curve-fitting statistical software (Anonymous, 2000).

Results and Discussion

The assay of lymphocyte proliferation showed that stimulation index using PHA mitogen was higher than Con-A mitogen (P<0.05). Stimulation index of mice lymphocyte fed horse milk lactoferrin and control with PHA mitogen were: 1.2911; 1.2250 respectively, whereas stimulation index of lymphocytes fed horse milk lactoferrin and control with Con A mitogen were: 0.9234; 0.9025 respectively. Concanavalin-A mitogen is specific for high mannose type

Figure 1. Stimulation index (SI) of lymphocyte in mice fed horse milk lactoferrin with PHA and Con-A mitogen.

Figure 2. Stimulation index (SI) of lymphocyte in mice fed horse milk lactoferrin with PHA mitogen.

glycan, whereas PHA mitogen is specific for complex type glycan. Horse milk lactoferrin has complex type glycan (Nishiyani, 2004), therefore PHA can bind that glycan and then horse milk lactoferrin bind their receptor on the surface of lymphocyte. Interaction between PHA, horse milk lactoferrin, and lactoferrin receptor leads lymphocyte was stimulated. Lactoferrin have specific receptor in the brush border intestinal, macrophage, and lymphocyte (Yuki et al., 1998). The other possibility, the role of lactoferrin on lymphocyte proliferation may be the result of lactoferrin binding to the specific DNA sequences and transcriptional activation. Specific binding of lactoferrin to the cell surface was followed by rapid internalization and subsequent localization of lactoferrin into the nucleus. The binding of lactoferrin to the promotore regions of specific genes is known to stimulate transcription of the genes (Fleet, 1995).

The proliferative response of mice fed horse milk lactoferrin on the day of 14th was higher than mice fed bovine milk lactoferrin with PHA mitogen. However, the proliferative response of mice fed bovine lactoferrin on the day of 28th was higher than mice fed horse milk lactoferrin and the control mice. Stimulation index of mice lymphocyte with PHA mitogen fed horse milk lactoferrin, bovine lactoferrin, and control on the day of 28th were 1.3512; 1.5495; 1.3562 respectively. On the day of 14th, horse milk lactoferrin stimulate of proliferative response, whereas the effect of bovine milk lactoferrin on proliferative response occur in the day of 28th. That differences of proliferative response may be the difference of the conformation between horse milk lactoferrin and bovine milk lactoferrin. The horse milk spolactoferrin has the closed conformation, with both the N and C lobes in closed forms. Therefore, horse milk lactoferrin was more stable than the bovine milk lactoferrin from proteolytic degradation in the gastrointestinal tract. Consequently, interaction with the immunocompetent cells was higher in the horse milk lactoferrin than bovine milk lactoferrin, and the result the proliferative response was faster in the lymphocyte of mice fed horse milk lactoferrin than bovine milk lactoferrin. The N lobe of three-dimensional structure of horse divergent lactoferrin appears to be well ordered and is more stable than the C lobe unlike in other lactoferrins, where the C lobe is the more
stable. In the absence of iron, each lobe of lactoferrin molecule can flex, allowing the cleft to open and shut, but when iron is bound, the cleft is locked shut (Brock, 1995). Thus, iron saturation enhances the conformational stability and resistance to denaturation (Agullia and Brock, 2001) and proteolytic degradation compared to the apo-form (Lounerdal and Iyer, 1995). However, the conformation of both lobes in the horse milk lactoferrin as determined to be in the closed conformation, similar to its iron-saturated forms (Sharma et al., 1999). Aplacotactin and partially saturated N-lobe forms being predominant in vivo (Agullia and Brock, 2001). In vitro T cell responses by spleen cells from mice treated for 6 weeks with 4.0 mg of bovine milk lactoferrin by intragastric gavage, and cultured for 48 h alone or in the presence of bovine milk lactoferrin or Con-A, showed higher thymidine uptake than the control mice (Steir et al., 2004). In contrast, bovine milk lactoferrin inhibits proliferative response of TH1 but not TH2 cell lines (Zimecki et al., 1996). The effect of lactoferrin on lymphocyte proliferation may be different between fresh lymphocyte from mice fed lactoferrin and cell lines.

The level of IL-2 and IL-4 in lymphocyte culture supernatant of mice fed horse milk lactoferrin were higher than the control mice (P<0.05). Level of IL-2 in lymphocyte culture of mice fed horse milk lactoferrin on the day of 7th, 14th, 21st and 28th were: 704.16; 496.43; 739.92; 321.38 pg/ml, respectively, whereas the IL-4 were: 376.93; 406.29; 333.77; 262.04.

Figure 3. Level of IL-2 in lymphocyte culture of mice fed horse milk lactoferrin.

Figure 4. Level of IL-4 in lymphocyte culture of mice fed horse milk lactoferrin.

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Horse milk lactoferrin was higher than the control mice (P<0.05). Level of serum IL-2 on the day of 7th, 14th, 21st and 28th were: 170.08; 143.68; 104.30; 151.80 pg/ml, respectively. The horse milk lactoferrin can stimulate production of IL-2 and IL-4. This study was similar to the study by Steir et al. (2004), that bovine milk lactoferrin can increase of lymphocyte proliferation and production of cytokine TH1 (IL-2) and TH2 (IL-4) with Con A mitogen. The level of IL-2 in lymphocyte culture was higher than IL-4, because IL-2 was produced for T cell growth factor.

Figure 5. Level of IL-4 in serum of mice fed horse milk lactoferrin.

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