Effect of IGF-I Rich Fraction from Bovine Colostral Whey on Murine Immunity

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ABSTRACT : Insulin-like growth factor-I (IGF-I) rich fraction, collected components between 1 kDa and 30 kDa, was fractionated from bovine colostral whey using an ultrafiltration membrane. IGF-I was confirmed in the collected IGF-I rich fraction by both SDS-PAGE and Western blotting. The concentration of IGF-I in the IGF-I rich fraction was 10 ng/mg protein. One hundred microliters of the reconstituted IGF-I rich fraction was intraperitoneally injected into ICR male mice for 2 weeks at 24 h intervals. The functions of peritoneal macrophages, including phagocytosis, interleukin (IL)-6 and tumor necrosis factor (TNF)-α production, and nitric oxide and hydrogen peroxide production, were enhanced significantly by the administration of the IGF-I rich fraction in a dose-dependent manner ($p<0.01$). The proliferation of Concanavalin (Con) A-stimulated and Lipopolysaccharide (LPS)-stimulated splenocytes was also determined to have been enhanced significantly by the administration of the IGF-I rich fraction in a dose-dependent manner ($p<0.01$). Our results indicate that the administration of IGF-I rich fraction obtained from bovine colostral whey enhances both innate and acquired immunity for ICR male mice. ***(Asian-Aust. J. Anim. Sci. 2006. Vol 19, No. 2 : 297-304)***

Key Words : IGF-I, Colostrum, Macrophage, Splenocyte, Immunity

INTRODUCTION

Colostrum is a nutritious complex biological fluid, and contains abundant biologically active components, such as growth and antimicrobial factors (Playford et al., 2000; Wenjun et al., 2004). Polypeptides, so-called milk growth factors (MGF), including insulin-like growth factor-I, II (IGF-I, II), epidermal growth factor (EGF), transforming growth factor (TGF), and platelet-derived growth factor (PDGF) are seen in much higher levels in the colostrum than in normal milk (Pakkanen and Alto, 1997). Among the MGFs, IGF-I plays a crucial role in the maturation of the gastrointestinal tract during infancy (Simmens et al., 1988).

IGF-I is known to be involved in such activities as wound healing, DNA replication, and cell proliferation and differentiation (Clemmons, 1998; Uruakpa et al., 2002). IGF-I is also an important growth factor with regard to the regulation of the immune system and inflammation (Heemskerk et al., 1999).

In this study, we investigated the effects of peritoneally injected IGF-I rich fraction on the production of cytokines and chemicals in the murine peritoneal macrophage, as well as splenocyte proliferation and NK cell activity in the mouse splenocyte.

MATERIAL AND METHODS

IGF-I rich fraction

Holstein colostrums, which were used to fractionate the IGF-I rich fraction, were collected within 24 h after parturition. The colostrum samples were centrifuged at 5,000 g and 4°C for 30 min. in order to remove the milk fat. Recovered skim milk was then acidified to pH 4.5 with 1N HCl, and then centrifuged at 5,000 g and 5°C for 30 min. According to Hossner and Yemm (2000), the recovered whey was passed through a 30 kDa and 1 kDa ultrafiltration cartridge (prep/scale-TFT, Millipore, USA) in order to separate the IGF-I rich fraction, which contains free-form IGF-I. All steps applied in the acquisition of the IGF-I rich fraction were conducted at a temperature of under 4°C. IGF-I in the ultrafiltered fractions was confirmed by both SDS-PAGE (Laemmli, 1970) and Western blotting (Hossenlopp et al., 1984). IGF-I content was measured by Sandwich enzyme-linked immunosorbent assay (Battelli et al., 1999).

Animals and injection of IGF-I rich fractions

ICR male mice (4 weeks old) were acquired from Orient (Korea), and five mice per group were randomly assigned to filtered cages in an environmentally controlled atmosphere (temperature 22°C; 55% relative humidity), with a 12-h light and 12-h dark cycle. Mice were fed on a standard diet (Pruina Korea, Korea) for 1 week prior to the injection of samples. After a 1 week adaptation period, the mice received intraperitoneal injections with 100 μl of reconstituted IGF-I rich fraction, IGF-I, and colostral whey in which protein levels were 0.001, 0.01, 0.1 and 1 μg, respectively, once per day for 2 weeks.

Tissue preparation

Peritoneal macrophages were obtained by the method described by Klimetzek and Remold (1980). Mice were

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anaesthetized with ether, after which time, the sterilized PBS was injected into the abdominal cavity. Then, sufficient peritoneal macrophages were harvested and then resuspended in RPMI 1640 containing 10% FBS. The suspended macrophages were cultured in a Teflon-coated culture dish for two hours at 5% CO_2, 37°C, and 95% relative humidity. Peritoneal macrophages prepared in this manner was washed twice with PBS. Macrophages detached by 300 μl 0.1 M EDTA were then resuspended in RPMI 1640, and 200 μl of suspended Mφ, 1×10^5 cells/well, was plated onto a 96-well microplate.

The spleens were aseptically removed, and placed in sterile endotoxin-free RPMI 1640 (Gibco, USA) media supplemented with 25 mM HEPES (Gibco, USA), 2 mM glutamine (Gibco, USA), 100 U/ml of penicillin, and 100 μg/ml of streptomycin (Gibco, USA) (Holsapple et al., 1984). Single cell suspensions were prepared by gentle disruption of the spleens between two sterile frosted slide glasses. The splenocytes were then isolated by centrifugation at 400×g, and the red blood cells were lysed with Gey's reagent. The splenocytes were washed twice with RPMI 1640, and viability was determined by trypan blue exclusion method. The splenocytes were suspended in RPMI 1640 with 10% heat-inactivated FBS. Then, 200 μg of suspended splenocytes, at 5×10^5 cells/well, were plated onto 96-well microplates.

Determination of phagocytosis

Phagocytosis of peritoneal macrophages was measured by zymosan particle and NBT (4-nitroblue tetrazolium chloride) reduction, as was described by Okimura et al. (1986) and Stossel (1973).

IL-6 measurement

Peritoneal macrophages were isolated and plated (1×10^5 cells/well) on 96-well microplates as described by Ding et al. (1988). Microplates were incubated for 18 h at 5% CO_2, 37°C, and 95% relative humidity, after which the supernatants were collected and stored at -70°C for analysis. IL-6 content was measured by sandwich ELISA assay.

TNF-α measurement

TNF-α was assayed as described by Flick and Girfford (1984). Peritoneal macrophages were isolated and plated (1×10^5 cells/well) on 96-well microplate, according to the method described by Ding et al. (1988).

Microplates were incubated for 24 h at 5% CO_2, 37°C, and 95% relative humidity, after which time, the supernatants were mixed with L929 cells and actinomycin D. TNF-α production was described as the death rate of L929 cells.

Nitric oxide (NO) measurement

As described by Ding et al. (1988), the microplates were incubated for 18 h at 5% CO_2, 37°C, and 95% relative humidity, after which time, the NO in the supernatant was measured according to the method described by Griess (Green et al., 1982).

Hydrogen peroxide measurement

Hydrogen Peroxide production from the peritoneal macrophages was measured by the method of Nathan and Root (1977), with slight modifications. Peritoneal macrophages were isolated and plated (1×10^5 cells/well) on 96-well microplates. The microplates were then incubated for 2 h at 5% CO_2, 37°C, and 95% relative humidity, after which, 50 μl of zymosan (1 mg/ml) was supplemented and incubated for 1 h, in order to allow the peritoneal macrophages to uptake zymosan. Then, 100 μl of fresh media and 50 μl of phenol red were added to the microplate, and incubated for 1 additional hour under the same incubation conditions, after which the optical density was measured at 540 nm, by an ELISA microplate reader. OD reduction was found to be proportional to hydrogen peroxide production. Hydrogen peroxide (%) levels were expressed as a percentage of control group values.

Splenocyte proliferation

Isolated splenocytes were plated at 5×10^5 cells/well on 96-well microplates. The microplates were incubated in the presence of T cell mitogen (Concanavalin A, 2.5 μg/ml) and B cell mitogen (Lipopolysaccharide, 10 μg/ml) for 48 h at 5% CO_2, 37°C, and 95% relative humidity (Landreth et al., 1992).

Determination of natural killer cell activity

Natural killer cell activity was measured according to its cytotoxicity to YAC-1 cells, as described by Ortaldo and Hermerman (1984) and calculated as follows:

$$\text{Cytotoxicity (\%)} = \frac{100-[(\text{NK cell abs}+\text{YAC-1 abs})-(\text{NK cell abs})]\times100}{\text{YAC-1 abs}}$$

Statistical analysis

Data were analyzed by Duncan's multiple range tests, using the SPSS ver. 10.0 package program. Data were reported as the means ± SEM. Significance was set at $p<0.01$ and <0.05.

RESULTS

Identification of IGF-I rich fraction and IGF-I assay

The 7,649 Da molecular weight of free IGF-I was identified in the fraction obtained between the 1 kDa and 30

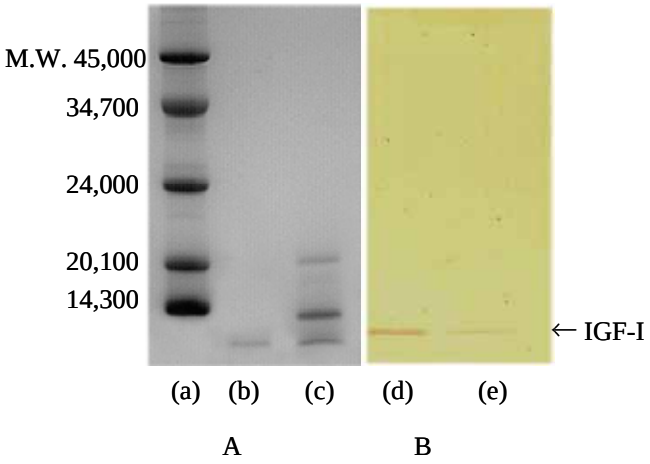

Figure 1. SDS-PAGE (A) and Western blotting (B) patterns of IGF-I rich fraction. (a): Low molecular weight marker (b): Standard IGF-I, (c): IGF-I rich fraction (d): Standard IGF-I (e): IGF-I rich fraction.

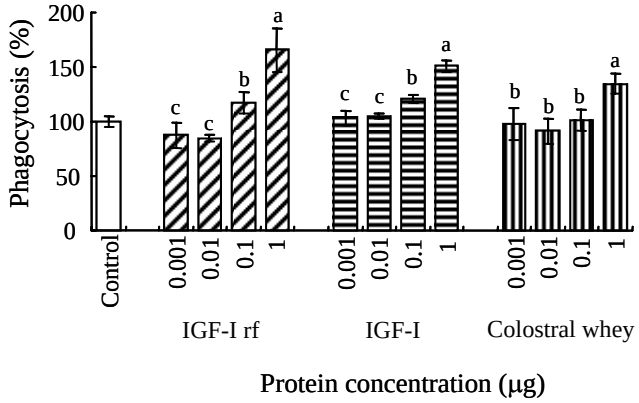

Figure 2. The effects of intraperitoneally injected IGF-I rich fraction separated from the colostral whey by ultrafiltration on phagocytosis production from murine peritoneal macrophages. Different letters on bars mean significant difference in same sample at $p<0.01$.

kDa ultrafiltration membrane by both SDS-PAGE (Figure 1A) and Western blotting (Figure 1B). As shown in Figure 1A, proteins smaller than 20 kDa were found, but proteins larger than 30 kDa were not found in the IGF-I rich fraction. These indicated that proteins larger than 30 kDa were effectively removed during the process of free IGF-I fractionation by ultrafiltration. Hossner and Yemm (2000) also reported that proteins smaller than 30 kDa and corresponding to IGF-I could be identified in the permeate recovered from diafiltration, using a membrane filter of 30 kDa cut-off molecular weight. Therefore, ultrafiltration applied to the separation of IGF-I was determined to be the appropriate method for fractionation of the IGF-I rich fraction from bovine colostrum. The concentration of IGF-I in the obtained IGF-I rich fraction was 10 ng for 1 mg of protein by enzyme-linked immunosorbent assay (Battelli et al., 1999).

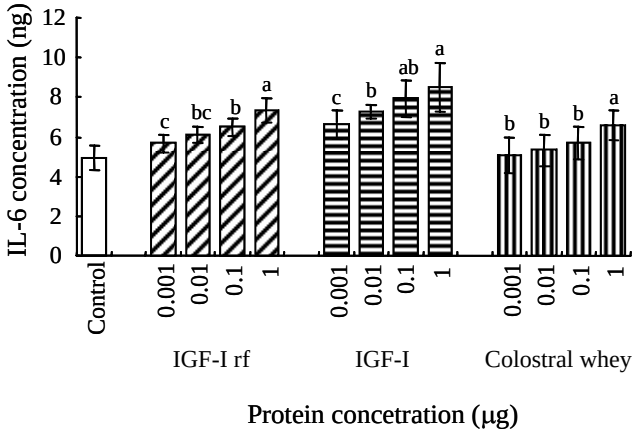

Figure 3. The effects of intraperitoneally injected IGF-I rich fraction separated from the colostral whey by ultrafiltration on *in vivo* IL-6 production from the murine peritoneal macrophages. Different letters on bars mean significant differences in the same sample at $p<0.01$.

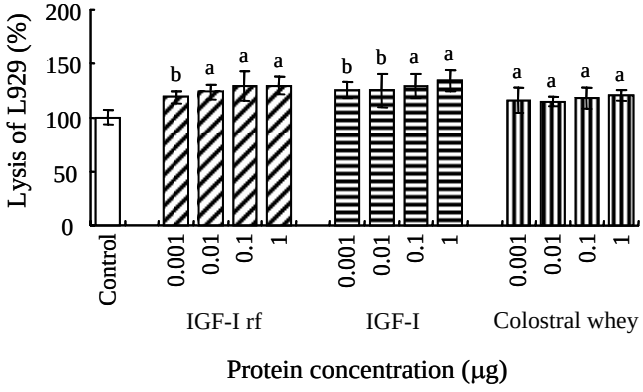

Figure 4. The effects of intraperitoneally injected IGF-I rich fraction separated from the colostral whey by ultrafiltration on TNF-α production from murine peritoneal macrophages. Different letters on bars mean significant differences in the same sample at $p<0.01$.

Effects of IGF-I on the phagocytosis of murine peritoneal macrophages

Peritoneally injected IGF-I rich fraction was found to exert a significant effect on the phagocytosis of peritoneal macrophages at 0.1 μg and 1 μg protein ($p<0.01$). The effects of this intraperitoneally injected IGF-I rich fraction on the phagocytosis of murine macrophages were similar to those associated with IGF-I. The phagocytosis of the murine macrophages did not appear to be significantly affected at 0.001 and 0.01 μg protein of the IGF-I rich fraction and IGF-I. The phagocytosis of peritoneal macrophage from all mice intraperitoneally injected with 1 μg level of protein from IGF-I rich fraction, IGF-I and colostral whey was significantly higher than that seen when the mice were intraperitoneally injected with less than 1 μg level protein ($p<0.01$).

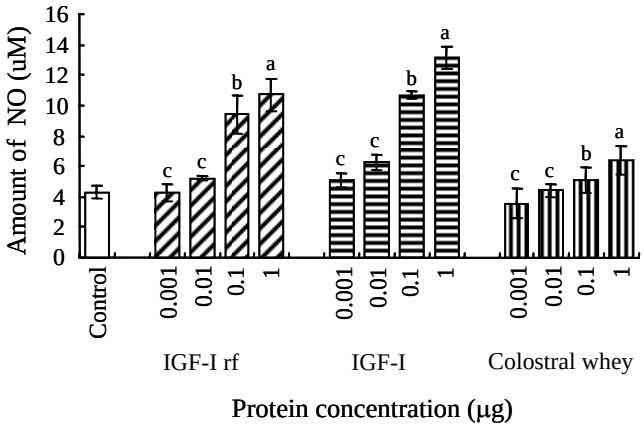

Figure 5. The effects of intraperitoneally injected IGF-I rich fraction separated from the colostral whey by ultrafiltration on NO production from the murine peritoneal macrophages. Different letters on bars mean significant difference in the same sample at $p<0.01$.

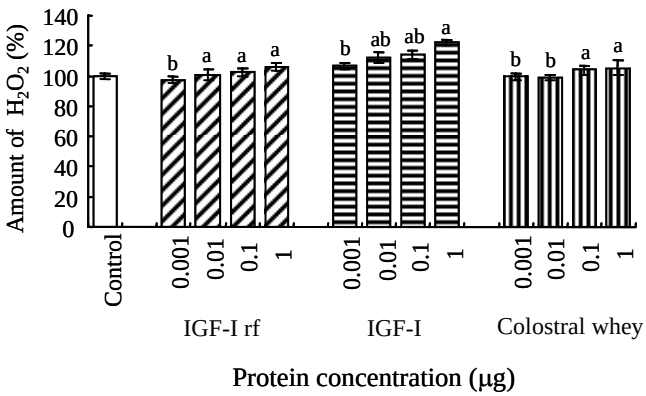

Figure 6. The effects of intraperitoneally injected IGF-I rich fraction separated from the colostral whey by ultrafiltration on H_2O_2 production from the murine peritoneal macrophages. Different letters on bars mean significant differences in the same sample at $p<0.01$.

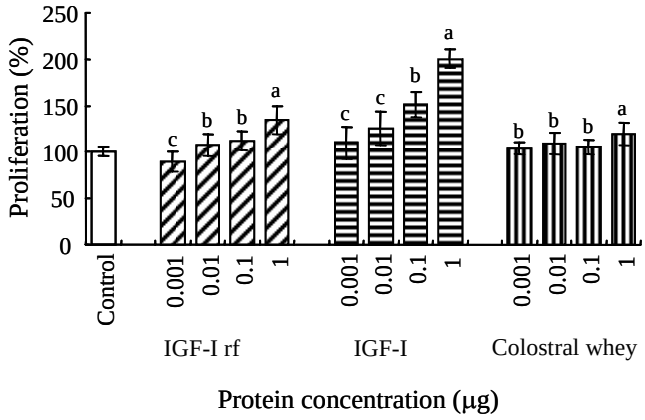

Figure 7. The effects of intraperitoneally injected IGF-I rich fraction separated from the colostral whey by ultrafiltration on the proliferation of the murine splenocytes stimulated with Concanavalin A. Different letters on bars mean significant differences in the same sample at $p<0.01$.

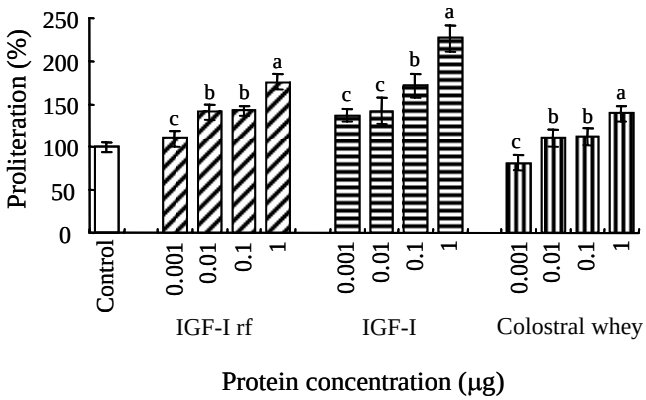

Figure 8. The effects of intraperitoneally injected IGF-I rich fraction separated from the colostral whey by ultrafiltration on the proliferation of murine splenocytes stimulated with LPS. Different letters on bars mean significant differences in the same sample at $p<0.01$.

IL-6 production and TNF-α

IL-6 production from the peritoneal macrophages of mice intraperitoneally injected with the 1 μg protein of IGF-I rich fraction was shown to be significantly more increased than that seen in mice injected with less than 1 μg protein of the IGF-I rich fraction ($p<0.01$). IL-6 production by the peritoneal macrophages from mice intraperitoneally injected with 1 μg of IGF-I was also found to be significantly increased ($p<0.01$). IL-6 production after injection with 1 μg protein of colostral whey was also observed to have significantly increased ($p<0.01$).

Intraperitoneally injected IGF-I rich fraction and IGF-I had a significant effect on TNF-α production from the murine peritoneal macrophages at 0.01, 0.1, and 1 μg protein of the IGF-I rich fraction, and 0.1 and 1 μg of IGF-I ($p<0.01$). However, we observed there to be no significant effects of colostral whey on the production of TNF-α.

Nitric oxide (NO) production and hydrogen peroxide production

Intraperitoneally injected IGF-I rich fraction at the levels of 0.1 μg and 1 μg protein had a significant effect on NO production from the murine peritoneal macrophages ($p<0.01$). NO production from the murine peritoneal macrophages was significantly augmented by the intraperitoneal injection of IGF-I at protein concentrations of 0.1 μg and 1 μg ($p<0.01$). The trend associated with NO production from the peritoneal macrophages due to the intraperitoneal injection of colostral whey was quite similar to those associated with the IGF-I rich fraction and IGF-I. However, intraperitoneally injected IGF-I rich fraction at 0.01, 0.1 and 1 μg protein concentrations clearly had a

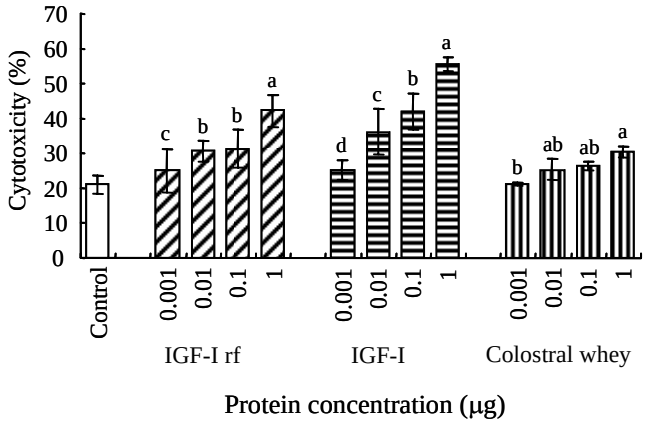

Figure 9. The effects of intraperitoneally injected IGF-I rich fraction separated from the colostral whey by ultrafiltration on the NK cell activity of murine splenocytes. Different letters on bars mean significant differences in the same sample at $p<0.01$.

significant effect on hydrogen peroxide production from the murine peritoneal macrophages ($p<0.01$). Hydrogen peroxide production from the peritoneal macrophages from mice intraperitoneally injected with the IGF-I rich fraction was shown to increase in a dose-dependent manner.

Intraperitoneally injected IGF-I at 1 μg protein concentrations exerted a significant effect on hydrogen peroxide production from the peritoneal macrophages ($p<0.01$). Hydrogen peroxide from the peritoneal macrophages from mice intraperitoneally injected with colostral whey at a 1 μg protein concentration was also shown to have been significantly enhanced ($p<0.01$).

Proliferation of splenocytes

IGF-I rich fraction had a more significant effect on the proliferation of Con A-stimulated splenocytes from mice intraperitoneally injected with 1 μg of protein than it did with less than 0.01 μg of protein ($p<0.01$). The proliferation of the Con A-stimulated splenocytes exhibited the most significant effects after the intraperitoneal injection of the IGF-I rich fraction at a protein level of 1 μg ($p<0.01$). The proliferation of the Con A-stimulated splenocytes from mice intraperitoneally injected with IGF-I was observed to be significantly more enhanced at 0.1 μg and 1 μg protein levels than at below 0.01 μg protein ($p<0.01$). However, colostral whey did not exert enhancing effects on the proliferation of Con A-stimulated splenocytes, except at a 1 μg protein level.

IGF-I rich fraction, IGF-I, and colostral whey all exerted significant effects on the proliferation of the LPS-stimulated splenocytes. The proliferation of LPS-stimulated splenocytes from mice which were intraperitoneally injected with 1 μg protein of the IGF-I rich fraction was more significantly increased than in mice which had been intraperitoneally injected with less than 1 μg protein of IGF-I rich fraction ($p<0.001$). However, The proliferation of LPS-stimulated splenocytes from mice intraperitoneally injected with 0.01 μg and 0.1 μg protein of the IGF-I rich fraction was more significantly enhanced than in mice which had been intraperitoneally injected with less than 0.01 μg protein of the IGF-I rich fraction ($p<0.01$). The proliferation of LPS-stimulated splenocytes from mice intraperitoneally injected with 1 μg protein level of IGF-I was also more affected than in those of mice injected less than 0.1 μg protein level of IGF-I ($p<0.01$). However, The proliferation of LPS-stimulated splenocytes from mice intraperitoneally injected with 0.1 μg protein level of IGF-I was more significantly increased than in those of mice mice intraperitoneally injected with less than 0.1 μg protein level of IGF-I ($p<0.01$). There was more significant effects on the proliferation of LPS-stimulated splenocytes from mice intraperitoneally injected with 0.01, 0.1, and 1 μg protein level of colostral whey than it did on those in mice which were intraperitoneally injected with less than 0.001 μg protein level of colostral whey ($p<0.01$).

Natural Killer (NK) cell activity

IGF-I rich fraction had the most significant effects on NK cell activity in the splenocytes from mice which were intraperitoneally injected with 1 μg protein of IGF-I rich fraction ($p<0.01$). The NK cell activity of the splenocytes from mice which were intraperitoneally injected with 0.01, 0.1, and 1 μg protein of the IGF-I rich fraction was more significantly enhanced than in mice which were intraperitoneally injected with 0.001 μg protein of the IGF-I rich fraction ($p<0.01$). IGF-I also had a more significant effect on NK cell activity in the splenocytes of mice which were intraperitoneally injected with 1 μg of protein ($p<0.01$). NK cell activity in the splenocytes of mice which had been injected with either 0.01 or 0.1 μg protein level of IGF-I was more significantly enhanced than the NK cell activity in the splenocytes of mice which had been intraperitoneally injected with 0.001 μg protein level of IGF-I ($p<0.01$). There was also a more significant effect of colostral whey on the NK cell activity of splenocytes from mice which had been intraperitoneally injected with 1 μg protein level of colostral whey than of those in mice which had been intraperitoneally injected with less than 1 μg protein level of colostral whey ($p<0.01$). There were similar trends with regard to the NK cell activity of the splenocytes from mice which had been intraperitoneally injected with the IGF-I rich fraction, IGF-I, and colostral whey in the same dose-dependent manner.

DISCUSSION

In order to characterize the effects of bovine colostral

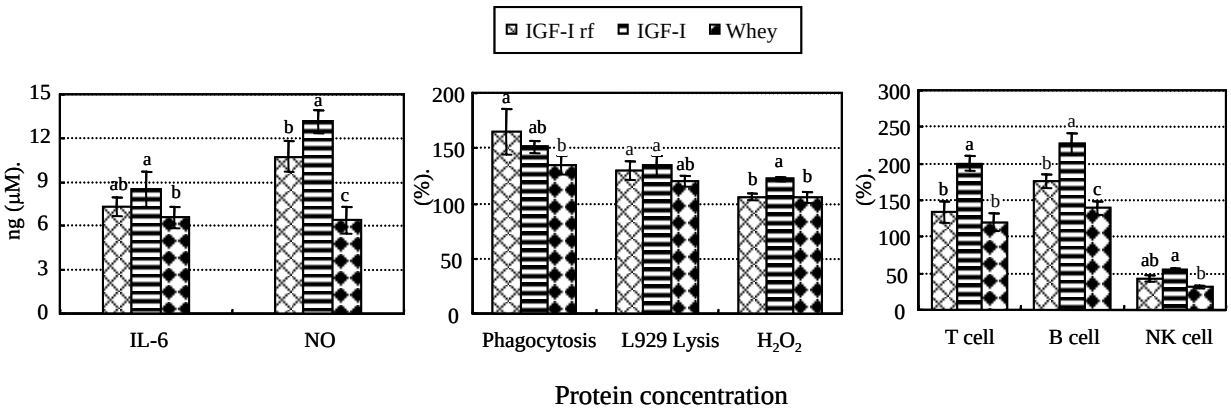

Figure 10. Comparison among immune responses from mice intraperitoneally injected with 1 μg protein level from IGF-I rich fraction, IGF-I and colostral whey. Different letters on bars mean significant differences in the same immune response at $p<0.05$.

whey fractions on immune response, we attempted to determine the effects of the IGF-I rich fraction from bovine colostrum, IGF-I, and colostral whey on phagocytosis, and the production of cytokines, NO, and hydrogen peroxide from the murine peritoneal macrophages, the proliferation of mitogen-stimulated splenocytes, and the NK cell activity of the splenocytes following the intraperitoneal injection of the IGF-I rich fraction, IGF-I, and colostral whey, at 24 h intervals for a period of 2 weeks. Our results confirmed that more enhanced immune response could be seen in the mice which had been intraperitoneally injected with IGF-I rich fraction. As shown in Figure 10, we found the IGF-I rich fraction enhanced immune response manifested in terms of NO production, phagocytosis, and TNF-α production in the peritoneal macrophages, and it appeared more profound in association with the proliferation of LPS-stimulated splenocytes than in those injected with the colostral whey ($p<0.05$). The proliferation of Con A, CD8+T cell mitogen, and LPS, B cell mitogen, stimulated splenocytes in the mice that had been intraperitoneally injected with IGF-I rich fraction was significantly lower than what was observed in the mice that had been injected with IGF-I ($p<0.05$).

Regarding the proliferation of splenocyte with colostral IGF-I, Ha et al. (2005) reported that colostral whey fraction containing IGF-I molecule affected positively the proliferation of mouse splenocyte. Frankie and Kitabchi (2004) reported that PHA stimulated human T lymphocyte expressed and produced IGF-I receptors on $CD4^+$ and $CD8^+$ human T cell surface and they also reported that the development of T cell receptors is a major prevention mechanism of T cell to various diseases. It was estimated that differences in the proliferation of splenocytes stimulated Con A and LPS were due to the binding affinity of IGF-I sources to IGF-I receptors expressed on the surface of T and B cells by the stimulation of mitogens.

Phagocytosis and secretions from peritoneal macrophages, including IL-6, NO, and TNF-α production, were similar between the IGF-I rich fraction and IGF-I-treated cases with the exception of hydrogen peroxide production. The effects of the IGF-I rich fraction on T cell and B cell enhancement based on the proliferation of the Con A-stimulated splenocytes and LPS-stimulated splenocytes were significantly lower than the same factors in the mice treated with IGF-I ($p<0.05$).

IGF-I, unlike growth hormone, fails to prime monocytes for the enhanced *in vitro* production of hydrogen peroxide in response to phorbol 12-myristate 13-acetate (PMA) (Warwick-Davies et al., 1995) but does stimulate the human neutrophils to secrete superoxide anions, which are necessary for phagocytosis (Fu et al., 1991).

IGF-I has been demonstrated to enhance phagocytosis of human polymorphonuclear leukocytes (Fu et al., 1991; Kooijman et al., 1992) and natural killer cell activity, and resulted in the induction of TNF-α production from the monocytes (Renier et al., 1996; Hagiwara et al., 2000). Tu et al. (1999) reported that IGF-I resulted in an increase in the level of interferon-gamma and IL-6 mRNA expression and protein production in the neonatal mononuclear cells. Wang and Koyama (2004) reported that IGF is a newly described growth factor which can affect the up-regulation of eNOS expression and phosphatidylinsitol 3-kinase.

The presence of IGF-I receptors on human T lymphocytes has always been reported. Tapson et al. (1988) reported that IGF-I bound with a high degree of affinity to the T lymphocytes, with a Kd of 0.12 nmol/l, and a mean of 330 receptor sites/cell for PHA-activated T lymphocytes, as well as a mean of 45 receptor sites/cell for the resting T lymphocytes. IGF-I also binds specifically binds to human B lymphocytes and B lymphoblast cell lines (Tapson et al., 1988; Stuart et al., 1991) and thymidine incorporation in response to IGF-I has also been demonstrated in both resting and activated T cells (Tapson et al., 1988)

This study demonstrates that the functions of macrophages, including phagocytosis and NO production of the peritoneal macrophages, could be enhanced by peritoneal macrophages from mice which had been

intraperitoneally injected with the IGF-I rich fraction. However, other macrophage functions, including IL-6, TNF-α and hydrogen peroxide production, were not significantly affected by the injection of the IGF-I rich fraction. B cell response was enhanced by intraperitoneal injection of the IGF-I rich fraction, but T cell response was not significantly affected. We have not attemped to elucidate the precise immunologiccal mechanism underlying the observed increases in some of the characteristics of nonspecific immunity and some specific immunity associated with the injection of IGF-I rich fraction from bovine colostrum. Therefore, a great deal more study will be required in order to demonstrate this exact mechanism with regard to the immune enhancing effects of the IGF-I rich fraction.

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