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Effect of Yak Milk Casein Hydrolysate on Th1/Th2 Cytokines Production by Murine Spleen Lymphocytes in Vitro

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Yak casein hydrolysate was derived from the enzymatic alcalase-hydrolysate of a typical northwestern China milk product called Qula. An in vitro study was conducted to examine their immunoregulatory effects on murine T cells, including Con-A-induced lymphoproliferation and splenocyte cell cycle, production, and messenger RNA (mRNA) expression of interleukin-2 (IL-2), interferon-γ (IFN-γ), and interleukin-4 (IL-4). The results showed that yak casein hydrolysate had lymphoproliferation activity on murine splenocytes and induced their cell cycle from the G1 to the S phase. It could increase Con-A-induced IL-2 and IFN-γ production in spleen cells, but a very weak or no effect was observed in the absence of Con-A. The present study also showed that it could markedly increase the production and mRNA expression of IFN-γ and IL-2, which are key cytokines for T helper 1 cell (Th1) cell development, in a dose-dependent manner. However, their effects on IL-4 secretion were not obvious, and the enhancement was much lower than that of IFN-γ and IL-2. All of these demonstrated that yak casein hydrolysate could increase type 1 cytokine production, thereby shifting the Th1/T helper 2 cell (Th2) balance toward a Th1-dominant phenotype, which meant that yak casein hydrolysate could indeed not only modulate the differentiation of helper T cell but also has the capacity to modulate the Th1/Th2 balance. Therefore, yak casein hydrolysate may be useful for the treatment of cell-mediated immune diseases.

KEYWORDS: Qula; yak (*Bos grunniens*) milk casein; lymphoproliferation; cytokines; mRNA expression

INTRODUCTION

T cells, including those that direct the killing of pathogen-infected cells (T-cytotoxic cells and natural killer cells) and those that regulate the immune response via cytokines (T-helper cells), can induce immune responses. Cytokines play an important role in mucosal humoral and cell-mediated immunities. T-helper cells are divided into two subgroups, of T helper 1 cell (Th1) and T helper 2 cell (Th2), according to their cytokine production profiles (*1*). The differentiation of naive $CD4^+$ T cells into Th1 or Th2 cells determines the specific immune responses of effector cells (*2*, *3*). These distinct subsets of helper T cells are responsible for specific immune functions. Th1 cells play a critical role in the regulation of cellular immunity through the secretion of interleukin-2 (IL-2), interferon-γ (IFN-γ), and TNF-β. On the other hand, Th2 cells regulate humoral immunity through the production of interleukin-4 (IL-4) and IL-10 (*4*, *5*). There is also a feedback control system between Th1 and Th2 cells for regulation of the response.

Yak milk is the most common product in northwestern China, such as Gansu, Xinjiang, and Tibet. However, in these areas, people only use it for the production of butter, and the byproduct (Qula, a kind of crude cheese, whose main component is casein) is not yet fully used. Therefore, improving the value of Qula has received great attention from the Chinese government. Fortunately, the casein content of Qula is high and more than 80%. The amino acid composition of yak milk casein are very similar to that of cow milk (*6*). Milk proteins are good sources of bioactive peptides, which possess many activities, such as immunoregulatory, opioid, antioxidant, or antihypertensive activities (*7*−*11*).

Our previous experiment showed that casein hydrolysate had multiple immunomodulatory activities, such as promoting lymphoproliferation responses (*10*), modulating murine peritoneal macrophages by inducing NO release, and tumoricidal activity to Jurkat cells of LPS-stimulated murine macrophages (*11*). The purposes of the present study are (i) to investigate the effects of yak milk casein hydrolysate on lymphoproliferation activity of murine spleen cells and (ii) to test the effects of yak milk casein hydrolysate on cytokine production of IL-2, IFN-γ, IL-4, and their messenger RNA (mRNA) expression by reverse transcriptase polymerase chain reaction (RT-PCR) analysis, to confirm the influence of yak casein hydrolysate in vitro on Th1/Th2 cytokine balance.

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MATERIALS AND METHODS

Materials. Qula (casein) was provided by Tongjian Co. (Gansu province, China); alcalase was purchased from Huaqiang Biochem. Inc. (activity, 5000U/g; Beijing, China). Con A (Sigma), PMS (Sigma), MTS (Promega), RPMI-1640 (Gibco, New York) were purchased. Penicillin G sodium salt and streptomycin sulfate were obtained from Amresco (USPgrade). RNase A (Sigma) and PCR primer (SBS Genetech, Beijing China) were purchased. IL-2, IFN-γ, and IL-4 kit were purchased from Jingmei Biotech (Shanghai, China); and Balb/c mice were purchased from Beijing Experimental Animal Center.

Preparation of Yak Milk Casein Hydrolysate. Yak milk casein was dissolved in demineralized preheated water and brought to the needed pH using 1 N NaOH. Casein was hydrolyzed by alcalase from *Bacillus licheniformis*. The hydrolysis condition was as follows: The substrate concentration was 6% (w/w), the enzyme dose was 2.5% (w/w, defined as enzyme mass/substrate mass $\times$ 100 %), the pH value was 8.0, and the temperature was 55 °C. At the end of the hydrolysis, the solution was immediately heated in a boiling water bath for 10 min to inactivate the enzyme. The unhydrolyzed casein was removed by an isoelectric precipitation method at pH 4.6. After centrifugation (3000*g*, 30 min), the supernatant was ultrafiltered on a 6000 Da molecular mass cutoff membrane (Sartorius Co., Göttingen, Germany) to remove enzyme and nonhydrolyzed material from the reaction mixture. The permeation part was collected and lyophilized.

Cell Culture and Assay for Cell Proliferation Activity of Yak Casein Hydrolysate. Balb/c mice, 18−20 g body weight, were killed by vertebral dislocation, and their spleens were removed aseptically. Spleen cells were washed with RPMI-1640 medium three times and adjusted to 2.0×10^6 cells/mL in RPMI-1640 medium supplemented with heat-inactivated fetal calf serum. The cell viability determined by a trypan blue (Sigma) exclusion test was above 98%.

The immunomodulating effect of yak casein hydrolysate was evaluated in vitro by measuring their impact on the spleen cell proliferation activity with a MTS colorimetric technique (*12*). Yak milk casein hydrolysate was dissolved in RPMI-1640. Unfractionated spleen cells in RPMI-1640 medium were added into 96 well flat-bottomed tissue culture plates followed by the addition of ConA (which is a kind of T-lymphocyte proliferation mitogen) at a final concentration of 5 μg mL^{-1}. For testing, 10 μL of casein hydrolysate at various concentrations was also incorporated into cultures in quadruplicate wells. Negative control culture wells (−ConA) received RPMI-1640 medium only. Positive control wells (+ConA) received both ConA and RPMI-1640. All casein hydrolysate solutions and ConA solutions were sterilized through 0.22 μm pyrogen-free filters (Millipore, Bedford, MA). Microplates were incubated at 37 °C in 5% CO_2 atmosphere and 95% humidity for 72 h. At the end of this period, the proliferation response of cells was measured with a colorimetric MTS assay by measuring spetrophotometrically at 490 nm with a Bio-Rad model Quant (Bio-tech Instrument Inc.) (*12*). The proliferation response of cultures containing hydrolysate was expressed as the ratio of sample OD to positive control OD (+ConA) (for simplification, it was expressed as S/P in all tables and figures in this paper).

Detection of Cytokines Production of Murine Splenocytes after Stimulation. Splenocytes (2×10^6 cells/mL) in 2 mL of RPMI-1640 medium in 12 well microplates were cultured in the presence of Con A, yak casein hydrolysate, or both. After they were cultured for certain period of time at 37 °C, the supernatants were collected and centrifuged to remove cells. They were stored at −20 °C until used.

Production of IL-2, IFN-γ, and IL-4 in splenocyte supernatants was measured by a sandwich enzyme-linked immunosorbent assay (ELISA) method (Jinmei Co., Shenzhen, China) following the procedure as described in the instructions. The results are expressed as mean values of picograms per milliliter with standard error in triplicate cultures.

Cell Cycle Phase Analysis. The cell cycle phase was analyzed with flow cytometric analysis of propidium iodine (PI) staining performed as described previously (*13*−*15*). In brief, splenocytes were adjusted to 2×10^6 cells/mL, and then, the cell suspension was placed in 12 well flat-bottomed plate with or without ConA. Yak casein hydrolysate (100 μg/mL) was added to the cells except for the negative control group (−ConA). The plate was incubated under 37 °C in 5% CO_2 atmosphere and 95% humidity for 48 h. At the end of this period, the cells were harvested by means of centrifugation, washed with phosphate-buffered saline (PBS), and then fixed with 70% ethanol overnight at 4 °C. The cells were centrifuged and washed with PBS and then suspended in 50 μg/mL PI solution containing 1 mg/mL RNase A and 0.1% Triton X-100 for 30 min in the dark to stain the cells' DNA. After the cells were washed three times with FACS buffer, the PI fluorescent intensity was analyzed on a FACScan (Becton-Dickinson, Franklin Lakes, NJ).

Determination of the Proper Coincubation Time of Yak Casein Hydrolysate with Spleen Cells for the Optimum Production of IL-2, IL-4, and IFN-γ. Yak casein hydrolysate was coincubated with spleen cells for 12, 24, 36, 48, 60, or 72 h. The release of IL-2, IL-4, and IFN-γ was quantified by the ELISA method (Jinmei Co.) as specified by the manufacturers.

Effect of Yak Casein Hydrolysate on IL-2, IL-4, and IFN-γ Production of Spleen Cells. Splenocytes were coincubated with varying concentrations of yak casein hydrolysate, i.e., 0, 4, 20, 100, and 500 μg/mL for the proper time. The production of IL-2, IL-4, and IFN-γ was quantified by the ELISA method (Jinmei Co.) as specified by the manufacturers.

Effect of Yak Milk Casein Hydrolysate on mRNA Expression of Cytokines. The total RNA from spleen cells was isolated and submitted to RT-PCR to investigate Th1 (IL-2 and IFN-γ) and Th2 (IL-4) cytokines gene expression. The total cellular RNA from murine splenocytes was isolated by a modified protocol of phenol/chloroform/isopropanol extraction (*16*). In brief, cells (5×10^6) were cocultured with 25 μg/mL of yak casein hydrolysate for 24 h with or without the presence of ConA. After stimulation, the collected cells were washed twice with PBS and then subjected to lysis with RNA-Beek (Leedo Laboratories, Houston, TX) as described by the manufacturer. After centrifugation, supernatants were extracted with a phenol−chloroform mixture. The extracted RNA was precipitated with isopropanol.

The total cellular RNA was obtained by centrifugation and redissolved in diethyl pyrocarbonate (DEPC)-treated H_2O. We calculated the concentration of the extracted RNA by measuring the optical density at 260 and 280 nm. If the ratio of OD_{260} to OD_{280} was higher than 1.8, it can be used for PCR detection. Nucleotide sequences of the primers used for amplification of cytokines were as follows: IL-2, RV 5′-GTG CTC CTT GTC AAC AGC GC-3′ and FW 5′-GAG CCT TAT GTG TTG TAA GC-3′; IFN-γ, RV 5′-CAT GAA AAT CCT GCA GAG CC-3′ and FW 5′-GGA CAA TCT CTT CCC CAC CC-3′.

RT-PCR Analysis. For reverse transcription, the total cellular RNA of samples was diluted to 0.1 mg/mL. Then, they were incubated with 19 mL of complementary DNA master mixture containing AMV reverse transcriptase (1 mL), RNase inhibitor (0.5 mL), $MgSO_4$ (5 mmol/L), 5 $\times$ PCR buffer, and deoxyribonucleotides (dGTP, dATP, dTTP, and dCTP). The reverse transcription product was used for PCR amplification of IL-2 and IFN-γ. The PCR conditions were as follows: the programmed period was 94 °C for denaturalization; 35 cycles of 94 °C/30 s, 60 °C/90 s, and 72 °C/60 s for amplification; 7 min at 72 °C for prolongation; and 60 min at 4 °C for shock.

After the reaction, the amplified product was taken out of the tubes and run on 1.8% agarose gel. The final PCR product was used for electrophoresis and stained with ethidium bromide. Photographs were taken of the gels.

Statistical Analysis. Results were expressed as the mean $\pm$ standard errors (SE) of three experiments in bioassays and were compared using analysis of variance (ANOVA test). Differences were significant at $p < 0.05$.

RESULTS

Lymphoproliferation Activity of Yak Casein Hydrolysate on Murine Spleen Lymphocytes. Splenocytes of Balb/c mice were incubated with yak casein hydrolysate at a concentration of 0−500 μg mL^{-1} in the presence of ConA, and the proliferation response was shown in **Figure 1**. Yak casein hydrolysate was found to significantly stimulate the proliferation of murine splenic lymphocytes in a dose-dependent pattern. The maximal

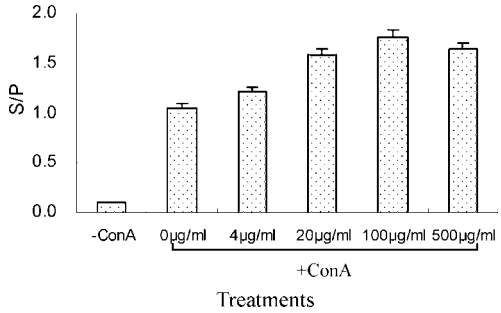

Figure 1. Lymphoproliferation activity of yak casein hydrolysate on murine spleen lymphocytes. All measurements were expressed as means ± SD of three independent experiments. The results were expressed as S/P, which meant the ratio of sample OD to "+ConA" OD.

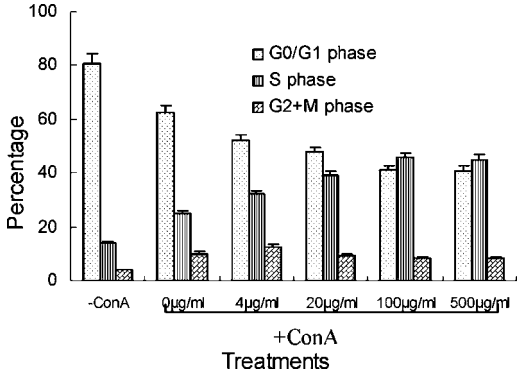

Figure 2. Effects of yak casein hydrolysate on cell cycle progression of Balb/c mice measured as G0/G1 phase, S phase, and G2/M phase percentage. Flow cytometry analysis was conducted using a Becton-Dickinson FACScan. The data from three independent experiments are calculated and demonstrated in a graphical representation.

lymphoproliferation effect was found with 100 μg mL^{-1} of the hydrolysate, achieving a 1.68 ± 0.04-fold increase when compared with no addition of yak casein hydrolysate. **Figure 1** also shows that when the addition dose of the hydrolysate is too high, higher than 500 μg mL^{-1} in this experiment, their lymphoproliferation activity decreases, which means that yak casein hydrolysate has dual immunoregulatory characteristics on murine splenocytes.

Effect of Yak Casein Hydrolysate on the Cell Cycle Phase of Murine Splenocytes. Flow cytometric cell cycle analysis of untreated and yak casein hydrolysate-treated cells was performed by PI staining, which is shown in **Figure 2**. Cell samples were analyzed for DNA content by flow cytometry. As compared with the control group, cells at the G0/G1 phase of yak casein hydrolysate-treated group decreased significantly, while nuclei in the S phase and the G2/M phase increased and showed a dose-dependent manner. As shown in **Figure 2**, after coincubation of 100 μg/mL yak casein hydrolysate with splenocytes in the presence of ConA, 40.9% of lymphocytes was identified as cells with G0/G1 DNA content, while 45.7 and 8.4% cells were in S and G2/M phases, respectively, which were insignificantly different from the resulting values of 500 μg/mL yak casein hydrolysate ($p > 0.05$). All of these results showed that yak casein hydrolysate could indeed promote lymphproliferation in the presence of ConA.

Determination of the Proper Coincubation Time of Yak Casein Hydrolysate with Spleen Cells for the Optimum Production of IL-2, IFN-γ, and IL-4. Yak casein hydrolysate was coincubated with spleen cells for different times of 12, 24, 36, 48, 60, and 72 h. The production of IL-2, IL-4, and IFN-γ was quantified as shown in **Figure 3**. Regarding the production of Th1-related cytokines, low levels of IL-2 and IFN-γ were detected in the murine splenocyte culture supernatant after 24 h of exposure to yak casein hydrolysate. IL-2 peaked at 48 h, but IFN-γ peaked after 60 h of coincubation. Comparing the production of IL-2, IFN-γ, and IL-4, yak casein hydrolysate induced very low levels of Th-2-related cytokine of IL-4, and it peaked at 48 h.

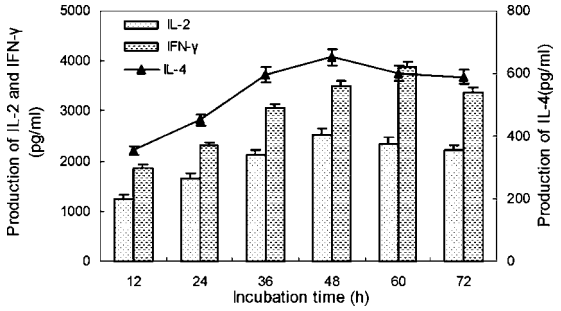

Figure 3. Change of IL-2, IFN-γ, and IL-4 release with the coincubation time of yak casein hydrolysate and spleen cells. The results are expressed as picograms per milliliter ± SD in duplicate.

Effect of Yak Casein Hydrolysate on Cytokine Production of Murine Spleen Cells. The effects of yak casein hydrolysate on cytokine secretion of splenocytes were assessed through the changes of IL-2, IFN-γ, and IL-4 release after an in vitro stimulation with or without the presence of ConA. In the absence of ConA, IL-2, IFN-γ, and IL-4 were secreted at a nearly inappreciable level as shown in **Figure 4**. While in the presence of ConA, yak casein hydrolysate led to a markedly increased release of these three kinds of cytokines by spleen cells, and the response was in a dose-dependent manner. The effect of various concentrations (0, 4, 20, 100, and 500 μg/mL) of yak milk casein hydrolysate on Con-A-induced production of IL-2, IL-4, or IFN-γ by nonfractionated murine spleen cells is shown in **Figure 4**. When yak casein hydrolysate was added to the spleen cells in combination with Con-A, the cytokine levels were markedly increased with increasing concentrations of the hydrolysate. IL-2 production was maximal at 100 μg/mL. When the concentration was higher than 500 μg/mL, the increasing effect of yak casein hydrolysate decreased. **Figure 4** also shows that the increasing effect of yak casein hydrolysate on the secretion of IL-4 is very slight and much lower than that of IL-2 and IFN-γ ($p < 0.001$), which indicates that yak casein hydrolysate mainly affects T helper cells through Th1 cell and maybe only induces cellular immunity.

mRNA Expression of IL-2 and IFN-γ. We tested the effects of yak casein hydrolysate on the switching of Th0 to Th1 or Th2 cytokine expression in spleen cells with or without ConA. For this purpose, total RNA from spleen cells was isolated and submitted to RT-PCR to investigate Th1 (IL-2 and IFN-γ) cytokine gene expression. The result is shown in **Figure 5**. It demonstrates that yak casein hydrolysate could increase the mRNA expression of IL-2 and IFN-γ in a dose-dependent manner. We concluded that the yak casein hydrolysate may modulate Th0 cells to the Th1 phenotype by regulating the secretion of Th1 cytokines.

DISCUSSION

Cytokines, low molecular weight proteins released from cells, can regulate important biological processes including cell

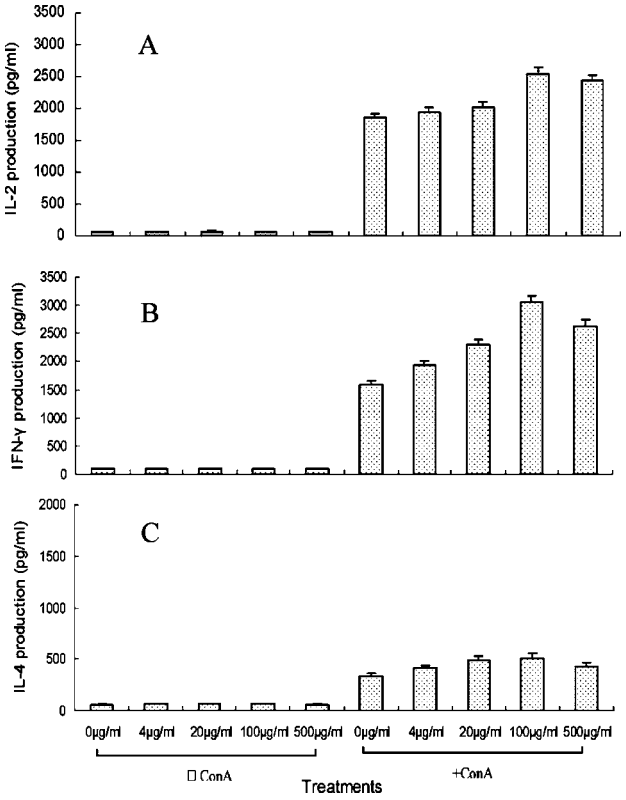

Figure 4. Effect of yak casein hydrolysate on the production of IL-2 (**A**), IL-4 (**B**), and IFN-γ (**C**) of spleen cells. Spleen cells (2×10^6/mL) of Balb/c mice were cultured with or without ConA at different concentrations of yak milk casein hydrolysate at 37 °C in a humidified 5% CO_2 atmosphere for 48 (IL-2 and IL-4) or 60 h (IFN-γ). The results are expressed as picograms per milliliter ± SD in duplicate. For the standard curves, the range of IL-2, IL-4, and IFN-γ was from 0 to 1000 pg/mL as required of the kit instructions. The values reported for the samples cultured without/with ConA were in the detection range of the standard curves.

growth, cell activation, inflammation, immunity, tissue repair, and fibrosis. IFN-γ, which is one of the T helper type 1 cytokines, is important for the activation of T cells and plays an important role in immunoregulation and cytotoxic response. Both IFN-γ and IL-2, two Th1 cytokines, control the differentiation of naive T helper cells into Th1 cells. While IL-4, a Th2 cytokine, controls the differentiation of naive T helper cells into Th2 cells. IFN-γ downregulates the secretion of certain Th2 cytokines, such as IL-4 (*17*).

Our previous study showed that resting T cells failed to produce IL-2 or proliferate in response to yak milk casein hydrolysate; that is, yak casein hydrolysate itself is noncytotoxic and nonproliferating to normal lymphocytes as well as tumor cell lines at a concentration range of 0−50 mg/mL (*18*). This study showed that yak casein hydrolysate was capable of inducing lymphocyte proliferation and caused G1 to S phase proliferation on murine-activated splenocytes. In this study, we examined the effect of yak milk casein hydrolysate on IL-2, IFN-γ, and IL-4 production of spleen cells with or without ConA. The result showed that yak casein hydrolysate significantly increased Con-A-induced IL-2 and IFN-γ production in spleen cells, while it had little effect on the IL-2 and IFN-γ production in the absence of ConA. One possible explanation for this synergistic activity of mitogen and yak casein hydrolysate is that Con A was capable of upregulating a putative receptor for yak casein hydrolysate, which would have caused

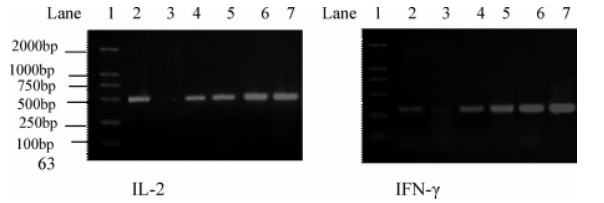

Figure 5. Effect of yak casein hydrolysate on the mRNA expression of IL-2 and IFN-γ. Lane 1 represents the standard marker. Lanes 2 and 3 represent −ConA and +ConA treatment, respectively. Lanes 4–7 represent the concentrations of yak casein hydrolysate, which were 4, 20, 100, and 500 μg/mL in the presence of ConA, respectively.

a greater lymphoproliferative response and cytokine secretion activity than the activity of yak casein hydrolysate alone (*19*).

The present study showed that yak casein hydrolysate had no obvious enhancing function on the production of IL-4 in the presence of either ConA or not. In other words, the increasing effect of yak casein hydrolysate on IL-4 release is much lower than on that of IL-2 and IFN-γ, which meant that yak milk casein hydrolysate mainly affected the production of Th1 cytokine, i.e., it preferentially activates IL-2- and IFN-γ-producing Th1 cells. It also showed that hydrolysate derived from milk casein can stimulate cytokine secretion of naive CD4+ T cells and differentiate into helper T cells into type-1 cells (Th1 cell) in a dose-dependent manner. Yak casein hydrolysate could serve as immunotherapeutic agents by selectively increasing the pool of activated T lymphocytes.

Activation of lymphoproliferation is relevant since lymphocytes produce cytokines that activate lymphocyte and macrophage functions. In our previous study, we showed that yak casein hydrolysate could increase the secretion of inducible nitric oxide (NOS) production in macrophages and inhibit the growth of Yac-1, Jurkat, and K_{562} tumor cells mediated by natural killer cells or macrophages (*18*). In this study, we showed that yak casein hydrolysate could increase the secretion of IL-2 and IFN-γ in the presence of ConA. IL-2, a major T-cell growth factor, can increase immunoglobulin synthesis and J-chain transcription in B cells, potently augment the cytolytic activity of nature killer (NK) cells, and induce the cytolytic activity of lymphokine-activated killer (LAK) cells. IFN-γ can induce an antiviral state and make cells resistant to virus infection. T-cell IFN-γ is frequently responsive to viral infections (*20*). The present study can partly explain the tumor-killing effect of casein hydrolysate in the presence of ConA.

In conclusion, the present work showed that yak casein hydrolysate had lymphoproliferation effects on murine splenocytes and caused their G1 to S phase proliferation. Results in the present study demonstrate that yak casein hydrolysate is an activator of murine splenocytes. It enhanced the secretion of Th1 cytokines IL-2 and IFN-γ. Whether it can play an important role in the prevention of disease associated with an increase in the number of Th2 (T helper type 2) cells and Th2 cytokines and a decrease in the number of Th1 (T helper type 1) cells and Th1 cytokines through differential regulation of T helper lymphocytes is under way in our lab. Through which signal transduction cascade that yak casein hydrolysate affects cellular proliferation needs further study.

ABBREVIATIONS USED

Th1, T helper 1 cell; Th2, T helper 2 cell; IL-2, interleukin-2; IL-4, interleukin-4; IFN-γ, interferon-γ; mRNA, messenger RNA; RT-PCR, reverse transcriptase polymerase chain reaction.

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