Characterization of Immune Responses Caused by Bovine Leukemia Virus Envelope Peptides in Sheep

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(Received 12 November 1998/Accepted 15 December 1998)

ABSTRACT. To study the immunomodulative activity caused by bovine leukemia virus envelope (BLV Env) peptide, sheep were immunized with two kinds of Th-epitope peptides, peptide 98 (BLV Env 98-117), and 61 (BLV Env 61–78). Four of eight immunized sheep showed specific proliferative responses against both of the peptide stimulations. To characterize the cells responding to the peptides, peptide-specific cells were established from the responding sheep by the continuous stimulation of peripheral blood mononuclear cells (PBMCs) with either peptide 98 or 61 in vitro. The peptide 98-specific cells consisted of CD4-positive cells, whereas the peptide 61-specific cells consisted of CD8-positive cells and MHC class II-positive cells. In addition, cytokine profile analysis indicated that the peptide 98-stimulated cells expressed IFN-γ but not IL-10, although the peptide 61-stimulated cells expressed IL-10 but not IFN-γ. These results show that BLV envelope peptides 98 and 61 can modulate immune responses of sheep lymphocytes in different ways and may contribute to the pathogenesis of BLV infection.—KEY WORDS: bovine leukemia virus, cytokine profile, envelope peptide, immunomodulation.


Bovine leukemia virus (BLV), the causative agent of enzootic bovine leukosis (EBL), is an oncogenic B-lymphotropic retrovirus. BLV can induce preneoplastic lymphocyte dysregulation and lymphoid neoplasia [18]. EBL causes significant economic problems for the dairy industry, and is a relevant model for studying retroviral disease progression. Most of the BLV-infected cattle remain clinically normal, classified as aleukemic (AL), while 30 % of infected animals progress to persistent lymphocytosis (PL), which is characterized by a polyclonal expansion of B-lymphocyte. Fewer than 5 % of infected animals eventually develop lymphosarcoma [5]. Despite the often long duration for disease transition, the mechanism for the progression is yet unknown.

Cytokine profiles (Th1/Th2) have been reported to contribute to the pathogenesis of a variety of viral infections such as human immunodeficiency virus (HIV) [4], Epstein-Barr virus (EBV) [20], as well as BLV [24, 25]. Pyeon and Splitter [25] have reported that IL-12 p40 mRNA was produced at the relatively high concentrations by freshly isolated peripheral blood mononuclear cells (PBMCs) from BLV-infected animals in the AL stage and at significantly reduced levels in PL animals. They also demonstrated that PBMCs from BLV-infected animals at the late-stage of the disease express considerably more IL-10 mRNA than animals which are not infected or are in the early stages of the disease [24]. Thus, they suggested that the type 1 immune response prevails in animals with the AL stage and the cytokine profile converts to the type 2 response in animals with the more progressed PL stage. Consequently, cytokine dysregulation, either through the direct effects of viral regulatory genes or a response to antigenic stimuli, may result in B-lymphocyte stimulation during BLV-induced persistent lymphocytosis. Notably, human T-lymphotropic virus (HTLV) infection of T-cells resulted in IL-2 and IL-2 receptor-gene activation via the promiscuous transactivation of cellular gene promoters by a viral protein, Tax [7, 9]. This activation, in turn, causes lymphoproliferative disorders similar to the PL of BLV. Supporting to this, the IL-2 activity in culture supernatants of concanavalin A (Con A)-stimulated PBMCs from BLV-infected animals with the PL stage was increased [28]. The expression of an inducible IL-2 receptor alpha chain, CD25, was also increased on lymphocytes from BLV-infected cattle [27]. Thus, increased expressions of IL-2 and IL-2 receptor on lymphocytes in BLV-infected animals may contribute to development and/or maintenance of persistent B-lymphocytosis.

Viral protein components play an important role in retrovirus-induced immune dysfunction [1, 8, 13, 23]. CKS-17, representing the conserved domain within the transmembrane envelope protein (Env) was reported to be immunosuppressive in vitro and in vivo [10, 11, 21]. This viral component inhibits production of type 1 cytokines e.g. interferon γ (IFN-γ), IL-2, and tumor necrosis factor α (TNF α), though augment type 2 cytokines IL-10 accumulation [10, 11]. We have previously shown that BLV also contains particular peptide sequences in the Env which causes immunomodulation including up-regulation of IL-2 production, and of responses to IL-2 stimulation in the BALB/c mice. In the present experiment, the immunomodulatory activity of the peptides which have been reported as Th-epitopes in sheep was investigated in peptide-inoculated sheep. The immune responses induced by these peptides were examined by analyzing the phenotypes of lymphocyte populations and the patterns of
cytokine production. We show that the different types of immunomodulations were induced by BLV Env peptides both which have been reported as Th-epitopes.

MATERIALS AND METHODS

Experimental animals: Twelve male Suffolk sheep (4 to 5 month old) were provided from Takikawa Animal Husbandry Experiment Station, Hokkaido, Japan. These sheep were castrated and divided into two groups. Eight animals were used for in vivo peptide priming, while four sheep were inoculated with a mock material as a control.

Peptide synthesis: The peptides used in this study were peptides 61 (PGQRRFGARAMVTYDCE; BLV Env 61–78, sheep helper T-cell epitope [61]), 98 (SQADQGFSFYYNHQILFLHLK; BLV Env 98–117, BALB/c mouse helper T-cell and B-cell epitope [2]), and R (SKVNQLYHAHFQPLSFQGL; rearranged sequence of peptide 98). These peptides were synthesized directly onto the core resin by the COOH-terminal amino acid by a peptide synthesizer (PSSM-8, Shimazu, Kyoto, Japan).

In vivo priming with epitope peptides: Eight sheep were primed with synthetic peptides 61 and 98. Mannan-coated liposomes containing these peptides were constructed according to the method described previously [22]. Empty mannan-coated liposome was used as a mock material. These materials were injected three times into sheep intramuscularly every 2 weeks.

Establishment of the epitope peptide-specific cell lines: PBMCs were collected from sheep No.1 by the Ficol-Conray method and cultured in RPMI medium containing 10 % fetal calf serum (FCS) (1 × 10^7 cells/ml) in the 24-well plate. These cells were stimulated with epitope peptides (peptides 98 or 61) at the final concentration of 40 μg/ml. After 1 week cultivation, the cells were harvested and re-adjusted at 1 × 10^5 cells/well in RPMI 1640 medium containing 10 % FCS in the 24-well plate. Concurrently, 60Co-irradiated (35 Gy) self-PBMCs (1 × 10^7 cells/well) were added to this culture as antigen-presenting cells, and the cells were stimulated with epitope peptides as same as the previous culture. The passages were repeated every 1 week, and after the third passage, the cells were used as peptide-specific cell lines for the following experiments.

Lymphocyte proliferation assay: The lymphocyte proliferation assay was performed as described previously [16]. Briefly, PBMCs collected by the Ficol-Conray method, or the epitope peptide-specific cell lines were cultured in RPMI medium containing 10 % FCS in flat-bottom microtiter plates (2 × 10^4 or 1 × 10^5 cells per well, respectively). In some cases, CD4- or CD8-positive cells were depleted from PBMCs by using mouse anti-sheep CD4 (17D), or CD8 (ST8) antibodies and subsequently, anti-mouse immunoglobulin (Ig)-coated magnetic beads (PerSeptive). These cells were stimulated with synthetic peptides at the concentration ranging from 1 to 100 μg/ml in the culture medium (200 μl per well). To block the expression of peptide antigens on the MHC class II molecules, monoclonal antibody, either BLMO4 (antibovine MHC class II) or Lc6 (anti chicken CD4; control), was added to the individual well (50 μl/well; totally 200 μl/well). Triplicate cultures were prepared for each concentration of peptide along with control cultures (without any antigens). The plates were incubated at 37°C under an atmosphere of 5 % CO2 for 5 days. Following cultivation, the cells were pulsed with 37 kBq of [methyl-3H] thymidine per well for 16 hr. Then, the cells were harvested onto glass-fiber filters and incorporation of [methyl-3H] thymidine into DNA was determined by liquid scintillation counter. Results were presented as stimulation index (SI; SI=cpm in tested sample/cpm in control cells).

The analysis of the cell-surface antigen expressions on the peptide-specific cells by flowcytometry: The cells were suspended at the concentration of 1 × 10^7 cells/ml in phosphate-buffered saline containing 0.03 % NaN3 (PBS/FCM). One hundred ml of the cell suspension was plated in the V-bottom 96-well plate, and cells were pelleted by centrifugation (1,500 rpm, 5 min). These cells were incubated for 30 min at 4°C with monoclonal antibodies, Lc6, 17D (anti-sheep CD4), ST8 (anti-sheep CD8), BLMO4, and IL-A29 (anti-workshop cluster (WC)1). Then, cells were washed once with PBS/FCM, and incubated for 30 min at 4°C with × 50 diluted FITC-conjugated goat anti-mouse Ig antibody (CAPPEL). Cells were washed twice with PBS/FCM and relative immunofluorescence of the cells was analyzed by a flow cytometer (Profile Analyzer; Coulter).

Detection of the cytokine mRNA expressions from peptide-specific cells: Total RNA was extracted from each cell lines after the passages by using the TRizol reagent (GIBCO BRL), and the concentrations of RNA were determined from A260. To synthesize the first strand of cDNA, 7 μg of total RNA was added to 30 μl of the reaction mixture (50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM MgCl2, 10 mM DTT) containing 1.3 pmol of the oligo d(T) primer (15 mer), 2 mM of dNTP mix (dATP, dGTP, dCTP, dTTP), 40 U of RNasin (Promega), 11 U of Rous associated virus 2 reverse transcriptase (TAKARA), and incubated at 45°C for 1 hr. The cDNA synthesized was diluted four times with distilled water, and used for PCR as template cDNA. The cDNA template (0.5–2.0 μl) was mixed with the reaction solution (10 mM Tris, pH 9.0, 50 mM KCl, 1.5 mM MgCl2) to the final volume of 20 μl containing 0.8 μl of dNTPs, 20 pmols each of sense/antisense primers, and 1 μl of Taq polymerase (Pharmas). The primers used were β-actin 5’ (ACCAACTGGGAGCACATGGAG), β-actin 3’ (GCAATTTGGGTGATACTG), IFN-γ 5’ (CTTGAGTCGTTCTGCTGGTATG), IFN-γ 3’ (CTCCTCCTGGTTCTGAGTAGTA), IL-10 5’ (GCAAGCTCTTGTGGCTTACG), and IL-10 3’ (CTCCTCCTGGTTCTGAGTAGTA). PCR reactions were performed in a thermal cycler (System 9700, Applied Biosystems) under the following conditions: one cycle for 3 min at 94°C, followed by 30 cycles of 45 sec at 94°C, 1 min at 60°C, and 1 min at 72°C, and the final extension cycle for 5 min at 72°C. Five
µl of the PCR products were analyzed on a 2 % agarose gel.

Epitope analysis of the peptide 98: The PBMCs obtained from sheep No.1 were stimulated with short-ranging 10-mer peptides in peptide 98–117 or peptide 98 or peptide R at a concentration of 20 µg/ml. The plates were incubated at 37˚C under an atmosphere of 5 % CO₂ for 5 days. Following cultivation, the cells were pulsed with 37 kBq of [methyl-³H] thymidine per well for 16 hr and were harvested onto glass-fiber filters. The incorporation of [methyl-³H] thymidine into DNA was determined by liquid scintillation counter.

RESULTS

Proliferative responses against epitope peptide stimulation in the immunized sheep: Eight peptide-immunized (Nos. 1–8) and four control (Nos. 9–12) sheep were examined for proliferative responses against each of the epitope peptides (peptides 98 and 61). Before the immunization, no sheep showed proliferative responses against these peptides (Fig. 1A, B). After three immunizations, four of eight (Nos. 1–4) sheep showed proliferative responses (SI>2.0) against peptide 98 (Fig. 1C). Proliferative responses against peptide 61 were detected in 7 of 8 peptide-immunized sheep (Nos. 1–7) though the other (No. 8) and 4 control sheep remained negative (Fig. 1D).

The different types of immunomodulations were induced by BLV Env peptides: To characterize each of the peptide-specific proliferating cells, peptide-specific cell lines were established by stimulating PBMCs from sheep No. 1 with each of the peptides as described in Materials and Methods. The expression of cell-surface antigens on these cells and original PBMCs were analyzed by flowcytometry (Fig. 2). Sixtyfour percents of the peptide 98-specific cells expressed CD4 molecules and the intensities of the expressions were also upregulated on their cell-surfaces (PBMCs; MFI=223.9, peptide 98-specific cells; MFI=677.5), indicating that peptide 98 activated the CD4-positive cells. However, the peptide 61-specific cells consisted of CD8-positive cells, MHC class II-positive cells and WC 1-positive cells. The expressios of CD8 and WC 1 molecules on the peptide 61-specific cells were significantly up-regulated (CD8; PBMC; MFI= 281.2, peptide 61-specific cells; MFI=661.8, WC 1: PBMC; 354.0, peptide 61-specific cells; MFI=613.3) compared to those of original PBMCs. Then, we examined
the expressions of cytokine mRNA in the peptide-stimulated cells by RT-PCR (Fig. 3). After the fourth passage, the total RNA was extracted from each of the cells and examined for the cytokine expression. The peptide 98-stimulated cells expressed IFN-γ but not IL-10, while the peptide 61-stimulated cells expressed IL-10 but not IFN-γ. These results indicate that peptides 98 and 61 of BLV Env can differently modulate immune responses of PBMCs in sheep. The mRNA expression of IL4 was induced in both of these cell lines, though significantly higher amount of IL-4 mRNA was induced in the peptide 61-stimulated cells than in the peptide 98-stimulated cells (data not shown).

Epitope analysis of the peptide 98–117: Since the cytokine profiles in the peptide 98-specific cells showed the type 1 response, which is supposed to be an important for the control of BLV infection [24, 25], we further characterized the peptide 98-stimulated lymphocyte responses. Proliferative responses against peptide 98 were dependent on CD4-positive cells because PBMCs depleted of CD4-positive cells diminished proliferative responses though PBMCs depleted of CD8-positive cells did not (data not shown). In addition, MHC class II molecule was involved in this response because anti-MHC class II antibody blocked the proliferation (data not shown). To determine the exact epitope in peptide 98, 9 of short overlapping 10 mer peptides were synthesized as described in Fig. 4B, and used for epitope mapping (Fig. 4). The peptide R, which is rearranged amino acid residues present in the peptide 98–117 and used as a control of peptide 98, showed no proliferation inducing activities. The proliferative responses were induced when stimulated with peptide 102 to 107, while no responses were induced when stimulated with peptide 101. This result indicate that isoleucine, at position 111, is critical for inducing the proliferative response. Similarly, peptide 108 fails to induce proliferative responses, indicating an important role for valine at position 107 in proliferation. Thus, valine and isoleucine in the peptide 98 are critical for its ability to induce proliferative response.

DISCUSSION

In the present study, we found different immunomodulative activities in the peptides derived from the BLV envelope glycoprotein. Interestingly, different types of cells responded to each of the peptides in one sheep. The proliferating cells stimulated by peptide 98 were CD4-positive cells, which secreted type 1 cytokines. In contrast, the proliferating cells stimulated by peptide 61 consisted of CD8-positive cells, WC 1-positive cells and mostly MHC class II-positive cells which may originate from B-cells and macrophages. This result suggests that peptide 61-stimulated CD8-positive cells secrete type 2 cytokines, and this activation may result in the expansion of B-cells.
Furthermore, CD8-positive cells which secrete type 2 cytokines were supposed to be associated with immunosuppressions in several infectious diseases [3, 19, 26, 29]. Thus, peptide 61 in BLV Env may be involved in the BLV-induced immunosuppressions by its induction of CD8-positive cells secreting type 2 cytokines.

CKS-17 is a heptadecapeptide corresponding to a region highly conserved in retroviral transmembrane proteins such as p15E of feline leukemia virus [10]. This peptide has been shown to differentially modulate type I and II cytokine productions through the elevation of intracellular cAMP levels [10–12], and supposed to play an important role in the development of retroviral infectious diseases. In present study, we showed that peptide 61 can up-regulate the IL-10 and/or MHC class II expression in sheep. In our previous experiment, we found that peptide 61 can induce spontaneous proliferation of spleen cells from the naive BALB/c mice and enhance IL-2 production. Interestingly, it was reported that in BLV-infected animals, higher up-regulated expressions of IL-10, IL-2, and MHC classII occurred in the PL stage compared to the AL stage animals [24, 27, 28]. Taken together, our hypothesis is that the viral envelope peptide 61 may be involved in the disease progression of the BLV infection by its modification of the cytokine responses, similar to CKS-17 in HIV-infection [10, 14, 15, 17].

The peptide 98 is reported to contain B-cell and helper T-cell epitopes for BALB/c mice [2]. In the present study, we found that peptide 98 also contained CD4-T-cell epitopes for sheep, and valine and isoleucine at positions 107 and 111, respectively, play a critical role in the induction of proliferative responses. The peptide 98 is thought to be a good candidate for the peptide-based BLV vaccine because this peptide can induce type I immunity (Fig. 3, and [22]) which could play an important role in the control of the disease progression of BLV infection [24, 25]. For the development of epitope-based vaccines, an analysis of the immune responses caused by the peptide 98 will be necessary, and has potential practical implications.

Overall, our findings suggest that the two peptide sequences in BLV Env could differentially modulate the sheep lymphocytes. One peptide (peptide 98) may be a good candidate for a peptide vaccine because it can induce specific type 1 cytokine responses, while the other (peptide 61) may contribute to the BLV-associated immunological abnormalities. This information would be helpful for understanding both the immunopathogenesis of BLV infection and development of an effective BLV vaccine.

ACKNOWLEDGMENTS. The authors wish to thank Dr. G.Ingram for critical review of the manuscript. This work was partly supported by Recombinant Cytokine’s Projects from the Ministry of Agriculture, Forestry and Fisheries, Japan (RCP-1997–2330).

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