Epitope-Mapping of Antigen-Specific T Lymphocyte in Cattle Immunized with Recombinant Major Piroplasm Surface Protein of Theileria sergenti

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ABSTRACT. The cellular immune responses against major piroplasm surface protein (MPSP) of Theileria sergenti were characterized. Three cattle were immunized with recombinant MPSP (C type) encapsulated by mannan-coated liposome. The proliferative responses of peripheral blood mononuclear cells (PBMC) against MPSP were detected in all immunized animals. Reverse transcriptase polymerase chain reaction (RT-PCR) analysis revealed that T cell lines derived from each animal expressed relatively high levels of interferon (IFN)-γ mRNA, low levels of interleukin (IL)-2, IL-10, and tumor necrosis factor (TNF)-α mRNAs, but no detectable level of IL-4 mRNA. From the results of T cell epitope-mapping, T-cell lines from two animals responded to DTSKFTPTVAHRLKHAEDLF (residues 61 to 80), while other animal responded to GTGKVYDFVGNFKVTKFE (residues 141 to 160). The MPSP-type specific response of a T-cell line was observed in the latter region of MPSP. These data suggest that immunization with cocktail vaccine consisting of different types of MPSP may be necessary in the field trial.

KEY WORDS: epitope-mapping, T-cell line, Theileria sergenti.

Theileria sergenti is a tick-born protozoan parasite of cattle that causes chronic anemia as intraerythrocytic piroplasms [17]. The live parasites in erythrocytes had previously been used as an effective vaccine in Japan [6], but its use is prohibited because of the transmission of infectious agents such as bovine leukemia virus [7]. Instead of using live vaccines, alternative methods for the control of this disease are desired.

MPSP, major piroplasm surface protein, is a glycoprotein with molecular size ranging from 32 to 34 kDa [14,15]. This molecule is widely conserved among different species of Theileria parasites [19]. The function of MPSP is still unknown, but it is presumed that it has a critical role in survival within the host. It was reported that cattle immunized with MPSPs of T. annulata designated as Tams1-1 and Tams1-2 encapsulated into immunostimulating complexes (ISCOMs) showed resistance against challenge infection with infected blood cells [5]. However, cattle immunized with DNA vaccine were also protected against challenge infection although specific antibodies were not detected. Similar observation was also taken in bovine babesiosis in which there was little correlation between antibody titers and protective immunity in animals immunized with parasite antigens or animals that had recovered from infection [16, 21, 23, 24]. It is reported that proliferative responses of peripheral blood mononuclear cells (PBMC) against purified piroplasm of T. sergenti were detected in infected animals [25]. These observations underscore the importance of cellular immunity in protection against these parasite infections.

The fact that an injection of piroplasms occasionally did not induce any protective immunity in calves suggested the presence of antigenically different strains of Theileria parasites in Japan. Among Theileria parasites isolated from different areas in Japan, genetic diversities were detected by Southern blotting using probes of genomic DNA clones and cDNA clones encoding a MPSP [14]. From the nucleotide sequence analysis of many field isolates, it was found that there are at least five allelic forms in MPSP genes [10], and most parasite stocks and field isolates consisted of mixed populations of parasites bearing different allelic form of MPSP genes [8, 12, 13].

Because the immunogenicity of T. sergenti MPSP for T cells and the nature of the helper T (Th)-cell responses against this antigen have not been previously determined, studies reported herein were undertaken to characterize Th-cell responses against T. sergenti MPSP in cattle immunized with recombinant MPSP. Th-cell lines derived from MPSP-immunized cattle were used for examination of cytokine profiles and identification of MPSP Th-cell epitopes.

MATERIAL AND METHODS

Construction, expression, and purification of recombinant MPSP. Recombinant MPSP was prepared as a histidine-tagged protein. MPSP gene of an experimental strain, Fukushima (C type) was amplified by polymerase chain reaction (PCR). Forward and reverse primer sequences located at the 5’ and 3’ end of the open reading frame of MPSP were modified to include a PstI site at the 5' end. The recombinant protein was purified using live vaccines, alternative methods for the control of infectious agents such as bovine leukemia virus [7]. Instead of using live vaccines, alternative methods for the control of this disease are desired.

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Generation of MPSP-specific T cell lines: MPSP-specific T cell lines were established from PBMC of MPSP-immunized animals 1 month after the final antigen inoculation. PBMC (4 × 10^7) per well were cultured in 24-well plates in a 1.5 ml volume of complete RPMI 1640 medium with 25 µg of recombinant MPSP per ml. After 7 days, cells were subcultured to a density of 5 × 10^5 cells per well and cultured with antigen and 2 × 10^5 fresh irradiated (35 Gy) autologous PBMC as a source of antigen-presenting cells (APC). T-cell lines were maintained by weekly stimulation with antigen and APC, and the cells were assayed for antigen-dependent proliferation 6 to 8 days following the last antigenic stimulation.

Lymphocyte proliferation assays: Lymphocyte proliferation assays were carried out in triplicate wells of a 96-well plate for 6 days with PBMC and for 3 days with T-cell lines. 3 × 10^5 PBMC were cultured in triplicate wells with antigen in a total volume of 200 µl of complete medium. T-cell lines were assayed 7 days after the last stimulation with antigen and APC. 1 × 10^5 cells were cultured in duplicate wells in a total volume of 200 µl of complete medium containing 2 × 10^5 autologous APC and antigen. In preliminary experiments, each of CD4+, CD8+, WC1+ T-cell populations were eliminated from PBMC by treatment with specific antibody and complement. PBMC were first incubated with either anti-(α) CD4 (ILA11) or αCD8 (ILA51) or αWC1 (ILA29) monoclonal antibodies (Mab) in PBS for 0.5 hr at 4°C. After washing, they were incubated with rabbit complement (dilution of 1/10 in RPMI 1640) for 1 hr at 37°C. After that, cells were washed three times with complete medium and assayed as above. To determine proliferation, cells were radiolabeled for the last 7 to 15 hr of culture with 0.25 µCi of [3H]thymidine, radioiodinated nucleic acids were harvested onto glass filters and radiocount incorporation was measured using a liquid scintillation counter. Results are presented as mean counts per minute (cpm) ± 1 standard error.

Stimulation of cells and detection of cytokine mRNA by reverse transcriptase (RT)-PCR: T-cell lines were obtained 7 days after the last stimulation with antigen and APC, washed twice in complete medium, and cultured at a concentration of 2 × 10^5 cells per ml in the presence of an equal number of autologous APC and 25 µg of recombinant MPSP per ml. After 12 hr of culture, total cellular RNA was prepared from the cells following lysis with Trizol reagent (GIBCO BRL, Tokyo). As a positive control for cytokine mRNA expression, RNA was prepared from bovine PBMC stimulated at a concentration of 2 × 10^5 cells per ml with ConA for 12 hr. For negative controls, RNA was prepared from culture of irradiated APC and antigen.

To evaluate mRNA expression of interferon (IFN)-γ, interleukin (IL)-2, IL-4, IL-10 and tumor necrosis factor (TNF)-α,
α, a RT-PCR assay was employed as described previously. First strand cDNA was synthesized 1 hr at 42°C in a 30 μl reaction volume which containing 10 μg total RNA in diethyl pyrocarbonate-(DEPC) dH2O, 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl2, 10 mM DTT, 10U RAV-2 Reverse Transcriptase (Takara, Tokyo), 40 U Rnasin (Promega KK, Tokyo), 0.5 mM dNTP, 100 pmol oligo dT, 3 mM Mg²⁺. Newly synthesized first strand cDNA was diluted 1:3 to increase pipetting accuracy. Three μl of cDNA was added to a 50 μl of reaction mixture containing 150 μM of each dNTP, 10 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM MgCl₂, 2.5 U Taq polymerase and each 0.5 μM sense/antisense primers. The primer pairs used for PCR were described elsewhere [4]. Samples were overlaid with mineral oil and PCR reactions were performed in a PCR thermocycler under the following conditions: one cycle 4 min at 94°C, followed by 28 cycles 1 min denaturation at 94°C, 1 min annealing at 60°C, 1 min extension at 72°C, and final 72°C 4 min extension cycle. Reaction products (10 μl) were visualized on ethidium bromide stained 1.5% agarose gels.

RESULT

Lymphocyte proliferative responses of MPSP-immunized cattle: MPSP-specific proliferation of PBMC was continuously detected in all three immunized cattle following the third antigen inoculation. The proliferative responses were dose-dependent and were not found against control TRX (Fig. 2) and in a calf inoculated with liposome alone (Data not shown). The antigen-specific proliferative response of calf No. 1 was reduced to the level of the control when CD4+ T-cell population was depleted by anti-CD4 Mab and complement treatment, whereas this reduction was not observed when CD8+ and WC1+ T-cell population were depleted (αCD4 Mab-treatment; 8279 ± 951 cpm, αCD8 Mab-treatment; 57244 ± 9216 cpm, αWC1 Mab-treatment; 51417 ± 14758 cpm, No treatment; 60087 ± 10763 cpm).

Establishment of MPSP-specific T-cell line and epitope mapping: Three T cell lines derived respectively from calf No. 1, 2, 3 and stimulated with recombinant MPSP were tested repeatedly for MPSP-specific proliferative responses. All the cell lines proliferated vigorously in a dose-dependent manner to recombinant MPSP but not to control TRX (Data not shown).

The T-cell epitopes recognized by cattle vaccinated with MPSP were determined based on the proliferative responses of T-cell lines derived from each animal to the set of 20-mer overlapping peptides. Individual animals showed differences in both the peptides which induced proliferation of their T-cell lines and the magnitude of the proliferation induced. T-cell line derived from calf No. 1 proliferated vigorously to peptide No. 7. T-cell line derived from calf No. 2 proliferated vigorously to peptide No. 7 and weakly to peptide No. 2. T-cell line derived from calf No. 3 proliferated vigorously to peptide No. 15 and weakly to peptide No. 2, 6, 23 (Fig. 3).

To determine whether these T-cell epitope are conserved among different types of MPSP, peptides of the regions corresponding to peptide No. 7 and No. 15 were further synthesized for MPSP of I, B-1 and B-2 (Fig. 1). T-cell line, No. 2 proliferated in a similar manner to all the types of peptide. T-cell line, No. 3 proliferated vigorously in a dose-dependent manner to the peptides of C and B-2 types but not to those of I and B-1 types (Fig. 4).

Cytokine expression by MPSP-specific T-cell lines: PCR analysis of mRNA for IL-2, IL-4, IL-10, IFN-γ, and TNF-α revealed that all T-cell lines expressed relatively high levels of IFN-γ and low levels of IL-2 and IL-10, but undetectable levels of IL-4. In No. 2 and No. 3 T-cell lines, mRNA expression of TNF-α were also detected at low levels (Fig. 5). Any cytokine expressions were not detected in irradiated
DISCUSSION

The pathogenicity of a group of *T. sergenti/buffeli/orientalis* is mainly caused by the piroplasms proliferating within the erythrocytes of the hosts. MPSP, which is expressed on the surface of the piroplasm, is thought to be one of important target molecules for subunit vaccine development. The studies on the host's immune response against MPSP have been focused on antibody responses [26, 27]. Recently, the role of cellular immunity has been emphasized in the protection against intraerythrocyte parasites such as *Babesia* spp. and *Anaplasma* spp. [2, 3]. In the present study, cellular reaction in animals immunized with recombinant MPSP were investigated.

![Fig. 3. The proliferative response of T cell lines derived from immunized cattle to stimulation with 20-mer synthetic peptides comprising regions of the MPSP sequence. The results are presented as the mean ± SD of duplication cultures. The horizontal line represents a stimulation index (SI) of 2, the cut off for a positive response. T cells (2 × 10^4) were cultured with autologue APC and 25 µg of peptide per ml for 3 days. Cells were radiolabeled for 7 hr, harvested, and counted.](image-url)
Immunization with recombinant MPSP encapsulated by mannan-coated liposome induced strong cellular responses against MPSP in all immunized animals. These cellular responses were thought to be restricted by MHC because proliferative responses of T-cell lines were not detected in co-culture with antigen and APC derived from different animals.

In many infectious diseases, cytokines produced by CD4+ Th cells are thought to play important roles on protective immunity against infectious agents. To define whether T-cell responses against MPSP are Th-1 or Th-2 type, mRNAs of cytokines in MPSP-specific T-cell lines were examined by RT-PCR. Both of Th-1 cytokines, IL-2 and IFN-γ, and Th-2 cytokine, IL-10 were detected at mRNA levels in all T-cell lines examined here. Interestingly, high level of IFN-γ was expressed, but mRNA of IL-4 was less than detectable levels. The expression of IL-10 mRNA by different subsets of Th cells has been previously reported and is not diagnostic of a Th2 response in cattle [1]. Taking all this data into consideration, we conclude that MPSP, administered in mannan-coated liposome, induce a predominant type-1 immune response in all three cattle examined. It is reported that MPSP is strongly recognized by antibodies produced in infected animals [9, 11, 18, 20]. Present data revealed that the immunogenic nature of MPSP and its ability to stimulate Th cells that express gamma interferon support its inclusion in a vaccine.

From the results of epitope mapping using truncated synthetic peptides of MPSP, each cattle reacted to a panel of truncated peptides in a different manner, although cattle Nos. 1 and 2 responded to the same peptide, No. 7, DTSKFTPTVAHRLKHAEDLF (residues 61 to 80). T-cell lines derived from cattle No.3 responded to peptide No.15, GTGKVYDFVGNFKVTVKVF (residues 141 to 160) but not to peptide No. 7. In the region corresponding to peptide No. 15, the diversity of amino acid sequences were observed among different types of MPSP. In cattle No. 3, T-cell line showed type-specific responses against peptides designed from amino acid sequences of each MPSP type. The diversity of MPSP may indicate limitations on it’s use as a subunit vaccine. However, most of MPSP types found in Japan are categorized into two major types, i.e. I and C. Within a type of MPSP, moreover, all DNA sequences investigated showed only a very small variation (more than 98% homology) [10]. Therefore, immunization with cocktail consisting of different types of MPSP may be effective. Further study to identify the epitope specific for each allelic variant of MPSP is necessary.

REFERENCES


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