NOTE  
Clinical Pathology

Cytokine and Inducible Nitric Oxide Synthase Gene Expressions in Peripheral Blood Mononuclear Cells and Related Clinical Characteristics in Theileria orientalis sergenti-Infected Calves

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ABSTRACT. Eight splenectomized calves were inoculated with Theileria orientalis sergenti (Tos)-infected tick gland homogenate (5 calves) or infected erythrocyte suspension (3 calves). Clinical characteristics were different in calves post-infection. Animals were divided into 3 groups on the basis of susceptibility as high, middle, and low. Increase in mRNA of IFN-γ, IL-2, and TNF-α was observed in peripheral blood mononuclear cells at the peak of infection and was seen to be related with pyrexia and parasitemia. Expression of IL-1, IL-4, and inducible nitric oxide synthase was not observed. Decreased plasma nitrite/nitrate level was observed in the groups. The results of this study indicate that Th1 response is the predominant response in Tos infection, and this response is also related with their clinical characteristics.

KEY WORDS: cytokine, RT-PCR, Theileria orientalis sergenti.

Cellular immune responses generated after antigen stimulation of lymphocyte populations can be characterized by the distinct cytokines that are produced [1]. CD4+ T cell clones are broadly divided into 2 subsets based on their cytokines production: Th1 (IL-2, IFN-γ, and TNF) or Th2 (IL-4, IL-5, IL-6, IL-10, and IL-13) [21]. The significance of apparent domination of a Th1 or a Th2 response has been shown to have particular relevance in response to many pathogens [23]. In Plasmodium infection, it has been suggested that the balance between Th1 and Th2 immune response determines the degree of parasitemia, level of anemia, clinical severity, presentation and/or outcome through direct or indirect reactions of cytokines and other physiologically active substances such as nitric oxide (NO) [14]. Bovine theileriosis, caused by Theileria orientalis sergenti (Tos), is one of the most serious diseases of grazing cattle in Japan. The main symptoms of this disease are fever and anemia. The cytokines produced during infection might play a critical role in the pathogenesis of this disease; however, currently there is very little information available about this relationship.

In the present investigation we evaluated the mRNA expression of cytokine and inducible nitric oxide synthase (iNOS) genes in peripheral blood mononuclear cells (PBMC) and plasma nitrite/nitrate levels to study the relationship of these parameters with the clinical characteristics of this disease.

Animals: Eight Holstein splenectomized calves were divided into two groups. The homogenate-inoculated group (HIG) comprised of 5 calves (Nos. 851, 868, and 869) that had been infected with Ikeda stock of Tos previously. The origin of each inoculum was from a calf (No. 853) that had been infected with Ikeda stock of Tos previously.

Blood sample preparation and RT-PCR: Ten ml of heparinized blood samples of all calves were collected from the jugular vein at 2 to 3 day intervals. PBMCs were separated by Ficoll-Conray (SG 1.081) gradient centrifugation, and these cells usually contained no more than 5 per cent neutrophils. Poly(A)RNA from the PBMCs was isolated using a mRNA purification kit from Amersham Pharmacia Biotec (Buckinghamshire, England). Ethanol-precipitated poly(A)RNA were finally dissolved in an appropriate volume of TEN (10 mM Tris-HCl pH 8.0, 10 mM sodium chloride, 1 mM EDTA) buffer. The concentration of Poly(A)RNA was determined by its absorbance at 260 nm.

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In the present investigation we evaluated the mRNA expression of cytokine and inducible nitric oxide synthase (iNOS) genes in peripheral blood mononuclear cells (PBMC) and plasma nitrite/nitrate levels to study the relationship of these parameters with the clinical characteristics of this disease.

Animals: Eight Holstein splenectomized calves were divided into two groups. The homogenate-inoculated group (HIG) comprised of 5 calves (Nos. 851, 868, and 869) that had been inoculated intravenously with 30 ml of fresh Tos-infected erythrocyte suspension (50 v/v in normal saline; total inoculated parasitized erythrocytes: approx. 7.5 × 1010). The origin of each inoculum was from a calf (No. 853) that had been infected with Ikeda stock of Tos previously.

Blood sample preparation and RT-PCR: Ten ml of heparinized blood samples of all calves were collected from the jugular vein at 2 to 3 day intervals. PBMCs were separated by Ficoll-Conray (SG 1.081) gradient centrifugation, and these cells usually contained no more than 5 per cent neutrophils. Poly(A)RNA from the PBMCs was isolated using a mRNA purification kit from Amersham Pharmacia Biotec (Buckinghamshire, England). Ethanol-precipitated poly(A)RNA were finally dissolved in an appropriate volume of TEN (10 mM Tris-HCl pH 8.0, 10 mM sodium chloride, 1 mM EDTA) buffer. The concentration of Poly(A)RNA was determined by its absorbance at 260 nm.

RNA-specific oligonucleotide primers used for each cytokine, β-actin used as a housekeeping gene, were the same as reported earlier [16]. The primers of iNOS were designed using published sequences and were synthesized on a DNA synthesizer (forward primer, 5′-TTGGGCCTG-GTACGGG-3′ and reverse primer, 5′-ATCTCCGCAAAT-GTAGAGGTG-3′). The reverse transcription-polymerase chain reaction (RT-PCR) was carried out as per the protocol of Takara Shuzo (Kyoto, Japan).

Semi-quantification of mRNA: Electrophoretic gel images were captured using a digital camera (DC120, Eastman Kodak, Rochester, NY), and the intensity of each band was analyzed by 1 D Image Analysis Software (Eastman Kodak). Semi-quantification of the mRNA of each cytokine
and iNOS was expressed as relative strength against intensity of the β-actin band.

Nitrite/nitrate detection in plasma: Nitrite/nitrate in plasma of Tos-infected calves was measured by the method of Misko et al. [18]. The fluorometric determination of nitrite is based on the fact that 2,3-Diaminonaphthalene (DAN) reacts with nitrite under acidic conditions to form 1-H-naphthotriazole, a fluorescent product. The intensity of the fluorescent signal was measured using a fluoroscent plate reader (SPECTRAFLUOR PLUS, TECAN, Grödig, Austria) with excitation and emission at 365 nm and 450 nm respectively.

Clinical characteristics and mRNA expression: The clinical characteristics varied among HIG calves, which were divided into two groups; high (Nos. 870, 893, and 901) and low (Nos. 897 and 900) susceptibility group. In high susceptibility group, the parasitized RBC were detected in the peripheral blood around 15 days post inoculation, and reached a peak parasitemia of 35–45 per cent. These calves showed high pyrexia (more than 40°C) at the peak of parasitemia. In low susceptibility group calves, the appearance of parasitized RBC in the peripheral blood was noticed on day 50 post inoculation, and showed a low peak of parasitemia (8 and 10%). Definite pyrexia was not observed in these calves. All the calves in EIG were showing moderate clinical characteristics, pyrexia (39–39.5°C) and parasitemia (20–25%), these were classified as middle susceptibility group. Typical clinical characteristics and patterns of cytokine mRNA expressions on PBMC in each group are shown in Fig. 1 and the summarized results of gene expressions at the crisis period are shown in Table 1. A definite increase in IFN-γ (3/3 calves), TNF-α (3/3 calves), and IL-2 (2/3 calves) was observed in the high susceptibility group. Slight increase in mRNA expressions of IFN-γ (3/3 calves), TNF-α (2/3 calves), and IL-2 (2/3 calves) was observed in the low susceptibility

Table 1. Cytokine and iNOS mRNA expression on peripheral blood mononuclear cells at the crisis period of Theileria orientalis sergenti-infected calves

<table>
<thead>
<tr>
<th>Susceptibility</th>
<th>IFN-γ</th>
<th>IL-2</th>
<th>IL-4</th>
<th>TNF-α</th>
<th>IL-1</th>
<th>iNOS</th>
</tr>
</thead>
<tbody>
<tr>
<td>870 H</td>
<td>+++</td>
<td>–</td>
<td>–</td>
<td>+++</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>893 H</td>
<td>+++</td>
<td>+</td>
<td>–</td>
<td>+++</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>901 H</td>
<td>+</td>
<td>+++</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>851 M</td>
<td>++</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>868 M</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>869 M</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>897 L</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>900 L</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

H, high susceptibility calves (parasitemia 35–45%, body temperature < 40°C); M, middle susceptibility calves (parasitemia 20–25%, body temperature 39–39.5°C); L, low susceptibility calves (parasitemia 8 and 10%, body temperature < 39°C; +++ indicates 50–150% strength against intensity of the β-actin band; ++ indicates 20–49% strength against intensity of the β-actin band; + indicates < 19% strength against intensity of the β-actin band.
In the middle susceptibility group, a moderate increase in IL-2 (2/2 calves), IFN-γ (1/2 calves), and TNF-α (1/2) was observed. There was no definite increase in mRNA expression of IL-4, iNOS, and IL-1, except in one calf (No. 851) during the examination period.

Plasma nitrite/nitrate: A typical pattern of plasma nitrite/nitrate in one calf of each susceptibility group is shown in Fig. 2. A decreasing tendency in plasma nitrite/nitrate with increase in parasitemia was observed in all groups, though the decrease was not significant statistically.

Cytokines may be the key determinants of severity and outcome in malaria [13, 27, 28], and may thus become the potential targets of therapeutic intervention provided their effect can be better understood. TNF-α has been reported to play a central role in malarial fevers and parasitemia [19, 21]. Malarial fever, the hallmark of a malarial infection, coincides with schizont rupture and the release of soluble antigen-carrying glycosyl-phosphatidyl inositol anchors that induce secretion of pyrogenic TNF-α from monocytes [10]. In lymphodestructive theileriosis, such as T. parva or T. annulata infection, hyperthermia appears at the schizont stage and persists up to the subsequent piroplasm stage or until the host dies [6, 7]. It was reported that the schizont stage of Tos is noted between day 4 and 8 after inoculation with sporozoite stabilate [12, 24]. However, during this period in our examination, definite fever was not observed, though a slight and transient increase in expression of TNFα was noticed in the high susceptibility calves. In our earlier examination too, a transient fever in the schizont stage was occasionally recorded even if a large amount of sporozoites were inoculated in naïve cattle (unpublished data). Therefore, the importance of the schizont stage in Tos infection might be insignificant pathogenically and mechanism of hyperthermia might be different from other protozoan infections.

In the present study, enhanced mRNA expressions of IFN-γ and TNF-α, and coincided with hyperthermia and high parasitemia. The presence of an antibody coating on parasites and parasitized erythrocytes has been presumed to increase the amount of cytokines produced by mononuclear phagocytic cells by enhancing the recognition of parasites and parasitized erythrocytes via their receptors for the Fc component of immunoglobulin. IFN-γ was also found to sensitize macrophages to opsonized particles by increasing the density of these receptors [11]. It has also been reported that cross-linking of Fc receptors on monocytes rapidly induces TNF release [3, 4]. The enhanced cytokine genes, which coincided with high parasitemia in this examination, might be the result of accelerated phagocytic activity in macrophages. Origin of observed hyperthermia at piroplasm stage also might be derived from this accelerated activity.

Protective immune responses against Plasmodium appear to be stage-specific, with cell-mediated immunity being important in the liver-stage (schizont stage) infections, whereas antibody-mediated immunity seems most protective for blood-stage (piroplasm stage) parasites [10]. However, schizont stage in this examination was not clear, and only expression of Th1 cytokine genes (TNF-α, IFN-γ, and IL-2) with no traces of IL-4 mRNA were observed at the piroplasm stage. This finding indicates that Th1 response is predominant in the piroplasm stage of Tos infection and is also related with their clinical characteristics (including parasitemia). This fact is suggesting that strong cell-mediated immune response is owing to their clinical severity. It is clinically well known in the field that cattle showing mild symptoms of the disease are apt to fall in recrudescence by subsequent challenge. This phenomenon might relate to antigen specific T cell immunity evoked by cell-mediated immune response [17]. However, details of this immunity, particularly study for T cell memory have not been performed. Further study will be needed to clarify the role of cell-mediated immune response in prevention of this disease.

In the present study, variations in clinical characteristics were seen in the HIG calves, though these calves were inoculated with the same amount and same strain of Tos stabiliates. The reason for this difference could not be established. A similar phenomenon observed in experimen-
nal infection with *T. parva* was reported with the reasoning that the cryopreservation procedure influenced the virulent effect of *T. parva* stabulate [15]. In Tos infection, number of infected intact parasites (sporozoite or piroplasma) is considered to be a determinant factor for subsequent outcome of the disease (Kamio T, unpublished data). One possible reason why EIG calves showed some clinical characteristics is based on this phenomenon, and due to introduction of fresh and large number of parasites. It is also reported that differences in the MHC haplotype among calves restrict *Theileria*-specific cytotoxic cells [20]; therefore, some parasite and/or host factors might affect these variations.

NO production in the body is regulated by two distinct enzyme systems, both of which use arginine as their initial substrate and give rise to citrulline and NO. One is a calcium-dependent system involving a constitutive NO synthase (NOS) while the other, which is likely to be more relevant for pathogenesis, involves an iNOS that is activated by cytokines. NO reacts with oxygen to produce nitrite and/or nitrate, which are termed as reactive nitrogen intermediates and are toxic to intraerythrocytic protozoa [9, 22]. In our study, a decrease in plasma nitrite/nitrate with progression in infection was observed in all susceptibility calves. Furthermore, no trace of the expression of iNOS mRNA was found in these calves. On *in vitro* examination, the addition of IFN-γ to mouse peritoneal macrophages [5] and bovine peripheral macrophages [9], induced nitrite secretion, and further addition of TNF-α increased nitrite secretion significantly. The expression of iNOS mRNA in PBMC and plasma nitrite/nitrate concentration was expected to increase at least in the high susceptibilities as per *in vitro* findings, but it was not so. Similar phenomena have been reported in *Trypanosoma*-infected cattle [9, 26], mice [8], and *Toxoplasma gondii*-infected mice [25] and a decrease in plasma L-arginine concentration was recorded. The depleted L-arginine levels were thought to be as a result of arginase activity of host cells and consumption of L-arginine by the parasite, and was considered to be a determinant factor for the modulation of NO production [8]. In our earlier experiment also, serum arginine concentration in Tos-infected calves decreased with the increase in parasitemia, despite that all the calves were fed the same amount of fodder during the examination (unpublished data). It is still not clear whether a phenomenon similar to that observed in *Trypanosoma* infection does occur in Tos infection or not and further studies concerning the relationship between the parasite metabolism and NO production in host cells are required.

In this examination, we used splenectomized calves because it is difficult to get the desired pathological condition in the laboratory without this operation. It is well known that spleen plays an important role in the prevention of infections caused by intraerythrocytic protozoa diseases, therefore; splenectomy was performed to increase the level of infection in this disease. It is now thus necessary to perform further examination using naturally infected calves to confirm this result.

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REFERENCES


