Cytokine Gene Expression in Response to SnSAG1 in Horses with Equine Protozoal Myeloencephalitis

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Equine protozoal myeloencephalitis (EPM) is a neurologic syndrome seen in horses from the Americas and is mainly caused by Sarcocystis neurona. Recently, a 29-kDa surface antigen from S. neurona merozoites was identified as being highly immunodominant on a Western blot. This antigen has been sequenced and cloned, and the expressed protein has been named SnSAG1. In a previous study, cell-mediated immune responses to SnSAG1 were shown to be statistically significantly reduced in horses with EPM in comparison to EPM-negative control horses. It therefore appears as though the parasite is able to induce immunosuppression towards parasite-derived antigens as parasite-specific responses are decreased. Isolated peripheral blood lymphocytes from 21 EPM (cerebrospinal fluid [CSF] Western blot-negative) horses with no clinical signs and 21 horses with clinical signs of EPM (CSF Western blot positive) were cocultured with SnSAG1 for 48 and 72 h, and the effect on cytokine production was investigated by means of reverse transcriptase PCR. Cytokines assayed include gamma interferon (IFN-γ), tumor necrosis factor alpha, interleukin (IL)-2, IL-4, and IL-6. β-Actin was used as the housekeeping gene. A Wilcoxon signed-rank test of the findings indicated that there was a statistically significant decrease in IFN-γ production after 48 h in culture for samples from horses with clinical disease. There was also a statistically significant increase in IL-4 production after 72 h in culture for samples from horses with EPM. These results further support the notion that this parasite is able to subvert the immune system in horses with clinical disease.

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Materials and Methods

Horses. The present study included 21 EPM-negative horses (with Western blot-negative cerebrospinal fluid [CSF] samples; EBI, Lexington, KY) and 21 horses with clinical signs of EPM and Western blot-positive CSF samples from Alabama and Florida (19).

Lymphoblastogenesis. Peripheral blood samples were collected by jugular venipuncture into sterile tubes containing EDTA, and lymphocytes were isolated by differential centrifugation on Histopaque-1083 (Sigma, St. Louis, Missouri). All in vitro procedures were carried out under aseptic conditions. Lymphocyte numbers were assessed in Turk's white blood cell counting fluid (19), and the concentration was adjusted to 2 × 10^6 lymphocytes/ml in RPMI 1640 medium (containing 10% heat-inactivated fetal calf serum, antibiotics, and l-glutamine) (Atlanta Biologicals, Norcross, Georgia). Lymphocytes (containing less than 10% contaminating monocytes or neutrophils) were placed in the wells of 96-well (100 μl/well) round-bottom plates coated with 10 μg/ml of SnSAG1 and incubated at 37°C in a humidified atmosphere containing 5% CO2 and 95% air for 48 h and 72 h (17). The cells were then harvested and pelleted in a microcentrifuge, resuspended in RNAlater (QIAGEN, Valencia, California), and stored frozen until assayed by RT-PCR.
RT-PCR. mRNA was isolated by using RNA STAT-60 total RNA/mRNA isolation reagent (Tel-Test, Friendswood, Texas) according to the manufacturer’s instructions. The RT-PCR was performed using an Access RT-PCR system kit (Promega, Madison, Wisconsin), also in accordance with the manufacturer’s instructions. Approximately 500 ng of cDNA was used per PCR. The following cytokines were assayed under conditions previously described: gamma interferon (IFN-γ), tumor necrosis factor alpha (TNF-α), interleukin (IL)-2, IL-6 with β-actin as the housekeeping gene (14), and IL-4 (7). These cytokines were chosen as they are representative of T helper 1 (Th1) or T helper 2 (Th2) immune responses. The level of gene expression was not quantified, and the results were expressed as the presence or the absence of gene transcripts as visualized on a 1.5% agarose gel (Fig. 1).

Statistics. A Wilcoxon signed-rank test was performed to evaluate any statistically significant increased or decreased differences between the cytokine transcripts expressed by EPM-negative and EPM-positive horses in response to SnSAG1 incubation after 48 h or 72 h.

RESULTS

The results of this study indicate a trend towards an induction of proinflammatory cytokines in response to Sarcocystis neurona infection. A statistically significant difference was reported for IFN-γ for both the 48-h (P = 0.005) and 72-h (P = 0.046) incubation periods, with a decrease in the expression of this cytokine gene in horses with clinical EPM compared with the expression of this gene in EPM-negative horses at 48 h. There was significantly less IFN-γ expression after 48 h of incubation than after 72 h of incubation, indicating a significant lag in expression of this very important cytokine by horses infected with S. neurona. In EPM-negative control horses, there was no evidence of IFN-γ expression after 72 h (Table 1), indicating that there is an early induction of this cytokine in response to this parasite (P > 0.005) in normal horses.

A statistically significant increase in IL-4 gene expression was also observed after 72 h (P = 0.102) of incubation in horses that were positive for EPM (Table 1). There was no statistically significant difference in the amount of IL-4 gene transcription after 48 or 72 h of incubation in the presence of SnSAG1 in EPM-negative horses.

There were no statistically significant differences in gene expression of any of the other cytokines regardless of the horses’ EPM status, although certain trends appeared to be developing. It would appear as though TNF-α, IL-2, and IL-6 expression levels decrease with time in EPM-negative horses but increase with time in EPM-positive horses.

DISCUSSION

S. neurona is able to induce immunosuppression towards parasite-derived antigens as parasite-specific blastogenic responses are decreased (19). The results of this study indicate that this immunosuppression is due, in part, to a trend towards Th1 cytokine gene suppression and Th2 cytokine gene induction. This supports other studies in which it has been shown that levels of cytokines that are important in stimulating cell-mediated responses to protozoan antigens such as Toxoplasma gondii and Leishmania spp. are decreased (17, 20). It is possible that local immunosuppression is being induced by the presence of S. neurona merozoites in horses with clinical EPM, and the extent of this suppression could be related to host genetic factors. This possibility could explain why not all infected horses develop clinical disease.

In the current study, IFN-γ expression was shown to be suppressed in horses with clinical signs of EPM, further supporting the suggestion that S. neurona is able to depress expression of this very important cytokine. From previous studies (19), it has been shown that lymphocyte blastogenesis still occurs after 72 h in response to SnSAG1, thus eliminating the possibility that this protein induces cell death. Infection of immunocompetent mice with S. neurona merozoites does not result in parasitemia or any signs of disease. However, IFN-γ knockout mice succumb to disease, and parasites can be recovered from severe combined immunodeficient Arabian foals, further indicating the importance of this cytokine in disease prevention (11, 15).

IFN-γ is critical in the development of a Th1 response and is produced mainly by T cells and natural killer cells. It has a wide range of biological functions, including antiviral functions, influencing cell-mediated cytoxicity, priming macrophages, and augmenting production of IL-12, a cytokine which has a pivotal role in upregulation of various immune responses (1).

Interleukin-4 is produced primarily by Th2 cells and also has many biological effects, such as inducing B lymphocytes to undergo immunoglobulin isotype switching to produce immunoglobulin E, suppressing production of TNF-α, and inhibiting macrophage synthesis of IL-12 (1). The fact that S. neurona is able to upregulate IL-4 gene expression suggests another path-
way by which this parasite may be subverting the immune response, resulting in clinical disease.

The observed trend of TNF-α, IL-2, and IL-6 expression decreasing with time in EPM-negative horses, while increasing with time in EPM-positive horses, could be because these cytokines are produced by macrophages, and the increased production could be due to an anamnestic type of response in EPM-positive horses already exposed to this parasite. Further study examining different incubation times may shed some light in this area.

In conclusion, there are many ways in which protozoan parasites evade host immune responses, and these include changes in cytokine patterns as seen in the present study (20). One can assume that there are many more underlying factors involved, such as genetics of the host, inoculum dose, concurrent infections, and strain of the parasite, as not all infected horses develop disease. Whatever predisposes horses towards disease development may be further exacerbated by the presence of the parasite. Further work in this field is ongoing.

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