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ABSENCE OF THE SP/SP RECEPTOR CIRCUITRY IN THE SP PRECURSOR KNOCKOUT MICE OR SP-RECEPTOR, NEUROKININ (NK1) KNOCKOUT MICE LEADS TO AN INHIBITED CYTOKINE RESPONSE IN GRANULOMAS ASSOCIATED WITH MURINE TAENIA CRASSICEPS INFECTION

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Abstract

Neurocysticercosis, caused by the cestode *Taenia solium*, is the most common parasitic infection of the human central nervous system that leads to seizures. *Taenia crassiceps* cysticercosis in mice is an experimental model for *Taenia solium* cysticercosis. Similar to the human infection, live parasites cause little or no granulomatous inflammation. Dying parasites initiate a granulomatous reaction. The neuropeptide, Substance P (SP), stimulates Th1 cytokine production. In the current studies, we determined if absence of SP/SP receptor circuitry in the SP precursor, preprotachykinin knockout or SP-receptor, neurokinin (NK1) knockout mice, affected granuloma cytokine production. We hence compared the levels of Th1 cytokines, IL-2 and IFN- γ , and levels of Th2/immunoregulatory cytokines, IL-4 and IL-10, by ELISA in *T. crassiceps*-induced granulomas derived from infected C57BL/6 wild type (WT) versus SP-Precursor knockout and NK1 knockout mice. We found that mean levels of IL-2, IFN- γ , IL-4, and IL-10 in infected, WT-derived granulomas were significantly higher than those of granulomas derived from infected SP-Precursor knockout or the NK1 receptor knockout mice. Levels of Th2/immunoregulatory cytokines, IL-4 and IL-10, were higher in early stage granulomas (histologically-staged on basis of evidence of parasite remnants) versus late stage granulomas (no parasite-remnants) of both knockouts, whereas the reverse was noted in WT-derived granulomas. These studies established that the absence of an SP/SP receptor circuitry in the SP precursor knockout mice or NK1 receptor knockout led to an inhibited cytokine response.

Neurocysticercosis, caused by the cestode *Taenia solium* is the most common parasitic infection of the human central nervous system that leads to epileptic-like seizures.

Neurocysticercosis was previously prevalent mostly in the developing countries such as India and China, and in countries of Africa and Latin America (Bharucha, 2003; deBittencourt et al., 1996; Jallon, 1997; Sanchez et al., 1999; Rajshekhar et al., 2003). It is now becoming an increasingly important cause of seizures in the United States due to immigration from Mexico, and Central and South America (Sorvillo et al., 1992; Ong et al., 2002).

Food contaminated with feces of a *T. solium* tapeworm carrier (containing eggs of the tapeworm) is one of the primary causes leading to neurocysticercosis (Carpio, 2002). Eggs hatch in the small intestine. Oncospheres that emerge, penetrate the gut wall, enter the blood

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stream, and migrate to various sites, including the brain, subcutaneous tissues, heart muscle, and eyes, where they mature into cysticerci. Viable parasites are capable of modulating and suppressing the host immune responses (White et al., 1997). Initially, viable cysticerci induce a minimal granulomatous inflammatory response. However, as the cysticerci die, they lose their ability to control the host's inflammatory response. The cyst wall is then infiltrated and surrounded by host inflammatory granulomatous cells composed primarily of mononuclear macrophages. Inflammatory cells are also known to enter the fluid of the parasite. This inflammatory response is associated with production of type 1 cytokines such as interferon gamma (IFN- γ), Interleukin-2 (IL-2), and Interleukin-12 (IL-12) (Restrepo et al., 1998). As the host response progresses, fibrosis permeates the cysticercus with collapse of the cyst cavity. Eventually, the parasite is replaced totally by fibrotic tissue, which may ultimately calcify.

Taenia crassiceps cysticercosis in mice is an experimental model for *Taenia solium* cysticercosis (Kunz et al., 1989; Larralde et al., 1990; Villa and Kuhn, 1991; Sciutto et al., 1995; Villa and Kuhn, 1997). In this model, inoculation intraperitoneally with 10 small parasite cysts of *T. crassiceps* leads to loading within 3–6 mo the entire peritoneal cavity *crassiceps* cysts that have been propagated by asexual, exogenous budding of mature cysticerci. As in the human infection, live cysticerci of *T. crassiceps* induce little or no granulomatous inflammation, whereas dying parasites initiate a granulomatous reaction. The mediators responsible for modulating the granulomatous cytokine responses are not known.

Bioactive substances that can modify granulomatous cytokine responses include substance P (SP), which is a neuropeptide involved in pain transmission (Honore et al., 2000). Neurons, endothelial cells, and cells of the immune system synthesize SP; receptors for SP are distributed throughout the body on neurons, endothelial cells, lymphocytes, and macrophages (Weinstock et al., 1988; Cook et al., 1994; Ho et al., 1997; Maggi, 1997; Goode et al., 1998). SP plays an important role in immune-mediated inflammatory processes. Normal induction of the granulomatous inflammation in other parasitic infections, e.g., murine schistosomiasis, requires binding of SP to its specific high affinity receptor (Blum et al., 1999). Substance P can also bind to NK2 and NK3 receptors with very low affinity. Most importantly, SP stimulates the production of the Th1 cytokine, IFN- γ (Blum et al., 1998; Weinstock and Elliott, 1998).

We previously examined mRNA and protein expression of Th1 cytokines, IL-2 and IFN- γ , and Th2/immunoregulatory cytokines, IL-4 and IL-10, in granulomas from infected wild type BALB/c mice by in situ hybridization and immunohistochemistry (Robinson et al., 1997). Early stage granulomas (characterized on basis of parasite remnants) predominantly express Th1 cytokines, IL-2 and IFN- γ , whereas Th2/immunoregulatory cytokines, IL-4 and IL-10, are expressed in the later stages (no parasite remnants). We also detected SP mRNA and protein predominantly in early stage granulomas (Robinson et al., 2002). The role of SP in the regulation of cytokine production in murine cysticercosis was not addressed. Therefore, in the current studies, we determined if mice devoid of SP or its receptor generate granulomas in cysticercosis with altered capacity to make cytokines.

MATERIALS AND METHODS

In vivo animal studies

Murine cysticercosis model—Granulomas were removed from the peritoneal cavities of female mice that had been infected for 3 mo after intra-peritoneal inoculation with 10 cysts of the ORF strain of *T. crassiceps*. Three groups of mice were included in the experiments: (1) wild type C57BL/6 mice; (2) preprotachykinin or SP precursor knockout mice (bred 10 generations onto the C57BL/6 background); and (3) SP receptor, neurokinin (NK1) knockout mice bred 10 generations onto the C57BL/6 background). Five to 8 infected mice from each of the 3 groups were used for this study. Eleven to 18 granulomas/mouse group were used for

the study. Granulomas associated with parasites were identified visually, removed from the peritoneal cavity, and divided into 2 portions; 1 portion was fixed in 4% paraformaldehyde to be utilized for histological staging, and the other was used for quantifying cytokine proteins by ELISA.

Granuloma staging

The portion of each granuloma that was fixed with 4% paraformaldehyde was paraffin-embedded and cut into 5- μ m sections, which were then stained with Giemsa and examined microscopically. We separated the granulomas into early and late stages based on the histological appearance of the degenerating parasite according to previously described methods (Robinson et al., 1997). Briefly, granulomas were classified as follows. Early stage granulomas were divided into stages 1 and 2; the former exhibited areas of intact tegument of the dead parasites, but also possessed areas with infiltration with host cells. Stage 2 granulomas displayed no areas of normal tegument of the parasite, infiltration with lymphocytes, but exhibited intact parasite morphology of the dead parasite, including a cyst cavity. Late stage granulomas were divided into stages 3 and 4; stage 3 granulomas revealed complete infiltration with host mononuclear cells and no evidence of a parasite cyst cavity, but a suggestion of the underlying degenerating parasite morphology. Stage 4 granulomas revealed only host cells and debris without clearly identifiable parasite elements.

Sandwich ELISA for the detection of cytokines

We determined protein levels of the different cytokines in 15–18 different granulomas derived from *T. crassiceps* infected wild type mice, 10–13 granulomas derived from *T. crassiceps* infected, NK1 knockout mice, and 9–11 granulomas derived from *T. crassiceps* infected Substance P knockout mice. To quantify cytokine protein, a portion of each granuloma was homogenized in PBS, followed by centrifugation at 14,000 RPM. Supernatant was analyzed for IL-2, IL-4, IL-10, and IFN- γ protein by sandwich ELISA (R&D Systems, San Diego, California) as per manufacturer's instruction. Results are expressed as pg/mg of total protein.

Statistical analysis

Statistical differences were determined using 1-way ANOVA, followed by pairwise comparison using Tukey's or Dunn's test as appropriate. Significance was set at $P < 0.05$.

RESULTS

Relative levels of Th1 cytokines, IL-2, and IFN- γ in infected, WT-derived granulomas compared to granulomas derived from infected SP precursor knockout or NK1 receptor knockout mice

Interleukin-2—The mean IL-2 levels in the granulomas derived from infected wild type mice were significantly higher than those from granulomas derived from infected NK1R knockout mice (ANOVA; $P < 0.05$) (Fig. 1A). Similarly, the mean IL-2 levels in the granulomas derived from infected wild type mice were significantly higher than those from granulomas derived from infected SP-Precursor knockout mice (ANOVA; $P < 0.05$) (Fig. 1B).

Interferon- γ —Similar to the Th1 cytokine IL-2, the IFN- γ levels in the granulomas derived from infected wild type mice were significantly higher than those of the granulomas derived from infected NK1R knockout or SP knockout mice (ANOVA; $P < 0.05$) (Fig. 2A).

Relative levels of Th1 cytokines, IL-2 and IFN- γ derived from different stages of granuloma from infected, wildtype, SP Precursor knockout or NK1 receptor knockout mice

Interleukin-2—There was no major difference in the amount of IL-2 in early versus late stage granulomas derived from infected, wild type, or SP knockout mice (ANOVA; $P>0.05$) (Fig. 1B). However, significant differences were noted when comparing IL-2 levels in early versus late stage granulomas from infected NK1R knockout mice (ANOVA; $P<0.05$) (Fig. 1B).

Interferon- γ —There was no major difference in the amount of IFN- γ in early versus late stage granulomas derived from infected, wild type or SP knockout mice (ANOVA; $P>0.05$) (Fig. 2B). In contrast, IFN- γ levels in the early granulomas derived from infected NK1R knockout mice was significantly higher than that of the late stage granulomas derived from infected NK1R knockout mice (ANOVA; $P<0.05$) (Fig. 2B).

Relative levels of Th2/immunoregulatory cytokines, IL-10 and IL-4 in infected, WT-derived granulomas compared to granulomas derived from infected SP knockout or NK1 receptor knockout mice

Interleukin-10—Similar to the Th1 cytokines, IL-10 levels in the infected, wild type derived granulomas was higher than that of the infected, NK1R knockout derived granulomas or SP-Precursor knockout derived granulomas (ANOVA; $P<0.05$) (Fig. 3A).

Interleukin-4—Similar to the Th1 cytokines and the regulatory cytokine IL-10, levels of the Th2 cytokine, IL-4 in the granulomas derived from infected wild type mice were higher than those of the granulomas derived from infected, NK1R knockout or the SP-Precursor knockout mice (ANOVA; $P<0.05$) (Fig. 4A).

Relative levels of Th2/immunoregulatory cytokines, IL-4 and IL-10, derived from different stages of granulomas from infected, wildtype, SP-Precursor knockout or NK1 receptor knockout mice

Interleukin-10—Although the IL-10 levels in the early granulomas derived from infected wild type mice were somewhat lower than the late stage granulomas derived from infected wild type mice, these differences were not statistically different (ANOVA; $P>0.05$) (Fig. 3B). In contrast to wild type mice, mean IL-10 levels in the early stage granulomas derived from infected NK1R knockout mice were higher than those of the late stage granulomas derived from infected NK1R knockout mice (ANOVA; $P<0.05$) (Fig. 3B). Also, similar to that of the NK1R knockout granulomas, it was noted that the mean IL-10 levels in the early stage granulomas derived from infected, SP-Precursor knockout mice were higher than that of the late stage SP-Precursor derived granulomas. However, the increase was not statistically significant (ANOVA; $P>0.05$) (Fig. 3B).

Interleukin 4—The mean IL-4 levels in the early granulomas derived from infected NK1R knockout mice was significantly higher than those of the late granulomas derived from infected NK1R knockout mice. (ANOVA; $P<0.05$) (Fig. 4B). Similarly, we noted that the mean IL-4 levels in the early stage granulomas derived from infected, SP-Precursor knockout mice were significantly higher than those of the late stage SP-Precursor derived granulomas (ANOVA; $P<0.05$) (Fig. 4B). The IL-4 levels in the early granulomas derived from infected, wild type mice were not significantly different from those of the late stage granulomas derived from infected wild type mice (ANOVA; $P>0.05$) (Fig. 4B).

DISCUSSION

In the current studies, we determined if the absence of SP and its specific receptor, NK1R, affects cytokine expression in peritoneal granulomas induced by murine *T. crassiceps*

infection. We thus measured cytokine content of granulomas that developed in infected C57BL/6, wild type, SP-Precursor knockout, and NK1R knockout mice. We noted that the levels of Th1 cytokines, IL-2 and IFN- γ , and Th2/immunoregulatory cytokines, IL-4 and IL-10, in the wild type granulomas were significantly higher than those of the SP-Precursor and NK1R, knockout granulomas.

Substance P is known to stimulate the production of Th1 cytokines (Blum et al., 1998; Weinstock and Elliott, 1998). We had thus anticipated that if SP is an important factor in the stimulation of Th1 cytokines; in the absence of SP-SP receptor interaction, a blunted Th1 response should occur. As anticipated, we noted that in the granulomas derived from both infected SP-Precursor and the NK1R knockout mice, there was significantly lower Th1 cytokine levels as compared to granulomas derived from infected, wild type mice.

Since there are no studies implicating the role of SP-NK1R interaction in Th2/immunoregulatory cytokine production, our results demonstrating a blunted Th2/immunoregulatory cytokine production in the absence of the SP-NK1 receptor interaction network were unexpected. We do not know why the Th2/immunoregulatory cytokine response is blunted in the absence of the SP-NK1 receptor interaction pathway.

In the current studies, we also observed that the levels of Th2/immunoregulatory cytokines, IL-4 and IL-10, were higher in early stage versus late stage granulomas of both the knockouts. Although not significant, the Th2/immunoregulatory cytokine levels were higher in late stage versus early stage granulomas of the wild type mice. We do not know the reason why the pattern of Th2/immunoregulatory cytokine expression is different from that of the wild type infected mice.

The present studies show that the absence of SP precursor (preprotachykinin) and the specific substance P receptor, NK1R, affects cytokine expression in peritoneal granulomas induced by *T. crassiceps*. Preprotachykinin encodes for substance P and neurokinin A. Therefore, preprotachykinin knockout mice do not produce either substance P or neurokinin A. However, because NK1 receptor is specific for substance P and not for neurokinin A, and the current studies demonstrate that absence of SP precursor (preprotachykinin) and the specific substance P receptor, NK1R, affects cytokine expression in peritoneal granulomas induced by *T. crassiceps*, it is logical to deduce that most if not all of the effects on altered cytokine production is due to substance P and not by neurokinin A.

It has been demonstrated by Metwali et al. (2004) that peptide hormones such as hemokinin can bind and activate the NK1 receptor at sites of chronic inflammation (Metcwili et al., 2004). Therefore, although the current studies suggest that SP may be an important mediator associated with cytokine production, there may be other peptide hormones like hemokinin that also bind and activate the NK1 receptor that may be associated with cytokine production. Therefore, it may be possible that in the NK1 receptor knockout mice, besides the obstruction of the SP mediated affects, there may be additional blockage via the effects of other possible mediators such as hemokinin.

These studies determined that the absence of an SP/SP receptor circuitry in the SP precursor knockout mice or NK1 receptor knockout led to an inhibited cytokine response.

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Figure 1.

Quantitative levels of Th1 cytokine (IL-2) in (A) granulomas derived from *Taenia crassiceps* infected, wild type versus SP-Precursor knockout and NK1 receptor knockout mice.

* $P < .05$, IL-2 levels in infected, SP-Precursor knockout-derived granulomas versus wild type-derived granulomas, † $P < .05$, IL-2 levels in infected, NK1 knockout-derived granulomas versus wild type-derived granulomas.

(B) Early versus late granulomas derived from *Taenia crassiceps* infected, wild type versus SP-Precursor knockout and NK1 receptor knockout mice.

* $P < .05$, IL-2 levels in infected, early stage versus late stage NK1 knockout derived granulomas.

Figure 2.

Quantitative levels of Th1 cytokine (IFN- γ) in (A) granulomas derived from *Taenia crassiceps* infected, wild type versus SP-Precursor knockout and NK1 receptor knockout mice.

* $P < .05$, IFN- γ levels in infected, SP-Precursor knockout-derived granulomas versus wild type-derived granulomas, † $P < .05$, IFN- γ levels in infected, NK1 knockout-derived granulomas versus wild type-derived granulomas. (B) Early versus late granulomas derived from *Taenia crassiceps* infected, wild type versus SP-Precursor knockout and NK1 receptor knockout mice.

* $P < .05$, IFN- γ levels in infected, early stage versus late stage NK1 knockout-derived granulomas.

Figure 3.

Quantitative levels of IL-10 in (A) granulomas derived from *Taenia crassiceps* infected, wild type versus SP-Precursor knockout and NK1 receptor knockout mice. * $P < 0.05$, IL-10 levels in infected, SP-Precursor knockout-derived granulomas versus wild type-derived granulomas, † $P < 0.05$, IL-10 levels in infected, NK1 knockout-derived granulomas versus wild type-derived granulomas. (B) Early versus late granulomas derived from *Taenia crassiceps* infected, wild type versus SP-Precursor knockout and NK1 receptor knockout mice. * $P < 0.05$, IL-10 levels in infected, early stage versus late stage NK1 knockout-derived granulomas.

Figure 4.

Quantitative levels of Th2 cytokine (IL-4) in (A) granulomas derived from *Taenia crassiceps* infected, wild type versus SP-Precursor knockout and NK1 receptor knockout mice.

* $P < .05$, IL-4 levels in infected, SP-Precursor knockout-derived granulomas versus wild type-derived granulomas, † $P < .05$, IL-4 levels in infected, NK1 knockout-derived granulomas versus wild type-derived granulomas.

(B) Early versus late granulomas derived from *Taenia crassiceps* infected, wild type versus SP-Precursor knockout and NK1 receptor knockout mice.

* $P < .05$, IL-4 levels in infected, early stage versus late stage SP-Precursor knockout-derived granulomas, † $P < .05$, IL-4 levels in infected, early stage versus late stage NK1 knockout-derived granulomas.