Sequential Expression of the Neuropeptides Substance P and Somatostatin in Granulomas Associated with Murine Cysticercosis

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Neurocysticercosis, a parasitic infection of the human central nervous system caused by *Taenia solium*, is a leading cause of seizures. Seizures associated with neurocysticercosis are caused mainly by the host inflammatory responses to dying parasites in the brain parenchyma. We previously demonstrated sequential expression of Th1 cytokines in early-stage granulomas, followed by expression of Th2 cytokines in later-stage granulomas in murine cysticercosis. However, the mechanism leading to this shift in cytokine response in the granulomas is unknown. Neuropeptides modulate cytokine responses and granuloma formation in murine schistosomiasis. Substance P (SP) induces Th1 cytokine expression and granuloma formation, whereas somatostatin inhibits the granulomatous response. We hypothesized that neuropeptides might play a role in regulation of the granulomatous response in cysticercosis. To test this hypothesis, we compared expression of SP and expression of somatostatin in murine cysticercal granulomas by using in situ hybridization and immunohistochemistry. We also compared expression with granuloma stage. Expression of SP mRNA was more frequent in the early-stage granulomas than in the late-stage granulomas (34 of 35 early-stage granulomas versus 1 of 13 late-stage granulomas). By contrast, somatostatin was expressed primarily in later-stage granulomas (13 of 14 late-stage granulomas versus 2 of 35 early-stage granulomas). The median light microscope grade of SP mRNA expression in the early-stage granulomas was significantly higher than that in the late-stage granulomas (*P* = 0.008, as determined by the Wilcoxon signed rank test). By contrast, somatostatin mRNA expression was higher at later stages (*P* = 0.008, as determined by the Wilcoxon signed rank test). SP and somatostatin are therefore temporally expressed in granulomas associated with murine cysticercosis, which may be related to differential expression of Th1 and Th2 cytokines.

Neurocysticercosis is a parasitic infection of the human central nervous system caused by the helminth *Taenia solium*. The most common clinical manifestation of neurocysticercosis is seizures. Neurocysticercosis is now recognized as a leading cause of seizures worldwide (6, 22, 23, 40). Although there are cases of human neurocysticercosis in which symptoms occur after a short asymptomatic period, seizures in neurocysticercosis most often occur after an asymptomatic period lasting 4 to 5 years (8). Thus, infection of the central nervous system alone does not explain the symptoms. Individuals who die of other causes can have viable cysticerci in their central nervous systems. By contrast, cysticerci from patients with seizures invariably demonstrate a prominent inflammatory infiltrate. Thus, seizures likely result not from parasitic infection per se but from the chronic granulomatous host response to dying cysts (7, 12, 14, 27, 40). Antiparasite drugs can be used to kill the parasites, but they also may worsen symptoms by stimulating the host inflammatory response in response to the death of the organisms.

Murine *Taenia crassiceps* cysticercosis has been used as an experimental model for human *T. solium* cysticercosis (18, 20, 32, 36, 37). As in the human infection, live parasites produce little or no inflammation, whereas dying parasites initiate a chronic granulomatous reaction. We have previously studied and grouped granulomas that are associated with murine cysticercosis into four stages based on the histologic appearance of the degenerating parasite; the early stages are characterized by dying degenerating parasite remnants, and in the later stages there are no clearly identifiable parasites (29). Early-stage granulomas were also found to predominantly express Th1 cytokines, whereas interleukin-4 (IL-4) was expressed in the later granulomas (29). The mediators leading to initiation and control of the granulomatous inflammation are unknown.

Recent studies have shown that neuropeptides modulate the host immune response to parasites. Substance P (SP) is a short polypeptide involved in pain transmission (it is especially associated with inflammation) (17). SP is made by nerves, endothelial cells, and cells of the immune system. Receptors for SP are widely distributed throughout the body on neurons, endothelial cells, and immunocytes, such as lymphocytes and macrophages (5, 38). Binding of SP to its specific receptor is required for the maximal granulomatous response in murine schistosomiasis (3). In addition, SP stimulates the production of cytokines, including gamma interferon (IFN-γ) and proinflammatory cytokines like IL-1β, and tumor necrosis factor alpha (TNF-α) (1, 2, 13, 26, 31).

Somatostatin, another neuropeptide, counteracts the effects of SP and mediates analgesic effects by inhibiting the pain induced by noxious agents (15, 16, 25, 28, 30). Macrophages, after stimulation by IFN-γ and TNF-α, produce large amounts of somatostatin, while lymphocytes express somatostatin re-
ceptrons (4, 9, 10, 39). Somatostatin analogues have anti-inflamma-
tory activities and suppress the production of proinflamma-
tory Th1 cytokines (19, 31). Schistosome-infected animals
treated with somatostatin analogues form smaller granulomas
(11). We therefore hypothesized that neuropeptides might play
a role in regulation of the granulomatous response in cystic-
ercosis. To test this hypothesis, we compared expression of the
neuropeptides SP and somatostatin after infection at different
granuloma stages.

MATERIALS AND METHODS

In vivo animal studies. Six- to 10-week-old female BALB/c mice were inocu-
lated with 10 cysts of the ORF strain of T. crassiceps suspended in Hank's
balanced salt solution (T. crassiceps cysts were kindly provided by Raymond E.
Kuhn, Wake Forest University). After 3 to 9 months, eight heavily parasitized
mice were sacrificed. Granulomas associated with parasites were identified visu-
ally and removed from the peritoneal cavity. Each granuloma was sectioned into
two portions. One portion was immediately fixed in 4% paraformaldehyde for
histological staging and examination of mRNA of the different bioactive medi-
ators. The other portion was frozen immediately in freezing media for examina-
tion of the SP and somatostatin protein. The portion of each granuloma that was
fixed with 4% paraformaldehyde (prepared in diethyl pyrocarbonate-treated
phosphate-buffered saline) was stored in 70% alcohol until it was sectioned. The
sections were used for in situ hybridization studies and histological staging
analysis performed by a previously described method (29). This study was ap-
proved by the Animal Research Committee at Baylor College of Medicine.

Granuloma staging. Histologic stages of the granulomas were determined as
follows. Stage 1 granulomas showed areas of histologically intact tegument of
dead parasites and other areas where there was infiltration of host cells. Stage 2
granulomas displayed no areas of normal tegument of the parasite with intact
morphology of dead parasites, including a cyst cavity with infiltration of lympho-
cytes. In stage 3 granulomas there was no evidence of a parasitic cyst cavity, but
there was a suggestion of the underlying degenerating parasitic morphology and
there was complete infiltration of host mononuclear cells. Stage 4 granulomas
had no clearly identifiable parasite elements and consisted of only host cells and
debris.

Cytokine cDNAs, plasmids, and preparation of 35S-labeled riboprobes. pBlue-
script SK(−) plasmids containing cDNA for murine SP precursor (preprotachy-
kinin) and somatostatin (provided by J. V. Weinstock, University of Iowa) were
prepared by using ion-exchange chromatography (Qiagen Inc., Chatsworth, Cal-
lif.) and a previously standardized protocol (29, 41). The plasmid cDNA were
linearized with appropriate restriction enzymes. Antisense and sense RNA probes
were synthesized by in vitro transcription by using T3 or T7 polymerases
along with 250 μCi of 35S-labeled UTP, 20 U of RNasin, 0.5 mM unlabeled ATP,
0.5 mM unlabeled GTP, 0.5 mM unlabeled CTP, 0.1 M dithiothreitol, and
transcription buffers in diethyl pyrocarbonate-treated water, using a commercial
kit (Amersham Life Science, Inc., Arlington Heights, Ill.) (29, 41). The template
was digested with RNase-free DNase; the labeled probe was precipitated with
hydridization buffer for 1 h and then with the 35S-UTP-labeled
probe in an in situ hybridization cocktail for 1 h. Adjacent serial sections
were examined with antisense (experimental) and sense (negative control)
probes. After hybridization, the slides were washed twice with 2× SSC (1× SSC is 0.15
M sodium chloride plus 0.015 M sodium citrate), incubated for 20 min with 50% formamide in 2× SSC, washed six times in decreasing concentrations of 2× SSC,
and digested with RNase (37°C, 30 min) to remove nonhybridized probe. The
slides were then immersed in autoradiographic emulsion NTB2 (Kodak Eastman
Co., Rochester, N.Y.) for 48 h at 24°C, developed with Kodak Dektol developer,
fixed with Kodak fixer, and counterstained with Giemsa stain. The optimal
concentration of probe that gave a positive signal with minimal background was
assessed for each probe by using murine brain sections as a positive control. The
sense strand of each probe was used as a negative control. Following in situ
hybridization, the slides were examined by bright-field microscopy, and the
number of cells overlaid with numerous silver granules was expressed as follows:
1+ (one or two positive cells per slide), 2+ (more than two positive cells per slide
but less than one positive cell per low-power field [×20 lens]), 3+ (about one
positive cell per low-power field but less than one positive cell per high-power
field [×40 lens]), or 4+ (one or more positive cells per high-power field).

Immunohistochemistry. Immunoperoxidase staining was performed on 5-μm
thick frozen or paraformaldehyde-fixed granuloma sections by using the avidin-
biotin method, an automated immunostainner (Biogenex), and polyclonal rabbit
antibody to murine SP (1:20; Chemicon, Temecula, Calif.) and somatostatin
(1:50; Chemicon). Murine brain tissue sections were used as a positive control.
Slides were considered positive if brown staining was noted within the cytoplasm
of cells at a level above the level of the nonspecific signal in tissue cells. Positive
slides were graded on a scale from 1+ to 3+ (1+, <10% of the leukocytes were
stained; 2+, 10 to 20% of the leukocytes were stained; 3+, >20% of the
leukocytes were stained).

RESULTS

Eight T. crassiceps-infected mice with 49 granulomas were studied. Nineteen granulomas were at stage 1, 16 granulomas
were at stage 2, 6 granulomas were at stage 3, and 8 granulo-
mas were at stage 4. In the present study, since the neuropep-
tide expression patterns seen in stage 1 and 2 granulomas do
not differ significantly from one another and, similarly, the
neuropeptide expression patterns seen in stage 3 and 4 gran-
ulomas do not differ significantly from one another, we
(grouped stage 1 and 2 granulomas together as early-stage gran-
ulomas and stage 3 and 4 granulomas together as late-stage granulomas. SP and somatostatin mRNA were expressed in the
granulomatous inflammations surrounding the dying cysts of T. crassiceps. SP mRNA was located primarily in the cells
along the interface between the parasite and the host in early-
stage granulomas (Fig. 1A). Somatostatin was found primarily in the late-stage granulomas in cells resembling monocytes
(Fig 2A).

Expression of SP mRNA was significantly more frequent in
the early granuloma stages than in the late granuloma stages;
34 of the 35 early-stage granulomas expressed SP precursor
mRNA, whereas only 1 of the 13 late-stage granulomas that
were studied for expression of SP precursor was positive. The
median light microscope grade for SP mRNA expression in the
early granulomas (stages 1 and 2) was significantly higher than
the median light microscope grade for SP mRNA expression in
the late granuloma stages (stages 3 and 4) (P = 0.008, as determined by the Wilcoxon signed rank test) (Table 1). In contrast, so-
matostatin mRNA was more frequent in late granuloma stages
than in early granuloma stages. Of 14 late-stage granulomas, 13
expressed somatostatin, compared to 2 of 35 early-stage gran-
ulomas. The median light microscope grade for somatostatin
mRNA expression in the late-stage granulomas was signifi-
cantly higher than the median light microscope grade for so-
matostatin mRNA expression in the early-stage granulomas
(P = 0.008, as determined by the Wilcoxon signed rank test)
(Table 1).

To confirm that mRNA expression correlated with synthesis of
the mature protein, representative frozen sections of gran-
ulomas from each of the four stages were studied by immuno-
histochemistry by using polyclonal antibody to SP or soma-
tostatin (Chemicon). The two early-stage granulomas both
expressed SP (Fig. 1B), whereas the two late-stage granulomas
did not express SP. By contrast, both the late-stage granulomas
expressed somatostatin protein (Fig. 2B), whereas both the
early-stage granulomas did not.
DISCUSSION

We observed differential expression of the neuropeptides SP and somatostatin in the granulomatous inflammations surrounding dying cysts of *T. crassiceps*. SP was preferentially expressed in early-stage granulomas, whereas somatostatin was expressed mainly in the late stages. These results suggest that SP and somatostatin may modify granulomatous inflammatory responses in cysticercosis. In murine schistosomiasis, normal induction of granulomatous inflammation requires binding of SP to its specific receptor (3). SP stimulates the production of cytokines, including IFN-γ, and proinflammatory cytokines like IL-1β, as well as TNF-α (1, 2, 13, 26, 31). Thus, SP is thought to be an important upregulator of the granulomatous response. By contrast, somatostatin analogues suppress the production of proinflammatory Th1 cytokines (19, 31). Infected animals treated with somatostatin analogues form smaller granulomas (11). This leads to a paradigm in which early expression of SP causes enhancement of the granulomatous response. As this response progresses, somatostatin is expressed, which in turn downregulates the response.

Significantly, a similar pattern of sequential expression of these neuropeptides occurred in our cysticercosis model. The association of SP with early-stage granulomas suggests that SP

![FIG. 1. Granuloma sections probed by in situ hybridization with 35S-labeled riboprobes for SP precursor. (A) Stage 1 granuloma surrounding a section of a *T. crassiceps* cyst, showing numerous positive cells after the section was probed with an antisense probe for SP precursor mRNA. The arrows indicate positive cells overlaid with multiple silver granules; the arrowhead indicates an intact cyst showing intact tegument, loose subtegmental tissue, and a central cyst cavity. Original magnification, ×100. The inset is a close-up of a few positive cells overlaid with multiple silver granules (original magnification, ×400). (B) Early-stage granuloma (stage 1), showing numerous positive cells expressing SP protein as revealed by immunohistochemistry. Original magnification, ×400.](http://iai.asm.org/)

![FIG. 2. Granuloma sections probed by in situ hybridization with 35S-labeled riboprobes for somatostatin. (A) Late-stage granuloma (stage 3), showing numerous positive cells after the section was probed with an antisense probe for somatostatin mRNA. The arrow indicates positive cells overlaid with multiple silver granules. Magnification, ×100. (B) Late-stage granuloma (stage 3), showing numerous positive cells expressing somatostatin protein as revealed by immunohistochemistry. Original magnification, ×200.](http://iai.asm.org/)
may play a similar role in cysticercosis by inducing early granuloma formation. The expression of somatostatin later is consistent with an anti-inflammatory role in the late stages of granuloma formation. While the present study was performed with heavily parasitized mice, we expect the same results for mice that are less heavily parasitized. In our previous studies, we noted that early-stage granulomas expressed predominantly Th1 cytokines, whereas Th2 cytokines were expressed only in the late stages (29). It is tempting to speculate that differential expression of granulomas may promote the Th1-to-Th2 cytokine shift in the granulomas surrounding dying T. crassiceps cysts.

Neuropeptides are known modulators of epileptogenic responses. SP can evoke epileptiform responses in neurons (24). Intrahippocampal administration of SP triggers status epilepticus in a process resembling human epilepsy (21). In contrast, somatostatin modulates classical neurotransmission and has anticonvulsant properties in experimental models of seizures (33–35). At present, the relevance of neuropeptides for the pathophysiology of neurocysticercosis is unknown. In preliminary studies, we have also observed induction of seizures in rodents by extracts derived from early murine cysticercal granulomas. Understanding which inflammatory modulators are present in the different stages of the granulomas, which mediators are epileptogenic, and which modulators inhibit seizure responses may open up possibilities for treatment of neurocysticercosis seizures with specific antagonists or analogues. Further studies are also required to demonstrate expression of neuropeptides in response to human infection with T. solium, within the central nervous system, and in association with seizures.

ACKNOWLEDGMENTS

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REFERENCES


TABLE 1. Correlation of SP and somatostatin mRNA expression with granuloma stage*

<table>
<thead>
<tr>
<th>Animal</th>
<th>SP mRNA expression score</th>
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*Slides were examined by bright-field microscopy, and the number of positive cells expressing SP or somatostatin mRNA were counted and scored as follows: 0, no positive cells on the entire slide; 1+, one or two positive cells per slide; 2+, more than two positive cells per slide but less than one positive cell per low-power field (×20 lens); 3+, about one positive cell per low-power field but less than one positive cell per high-power field (×40 lens); or 4+, one or more positive cells per high-power field. The median light microscope scores for SP and somatostatin mRNA expression were calculated for the early stages (1 and 2) and the late stages (3 and 4) for each animal. The differences between the median scores for the early and late stages were compared by using the Wilcoxon signed rank test. 

P = 0.008 (Wilcoxon signed rank test) for expression of SP mRNA in early versus late stages.


