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Granuloma Cytokines in Murine Cysticercosis

PREMA ROBINSON, ROBERT L. ATMAR, DOROTHY E. LEWIS, AND A. CLINTON WHITE, JR.*

Departments of Microbiology & Immunology and Medicine, Baylor College of Medicine, Houston, Texas

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Neurocysticercosis, caused by *Taenia solium*, is one of the most common causes of seizures worldwide. The symptoms result from granulomatous inflammation associated with dying cyst forms of the parasite. Although the invasive larvae can be killed by immune serum plus complement, immunity to the cyst stage depends on a cellular response. This dichotomous immune response is reminiscent of the extremes of the immune response associated with T helper 1 (Th1) and Th2 cytokine profiles. To characterize the cytokine response in cysticercosis, granulomas were removed from the peritoneal cavity of mice infected with *Taenia crassiceps* cysts and examined for cytokine message by in situ hybridization using ^{35}S-labeled RNA probes. The granulomas were staged based on histologic appearance of the degenerating parasite. Message for gamma interferon (IFN-γ) was identified by light microscopy in 11 of the 12 granulomas, and interleukin-2 (IL-2) message was identified in 9 of the 12. By laser scanning confocal microscopy, significantly increased IFN-γ and IL-2 pixel intensity was identified in nearly all of the granulomas from early histologic stages. Message for IL-4 was seen in 6 of the 12 granulomas. Only granulomas with complete destruction of the parasite architecture displayed more than minimal amounts of IL-4 message by light microscopy, and only 2 of 12 granulomas had IL-4 pixel intensity significantly above background. Only minimal amounts of IL-10 message were detected in 4 of 11 granulomas. Thus, early granulomas in cysticercosis are predominantly associated with a Th1 response, whereas later granulomas, in which parasite destruction is complete, have a mixture of Th1 and IL-4. The Th1 response appears to play an important role both in the pathogenesis of disease as well as in the clearing of the parasites, with IL-4 involved in downregulation of the initial response.

Neurocysticercosis, caused by infection of the central nervous system with the metacestode (cyst) stage of *Taenia solium*, is a common cause of seizures worldwide (13, 43, 44). To complete its life cycle, the metacestode form must survive for months in the tissues of the intermediate host. Viable cysticerci from pig muscles have minimal surrounding inflammation, but dying cysts are associated with a chronic granulomatous reaction (2, 3). Similarly, pigs vaccinated with parasite antigens have intense granulomatous inflammation around dying cysts (29). Symptomatic human neurocysticercosis follows an asymptomatic period typically lasting 4 to 5 years (7). While autopsies of patients with asymptomatic infection reveal little or no inflammation surrounding the cysts, both neuroimaging studies and histopathological specimens from symptomatic patients reveal evidence of inflammation, which consists of a chronic granulomatous reaction, associated with the cysts and progresses through a series of stages, eventually leading to parasite clearance (8, 10, 36). Symptomatic disease is thought to result when the dying parasite can no longer control the host inflammatory response (10, 44).

Active infection is associated with increased immunoglobulin production (42, 46). Furthermore, infection with the cyst stage is associated with concomitant antibody-mediated immunity to the oncosphere (invasive larval) stage (28, 30, 44). The two poles of the immune response, immunoglobulin production and humoral immunity to the oncosphere versus granulomatous response to the dying metacestode, are reminiscent of the responses associated with the two patterns of T-cell cytokines (T helper 1 [Th1] and Th2) found in other parasitic infections (32, 33, 45).

* Corresponding author. Mailing address: Department of Medicine, Baylor College of Medicine, One Baylor Plaza, Room 561E, Houston, TX 77030. Phone: (713) 798-6846. Fax: (713) 790-0681. E-mail: arthurw@bcm.tmc.edu.

Taenia crassiceps cysticercosis in mice has been used as an experimental model for *T. solium* cysticercosis (16, 19, 37, 41, 42). We and others have found granulomas surrounding dying *T. crassiceps* cysts, a pathologic process resembling that seen in human neurocysticercosis (10, 36). Little is known about the immune mechanisms involved in cysticercal granuloma formation, the type of cytokines produced, or their role in the evolution of the disease.

MATERIALS AND METHODS

Parasites and animals. The ORF strain of *T. crassiceps* was provided by Raymond Kuhn (Wake Forest University). Six 6- to 10-week-old female BALB/c mice were inoculated with 10 cysts suspended in Hanks balanced salt solution. After 3 to 14 months, the heavily parasitized mice were sacrificed. Granulomas associated with parasites were identified visually, removed from the peritoneal cavity, and fixed with 4% paraformaldehyde prepared in diethylpyrocarbonate-treated phosphate-buffered saline (1 to 2 h, 23°F). The slides were washed with phosphate-buffered saline prior to storage in 70% alcohol.

Cytokine cDNAs, plasmids, and preparation of ^{35}S-labeled riboprobes. Plasmids containing cDNA for murine interleukin-2 (IL-2), gamma interferon (IFN-γ), IL-4, and IL-10 were provided by Fred Heinzel (Case Western Reserve University). The cytokine cDNA-containing plasmids, insert sizes, restriction enzymes, and RNA polymerases used for transcription of the antisense and sense RNA probes are listed in Table 1. Plasmids were prepared from bulk cultures of transformed *Escherichia coli* DH5α (Gibco BRL Life Technologies Inc., Gaithersburg, Md.) by alkaline lysis and ion-exchange chromatography (Qiagen kit; Qiagen Inc., Chatsworth, Calif.). After linearization of the plasmid cDNA with the appropriate restriction enzymes, sense and antisense RNA probes were generated by in vitro transcription using either T7, T3, or SP6 RNA polymerase along with 250 μCi of [^{35}S]UTP, 20 U of RNAsin, 0.5 mM unlabeled nucleoside triphosphates (ATP, GTP, and CTP), 0.1 M dithiothreitol (DTT), and transcription buffer in diethylpyrocarbonate-treated water, using a commercially available kit (Amersham Life Science, Inc., Arlington Heights, Ill.). The template was digested with RNase-free DNase, followed by precipitation with alcohol in the presence of salt. Labeled probe was washed once with 70% ethanol, suspended in 0.1 M DTT, and stored at −70°C.

In situ hybridization. Paraffin-embedded sections of granulomas were deparaffinized by treatment with xylene, rehydrated with decreasing concentrations of ethanol (90 through 70%), acetylated, and glycinated. After being washed with 2× SSC (1× SSC is 0.15 M sodium chloride plus 0.015 M sodium citrate), the sections were digested with proteinase K (37°C, 10 min), washed with 2× SSC, and refixed with 4% paraformaldehyde. Sections were then incubated with the

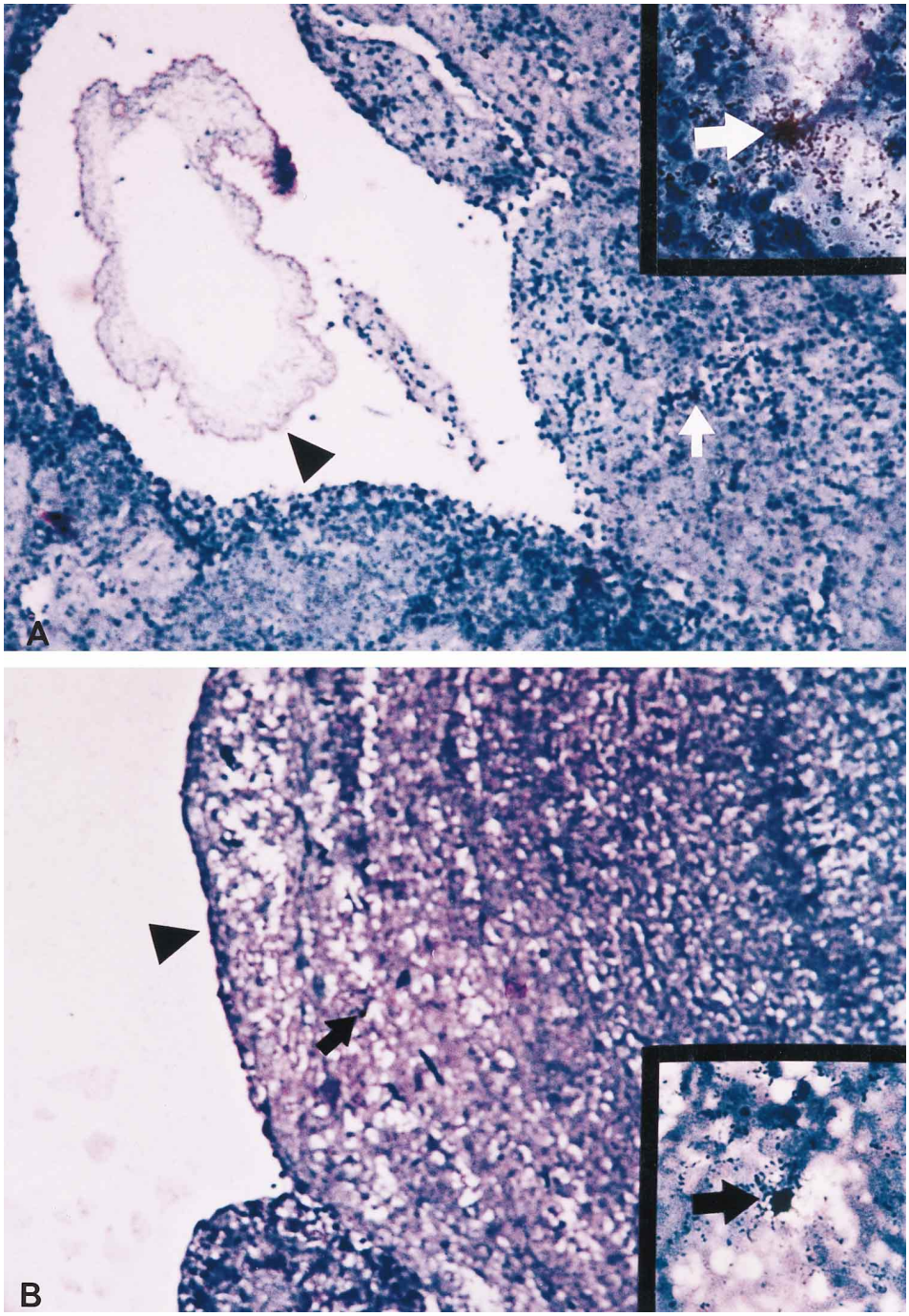

FIG. 1. (A) A stage 1 granuloma (granuloma 1B) surrounding a section of a *T. crassiceps* cyst was probed by in situ hybridization with ^{35}S-labeled antisense cRNA for IFN-γ. A section through the intact cyst shows the intact tegument (arrowhead), loose subtegmental tissue, and a central cyst cavity (histologic stage 1). After in situ hybridization with an antisense probe for IFN-γ, a single positive cell overlaid with multiple silver granules was visible in this field (small arrow; original magnification, ×100). The inset shows a closeup of the positive cell overlaid with multiple silver granules (large arrow; original magnification, ×500). (B) A stage 2 granuloma (granuloma 5A) from a mouse infected with *T. crassiceps* cysts was probed by in situ hybridization with ^{35}S-labeled antisense cRNA for IL-10. Sections through the granuloma revealed partial degradation of the tegument (arrowhead), with marked thickening of the cyst wall (histologic stage 2). The cyst also continued to contain an intact cyst cavity (not shown). After in situ hybridization with antisense probe for IL-10, a single positive cell overlaid with multiple silver granules was visible in this field (small arrow; original magnification, ×100). The inset shows a closeup of the positive cell overlaid with multiple silver granules (large arrow; original magnification, ×500).

^{35}S-labeled probe in hybridization cocktail (50% formamide, 10 mM DTT, 50% dextran sulfate, 10 mM Tris-HCl [pH 7.4], 0.5 mg of yeast tRNA, 10 μl of 100× Denhardt's solution, 300 mM NaCl, 1 mM EDTA [pH 8.0]; 3 h). The temperature and concentration were optimized for each probe, using concanavalin A-stimulated murine spleen cells as a positive control (IL-2, IL-4, and IFN-γ) and the sense strand of each probe as a negative control. For IL-10, spleen sections of *T. crassiceps*-infected mice were used as the positive control. After hybridization, the slides were washed twice with 2× SSC, incubated for 20 min with 50% formamide in 2× SSC, washed six to eight times in 2× SSC, and digested with RNase (37°C, 30 min) to remove nonhybridized probe. The slides

TABLE 1. Cytokine cDNAs, plasmids, restriction enzymes used to linearize the plasmids, and RNA polymerases used to prepare cytokine cRNA probes[a]

Probe	Insert size (bp)	Restriction enzyme		Plasmid	RNA polymerase	
		Antisense	Sense		Antisense	Sense
IL-2	345	*Bam*HI	*Hin*dIII	pBluescript II SK+	T7	T3
IL-4	400	*Eco*RI	*Hin*dIII	pGEM-1	SP6	T7
IL-10	415	*Bam*HI	*Hin*dIII	pGEM-3Z	SP6	T7
IFN-γ	430	*Eco*RV	*Eco*RI	pGEM-3Z	SP6	T7

[a] The plasmids containing cytokine cDNAs were provided by Fred Heinzel (Case Western University).

were immersed in autoradiographic emulsion NTB2 (Kodak Eastman Co., Rochester, N.Y.; 48 h, 23°C), developed with Kodak Dektol developer, fixed with Kodak fixer, and counterstained with Giemsa stain. The slides were examined by bright-field microscopy, and the number of cells overlaid with 25 or more grains per high-power field was determined. The concentration of probe which gave an optimal positive signal with minimal background was assessed for each probe. Optimal concentrations were 100 ng/ml for the IFN-γ probe and 500 ng/ml for IL-2, IL-4, and IL-10 probes.

Quantitation of cytokine signal. Pixel intensity was quantitated by laser scanning confocal microscopy (LSCM) as previously described (26, 31). Briefly, granuloma sections hybridized with a sense or antisense cRNA probe were imaged with an MRC-500 laser scanning confocal microscope (Bio-Rad Laboratories, Richmond, Calif.). Areas of suspected hybridization to the labeled riboprobe were initially localized by light microscopy. These areas were then confirmed by LSCM operated in the reflected light mode. Optical sections were taken at 0.5-μm increments through the specimens. Positive hybridization was confirmed if the silver grains remained in the confocal image of the optical sections above the level of nonspecific background. The number of pixels in four or five areas of each section was determined. Pixel numbers on sections probed with antisense and sense probes were compared by *t* test or, when only a few strongly positive areas were found, by Wilcoxon rank sum test. *P* values of ≤0.05 by one-tail tests were considered significant.

Histologic staging. Giemsa-stained slides were examined by light microscopy and staged by an observer masked to other data. The histopathology of the granulomas was classified into four stages. Stage 1 granulomas showed areas of histologically intact parasite tegument but other areas with infiltration with host cells. Stage 2 granulomas displayed no areas of normal tegument, infiltration with lymphocytes, but intact parasite morphology including a cyst cavity. Stage 3 granulomas revealed complete infiltration with host mononuclear cells, no cyst cavity, but a suggestion of the underlying parasite morphology. Stage 4 granulomas revealed only host cells and debri without clearly identifiable parasite elements.

RESULTS

Twelve granulomas were removed from six mice infected with *T. crassiceps* cysts. Two granulomas from mouse 6 and one from mouse 1 were stage 1 (Fig. 1A; Table 2). Two granulomas from mouse 5 were stage 2 (Fig. 2B). Four granulomas from three mice were stage 3 (Fig. 2A; Table 2). Three granulomas from three different mice were stage 4 (Fig. 2B; Table 2). Histologic stage did not correlate with the time from infection until the mouse was sacrificed. Message for IFN-γ was identified by light microscopy in 11 of the 12 granulomas. In the positive granulomas, message was detected in one to eight strongly positive cells at the periphery of the granuloma (Fig. 1A). The granulomas with the most cells positive for IFN-γ message belonged to groups 2 and 3 (Table 2; Fig. 3A). Quantitation of pixel intensity by LSCM revealed message for IFN-γ significantly above background in 9 of the 12 granulomas (Table 2). All of the stage 1 and 2 granulomas had significantly increased IFN-γ message compared with four of seven granulomas for stages 3 and 4 (Fig. 3B; Table 2). The pixel intensity also tended to be higher in these early stages (Table 2).

Message for IL-2 was identified by light microscopy in 9 of the 12 granulomas (Fig. 2A; Table 2). IL-2 message was found in 1 to 11 strongly positive cells at the periphery of the gran-

TABLE 2. Cytokine expression in granulomas from mice infected with *Taenia crassiceps*, quantitated as mean number of pixels in a defined area by confocal microscopy and also by the number of positive cells by light microscopy

Granuloma	Duration of infection (mo)	Histologic stage	Probe	IFN-γ		IL-2		IL-4		IL-10	
				χ Pixels[a]	Positive cells	χ Pixels	Positive cells	χ Pixels	Positive cells	χ Pixels	Positive cells
1A	3	3	Antisense	**38.8**	1	**90**	6	2.8	0	19.3†	1
			Sense	**20.6**		**2.3**		5.0		6.2	
1B	3	1	Antisense	**86.2**	3	**97.2**	2	23.8	0	30.8	0
			Sense	33.6		**25.6**		22.0		25.2	
2A	5	4	Antisense	99.9†	3	**133.6**	11	16.9†	2	3.34	0
			Sense	6.1		**1.1**		5.5		3.3	
2B	5	3	Antisense	87.8	5	54.0	2	42.4	0	**54.2**	1
			Sense	78.2		30.2		58.8		**27.4**	
3	7	4	Antisense	**103**	2	**30.8**	3	**33.6**	5	ND[b]	0
			Sense	**13.3**		**1.1**		**2.3**		ND	
4A	10	4	Antisense	4.4	0	25.9	0	32.5	1	19.0	1
			Sense	3.1		35.9		36.3		5.3	
4B	10	3	Antisense	**43.6**	5	78.0	1	**54.4**	1	13.2	0
			Sense	**18.4**		70.8		**20.2**		42.6	
4C	10	3	Antisense	**44.8**	8	48.6	1	20.8	0	133.8†	0
			Sense	**9.8**		22.4		21.8		92.0	
5A	11	2	Antisense	**73.4**	5	**72.4**	6	67.6	1	**77.0**	1
			Sense	**11.4**		**11.4**		53.2		**45.8**	
5B	11	2	Antisense	**97.8**	3	**149.4**	6	17.8	0	21.2	0
			Sense	53		**48.2**		23.8		30.2	
6A	14	1	Antisense	**132**	1	185	0	61.8	0	23.2	0
			Sense	**32**		171		70.4		22.2	
6B	14	1	Antisense	**104.2**	1	**224.8**	0	82.6	1	155.4	0
			Sense	**18.4**		**162.8**		63.4		111.6	

[a] Pixel number was compared for sections probed with antisense (positive) and sense (negative control) probes by using a one-tailed *t* or Wilcoxon rank sum test. Granulomonas with significantly increased pixel intensity ($P \leq 0.05$) are shown in boldface type. †, $P > 0.05$ but < 0.1.

[b] ND, not determined.

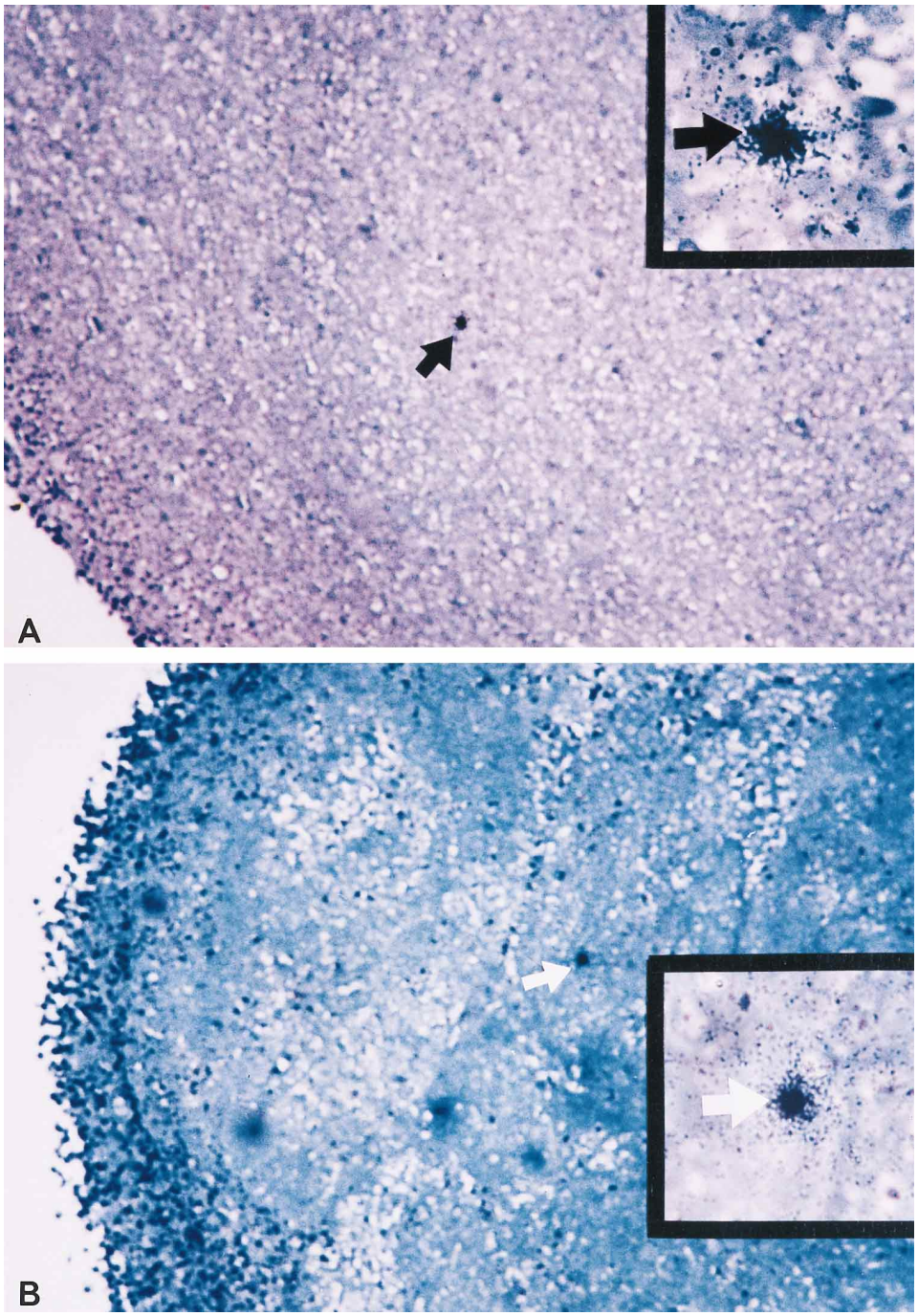

FIG. 2. (A) A stage 3 granuloma (granuloma 1A) from a mouse infected with *T. crassiceps* cysts was probed by in situ hybridization with ^{35}S-labeled antisense cRNA for IL-2. Sections through the granuloma revealed complete degradation of the tegument, with marked thickening and infiltration of the cyst wall and disappearance of the cyst cavity (histologic stage 3). After in situ hybridization with antisense probe for IL-10, a single positive cell overlaid with multiple silver granules was visible in this field (small arrow; original magnification, ×100). The inset shows a closeup of the positive cell overlaid with multiple silver granules (large arrow; original magnification, ×500). (B) A stage 4 granuloma (granuloma 3) from a mouse infected with *T. crassiceps* cysts was probed by in situ hybridization with ^{35}S-labeled antisense cRNA for IL-4. Sections through the granuloma revealed complete degradation of parasite, necrosis, and infiltration with numerous host cells (histologic stage 4). After in situ hybridization with an antisense probe for IL-4, a single positive cell overlaid with multiple silver granules was visible in this field (small arrow; magnification, ×71). The inset shows a closeup of the positive cell overlaid with multiple silver granules (large arrow; original magnification, ×500).

uloma. We detected IL-2 message in only one of three stage 1 granulomas by light microscopy. When examined by LSCM, however, a second granuloma had significantly increased signal. By light microscopy, most of the granulomas had several cells positive for IL-2 message (Table 2; Fig. 3A). Pixel intensity was significantly above background for 7 of the 12 granulomas, including 4 of 5 stage 1 and 2 granulomas and 3 of 7 stage 3 and 4 granulomas (Fig. 3B; Table 2). Signal intensity tended to be higher in early stages (Table 2).

Only small amounts of message for the Th2 cytokines IL-4 and IL-10 were detected by light microscopy (Fig. 1B and 2B; Table 2). Message for IL-4 was identified in only 6 of the 12

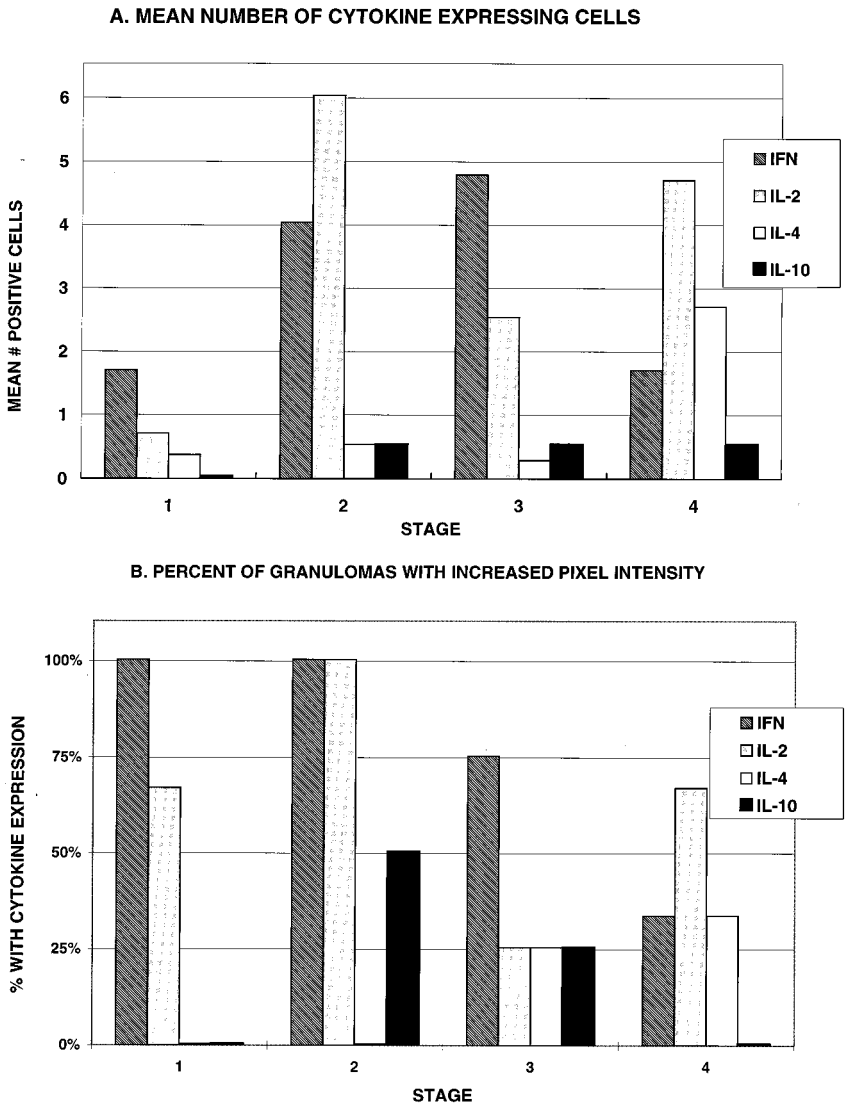

FIG. 3. Comparison of cytokine expression by in situ hybridization and histologic stage for granulomas from mice infected with *T. crassiceps*. (A) The number of cells positive for each cytokine was assessed by light microscopy. The mean number of positive cells for each cytokine is plotted by histologic stage. (B) The pixel intensity was compared for slides probed with antisense (positive) and sense (negative control) probes. Pixel intensity was assessed by LSCM. The percentage of granulomas with statistically significant increases in cytokine pixel intensity for each cytokine is plotted by histologic stage.

granulomas. All of the granulomas with more than one cell positive for IL-4 message were histologic stage 4. One granuloma each of stages 1, 2, and 3 had message for IL-4. When examined by LSCM, only 2 of 12 granulomas had IL-4 message significantly above the control level, including 1 granuloma each for stages 3 and 4 (Table 2; Fig. 3B). By light microscopy, message for IL-10 was identified in only 4 of the 12 granulomas, with only one cell positive per granuloma. In only two of these was pixel intensity significantly above background. In contrast, both IL-4 and IL-10 mRNAs were easily identified in the positive controls.

DISCUSSION

We identified message for the Th1 cytokines IL-2 and IFN-γ in most of the granulomas associated with dying *T. crassiceps* cysts. We also found small amounts of message for the Th2 cytokines IL-4 and IL-10. For the most part, granulomas with positive cells by light microscopy also had significantly increased pixel intensity by LSCM. In a few cases, a few strongly positive cells were detected only by light microscopy or granulomas with numerous cells with low signal intensity were positive only by confocal microscopy. In a preliminary immunochemistry study of brain biopsies from patients with neurocysticercosis, a similar cytokine profile was noted (34).

There was a strong correlation between histologic stage and cytokine expression. Stage 1 and 2 granulomas, in which the parasite is at least partially intact, displayed significant expression of Th1 cytokines and minimal amounts of IL-4 and IL-10. Stage 3 granulomas continued to express Th1 cytokines but also displayed small amounts of either IL-4 or IL-10 message. Finally, stage 4 granulomas had variable expression of Th1 cytokines but consistent low-level expression of either IL-4 or IL-10. Overall, IFN-γ was expressed early and decreased with advancing histologic stage. Thus, the Th1 response is the predominant response in early granulomas. In contrast, IL-4 as well as a Th1 response were identified in the later granulomas,

after the parasite was destroyed. We found no correlation between histologic stage or cytokine expression and the duration of infection. Because *T. crassiceps* cysts can reproduce within the host, there is a wide range of ages of parasites found in a single host. Similarly, the granulomas may develop early or later. Thus, duration of infection may not correlate with the age of either the parasite or the granuloma.

These data suggest a temporal response in which an early Th1 response causes parasite destruction. After the parasite is destroyed, IL-4 is expressed. Thus, IL-4 may play a role in modulation and downregulation of the inflammatory response. A similar temporal modulation of the cytokine response has been noted in granulomas associated with schistosomiasis. Early granuloma formation surrounding the schistosome egg is associated with both IFN-γ and IL-4 (Th0 pattern) followed by predominantly Th2 expression after modulation of the initial response (32, 45, 47).

Viable *Taenia* cysts are known to suppress the host inflammatory response. For example, viable cysticerci from pig muscle have little surrounding host inflammation (3). A similar pathologic picture is associated with asymptomatic cysticercosis found in human autopsy specimens from individuals who died of other causes (10, 36). In contrast, cysts from patients with symptomatic disease display a prominent inflammatory response, including lymphocytes, eosinophils, granulocytes, and plasma cells (8, 10, 36).

A number of parasite molecules which modulate the host response have been described (44). Taeniaestatin, a parasite serine protease inhibitor, inhibits complement activation, downregulates lymphocyte proliferation, blocks cytokine production, and interferes with neutrophil function (20, 21, 39). Paramyosin inhibits the function of the classical pathway of complement (17, 18). Sulfated polysaccharides activate complement away from the parasite (11, 25). Cysts also produce glutathione *S*-transferase and other molecules to detoxify reactive oxygen intermediates (23, 24). *Taenia* cysts are also known to produce small molecules that suppress host inflammation (40) as well as prostaglandins (22).

Studies done with other cestodes have shown that a vigorous antibody response is associated with active infection (1, 46). The cyst stages of the parasites are resistant to killing by the antibody (38). Instead cysts are shown to actively bind and take up immunoglobulin (4, 6, 12, 15). The parasite surface and cyst fluid contain host immunoglobulin. Some of the immunoglobulin binds to parasite antigens, but most of it is not specific for the parasite (27). Host immunoglobulins are known to be slowly degraded, perhaps serving as a source of amino acids (4, 6). In contrast, the humoral immune response plays a critical role in the immune response to the oncosphere stage (44). For example, specific antibody can kill the invasive oncosphere in vitro (30). Similarly, animals can be protected against an egg challenge by passive immunization with specific antibody (28). Antibody has been used to identify clones of recombinant antigens, which then have been used as vaccines to provide protection against egg infections (14). A vaccine against ovine cysticercosis that uses recombinant *Taenia ovis* oncosphere antigens is commercially available in New Zealand (35).

Studies have examined Th2 cytokines in active cestode infection. Estes and Teale demonstrated that the increased immunoglobulin G response to *Metacestoides corti* is driven primarily by IL-6 (9). Similarly, Villa and Kuhn demonstrated that splenic T cells from mice infected with viable *T. crassiceps* produce the Th2 cytokines IL-4 and IL-10 (42). They noted increased expression of Th2 cytokines in spleen cells (near the cite of infection) but a less prominent response in the blood. This Th2 response appears to be actively stimulated by the parasite.

In contrast to the Th2 pattern associated with viable parasites, we observed predominantly Th1 cytokines in granulomas associated with dying parasites. These data suggest that dying parasites can no longer direct the host immune response. When the Th1 response escapes parasite suppression, the resulting granulomatous immune response leads to clearing of the parasites. This Th1 granulomatous response is likely to be involved in the pathogenesis of symptomatic human infection, in which active inflammation is the major cause of parenchymal disease. As the parasite is destroyed, the response is modulated to involve greater Th2 cytokine expression, perhaps as a means of downregulating the granulomatous response, similar to that seen in schistosomiasis (32, 45). This process could be involved in healing. However, it may also be involved in scarring that can occur following the death of the parasite.

Immunity to the oncosphere stage is antibody mediated and driven by Th2 cytokines. This contrasts with the apparent role of Th1 cytokines and granulomas in the elimination of the cyst stage. These data further reinforce the concept of stage-specific immunity in *Taenia* infections (5). Attention to stage-specific antigens and immune responses will be essential for the development of effective vaccines for human cysticercosis.

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