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Pattern of Proinflammatory Cytokine mRNA Expression during *Trichinella spiralis* Infection of the Rat

ANDREW W. STADNYK[1,2]* AND JOY A. KEARSEY[2]

Departments of Pediatrics[1] and Microbiology and Immunology,[2] Dalhousie University, Halifax, Nova Scotia, Canada

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Trichinella spiralis **occupies an intramulticellular niche in the small intestinal epithelium, and thus we examined the intestine and gut-associated tissues for proinflammatory cytokines during the infection. We document the patterns of interleukin-1 (IL-1), IL-6, gamma interferon, and tumor necrosis factor alpha mRNA expression in the duodenum, jejunum, Peyer's patches, mesenteric lymph node, spleen, and liver in *T. spiralis*-infected rats. By reverse transcription-PCR detection of mRNAs, IL-1β was found increased in the jejunum but only on day 2. The jejunal IL-1β increase was attributed to the epithelium by isolating epithelial cells and then depleting them of intraepithelial lymphocytes prior to analysis. The only cytokine for which mRNA was substantially increased in tissues later in infection was tumor necrosis factor alpha in the spleen and, to a lesser extent, in the mesenteric lymph node. In fact mRNA levels for some cytokines declined below uninfected levels in some organs during the infection. IL-1 may be important in the initiation of the intestinal inflammatory response to this infection.**

Rodent models of intestinal inflammatory diseases have indicated that the epithelium may be a source of proinflammatory cytokines, including interleukin-1β (IL-1β). Radema et al. (25) showed by in situ hybridization that rat crypt colonocytes became positive for IL-1β mRNA within 4 h of acute colitis induced by acetic acid, and Cominelli et al. (5) attributed early increases in IL-1 to the intestinal epithelium in a rabbit model of immune complex-induced colitis. In both of these models the IL-1 expression was early and transient in relation to the subsequent cellular changes. A transient pattern of epithelial IL-1 expression may partially explain why there are few reports of this cytokine in the epithelium from intestinal biopsies of human inflammatory bowel disease (19, 24, 37) and why the issue of epithelial cells as a source of IL-1 is unsettled (38). It is not known whether the small intestinal epithelium responds to injury with similar cytokines as the colonic epithelium, and little is known about the cytokines resulting from infection in either bowel.

We are interested in cytokine changes during inflammation due to infection and how they relate to the systemic acute-phase protein (APP) response (31). We began to examine intestinal helminth infections with the outlook that the APPs might be indicators of intestinal inflammation. We reported the strikingly different APP patterns due to two intestinal nematode infections of the rat: *Nippostrongylus brasiliensis* infection led to significant positive APP changes coincident with inflammation in the host's lungs (day 2) and a second, greater response while the infection was in the small intestine (days 6 to 14), yet only a small increase occurred on day 2 of the *Trichinella spiralis* infection and there were no positive changes in the same proteins during days 6 to 14 (29). On the other hand, the concentrations in serum of negative APPs (albumin and $\alpha_{2\mu}$ globulin) and liver mRNAs encoding these molecules did decline with similar time course kinetics during the 2nd week of both infections (28, 29). We concluded that infection of the small intestine without prior mucosal inflammation (e.g., the lung stage of *N. brasiliensis*) failed to elicit the systemic positive APP response.

IL-1 and IL-6 are both potent stimulating agents for APP synthesis by hepatocytes (1). Both were detectable, by bioassays, in the uninfected rat small intestine (28), possibly contributing to the basal level of APP production by the liver. Increased IL-6 and IL-1 were detectable in the lungs and intestines of *N. brasiliensis*-infected rats, in parallel with changes in the APPs (3, 8, 29), but both molecules were lower than normal (per 10^6 cells) in *T. spiralis*-infected rat intestine on days 7 and 10 postinfection (28). Tumor necrosis factor alpha (TNF-α) and, to a lesser extent, gamma interferon (IFN-γ) will effect the negative APP changes (14), and neither has been reported in the helminth-infected rat intestine. Thus, neither IL-1 nor IL-6 appears to be increased during the time of infection when the greatest cellular and pathological changes are occurring in the *T. spiralis*-infected gut. Here we report the cytokine mRNA changes from a number of organs during *T. spiralis* infection of the rat, in case there may be transcription without translation, but also with an emphasis on the epithelium early during the infection, as the worm is an intramulticellular parasite of the epithelial cells.

MATERIALS AND METHODS

Animals and infection. *T. spiralis* was maintained by passage in CBA/J mice (Jackson Laboratories, Bar Harbor, Maine). Infected mouse carcasses were digested in pepsin-HCl to release the larvae for infection of rats. Lewis strain male rats (Harlan Sprague Dawley, Indianapolis, Ind.) were used in all experiments. Lightly anesthetized rats were infected with 2,000 larvae each, administered with a feeding tube. In a typical experiment rats were infected in the midafternoon, and the tissues were harvested in the midmorning on subsequent days (considered days 1, 2, 3, etc.).

Tissues and RNA. Samples of the following tissues were examined for cytokine mRNA: liver, spleen, mesenteric lymph node (MLN) Peyer's patches (PP) (from the first 20 cm of intestine), duodenum (1 cm from the pyloric sphincter), and jejunum (10 cm from the pyloric sphincter). Total cellular RNA was extracted from homogenized tissues or cells (see below) by the method of Chomczynski

* Corresponding author. Mailing address: Infection and Immunology Research Laboratory, IWK—Grace Health Centre, 5850 University Ave., Halifax, Nova Scotia, Canada B3J 3G9. Phone: (902) 428-8491. Fax: (902) 428-3217. Electronic mail address: astadnyk@is.dal.ca.

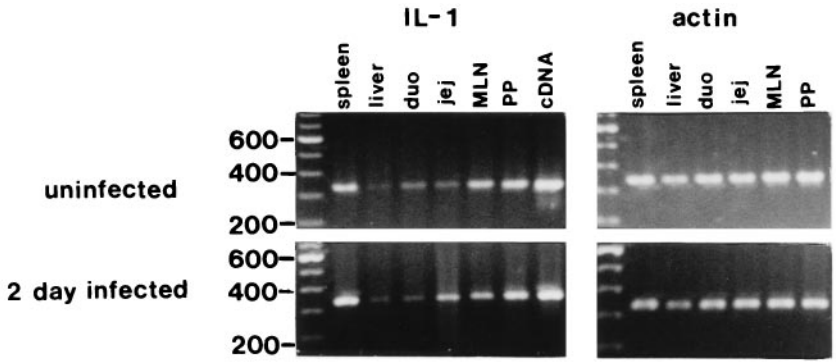

FIG. 1. RT-PCR products of rat tissues from day 2 of infection, showing that increased IL-1β mRNA is limited to the jejunum and that IL-1β mRNA levels decline in the MLN. Abbreviations: duo, duodenum; jej, jejunum.

and Sacchi (4), but with a second phenol-chloroform extraction added. The final pellet of RNA was resuspended in sterile water, heated to 65°C for 15 min, and finally cooled to room temperature; the optical density at 260 nm was then determined.

In order to examine the epithelium alone, we used the "swollen sac" method of Wieser as modified by Traber et al. (35) but collected only three fractions. The freshly isolated cells were passed through loosely packed nylon wool columns to enrich for viable cells; then the number of cells was determined. Some cells were reserved for cytocentrifuge preparations, and the remainder was homogenized in solution D (4) of the RNA extraction. Cytocentrifuge preparations were stained with a modified Wright's stain (Diff-Quik; Baxter Healthcare Corp., Miami, Fla.) and examined by light microscopy. To exclude the possibility that cytokines detected in the epithelial fractions were due to intraepithelial lymphocytes (IEL), we isolated epithelial cells by a method intended to enrich the cells for IEL, which we describe in detail elsewhere (11). Briefly, segments of the intestine were everted and filled with media, the ends were ligated, and then the segments were vortexed as sacs. The IEL were enriched by multiple filtering steps followed by centrifugation, first through a single-step 30%, then a discontinuous 45/75% (vol/vol) Percoll density gradient (Pharmacia, Nutley, N.J.). Cells recovered from the top of the 30 and 45% Percoll steps were pooled and considered IEL depleted. The IEL were enriched at the 45/75% interface. The tissue that remained after the segments were vortexed and the loose cells were decanted was referred to as epithelium depleted.

cDNA synthesis-RT. Total cellular RNA was examined for specific cytokine mRNAs by the reverse transcription (RT)-PCR method. RT of 1 or 5 μg of RNA was undertaken by using Moloney murine leukemia virus reverse transcriptase according to the manufacturer's protocol (Life Technologies, Burlington, Ontario, Canada) but with 0.5 mM deoxynucleoside triphosphates (Pharmacia) and 0.8 μg of random hexamers (Promega, Madison, Wis.). The reaction mixture was spiked with 5 μCi of [^{32}P]dCTP (ICN BioMedicals, Inc., Irvine, Calif.) (specific activity = 3,000 Ci/mmol), and then the reaction was carried out at 37°C for 1 h; finally, the reaction mixture was heated to 94°C for 5 min to inactivate the enzyme. The radiolabel incorporated into first-strand cDNA was determined by trichloroacetic acid precipitation. The product was stored frozen until it was used in the PCR. The first-strand cDNA from a single RT reaction provided sufficient template for multiple cytokine and β-actin analyses.

PCR. Cytokine sequences suitable for use as primers in the PCR were determined from the published gene or cDNA sequences by using the algorithm designed by Lowe et al. (17). Where the gene sequence was available, primers were made to span an intron in order to discriminate amplification of cDNAs from that of contaminating genomic DNA. This was especially useful for the β-actin primers, for which it was planned that a fragment resulting from amplification of genomic DNA would not be much larger than the product amplified from the cDNA (550 bp and 331 bp, respectively), so as to maximize the likelihood of detecting genomic DNA. The sequences of most of the primer pairs are published elsewhere (11). The primer sequences for sucrase and isomaltase, reading 5′ to 3′, were CACAGGCAATATGTGAAGAGC and TTGGTTTGA GTACACCAGTGG, respectively.

Fifty thousand (acid precipitable) counts per minute of the cDNA was used as the template in the PCR. The volume of cDNA necessary to achieve 50,000 cpm was corrected to take into account the decay in ^{32}P, and all analyses were completed within 1 week of the RT reaction. The PCR buffer mixes were prepared in batches and included a reaction buffer given in greater detail elsewhere (11). The PCR was undertaken in a BioOven thermocycler (BioTherm Corp., Arlington, Va.) programmed to cycle between 92, 60, and 72°C, each for 30 s. Eight microliters of the PCR product was run on a 1 or 1.5% agarose gel impregnated with 0.5 μg of ethidium bromide per ml. Polaroid snapshots of the stained products were taken under UV exposure with Kodak 667 film. Where it was desirable to measure and quantify the amplicon products, a Mac LC II densitometer, "one scanner," was used to digitize the Polaroid image, and then the band intensities were transformed by using Ofoto scanning software. The numerical values for cytokine band intensities from different days of infection for the same cytokine were first corrected for any differences between infected and uninfected β-actin product density. With this method of empirical calibration, the relative changes in mRNA levels between days of infection for the same cytokine are indicated by a +, ++, etc., where the addition of a + represents a twofold change (see Table 1).

RESULTS

The proximal jejunum is the site of infection by *T. spiralis*; the infective larvae penetrate epithelial cells and then molt to become young adults in about 36 h (6). Lymphocyte proliferation in the intestine is detectable by this time, and immune cells are recoverable from the thoracic duct by 72 h (2). Considering these early manifestations of lymphocyte activation, we examined whole-organ RNA for proinflammatory cytokines on days 1 and 2 of the infection. There were no remarkable changes detected in any of the cytokine mRNAs examined in any tissue on day 1. In contrast, the day 2 jejunum showed increased IL-1β and the same cytokine declined in the MLN (Fig. 1).

T. spiralis occupies an intramulticellular niche in the small intestinal epithelium (Fig. 2A), so in order to determine whether the IL-1β changes occurred within the epithelium or the lamina propria, we isolated epithelial cells using a method that is intended to sequentially strip the cells from the villus tip to the crypt. Columnar enterocytes were stripped off in sheets or clumps. As shown by trypan blue staining, samples of the cells were more than 85% viable before the addition of lysing solution. Despite this apparent high viability, many cells seemed to lose their columnar shape and nuclei disintegrated during or following cytocentrifugation (Fig. 2B). The epithelial cells comprised more than 90% of the total cell differential, with some granulocytes and mononuclear cells present, as observed by light microscopy (Fig. 2B). Sucrase/isomaltase mRNA was detected by RT-PCR in all fractions (not shown). Epithelial cell preparations from infected rats resembled those from uninfected rats.

After pooling the fractions, we further characterized the freshly isolated epithelial cells from uninfected rats using indirect immunofluorescence and flow cytometry. There were essentially no cells positive for either macrophage marker, ED-1 or ED-2 (27), which is important, since we have reported that intestinal macrophages are likely to be subepithelial (30). Less than 5% of the cells stained for the common leukocyte marker, MRC OX-1, but about 40% stained positive for class II major histocompatibility complex (MRC OX-6) in a broad peak, indicating considerable variation in the surface expres-

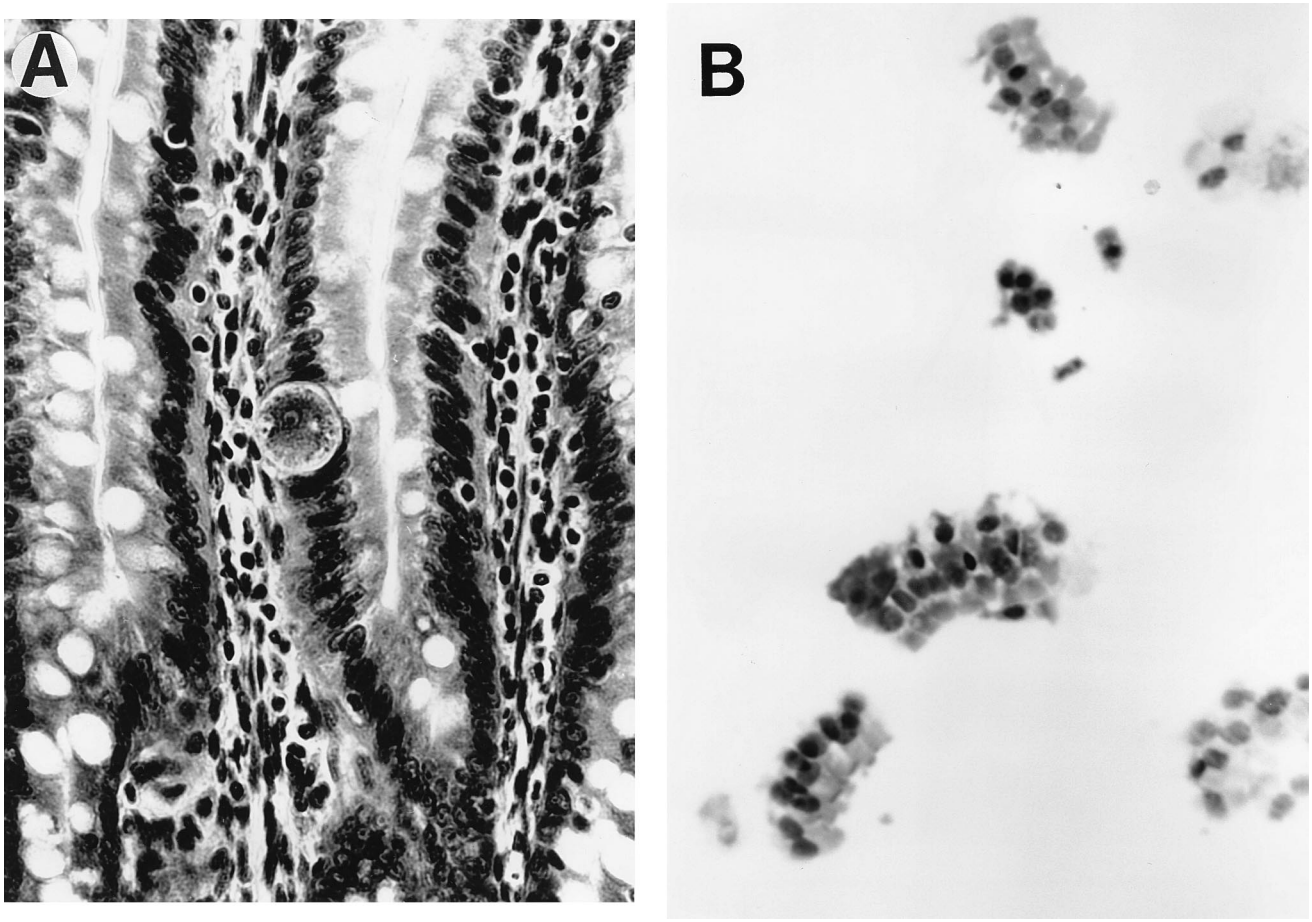

FIG. 2. Photomicrographs showing a cross section of a worm in the epithelium of the jejunum on day 2 of infection (A) and epithelial cells collected from infected rats on day 2 by the swollen sac method outlined by Traber et al. (35) (B). Judging from morphology, more than 90% of the cells are nonhematopoietic epithelial cells, the most abundant of which is the enterocyte, shown here.

sion of this antigen (not shown). The results were essentially the same for preparations made from infected epithelial cells harvested on day 2.

Total cellular RNA was prepared from the three fractions, from rats infected for up to 3 days, without further separation of cell types and was subjected to RT-PCR. As shown in Fig. 3, IL-6 mRNA was detectable in all of the fractions from uninfected rats and remained unchanged during the infection. A low level of IL-1β mRNA was detected in the uninfected cells, but this increased sharply, about 50-fold as estimated by densitometry, in the day 2 preparations. IL-1α was at the threshold of detection in uninfected cells and remained unchanged on day 2. In view of the low epithelial signal from the uninfected rat in Fig. 3, the considerable IL-1β mRNA detectable in the normal rat jejunum (Fig. 1) must be present in the lamina propria. The epithelial mRNA on day 2 then amounted to a small increase in total jejunal IL-1β, since the increase in the whole-organ mRNA signal was only about threefold. The mRNA pattern for IL-1 and IL-6 on days 1 and 3 of infection (not shown) resembled that of the uninfected animals.

In order to evaluate whether lymphocytes were responsible for the IL-1β mRNA detected on day 2, IEL were isolated from *T. spiralis*-infected rat intestines harvested 2 days postinfection. We were able to enrich the cells to 88% lymphocytes by morphology, and the ungated population consisted of 72% αβ T-cell receptor$^+$, 69% CD8α$^+$ cells as detected by flow cytometry. Total RNA was prepared from these cells, and mRNAs were detected by RT-PCR with primers for β-actin, IL-1β, and IFN-γ. Figure 4 shows that despite the processing, the IL-1β PCR product was still attributable to epithelial cells depleted of IEL and not to IEL directly. In repeats of this experiment we have detected IL-1β in uninfected IEL preparations (11), but it has never been increased in IEL from infected animals. On the other hand, only IEL yielded a positive signal by RT-PCR for IFN-γ (Fig. 4).

The period of greatest cellular and histopathological changes in the intestine due to the infection, including villus cell atrophy, crypt cell hyperplasia, and the attendant physiological changes, is between days 6 and 14 (26). Therefore, rats infected with 2,000 *T. spiralis* larvae were killed on days 4, 7, 10, and 14 or 17 and were examined for proinflammatory cytokine mRNA. In the uninfected rat, IL-1β and IL-6 transcripts were clearly detectable in all tissues except the liver and both were most abundant in the spleen. IL-1α was found in the uninfected PP, MLN, and spleen and was at the threshold of detection (very faint band) in the duodenum, jejunum, and liver. IFN-γ was also detected faintly in the MLN and spleen in uninfected rats. TNF-α was detected faintly in the uninfected MLN, spleen, and PP. Changes in mRNA relative to uninfected animals, pooled from three time course experiments, are summarized in Table 1. Generally, these cytokines showed unremarkable changes during the latter period of infection.

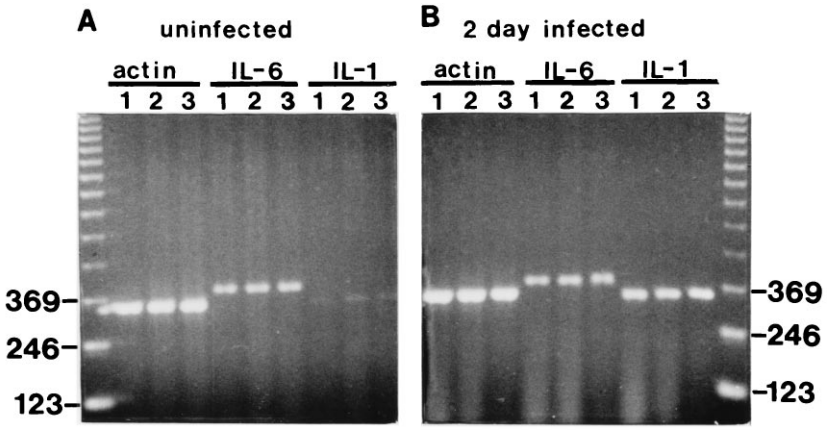

FIG. 3. (A) Ethidium bromide-stained gel of products from RT-PCR amplification of uninfected rat epithelial cell RNA. (B) Examination of similar epithelial fractions from day 2 of infection, showing increased IL-1β mRNA. Neither IL-1α nor IL-6 increased during the first 3 days of infection. Lanes 1 through 3, 15-, 10-, and 5-min incubations, respectively.

Increases in PCR products at day 4 versus day 0 were generally on the order of twofold. Declines in IL-1β and IL-6 mRNAs were detected in some tissues (Table 1). IL-1α levels increased slightly (less than twofold) in the duodenum, MLN, liver, and spleen on day 4 but remained at this level until day 10 only in the MLN. IFN-γ became detectable in the intestine and PP by day 4 and was still detectable as late as day 17 in the PP. The only cytokine to show a large increase was TNF-α. TNF-α transcripts increased in the MLN up to day 10 and also showed steady increases from a low or undetectable signal in the spleen until day 7, declined on day 10, and returned again on day 14 or 17. The day 10 decline in splenic TNF-α mRNA was consistent in repeats of the experiment, but it was not always reduced to undetectable levels. The cytokine mRNAs detected on day 14 were similar to those detected on day 17. Despite the steady increase in TNF-α mRNA, we have failed in two experiments to detect TNF-α bioactivity in the sera (collected from the tail vein) of infected rats by the sensitive L-M bioassay (21).

DISCUSSION

Helminth infections of rodents have become popular models for studying lymphocyte activation, yet little is known about proinflammatory cytokine changes that likely precede lymphocyte activation. Egwang et al. (8) first described the pattern of "lymphocyte activating factor" and "hepatocyte stimulating factor" secreted by macrophages from the *N. brasiliensis*-infected rat lung. Production of these cytokines by the alveolar macrophages persisted beyond day 8 (8). Recently the lung stage of *N. brasiliensis* was shown to also induce changes in TNF-α production (3). We reported APP increases during the *N. brasiliensis* infection, which imply increased IL-6 and IL-1, and compared the rat response to *N. brasiliensis* with that to *T. spiralis* (28, 29). Two episodes of APP changes were detected in the *N. brasiliensis*-infected rats coincident with pathology in the lungs and then in the intestine; however, only a slight increase in some proteins was detected on day 2 in the serum of *T. spiralis*-infected animals, and only the negative changes were detected later during the infection. We now present evidence that a brief increase in IL-1β during *T. spiralis* infection may be responsible for the early APP change. In addition, IL-1 may serve directly as a neutrophil chemoattractant or facilitate leukocyte emigration by inducing adhesion molecule expression on endothelial cells and stimulating chemokine production (7). IL-1 is also important in T-cell activation and has been shown to stimulate Th2 cells in the mouse (16). This property in the rat would be compatible with the appearance of protective T cells by day 3 in the MLN and thoracic duct (15) and the host's response with high levels of immunoglobulin E and eosinophils (26).

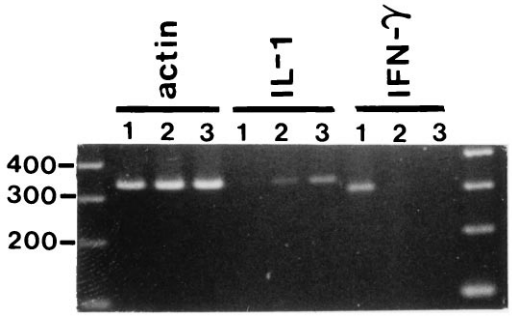

FIG. 4. RT-PCR products amplified from enriched IEL (lanes 1), IEL-depleted epithelial cells (lanes 2), and the remainder of the tissue after removal of the epithelium (lanes 3). All of the samples were collected from an infected rat on day 2. IL-1 refers to IL-1β.

The conclusion that the IL-1β is epithelial cell derived is inferred from the lack of a signal from highly enriched IEL and from the presence of IL-1β in IEL-depleted epithelium. Our phenotypic examination of the isolated epithelial cells by flow cytometry makes it unlikely that the IL-1β mRNA was a product of contaminating macrophages. Also, the pulse character of the IL-1β changes seems to be more typical of epithelial cells than of a macrophage source, for which a longer period of production is characteristic. We have some evidence from work with epithelial cell lines that these cells readily secrete IL-1β (unpublished data), and there may be difficulties in the detection of the epithelial IL-1β by immunohistochemistry. The RT-PCR data represent the average mRNA content of all cells recovered from all villi along the resected segment of intestine. Thus the precise identity of the cell or cells expressing the IL-1β during this infection remains to be determined. The fact that we did not detect a villus-to-crypt gradient in mRNA might indicate that the increase started before the day 2 time point and that cells were stimulated while in the crypt but rose up the villi by the time the rats were killed. This was

TABLE 1. Relative cytokine mRNA changes due to *T. spiralis* infection of the rat

Site	Relative change in mRNA level between days of infection for:																									
	IL-1α					IL-1β					IL-6					TNF-α					IFN-γ				
	0	4	7	10	17[a]	0	4	7	10	17	0	4	7	10	17	0	4	7	10	17	0	4	7	10	17
Duodenum	±[b]	+	+	+	±	+	+	+	+	+	+	+	+	+	+	−[c]	−	−	−	−	−	±	±	−	−
Jejunum	±	+	+	+	±	+	+	+	±	+	+	+	+	+	+	−	−	−	−	−	−	±	±	±	−
PP	+	+	+	+	+	++	++	++	+	++	+	+	+	+	+	±	+	+	±	++	−	±	±	±	±
MLN	+	++	++	++	++	++	++	++	+	++	+	+	+	+	+	±	±	+	+	±	+	+	±	−	+
Spleen	+	++	+	+	++	++	++	++	+	++	++	++	++	+	++	−	±	+	±	++	+	−	−	−	±
Liver	±	+	+	+	+	±	±	±	±	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−

[a] Day 14 was similar to day 17.
[b] ±, faintly detectable.
[c] −, undetectable.

the pattern observed with the IL-1β staining by in situ hybridization in the acetic acid-induced colitis model (25). Also, we used longer incubations and collected only three fractions of cells, which may have resulted in greater overlap of cells representing the crypt-to-villus gradient.

The RT-PCR results reported for the uninfected rat intestine support our earlier observations based on bioassay determinations, i.e., that there is constitutive production of IL-1 and IL-6. Now we can add that a portion of the IL-6 is derived from the epithelium and the IL-1 is derived from the lamina propria. The rat intestinal epithelial cell line IEC-6 was shown to spontaneously secrete IL-6 and to respond to IL-1β with increased secretion (20). The IL-1-driven increase in IL-6 does not appear to occur in vivo, as the IL-6 mRNA levels were unchanged following the (day 2) IL-1β pulse in the epithelium reported here. We interpret the finding that the IL-1β changes occurred on the 2nd day of infection to mean that these changes are not directly due to infection of epithelial cells by the worms. We detected neutrophils in day 2 IEL preparations, and we are more closely exploring the possibility that recruitment of these cells stimulates IL-1β. We also intend to test mRNA from the infected epithelium for various chemokines.

A decline in some mRNAs was observed in some tissues, likely because of the dilution of the resident cells by infiltrating cells that do not produce IL-1 or IL-6. For example, IL-1β mRNA declined in the MLN, which is infiltrated by cells from the intestine during *T. spiralis* infections (15). Another possible explanation for the decline in IL-1β in the MLN is a specific inhibition of this cytokine by other cytokines, such as IL-4 (36), possibly produced by the cells migrating through the node. We did not measure IL-4 in the whole MLN, but Matsuda et al. (18) failed to detect an increase in IL-4 mRNA in MLN cells before day 3 in *N. brasiliensis*-infected rats.

Neither IL-1 nor IL-6 showed increases in mRNA later during infection. However, TNF-α did show increases in mRNA during the 2nd week of infection. An increase in splenic TNF-α mRNA began on day 4 but was interrupted on day 10. TNF-α mRNA increased, but to a lesser extent, in the MLN. Our finding of increased TNF mRNA is compatible with previously reported evidence for the increased protein, including increased resistance to tumors in infected animals (22), negative APP changes (28), and the cachexia that may occur (typically with higher numbers of worms). It was surprising that TNF-α was not detected in the intestinal tissues, since Paneth cells reportedly have mRNA for TNF-α (13, 34). Whether rat Paneth cells have mRNA for TNF-α or whether our result is due to technical limitations is unclear. Injection of TNF into mice led to an enteropathy that included crypt cell hyperplasia and villus atrophy (9), although TNF may act indirectly by increasing platelet-activating factor (32). Although the levels of platelet-activating factor have not been examined in *T. spiralis*-infected rats, they are not increased in *N. brasiliensis*-infected rats until after worm expulsion (10). The striking decline of TNF-α mRNA observed on day 10 in spleen but not MLN from infected rats remains to be understood. The activity of the muscle-stage larvae may effect a systemic cytokine depression, which may explain why there is no positive APP response during the penetration of muscle cells by these worms, also beginning by the 2nd week of infection. Despite our inability to detect TNF in infected rat sera by a bioassay, experiments intended to inhibit this mediator are worthwhile, considering its effects on intestinal physiology.

IFN-γ mRNA was generally undetectable and changed little during the infection at the whole-organ level. Infection of mice with *Heligmosomoides polygyrus* also led to small (twofold) increases of IFN-γ in the PP but levels actually declined in the MLN, as measured by RT-PCR (33). Matsuda recently reported a slight decline in IFN-γ mRNA levels, first detectable at day 7, in single-cell suspensions of MLN from *N. brasiliensis*-infected rats (18). The significance of subtle changes in vivo or in nonstimulated cells ex vivo (unlike the reports of increased cytokines from ex vivo mitogen-stimulated cells [12, 23]) needs to be better understood. IFN-γ is a hallmark of Th1 activity, but it is not clear from the slight increases observed in the whole-organ RNA specimens that there is a local Th1-like response. The work of Matsuda et al. (18) clearly implicates Th2 cytokines in the MLN, but unfortunately they did not show results for other mucosal sites. A study similar to that of Matsuda et al. (18) remains to be done in *T. spiralis*-infected rats.

To recapitulate, we show that epithelial cells are likely a source of IL-1β early during the infection of rats with the helminth *T. spiralis* and that increased TNF-α, most clearly in the spleen and MLN, likely contributes to the pathophysiology typical of the 2nd week of infection.

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