INFECTION AND IMMUNITY, Jan. 1995, p. 289–296 Vol. 63, No. 1
0019-9567/95/$04.00+0

Persistence of Local Cytokine Production in Shigellosis in Acute and Convalescent Stages

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Received 2 May 1994/Returned for modification 29 July 1994/Accepted 28 September 1994

Shigella* infection is accompanied by an intestinal activation of epithelial cells, T cells, and macrophages within the inflamed colonic mucosa. A prospective study was carried out to elucidate the cytokine pattern in *Shigella* infection linked to development of immunity and eradication of bacteria from the local site and also to correlate the cytokine profile with histological severity. An indirect immunohistochemical technique was used to determine the production and localization of various cytokines at the single-cell level in cryopreserved rectal biopsies from 24 patients with either *Shigella dysenteriae* type 1 ($n = 18$) or *Shigella flexneri* ($n = 6$) infection. The histopathological profile included presence of chronic inflammatory cells with or without neutrophils and microulcers in the lamina propria, crypt distortion, branching, and less frequently crypt abscesses. Patients had significantly higher ($P < 0.005$) numbers of cytokine producing cells for all of the cytokines studied, interleukin-1α (IL-1α), IL-1β, IL-1ra, tumor necrosis factor alpha (TNF-α), IL-6, IL-8, IL-4, IL-10, gamma interferon, TNF-β, and transforming growth factor β_{1-3}, in the biopsies than the healthy controls ($n = 13$). The cytokine production profile during the study period was dominated by IL-1β, transforming growth factor β_{1-3}, IL-4, and IL-10. Significantly increased frequencies of cytokine-producing cells ($P < 0.05$) were observed for IL-1, IL-6, gamma interferon, and TNF-α in biopsies with severe inflammation in comparison with those with mild inflammation. During the acute stage of the disease, 20 of 24 patients exhibited acute inflammation in the rectal biopsies and the cellular infiltration was still extensive 30 days after the onset of diarrhea, although the disease was clinically resolved. In accordance with the histological findings, cytokine production was also upregulated during the convalescent phase; there was no significant difference ($P > 0.05$) in the incidence of cytokine-producing cells between acute (2 to 8 days after the onset of diarrhea) and convalescent (30 days after onset) stages.

Shigellosis or bacillary dysentery is one of the most commonly encountered diarrheal diseases in Bangladesh and other developing countries. Usually a self-limiting infectious colitis, shigellosis is estimated to be responsible for 650,000 deaths annually in the world, a majority of them occurring in the poor areas of developing countries (16a). Clinical presentation of shigellosis includes frequent bloody mucoid stool, abdominal cramps, and rectal tenesmus. The pathogenesis of bacillary dysentery involves invasion of colonic mucosa by shigellae, intracellular multiplication, and spreading to adjacent cells (21). The invasive process triggers a local inflammatory reaction leading to abscesses and ulceration of the colonic mucosa (9). The site of *Shigella* infection is the large bowel, which is also a potential source of local cytokine production. Production of interleukin-1β (IL-1β) (9, 36), IL-6 (26, 36), tumor necrosis factor alpha (TNF-α) (36), IL-2 (9, 17), IL-4 (25), IL-3 (31), IL-5 (25), IL-8 (17), transforming growth factor β (TGF-β) (25), and granulocyte-macrophage colony-stimulating factor (31) has been demonstrated in inflammatory bowel diseases. These cytokines appear to be key mediators for the tissue damage as well as for the immune responses in inflammatory bowel diseases. In addition, cytokines may play a role in many of the systemic disease manifestations in shigellosis, which include fever, anorexia, hypoglycemia, hypoalbuminemia, and hyponatremia in most patients. Two recent studies have shown enhanced IL-6 and TNF levels in serum and stool (15, 28) of children infected with *Shigella dysenteriae* type 1. However, no studies on the production and localization of cytokines in the gut mucosa of patients with shigellosis have been reported.

Cytokines are interregulatory proteins that mediate multiple immunologic and nonimmunologic biological functions. The cytokine profile of the innate immune response can influence the profile of the subsequent specific immune response. Insufficient production of cytokines or the inability to adequately respond to various immunomodulatory cytokines may result in a state of immunosuppression. Conversely, excess production of cytokines may cause severe shock, autoimmune diseases, or immunopathology associated with a given disease. The purpose of the study was to investigate the natural cause of immunity by defining the initiating cytokine profile linked to the development of immunity in shigellosis. In addition, an under-

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TABLE 1. Cytokine-specific antibodies used

Cytokine	Antibody	Isotype	Producer	Reference
IL-1α	1277-89-7	Mouse IgG1	H. Towbin, Ciba-Geigy, Basel, Switzerland	6
	1277-82-29	Mouse IgG1		
	1279-143-4	Mouse IgG1		
IL-1β	2-D-8	Mouse IgG1	H. Towbin, Ciba-Geigy, Basel, Switzerland	6
IL-1β		Mouse IgG1	Genzyme Corp.	29
IL-1ra	1384-92-17-19	Mouse IgG1	H. Towbin	6
IL-4	MP4-25D2	Rat IgG1	J. Abrams, DNAX, Palo Alto, Calif.	1
IL-6	MQ2-6A3	Rat IgG	J. Abrams, DNAX, Palo Alto, Calif.	1
IL-6		Mouse IgG1	Genzyme Corp.	Our work
IL-8	NAP-1	Mouse IgG1	M. Ceska, Sandoz, Vienna, Austria	2
IL-10	JES3-19F1	Rat IgG2a	J. Abrams	1
	JES3-12G8	Rat IgG2a		
TNF-α	MP9-20A4	Rat IgG	J. Abrams	1
TNF-β	LTX 21	Mouse IgG2b	G. Adolf, Bender, MedSystems, Vienna, Austria	7
	LTX 22	Mouse IgG1		
IFN-γ	DIK 1	Mouse IgG1	G. Andersson, KABI, Stockholm, Sweden	4
IFN-γ		Mouse IgG1	Genzyme Corp.	Our work
TGF-β1	96	Polyclonal rabbit IgG	K. Miyazono, Ludwig, Institute, Uppsala, Sweden	30
TGF-β2	94			
TGF-β3	95			
TGF-β1–3		Mouse IgG1	Genzyme Corp.	8

standing of the regulation of the immune response in the gut may pave the way for the development of efficacious *Shigella* vaccines.

An immunohistochemical study was performed to determine the production and localization of various cytokines at the single-cell level in the rectal mucosa in cryopreserved tissue obtained from *Shigella*-infected patients during the acute and convalescent stages. In addition, the histopathologic features of the tissues were examined, and attempts were made to correlate the two approaches.

MATERIALS AND METHODS

Patient population. The study was conducted at the Clinical Division of the International Center for Diarrhoeal Diseases Research, Bangladesh (ICDDR,B), which serves mainly a low-socioeconomic-class population. The study protocol was approved by the ethical review committee of ICDDR,B. Adult male patients with complaints of dysentery with 2 to 5 days' duration and confirmed by stool microscopy (>50 fecal leukocytes and 20 to 50 erythrocytes per high-power field [×400]) were screened for inclusion in the study. Fresh stools were examined by microscopy for protozoa or ova and by culture for *Shigella*, *Salmonella*, and *Aeromonas* spp., *Vibrio cholerae*, and *Campylobacter jejuni*. A total of 24 patients (with an age range of 18 to 48 years) with culture-confirmed shigellosis were finally enrolled in the study. In 19 of the patients, *S. dysenteriae* type 1 was isolated from stools; in 2, *Shigella boydii* types 12 and 15, respectively were copathogens; in 5, *Shigella flexneri* was the identifiable enteropathogen. Prior to recruitment, an informed consent was obtained from each patient in accordance with the guidelines of the ethical review committee at ICDDR,B. These patients were treated with nalidixic acid or pivmecillinam as determined by the antibiotic sensitivity pattern. Clinical evaluation of the patients included physical examination, assessment of fever, pulse and blood pressure, stool frequency, and dehydration. Laboratory analyses included hemoglobin, C-reactive protein and creatinine concentration, hematocrit, total and differential leukocyte count, and platelet count in blood; and concentration of total protein and albumin in serum and stool.

Rectal biopsies were obtained at proctoscopy (long-speculum Anoscope; Welch Allyn series 31610) within 48 h of admission without prior use of enema. Patients were released from the hospital within 6 to 7 days when diarrhea subsided. These patients were requested to return for a follow-up visit 30 days from the time of presentation for medical attention. The disease in the patients was clinically resolved. At that time, another proctoscopy was performed to collect rectal biopsies from each patients. At least two biopsies were taken from each patient on each occasion. One specimen was fixed and processed for conventional histology. The other was snap-frozen in liquid nitrogen and processed for immunohistochemistry (see below).

Control subjects. Thirteen healthy, adult males matching the socioeconomic status of the patients residing in slums in and around the capital city were recruited as controls. Their ages ranged from 18 to 39 years. Control subjects were chosen on the basis of normal clinical history, physical examination, and the investigations carried out as for patients to exclude those with any infection. Proctoscopy was performed once, and biopsies were obtained and processed as for patients.

Immunohistochemistry. Rectal tissues embedded in OCT compound (Tissue-Tek; Miles, Elkhart, Ind.) were sectioned as 8-μm-thick sections and mounted on gelatin-coated glass slides. Sections were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS [pH 7.4]; Sigma, St. Louis, Mo.) for 15 min, washed in PBS, air dried, and kept at −20°C until used. Slides were rehydrated in balanced salt solution (BSS; Gibco Ltd., Paisley, Scotland) containing Ca^{2+} and Mg^{2+} supplemented with 0.01 M *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer, and the endogenous peroxidase activity was blocked by 15 min of incubation in 1% H_2O_2 in BSS. After three washes in BSS, the sections were treated with 0.1% saponin (Sigma) in BSS for 5 min for permeabilization of the cell membrane and the Golgi complex (33). Thereafter, the slides were incubated overnight at 4°C with a panel of cytokine-specific monoclonal antibodies (MAbs) or affinity-purified polyclonal antibodies at a concentration of 2 to 5 μg/ml (MAbs from Genzyme were used at a concentration of 40 μg/ml). To prevent nonspecific binding, the slides were incubated with 1% normal goat serum for 15 min and then rinsed three times in BSS-saponin. Biotinylated goat anti-mouse immunoglobulin G1 (IgG1) and IgG2b (Caltag Laboratories, South San Francisco, Calif.) diluted 1:300 and biotinylated goat anti-rat antibody (Vector Laboratories, Burlingame, Calif.) diluted 1:100 were used as secondary antibodies (adsorbed against human Ig) for 30 min in room temperature. After further washing, the sections were treated with an avidin-biotin-horseradish peroxidase complex (Vectastain, ABC-HP-kit; Vector Laboratories) for 30 min, rinsed thrice, overlaid with substrate chromagen (Vector Laboratories), and incubated for 10 min to allow for color development according to the manufacturer's instructions. The reaction field was blocked by three washes in BSS, and sections were dehydrated by a 1-min treatment in 50% ethanol–50% water followed by 99.5% ethanol. Slides were counterstained with Mayers' hematoxylin and mounted in a glycerin buffer.

Cytokine-specific antibodies. The cytokine-specific antibodies used are described in Table 1.

Irrelevant isotype-matched MAbs, goat anti-mouse IgG subclass-specific (Caltag Laboratories) and goat anti-rat IgG (Vector Laboratories) antibodies were used in controls for non-specific staining reactions.

Recombinant and natural cytokines. To test for staining specificity, highly purified natural or recombinant produced cytokines were used to block specific cytokine staining. The primary antibody (2 to 5 μg/ml) was incubated with corresponding natural or recombinant cytokine in a 10-fold excess concentration (20 to 50 μg/ml) at 4°C overnight. Staining with the complex was performed as previously described. The following cytokines were used: natural IL-1α (C. Heusser, Basal, Switzerland) and IL-1β (C. Dinarello, Boston, Mass.), recombinant IL-1ra (H. Towbin, Basel, Switzerland), IL-4, IL-6, and IL-8 (Genzyme Corp., Boston, Mass.), natural IL-10 (J. Abrams, Palo Alto, Calif.), recombinant TNF-α and TNF-β (Bayer Inc., Hannover, Germany), recombinant gamma interferon IFN-γ (Boehringer Ingelheim Inc., Vienna, Austria), bovine TGF-β1 (C. Snapper, Bethesda, Md.), and recombinant TGF-β2 (Genzyme).

Quantification of immunostains. Cytokine-producing areas were identified in a Reichert-Jung microscope (Polyvar 2; Reichert-Jung, Vienna, Austria) under

×100 magnification. The entire tissue section was examined. Stained cells were counted under ×400 magnification. The area of each section was measured by a computerized image analyzer (Quantimet 570; Leica, Cambridge Ltd., Cambridge, England). The average tissue area observed for each section was approximately 12.2×10^5 μm^2.

Lamina propria. Several high-power fields were examined, and the number of stained cells in the lamina propria in each field was counted. Thus, average number of stained cells in the lamina propria was determined for each patient.

Crypts. Crypts were examined for stained cells and for specific cytokine-producing areas within the crypts. Results were expressed as percentages of patients with positively staining crypts.

Cells within the lamina propria and transmigrating in crypts were grouped morphologically as either polymorphonuclear leukocytes, mononuclear cells, or endothelial cells. Mononuclear cells therefore include both lymphocytes and mononuclear phagocytes. Only cells with a specific localized intracytoplasmic staining appearance were enumerated.

Histopathology. Formalin-fixed, parrafin-embedded slides were sectioned at 6 μm and stained with hematoxylin and eosin. Coded sections from each specimen were examined independently by two investigators who were unaware of the culture report and the clinical profile of the patient. For evaluation of the biopsy specimens, histopathological features as described by Anand et al. (3) and modified by Kärnell et al. (18) were selected. The criteria were as follows. (i) Mucosal erosions were considered only when neutrophils were present at the site of damage. (ii) Cellular infiltrate within the lamina propria was subjectively assessed for increase in total number and relative number of neutrophils and mononuclear cells. (iii) Distorted crypt architecture was considered if there was crypt branching or when the regular, parallel array of crypts was deranged. Thus, histopathological changes were graded as mild, moderate, or severe. Normal histology or chronic inflammation was graded as 0. Mild colitis was diagnosed when the epithelial lining was intact with some cellular infiltrate and an increase in inflammatory cells in the lamina propria and edema. Specimens with moderate changes had focal erosions of the surface and a pronounced increase in cellular infiltrate in the lamina propria with marked depletion of mucus. Severe colitis was diagnosed in presence of diffuse mucosal erosion with surface exudate and crypt abscesses and thrombus within capillaries.

Statistical analysis. Statistical analysis were performed by using JMP, a computer system supplied by SAS Institute, Inc. Wilcoxon/Kruskal-Wallis test, Student's *t* test, and chi-square test with Yates' correction were used wherever applicable. A probability value lower than or equal to 0.05 was considered the criterion for a significant difference.

RESULTS

Histopathological analysis. (i) Patients. Biopsies obtained during the active stage of the disease showed acute inflammation in 20 of 24 biopsies, and 4 specimens were normal. Of 20 biopsies with acute inflammation, 11 showed moderate to severe and 9 showed mild inflammation; 3 of these 9 had also chronic inflammation. During the convalescent stage, 3 patients did not return for follow-up. Acute inflammation was observed in the day 30 specimens in 18 of 21 biopsies (85.7%); 6 of them showed moderate to severe inflammation. Twelve patients exhibited mild inflammation in the rectal biopsies; six among them had chronic inflammation. Only three patients (14%) had normal histology.

(ii) Controls. Twelve of thirteen specimens were found to have normal histology. A mild inflammation was observed in one individual, although no enteropathogens could be cultured from the stool specimens.

Expression of cytokine staining in rectal tissues. Immunohistochemical staining of cryostat sections of rectal biopsies from patients showed expression of various cytokines in the inflamed tissues. The intracellular cytokine staining pattern for most interleukins was observed to be cytoplasmic. In some cells, the staining was localized with a juxtanuclear position in the cytoplasm (Fig. 1A), as has also been demonstrated on in vitro-activated cytokine-producing cells in suspension (Fig. 1B) (33). This finding indicates that even in cryopreserved tissue, cytokine accumulation occurs in the Golgi complex in cytokine-producing cells. It has been reported that all cytokines studied so far with the exception of the IL-1 family have hydrophobic amino acid binding sequences directing secretion through the Golgi-endoplasmic route (Fig. 1C) (33). Immunostaining of IL-1α, IL-1β, and IL-1ra in rectal tissues revealed a diffuse nuclear and cytosolic compartmentalization without a local intracellular accumulation (Fig. 1D). Recent studies (35) have shown that the intracellular transport of IL-1α and IL-1β and part of IL-1ra follows a pathway different from that for the cytokines examined in this study. Besides the stained cells, the immunoreactivity extended over a large extracellular area encompassing many producer cells. The detection of extracellular cytokines may be due to a binding to low-affinity receptors in the local areas or to extracellular matrix components after the secretion. The specificity of the immune reaction was confirmed by complete abolition of the reactivity, which was achieved by preincubating cytokine-specific MAbs with the corresponding human cytokines.

Comparison of cytokine production in biopsies from patients and controls. Concomitant production of IL-1α, IL-1β, IL-1ra, TNF-α, IL-6, IL-8, IL-4, IL-10, IFN-γ, TNF-β, and $TGF\beta_{1-3}$ was found in most patients during the acute stage of shigellosis (Table 2). The cytokine production profile was dominated by IL-1β, $TGF\beta_{1-3}$, IL-4, and IL-10, but IL-8, TNF-α, and IFN-γ were also extensively expressed. Altogether, 82% of the patients expressed more than three cytokine in the cryopreserved rectal biopsies. Staining of rectal biopsies from healthy controls revealed a significantly lower incidence of cytokine-expressing cells ($P < 0.005$) or no reactivity in the tissues (Fig. 1E). Rarely, a few cells stained for IL-4, IL-1ra, IFN-γ, or TGF-β. The morphology of the stained cells was similar to that observed in patients. The only consistent and strongly positive reactivity in the controls was observed for IL-8, which was confined to crypts and occasionally to the mucosal surface (Fig. 1F).

Localization of cytokine-producing cells. The localizations of immunoreactivity of the cytokines in the rectal mucosa from patients with acute *Shigella* infection are summarized in Table 3. In general, there was a higher incidence of IL-1β-containing cells in comparison with IL-1α-synthesizing cells in the lamina propria. Both mononuclear cells and polymorphonuclear leukocytes, which were scattered throughout the lamina propria in small clusters and were also located in the surface and crypt epithelium, stained for IL-1β (Fig. 1D). Some clusters contained cells producing different cytokines as detected by staining serial sections. Frequently endothelial cells of capillaries and venules also expressed IL-1β (Fig. 2A). IL-1β-producing cells could also be detected in lymphoid aggregates. Immunoreactivity of IL-1ra and IL-8 was confined mainly to the crypt lumen and could only rarely be detected in the lamina propria (Fig. 1F). The consistent and extracellular localization of IL-8 suggested a localized storage depot in all of the stained biopsies. In the lamina propria, cells producing TNF-α (Fig. 2B), TNF-β, IL-6, IL-4, and IL-10 were mainly located in clusters with a patchy distribution. In particular, IL-4, IL-6, IL-10, and TGF-β showed, in addition to a localized cytoplasmic staining, an extracellular distribution of pools of cytokines. IL-4-producing cells could be visualized in lymphoid aggregates (Fig. 2C). IL-6- and TGF-β-producing endothelial cells were less frequent. The frequency of IFN-γ-producing cells was lower in comparison with other studied cytokines and always strictly intracellular.

Comparison between histopathological grading of colitis and cytokine production in individual cells. In general, the cytokine production in rectal biopsies from patients with *Shigella* infection correlated with the severity of histological grading (Table 2). For the whole study period, a significantly elevated incidence of cytokine-producing cells was noticed for IL-1, IL-6, IFN-γ, and TNF-α in cases with severe inflammation versus those with mild inflammation ($P < 0.05$). During the acute phase, the number of IL-1β-containing cells in biop

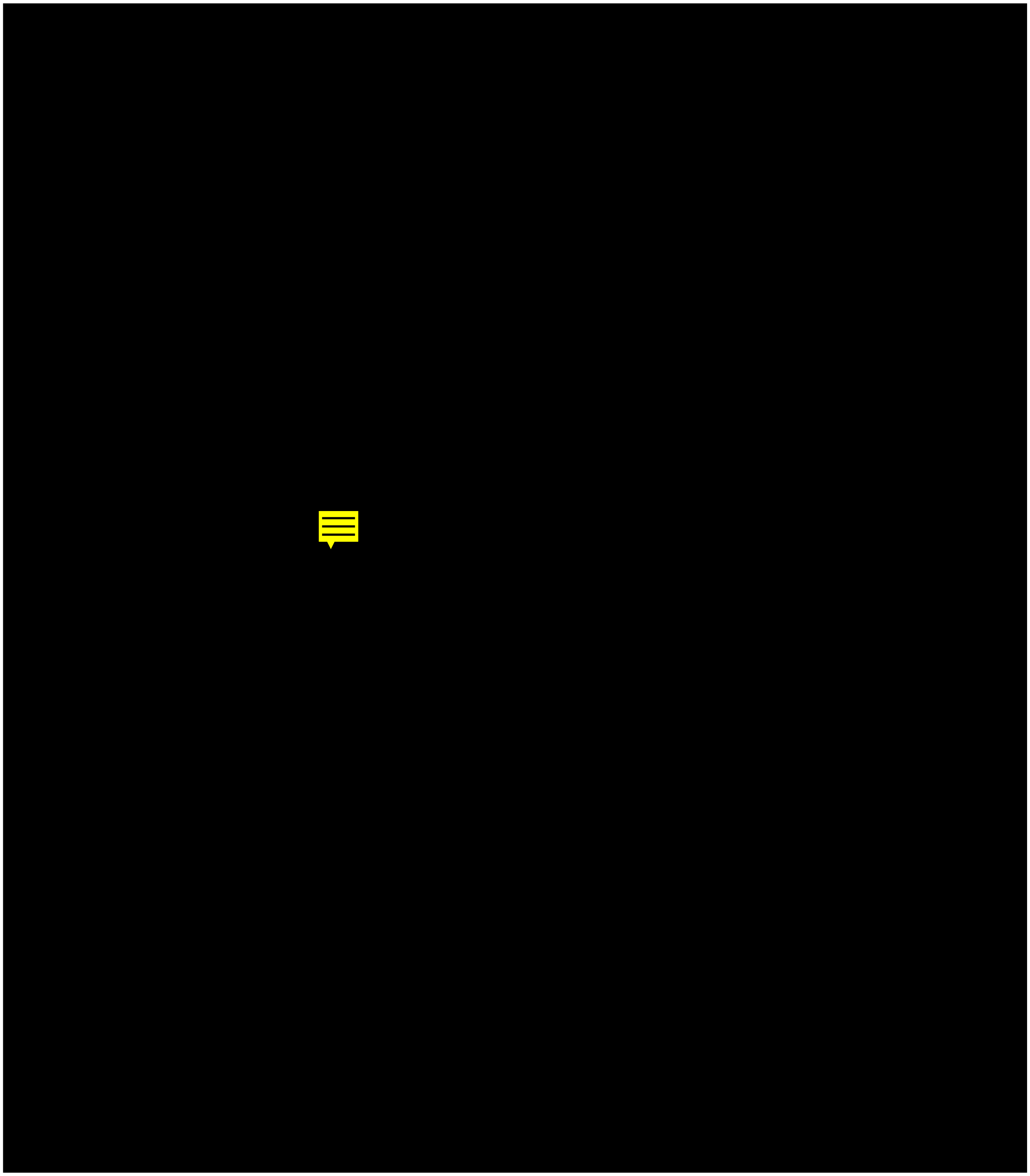

FIG. 1. Color video photographs illustrating immunoperoxidase staining for cytokine-producing cells in cryopreserved rectal tissues obtained from *Shigella*-infected patients; the sections were counterstained with hematoxylin. (A) The local juxtanuclear deposition of IL-4 in the producer cells stained by the immunoperoxidase DAB technique indicates accumulation of IL-4 in the Golgi organelle (arrow). Original magnification, ×500. (B) A clone (Cos) of cells transfected with the gene for human IL-10. All cells are IL-10 producers. Note the juxtanuclear deposition of peroxidase due to accumulation of IL-10 in the Golgi organelle (arrow). The cells were counterstained with hematoxylin. (C) Lipopolysaccharide-stimulated peripheral blood mononuclear cells harvested after 3 h of culture. Note the perinuclear and cytoplasmic localization of IL-1α in the monocytes. Cells were stained with peroxidase and counterstained with hematoxylin. (D) IL-1β-containing cells in the crypt epithelium (arrows). Note the entrapping of cytokine in the extracellular area close to the synthesizing cells in the lamina propria. Original magnification, ×800. (E) Rectal biopsy specimen from a healthy control showing no detectable staining for any of the studied cytokines except IL-8. Original magnification, ×200. (F) Prominent IL-8 positivity expressed in the crypts and surface epithelium. Original magnification, ×125.

TABLE 2. Numbers of cytokine-producing cells per section[a] in rectal biopsies from patients with shigellosis: comparison between mild and severe colitis

Cytokine	Histology, day 1				Histology, day 30			
	No. of cytokine-producing cells/sections			P value	No. of cytokine-producing cells/sections			P value
	Mean (severe + mild)	Severe (2–3°)	Mild (0–1°)		Mean	Severe (2–3°)	Mild (0–1°)	
IL-1α	31.2	10(0–75) [8/11]	3(0–13.5) [6/13]	0.07	26.3	21(3–97) [4/6]	0(0–30) [6/15]	0.08
IL-1β	37	60(0–100) [8/11]	5(0–55) [6/13]	0.09	51.2	87(0–212) [4/6]	5(0–75) [10/15]	0.07
TNF-α	23.5	5(0–50) [7/11]	0(0–28) [6/13]	0.33	5.4	6(0–15) [4/6]	0(0–5) [4/15]	0.04*
IL-6	31	35(18–75) [9/11]	0(0–45) [7/13]	0.01*	36.8	56(8–125) [5/6]	5(0–30) [9/15]	0.02*
IFN-γ	16	5(0–35) [6/11]	0(0–8.2) [5/13]	0.04*	18.8	2.5(0–22.5) [3/6]	0(0–25) [5/15]	0.8
TNF-β	20	7(0–50) [6/11]	5(0–11.5) [7/13]	0.7	19.5	0(0–6) [1/6]	8(0–35) [8/15]	0.2
IL-4	29.5	25(0–90) [6/11]	5(0–29) [8/13]	0.27	41	60(19–137.5) [5/6]	15(0–30) [9/15]	0.08
IL-10	30.5	5(0–30) [5/11]	5(0–26.5) [6/13]	0.9	26.5	20(0–39) [4/6]	6(0–50) [11/15]	0.8
TGF-β	38.7	25(0–75) [7/10]	25(4.5–76.5) [9/13]	0.97	72.8	52.5(19–116) [6/6]	50(25–100) [14/15]	0.8
IL-1ra	33	45 [5/11]	23 [3/13]	0.2	81	83 [5/6]	73 [11/15]	0.6
IL-8	100	100 [11/11]	100 [13/13]	ND	100	100 [6/6]	100 [15/15]	ND

[a] Given as median, first, and third quartiles (25 and 75% points shown in parentheses). The average studied area of each section was 12.2 by 10^5 μm^2 (±10%). *P* values were determined by Wilcoxon/Krushkal-Wallis test and chi-square test as described in Materials and Methods. Asterisks indicate significant *P* values. Numbers in brackets indicate number of biopsies stained positive out of total number of patients studied. Biopsies staining for IL-1ra and IL-8 are expressed as percentages as described in Materials and Methods. ND, not done.

sies with severe inflammation was not significantly increased ($P = 0.07$) compared with the incidence in biopsies with mild inflammation. A similar picture was obtained for IL-1α-producing cells. Numbers of IL-6-producing cells were significantly higher in tissues with severe colitis than those with mild colitis during both the acute and convalescent phases ($P = 0.01$ and 0.02, respectively) (Fig. 3A). IFN-γ-producing cells were significantly higher in number ($P = 0.04$) and in staining intensity in biopsies with moderate to severe inflammation than in those with mild inflammation during the acute stage. The incidence of TNF-α-producing cells in biopsies with moderate to severe colitis was significantly higher ($P = 0.04$) than in biopsies with mild inflammation during the convalescent phase. No difference was observed in the frequency of cells producing TNF-β, TGF-β, IL-10, IL-8, and IL-4 in the rectal tissues during either the acute or convalescent stage.

Persistent incidence in rectal biopsies of cytokine-producing cells in acute and convalescent phases. Comparison between day 1 and day 30 specimens in terms of cytokine-containing cells showed that expression of IL-1ra in day 30 biopsies was remarkably and significantly higher than that in day 1 specimens, both in mild and in severe colitis ($P < 0.002$). This may be due to a counterregulatory effect and may be linked to regression of clinical symptoms. Similarly, the numbers of TGF-β-producing cells was significantly higher in day 30 specimens compared with day 1 specimens in severe inflammation, whereas in mild inflammation, no change in the frequency was observed (Fig. 3B). Other studies have also pointed to TGF-β being involved in acute inflammatory responses (13). No significant difference was noted in the incidence of the cells producing IL-1α, IL-1β, IL-4, IL-6, IL-10, TNF-α, TNF-β, and IFN-γ in day 1 biopsies compared with day 30 biopsies. Thus, consistent with the histological findings, cytokine-producing cells were still upregulated even 30 days after the onset of disease.

TABLE 3. Localizations of cytokine immunoreactivity in rectal biopsies obtained from patients with acute shigellosis[a]

Cytokine	Localization in rectal biopsies			
	Mucosal surface	Lamina propria	Crypts	Vascular cells
IL-1α		MNC, PMN		
IL-1β	MNC, PMN	MNC, PMN	MNC, PMN	EC
IL-1ra		MNC	Ep	
TNF-α		MNC	MNC	
IL-6	Ep	PMN, MNC	Ep	EC
IL-8	Ep	Ep, MNC	Ep	
IFN-γ	MNC	MNC	MNC	
TNF-β	MNC	MNC	MNC	
IL-4		MNC	MNC	
IL-10		MNC	MNC	
TGF-β	Ep	MNC, PMN	Ep	EC

[a] Cytokine-producing cells were stained as described in Materials and Methods in cryopreserved 8-μm sections. Abbreviations: MNC, mononuclear cells; PMN, polymorphonuclear leukocytes; Ep, epithelial cells; EC, endothelial cells.

DISCUSSION

Previous studies have indicated that immunohistochemistry using cytokine-specific antibodies with the combination of peraformaldehyde for fixation and saponin for permeabilization (5, 33) permitted optimal access of the MAbs to intracellular cytokine stores. These findings were also verified in this study, which revealed localization of the cytokine-producing cells within cryopreserved tissue obtained from *Shigella*-infected patients (Fig. 1). The staining specificity was supported by ablating the staining by using cytokine specific MAbs preincubated with recombinant cytokines. Frequent detection of cytokine-containing cells in the rectal tissue with a perinuclear accumulation suggests intracellular production rather than uptake of cytokines (13). This staining morphology was never achieved in the cultured cells which were exposed to passive addition of natural or recombinant cytokine products (1). Further evidence for this statement was provided in our study (5) by demonstrating localization of the cytokines to the Golgi organelle, using MAbs specific to the Golgi complex. Supporting evidence comes from a recent study showing a correlation of the gradual juxtanuclear appearance of the specific cytokine staining pattern in cultured cells with the increasing concentration of extracellular cytokine measured by enzyme-linked

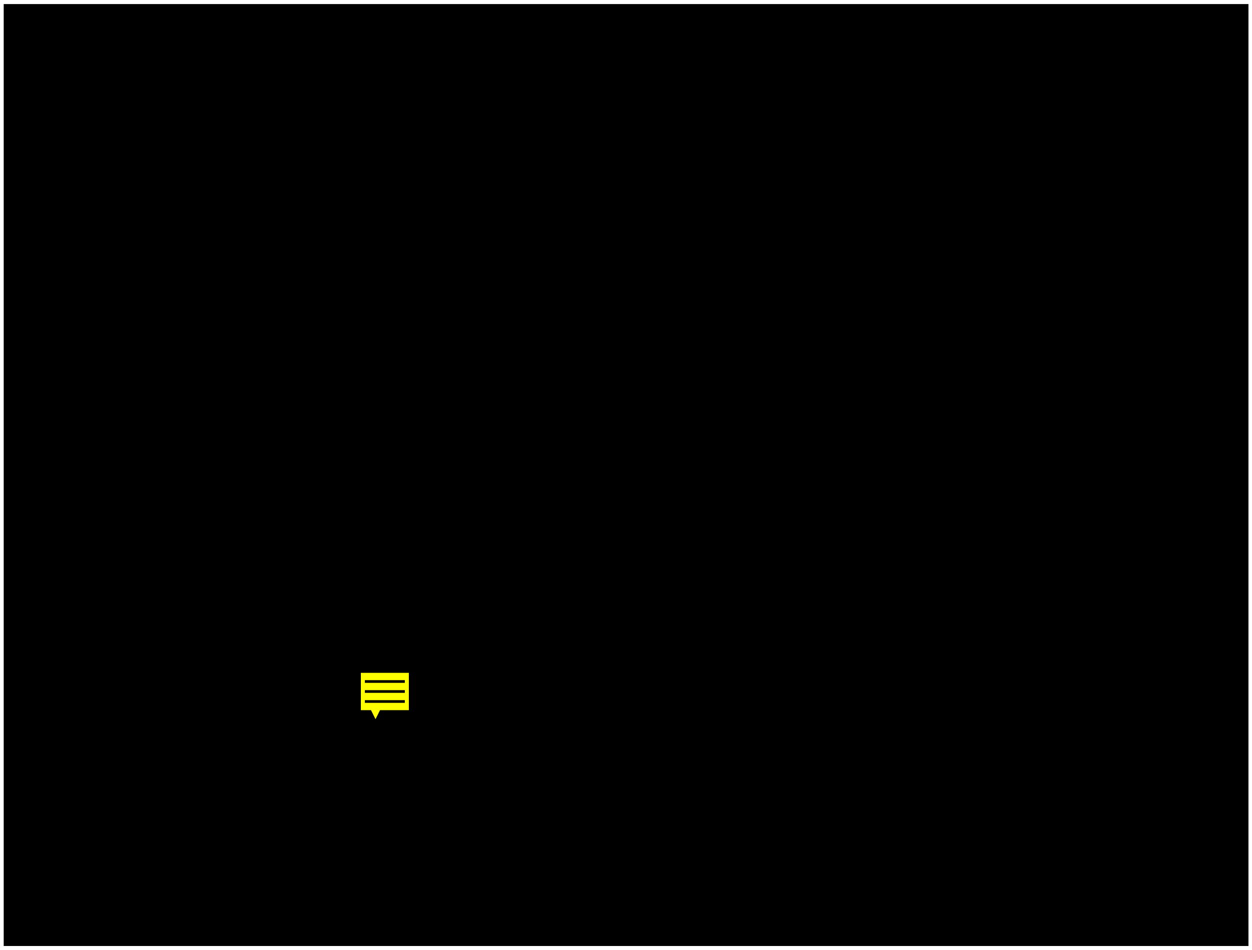

FIG. 2. (a) Expression of IL-1β on the vascular endothelium (arrow). Original magnification, ×320. (B) TNF-α-synthesizing cells in the lamina propria. Adjacent crypts show accumulation of TNF-α within the lumen of the crypts. Original magnification, ×320. (C) IL-4-producing cells in the lymphoid aggregates. Original magnification, ×200.

immunosorbent assay and with the appearance of mRNA transcripts identified by reverse transcription PCR (13).

There has been much speculation about the importance of cytokines in the pathogenesis of shigellosis in recent years (15, 22, 23, 28, 38). We demonstrated in this study the presence of enhanced cytokine production in inflamed tissues from patients infected with *S. dysenteriae* type 1 and *S. flexneri*. The study also describes the localization of cytokine production in rectal biopsies obtained during early and late stages of the disease and provides a histopathological context. A significant difference in the number of cells producing cytokines such as IL-6, TNF-α, and IFN-γ could be observed between tissues with severe and mild colitis, with excessive production linked to the histological severity of disease. A relative increase in the number of IL-1α- and IL-1β-containing cells was also observed in severe inflammation, although no significant difference ($P = 0.07$) could be shown. This finding emphasizes that there is increased expression of proinflammatory and regulatory cytokines in the severe form of colitis caused by shigellae.

The absence or rare detection of cytokine-producing cells in the healthy controls and the dramatic increase ($P < 0.005$) in the number of cytokine-producing cells during shigellosis reflect the actual events occurring in the mucosa under physiological and pathological conditions. Our results, based on identification of single cytokine-producing cells, have the limitation that they provide a momentary picture of cytokine expression in *Shigella* infection. Therefore, in order to understand the complete clinical picture in *Shigella* infection, consecutive biopsies were required with kinetic analysis particularly at the onset of disease and in the convalescent stage.

Shiga toxin has been shown to mediate direct damage to vascular endothelial cells (38). Extensive tissue damage in the colonic mucosa in experimentally infected monkeys was shown to be related to Shiga toxin production by virulent wild-type strains (14). Studies with animal models also showed induction of vascular lesions or Shwartzman-like reaction by the lipid A moiety of bacterial endotoxin leading to severe diarrhea (24). The paucity of *Shigella* bacteria in the inflamed tissue and widespread prevalence of vascular lesions in the lamina propria suggested that absorbed Shiga toxin may play a role in causing the lesion either as a part of a local Shwartzman reaction or as a release of cytokines such as TNFα, IL-6, and IL-1β elicited by endotoxin, or it exerts a synergistic effect combined with exotoxins (22). Our study further strengthens the view that *Shigella*-associated toxins may induce vasculitis, as we have demonstrated the immunohistochemical localization of IL-6 and IL-1β in the endothelial cells within the lamina propria. IL-6 is known to cause vascultis; IL-1β induces hyperadhesiveness of endothelium for leukocytes (12) and has been shown to induce a Shwartzman-like response (27). Ex-

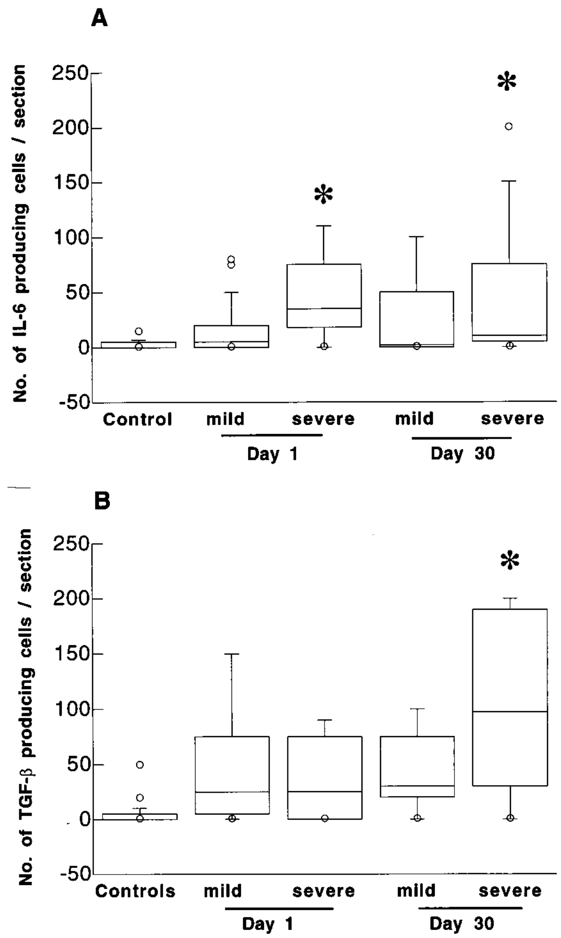

FIG. 3. Frequencies of cytokine-producing cells in rectal biopsies from patients with *Shigella* infection on days 1 and 30 after the onset of diarrhea and in healthy controls. (A) IL-6-producing cells; (B) TGF-β-producing cells. Each box encloses 50% of the data, with the median value displayed as a line. The lines extending from the top and the bottom of each box mark the minimum and maximum values within an acceptable range. The outlier is displayed as an individual point. The asterisks indicate a significant increase in numbers of producer cells ($P < 0.05$) in the rectal mucosa.

tensive infiltration of the colonic mucosa by polymorphonuclear leukocytes and macrophages during shigellosis may be related, besides contact with the bacteria, to the alteration in the vascular compartment of the colonic mucosa (14). These infiltrated macrophages and polymorphonuclear leukocytes may produce more cytokines, leading to ulceration and further tissue necrosis. Little information is currently available on the role of cytokines in the pathogenesis of shigellosis. Klimpel et al. (20) showed that *S. flexneri*-infected fibroblasts were susceptible to the cytotoxic action of TNF-α. TNF-α was shown to inhibit the invasiveness of *S. flexneri* (11); moreover, the action of TNF was not dependent on continuous protein synthesis for its anti-invasive effect. Recepters for TNF-α were shown to be present on *S. flexneri* bacteria, and the virulence property of a bacterium could be altered as a result of cytokine binding (23).

It was of particular interest to examine the histopathological profile in relation to the duration of illness in *Shigella* infection. All patients were treated with antibiotics (nalidixic acid or pivmecillinam) as soon as they were admitted to the hospital; the patients were free of symptoms when released 6 to 7 days after admission. Although the disease in patients was clinically resolved, the persistence of inflammation, presence of chronic inflammatory cells, and crypt distortion or branching in the day 30 biopsies simulate the histologic picture of inflammatory bowel diseases (10). Moreover, the probable role of the antibiotic regimen is difficult to assess. Production of various cytokines in the rectal biopsies during acute and convalescent periods, with the exceptions of TGF-β and IL-1ra, was not significantly different between the two periods. Cytokine release in acute and convalescent stages was a sustained phenomenon throughout the clinical course, which could maintain an acute inflammatory response in the mucosa. Circumstantial association of some cytokines with tissue damage was reinforced by demonstrating the colocalization of specific cytokines within the abscess itself. Polymorphonuclear leukocytes and monocytes present in the crypt abscess were clearly seen to contain IL-1β and TNF-α, respectively. The healing process in shigellosis may also be critical in the resolution of tissue damage. In the later stage of the disease, healing proceeds into a persistent chronic phase characterized by continuous infiltration of monocytes secreting various cytokines (IL-1, TGF-β_{1-3}, IL-10, and IL-6), which may play an important role in the host humoral defense. The demonstration of cytokine production in the convalescent-phase biopsies is supporting evidence that these cytokines are involved in the inflammatory responses in shigellosis and in line with the finding that most cases were not histologically normal. The persistence of inflammation over the long term may effect the linear growth pattern in children, which could be an important factor for the development of malnutrition in developing countries (16).

It should be noted that in a few of the inflamed biopsies, cytokine expression could not be detected. Histologically normal biopsies were also obtained from severely sick patients with bloody stools. This was most likely not caused by technical problems, as all biopsies were taken in a uniform way and strict measures were taken to include technically adequate specimens. A more ready explanation is the fact that damage in shigellosis is focally distributed (19), and therefore more than one frozen section and preferably several biopsies should be examined to ensure a representative assessment.

There are accumulating data suggesting the existence of TH1 and TH2 in T-cell subsets in mice and perhaps also in humans (32). Preferential activation of TH1 or TH2 depends on the infectious agent, infective dose, and antigen presentation (34), and thus there are different patterns of cytokine expression in different compartments as well as in individuals with different severities of disease. This study does not show a selective pattern of cytokine production; instead, the lymphokines reflect induction of local humoral as well as cytotoxic immune responses. The general upregulation of all of the studied cytokines seemed to be correlated with the diseased state. Acute shigellosis induced a significant local production of a vast number of cytokines (IL-1α, IL-1β, TNF-α, IL-6, IFN-γ, TNF-β, IL-4, IL-10, TGF-β, IL-1ra, and IL-8) in the rectum. The production of these cytokines was not normalized even 30 days after the onset of disease. Severe disease was associated with a significantly increased production of IL-1β, IL-6, TNF-α, and IFN-γ compared with cases with mild histopathology. These findings indicate that local cytokine production perhaps mediated by various *Shigella* toxins is associated with the pathogenesis of shigellosis.

ACKNOWLEDGMENTS

We thank Pradeep Chandra Dash for his assistance in recruiting patients and control subjects and acknowledge the gift of cytokine-specific antibodies from Harry Towbin (Basel), Miroslav Ceska (Vienna), J. Abrams (Palo Alto), G. Adolf (Vienna), Gudrun Andersson

(Lund), and Kohei Miyazono (Uppsala). The skilled technical assistance of Caroline Ekberg and Sushma Pashi is gratefully acknowledged.

This study was supported by the Swedish Agency for Research Cooperation with developing countries (SAREC), ICDDR,B, the Medical Research Council (grants 9082 and 10850), and the National Cancer Institute (grants 2490 and 2766). The ICDDR,B is supported by countries and international agencies which share its concern for the health problems of developing countries. Current donors include the aid agencies of the government of Australia, Bangladesh, Belgium, Canada, Denmark, France, Japan, The Netherlands, Norway, Saudi Arabia, Sweden, Switzerland, the United Kingdom, and the United States; international organizations including the United Nations Development Programme, United Nations Children's Fund and the World Health Organization; and private foundations including the Ford Foundation and the Sasakawa Foundation.

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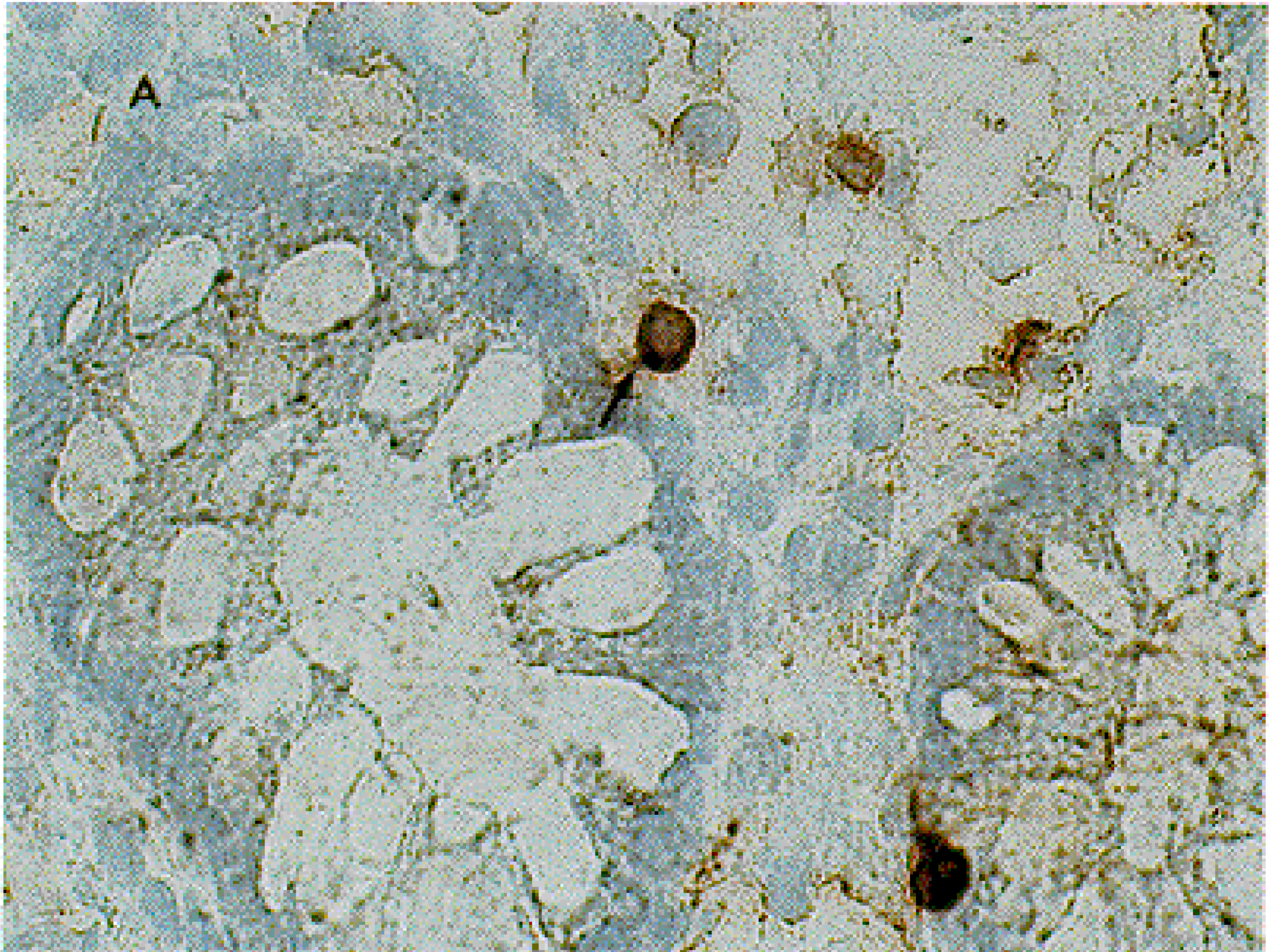
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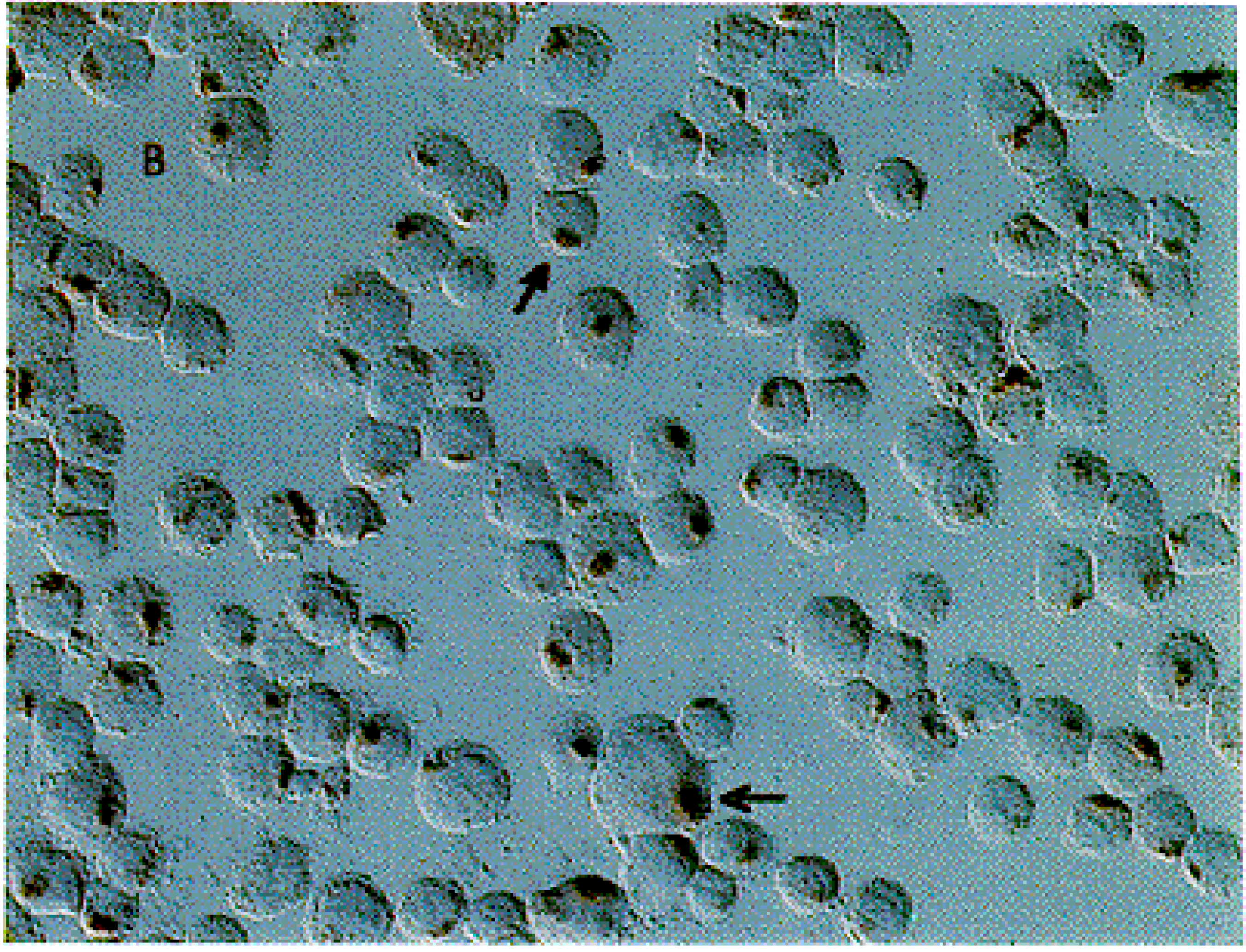
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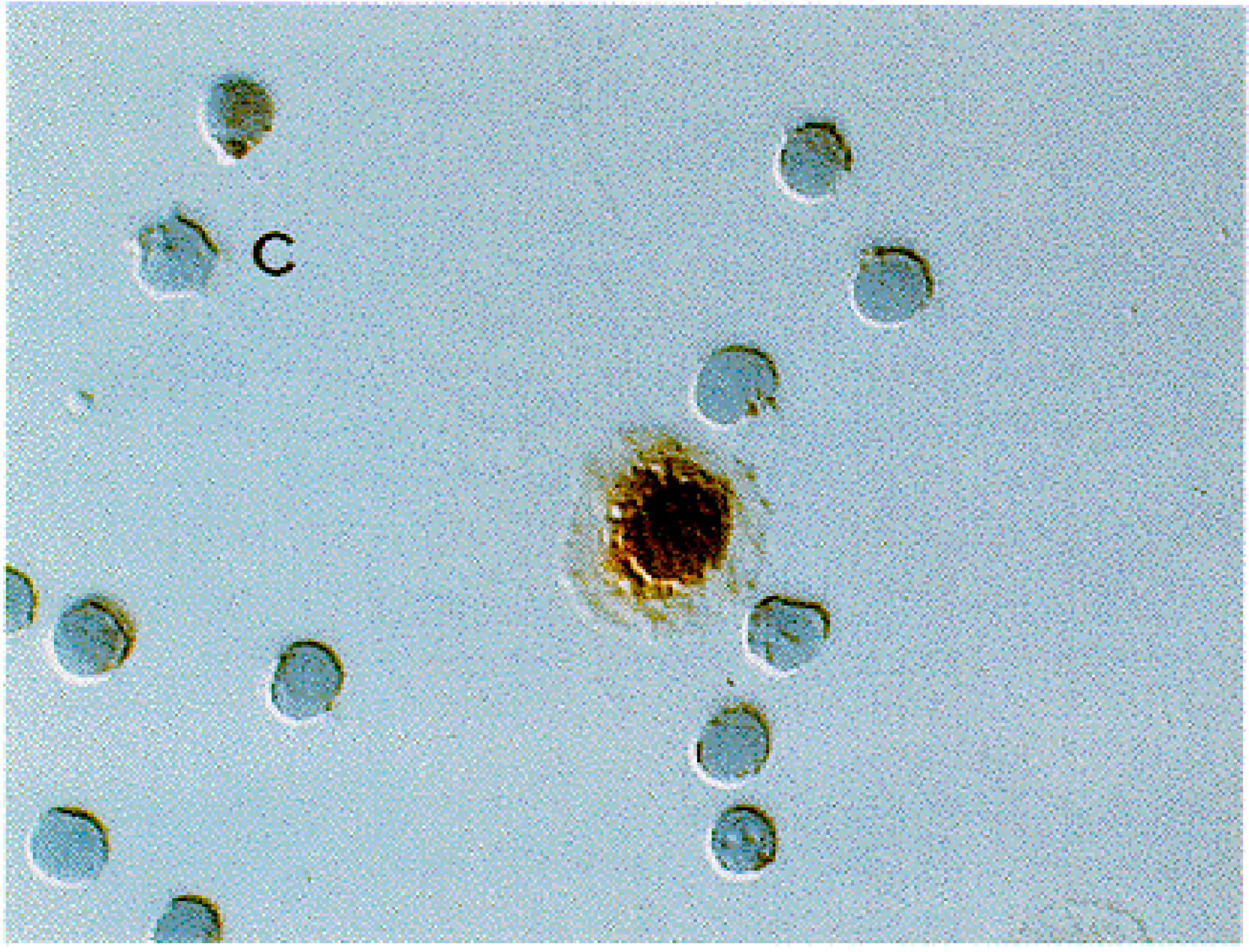
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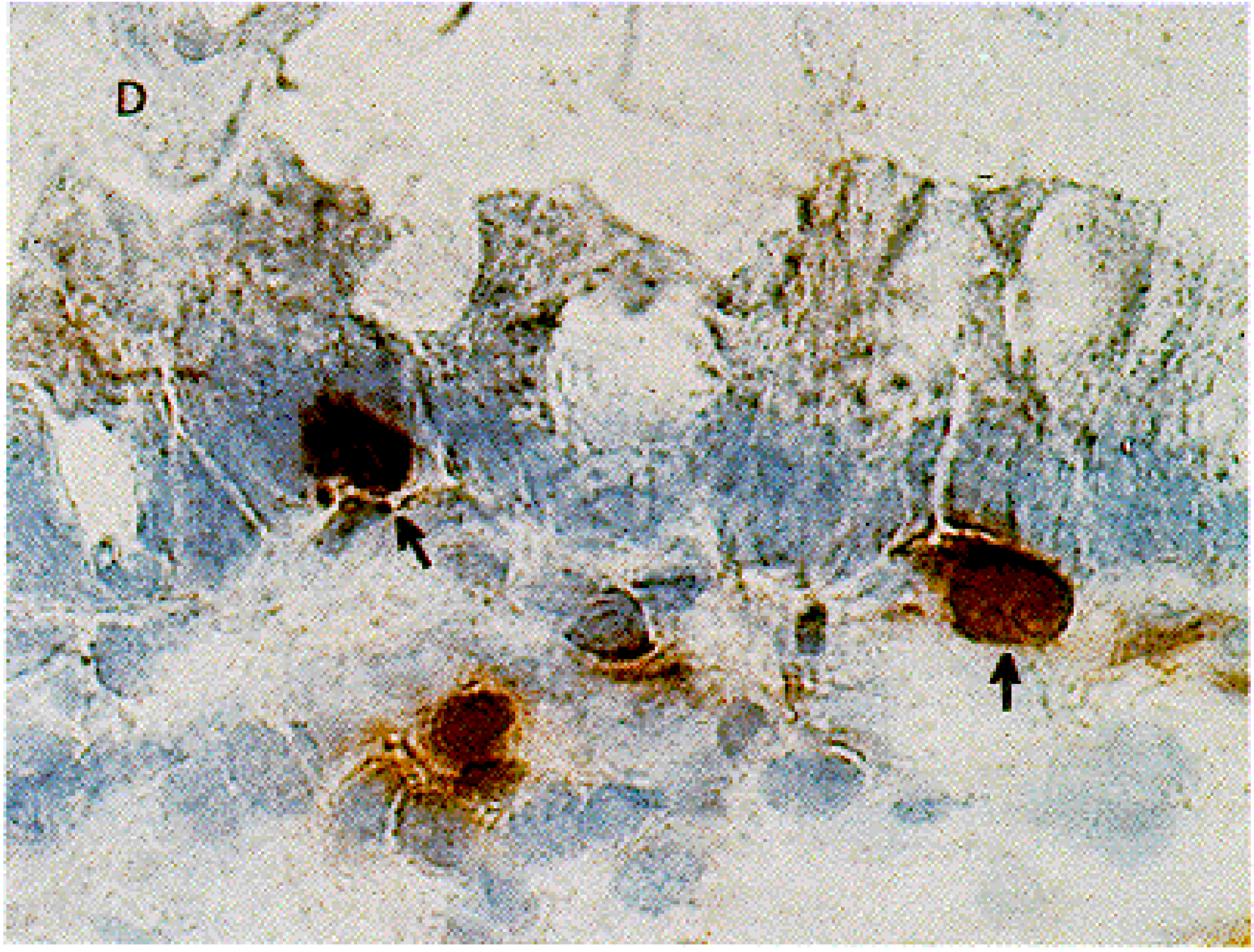
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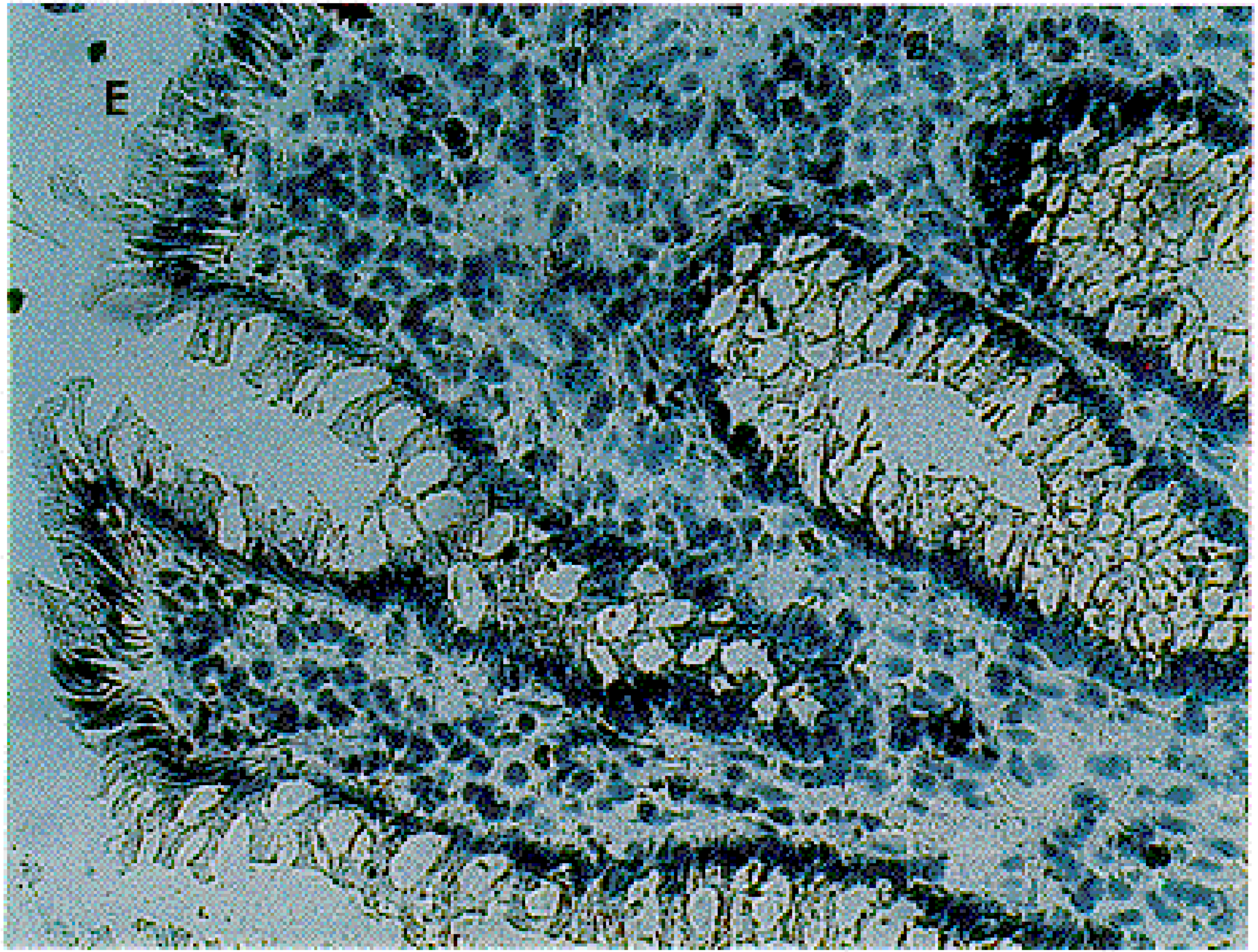
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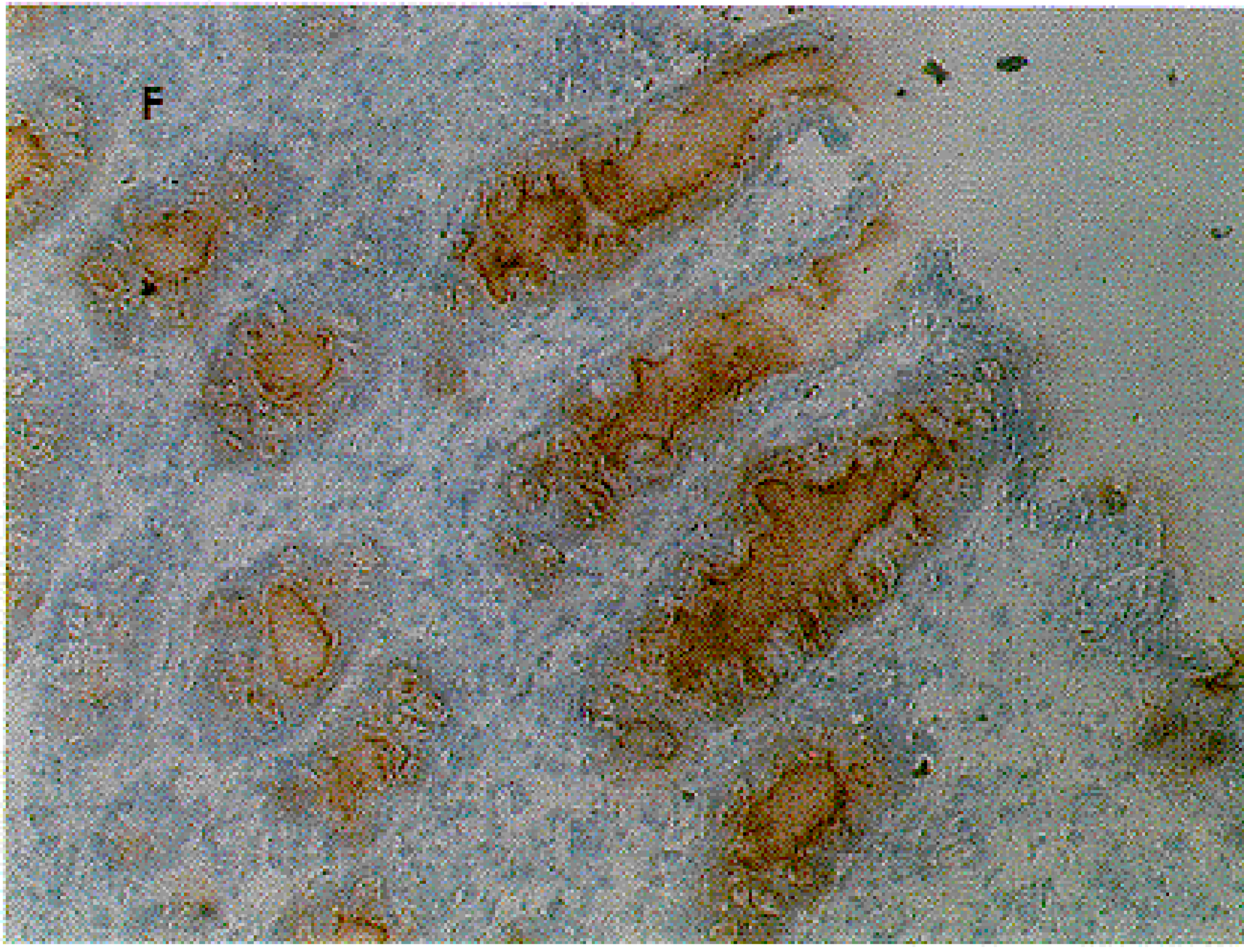
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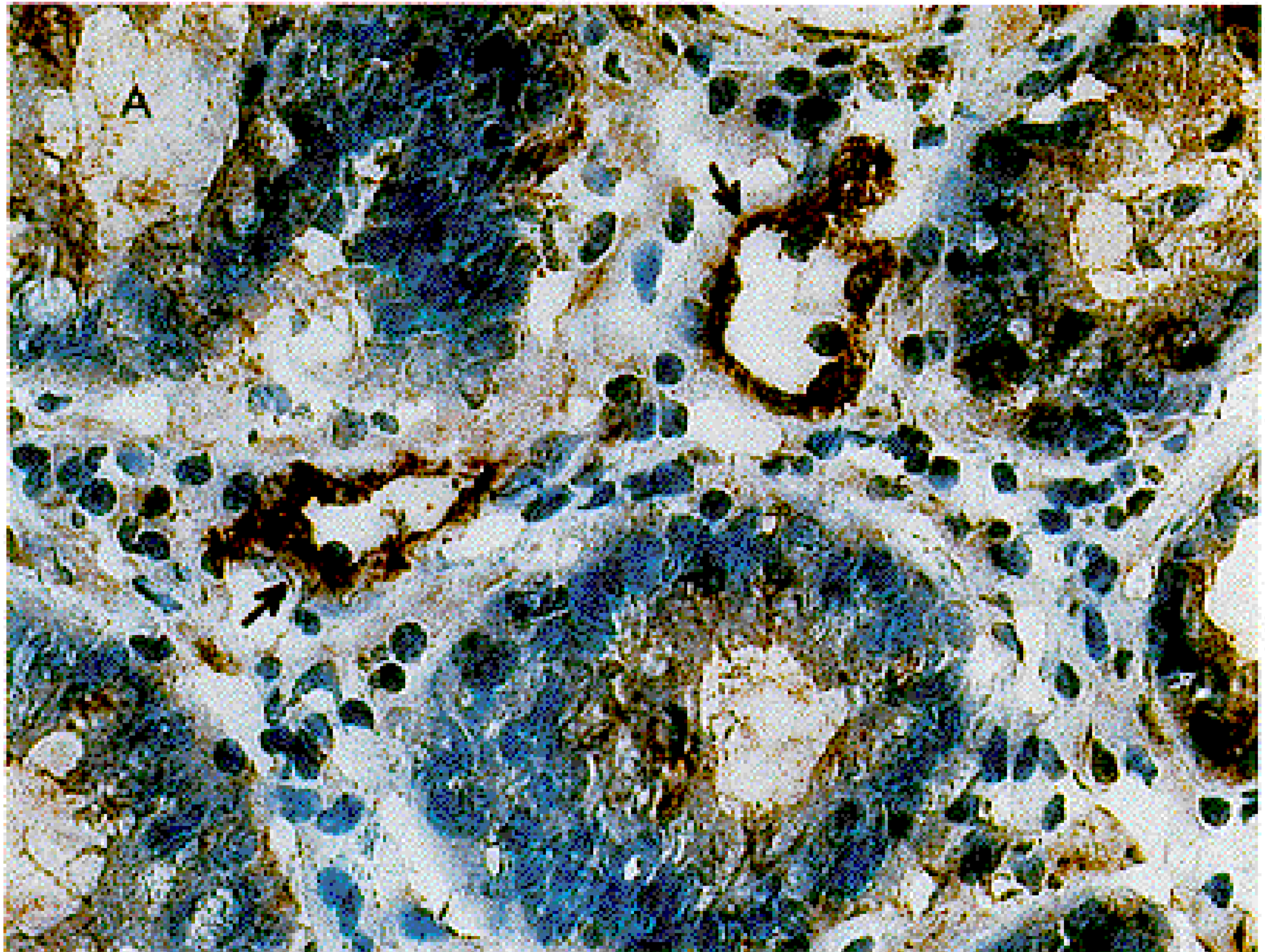
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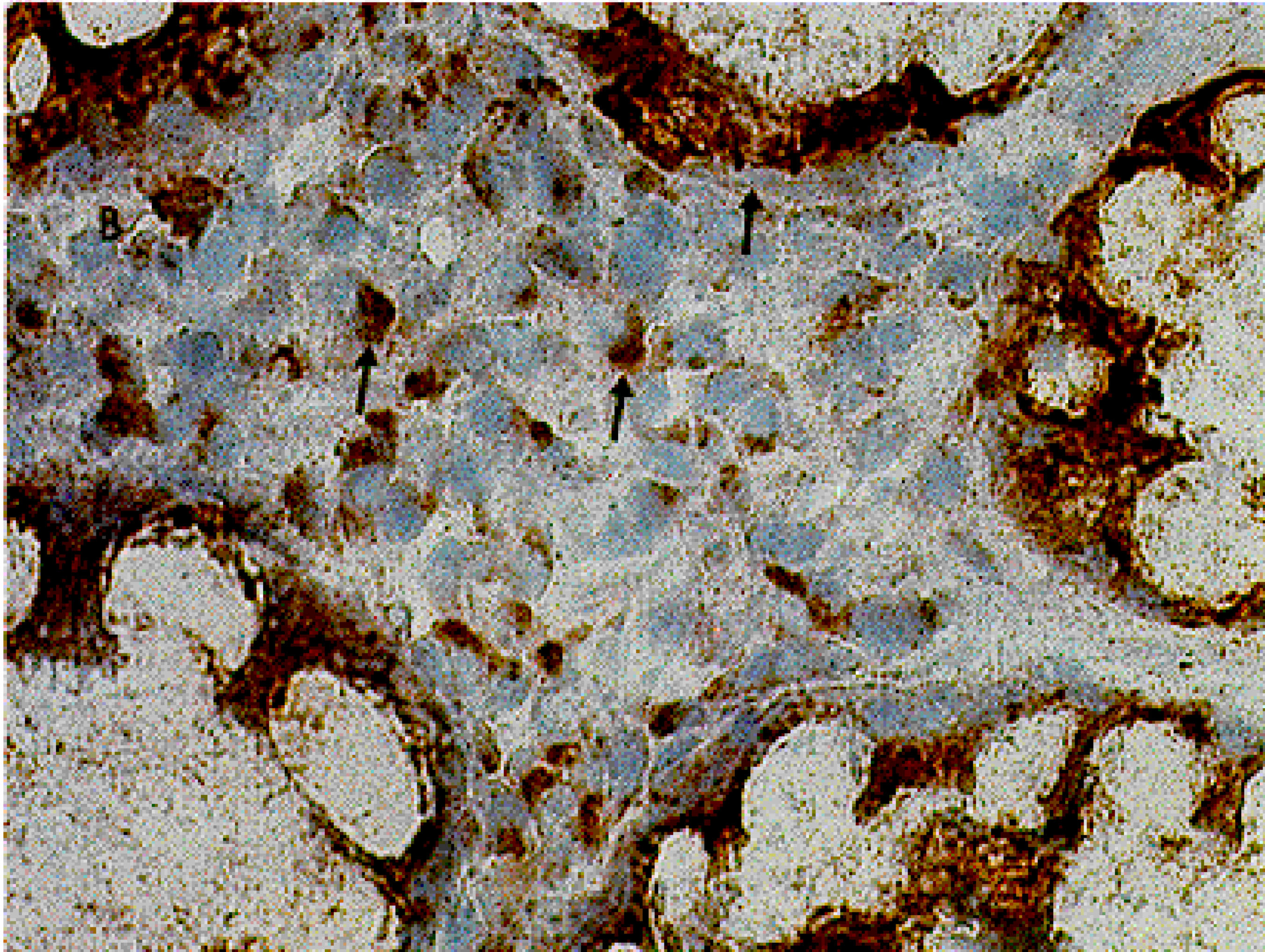
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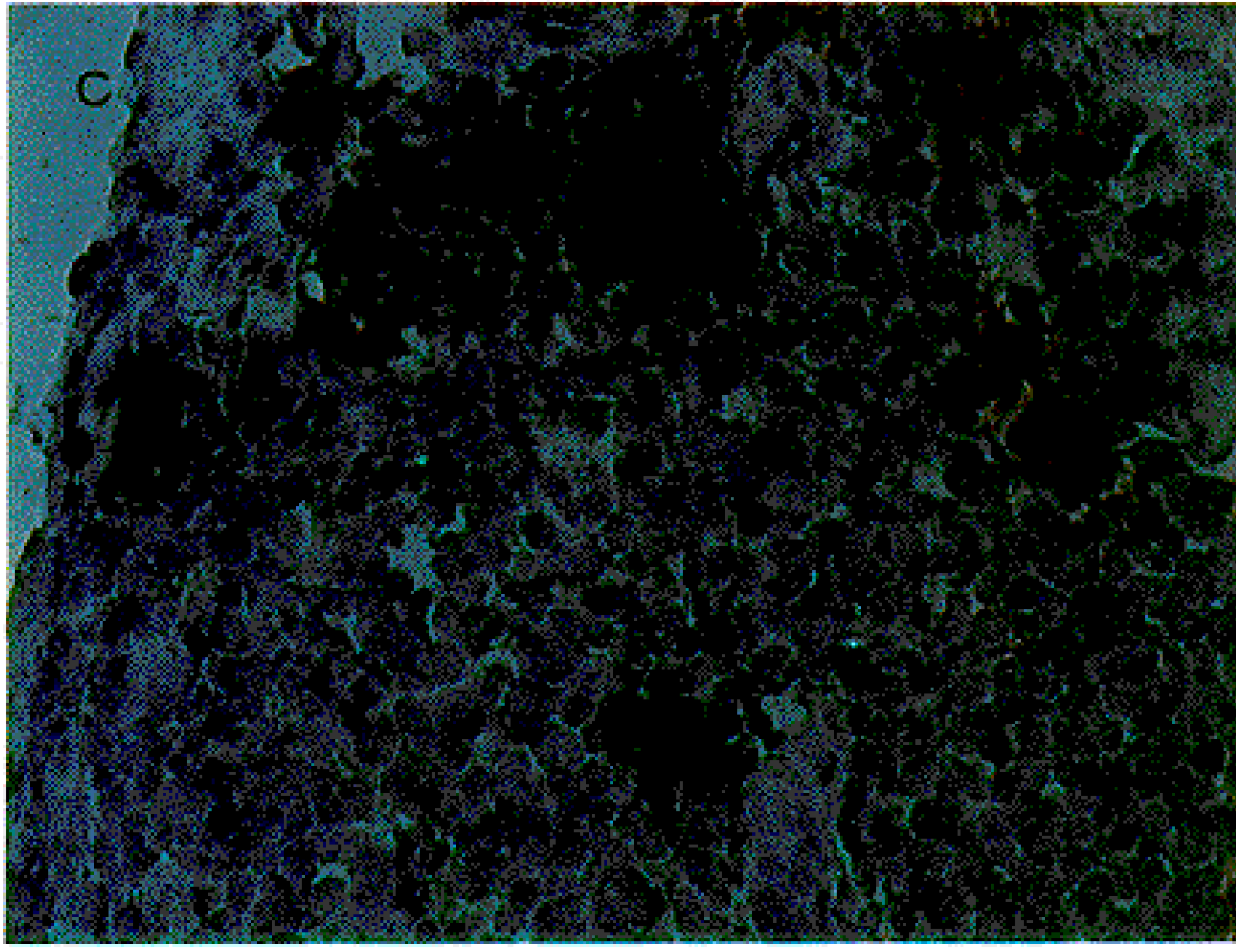
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