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In Situ Production of Gamma Interferon, Interleukin-4, and Tumor Necrosis Factor Alpha mRNA in Human Lung Tuberculous Granulomas

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Human tuberculous granulomas from five adults undergoing surgery for hemoptysis were analyzed by nonradioactive in situ hybridization for tumor necrosis factor alpha (TNF-α), gamma interferon (IFN-γ), and interleukin-4 (IL-4) gene expression. All of the patients produced TNF-α mRNA. Three patients stained positive for both IFN-γ and IL-4 mRNA; the other two stained positive for IFN-γ but not IL-4 mRNA. Heterogeneity between the granulomas was observed in those patients staining positive for both IFN-γ and IL-4 mRNA; these patients exhibited granulomas having IFN-γ and not IL-4 mRNA as well as granulomas positive for both cytokine mRNAs. There was no evidence of caseation in these granulomas, and the cytokine patterns may represent events in the evolution of the granuloma. However, in those granulomas exhibiting caseous necrosis, very little IFN-γ or IL-4 mRNA was observed, implying that progression of the granuloma is accompanied by a down regulation of T-cell responses. TNF-α mRNA expression was highest in patients with both IFN-γ and IL-4 mRNA. Populations of CD68 positive macrophage-like cells within the granulomas produce mRNA for TNF-α, IFN-γ, and IL-4. This implies that macrophages within the tuberculous granuloma may not be dependent on T-cell cytokines for modulation of their function but may be able to regulate their own activation state and that of the surrounding T cells. These findings have implications on the delivery of immunotherapies to patients with tuberculosis.

Mycobacterium tuberculosis is a facultative intracellular pathogen which can survive and replicate within the host macrophage. The majority (about 90%) of individuals are relatively resistant to *M. tuberculosis* and mount an effective cell-mediated immune response, which results in the development of delayed-type hypersensitivity reactions in the skin but no manifestations of clinical disease. In susceptible individuals, however, an inadequate immune response allows the clinical disease of tuberculosis (TB) to develop. In the majority of patients who develop TB, the symptoms are restricted to the lung (pulmonary TB); however, some patients develop extrapulmonary TB, where the mycobacteria escape from the lungs and colonize other organs.

The host immune response plays a crucial role in determining which outcome results from the encounter between the host and the pathogen. There have been many studies of cell-mediated immunity in patients with TB or other granulomatous diseases and in subjects exposed to *M. tuberculosis* but exhibiting no symptoms. Studies of the peripheral circulation and the site of pathology, e.g., the tuberculous effusion, bronchoalveolar lavage fluid, and lymph node biopsies, have been carried out (3, 12, 17). There is general consensus that $CD4^+$ T cells play a pivotal role in protective immunity but controversy over the relative contributions of gamma interferon (IFN-γ)-secreting T cells (T helper cell type 1 [TH1]) and T cells secreting predominantly interleukin-4 (IL-4) (TH2) (18, 21). Other T-cell subsets such as $CD8^+$ and γδ T cells are also capable of making IFN-γ and/or IL-4 and probably contribute to the observed protective immune response (7, 10).

TH1 and TH2 T cells were first described in the mouse, where polar immune responses are readily observed. TH1 T cells produce IFN-γ (among other cytokines) and stimulate cell-mediated immune responses, TH2 T cells produce IL-4 and IL-5 and stimulate humoral immunity, and TH0 cells make detectable levels of both IFN-γ and IL-4. TH2 cytokines inhibit the proliferation and cytokine secretion of TH1 cells and vice versa (14). However, in humans the immune response appears less polar and a clear division between TH1 and TH2 is less obvious. Leprosy (causative agent, *Mycobacterium leprae*) has been described as a polar disease, with tuberculoid leprosy patients exhibiting paucibacillary disease with a well-developed TH1 response (20). The T cells of these patients proliferate vigorously in response to mycobacterial antigens, and large amounts of IFN-γ are present within the lesions. However, lepromatous leprosy patients have a high bacillary load and a poor proliferative response to mycobacterial antigens (20). Whether this is due to a TH2 response or merely a lack of effective TH1 is debatable. Attempts have been made to extrapolate these observations in leprosy to patients with TB. The variability of cytokine profiles found in patients with TB (18, 21) may result from in vitro artifacts arising during the generation of the T-cell lines or clones used in these studies. Alternatively, these findings could reflect a real spectrum of immune responses occurring in these patients.

The mRNA for IFN-γ and IL-10 was found to be increased in lymph node biopsies from TB patients, and IL-4 mRNA expression was low compared to controls (1, 11). In contrast, only TH1 cytokines were detected and IL-4 was absent in

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TABLE 1. Clinical details of patients and in situ hybridization of their granulomas

Patient[a]	Age (yr)/sex	Time (yr) since previous TB	Preoperative treatment duration (mo)	Chest radiograph[b]	Reaction[c]			
					TNF-α	IFN-γ	IL-4	β-Actin
A1	39/M	3	2	RUL	++	+++	+	+++
A2	35/M	15	3	RUL	+++	+++	++	+++
A3	26/F	3.4	2	LUL	+++	+++	−	+++
A4	23/M	3	2	LUL	++	++	−	+++
A5	31/M	5	3	LUL	+++	++	++	+++

[a] All patients had been treated before surgery with rifampin-isoniazid-pyrazinamide.
[b] RUL or LUL, right or left upper lobe cavitation.
[c] +++, strong positivity; ++, intermediate positivity; +, weak staining; −, no staining.

lymph node biopsies from sarcoidosis patients (1). Immunohistochemical analyses of IL-12, IFN-γ, and IL-10 confirmed the reverse transcription-PCR (RT-PCR) findings, but immunostaining for IL-4 was not performed (11). It therefore appears from these studies that TB elicits a predominantly TH1 response, with low but detectable levels of IL-4 mRNA. These studies left certain questions unanswered: where are the cytokines produced in relation to each other, which cells are responsible for their production, and how does the situation in the lymph node (1, 11) compare with that in the infected lung?

To address these issues, we studied the pattern of cytokine production in the human lung granuloma itself, using paraffin-embedded tissue. Riboprobes for human tumor necrosis factor alpha (TNF-α), IFN-γ, and IL-4 were generated and used to detect mRNA in granulomas from five patients with pulmonary TB. RNA-RNA in situ hybridization allows direct localization of mRNA to its cellular compartment without prior manipulation of the RNA, thus preserving the morphology of the tissue (8, 13). Three of the patients were found to exhibit detectable levels of mRNA for both IFN-γ and IL-4. However, two patients were negative for IL-4 mRNA and positive for IFN-γ mRNA. All patients produced mRNA for TNF-α. Furthermore, CD68-positive cells, with a macrophage morphology, were found to produce mRNA for the traditionally T-cell-specific cytokines IFN-γ and IL-4.

MATERIALS AND METHODS

Tissue specimens. Adult lung tissue was obtained from five patients undergoing surgery for hemoptysis at Tygerberg Hospital. There were four males and one female with acute TB with upper lobe cavitation, and all five received treatment for between 2 and 3 months prior to surgery (Table 1). Diagnosis was confirmed by Ziehl-Neelsen staining, and all patients were culture positive for drug-sensitive *M. tuberculosis*. All patients were human immunodeficiency virus negative, and they all received a blood transfusion prior to the surgery. The patients had reactivation or reinfection disease, as they had all been successfully treated for tuberculosis between 3 and 15 years previously (Table 1). Directly after surgery, tissue was selectively dissected for formaldehyde fixation. All patients successfully completed their anti-TB therapy; after a minimum of 2 years, none has returned to hospital for further treatment. Informed consent was obtained from all patients, and the study was approved by the Stellenbosch University Ethical Review Committee.

Preparation of riboprobes. Peripheral blood mononuclear cells (PBMCs) were isolated from 10 ml of blood obtained from a healthy volunteer. The PBMCs were stimulated with phytohemagglutinin for 18 h prior to RNA extraction. Total RNA was extracted from the cells using the Tri-Reagent (Sigma Aldrich) and quantified by measurement of absorbance at 260 nm. The RNA was shown to be undegraded following electrophoresis of an aliquot in a 1% agarose gel containing 8% formaldehyde and visualization of the RNA by ethidium bromide staining. cDNA and PCR products for IL-4, TNF-α, IFN-γ, and β-actin were subsequently prepared from 2 μg of RNA, using the Titan (one-tube RT-PCR) system (Boehringer Mannheim, Mannheim, Germany). PCR conditions and primer sequences were as published elsewhere (8, 13). The PCR products were blunt-end cloned into the vector pGEM7Zf (Promega U.K.) which had been digested with *Sma*I (Boehringer Mannheim). The clones were then sequenced (Table 2) to confirm the DNA sequence, as well as to ascertain the orientation of the PCR product in order to synthesize sense and antisense riboprobes. After digestion with *Pvu*II, these fragments were separated by electrophoresis on 1% low-melting-point temperature agarose gels and isolated from the gel by using the Boehringer Mannheim DNA extraction protocol. T7 and SP6 RNA polymerases were used to transcribe antisense and sense digoxigenin-labeled RNA as instructed by the manufacturer (Boehringer Mannheim). Labeling of the probes was confirmed by Northern blot analysis. Filters were first incubated with antidigoxigenin alkaline phosphatase-conjugated $F(ab')_2$ fragments (Boehringer Mannheim), washed, and then incubated with BCIP-NBT-INT (5-bromo-4-chloro-3-indolylphosphate–nitroblue tetrazolium–iodonitrotetrazolium violet; Dako, Glostrup, Denmark) (4).

RNA-RNA in situ hybridization. As previously described (5), paraffin-embedded lung tissue was cut into 5-μm sections using a microtome. Consecutive sections were applied to RNase-free slides previously coated with aminopropyltriethoxysilane (5 μg/ml; Sigma Aldrich). Sections were deparaffinized in xylene, rehydrated through graded ethanols and diethyl pyrocarbonate-treated water, and finally incubated in phosphate-buffered saline (PBS). The sections were treated with 1 μg of proteinase K per ml in 10 mM Tris-HCl (pH 7.5)–5 mM EDTA for 45 min at 37°C. After being washed with PBS, the sections were refixed in 0.4% paraformaldehyde and acetylated in a 400:1 (vol/vol) solution of

TABLE 2. Sequences of cytokine riboprobes used in in situ hybridization

Riboprobe	Length (bp)	Sequence (5′–3′)
TNF-α	124	TCTCGAACCCCGAGTGACAAGCCTGTAGCCCATGT TGTAGCAAACCCTCAAGCTGAGGGGCAGCTCCAGTG GCTGAACCGCCGGGCCAATGCCCTCCTGGCCAATGGTGTGGAGCTGAGAGATA
IFN-γ	356	AGTTATATCTTGGCTTTTGAGCTCTGCATCGTTTTGGGTTCTCTTGGCTGTTACTGCCAGGACCCATATGTAC AAGAAGCAGAAAACCTTAAGAAATATTTTAATGCAGGTCATTCAGATGTAGCGGATAATGGAACTCTTTT CTTAGGCATTTTGAAGAATTGGAAAGAGGAGAGTGACAGAAAAATAATGCAGAGCCAAATTGTCTCCTT TTACTTCAAACTTTTTAAAAACTTTAAAGATGACCAGAGCATCCAAAAGAGTGTGGAGACCATCAAGGA AGACATGAATGTCAAGTTTTTCAATAGCAACAAAAAGAAACGAGATGACTTCGAAAAGCTGACTAATTAT TCGGT
IL-4	317	CTTCCCCCTCTGTTCTTCCTGCTAGCATGTGCCGGCAACTTTGTCCACGGACACAAGTGCGATATCACCTTAC AGGAGATCATCAAAACT TTGAACAGCCTCACAGAGCAGAAGACTCTGTGCACCGAGT TGACCGTAACAG ACATCTTTGCTGCCTCCAAGAACACAACTGAGAAGGAAACCTTCTGCAGGGCTGCGACTGTGCTCCGGCA GT TCTACAGCCACCATGAGAAGGACACTCGCTGCCTGGGTGCGACTGCACAGCAGT TCCACAGGCACAA GCAGCTGATCCGATTCCTGAAACGGCTCGACAGGAA

TABLE 3. Cytokine patterns of individual granulomas from five patients

Patient	Total no.	Granulomas/section (% of total)						
		IFN-γ positive	TNF-α positive	IL-4 positive	With caseous necrosis	TH2	TH1	TH0
A1	6	100	83	33	33	0	67	33
A2	7	86	71	43	29	0	43	43
A3	12	75	58	0	50	0	75	0
A4	11	82	64	0	45	0	82	0
A5	9	67	78	44	33	0	22	44

triethanolamine-acetic anhydride for 10 min. The slides were then rinsed in PBS, dehydrated in graded ethanols, and air dried before hybridization. Sections were incubated for 30 min at 50°C in a prehybridization mixture containing 25% dextran sulfate, 25 mM Tris-HCl (pH 8.0), 2.5× Denhardt's solution, 2.5 mM EDTA, 25 mM dithiothreitol, 1.25 mg of herring sperm DNA per ml, 0.06 mg of tRNA per ml, and 50% deionized formamide. Digoxigenin-labeled riboprobes (5 ng/μl) were added to a hybridization mixture containing 20% dextran sulfate, 12.5 mM Tris-HCl (pH 8.0), 2.5× Denhardt's solution, 2.5 mM EDTA, 12.5 mM dithiothreitol, 0.01 mg of herring sperm DNA per ml, 0.002 mg of tRNA per ml, and 50% deionized formamide. The sections were hybridized for 18 h at 50°C in a humidified chamber, after which they were washed twice in 2× SSC (saline sodium citrate; 1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) for 15 min each time at room temperature and then twice at 43°C with 0.1× SSC for 15 min each time. The slides were incubated for 5 min in 100 mM Tris-HCl (pH 7.5)–150 mM NaCl (buffer A), placed in buffer A containing 2% normal sheep serum, and washed in buffer A plus 0.05% Tween 20. Antidigoxigenin antibody conjugated to alkaline phosphatase (Boehringer Mannheim) was incubated with the sections for 30 min at room temperature, and the signal was detected using BCIP-NBT-INT (Dako). After the brown color had appeared (up to 60 min), the slides were counterstained with Mayer's hematoxylin (Sigma Aldrich) for 15 s, rinsed in distilled water, and mounted with Dako Faramount, after which the slides were viewed under a light microscope.

Dual labeling. To identify macrophages, we used a double-staining technique which entailed first doing the in situ hybridization (as described above) and then incubating the sections with CD68 (Dako). Directly after in situ hybridization, the slides were rinsed in distilled water for 5 min, after which nonspecific proteins were blocked with 5% milk powder in PBS in 0.5% Triton X-100 for 30 min at room temperature. The slides were then incubated with CD68 diluted 1:400 in goat serum for 1 h at room temperature. In standard immunohistochemical staining, this antibody requires a trypsin pretreatment in order to unmask the antigen. However, the proteinase K used in the in situ staining was able to substitute for this step, and no further proteolysis was required. The slides were placed in three washes of PBS, 5 min each, after which the secondary antibody (biotinylated goat anti-mouse; Dako) diluted 1:100 in 3% goat serum in 0.5% Triton X-100 was applied to the sections, and the slides were incubated at room temperature for 1 h. The sections were again washed three times for 5 min each in PBS. The sections were then incubated with streptavidin conjugated to alkaline phosphatase (Vector Laboratories, Burlingame, Calif.) for 30 min, washed in PBS, and then incubated with a solution of fast red (Vector Laboratories) for 30 min. The slides were counterstained with hematoxylin and mounted in Dako Faramount.

Photography. The images were captured using a Zeiss microscope fitted with a video camera. To maintain comparability between slides, the light parameters were optimized for the actin-stained slide and then kept constant for all subsequent slides. The images were saved using Adobe Acrobat.

Assessment of slides. Digoxigenin nonradioactive in situ hybridization is an empirical staining method and cannot be accurately quantitated. The results were therefore graded according to the brown color reaction as +++ (strong positivity), ++ (intermediate positivity), + (weak positivity), and − (no signal). Slides were assessed by three observers including a pathologist and were graded as described above. The slides for each cytokine (TNF-α, IFN-γ, and IL-4) were analyzed for each patient in triplicate.

RESULTS

Histobacteriology. The five patients presented with caseating necrosis, liquefaction, and cavitation. Ziehl-Neelsen staining revealed low levels of acid-fast bacilli in the lungs of all patients (not shown), even though all had received at least 2 months of multidrug therapy prior to surgery.

In situ hybridization. (i) General assessment of patient granulomas. Scanning of triplicate sections for each cytokine at low-power magnification allows an overall assessment of the relative staining of the tissue. Table 1 summarizes these results. Three patients (A1, A2, and A5) stained positive for IL-4 and IFN-γ. Two patients (A3 and A4) stained positive for IFN-γ and negative for IL-4 mRNA. All patients were positive for TNF-α mRNA.

(ii) Analysis of individual granulomas in all five patients. The individual granulomas for each patient were scored for the presence of cytokine mRNA and caseous necrosis (Table 3). The number of granulomas per section ranged from 6 to 12, and the majority of these stained positive for IFN-γ and TNF-α mRNA. The IL-4 staining was negative in two patients (A3 and A4) and present in three. TNF-α mRNA staining was highest in those patients with some IL-4-positive granulomas. No granulomas which stained positive for IL-4 and negative for IFN-γ were observed; in fact, IL-4 mRNA was always detected in granulomas with low levels of IFN-γ staining. Granulomas with caseous necrosis were generally larger than those without central necrosis and were either negative or only weakly positive for IFN-γ and IL-4 mRNA. It appears that the necrotic granulomas were more abundant in those patients with no evidence of IL-4 staining. In general, necrotic granulomas were negative to intermediately positive for TNF-α mRNA. Cytokine mRNA staining, where present, was generally in the periphery of the granulomas and not in or around the necrotic center (see Fig. 3c and e), despite the periphery being positive for β-actin mRNA (see Fig. 3b). In summary, the two patients with IFN-γ-positive, IL-4-negative patterns of cytokine production expressed lower levels of TNF-α mRNA and had more necrotic granulomas than the three patients whose granulomas were positive for both IFN-γ and IL-4.

(iii) Detailed comparison of different granulomas in a single patient. A more in-depth analysis of a tissue section from one patient (A5), which is representative of the three cases staining positive for all three cytokine mRNAs, is illustrated in Fig. 1 to 4. This patient had granulomas staining positive for both IFN-γ and IL-4 (44% of granulomas) as well as granulomas positive for IFN-γ and negative for IL-4 (22% of granulomas). All granulomas were negative when stained for the sense probes of all cytokines (IFN-γ sense probe is illustrated [Fig. 1a, 2a, 3a, and 4a]). However, they all stained positive for β-actin mRNA (Fig. 1b, 2b, 3b, and 4b), indicating that mRNA had not been degraded.

The first granuloma (granuloma 1 [Fig. 1]) has not yet begun to form caseous necrosis at the center and can be considered relatively newly formed. It contains significant amounts of IFN-γ mRNA (Fig. 1d), but IL-4 mRNA is hardly detectable (Fig. 1c). mRNA for the proinflammatory cytokine TNF-α (Fig. 1e) is also produced.

The second granuloma (granuloma 2 [Fig. 2]), from the same tissue section, is also relatively new and shows no evidence of necrosis. Both IL-4 and IFN-γ mRNA are observed (Fig. 2c and d, respectively), as well as TNF-α mRNA (Fig. 2e). While in situ hybridization is not quantitative, it should be possible to compare the intensity of staining for a particular cytokine mRNA between different granulomas. Furthermore, using the β-actin staining as a reference, one can also express the cytokine mRNA staining as a function of the β-actin stain-

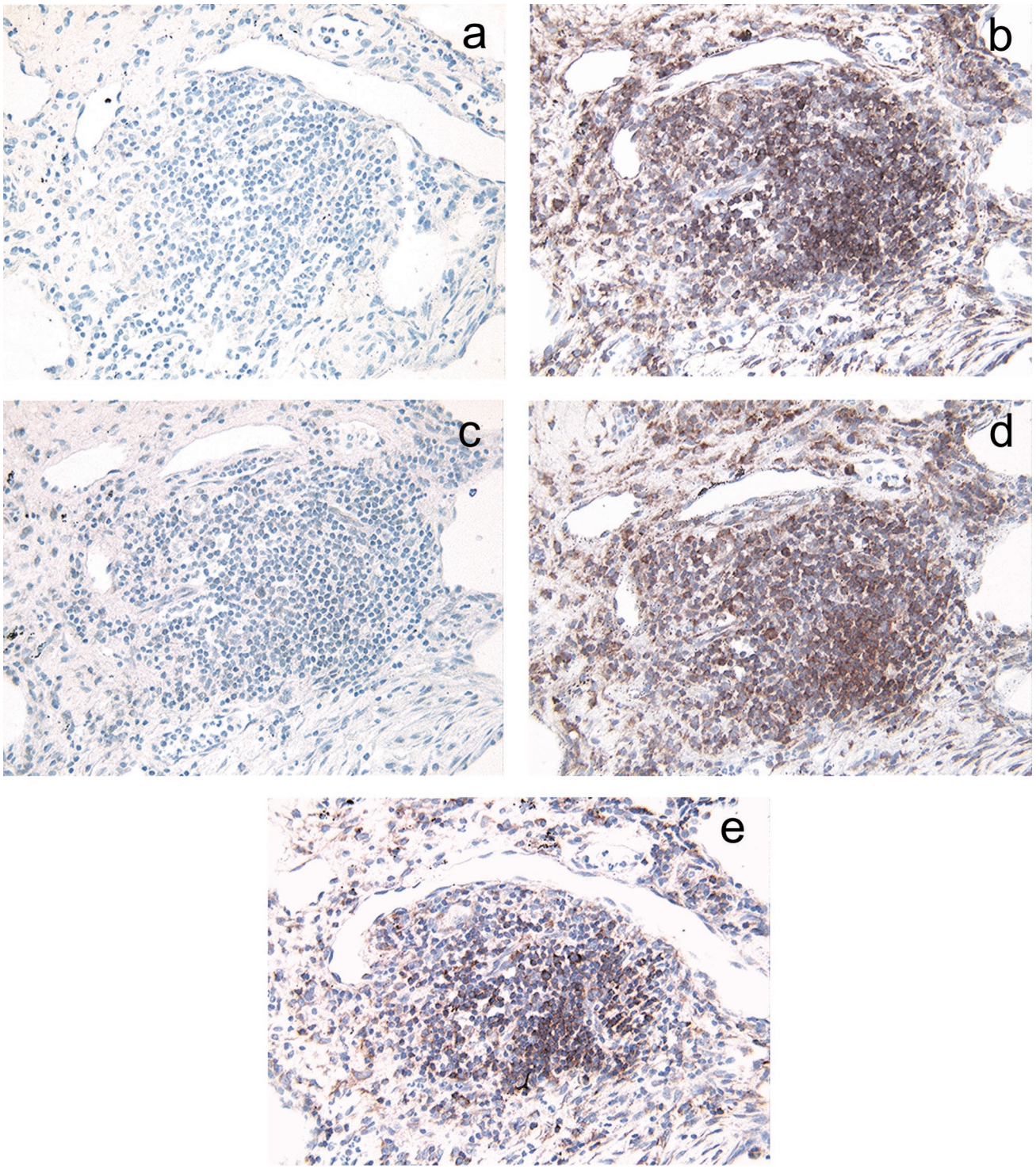

FIG. 1. In situ hybridization of sections through granuloma 1 from patient A5. This exudative granuloma shows no signs of necrosis. Nuclei stain blue, and cells staining positive for in situ hybridization have brown cytoplasm. Negative IFN-γ sense riboprobe (a) and positive β-actin riboprobe (b) sections are shown. The granuloma is slightly positive for IL-4 (c), is very positive for IFN-γ (d), and shows intermediate staining for TNF-α (e). Magnification, ×200.

ing and thereby compare different cytokine mRNAs within and between granulomas. This assumes that any mRNA degradation during the fixation process is more or less uniform for all mRNA species. Thus, the amount of IFN-γ mRNA in granuloma 2 appears less than that observed in granuloma 1 (Fig. 1d and 2d), where IL-4 mRNA was almost undetectable (Fig. 1c). This would suggest that there could be a reciprocal relationship between IL-4 and IFN-γ mRNA production. The relative amount of TNF-α mRNA in granuloma 2 (Fig. 2e) is increased compared to that observed in granuloma 1 (Fig. 1e). The

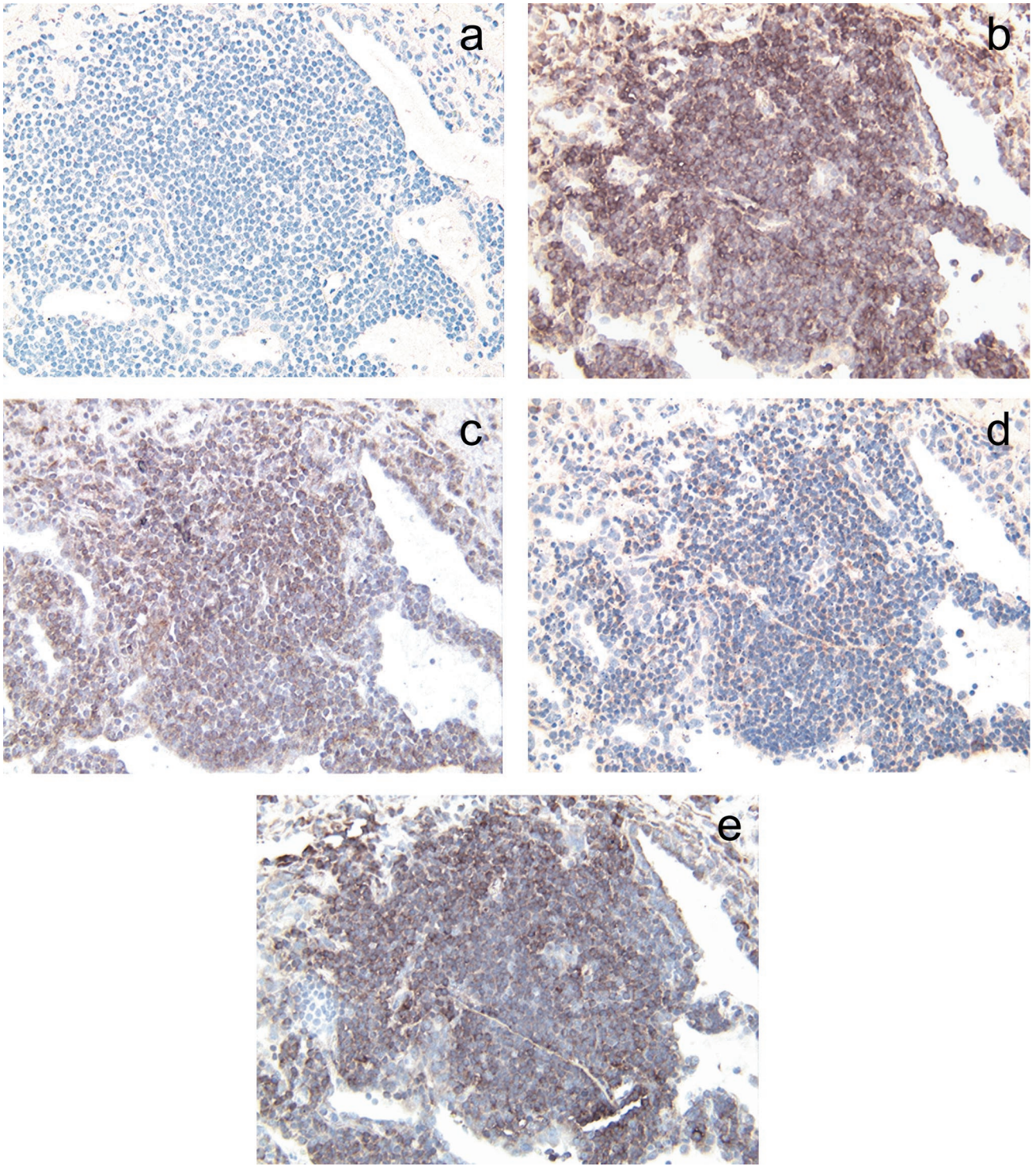

FIG. 2. In situ hybridization of sections through granuloma 2 from patient A5. This exudative granuloma shows no signs of necrosis. Nuclei stain blue, and cells staining positive for in situ hybridization have brown cytoplasm. Negative IFN-γ sense riboprobe (a) and positive β-actin riboprobe (b) sections are shown. The granuloma is positive for IL4 (c), is weakly positive for IFN-γ (d), and shows positive staining for TNF-α (e). Magnification, ×200.

cytokine pattern of granuloma 2 (Fig. 2) differs from that of granuloma 1 (Fig. 1), in that TNF-α is strongly positive, IL-4 is intermediate, and IFN-γ is weak.

The third granuloma (granuloma 3 [Fig. 3]), also in the same tissue section, has caseous necrosis at its center, probably indicating a more mature granuloma. It is positive for TNF-α mRNA (Fig. 3e) but has comparatively low levels of both IL-4 and IFN-γ (Fig. 3c and d, respectively).

(iv) Comparison of different cells within a single granuloma. It is possible to morphologically distinguish some cell types,

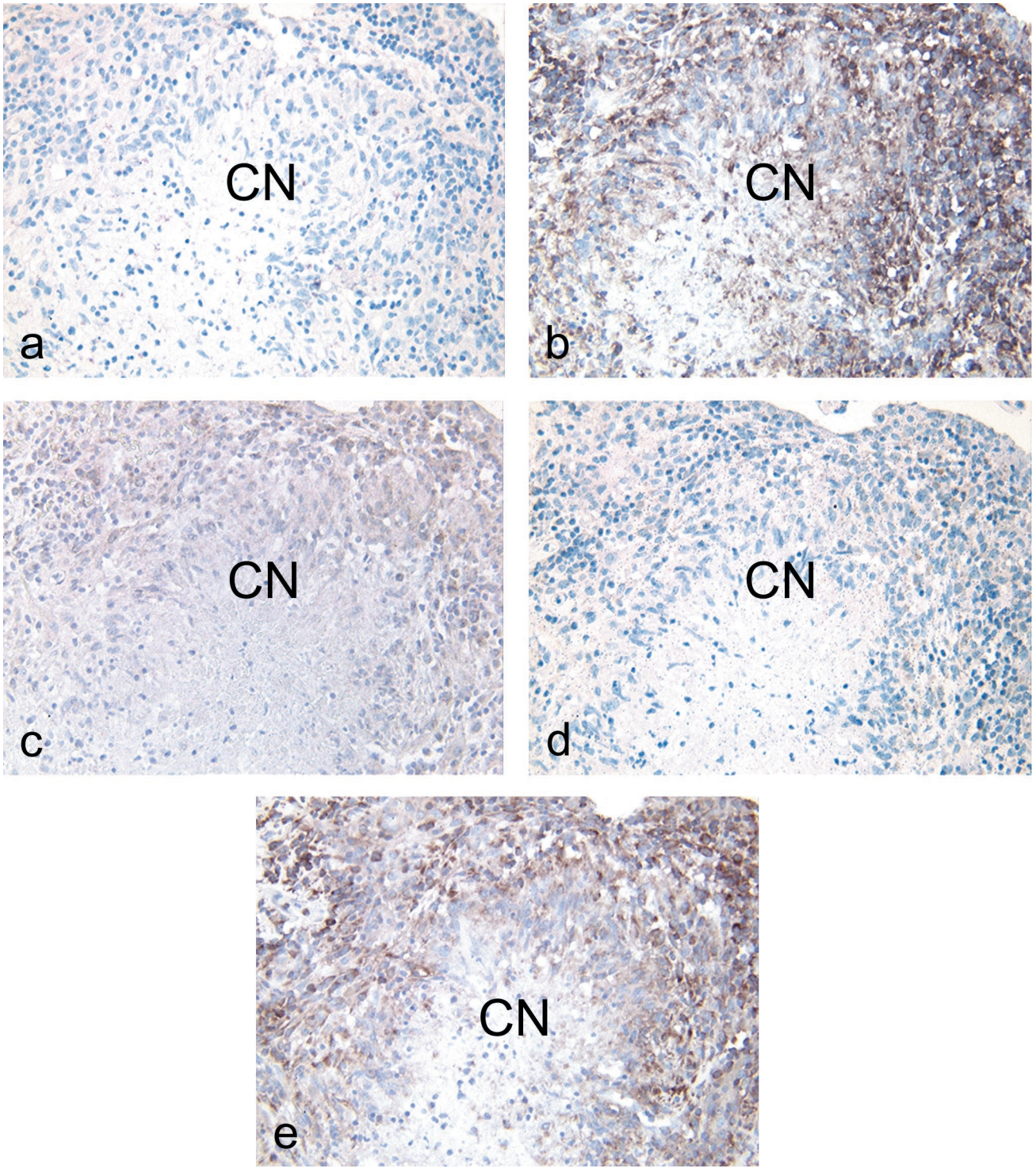

FIG. 3. In situ hybridization of sections through granuloma 3 from patient A5. The granuloma is larger than those in Fig. 1 and 2 and has caseous necrosis at its center (CN). Nuclei stain blue, and cells staining positive for in situ hybridization have brown cytoplasm. Negative IFN-γ sense riboprobe (a) and positive β-actin riboprobe (b) sections are shown. The granuloma is weakly positive for IL-4 (c) and IFN-γ (d) and shows positive staining for TNF-α (e). Magnification, ×200.

e.g., lymphocytes and macrophages, at higher magnification (Fig. 4). As expected, both lymphocytes and macrophages stained positive for TNF-α mRNA (Fig. 4e), and lymphocytes stained positive for IL-4 (Fig. 2c and 4c) and IFN-γ mRNA (Fig. 4d). However, cells with the morphology of macrophages also stained positive for IL-4 (Fig. 4c) and IFN-γ (Fig. 4d) mRNA. It was not possible to ascertain if the same lymphocyte or macrophage is making mRNA for a selection of cytokines or for only one cytokine at a time.

To confirm that macrophages were indeed producing

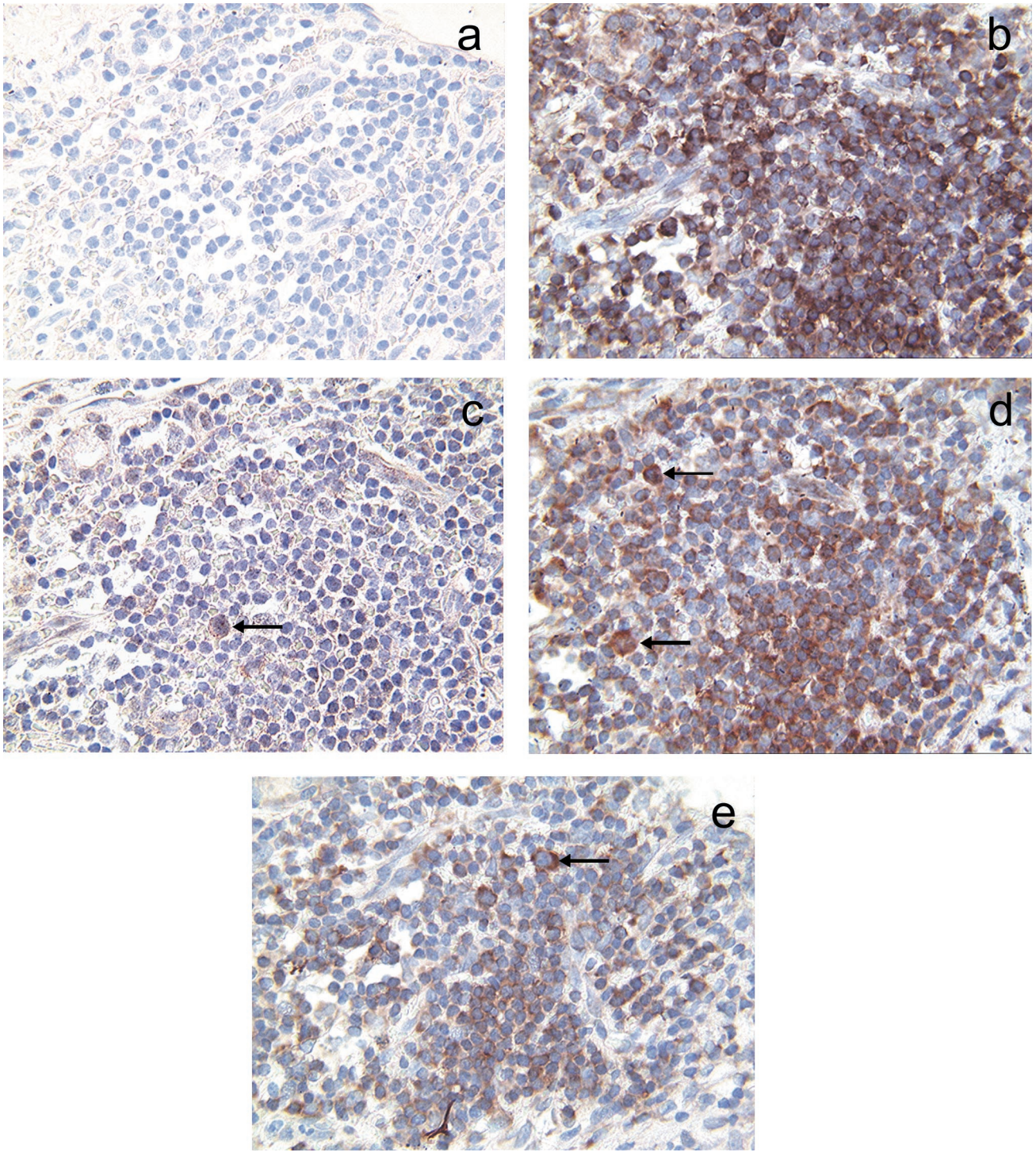

FIG. 4. In situ hybridization of sections through granuloma 1 at higher power. Nuclei stain blue, and cells staining positive for in situ hybridization have brown cytoplasm. Negative IFN-γ sense riboprobe (a) and positive β-actin riboprobe (b) sections are shown. Cells staining positive for IL-4 mRNA are visible (c). A cell with the morphology of a macrophage is stained for IL-4 mRNA (arrow). Other cells with less cytoplasm also stain positive, and these are likely to be lymphocytes. Similarly for IFN-γ (d), several cells with a large cytoplasm stain positive (arrows), as do cells with less cytoplasm. Cells with the morphology of both lymphocytes and macrophages (arrow) stain positive for TNF-α mRNA (e). Magnification, ×400.

mRNA for IFN-γ and IL-4, we performed a dual-labeling procedure, combining in situ hybridization with immunohistochemical staining of the myeloid-specific marker CD68 (Fig. 5). Cells staining positive for CD68 alone are pink (Fig. 5a), while those staining positive for IFN-γ and IL-4 alone are brown (Fig. 4c and d). However, colocalization of CD68 and IL-4 results in a dark red color (Fig. 5b). Similarly for IFN-γ, colocalization of CD68 and IFN-γ results in a dark red color

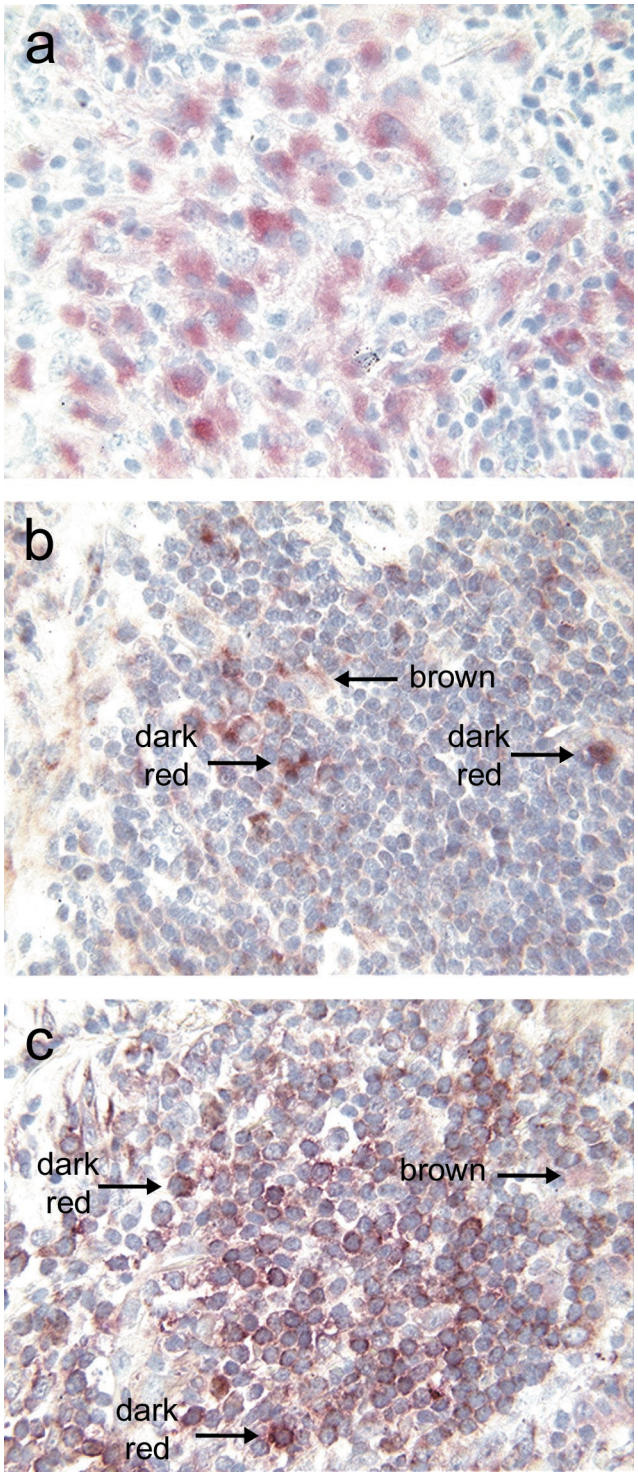

FIG. 5. Dual labeling. Immunohistochemical staining of CD68 shows pink cells which are macrophages (a). Colocalization of CD68 and IL-4 mRNA results in dark red staining, and CD68-negative cells producing IL-4 mRNA are brown (b). Similarly, colocalization of CD68 and IFN-γ mRNA results in a dark red staining, and IFN-γ staining alone is brown (c). Magnification, ×400.

(Fig. 5c). Thus, our results suggest that within human tuberculous granulomas, myeloid cells (which are probably macrophages) appear to produce mRNA for the cytokines IFN-γ and IL-4. Dual labeling for TNF-α was not performed.

DISCUSSION

These studies constitute the first observations of TNF-α, IFN-γ, and IL-4 mRNA production within the granulomas from lungs of patients with pulmonary TB. In situ hybridization maintains the tissue morphology and allows patterns of gene expression to be localized to certain tissues and cell types. This is advantageous over RT-PCR, which gives only total cytokine mRNA level, without any information as to site of production within the tissue. Immunohistochemistry is somewhat limited by the availability of antibodies that work reliably within paraffin-embedded tissues. In situ hybridization has revealed that each granuloma within each patient is a microenvironment in which unique patterns of cytokine production are observed. Furthermore, the actual cell types involved in producing the cytokines can be identified, and this has shown that both lymphoid and myeloid cells within the granuloma are capable of producing mRNA for the traditional T-cell cytokines IFN-γ and IL-4. Thus, this study confirms previous observations that patients with TB produce large amounts of TNF-α and IFN-γ and low levels of IL-4 (11). However, it is obvious from the present study that the production of these cytokines is closely regulated within the microenvironment of the granuloma and that the IFN-γ and IL-4 are produced not solely by T cells but also by CD68-positive myeloid cells.

Two of the patients described here exhibited granulomas staining positive for TNF-α and IFN-γ but negative for IL-4 mRNA. According to the TH1/TH2 dichotomy (14), these patients would therefore be considered as mounting an appropriate TH1 response (18, 21). However, three of the patients had granulomas staining positive for all three cytokine mRNAs. These patients would be classified TH0 (or mixed TH1/TH2). However, the fact that both TH1 and TH0 granulomas were present in these patients casts doubt on this strict classification. The presence of IL-4 in patients with TB is thought to be indicative of an inappropriate immune response and therefore of a poor prognosis. The three patients with a TH0 pattern of cytokine gene expression may therefore be expected to have a poor outcome compared to the two patients with a TH1 cytokine pattern. However, there was no indication of this in the patient records. All five patients were successfully treated for TB and have remained disease free for at least 2 years postsurgery. This suggests that the presence of IL-4 may not be an indicator of poor prognosis in such patients but rather may be an integral feature of tuberculous granuloma formation with a role in controlling tissue damage.

The evolution of the granuloma in the human was studied in the preantibiotic era by Canetti (2), who investigated the histology and the bacterial load of granulomas from 30 patients postmortem. This resulted in a classification of the granuloma, which may reflect a chronology of events. The preexudative and exudative stages have a mononuclear cell infiltrate and few detectable bacilli. Caseous necrosis at the center of the granuloma follows, with progressively fewer bacilli visible. The lesion can resolve by fibrin deposition, sclerosis, and calcification, with disappearance of the acid-fast bacilli. Alternatively, in some lesions, the caseum liquefies and foci of bacilli reappear in the liquefying areas. Rupture into a bronchus results in expulsion of the contents, and the patient becomes infectious. Canetti (2) observed that all of these stages were detectable in all patients studied. This would imply that the pathology observed is due to a continuous process of infection, granuloma formation, necrosis, liquefaction, rupture, and reseeding.

The patients described in this study had advanced TB; they were undergoing surgery for hemoptysis and had radiological evidence of cavitation. Therefore, the extent of their disease is

comparable to that of the patients described by Canetti (2). We observed the exudative granuloma and the necrotic stage in our patients, and we can assume that liquefaction and rupture of the lesions is also taking place. Individual exudative granulomas within the same patient exhibited unique patterns of cytokine production. Necrotic granulomas stained only weakly for IFN-γ and IL-4, despite remaining positive for β-actin mRNA staining. These different cytokine patterns may represent a subtlety of the evolutionary process of the granuloma described above (2). It is possible that initially a vigorous IFN-γ response is observed during the exudative stage, which results in containment of mycobacterial replication. This may subsequently be diminished, in order to avoid tissue damage due to delayed-type hypersensitivity, and is reflected by a relative increase in IL-4 and decrease in IFN-γ cytokine mRNA. By the time necrosis is established, the IFN-γ and IL-4 response has virtually abated. An alternative interpretation is that the different cytokine profiles represent isolated events, each with its own natural history and resulting in separate outcomes. IFN-γ-positive granulomas may successfully contain the infection, while IL-4 positive granulomas may go on to liquefy and cavitate. It is the sum total of these individual host-pathogen interactions which results in the pathology observed in the patient.

It has been suggested that the effect of TNF-α is influenced by the cytokine milieu of the surrounding tissue (15, 16). Introduction of TNF-α into a site of mycobacterial infection exhibiting a TH1 response is beneficial and enhances pathogen control in a mouse model of tuberculosis. However, TNF-α in a site with a TH0 or TH2 response causes a significant increase in tissue pathology (15, 16). In contrast, all five of our patients exhibited significant pathology and produced large amounts of TNF-α against both an IL-4 positive and an IL-4 negative background. At the level of the individual granuloma, the situation may be more complicated. Those patients exhibiting the highest percentage of TNF-α-positive granulomas also have IL-4-positive granulomas and a lower percentage of granulomas with caseous necrosis. TNF-α appears to positively correlate with IL-4 gene expression and to negatively correlate with caseous necrosis.

The observation that IFN-γ mRNA is produced by CD68-positive human myeloid cells during infection by *M. tuberculosis* has many implications for the immunopathogenesis of the disease as well as for strategies for immunotherapy. Fenton et al. (6) showed that human alveolar macrophages produce IFN-γ mRNA in vitro upon infection with *M. tuberculosis* and that IFN-γ acts in an autoregulatory manner. Prior to this observation, it had been accepted that alveolar macrophages were inefficient at controlling initial infection and that only after recruitment of lymphocytes producing IFN-γ did a protective response occur (6). More recently, Wang et al. (19) have shown that macrophages from mice infected with *M. bovis* BCG produce IFN-γ in an IL-12-dependent fashion. Our finding that CD68-positive cells in the human tuberculous granuloma also produce IFN-γ suggests that the macrophage may be able to autoregulate its activation state at the site of infection and pathology in tuberculosis.

Recently, Kelleher et al. (9) illustrated that mouse dendritic cells can produce IL-4 in response to infection by Rauscher leukaemia virus (RLV). This IL-4 production by dendritic cells was abrogated by addition of IL-12 (9). Similarly, we show that CD68-positive cells within the human tuberculous granuloma produce mRNA for IL-4. It therefore appears that IL-12 may reciprocally modulate myeloid cells to produce either of the classic TH1- or TH2-associated cytokines. Furthermore, intracellular pathogens, both viral and bacterial, may be able to exploit this mechanism to their advantage.

The challenge for the future is to determine how *M. tuberculosis* is able to subvert the host immune response within the granuloma and turn the full destructive power of the cell-mediated immune response away from the pathogen and onto the host itself, resulting in liquefaction, cavitation, and further dissemination. Effective immunotherapies will have to focus on the evasion mechanisms of *M. tuberculosis* in order to swing the balance back in the host's favor and eradicate the pathogen without inflicting further damage to the host.

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