Cytokine Patterns in Tuberculous and Sarcoid Granulomas

Correlations with Histopathologic Features of the Granulomatous Response[1]

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Cytokines play an important role in granuloma formation, but the extent that cytokine profiles are similar in different granulomatous diseases and whether differences in the histopathologic features of the granulomatous response results from differences in cytokine production have not been evaluated. To investigate these questions, we used RT-PCR to quantify the expression of mRNAs coding for 16 cytokines in granulomatous lymph nodes from patients with tuberculosis and sarcoidosis and from control tissues, and we sought correlations between the level of expression of these cytokines and the histopathologic features of the granulomas. Expression of mRNAs coding for a number of cytokines (IL-1β, IFN-γ, TNF-α, granulocyte-macrophage (GM)-CSF, IL-12 (p40), and lymphotoxin-β) was increased in tuberculous and sarcoid granulomas compared with that of control tissues. All sarcoid granulomas were shown to express a Th1 pattern of cytokine mRNAs, while tuberculous lymph nodes expressed either a Th1 or a Th0 profile. GM-CSF and lymphotoxin-β mRNAs were more abundant in sarcoid than in tuberculous granulomas, whereas IL-8 mRNA was strongly expressed only in tuberculous lymph nodes. Strong expression of GM-CSF, TNF-α, and IL-8 by granulomas was shown to be correlated, respectively, with the presence of florid granulomatous lesions, the absence of central necrosis, and the presence of neutrophil infiltration. These results demonstrate that the formation of tuberculous and sarcoid granulomas in humans is associated with the expression of characteristic cytokine profiles and indicate that the expression of certain cytokines is associated with the development of specific pathologic features in the resulting granulomas. *The Journal of Immunology,* **1997, 159: 3034–3043.**

Granuloma formation, a critical event in the immune response against a variety of intracellular pathogens, is an essential component of normal host defense (1, 2). The formation of granulomas can also contribute to the destruction of normal tissues and thereby participate in the pathogenesis of diseases such as sarcoidosis, a multisystem disorder of unknown etiology, resulting from the uncontrolled development of noncaseating granulomas at the sites of involvement (3–5).

Granulomas are dynamic structures composed of multiple cell types, and all immune granulomas share in common the presence of a central accumulation of mononuclear cells that is surrounded by and infiltrated with activated T lymphocytes (5). Nevertheless, both the extent of granuloma formation and the morphologic features of the resulting granulomas are subject to considerable variability in different individuals, and this variability may be associated with differences in the ability of the reaction to eliminate intracellular pathogens and/or contribute to tissue pathology. For example, a wide spectrum of granulomatous reactions can be seen in patients with tuberculosis (2, 6). At one extreme are immunocompromised individuals who develop a weak granulomatous response in which extensive tissue necrosis and large numbers of mycobacteria are present. At the other end of the spectrum are patients with indolent paucibacillary forms of tuberculosis characterized by the presence of florid noncaseating granulomatous reactions containing few detectable organisms, lesions that are morphologically similar to those seen in sarcoidosis (3, 6). Most tuberculosis patients fall between these two extremes, and both caseating granulomas and mycobacteria are observed at the site of infection, often in inverse proportions (2, 6).

The factors controlling the formation of granulomas are only partially defined, but studies using a variety of animal models indicate that cytokines play a prominent role (1, 7–11). Interestingly, however, considerable plasticity has been observed in the types of cytokines that support granuloma formation. Thus, in response to *Mycobacterium tuberculosis* and *Mycobacterium leprae* infections, the production of Th1 cytokines (IL-2, IFN-γ, TNF-β) has been found to be predominant, whereas production of Th2 cytokines (IL-4, IL-5, IL-10) in this setting is associated with impaired granuloma formation and reduced resistance (12–16). In contrast, granulomatous responses to schistosome egg Ags are associated with the abundant production of Th2 cytokines, and inhibition of IL-4 can essentially block the granulomatous response (9, 17, 18). Similarly, depending on the model studied, the inflammatory cytokine TNF-α has been suggested to be essential for granuloma formation (7, 8), participate only at certain stages (19), or have little impact on the process (11).

Less information is available concerning the cytokines participating in granuloma formation in the course of human diseases. A variety of mediators have been shown to be released in vitro and in vivo by immune and inflammatory cells from patients with granulomatous lung diseases such as tuberculosis and sarcoidosis (4, 5, 10, 20–25), although the profile of cytokines produced by the granulomatous lesions has not been evaluated extensively (26–28).

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In this context, we hypothesized that the profile of cytokines produced in different granulomatous diseases would be distinct and that the variability in granulomatous responses seen in different individuals, including differences in such features as the extent of necrosis and the intensity of the granulomatous response, could be explained by quantitative or qualitative differences in the types of cytokines produced. To test this idea, we have used reverse transcription (RT)-PCR[3] to evaluate the expression of mRNAs coding for 16 cytokines in granulomatous lesions from patients with tuberculosis and sarcoidosis and compared the level of expression with that observed in control tissue to identify the cytokines intensely expressed in the course of these different granulomatous reactions. In addition, we sought evidence for a relationship between the level of expression of these cytokines (as evaluated both by RT-PCR and by immunohistochemistry) and the histopathologic features of the granulomatous response, which were quantified using image analysis techniques.

Methods

Tissue specimens

Patients with tuberculosis. Lymph nodes (cervical (n = 6), supraclavicular (n = 1), mediastinal (n = 1)), were obtained at the time of surgical biopsy from eight patients with tuberculosis. All of these patients (five men and three women, mean age 46 ± 19 yr) had a positive tuberculin skin test, and none was seropositive for HIV. The diagnosis of tuberculosis was based on the isolation of *M. tuberculosis* from a clinical specimen and/or the presence of typical granulomas with central caseous necrosis in tissue obtained by biopsy. No patient was receiving antimycobacterial therapy at the time of the biopsy.

Patients with sarcoidosis. Lymph nodes (supraclavicular (n = 4), cervical (n = 1), mediastinal (n = 1)) were obtained from six patients with sarcoidosis (one man and five women, mean age 41 ± 14 yr) as a part of their diagnostic evaluation. The diagnosis was based on previously described criteria (29). None of the patients was receiving corticosteroids at the time of evaluation.

Control subjects. Grossly normal mediastinal lymph nodes obtained from five patients (five men, mean age 59 ± 9 yr) at the time of thoracic surgery for localized primary lung carcinoma (n = 4) or chronic organizing pneumonia (n = 1) were used as control specimens. These lymph nodes, taken at a site distant from the pathologic process, had normal architecture in all cases, although histologic examination showed mild to moderate follicular hyperplasia. No tumor cells were observed in the samples obtained from patients with localized lung carcinoma.

Processing of biopsies and morphologic assessment

Lymph node biopsies were divided into two fragments. One fragment was fixed in Bouin-Hollande solution, processed by routine techniques, and stained with hematoxylin and eosin. The other fragment was immediately frozen and stored in liquid N_2 until use for isolation of RNA or immunohistochemical evaluation. The histopathologic features of the frozen specimens were also evaluated using cryostat sections stained with hematoxylin and eosin. In all cases findings were similar to those observed on routinely processed material, indicating that tissues used for evaluation of cytokine mRNA profiles, immunohistochemical staining, and assessment of histologic features were comparable.

Histopathologic features of tuberculous and sarcoid granulomas were quantified as follows using a Biocom 200 (Les Ulis, France) image analysis system. To quantify the extent that lymph nodes were involved by granulomatous lesions, the entire surface of each section (70 ± 47 mm^2) was analyzed. Contours corresponding to granulomas were traced, and the percentage of the total surface occupied by granulomas was determined. Granulomas could be divided into two distinct groups according to the intensity of the granulomatous response: specimens with florid granulomas, in which 75 to 95% of the total surface was occupied by granulomas (86 ± 8%, six sarcoid and five tuberculous lesions); and biopsies containing only focal granulomas (23 ± 3% of the surface occupied by granulomas, three tuberculous lesions). The surface of the granulomatous lesions occupied by caseous necrosis was determined by analogous techniques. Tuberculous granulomas could be separated into those containing limited central necrosis (n = 4, 3 ± 1% necrosis) and samples demonstrating extensive caseous necrosis (n = 4, 71 ± 9% necrotic tissue). The extent of neutrophil infiltration in tuberculous lesions was graded as: 0, absent; +, rare or occasional neutrophils; ++, extensive infiltration. Each specimen was evaluated independently by two different observers; complete agreement in scoring was obtained for all samples.

Isolation of RNA and cDNA synthesis

Total RNA from each sample was isolated using RNAzol (Bioprobe Systems, Montreuil-sous-Bois, France) (30) and quantified by measurement of absorbance at 260 nm. All samples were demonstrated to contain undegraded RNA as assessed by electrophoresis of an aliquot of each preparation into 1% agarose gels and visualization of the products following ethidium bromide staining. To synthesize cDNA, 3 μg of total RNA were incubated in the presence of Moloney murine leukemia virus reverse transcriptase (Life Technologies, Inc., Gaithersburg, MD) and 1.25 μM random hexanucleotide primers (Pharmacia Biotech, Uppsala, Sweden) for 1 h at 42°C using reaction conditions described by the manufacturer (Life Technologies), and samples were stored at −80°C.

Quantification of cDNA

To permit comparisons of cytokine mRNAs in different samples by RT-PCR, it was important that an equivalent amount of cDNA from each sample was used. Therefore, the amount of cDNA corresponding to the abundant ribosomal protein S14 was quantified in each sample, and subsequent comparisons of cytokine cDNAs were made using aliquots of the different samples that contained equivalent amounts of S14 cDNA. S14 cDNA was quantified by competitive PCR as described, using an internal standard that differed from the the target sequence due to the presence of a 4-bp deletion (31). The relative amounts of amplification products corresponding to the sample and the internal standard were quantified as previously described using an Automated Laser Fluorescent DNA Sequencer (ALF, Pharmacia) (32).

To evaluate the reproducibility of this method, cDNA from 5 samples was quantified on 3 or more occasions spanning a 3-month interval. The coefficient of variation (SD/mean × 100) observed in these studies was <30% in all cases. To test the accuracy of the method, aliquots containing equivalent amounts of cDNA as determined by this approach from all 19 samples evaluated in these studies were used in parallel reactions to amplify cDNA coding for the S14 ribosomal protein. Amplification products were sampled throughout the amplification reaction as described below, electrophoresed into agarose gels, and visualized by ethidium bromide staining. As expected for samples containing similar amounts of cDNA, bands of equal intensity were obtained for each specimen, independent of whether the products were obtained during the exponential or plateau phases of the amplification reaction (data not shown). Similar results were obtained when equivalent amounts of cDNA from these samples were used to amplify a fragment of β actin cDNA (data not shown).

Quantification of cytokine cDNAs

For amplification of cytokine cDNAs, reaction mixtures (final volume 100 μl) contained 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.75 mM $MgCl_2$, 200 μM concentrations of each deoxynucleotide triphosphate, 10 pmol of each oligonucleotide primer (Table I), 2.5 U of *Taq* Polymerase (Life Technologies), and cDNA. For each cytokine, all samples (n = 19) were amplified during the same PCR, and an equivalent amount of cDNA, quantified as described above, was used in each reaction. Samples were heated to 80°C before adding *Taq* polymerase to reduce the formation of nonspecific amplification products. Cycling parameters were as follows: denaturation, 95°C for 1 min; annealing, 60°C (for IL-4, IL-5, and IL-6) or 55°C (all other cytokines) for 1 min; extension, 72°C for 1 min. On four occasions during the amplificiation reaction, the thermocycler (Perkin-Elmer, Norwalk, CT) was maintained at 72°C for the time necessary to remove a 20-μl aliquot from each reaction mixture, before resuming the amplification reaction. For each cytokine, preliminary experiments were performed to define the optimal numbers of cycles for this sampling such that aliquots would be obtained during the exponential phase of the PCR reaction. In most cases, sampling was performed at 25, 30, 35, and 40 cycles. In all

[3] Abbreviations used in this paper: RT-PCR, reverse transcription-polymerase chain reaction; LT-β, lymphotoxin-β; GM-CSF, granulocyte-macrophage colony-stimulating factor; RA, receptor antagonist.

Table I. *Sequence of primer pairs used for amplification of cytokine cDNAs*

IL-1β	5′ GCT GAT GGC CCT AAA CAG ATG A	5′ TTG CTG TAG TGG TGG TCG GAG AT
IL-1RA	5′ AAG ATG TGC CTG TCC TGT GTC AA	5′ TGT TCT CGC TCA GGT CAG TGA TG
IL-2	5′ CAG GAT GCT CAC ATT TAA GTT TTA CA	5′ CTC GAG AGG TTT GAG TTC TTC TTC TA
IL-4	5′ CTG CTT CCC CCT CTG TTC TTC	5′ CTG CTT GTG CCT GTG GAA CT
IL-5	5′ TCG AAC TCT GCT GAT AGC CAA TGA	5′ TCC AGT GTG CCT ATT CCC TGA AA
IL-6	5′ CAG CCA CTC ACC TCT TCA GAA C	5′ GCC TCT TTG CTG CTT TCA CA
IL-8	5′ GAC CAC ACT GCG CCA ACA CA	5′ TCT CAG CCC TCT TCA AAA ACT TCT
IL-10	5′ TGA TGC CCC AAG CTG AGA ACC	5′ TAG AGT CGC CAC CCT GAT GTC
IL-12 (p35)	5′ CTT CAC CAC TCC CAA AAC CTG	5′ CCA GGC AAC TCC CAT TAG TTA TG
IL-12 (p40)	5′ GCT TCT TCA TCA GGG ACA TCA	5′ GCT GAG GTC TTG TCG GTG AA
IL-13	5′ CAA CAT CAC CCA GAA CCA GAA G	5′ CAG AAT CCG CTC AGC ATC CTC
GM-CSF	5′ GGC GTC TCC TGA ACC TGA GTA G	5′ GTC GGC TCC TGG AGG TCA AA
TGF-β	5′ AGT TGT GCG GCA GTG GTT GA	5′ GAA CCC GTT GAT GTC CAC TTG
TNF-α	5′ TTG TTC CTC AGC CTC TTC TCC T	5′ GAG GGC TGA TTA GAG AGA GGT CC
TNF-β	5′ CCT CAA ACC TGC TGC TCA CC	5′ GAG AGA ATT GTT GCT CAA GGA GAA A
IFN-γ	5′ CGA GAT GAC TTC GAA AAG CTG ACT	5′ CCT TTT TCG CTT CCC TGT TTT A
LT-β	5′ GAT CAG GGA GGA CTG GTA ACG	5′ TCC TCT GGC AGC TTC TGA AA
S14	5′ ATC AAA CTC CGG GCC ACA GGA	5′ GTG CTG TCA GAG GGG ATG GGG
CD3δ	5′ GGA GTC TTC TGC TTT GCT GGA CA	5′ CGA TCT CGG AGG GGC TGA TAG

Table II. *Sequences of probes used to identify cytokine cDNA amplification products*

IL-1β	5′ ACA GAT GAA GTG CTC CTT CC
IL-1RA	5′ TGT CAA GTC TGG TGA TGA GA
IL-2	5′ TGA AAC ATC TTC AGT GTC TAG A
IL-4	5′ CAA GAA CAC AAC TGA GAA GG
IL-5	5′ TCC TGT TCC TGT ACA TAA AAA T
IL-6	5′ AAG GAG ACA TGT AAC AAG AGT
IL-8	5′ GAT GGA AGA GAG CTC TG T CTG
IL-10	5′ TGG ACA ACT TGT TGT TAA AGG
IL-12 (p35)	5′ TAT CAC AAA AGA TAA AAC CAG
IL-12 (p40)	5′ TAC TCC ACA TTC CTA CTT CT
IL-13	5′ GTA TGG AGC ATC AAC CTG AC
TNF-α	5′ TGC TGC ACT TTG GAG TGA T
TNF-β	5′ AGA ACT CAC TGC TCT GGA G
GM-CSF	5′ GCT GAG ATG AAT GAA ACA GT
IFN-γ	5′ AAC TGA CTT GAA TGT CCA ACG
TGF-β	5′ GAC AGC AGG GAT AAC ACA CT
LT-β	5′ CAG CAA GGA CTG GGG TTT C
CD3δ	5′ ACA CAA GCT CTG TTG AGG AAT

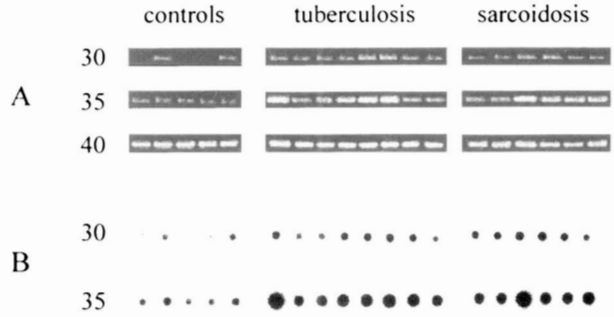

FIGURE 1. Quantification of cytokine cDNA. An equivalent amount of cDNA from each sample was amplified using specific primers for IL-1β. The reaction was interrupted briefly after 30, 35, and 40 cycles, to permit sampling of the reaction products. *A*, Amplification products obtained after the indicated number of cycles were electrophoresed into agarose gels, stained with ethidium bromide, and visualized by illumination with UV light. During the exponential phase of the reaction (30 and 35 cycles), differences in the intensity of the bands are seen in the different samples but are no longer seen when the amplification reaches the plateau phase (40 cycles). *B*, The same amplification products were bound to nylon membranes in a dot blot format and hybridized with a radiolabeled probe specific for IL-1β. The amount of radioactivity bound was quantified by electronic autoradiography.

experiments, negative controls (buffer without cDNA or samples of mRNA that had processed as for the reverse transcriptase reaction, but incubated in the absence of enzyme) were included and always gave negative results. Amplification products were analyzed by electrophoresis into 2% agarose gels and visualized by ethidium bromide staining.

To quantify the expression of a given cytokine in the different samples, 10-μl aliquots of the amplification products obtained at two successive times during the exponential phase of the PCR reaction were added to 190 μl of 0.4 M NaOH containing 25 mM EDTA, heated at 95°C for 2 min, and applied to Hybond-N nylon membranes (Amersham Corp., Arlington Heights, IL) in a dot blot format. For each cytokine, an oligonucleotide probe complementary to a sequence within the amplified fragment (Table II) was prepared by labeling the 5′ end with [γ-^{32}P]ATP (3000 Ci/mmol, Amersham) using T4 polynucleotide kinase (Life Technologies). Dot blots were hybridized with the corresponding probe overnight at 50°C (150×10^3 cpm/ml) and washed as previously described (33). The radioactivity bound was determined using electronic autoradiography (Instant Imager, Packard, Meriden, CT), and results are expressed as cpm. Comparison of the results obtained for the two samples taken during the exponential phase of the amplification reaction showed them to be concordant in all cases. A representative experiment is illustrated in Figure 1. Quantification by this approach required that under the conditions used, the probe bound only to the specific amplification product. To demonstrate that this was the case, aliquots of the amplification products were electrophoresed into 2% agarose gels, transferred to Hybond-N nylon membanes, and hybridized with the labeled probe as described for dot blots. In all cases, a single band of the appropriate m.w. was observed (data not shown).

Quantification of cDNA coding for the CD3δ chain

To evaluate the number of T lymphocytes infiltrating the granulomatous and control lymph nodes, equivalent amounts of cDNA (as determined by quantification of S14 cDNA) from the different biopsies were amplified using a pair of primers recognizing the cDNA for the δ chain of CD3. PCR and quantification of the amplification products were performed as described above for cytokine cDNAs using the oligonucleotide primers and probes shown in Tables I and II.

Immunohistochemical techniques

mAbs recognizing GM-CSF (Genzyme Diagnostics, Cambridge, MA), IL-8 (Genzyme), and TNF-α (Serotec Ltd, Kidlington, U.K.) were used to detect the expression of cytokines in tissue sections using previously described techniques (34). Briefly, 4-μm frozen sections were reacted with appropriate dilutions of Abs and washed, and positive cells were revealed by sequential reaction with alkaline phosphatase anti-alkaline phosphatase Ab complexes and the fast red substrate (APAAP kit system; Dakopatts, Glostrup, Denmark). No positive cells were identified when primary Abs were replaced by isotype-matched control mAbs.

Statistical methods

Unless otherwise stated, all results are presented as mean ± SD. For measurement of cytokine cDNAs, all steps in the procedure (amplification,

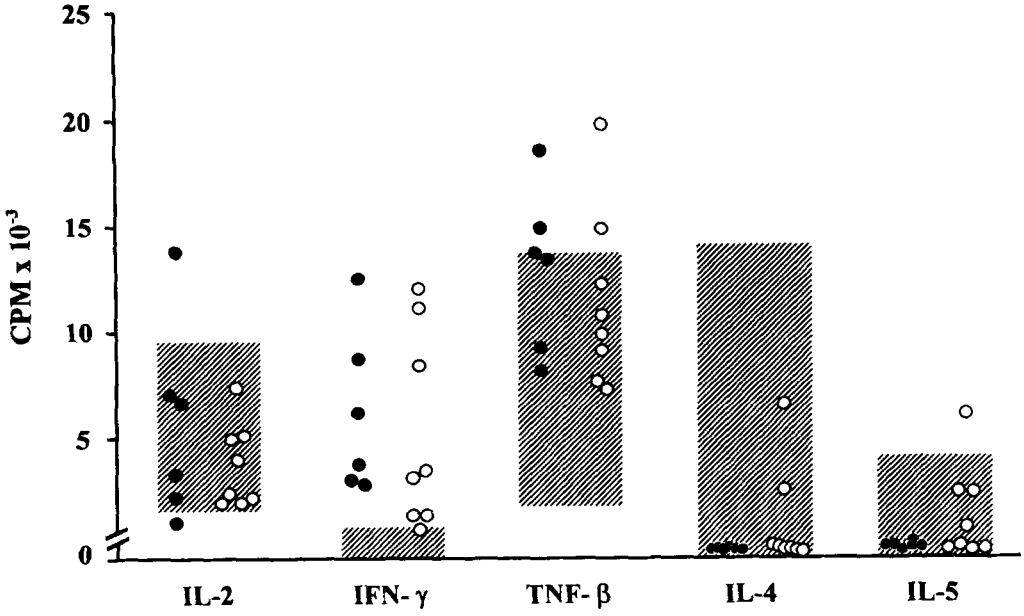

FIGURE 2. *T* helper pattern of cytokine mRNAs in lymph nodes from patients with granulomatous diseases and control subjects. Results of dot blots for the indicated cytokines, expressed in cpm, are shown for patients with sarcoidosis (solid symbols) and tuberculosis (open symbols). The shaded area represents the range of values observed for controls.

preparation of dot blots, hybridization, and autoradiography) were performed in parallel for all 19 samples. For each cytokine, the binding of radiolabeled probe to dot blots (cpm) was compared between the three groups of patients using one-way analysis of variance. $p < 0.05$ was considered significant.

Results

T helper profile of lymphocytes in granulomatous and control lymph nodes

To evaluate the T helper profile of lymphocytes present in tuberculous and sarcoid lesions, the expression of cytokine mRNAs useful in defining Th1 (IL-2, IFN-γ, TNF-β) and Th2 (IL-4, IL-5) lymphokine patterns were evaluated. Examination of PCR products on agarose gels indicated that mRNAs coding for IL-2, IFN-γ, and TNF-β were constantly expressed in both sarcoid and tuberculous granulomas. Quantification of expression by dot blotting, however, revealed that IL-2 mRNA was not present in greater amounts in granulomatous tissues than in control lymph nodes (controls 5,392 ± 3,572 cpm; tuberculosis 3,736 ± 1,966 cpm; sarcoidosis 5,663 ± 4,647 cpm, $p > 0.2$). The failure to detect increased amounts of IL-2 mRNA in granulomatous specimens could not be attributed to a lower density of T cells in these biopsies compared with that of normal lymph nodes, because no significant difference in IL-2 mRNA expression was observed comparing the three groups when the results were expressed relative to the amount of mRNA for CD3δ, a mRNA expressed specifically by T cells ($p > 0.2$, data not shown). In contrast, the expression of mRNA coding for IFN-γ was considerably greater in both tuberculous and sarcoid granulomas than in control lymph nodes, which expressed very low levels of this cytokine mRNA (Fig. 2) (controls 301 ± 273 cpm; tuberculosis 5,206 ± 4,652 cpm; sarcoidosis 6,139 ± 3,838 cpm, $p < 0.01$). The level of expression of TNF-β mRNA in some of the biopsies containing granulomas was greater than that observed in normal lymph nodes (Fig. 2), but when all samples were considered, the expression of TNF-β mRNA was not significantly different comparing granulomatous lesions and control tissue (controls 7,861 ± 4,462 cpm; tuberculosis 11,420 ± 4,165 cpm; sarcoidosis 12,980 ± 3,819 cpm, $p = 0.06$).

Unlike Th1 cytokine mRNAs, which could be detected in all granulomatous lymph nodes, mRNAs for Th2 cytokines were detected in only some samples. Lymph nodes from four of the eight patients with tuberculosis studied expressed detectable amounts of IL-4 and/or IL-5 mRNA (Fig. 2). Th2 cytokine mRNA was identified in tuberculous lymph nodes that were completely replaced by granulomatous lesions, suggesting that T cells within the granulomas participated in Th2 cytokine production. Production of detectable Th2 cytokine mRNAs in tuberculous granulomas, however, was not associated with a reduction of Th1 cytokine mRNAs in samples from these patients. Two of the five control lymph nodes also expressed IL-4 mRNA, one of which expressed IL-5 mRNA, and in these individuals, the level of mRNA production was comparable with that seen in tuberculosis. In contrast, expression of mRNAs for the Th2 cytokines IL-4 and IL-5 was extremely low in all sarcoid granulomas. Thus, a Th1 pattern was constantly observed in sarcoid biopsies, whereas tuberculous lymph nodes expressed either a Th1 ($n = 4$) or a Th0 profile ($n = 4$).

Expression of cytokine mRNAs in granulomatous and control lymph nodes

Among the 16 cytokines evaluated, only mRNAs coding for IFN-γ, IL-12 (p40), IL-1β, TNF-α, GM-CSF, and lymphotoxin-β (LT-β) were significantly more abundant in tuberculous and sarcoid granulomas than in control biopsies (Figs. 3 and 4*A*; $p < 0.05$ for IL12 (p40), $p < 0.01$ for all other cytokines by analysis of variance). Cytokine mRNA expression was not significantly different in lymph nodes obtained from the different anatomic sites. Expression of at least moderate levels of these cytokine mRNAs could be detected in control lymph nodes. Nevertheless, very little overlap was observed in the level of expression of these mRNAs in control and granulomatous tissues (Fig. 3). The average fold increase in expression comparing control and granulomatous lymph nodes ranged from 2-fold (IL-1β) to 19-fold (IFN-γ). The

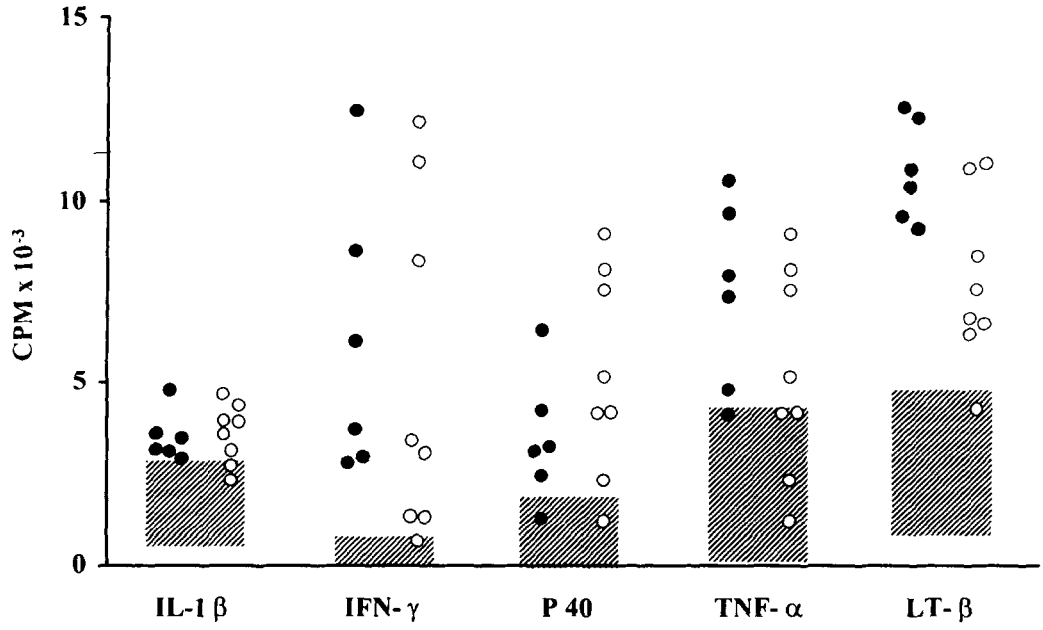

FIGURE 3. Cytokines significantly increased mRNA expression in samples from patients with sarcoidosis and tuberculosis. Results of dot blots for the indicated cytokines, expressed in cpm, are shown for patients with sarcoidosis (solid symbols) and tuberculosis (open symbols). The shaded area represents the range values observed for controls. Levels of expression of IL-1β, IFN-γ, IL-12 (p40), TNF-α, and LT-β mRNAs were significantly increased in lymph nodes from patients with granulomatous disease than in those from controls.

Table III. *Cytokine mRNAs expressed in similar amounts in lymph node biopsies from control subjects, patients with sarcoidosis and patients with tuberculosis*[a]

	Expression of Cytokine mRNAs (cpm ± SD)		
Cytokine	Controls	Sarcoidosis	Tuberculosis
IL-1RA	388 ± 543	969 ± 494	1,079 ± 900
IL-2	5,392 ± 3,572	5,663 ± 4,697	3,736 ± 1,966
IL-6	276 ± 399	2,004 ± 2,532	2,147 ± 3,228
IL-10	4,864 ± 3,531	8,144 ± 4,609	11,616 ± 6,444
IL-12 (p35)	417 ± 336	1,056 ± 473	1,162 ± 1,474
IL-13	1,562 ± 1,357	3,893 ± 6,006	4,111 ± 5,384

[a] Results of dot blots for the indicated cytokines, expressed in cpm, are shown for control subjects (n = 5), patients with sarcoidosis (n = 6), and patients with tuberculosis (n = 8).

expression of IFN-γ mRNA correlated with that of IL-12 (p40) mRNA (data not shown), but no other evidence for interdependence between the expression of these mRNAs was found.

The level of expression of TGF-β and TNF-β mRNAs also appeared to be somewhat higher in granulomas compared with that observed in control lymph nodes, but this average twofold difference did not achieve significance when the three groups were compared by analysis of variance (p = 0.06 for both cytokines). A number of cytokine mRNAs were studied the expression of which was not different in lymph nodes from tuberculosis patients, sarcoid patients, and controls. Included in this category of cytokines were IL-1RA, IL-2, IL-6, IL-10, IL-12 (p35), and IL-13 mRNAs (Table III).

Cytokine mRNAs differently expressed in tuberculous and sarcoid lymph nodes

In most cases, increased expression of a given cytokine was found to be coordinate in tuberculous and sarcoid granulomas. In addition to the differences observed in the Th cytokine profiles described above, expression of mRNAs coding for GM-CSF, LT-β, and IL-8 were quantitatively different comparing sarcoid and tuberculous granulomas. The expression of GM-CSF and LT-β mRNAs were significantly higher in sarcoid than in tuberculous granulomas (Figs. 3 and 4*A*; GM-CSF: tuberculosis 15,920 ± 8,075 cpm; sarcoidosis 23,415 ± 3,848 cpm; $p < 0.05$; LT-β: tuberculosis 7,781 ± 2,324 cpm; sarcoidosis 10,846 ± 1,353 cpm, $p < 0.01$). Conversely, increased expression of IL-8 mRNA was observed only in tuberculous lymph nodes, whereas sarcoid lesions and control tissues expressed similar amounts of this cytokine mRNA (Fig. 5*A*; tuberculosis 18,480 ± 12,251 cpm; sarcoidosis 7,263 ± 4,285 cpm; controls 6,203 ± 6,137 cpm, $p < 0.05$ comparing tuberculous and sarcoid biopsies).

Cytokine mRNA expression and histopathologic features of the granulomatous response

To seek relationships between the cytokines studied and the pathologic features of the granulomas, various histologic features of the biopsies were quantified using image analysis techniques. Comparison of these results and the level of expression of cytokine mRNAs revealed several interesting correlations.

The level of expression of GM-CSF mRNA was found to be tightly linked to the intensity of the granulomatous response. All of the lymph nodes from sarcoid patients evaluated in the present study contained histologically similar florid lesions, in which characteristic noncaseating granulomas, composed of epithelioid cells and giant cells and numerous surrounding lymphocytes, had almost entirely replaced the pre-existing normal architecture (87 ± 7% of the surface occupied by granulomas, n = 6). All these specimens expressed considerable amounts of GM-CSF mRNA (Fig. 4*A*; 23,415 ± 3,848 cpm). In contrast, biopsies from tuberculosis patients could be divided into those containing florid granulomas (87 ± 11% of the surface occupied by granulomas, n = 5) and tuberculous lymph nodes containing more focal granulomatous lesions (23 ± 3%, n = 3). Tuberculous lymph nodes with florid granulomas expressed levels of GM-CSF mRNA comparable with

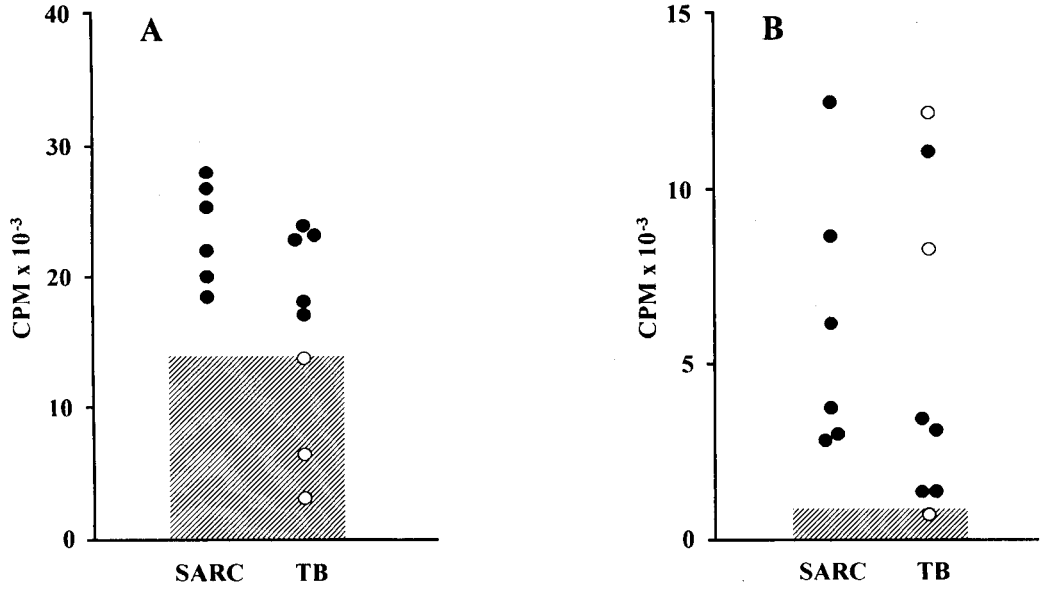

FIGURE 4. Expression of GM-CSF and IFN-γ mRNAs in tuberculous and sarcoid granulomas. Results of dot blots for GM-CSF (*A*) and IFN-γ (*B*) mRNA, expressed in cpm, are shown for patients with sarcoidosis (SARC) and tuberculosis (TB). Biopsies from patients with tuberculosis that contained florid granulomatous lesions (solid circles) and biopsies containing only focal granulomatous lesions (open circles) as determined by quantitative image analysis techniques are indicated. The shaded areas represent mean ± 2 SD of results obtained for control lymph nodes.

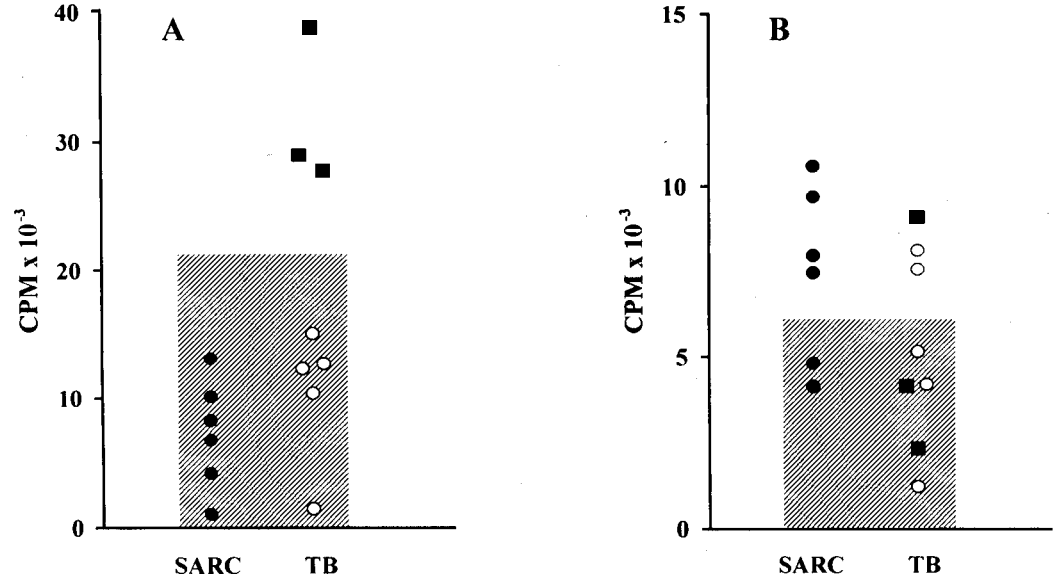

FIGURE 5. Comparison of the expression of IL-8 and TNF-α mRNAs and the presence of neutrophils in the granulomatous lymph nodes. Results of dot blots for IL-8 (*A*) and TNF-α (*B*) mRNAs, expressed in cpm, are shown for patients with sarcoidosis (SARC) and tuberculosis (TB). Biopsies from patients with tuberculosis that contained extensive (2+) neutrophil infiltration (solid squares) and little or no (0–1+) neutrophil infiltration (open circles) are indicated. The shaded areas represent mean ± 2 SD of results obtained for control lymph nodes.

those seen in sarcoid granulomas. Focal tuberculous lesions contained significantly lower levels of GM-CSF mRNA (Fig. 4*A*; florid granulomas 21,019 ± 3,174 cpm; focal granulomas 7,420 ± 5,891 cpm, $p < 0.01$), levels that were not different from that of control tissues (5,163 ± 4,332 cpm). Immunohistochemical studies confirmed that GM-CSF protein was present in considerable amounts in both epithelioid cells and the lymphocytes surrounding and infiltrating the lesions (Fig. 6*A*). The difference in GM-CSF expression in lesions containing florid and localized granulomas could not be explained merely by differences in the "intensity" of the immune reaction, because the level of expression of the other cytokines found to be elevated in these granulomatous lesions (e.g., IFN-γ, Fig. 4*B*) was not significantly different in the two groups.

The expression of IL-8 mRNA was correlated with the extent of neutrophil infiltration. Sarcoid granulomas, in which neutrophils were rarely observed or absent, expressed amounts of IL-8 mRNA that were not significantly different from that of control tissues (Fig. 5*A*; sarcoidosis 7,263 ± 4,285 cpm; controls 6,263 ± 6,137 cpm, $p > 0.2$). Similarly, the levels of IL-8 mRNA observed for tuberculous granulomas containing little or no neutrophil infiltration (graded 0 or 1+) fell within the range of values seen in normal lymph nodes (Fig. 5*A*, $p > 0.2$ compared with controls). In contrast, the three tuberculous granulomas that contained numerous

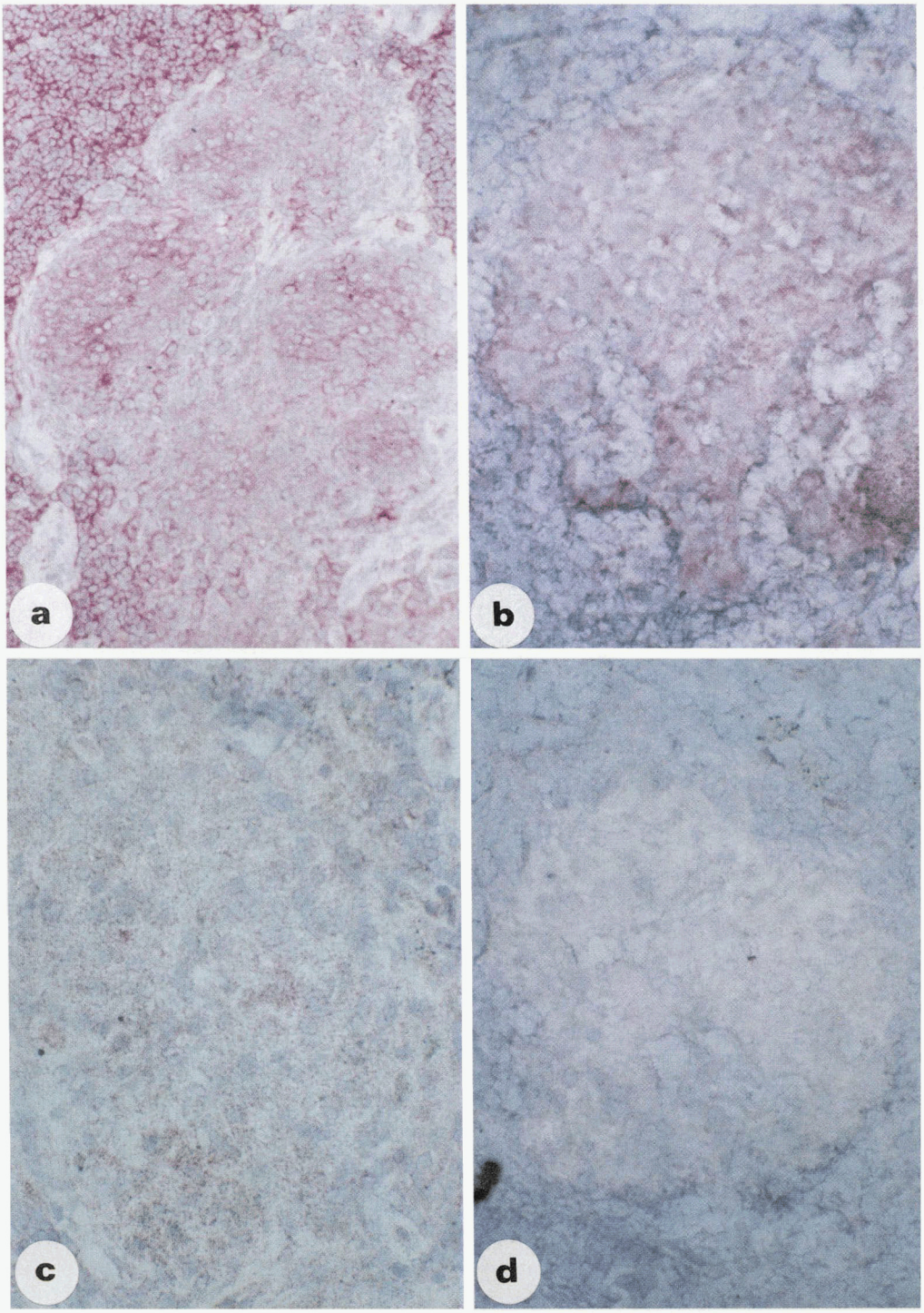

FIGURE 6. Evaluation of cytokine expression in granulomas using immunohistochemical techniques. *a*, Immunostaining for GM-CSF in a lymph node containing florid granulomas. Both epithelioid cells and the lymphocytes surrounding and infiltrating the lesions are strongly stained (original magnification ×125). *b*, Immunostaining for IL-8 in a tuberculous granuloma heavily infiltrated by neutrophils; an intense positive reaction is present. *c*, The intensity of the reaction with the anti-IL-8 Ab is clearly weaker in a tuberculous granuloma containing few neutrophils. *d*, No immunostaining was observed when a section from the same biopsy shown in *b* was reacted with an isotype-matched control Ab (*b–d*, original magnification ×250).

neutrophils (graded 2+) expressed considerably higher amounts of IL-8 mRNA than granulomas containing few neutrophils or control lymph nodes (31,915 ± 6,048 cpm, $p < 0.01$ for all comparisons). Granulomas heavily infiltrated by neutrophils were also shown to strongly express IL-8 protein by immunohistochemical techniques, whereas granulomas with little or no neutrophil infiltration expressed only low levels of this cytokine (Fig. 6, *b–d*). Although TNF-α also expresses neutrophil chemotactic activity in vitro (35), no correlation was found between the expression of this cytokine mRNA and the infiltration of granulomas by neutrophils (Fig. 5*B*).

The expression of TNF-α mRNA was inversely correlated with the extent of necrosis present in granulomas. Thus, sarcoid lesions, none of which manifested necrosis, and tuberculous lymph nodes

Table IV. *Cytokine mRNA expression and the extent of necrosis in granulomatous lymph nodes*[a]

	Expression of Cytokine mRNA (cpm ± SD)		
		Tuberculosis	
Cytokine	Sarcoidosis	Limited necrosis	Extensive necrosis
TNF-α	7,460 ± 2,576	7,271 ± 2,144	3,221 ± 1,775[b]
LT-β	10,846 ± 1,353	9,104 ± 2,222	6,459 ± 1,732[b]
IL-1	3,520 ± 675	3,754 ± 707	3,447 ± 982
IFN-γ	6,139 ± 3,838	4,314 ± 2,399	6,097 ± 5,185

[a] Cytokine mRNA expression was quantified by RT-PCR, and results are expressed as cpm ± SD for patients with sarcoidosis ($n = 6$) and patients with tuberculosis ($n = 8$). The percentage of the surface of granulomatous lesions occupied by caseous necrosis was determined by quantitative image analysis and permitted the identification of two distinct groups of tuberculous lesions, those with extensive necrosis (71 ± 9%, $n = 4$) and those with limited necrosis (3 ± 1%, $n = 4$). Sarcoid granulomas did not contain necrotic areas.

[b] $p < 0.05$ compared with tuberculous lesions with limited necrosis and sarcoid granulomas. $p > 0.2$ for all other comparisons.

containing limited necrosis expressed higher amounts of TNF-α mRNA than tuberculous granulomas demonstrating important caseous necrosis (Table IV). Immunohistochemical studies demonstrated that necrotic granulomas were clearly less well stained by the anti-TNF-α mAb than lesions demonstrating little caseous necrosis (data not shown). A similar correlation was observed for the expression of LT-β mRNA (Table IV). In contrast, the expression of other cytokine mRNAs, such as IL-1β or IFN-γ, did not vary as a function of the extent of necrosis present in tuberculous granulomas.

Discussion

In the present study, the profile of cytokine mRNAs expressed by sarcoid and tuberculous granulomas was evaluated by RT-PCR and correlated with histologic features of the granulomas. These results demonstrated that: 1) a Th1 pattern of cytokine mRNA expression was typically observed in sarcoid granulomas, whereas tuberculous lymph nodes expressed either Th1 or Th0 profile of cytokines; 2) among the 16 cytokines studied, the expression of mRNAs coding for IL-1β, IFN-γ, TNF-α, GM-CSF, IL-12 (p40), and LT-β was increased in both tuberculous and sarcoid granulomas compared with that of control tissues; 3) GM-CSF and LT-β were more abundant in sarcoid than in tuberculous granulomas, whereas IL-8 was predominantly expressed in tuberculous lesions; and 4) the production of several cytokines could be correlated with different pathologic features of the granulomatous lesions.

A Th1 cytokine pattern was constantly observed in sarcoid lymph nodes. To our knowledge, this is the first study in which the T helper profile of lymphocytes present in granulomatous tissues from these patients has been evaluated, although a previous study have shown a Th1 cytokine profile of T cells recovered by bronchoalveolar lavage from sarcoid patients (25). A pure Th1 cytokine pattern has also been observed in the tuberculoid form of leprosy, a disease in which the pathologic features of the granulomatous lesions are similar to those of sarcoid granulomas (3, 12). In contrast, both Th1 and Th0 patterns of cytokine mRNA expression were observed in granulomas from patients with tuberculosis. Although the Th pattern within tuberculous granulomatous lesions has not been previously evaluated, these results are consistent with prior evidence for heterogeneity in the pattern of cytokines produced in response to *M. tuberculosis*. Thus, although cells infiltrating tuberculin skin test reactions were reported to preferentially transcribe mRNAs coding for IFN-γ and IL-2, a Th0 pattern was also present in some specimens (15). Similarly, T cell clones derived from human peripheral blood recognizing *M. tuberculosis* Ags can express either the Th1 or Th0 phenotype (14, 36, 37).

The reason that a Th0 pattern can occur in tuberculous granulomas but not sarcoid granulomas remains unclear. A variety of cytokines, such as IFN-γ, IL-12, and IL-10, can influence T cell phenotype (38–40). Expression of IL-12 mRNA was increased in granulomatous lesions, and IL-12 has previously been shown to be produced by immune/inflammatory cells recovered by bronchoalveolar lavage from sarcoid patients (25) and to be present in pleural fluid of patients with tuberculosis (22). IL-12 is a potent inducer of IFN-γ production (39), and a correlation between the expression of IL-12 (p40) and IFN-γ mRNAs was observed here for both sarcoid and tuberculous granulomas. The expression of mRNAs coding for IL-12, IFN-γ, and IL-10 was not different, however, comparing patients with sarcoidosis and tuberculosis, and did not distinguish tuberculosis patients whose biopsies expressed a Th1 or Th0 profile. Thus, although these cytokines are undoubtedly important in determining T cell phenotype, other factors appear to contribute in these granulomatous diseases. The intensity of the Th1 response of peripheral blood T lymphocytes to *M. tuberculosis* has been shown to be reduced in immunocompromised patients (16), but none of our patients had conditions associated with immunosuppression. Studies in experimental animals infected with *Schistosoma mansoni* or *M. tuberculosis* have demonstrated that Th profiles can evolve in the course of infection (9, 17, 18, 41, 42) and could account for the variability observed here for patients with tuberculosis.

Granulomatous samples were characterized by the abundant expression of mRNAs coding for IL-1β, IFN-γ, TNF-α, GM-CSF, IL-12 (p40), and LT-β. Spontaneous production of certain of these cytokines by immune and inflammatory cells recovered from patients with sarcoidosis (4, 5, 23, 25) or tuberculosis (10, 20–22, 24) have previously been described, and IFN-γ, IL-1β, and TNF-α have been shown by immunohistochemical techniques to be present in sarcoid granulomas (26, 27). The results reported here extend the list of cytokines for which mRNA expression is increased in granulomatous lesions in humans to include GM-CSF, IL-12, and LT-β, and, as discussed below, to give additional insights into the role of these cytokines in the course of granulomatous responses.

First, GM-CSF was the only cytokine identified the expression of which was clearly linked with the presence of a florid granulomatous response. A number of studies have suggested that GM-CSF stimulates host defense against intracellular parasites. Thus, this cytokine enhances antimicrobial activity against a variety of pathogens in vitro, including *Mycobacterium avium* (10). In addition, increased levels of GM-CSF production in vivo has been shown to be associated with increased resistance of mice to *Listeria* infection (43), and administration of anti-GM-CSF Abs to mice infected with *Leishmania donovani* exacerbated visceral infection and parasite burden (44). Less information is available concerning the role of GM-CSF in the formation of tuberculoid granulomas. The salutory effect of GM-CSF on antileishmanial activity in normal mice appears to occur by mechanisms independent of an effect on granuloma formation (44). Other evidence, however, supports our findings that GM-CSF may directly promote granuloma formation. Thus, this cytokine is known to stimulate the proliferation and differentiation of monocyte precursors and to induce their mobilization into tissues, activities that might foster granuloma formation, and GM-CSF treatment of pale ear C57BL/6 (*ep/ep*) mice, which are particularly sensitive to *L. donovani*, partially corrected the abnormal granuloma formation seen in these immunodeficient animals following *Leishmania* infection (45).

Additional studies are needed to demonstrate that GM-CSF directly stimulates the formation of tuberculoid granulomas in humans and define the mechanisms involved.

In contrast to GM-CSF, the expression of IFN-γ, IL-1β, and TNF-α, although increased in both sarcoid and tuberculous granulomas, did not distinguish biopsies with florid granulomas from those with more localized lesions. Previous work has implicated these cytokines in granuloma formation induced by mycobacterial Ags in murine models (1, 2, 7–9, 46–48). Nevertheless, although initially delayed, extensive granuloma formation ultimately occurs in mice with targeted disruption of genes coding for IFN-γ, IFN-γ receptors, or TNF-α receptors (19, 46, 47). Thus, although these cytokines are very important in the activation of bactericidal activity of macrophages and appear to influence the early stages of granuloma formation, these results, like our findings, suggest that the level of production of these cytokines is not a major determinant of the extent of the granulomatous response occurring in response to established disease.

Our results also provide insights into the mechanisms responsible for central necrosis, a typical feature of tuberculous but not sarcoid granulomas. It remains controversial whether or not necrosis is beneficial. Mycobacteria are thought to proliferate poorly in caseous material, and the appearance of caseous necrosis correlates temporally with the end of log phase growth of *M. tuberculosis* in guinea pigs, findings compatible with a beneficial role (49). Cytotoxicity produced by cytokines, including TNF-α and LT, have been suggested to participate in this process (2, 50). We found, however, that level of expression of mRNAs coding for TNF-α and LT-β, two members of the TNF ligand family (51), correlated inversely with the extent of caseous necrosis present in tuberculous lesions. Furthermore, necrotic granulomas stained less well with anti-TNF-α Abs than sarcoid granulomas or tuberculous lymph nodes containing little necrosis. The reduced expression of these cytokines was not the result of necrosis per se, since the expression of mRNAs for all other cytokines tested was independent of the extent of necrosis in the tissue samples. Thus, these results do not support the idea that cytokines of the TNF ligand family are directly responsible for necrosis in tuberculous granulomas. Our findings are more compatible with the idea that caseous necrosis may be a deleterious process resulting from the killing of macrophages that have received insufficient activation signals to successfully resist mycobacterial infection. Consistent with this idea, mice, which are resistant to mycobacterial infection, form granulomas that do not contain central necrosis unless the animals are rendered susceptible to infection. In this regard, inhibition of the action of TNF-α results in the formation of necrotic granulomas in these animals (19). Furthermore, TNF-α has previously been shown to improve the antimycobacterial activity of both murine and human macrophages, which could explain why patients with lower levels of expression of this cytokine could be more prone to the development of necrosis (52–54). The possible role of LT-β in macrophage activation has not been examined, but our finding that the expression of LT-β mRNA was also inversely correlated with the presence of necrosis gives impetus to the evaluation of this possibility.

Finally, both IL-8 mRNA and protein expression were found to be increased only in tuberculous granulomas heavily infiltrated by neutrophils. These results are consistent with the known potent chemotactic activity for neutrophils of this cytokine (55). Human alveolar macrophages and blood mononuclear cells have been reported to release IL-8 in response to *M. tuberculosis* (56), but no previous data are available concerning expression in human granulomas. IL-8 has also been shown to express chemotactic activity for lymphocytes, and it has been suggested that this cytokine may contribute to granuloma formation. We did not find any statistical difference in the expression of IL-8 mRNA comparing sarcoid granulomas, tuberculous granulomas with little neutrophil infiltration, and control tissues. Thus, our results suggest that IL-8 is not critical for granuloma formation. Compatible with these findings, IL-8 was also shown to participate in delayed-type hypersensitivity in a rabbit model, but this effect was largely dependent on its ability to induce neutrophil influx (57).

The present study demonstrates that the formation of granulomas in humans is associated with the expression of characteristic cytokine profiles. Most of the cytokine mRNAs associated with granuloma formation identified here were expressed in increased amounts in both sarcoid and tuberculous granulomas, suggesting that the process of granuloma formation in these two diseases may involve shared mechanisms. Several differences in cytokine profiles in the two diseases were also identified. Our data also indicate that the expression of certain cytokines is associated with the development of specific pathologic features in the resulting granulomas, suggesting that the modulation of cytokine expression may be useful in improving the clinical outcome of patients with granulomatous disorders.

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