T-Cell Receptor Repertoire and Cytokine Pattern in Granuloma Annulare: Defining a Particular Type of Cutaneous Granulomatous Inflammation

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Granuloma annulare is a common granulomatous infiltration of the skin of unknown etiopathogenesis. We analyzed granuloma annulare biopsies in 11 patients and could find in all patients significant numbers of CD4-T cells. These cells showed a broad usage of the different T cell receptor Vβ families and a rather unbiased repertoire when the complementary determining region 3 spectra were analyzed by the Immunoscope technique. Comparison with the peripheral blood mononuclear cell repertoire, however, identified in all patients few skin-specific expansions, which were for one patient also present in two distinct skin sites. Extensive sequence analysis of the complementary determining region 3 region confirmed the presence of a limited number of skin-specific expansions together with various nonspecific T cell infiltrations. Analysis of the intralesional cytokine expression revealed abundant production of interleukin-2, which was not dominant in granulomas from leprosy patients and was not reflected by the cytokine profile in peripheral blood mononuclear cells. These results demonstrate the capacity of the granulomatous response to recruit T cells in high numbers with only few clones expanding specifically. The high local production of interleukin-2 might thereby play an important role in the nonspecific attraction of T cells to the granulomatous site. ***Key words: clonal expansions/cytokines/recruitment/skin/T cell receptors. J Invest Dermatol 118:957–966, 2002***

The formation of granulomas represents a particular immune defense mechanism, which is mounted to prevent the spreading of invading microorganisms to other body compartments and which has been exhaustively studied in the case of mycobacterial infections. Granulomas, however, are also found in idiopathic diseases such as sarcoidosis or Crohn's disease. The composition of the invading cells is variable but in almost all cases granuloma formation depends on the concomitant recruitment of antigen presenting cells, namely macrophages and dendritic cells, and lymphocytes with a dominance of T cells and in some cases natural killer cells.

Granuloma annulare (GA) is a granulomatous skin disease of unknown origin, which shows typical clinical and histologic features. Clinically, two forms have been characterized, localized GA and disseminated GA. The etiopathogenesis of GA has remained obscure. Some studies suggested an association with diabetes (Muhlbauer, 1980) and more recently with infectious agents (Aberer *et al*, 1999). The disseminated form of the disease seems to show increased prevalence among HIV patients (Toro *et al*, 1999; O'Moore *et al*, 2000).

The granulomatous infiltrate in GA is histologically characterized by the palisading infiltration of mononuclear cells around degraded collagen fibers and mucin infiltrates. The composition of the mononuclear infiltrate is variable, but T lymphocytes have been identified in significant numbers (Muhlbauer, 1980). The latter mostly include CD4+ cells and a few CD8+ cells (Buechner *et al*, 1983; Modlin *et al*, 1984a; 1984b), which show signs of activation (Fayyazi *et al*, 2000).

The role of the infiltrating T cells in the induction and perpetuation of GA has not been elucidated in detail. The clinical finding that T-cell-directed immunosuppressive approaches such as intralesional corticosteroid administration (Sparrow and Abell, 1975) or highly efficient ultraviolet A irradiation (Muchenberger *et al*, 1997; Schmutz, 2000) often rapidly resolve the granulomatous lesions may suggest a causative association.

Little if anything, however, is known about the nature of the stimulus driving T cell infiltration and expansion in GA.

In cases of infectious granulomas, antigen-specific T cells can be identified and expanded using their capacity to proliferate when stimulated by the respective pathogen-associated antigen(s). This technique, however, is not applicable when studying granulomas of unknown origin as in GA. We therefore chose a different approach to identify the nature of GA-infiltrating lymphocytes using immunohistochemistry, confocal microscopy, reverse transcription polymerase chain reaction (RT-PCR) and the Immunoscope

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Abbreviations: CDR3, complementary determining region 3; GA, granuloma annulare; NKT, natural killer T cells.

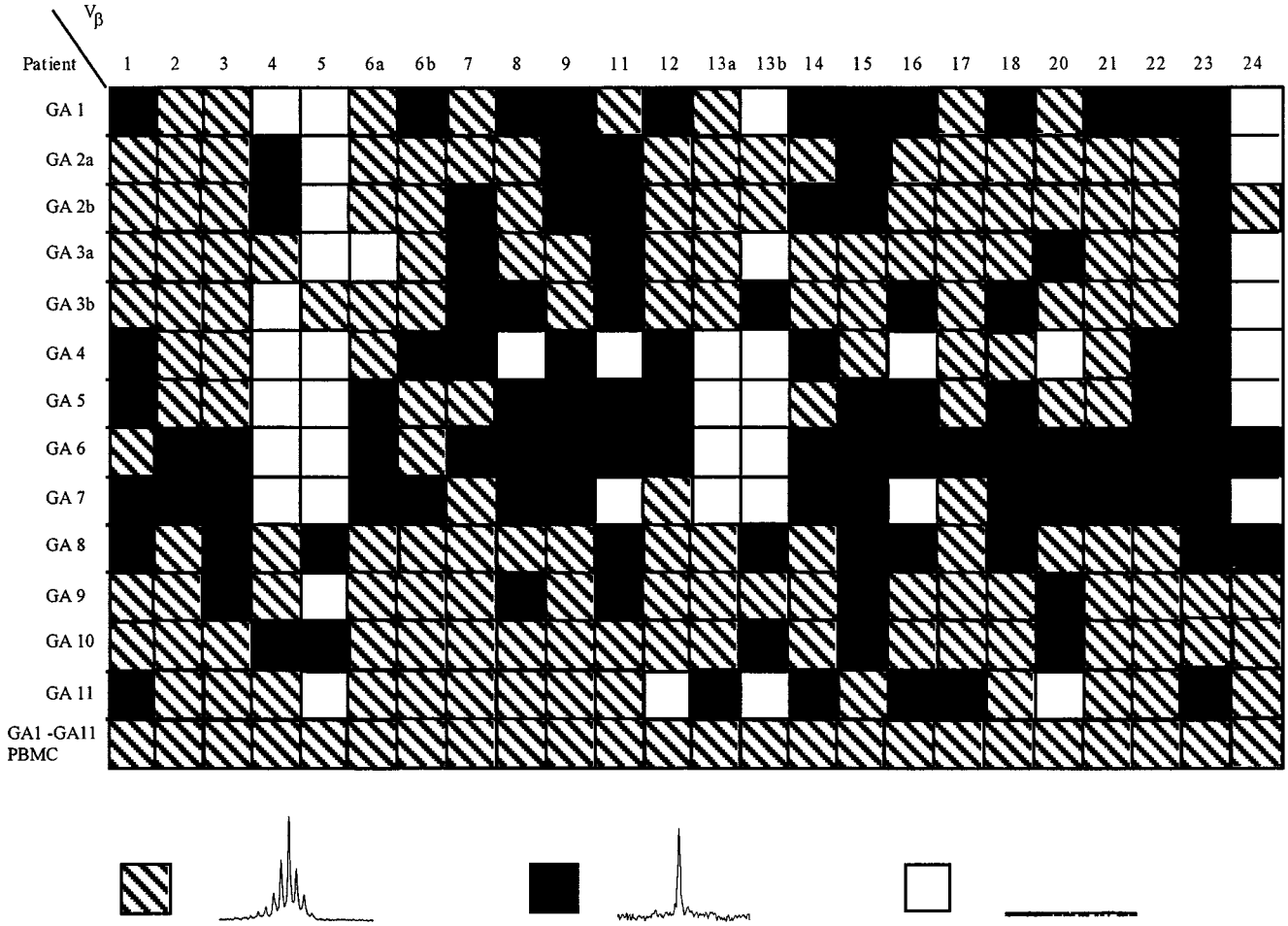

Figure 1. Distribution pattern of the various TCR-Vβ families within the different GA biopsies and the corresponding PBMC of the patients. Gausian- or comb-like shaped profiles represent polyclonal expansions (*scattered box*) whereas single-peak shaped expansions are found in cases of oligoclonal or monoclonal T cell populations (*black box*). If no PCR product was detected for the respective Vβ families, an open box is given. We could not find restrictions or predominant biases in the usage of Vβ chains within the cutaneous granulomas with the exception of Vβ23, which showed peak-shaped expansions in several patients.

technique, combined with extensive sequencing analysis. The results clearly imply that patients with GA recruit a variety of T cell receptor β (TCR-β) families without obvious restriction into their granulomatous lesions. In every patient, however, we could find skin-specific rearrangements on expansion, which were not detected in peripheral blood mononuclear cells (PBMC). In one patient identical expansions were found in two different granulomatous lesions suggesting antigen-driven T cell populations as a possible mechanism of T cell infiltration in GA. We also observed a high expression of interleukin-2 (IL-2) within the lesions, which might explain why a large nonspecific T cell population is recruited in parallel.

MATERIALS AND METHODS

Patients Eleven (10 female, one male; age range 23–67 y) patients presenting at the Department of Dermatology, Technical University Munich, were included in the study. All patients with the exception of patient GA9 presented with the disseminated form of GA. For two patients (GA2 and GA3) we obtained two biopsies at different body sites. For both patients the second biopsy (GA2b and GA3b) was taken 2 wk after cessation of an unsuccessful high-dose ultraviolet A treatment at a localization distinct from the first biopsy. The diagnosis of GA was made following clinical and histologic criteria. After informed consent had been obtained, 4 mm punch biopsies were obtained from lesional sites after local anesthesia and specimens were split for immunohistochemical and molecular biology analysis. The biopsies were snap-frozen and stored in liquid nitrogen until further preparation. On the same day PBMC were obtained by purifying blood samples using a Ficoll gradient (Pharmacia, Freiburg, Germany). cDNAs from leprosy patients analyzed in a previous study (Mempel *et al*, 2000) were included for comparison of lesional cytokine transcription.

Immunohistochemistry Frozen sections were analyzed for the expression of CD3, CD4, CD8, and panTCR-αβ, using commercially available monoclonal antibodies (Dako, Hamburg, Germany) following the APAAP technique with phenol red as indicator agent.

The percentage of positive cells was calculated by determining the number of stained cells among the total number of cells within the granulomas (e.g., hematoxylin-stained nuclei).

Double immunofluorescent staining using confocal laser microscopy Double immunostaining was carried out to determine the phenotype of the predominant T cell population carrying the TCR-Vβ chain of interest, which was found on expansion in the Immunoscope technique. Cryostat sections were incubated with the appropriate anti-TCR-Vβ monoclonal antibodies (Immunotech, Marseille, France) followed by goat cyanine-3-conjugated antimouse IgG staining (Sigma, St. Quentin Fallavier, France). In a second step, sections were stained with fluorescein-labeled anti-CD4 and anti-CD8 (Dako, Trappes, France) and were analyzed using a confocal laser microscope (MRC 1000 laser confocal system, Bio-rad, Ivry sur Seine, France).

RNA extraction and cDNA preparation Specimens were disrupted in Trizol (Gibco, Cergy Pontoise, France), using a Polytron homogenizer (skin biopsies) or by repeated pipetting (PBMC). RNA extraction and cDNA preparation were carried out following standard

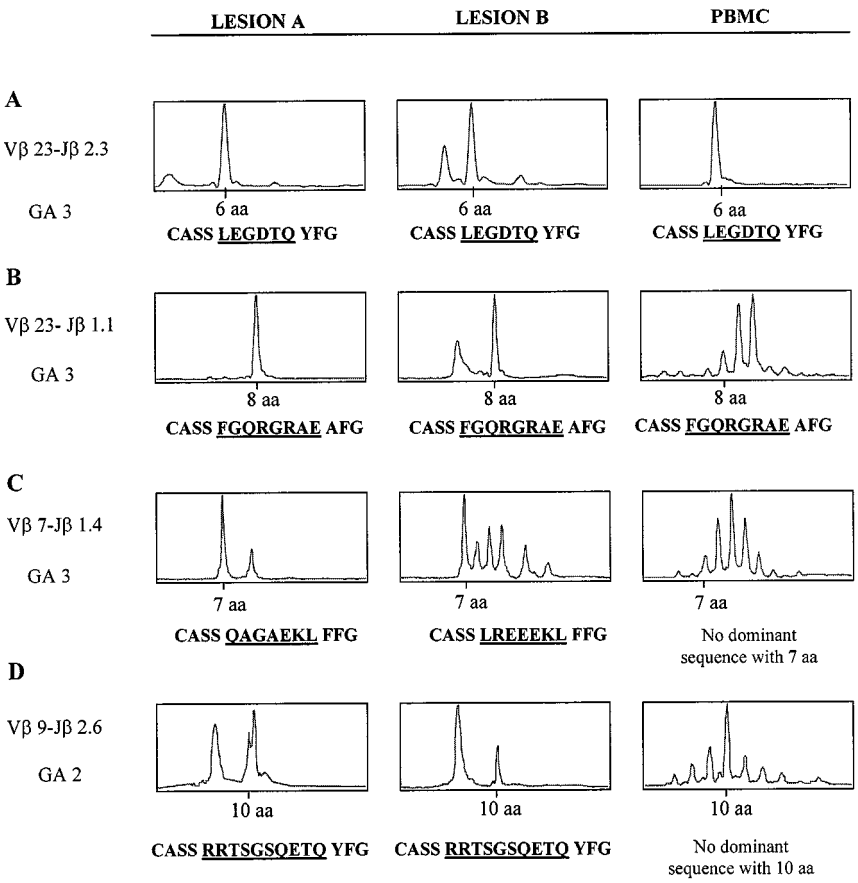

Figure 2. The typical situation of peak-shaped expansions found in the GA lesions of our patients as evidenced in the two patients GA2 and GA3 in which we could obtain biopsies from two different granulomatous sites. At the bottom of the graph the obtained Vβ–Jβ sequence is given with the CDR3 region underlined. When looking for monoclonal or oligoclonal expansions representing distortions of the Immunoscope profiles we found peaks that were present in both biopsies and the blood. These peaks exclusively used an identical CDR3 sequence in all three samples and can thus not be considered as skin specific (an example is given for the Vβ23–Jβ2.3 rearrangement of 6 aa in *A*). For other rearrangements we found dominant peaks in the two skin lesions with a more polyclonally shaped CDR3 distribution pattern in the blood. Upon sequence analysis, however, we found a dominant sequence for the corresponding CDR3 size in the blood sample, which was found identical in the skin lesions. Again, these expansions were considered as systemically activated and not to reflect skin-specific processes (an example is given for the Vβ23–Jβ1.1 rearrangement in *B*). Skin-specific rearrangements were identified by their peak-like patterns in the Immunoscope together with a unique sequence, which was not on expansion in the PBMC. In patient GA3 the two dominant expansions for the Vβ7–Jβ1.4 rearrangement in lesion A and lesion B differed in their exact CDR3 sequence and thus represent two different but similar expansions using the same rearrangement (*C*), whereas for the Vβ9–Jβ2.6 rearrangement of 10 aa in patient GA2 we found the identical clone on expansion in the two skin lesions, which was not detectable in the blood (*D*).

protocols using AMV Reverse Transcriptase from Roche (Meylan, France) and random dT primers.

TCR-β chain analysis The Immunoscope technique to analyze approaching complementary determining region 3 (CDR3) length and distribution has been described elsewhere (Pannetier *et al*, 1995). Briefly, standardized amounts of cDNA (i.e., the product of the reverse transcription of 10 μg of total RNA) were PCR amplified using each of the 24 Vβ-specific probes and a common Cβ-specific probe. Each Vβ–Cβ PCR product was analyzed by electrophoresis in an agarose gel. For CDR3 length diversity and Jβ usage, PCR amplified products were submitted to five cycles of primer extension using an internal, fluorescent, Cβ-specific probe or one of the Jβ-specific primers. The labeled material was loaded on a sequencing gel and analyzed using an automatic sequencer (Applied Biosystems) equipped with a computer program (Immunoscope, Applied Biosystems) that enables the determination of the intensity of fluorescence of each band as well as its actual size. The results are depicted as peaks whose areas are proportional to the amount of material and whose location is dictated by the length of the CDR3 region. The size distribution of the Vβ–Cβ is Gaussian in the case of nonactivated or polyclonally activated lymphocytes, whereas proliferating T cells generate a nonGaussian distribution with amplified peaks corresponding to clones using a definite CDR3 length within a Vβ–Cβ or a Vβ–Jβ combination. The lower limit of detection for this technique has been shown to be one specific cell in 10,000 (Lim *et al*, 1996). The primers used have been designed to have similar affinities for the different Vβ chains (Genevee *et al*, 1992).

Primers and PCR conditions for detection of the natural killer T cell (NKT) associated invariant Vα chain For detection of Vα24-invariant NKT we used a Vα24–Cα PCR (Genevee *et al*, 1992) followed by a run-off with the NKT-specific clonotypic Jα18 primer (5′-GCCTCCCCAGGGTTGAGCCTCTG-3′). For the primary PCR cDNA was amplified using 40 cycles (30 min 94°C, 30 min 60°C, 30 min 72°C) followed by five cycles (30 min 94°C, 30 min 60°C, 30 min 72°C) with the fluorescent run-off primer.

Cloning and sequencing of PCR products For cloning purposes cDNA products were amplified with the appropriate Vβ–Jβ primers using Pfu-Polymerase. PCR products were then ligated into a commercially available vector (Zero blunt, Invitrogen, Groningen, The Netherlands) and transformed into *Escherichia coli* (Invitrogen).

Amplifications were carried out using the M13 primer and standard protocols (Perkin Elmer) and analyzed using the ABI-Prism 373 software (Applied Biosystems).

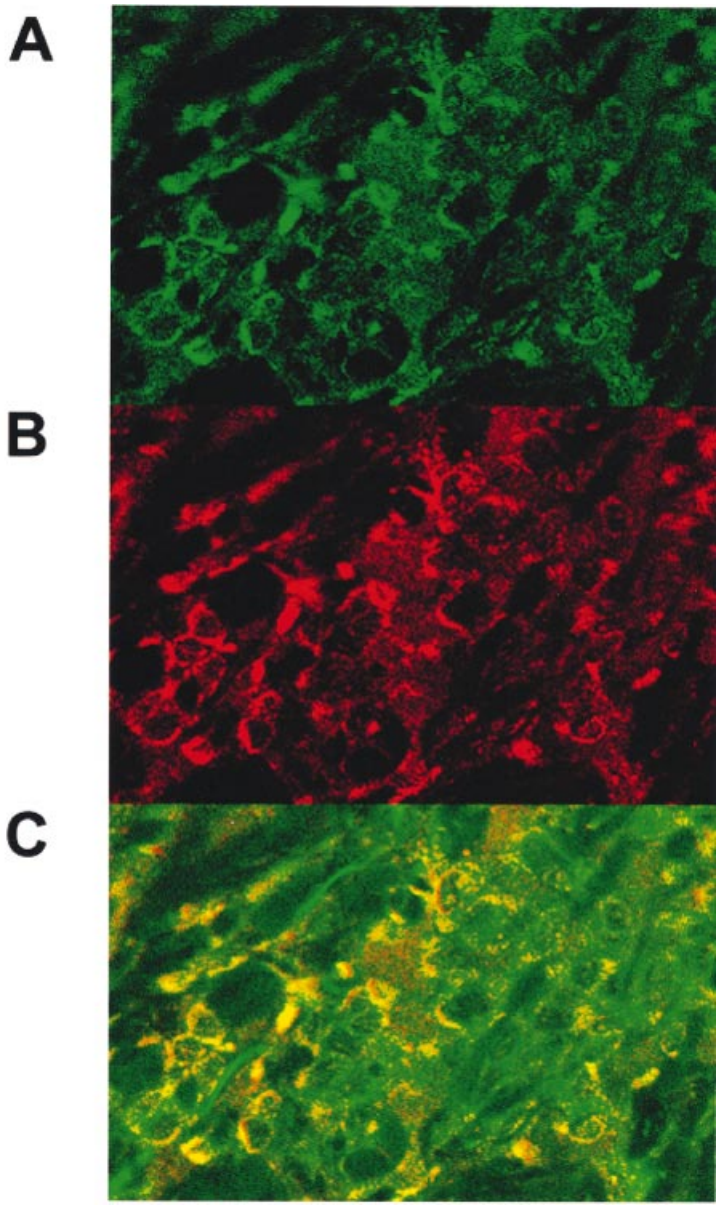

Figure 3. The TCR-Vβ families found on expansion in skin lesions by the Immunoscope technique (Table I) were analyzed for their expression of coreceptors with the confocal microscopy technique. Therefore the respective TCR-Vβ chains were stained using specific monoclonal antibodies together with anti-CD4 and anti-CD8. The figure gives the staining pattern for the skin lesion of patient GA6 with anti-CD4 (green labeling, *A*) and anti-Vβ21 (red labeling, *B*). The staining shows overlapping for Vβ21 and CD4 when overlying the pictures. This preferred association of the TCR-Vβ of interest with CD4 was found in all analyzed patients. Due to limitations in biopsy material only eight patients were analyzed using this technique.

Quantitative analysis of cytokine production For quantification of cytokine transcripts we used the CYBR green technique for quantitative RT-PCR analysis (Perkin Elmer). The following primer pairs were taken from Kammula *et al* (1999) or designed using the appropriate Perkin Elmer software to span at least one intron. All primer pairs have been validated for their applicability in the CYBR green technique using standard dilutions of cDNA derived from lipopolysaccharide-stimulated PBMC: glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5′-TGAAGGTCGGAGTCAACGG-3′ (sense), 5′-AGAGTTAAAAGCAGCCCTGGTG-3′ (antisense); IL-2, 5′-ACCAGGATGCTCACATTTAAGTTTT-3′ (sense), 5′-GAGGTTTGAGTTCTTCTTCTAGACACTG-3′ (antisense); IL-4, 5′-AACAGCCTCACAGAGCAGAAGACT-3′ (sense), 5′-GCCCTGCAGAAGGTTTCCTT-3′ (antisense); IL-5, 5′-TGTTCCTGTACATAAAAATCACCAACT-3′ (sense), 5′-TCCACAGTACCCCCTTGCAC-3′ (antisense); interferon-γ (IFN-γ), 5′-AGCTCTGCATCGTTTTGGGTT-3′ (sense), 5′-GTTCCATTATCCGCTACATCTGAA-3′ (antisense); tumor necrosis factor α (TNF-α), 5′-TTCCCCAGGGACCTCTCTCTAATC-3′ (sense), 5′-GCTTGAGGGTTTGCTACAACATG-3′ (antisense).

The results are expressed as the ratio of the amount of specific cDNA for the cytokine of interest compared to the amount of GAPDH-specific cDNA. The values were obtained using the formula $2^{-\Delta CT}$ in which ΔCT represents the difference in cycles used by the two PCRs to reach a threshold within the exponential phase of the PCR.

RESULTS

Immunohistochemistry The skin biopsies of all 11 patients included in the study were evaluated for typical histopathologic findings of GA (by standard hematoxylin and eosin staining) and for the nature of the infiltrating lymphocytes by immunostaining with anti-CD3, anti-CD4, anti-CD8, and anti-TCR-αβ. Hematoxylin and eosin staining revealed significant numbers of mononuclear cells within the cutaneous granulomas, and immunohistochemistry

Table I. Skin specific rearrangements and CDR3 sequences found in GA patients

Patient/lesion	Vβ chain	Vβ	CDR3	Jβ	Jβ segment	Frequency/skin	Frequency/PBMC
GA1	VB 1	CAS	SVSSALDGY	TFG	JB 1.2	43/72	0/45
GA2a/GA2b	VB 9	CAS	SRRTSGSQETQ	YFG	JB 2.5	8/29	0/45
GA3a	VB 7	CAS	SQAGAEKL	FFG	JB 1.4	24/37	0/48
GA3b	VB 7	CAS	SLREEEKL	FFG	JB 1.4	12/37	0/48
GA4	VB 23	CAS	SSWANPQETQ	YFG	JB 2.5	26/58	0/21
GA5	VB 16	CAS	SQEATDT	YFG	JB 2.3	20/25[a]	0/22
GA6	VB 21	CAS	SFARTSGAEQ	FFG	JB 2.1	25/26[a]	0/49
GA7	VB 20	CA	WGDGMNTEA	FFG	JB 1.1	21/23	0/67
GA8	VB 23	CAS	SLSPHSYEQ	YFG	JB 2.7	34/40	0/16
GA9	VB 8	CAS	SFQGTGEL	FFG	JB 2.2	5/26	0/42
GA10	VB 15	CA	TSDLTSGRADEQ	FFG	JB 2.1	3/5	0/17
GA11	VB 15	CA	TSDRDPGDEQ	FFG	JB 2.1	6/24	0/58

[a]In these patients the same amino acid sequence was encoded by two different sequences at the nucleotide level.

showed a predominance of CD4+, TCR-αβ lymphocytes in the lesions. These results confirmed previous findings (Modlin *et al*, 1984a; 1984b) that demonstrated a T lymphocyte infiltrate in GA, mainly of the CD4 phenotype.

GA lesions show a diverse TCR-β repertoire with clonal expansions Next we analyzed the usage of the different TCR-β chains within the granulomatous lesions and the PBMC of the patients by RT-PCR and the Immunoscope technique. Most of the patients showed a rather diverse usage of the various β-chains together with some peak-shaped expansions in the Immunoscope analysis, which are indicative of clonal expansions within the lesions. Analysis of the PBMC from all GA patients showed transcription of all Vβ families with occasional peaks, also most probably due to clonal expansions within the peripheral T cell population. **Figure 1** gives the TCR-Vβ chain usage within the GA patients.

To further define the nature of the T cells showing peak-like expansion in the Immunoscope analysis, we chose various rearrangements and determined the usage of Jβ segments associated with these peaks. Most of these expansions were associated with one specific Vβ–Jβ rearrangement of a single CDR3 size, which was used for further analysis.

We then compared the TCR rearrangement in the skin to the respective equivalent within the patients' PBMC. This analysis aimed to identify expansions seen in the lesions and not in the PBMC.

For all patients we could find several rearrangements within their skin lesions that were highly suggestive of a clonal expansion in the CDR3 spectra typing analysis and that showed a Gaussian-like polyclonal distribution pattern within their PBMC. These rearrangements were chosen as candidates for skin-specific expansions. For several rearrangements that showed peak-like distributions in the skin, however, we could identify the identical peak corresponding to the same CDR3 size within the PBMCs. These expansions were considered as reflecting a systemic expansion infiltrating the skin by a nonspecific mechanism. **Figure 2** gives an example for patients GA2 and GA3 from whom we obtained two different biopsies.

Interestingly, many of the GA patients included in our study showed peak-like expansions for the Vβ23 chain in the skin. Upon analysis of the associated Jβ chain and the corresponding CDR3 size, however, we could not identify identical expansions common to several patients. Thus, we identified a dominant 9 aa peak within the Vβ23–Jβ1.2 rearrangement of patient GA1, a dominant 9 aa peak within the Vβ23–Jβ2.3 rearrangement in patient GA2 (lesions A and B), an expanded peak of 8 aa within the Vβ23–Jβ1.1 and of 6 aa within the Vβ23–Jβ2.3 rearrangement in patient GA3 (lesions A and B), two dominant peaks of 5 aa and 10 aa in the Vβ23–Jβ2.5 rearrangement of patient GA4, an expanded peak of 10 aa in the Vβ23–Jβ2.7 rearrangement of patient GA8, and two 7 aa and 10 aa peaks within the Vβ23–Jβ2.5 rearrangement of patient GA11. Further sequence analysis of the corresponding CDR3 region, however, showed no similarities shared by the Vβ23-positive rearrangements.

Double labeling of GA-associated lymphocytes In order to evaluate the nature of the infiltrating T lymphocytes we investigated the phenotype of the dominant Vβ-expressing T cells identified in the Immunoscope technique. Therefore we performed double staining with the respective TCR-Vβ antibodies and anti-CD4/anti-CD8 antibodies and analyzed the results by confocal microscopy. This technique revealed in all investigated patients significant numbers of cells scoring positive for the Vβ family detected in the Immunoscope technique. When overlying the pattern of Vβ staining with the pattern for CD4/CD8 staining we found a good correlation with anti-CD4 staining indicative of a CD4 phenotype of the respective Vβ-expressing T lymphocytes within the lesions. **Figure 3** gives an example for staining with anti-Vβ21 and anti-CD4 in the lesion of patient GA6.

Sequence analysis shows skin-specific T cell populations in GA with no similarities between patients We could thus identify in all GA patients at least one expanded Vβ–Jβ rearrangement in skin samples but not in the blood of the patients by Immunoscope analysis. To screen for possible common features within the hypervariable region of the TCR-β chain we determined the exact sequence of the CDR3 region of the expanded T cell populations by sequencing the corresponding rearrangements. For this purpose the respective rearrangements showing peak-shaped expansions were cloned into *E. coli* and at least 50 randomly selected colonies were sequenced. We obtained in all patients dominant (i.e., repeatedly identified) sequences corresponding to the peaks of the suspected size after CDR spectra typing by Immunoscope. These dominant sequences were considered to reflect clonal expansions within the skin.

When comparing the obtained sequences suggestive for clonal expansions in the 11 GA patients, however, we found no common features or similarities between different patients. **Table I** shows the identified sequences in the investigated patients. For some of the patients (GA5, GA6) the same dominant amino acid sequence on expansion in the skin lesion was encoded by different nucleotide sequences arguing for an antigenic selection process.

The existence of skin-specific expansion of T cell clones was further confirmed by observations made with patients GA2 and GA3. In patient GA2 we identified the same expanded rearrangement (Vβ9–Jβ2.6, 10 aa) in both granulomatous lesions, but not in PBMC, indicative for a selection process specific for the two skin sites. In patient GA3, however, the situation was more complicated. We detected several expansions common to both that were also expanded in the blood (Vβ23–Jβ1.1, 6 aa; Vβ23–Jβ2.3, 6 aa). One dominant rearrangement was found in both lesions (Vβ7–

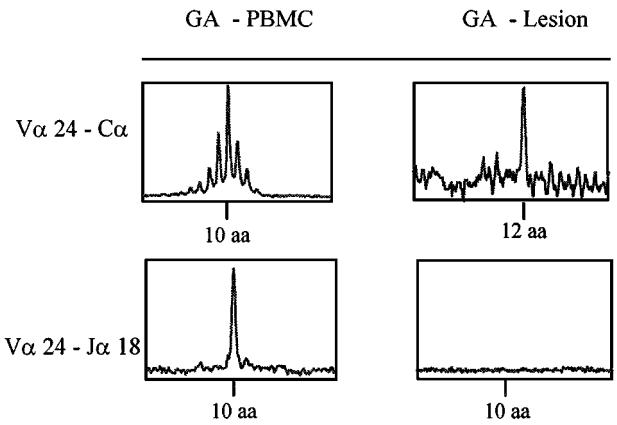

Figure 4. Lack of NKT infiltration into granuloma annulare lesions. We could detect the $V\alpha24_{inv}$-associated rearrangement (Vα24–Jα18, 10 aa) in all PBMC samples (*left panel*) but in none of the GA lesions, although some of them showed infiltration of Vα24-bearing T cells (*right panel*), indicating that significant numbers of NKT do not infiltrate GA lesions.

Jβ1.4, 7 aa) and not in PBMC, but it displayed a different CDR3 sequence (**Fig 2**, **Table I**).

$V\alpha24_{inv}$ NKT are absent in GA We have recently identified the presence of $V\alpha24_{inv}$ NKT in the lesions of T-cell-reactive leprosy patients (Mempel *et al*, 2000). These cells have additionally been implicated in the development of murine cutaneous granuloma formation induced by mycobacterial cell wall extracts (Apostolou *et al*, 1999). In order to screen for $V\alpha24_{inv}$ NKT we looked for the invariant Vα24–Jα18 rearrangement in both lesions and PBMC. Surprisingly, notwithstanding the broad usage of Vβ chains within the different patients and the presence of $V\alpha24_{inv}$ NKT in the blood of all GA patients we could not identify the canonical rearrangement within the lesions of any GA patient (**Fig 4**).

Quantitative PCR analysis shows high levels for IL-2 within GA lesions The persistence and expansion of lymphocyte populations within a site of cutaneous inflammation is often associated with the expression of cytokines favoring the development of either TH1 or TH2 associated immune responses. In the case of cutaneous granulomas, a well-described pathology is represented by the granulomas following infection with *Mycobacterium leprae* presenting a TH1 pattern with high amounts of IFN-γ and TNF-α for the T-cell-reactive response and a TH2 pattern with IL-4 and IL-5 transcription for the anergic response (Yamamura *et al*, 1991). We therefore included well-characterized leprosy patients (Mempel *et al*, 2000) in our studies for cytokine transcription together with the skin biopsies from GA patients. As shown in **Fig 5**(***b***), all patients with a T-cell-reactive disease pattern (e.g., tuberculoid leprosy and reversal leprosy) expressed a TH1-dominated cytokine expression (high amounts of TNF-α and IFN-γ) whereas all patients with the anergic form of the disease (lepromatous leprosy) expressed a TH2-dominated pattern (predominantly IL-4 and IL-5).

To our surprise, the cytokine profiles in GA lesions were strikingly different. As shown in **Fig 5**(***a***) the majority of GA patients displayed high transcription levels of IL-2 compared with the other cytokines. Interestingly this dominance was not mirrored by cytokine expression within PBMC where almost all patients presented with TNF-α as the predominantly expressed cytokine (**Fig 5*c***). This analysis showed that elevated IL-2 production was restricted to the skin and might explain the high number of T lymphocytes found in the lesions.

DISCUSSION

Our study confirms previous reports that significant numbers of T lymphocytes infiltrate the lesions of GA. These lymphocytes are predominantly CD4 positive. In order to elucidate the specificity of the invading lymphocyte populations we decided to screen the TCR-β repertoire within the granulomatous lesions compared to PBMC.

Three major possibilities for the composition of T cells in a given body compartment have been described. First, the homing of T lymphocytes to an inflamed site can be dictated by the dominance of local-antigen-specific T cells, which show a very restricted repertoire of their TCR and are suspected to be expanded by an antigen-specific process. Second, the infiltration of a minor antigen-specific T cell population can recruit, by means of nonspecific inflammatory signals (like cytokines and chemokines), a larger number of nonspecific T cells into the granulomatous site. In this situation the local TCR repertoire would be expected to show some antigen-driven biases together with large numbers of nonspecific cells and a polyclonal TCR pattern.

Third, there can be an infiltration of a totally unspecific T cell population with no restriction or biases in the use of their TCR repertoire. In this case the local T cell population would be expected to mirror more or less the T cell repertoire in the periphery.

Our results showed a very broad usage of Vβ families with only limited bias in all patients. Upon a refined analysis we could find in all patients a small number of skin-specific clones on expansion together with numerous non-skin-specific infiltrates as identified and confirmed by the combination of the Immunoscope technique and intensive sequence analysis.

This situation reflects a model for T cell infiltration into an inflammatous site that has been previously proposed for autoimmune diseases (Steinman, 1996) or infection of specific body compartments (Musette *et al*, 1995) and has been adapted by Hogan *et al* (1999) for infectious granulomas. In this so-called ''smart bomb model'' the selective infiltration of a limited number of site-specific T cells is followed by a large number of nonspecific T cells due to increased production of chemokines and/or cytokines. The T cell pattern in GA lesions therefore seems to differ profoundly from cutaneous granulomas recently analyzed in sarcoidosis and leprosy. In these diseases, infiltration of T cells is restricted and in T-cell-reactive leprosy shows a strong bias towards a limited set of Vα (Mempel *et al*, 2000) and Vβ (Wang *et al*, 1993) families. Of course, the skin of leprosy patients is invaded by a specific pathogen and the T cell repertoire at the site of infection is expected to show signs of antigen stimulation. In cutaneous sarcoidosis, however, a pathogen has not yet been identified and the granulomatous response has been described to show a similar histopathologic pattern to GA (Umbert and Winkelmann, 1977). The repertoires of the infiltrating T cells in the two conditions are strikingly different, however. Whereas the T cells in lesions of cutaneous sarcoidosis exhibit many antigen-driven-like expansions within their granulomas associated with only few nonspecific T cells (Mempel *et al*, 2000), the T cells in GA exhibit a broader spectrum of TCR specificities, consistent with a small number of disease-initiating clones nonspecifically recruiting a larger number of other T cells.

In addition, we found no common features of antigen-driven TCR-Vβ rearrangements (on the sequence level) between the different GA patients, even when looking at expansions generated in the same Vβ–Jβ rearrangements (e.g., Vβ9–Jβ2.1 in patients GA10 and GA11), indicating that T cell reactivity in GA is primarily an individual response without the existence of public responses.

This T cell pattern reflects the situation in antigen-driven processes with parallel infiltration of numerous nonspecific clones. The possibility of an underlying superantigen-driven process also seems unlikely as the dominant TCR-Vβ rearrangements strongly varied between the different patients.

In order to search for a possible mechanism for our findings, we analyzed the intralesional transcription for TH1- or TH2-associated cytokines by real-time quantitative PCR. In contrast to the previously described situation in leprosy in which the successful T cell response is associated with a dominant TH1 pattern and the

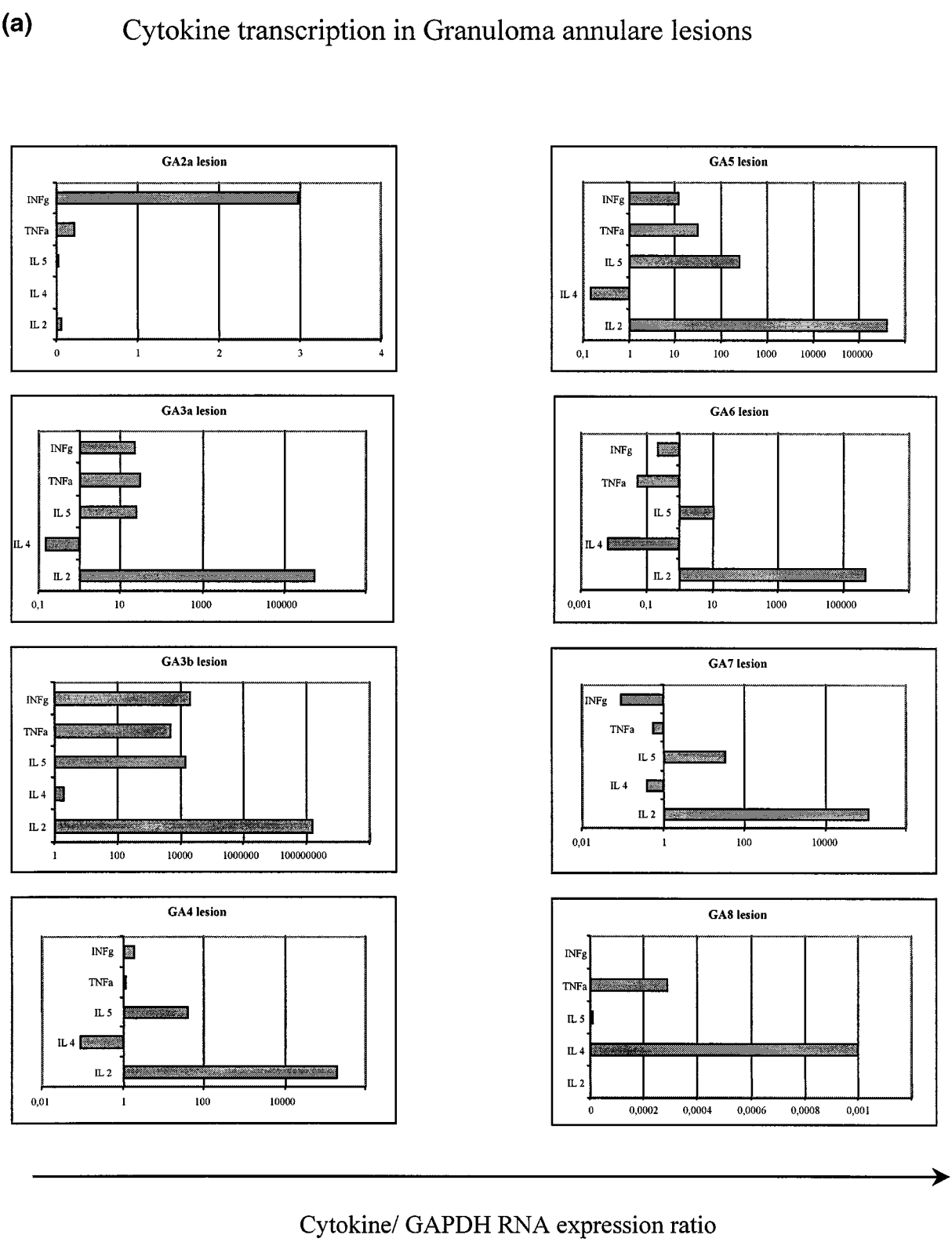

Figure 5. Cytokine transcription within various granulomas and PBMC of GA patients. Due to limitations in material we could include only eight granulomas from GA patients. In order to reveal the dominant cytokine milieu possibly directing the local immune response into a TH1- or TH2-associated response pattern, the transcription level is expressed as the ratio of the cytokine of interest and GAPDH as described in *Materials and Methods*. (*a*) In GA lesions we could find a very high transcription of IL-2 compared to other cytokines. That such a cytokine profile is not simply some methodologic artefact is confirmed by the analysis of granuloma samples from leprosy patients in which the T-cell-reactive form (e.g., tuberculoid (T) and reversal (R) leprosy) showed dominant transcription of IFN-γ and TNF-α, whereas the T-cell-anergic form (lepromatous (L) leprosy) showed a predominant transcription of IL-4 and IL-5 (*b*) as described previously (Yamamura *et al*, 1991). The excessive production of IL-2 in GA lesions was not due either to an increase of IL-2 transcription in PBMC as we found in almost all patients TNF-α as the dominant cytokine and to a lesser extent IL-4 when analyzing the blood samples (*c*). **Figures 5b, 5c,** overleaf.

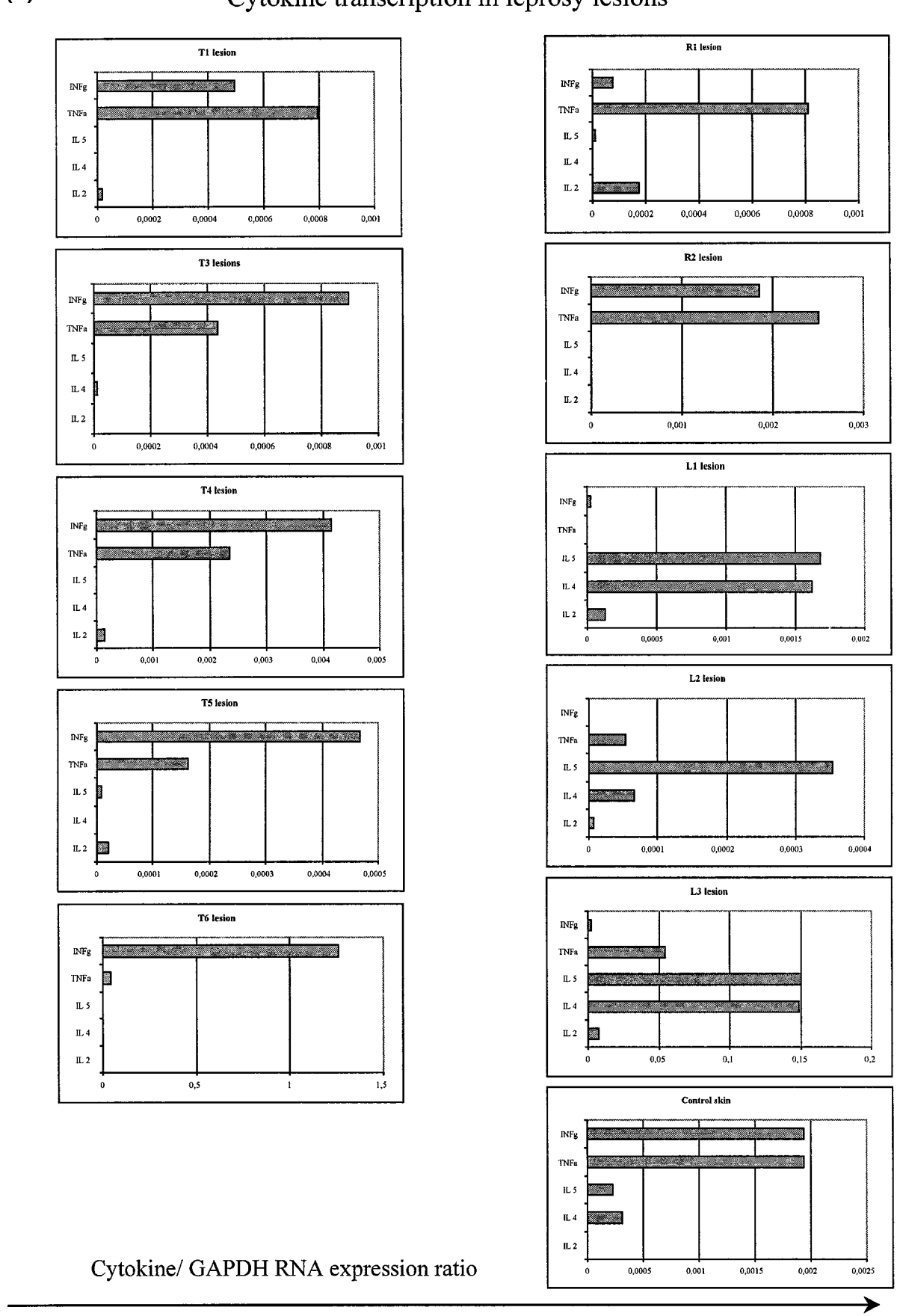
(b)
Cytokine transcription in leprosy lesions
T1 lesion
INFg
TNFa
IL 5
IL 4
IL 2
0
0,0002
0,0004
0,0006
0,0008
0,001
T3 lesions
0,0002
0,0004
0,0006
0,0008
0,001
T4 lesion
0,001
0,002
0,003
0,004
0,005
T5 lesion
0,0001
0,0002
0,0003
0,0004
0,0005
T6 lesion
0,5
1
1,5
R1 lesion
R2 lesion
0,001
0,002
0,003
L1 lesion
0,0005
0,0015
L2 lesion
L3 lesion
0,05
0,1
0,15
0,2
Control skin
0,0025
Cytokine/ GAPDH RNA expression ratio

Figure 5b

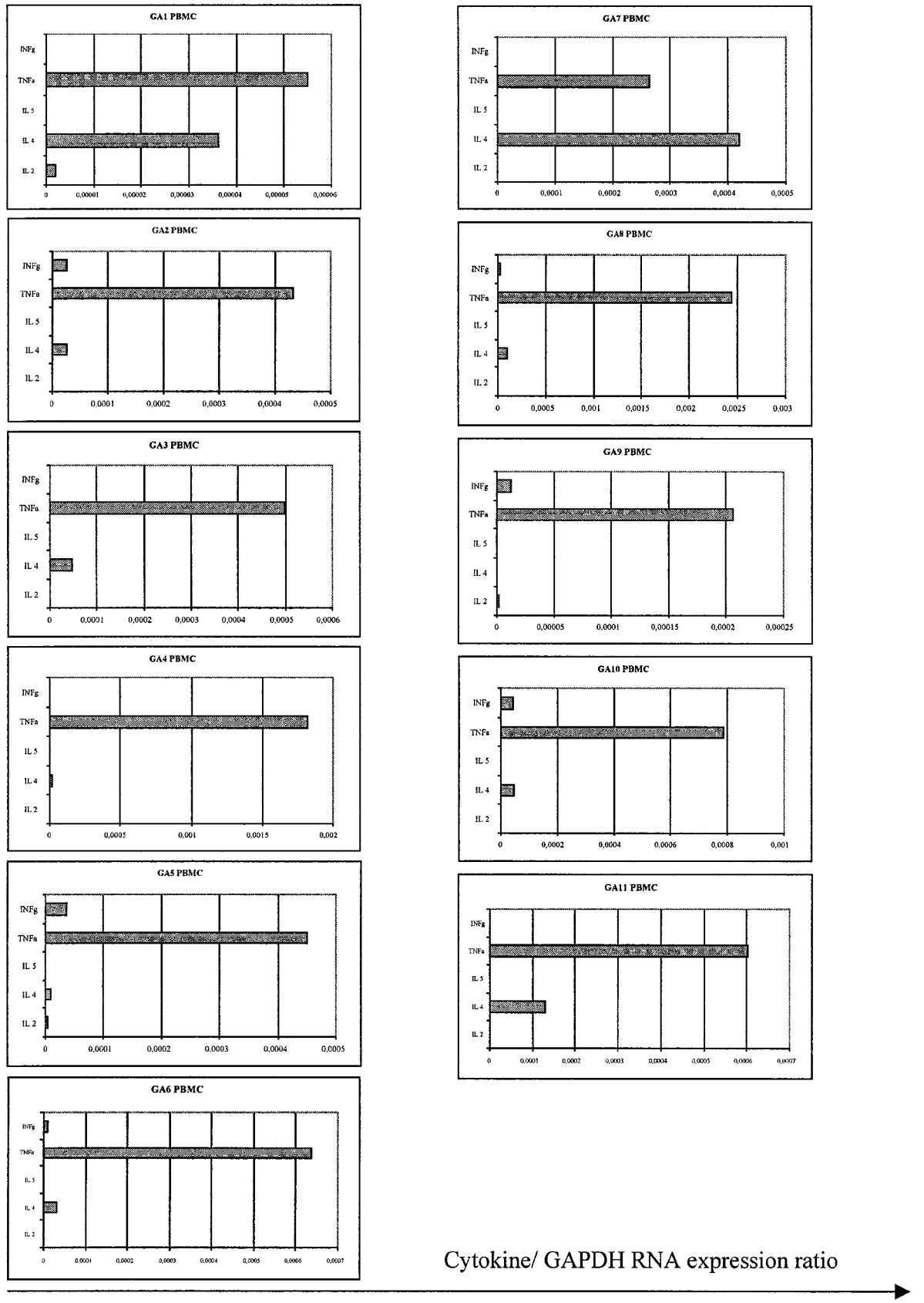
(c)
Cytokine transcription in PBMC of GA patients
GA1 PBMC
GA2 PBMC
GA3 PBMC
GA4 PBMC
GA5 PBMC
GA6 PBMC
GA7 PBMC
GA8 PBMC
GA9 PBMC
GA10 PBMC
GA11 PBMC
INFg
TNFa
IL 5
IL 4
IL 2
Cytokine/ GAPDH RNA expression ratio

Figure 5c

anergic response follows rather a TH2 type (Yamamura *et al*, 1992), we found in almost all of our GA patients a dominant transcription of IL-2, a finding reported several years ago using monoclonal anti-IL-2 antibodies (Modlin *et al*, 1984a). In this respect the situation in GA seems to differ profoundly from granulomas found in pulmonary sarcoidosis (Bergeron *et al*, 1997). This prevalence was not due to a high general upregulation within the patients' leukocytes as we could not find the same bias within PBMC, but seemed rather to reflect locally activated T cell populations. This local production of IL-2, a cytokine known to induce lymphocyte proliferation, could well explain the particular composition of the lymphocyte repertoire in our patients. IL-2 would thereby provide a second signal for the recruited lymphocytes and help to maintain the local T cell activation. Although recently TNF-α and IFN-γ have been found to be expressed in GA (Fayyazi *et al*, 2000) in most of the biopsies we analyzed the level of transcription of these cytokines was clearly below IL-2 expression, thus rendering unlikely a exclusive role in the inflammatous granulomatous process.

Another interesting finding in our GA patients is the absence of NKT as evaluated by the absence of the canonical Vα24–Jα18 transcript. These cells have been found in the T-cell-reactive forms of leprosy and their mouse homologs have been identified as being crucial in the development of cutaneous granulomas induced by mycobacterial glycolipids (Apostolou *et al*, 1999; Mempel *et al*, 2000). In this aspect, GA seems to follow the pattern in cutaneous sarcoidosis, which would further enforce the absence of a specific pathogen in both diseases.

In summary, we found in our patients with GA a T cell response characterized by the combination of few skin-specific clones together with many nonspecific infiltrations. The extremely high production of IL-2 could be an explanation for this particular situation. The GA-associated T cell pattern thus represents a particular type of granuloma composition.

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REFERENCES

Aberer E, Schmidt BL, Breier F, Kinaciyan T, Luger A: Amplification of DNA of borrelia burgdorferi in urine samples of patients with granuloma annulare and lichen sclerosus et atrophicus. *Arch Dermatol* 135:210–212, 1999

Apostolou I, Takahama Y, Belmant C, *et al:* Murine natural killer T (NKT) cells contribute to the granulomatous reaction caused by mycobacterial cell. *Proc Natl Acad Sci USA* 96:5141–5146, 1999

Bergeron A, Bonay M, Kambouchner M, *et al*: Cytokine patterns in tuberculous and sarcoid granulomas: correlations with histopathologic features of the granulomatous response. *J Immunol* 159:3034–3043, 1997

Buechner SA, Winkelmann RK, Banks PM: Identification of T cell subpopulations in granuloma annulare. *Arch Dermatol* 119:125–128, 1983

Fayyazi A, Schweyer S, Eichmeyer B, Herms J, Hemmerlein B, Radzun HJ, Berger H: Expression of IFNgamma, coexpression of TNFalpha and matrix metalloproteinases and apoptosis of T lymphocytes and macrophages in granuloma annulare. *Arch Dermatol Res* 292:384–390, 2000

Genevee C, Diu A, Nierat J, *et al*: An experimentally validated panel of subfamily-specific oligonucleotide primers (V alpha 1-w29/V beta 1-w24) for the study of human T cell receptor variable V gene segment usage by polymerase chain reaction. *Eur J Immunol* 22:1261–1269, 1992

Hogan LH, Weinstock JV, Sandor M: TCR specificity in infection induced granulomas. *Immunol Lett* 68:115–120, 1999

Kammula US, Lee KH, Riker AI, Wang E, Ohnmacht GA, Rosenberg SA, Marincola FM: Functional analysis of antigen-specific T lymphocytes by serial measurement of gene expression in peripheral blood mononuclear cells and tumor specimens. *J Immunol* 163:6867–6875, 1999

Lim A, Toubert A, Pannetier C, Dougados M, Charron DPK, Even J: Spread of clonal T cell expansions in rheumatoid arthritis patients. *Hum Immunol* 48:77–83, 1996

Mempel M, Flageul B, Suarez F, *et al*: Comparison of the T cell patterns in leprous and cutaneous sarcoid granulomas. Presence of Valpha24-invariant natural killer T cells in T cell-reactive leprosy together with a highly biased T cell receptor Valpha repertoire. *Am J Pathol* 157:509–523, 2000

Modlin RL, Horwitz DA, Jordan RR, Gebhard JF, Taylor CR, Rea TH: Immunopathologic demonstration of T lymphocyte subpopulations and interleukin 2 in granuloma annulare. *Pediatr Dermatol* 2:26–32, 1984a

Modlin RL, Vaccaro SA, Gottlieb B, et al: Granuloma annulare. Identification of cells in the cutaneous infiltrate by immunoperoxidase techniques. *Arch Pathol Laboratory Med* 108:379–382, 1984b

Muchenberger S, Schopf E, Simon JC: Phototherapy with UV-A-I for generalized granuloma annulare. *Arch Dermatol* 133:1605, 1997

Muhlbauer JE: Granuloma annulare. *J Am Acad Dermatol* 3:217–230, 1980

Musette P, Bureau JF, Gachelin G, Kourilsky P, Brahic M: T lymphocyte repertoire in Theiler's virus encephalomyelitis: the nonspecific infiltration of the central nervous system of infected SJL/J mice is associated with a selective local T cell expansion. *Eur J Immunol* 25:1589–1593, 1995

O'Moore EJ, Nandawni R, Uthayakumar S, Nayagam AT, Darley CR: HIV-associated granuloma annulare (HAGA): a report of six cases. *Br J Dermatol* 142:1054–1056, 2000

Pannetier C, Even J, Kourilsky P: T cell repertoire diversity and clonal expansions in normal and clinical samples. *Immunol Today* 16:176–181, 1995

Schmutz JL: PUVA therapy of granuloma annulare. *Clin Exp Dermatol* 25:451, 2000

Sparrow G, Abell E: Granuloma annulare and necrobiosis lipoidica treated by jet injector. *Br J Dermatol* 93:85, 1975

Steinman L: A few autoreactive cells in an autoimmune infiltrate control a vast population of nonspecific cells: a tale of smart bombs and the infantry. *Proc Natl Acad Sci USA* 93:2253–2256, 1996

Toro JR, Chu P, Yen TS, LeBoit PE: Granuloma annulare and human immunodeficiency virus infection. *Arch Dermatol* 135:1341–1346, 1999

Umbert P, Winkelmann RK: Granuloma annulare and sarcoidosis. *Br J Dermatol* 97:481–486, 1977

Wang XH, Ohmen JD, Uyemura K, Rea TH, Kronenberg M, Modlin RL: Selection of T lymphocytes bearing limited T cell receptor beta chains in the response to a human pathogen. *Proc Natl Acad Sci USA* 90:188–192, 1993

Yamamura M, Uyemura K, Deans RJ, Weinberg K, Rea TH, Bloom BR, Modlin RL: Defining protective responses to pathogens: cytokine profiles in leprosy lesions. *Science* 254:277–279, 1991