Mycobacterium tuberculosis Complex and Mycobacterial Heat Shock Proteins in Lymph Node Tissue from Patients with Pulmonary Sarcoidosis

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We demonstrated that not whole *Mycobacterium tuberculosis* but its particular antigens, $hsp70_{Mtb}$, $hsp65_{Mtb}$, and $hsp16_{Mtb}$, are present in lymph node tissues of patients with sarcoidosis (SA). $hsp16_{Mtb}$ occurs in the early stage of SA, whereas $hsp70_{Mtb}$ occurs in stage II of SA. $hsp65_{Mtb}$ is highly expressed in the capillary vessels in lymph node tissues in patients with SA.

Sarcoidosis (SA) is a granulomatous disorder of an unknown etiology. Because tuberculosis (TB)-like granulomatous inflammation is the histological hallmark of sarcoidosis, the participation of mycobacteria in the pathogenesis of SA is suggested (1, 7). Data obtained from the studies that used PCR techniques for the detection of *Mycobacterium tuberculosis* DNA are inconsistent (7). Some of molecular analyses revealed strong signals of *M. tuberculosis* heat shock protein 65 ($hsp65_{Mtb}$) in the sarcoid specimens (3). Enhanced titers of serum antibody to hsp_{Mtb} have been also described for patients with several autoimmune disorders, latent mycobacterial infection, and sarcoidosis (6, 12).

Heat shock proteins are often the target of T-cell- and hu-

moral-mediated immune responses to infections and may provide a link between the infection and autoimmunity caused by T-lymphocyte cross-reactivity between *M. tuberculosis* and human hsp70, hsp65, and hsp16 (46%, 60%, and 18% homologies, respectively) (4, 12).

To investigate mycobacterial antigen(s) involvement in SA, we determined the presence of *M. tuberculosis* complex (MTBC) and hsp_{Mtb} in frozen lymph node tissues from patients with pulmonary SA and controls (four patients with metastatic non-small-cell lung cancer, five patients with nonspecific lymphadenopathy as a negative control, and one patient with lung tuberculoma as a positive control). The diagnosis of SA was established by the clinical picture: no evidence

TABLE 1. The immunohistochemical analysis of expression of *M. tuberculosis* heat shock proteins $hsp70_{Mtb}$, $hsp65_{Mtb}$, and $hsp16_{Mtb}$ in lymph node tissues of patients with SA

Antibody	No. (%) of SA patients with immunopositive cells in lymph node tissue, with indicated intensity of antibody reaction[a]													
	PSH and granulomas				Lymphocytes					PSH (n = 5)		Granulomas (n = 20)	
	Stage I (n = 8)		Stage II (n = 17)		Stage I (n = 8)			Stage II (n = 17)					
	1	2	1	2	0	1	2	1	2	1	2	1	2
Anti-hsp70$_{Mtb}$	4 (50)	4 (50)[b]	4 (24)	13 (76)[b,d]	0	2 (25)	6 (75)[b]	2 (12)	15 (88)[b]	3 (60)	2 (40)[b]	5 (25)	15 (75)[b,e]
Anti-hsp65$_{Mtb}$	6 (75)	2 (25)	14 (82)	3 (18)	3 (37.5)	3 (37.5)	2 (25)	13 (76)	4 (24)	5 (100)	0	16 (75)	4 (25)[b,e]
Anti-hsp16$_{Mtb}$	2 (25)	6 (75)[b,c]	3 (18)	14 (82)[b]	0	0	8 (100)[b,c]	3 (18)	14 (82)[b]	2 (40)	3 (60)[b,c]	3 (15)	17 (85)[b,e]

[a] Data were analyzed using the computer program STATISTICA for Windows, version 7.1 (StatSoft), using the chi-square test with the Yates correction for repeated measurements at a P value of ≤ 0.05.
[b] $P < 0.0003$ for comparison of $hsp65_{Mtb}$ versus $hsp70_{Mtb}$ and $hsp16_{Mtb}$.
[c] $P < 0.004$ for comparison of $hsp16_{Mtb}$ versus $hsp70_{Mtb}$.
[d] $P < 0.0001$ for comparison of $hsp70_{Mtb}$ in stage I versus stage II of SA.
[e] $P < 0.0001$ for comparison of $hsp70_{Mtb}$, $hsp65_{Mtb}$, and $hsp16_{Mtb}$ in granulomas versus PSH.

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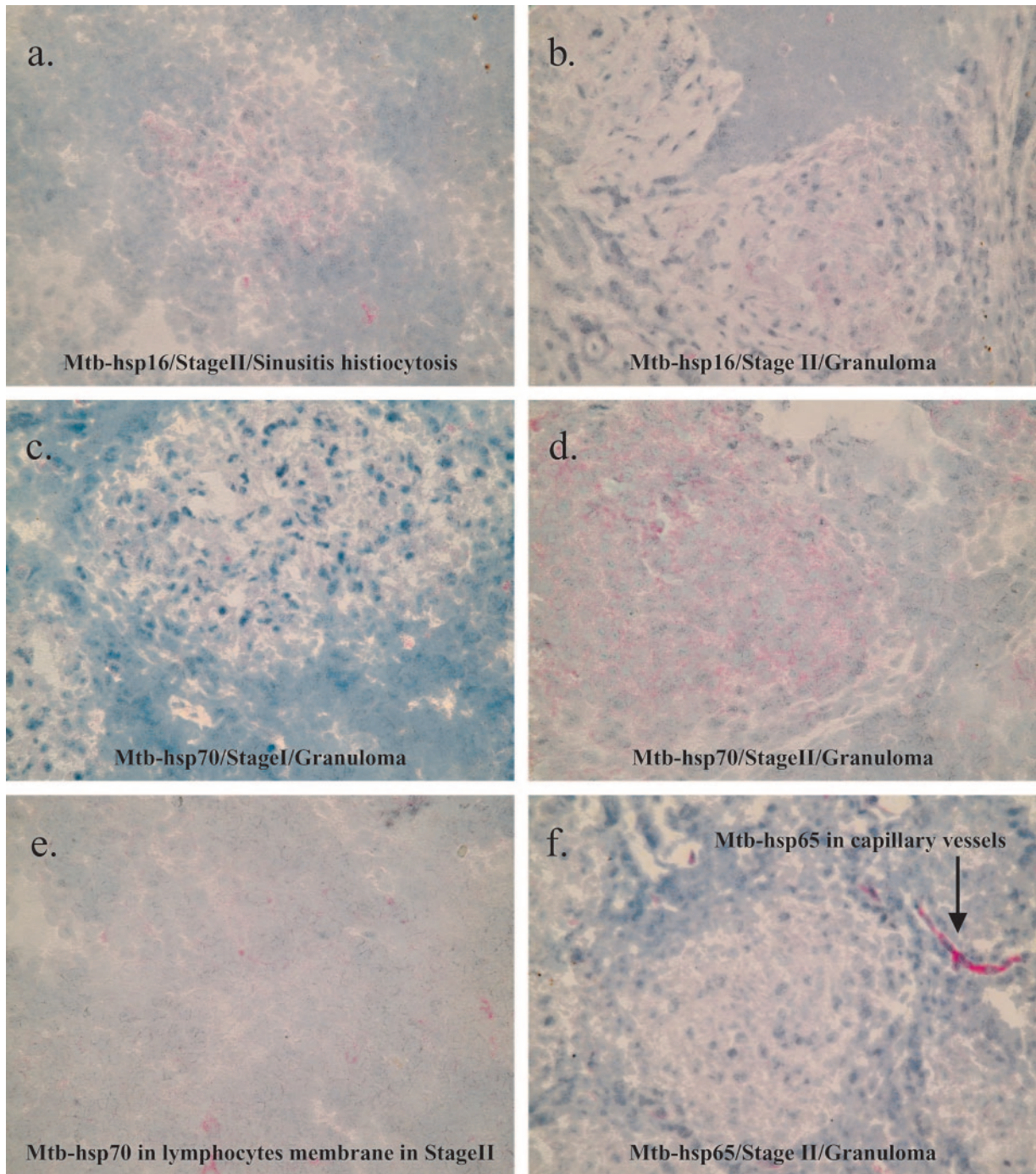
FIG. 1. The expression of *M. tuberculosis* (Mtb) heat shock proteins hsp70, hsp65, and hsp16 in the tested lymph node tissues from patients with sarcoidosis.

of current infection by *M. tuberculosis*, assessed by culture, and histological demonstration of noncaseating granulomas (43 SA patients) and pregranulomatous phase (PSH) (7 SA patients) in scalenobiopsy specimens of lymph node tissues. High-resolution computed tomography was used to diagnose stage I (bilateral hilar lymphadenopathy; 25 patients) and stage II (bilateral hilar lymphadenopathy and parenchymal infiltrations; 25 patients) of sarcoidosis. The diagnosis of TB was

established on the clinical findings, chest radiograph, positive results of culture of sputum, and the histological findings of caseous necrosis. The diagnosis of the metastatic non-small-cell lung cancer was established on the clinical, high-resolution computed tomography, and histological examinations. Non-specific lymphadenopathies were diagnosed on the basis of clinical and radiological signs followed by histological and microbiological results (no acid-fast bacilli, PCR, culture of the

M. tuberculosis strain, fungi, and atypical cells). All patients and the controls were vaccinated with *Mycobacterium bovis* bacillus Calmette-Guérin (BCG). None of them had either TB and SA familial history or contact with a patient(s) with active tuberculosis.

Tissue biopsy specimens were obtained as part of routine diagnostic procedures and preserved by cryopreservation. Histological lesions in each lymph node and lung tuberculoma were routinely diagnosed using hematoxylin and eosin-stained sections.

The BD ProbeTec (Becton Dickinson Diagnostic Instruments) system with the *M. tuberculosis* IS6110 complex-specific primers was performed in 50 SA patients and 10 controls according to the manufacturer's instructions (2). IS6110 of *M. tuberculosis* used in this study is more specific for *M. tuberculosis* than for the *M. bovis* BCG strain or *M. avium* complex (10). The results greater than 20 relative light units (RLU) were considered positive for MTBC, whereas MTBC results less than 20 RLU were considered negative if the internal control was greater than 10 RLU (2).

The 4-μm-thick cryosections from 25 SA patients and 10 control patients were immunolabeled for mycobacterial hsp using the monoclonal antibodies (Abs) against hsp70$_{Mtb}$, hsp65$_{Mtb}$, and hsp16$_{Mtb}$ (LIONEX Diagnostics and Therapeutics GmbH, Germany) with a three-layer APAAP protocol (DAKO, Dakopatts, Denmark). Control monoclonal Ab included isotype-matched irrelevant Ab and positive labeling controls. Intensities of antibody reaction were assessed semiquantitatively with the following scores: 0, no immunoreactivity; 1, weak intensity of reaction; and 2, strong intensity of reaction.

In the current study, there were no positive signals for *M. tuberculosis* IS6110 complex-specific primers in the negative control group. We detected the presence of MTBC DNA only in 3 of 50 SA patients (6%), which is consistent with the results of other authors (7). In contrast, some authors have obtained positive PCR results in over half of tested individuals with SA by using the IS6110 sequence. In the other studies (3, 7), no positive signals for *M. tuberculosis* were reported. A possible explanation for this is that the assay for IS6110 may not have been sufficiently sensitive to detect the very small quantity of *M. tuberculosis* genome in tissue from SA patients or that *M. tuberculosis* is present but the strains do not contain IS6110. Another possible explanation for the negative molecular results is that a small numbers of organisms provoke an intense inflammatory response, analogous to tuberculoid leprosy (3, 4). It is also suggested that the agents associated with sarcoidosis are not whole mycobacteria but their antigens, e.g., mycobacterial hsp (7, 8). Moreover, in the genetically predisposed hosts, exposure to a persistent antigen(s), including mycobacterial hsp, triggers an increased local cellular immune response, leading to granuloma formation (1, 4).

The immunohistochemical analysis of our series revealed the expression of hsp70$_{Mtb}$, hsp65$_{Mtb}$, and hsp16$_{Mtb}$ in all 25 lymph node tissues and tuberculous granuloma from patients with SA and in one nonspecific lymphadenopathy case with only weak hsp70$_{Mtb}$ reactivity. The rest of the eight control cases were not hsp reactive.

In SA patients, hsp70$_{Mtb}$, hsp65$_{Mtb}$, and hsp16$_{Mtb}$ reactivity was found in PSH, granulomas, and surrounding lymphocytes;

however, there were different levels of intensity (Table 1). The comparison of all tested mycobacterial hsp expression levels between PSH and granulomas revealed higher reactivity of hsp70$_{Mtb}$, hsp65$_{Mtb}$, and hsp16$_{Mtb}$ in granulomas. The hsp16$_{Mtb}$ expression was significantly more intense than that of hsp70$_{Mtb}$ in PSH (Fig. 1c) and in the lymphocyte membrane (Fig. 1e). In tested areas of lymph nodes, the hsp70$_{Mtb}$ and hsp16$_{Mtb}$ expression levels were significantly higher than that of hsp65$_{Mtb}$. Analysis of hsp$_{Mtb}$ occurrence in stage I and II of SA revealed that levels of expression of hsp70$_{Mtb}$ and hsp16$_{Mtb}$ were significantly higher than hsp65$_{Mtb}$ expression in both stages. The hsp70$_{Mtb}$ expression was significantly higher in stage II than in stage I (Fig. 1c and d), whereas hsp16$_{Mtb}$ expression levels were comparable in both stages of SA. Furthermore, the hsp16$_{Mtb}$ reactivity was significantly more intense than that of hsp70$_{Mtb}$ in stage I and was comparable to that of hsp70$_{Mtb}$ in stage II. It was worthy to notice that hsp65$_{Mtb}$ reactivity was significantly more intense than hsp70$_{Mtb}$ and hsp16$_{Mtb}$ in the capillary vessels in lymph node tissues (Fig. 1f).

Our results suggest that hsp65$_{Mtb}$ and hsp16$_{Mtb}$, being implicated in the stationary phase of *M. tuberculosis* or in the forming of immune complexes in TB (9, 11), could be also induced in the early immune response in SA. We reported before (6) on enhanced serum hsp70$_{Mtb}$ concentrations in the same group of patients, especially with stage II of SA. The higher expression levels of hsp70$_{Mtb}$ and hsp16$_{Mtb}$ in comparison to hsp65$_{Mtb}$ in lymph node tissues of the patients with stage II sarcoidosis could be caused by sequestration of hsp65$_{Mtb}$ antigens in the bound form, for example, in immune complexes in membrane of vessels (5).

In summary, our molecular analysis suggests that not whole *M. tuberculosis* cells but their particular antigens, e.g., hsp70$_{Mtb}$, hsp65$_{Mtb}$, and hsp16$_{Mtb}$, could participate in the pathogenesis of sarcoidosis. The occurrence of hsp16$_{Mtb}$ seems to be associated with the early stage of SA, whereas hsp70$_{Mtb}$ is associated with stage II of the disease. hsp65$_{Mtb}$ is highly expressed in the capillary vessels in lymph node tissues in patients with SA.

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