INFECTION AND IMMUNITY, Apr. 1991, p. 1434–1441
0019-9567/91/041434-08$02.00/0

Vol. 59, No. 4

Association between the 65-Kilodalton Heat Shock Protein, *Streptococcus sanguis*, and the Corresponding Antibodies in Behcet's Syndrome

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Received 29 June 1990/Accepted 30 January 1991

The etiology of Behcet's syndrome (BS) is unknown, but a number of streptococcal species have been implicated. A hypothesis was postulated that a shared antigen, such as a stress protein, might account for some of these findings. Indeed, a rabbit antiserum against a 65-kDa heat shock protein of *Mycobacterium tuberculosis* revealed a corresponding 65-kDa band with all six *Streptococcus sanguis* strains examined and *S. pyogenes* but not with *S. salivarius*. By applying a panel of nine monoclonal antibodies to the mycobacterial 65-kDa heat shock protein, an approximately 65-kDa antigen was identified in the uncommon serotypes of *S. sanguis* ST3 and H.83 and one with a different M_r was identified in KTH-1 and *S. pyogenes*. Monoclonal antibodies Y1.2, C1.1, II H9, and ML30, which reacted with these streptococci, recognize residues 11 to 27, 88 to 123, 107 to 122, and 276 to 297 of the 65-kDa heat shock protein, respectively, suggesting that these residues are conserved among some uncommon serotypes of *S. sanguis* and *S. pyogenes*. Immunoblot analyses of sera from patients with BS for immunoglobulin A (IgA) and IgG antibodies revealed bands of 65 to 70 kDa with the mycobacterial heat shock protein, *S. sanguis* strains, and *S. pyogenes*, although these reactivities were also found to a lesser extent in controls. A 65- to 70-kDa band was found more frequently with *S. sanguis* KTH-2 or KTH-3 and IgA in serum from patients with BS than with serum from controls ($P < 0.02$). Antibodies in serum were then studied by a radioimmunoassay, and in patients with BS this revealed significantly raised IgA antibodies to the recombinant 65-kDa mycobacterial heat shock protein and to soluble protein extracts of *S. sanguis* ST3, KTH-1, KTH-2, and KTH-3. Whereas significant anti-65-kDa heat shock protein and anti-*S. sanguis* ST3 antibodies were also found in sera from patients with rheumatoid arthritis and recurrent oral ulcers, the anti-*S. sanguis* KTH-1, KTH-2, and KTH-3 antibodies were confined to BS. The results are consistent with the hypothesis that some of the streptococcal antigens are associated with heat shock or stress proteins, which will need to be formally established by isolating heat shock proteins from streptococci.

Stress proteins or heat shock proteins (HSPs) are a highly conserved immunoreactive group of proteins found in microorganisms and animal tissues. In addition to heat, other stressful conditions induce stress proteins, especially anoxia, heavy metal ions, exposure to H_2O_2, and infection by DNA or RNA viruses (37). The stress proteins have been well conserved during evolution, probably on account of their function in responding to increased temperatures and other environmental stresses (37).

Although there are a number of families and functions of immunoreactive stress proteins (reviewed in references 9 and 42), the 65-kDa protein originally identified in *Mycobacterium leprae* (13, 15) is of particular interest. The antigen cross-reacts with other mycobacteria, such as *M. tuberculosis* (4). The 65-kDa protein is an immunodominant antigen that induces circulating antibodies and T-cell responses in human subjects (3, 6, 12, 26, 28, 36, 50). An important finding was that the 65-kDa stress protein is a common antigen found in gram-negative and gram-positive bacteria (22, 49). The 65-kDa antigen isolated from *M. tuberculosis* shows 50 to 60% amino acid sequence homology with the *Escherichia coli groEL* gene (46, 57). Of great significance is the observation that stress proteins are found in mammalian cells that show very significant sequence homology with the 65-kDa protein (24).

An association between stress proteins and a number of immunological disorders and diseases has been suggested, apart from their role in any of the infections the parent organism may cause (27, 56). *M. tuberculosis* can cause adjuvant arthritis in rats, and adoptive transfer of T-cell clones from those rats can induce arthritis in irradiated recipient rats (20, 55). Some of these T-cell clones react with the 65-kDa antigen and with cartilage proteoglycans, suggesting that the 65-kDa antigen is involved in patients with rheumatoid arthritis (55). Indeed, a T-cell clone that was TCR-$\gamma\delta^+$ and reacted with the 65-kDa HSP from *M. bovis* was recently isolated from the synovial fluid of a patient with rheumatoid arthritis (18).

Behcet's syndrome (BS) is a disease affecting a spectrum of tissues with a number of immunological manifestations (32, 33). The etiology is unknown, but two microbial agents have been implicated. The herpes simplex virus type 1 genome and nonpermissiveness for viral growth were found in circulating leukocytes (2, 7, 11). Four species of streptococci—*Streptococcus sanguis*, *S. pyogenes*, *S. faecalis*, and *S. salivarius*—have been suggested as etiological agents of BS (21, 25, 40). This multiplicity of streptococci raised the

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TABLE 1. Groups of patients used in the investigations

Group	No. of persons
Healthy controls	53
BS	64
Mucocutaneous	17
Arthritic	16
Neurological	9
Ocular	22
Recurrent oral ulcers	34
Minor aphthous	15
Major aphthous	11
Herpetiform	8
Recurrent herpes labialis	8
Rheumatoid arthritis	41

possibility that a common antigen, such as a stress protein, might account for these diverse observations (34).

The objectives of this investigation were to find out whether monoclonal antibodies (MAbs) to the 65-kDa HSP react on Western immunoblotting with a number of streptococcal species implicated in the etiology of BS. This was followed by examination of sera from patients and controls for antibodies to the 65-kDa recombinant stress proteins isolated from *E. coli* (48), antibodies to a 64-kDa protein isolated from *M. bovis* (6), or antibodies to the streptococci. Furthermore, oral mucosa homogenates were examined in view of the early reports of autoimmune responses (8, 29, 30, 41, 44).

MATERIALS AND METHODS

Subjects. The series of subjects consisted of 147 patients, of whom 64 had BS, 34 had recurrent oral ulcers (ROU), 41 had rheumatoid arthritis, and 8 had recurrent herpetic infection of the lips. A group of 53 healthy subjects, matched for age and sex, were included as controls. The patients with BS were divided into four groups (Table 1) based on criteria laid down previously (35). The 34 patients with ROU were further divided into 15 with minor and 11 with major aphthous ulcers and 8 with herpetiform ulcers, as defined previously (31).

Streptococci, herpes simplex virus, and antigens. A recombinant *M. bovis* 65-kDa protein expressed in *E. coli* was prepared as described previously (48). A number of *S. sanguis* serotypes and the uncommon serotypes found in BS are described in Table 2. We also examined beta-hemolytic streptococci of groups A and D and *S. salivarius*, *S. faecalis*, *S. mutans*, *S. sobrinus*, *S. milleri*, *S. mitis*, *S. mitior*, and *S. bovis* (Table 2). Most of these were used in Western immunoblots, and three *S. sanguis* strains were applied in a radioimmunoassay.

Fetal oral mucosa was removed from an approximately 22-week-old fetus. The tissue was cut into small fragments, homogenized, and centrifuged as described previously (29). Both the supernatant and the deposit were used for immunoblotting.

MAbs against the 65-kDa HSP. The MAbs used and the peptide sequences they recognize are shown in Table 3. All MAbs were kindly provided by the World Health Organization, except ML30, which was a gift from J. Ivanyi. Antiserum to the 65-kDa mycobacterial HSP was raised by one of us (T.S.) in rabbits by using protein purified from *M. bovis* BCG.

Western immunoblots. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was done in a vertical slab gel apparatus (LKB). An SDS–3% polyacrylamide stacking gel in Tris HCl buffer (pH 6.8) was poured with a 10-place comb in situ, and 10 μg of the 65-kDa HSP, mucosal homogenates, or herpes simplex virus type 1 and 40 μl of a standardized number of streptococci (optical density of 1.8 at 540 nm) were loaded. Only 1 μg of HSP was used with the MAb and rabbit antiserum. Prestained molecular weight markers (Gibco, BRL) were included in the gels. The stacked antigens were separated on an SDS–10% polyacrylamide gel in Tris-glycine buffer (pH 8.8). SDS-polyacryl-

TABLE 2. Species, types, and sources of streptococci and 65-kDa bands produced by Western blotting with rabbit antiserum

Organism	Type	Strain	Source	65-kDa band[a]
S. sanguis	I	7863	NCTC[b]	+
S. sanguis	II	7864	NCTC	+++
S. sanguis	I-II	7865	NCTC	−
S. sanguis		11086	NCTC	+
S. sanguis KTH-1		49298	ATCC[c]	++
S. sanguis KTH-2		49296	ATCC[c]	+
S. sanguis KTH-3		49295	ATCC[c]	++
S. sanguis KTH-4		49297	ATCC[c]	+
S. sanguis ST3			ATCC[c]	+++
S. sanguis H.83			ATCC[c]	+++
S. mutans	c	Guy's	J. Caldwell and T. Lehner	−
S. sobrinus	d	OMZ176	B. Guggenheim	+
S. milleri		10708	NCTC	±
S. mitis		OMZ100	B. Guggenheim	−
S. mitior		10712	NCTC	++
S. bovis		8177	NCTC	−
S. salivarius		8618	NCTC	−
S. faecalis	α	21C	NCTC	+++
S. faecalis	β	4949	NCTC	+++
S. pyogenes	Group A	8198	NCTC	+++

[a] Visual assessment of the 65-kDa band.
[b] National Culture Type Collection, Public Health Laboratories, London.
[c] From Y. Mizushima (40). ATCC, American Type Culture Collection.

TABLE 3. MAbs to the 65-kDa HSP used in this investigation and their binding sites

MAb	Sequence of binding region[a]	Residues[b]	Original source	References
Y1.2	ARRGLERGLNSLADAVK	11–27	V. Mehra and B. Bloom	1, 13
C1.1	NSLADAVKVTLGPKGRNVVLEKKWGAPTITNDGVS	88–123	V. Mehra and B. Bloom	13, 38
II H9	AAGANPLGLKRGIEKA	107–122	T. Buchanan	1, 4
TB 78	—[c]	170–236	J. Ivanyi	5, 51
ML30	KAPGFGDRRKAMLQDMAILTGA	275–295	J. Ivanyi	1, 23
III E9	KLKLTGDEA	423–437	T. Gillis	1, 4
II C8	EYEDLLKAGVAD	480–493	T. Buchanan	1, 4
III C8	DPTGGMGGMDF	527–541	T. Buchanan	1, 4
IV D8	KLKLTGDEA	423–437	T. Gillis	1, 4

[a] The binding regions of the MAbs were defined by determining either the smallest peptide that inhibited binding of the MAb to the purified *M. leprae* 65-kDa protein (Y1.2, II H9, ML30, III E9, II C8, III C8, and IV D8; reference 1) or the ability of the MAbs to bind to portions of the 65-kDa protein expressed by recombinants containing only a portion of the *M. bovis* BCG gene (TB 78; reference 51) or the *M. leprae* gene (C1.1; reference 38). The precise residues involved in antibody binding were not determined.

[b] Residues are numbered as shown by Shinnick et al. (45).

[c] —, See reference 45 or 50 for sequence.

amide gel electrophoresis was run for 4 h with cooling at a constant current of 25 mA per gel. At the end of the run, the gels were cut prior to blotting. Samples were transferred to nitrocellulose paper by using the Bio-Rad Trans-Blot apparatus (52). The transfer was run for 16 h with cooling at a constant current of 100 mA, which was increased to 220 mA for the last 2 h. The nitrocellulose sheets were cut and blocked in 100 ml of phosphate-buffered saline (PBS) (pH 7.4) containing 10% dried skim milk protein (Marvel) and 0.1% NaN_3 for 5 h at room temperature on an orbital shaker. After blocking, the nitrocellulose strips were incubated with 10 ml of rabbit or mouse anti-HSP MAb diluted to between 1:100 and 1:1,000, depending on potency, and left for 16 h at 4°C with constant rocking. The diluting buffer contained 1% dried skim milk protein, 1% bovine serum albumin, 0.05% Tween 20, and 0.01% NaN_3 in PBS. The blotted strips were then washed three times in excess PBS and 0.05% Tween 20 (1 h) at room temperature on an orbital shaker. Bound MAb was developed by using 10 ml of iodinated goat anti-mouse immunoglobulin G (IgG) (Jackson Laboratory) or goat anti-rabbit IgG (Tago, TCS) diluted to 0.1 μg/ml, giving 5×10^5 cpm/ml in diluting buffer. The strips were rocked for 2 h at room temperature and then washed as before and allowed to dry at room temperature before autoradiography was carried out for 72 h. Normal rabbit serum and ascitic fluid from mice injected with NSI myeloma cells were included as negative controls for nonspecific binding of the labeled goat anti-mouse or -rabbit antibodies.

The method used with human sera for nitrocellulose blots was similar, except that the serum was diluted 1:10 in diluting buffer without 1% dried skim milk protein and filtered through a 0.8-μm-pore-size filter (Flow) and then the milk was added to 1%. The human antibodies were developed by using 20 ml of iodinated goat anti-human IgA (α chain specific; Sigma) or goat anti-human IgG (γ chain specific; Sigma) diluted to 0.1 μg/ml, giving 5×10^5 cpm/ml in diluting buffer. Autoradiography with the human antibodies was done for 24 h.

Solid-phase radioassays. IgG and IgA anti-65-kDa antibodies in human serum were assayed by a solid-phase radioassay (47). Briefly, Remova-U polystyrene wells (Dynatech Laboratories, Inc., Alexandria, Va.) were coated with 100 μl of a solution of 5 μg (dry weight) of the 65-kDa HSP per ml of PBS. The wells were then blocked with 1.0% bovine serum albumin in PBS–0.05% Tween 20–0.1% NaN_3 for 1 h at 37°C. After three washes with PBS–0.05% Tween 20, the wells were incubated in duplicate with sera diluted 1:20 and 1:40 in blocking buffer for 1.5 h at 37°C. The wells were washed three times, and bound IgG or IgA antibodies were detected by incubation for 1.5 h at 37°C with 200 μl of ^{125}I-labeled affinity-purified goat anti-human IgG or IgA at 0.1 μg/ml, giving 5×10^5 cpm. The bound ^{125}I-labeled antiserum was counted in a gamma counter (Hydragamma 16; Innotron). The results were expressed as mean percentages of radioactivity bound. Controls included in each experiment were antigen-coated wells without serum or with reference immune and control sera. The specificity of the assay was established by competitive inhibition.

The method used for radioassays with *S. sanguis* coated onto the wells was the same, except that a soluble extract from these bacteria was first prepared. Whole cells were treated with the enzyme mutanolysin (Sigma), which digests the peptidoglycan of the cell wall (16). The lysed cells were then centrifuged at 1,000 × *g* for 10 min at room temperature, and any cells still intact were pelleted and discarded. The supernatant was then centrifuged at 10,000 × *g* for 10 min at room temperature. The supernatant contained the soluble extract, and the protein concentration was determined by using Coomassie blue (Bio-Rad protein assay kit).

Statistical analysis. Antibody binding greater than the mean of healthy control sera plus 2 standard deviations was considered a significant increase in antibodies. Statistical analysis between the healthy control, BS, and disease control groups was done by using the chi-square test.

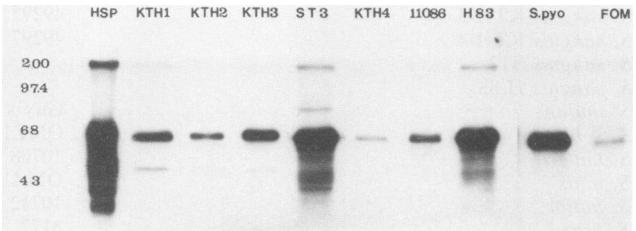

FIG. 1. Immunoblot analysis with rabbit antiserum of the 65-kDa HSP and streptococcal and oral mucosal antigens. The slots were loaded with the 65-kDa HSP; *S. sanguis* KTH-1, KTH-2, KTH-3, KTH-4, 11086, H.83, and ST3; *S. pyogenes* 8198 (S.pyo); and fetal oral mucosal antigen (FOM). Rabbit antiserum was diluted to 1 part in 500, and 1 ml was used per loaded antigen. The numbers on the left indicate molecular masses in kilodaltons.

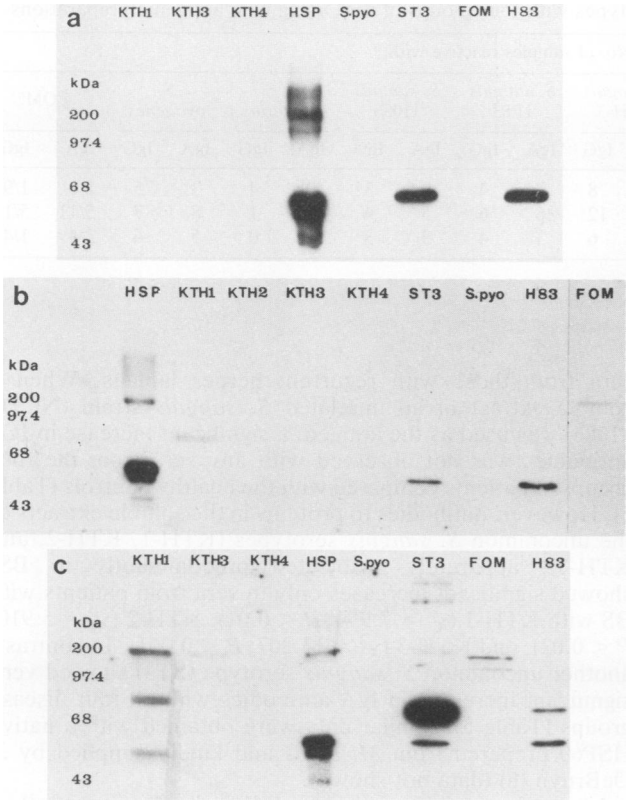

FIG. 2. Immunoblot analysis of cross-reactivity with MAbs. The slots were loaded with the 65-kDa HSP; *S. sanguis* KTH-1, KTH-2, KTH-3, KTH-4, H.83, and ST3; *S. pyogenes* 8198 (S.pyo); and fetal oral mucosal antigen (FOM). MAbs II H9 (a), C1.1 (b), and ML30 (c) were diluted to 1 part in 100 (II H9) or 1 part in 500 (C1.1 and ML30), and 1 ml of each was used per loaded antigen.

RESULTS

Immunoblot analysis with rabbit antiserum of the 65-kDa HSP and streptococcal and oral mucosal antigens. A 65-kDa band was found by blotting *S. sanguis* NCTC 11086, KTH-1 to KTH-4, ST3, and H.83 and *S. pyogenes* with polyclonal rabbit antimycobacterial HSP serum (Fig. 1; Table 2). However, *S. salivarius* failed to be blotted by the anti-HSP serum. Fetal oral mucosa also showed a band of about 65 kDa with the antiserum.

Immunoblot analysis with MAbs. The reactions were much more selective with the nine MAbs used. The strongest 65-kDa band was obtained with MAb II H9 (Fig. 2a), and a weaker band was obtained with C1.1 (Fig. 2b) against *S. sanguis* ST3 and H.83, which are associated with BS. A weak 65-kDa band resulted between MAb YI.2 and *S. pyogenes* but not the *S. sanguis* strains (data not shown). *S. sanguis* KTH-1 also reacted with MAb C1.1 (Fig. 2b) and Y1.2 to yield an 85-kDa band. MAb ML30 yielded a 65-kDa band with *S. sanguis* ST3 and H.83 and another band of about 70 kDa, which was very strong, with ST3 (Fig. 2c). KTH-1 reacted with ML30 and gave three bands of about 190, 75, and 55 kDa (Fig. 2c). MAb III C8 reacted with H.83, but the band was much weaker than that obtained with MAb II H9 (data not shown). Fetal oral mucosa reacted with MAbs Y1.2, C1.1, ML30, and IV D8, yielding bands of about 100 and 190 kDa (Fig. 2b and c). The other three MAbs to the

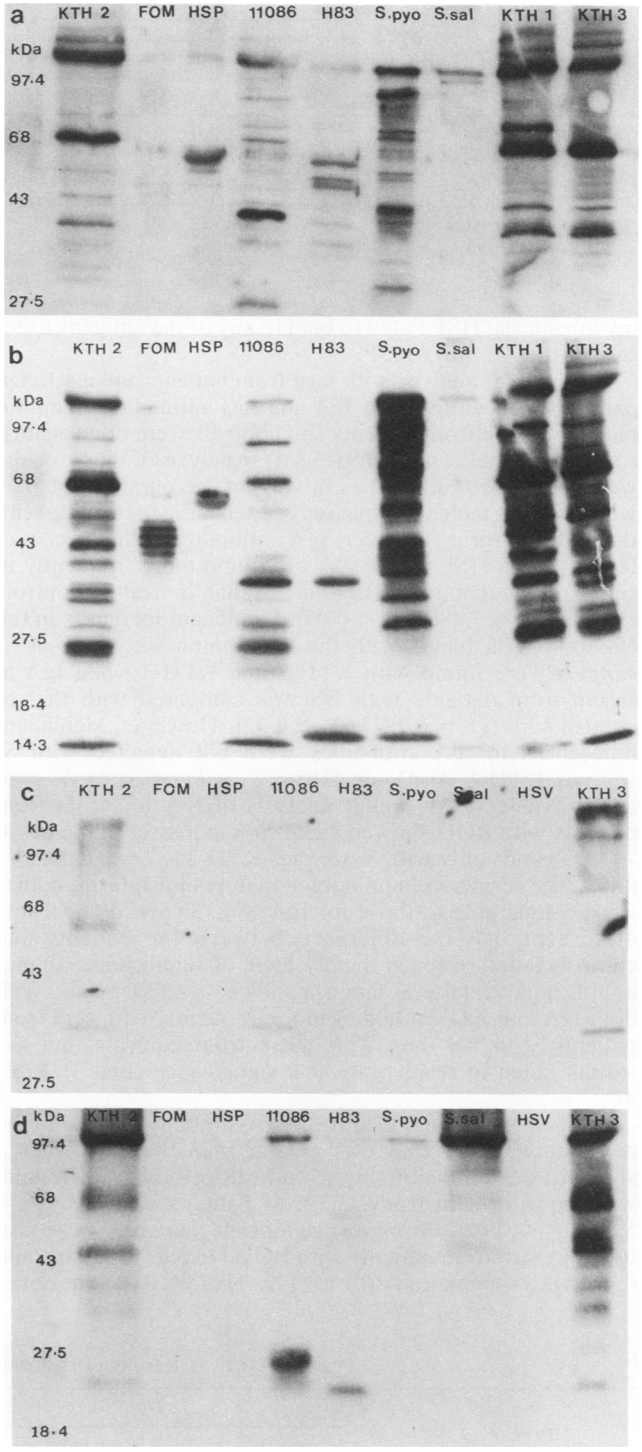

FIG. 3. Immunoblot analysis with human sera and a selected panel of antigens for IgA and IgG antibodies. The slots were loaded with the 65-kDa HSP; *S. sanguis* KTH-2, KTH-3, 11086, and H.83; *S. pyogenes* 8198 (S.pyo); *S. salivarius* 8618 (S.sal); herpes simplex virus type 1 McIntyre strain (HSV); and fetal oral mucosal antigen (FOM). Panels: a and b, respectively, IgA and IgG antibody reactions of serum from a patient with BS; c and d, respectively, control serum IgA and IgG antibody reactions.

TABLE 4. Western blot reactivities of 65- to 70-kDa IgA and IgG isotypes with four groups of sera using nine antigenic preparations

Serum source	No. of samples	No. of samples reactive with:																	
		S. sanguis HSP		*S. sanguis* KTH-1		*S. sanguis* KTH-2		*S. sanguis* KTH-3		*S. sanguis* H.83		*S. sanguis* 11086		*S. salivarius*		*S. pyogenes*		FOM[a]	
		IgA	IgG	IgA	IgG	IgA	IgG	IgA	IgG	IgA	IgG	IgA	IgG	IgA	IgG	IgA	IgG	IgA[b]	IgG[b]
Controls	10	3	7	8/9	8/10	6	8	6	8	2	4	0	3	0	4	3	5	2/9	1/9
Patients with BS	13	9	12	13	12	13	13	13	12	6	6	5	4	2	1	8	7	5/11	5/11
Patients with ROU	6	2	5	6	6	4	6	6	6	3	4	3	3	1	0	5	6	2/4	1/4

[a] FOM, Fetal oral mucosa.
[b] 43-kDa band only.

mycobacterial HSP failed to bind to any of the antigens used, except the *M. bovis* HSP.

Immunoblot analysis with sera from patients and a selected panel of seven antigens for IgA and IgG antibodies. Immunoblots with sera from patients and controls were done against a selected panel of antigens (Fig. 3). Analysis of these results was fraught with difficulties in view of the number of bands with different molecular masses of each antigen reacting with the different sera. However, IgA antibodies to the mycobacterial 65-kDa HSP (Table 4) were found more frequently in sera from patients with BS (9 of 13) than in healthy controls (3 of 10; $\chi^2 = 3.4862$; $P < 0.05$). Significant increases in the 65- to 70-kDa bands with the uncommon serotypes of *S. sanguis* were found with KTH-2 and KTH-3 when IgA in serum from patients with BS was compared with that of control sera ($\chi^2 = 6.2947$; $P < 0.02$). However, significant differences in IgA antibodies were not detected with *S. sanguis* KTH-1, H.83, or 11086; *S. salivarius*; or *S. pyogenes* (Table 4). A similar analysis of IgA in serum from patients with ROU showed significant increases in the 65- to 70-kDa bands only with *S. sanguis* KTH-3 ($\chi^2 = 5.7600$; $P < 0.02$). The results of immunoblot analyses for IgG antibodies were comparable to those for IgA (Fig. 3b and d and Table 4), except that the difference between the patients and controls failed to reach the 5% level of significance. Immunoblotting of fetal oral mucosa showed a 43-kDa band with both IgA and IgG antibodies more frequently with sera from patients with BS than with those from controls, but the results failed to reach statistical significance (Fig. 3; Table 4).

Antibodies to the 65-kDa HSP. IgA antibody binding to the recombinant HSP that was greater than the mean plus 2 standard deviations of sera from healthy subjects (Table 5) was found in sera from 23.3% of patients with BS ($\chi^2 = 5.567$; $P < 0.02$). However, significant increases were also found in sera from patients with ROU (26.5%; $P < 0.01$) and those with rheumatoid arthritis (29.3%; $P < 0.01$) but not in sera from those with recurrent herpes labialis. When a soluble extract of an unrelated *S. sanguis* strain (NCTC 11086) was used as the antigen, a significant increase in IgA antibodies was not observed with any sera from the four groups of patients compared with the healthy controls (Table 5). However, antibodies to proteins in the soluble extracts of the uncommon *S. sanguis* serotypes (KTH-1, KTH-2, and KTH-3), apparently associated predominantly in BS, showed significant increases only in sera from patients with BS with KTH-1 ($\chi^2 = 7.954$; $P < 0.01$), KTH-2 ($\chi^2 = 5.910$; $P < 0.02$), and KTH-3 ($\chi^2 = 13.207$; $P < 0.001$). In contrast, another uncommon *S. sanguis* serotype (ST3) showed very significant increases in IgA antibodies with all four disease groups (Table 5). Similar data were obtained with a native HSP60 prepared from *M. bovis* and kindly supplied by J. DeBruyn (6) (data not shown).

In sera from healthy subjects, IgG antibodies greater then the mean plus 2 standard deviations were found less frequently than IgA antibodies at that level (Table 6). The 5% level of significance was reached only with the HSP in 17.7% of sera from patients with BS. However, none of the four uncommon *S. sanguis* strains or NCTC 11086 showed a significant increase in IgG antibodies in patients with BS (Table 6). Surprisingly, a very significant increase in IgG anti-ST3 antibodies was found with sera from patients with rheumatoid arthritis ($\chi^2 = 12.233$; $P < 0.001$) and, to a lesser extent, KTH-3 ($\chi^2 = 4.755$; $P < 0.05$).

DISCUSSION

Recently, four species of streptococci, *S. pyogenes*, *S. sanguis*, *S. faecalis*, and *S. salivarius*, have been implicated in the etiology of BS (40). We have raised the possibility that a common streptococcal antigen is involved. Indeed, an approximately 65-kDa protein was found on Western blots (Fig. 1) between rabbit antiserum to the 65-kDa mycobacterial HSP and *S. sanguis* serotypes I and II, as well as the

TABLE 5. Results of radioassays for IgA antibodies in human sera

Disease	No. with results greater than control mean + 2 SD, total no. tested (% positive)					
	65-kDa HSP	*S. sanguis* NCTC-11086	*S. sanguis* KTH-1	*S. sanguis* KTH-2	*S. sanguis* KTH-3	*S. sanguis* ST3
None (healthy controls)	3, 51 (5.9)	3, 52 (5.8)	2, 30 (6.7)	3, 50 (6.0)	2, 51 (3.9)	1, 24 (4.1)
Recurrent herpes	0, 8 (0)	0, 8 (0)	0, 6 (0)	0, 8 (0)	0, 7 (0)	4, 5 (80)[c]
ROU	9, 34 (26.5)[a]	0, 32 (0)	0, 24 (0)	3, 24 (12.5)	3, 31 (9.7)	7, 19 (36.8)[b]
BS	14, 60 (23.3)[b]	5, 61 (8.2)	11, 30 (36.7)[a]	14, 62 (22.6)[b]	19, 62 (30.6)[c]	12, 24 (50.0)[c]
Rheumatoid arthritis	12, 41 (29.3)[a]	7, 38 (18.4)	3, 30 (10.0)	2, 39 (5.1)	3, 39 (7.7)	12, 24 (50.0)[c]

[a] $P < 0.01$.
[b] $P < 0.02$.
[c] $P < 0.001$.

TABLE 6. Results of radioassays for IgG antibodies in human sera

Disease	No. with results greater than control mean + 2 SD, total no. tested (% positive)					
	65-kDa HSP	*S. sanguis* NCTC-11086	*S. sanguis* KTH-1	*S. sanguis* KTH-2	*S. sanguis* KTH-3	*S. sanguis* ST3
None (healthy controls)	3, 53 (5.7)	5, 53 (9.4)	1, 30 (3.3)	2, 53 (3.8)	3, 40 (7.5)	1, 23 (4.3)
Recurrent herpes	0, 7 (0)	1, 6 (16.0)	0, 6 (0)	7, 0 (7.0)	1, 8 (12.5)	1, 5 (20.0)
ROU	4, 31 (12.9)	5, 17 (29.4)	1, 24 (4.2)	0, 31 (0)	6, 32 (18.8)	2, 19 (11.5)
BS	11, 62 (17.7)[a]	11, 61 (18.0)	3, 30 (10.0)	8, 62 (12.9)	9, 50 (18.0)	2, 24 (8.3)
Rheumatoid arthritis	6, 38 (15.8)	1, 18 (5.6)	1, 30 (3.3)	0, 38 (0)	8, 30 (26.7)[a]	12, 24 (50.0)[b]

[a] $P < 0.05$.
[b] $P < 0.001$.

uncommon strains KTH-2, KTH-4, ST3, and H.83, which are thought to be involved in BS (39). Examination of nine MAbs to the 65-kDa mycobacterial HSP revealed that MAbs C1.1, II H9, and ML30 yielded 65-kDa bands with *S. sanguis* ST3 and H.83 (Fig. 2). These MAbs are directed against residues 88 to 123, 107 to 122, and 275 to 295, respectively, of the 65-kDa protein (1). *S. sanguis* KTH-1 reacted with MAb Y1.2, which recognizes residues 11 to 27 (38), and MAbs C1.1 and ML30 (Fig. 2b and c). *S. pyogenes* reacted with MAb Y1.2, suggesting that the cross-reactivities between the different species of streptococci invoked in the etiology of BS and the 65-kDa stress protein occur at different portions of the 540-amino-acid peptide. Although some of the other streptococci, such as *S. sanguis* KTH-2 and KTH-4, showed a 65-kDa band with rabbit anti-65-kDa protein serum, this was not detected with any of the nine MAbs. These MAbs recognize only about 30% of the entire length of the 65-kDa HSP, so that the streptococcal antigens may share some of the remaining 70% of the residues of the 65-kDa protein and, indeed, other stress proteins.

Significantly raised IgA antibodies to the recombinant 65-kDa mycobacterial HSP and the soluble extracts of *S. sanguis* ST3, KTH-1, KTH-2, and KTH-3 were found in patients with BS. However, as expected, significantly raised IgA antibodies to the HSP were also found in sera from patients with rheumatoid arthritis and ROU (Table 5). Surprisingly, IgA antibodies to *S. sanguis* ST3 were also significantly increased in patients with rheumatoid arthritis and ROU but those to KTH-1, KTH-2, and KTH-3 were not. These findings suggest that IgA antibodies to *S. sanguis* KTH-1, KTH-2, and KTH-3 are significantly elevated only in patients with BS, although the proportions were rather low, reaching only 36.7, 22.6, and 30.6%, respectively (Table 5). Immunoblot analysis confirmed that a band of 65 to 70 kDa was found with KTH-2 or KTH-3 more frequently with IgA in sera from patients with BS than with sera from controls ($P < 0.02$). It should be noted that finding the 65- to 70-kDa bands by blotting *S. sanguis* with sera of patients with BS does not establish that these are HSPs.

The results obtained with IgG antibodies were less striking, and only sera from patients with BS showed elevated anti-65-kDa protein antibodies, reaching the 5% level of significance (Table 6). The four uncommon serotypes of *S. sanguis* failed to yield significant increases in IgG antibodies with sera from patients with BS, but surprisingly, ST3 ($P < 0.001$) and, to a lesser extent, KTH-3 ($P < 0.05$) showed elevated titers with sera from patients with rheumatoid arthritis (Table 6). Immunoblot analysis also showed a greater frequency of 65- to 70-kDa bands with IgG in sera from patients with BS than in those of controls, although this failed to reach the 5% level of significance.

It is of interest that the 65-kDa antigen appears to be involved in rheumatoid arthritis, as raised levels of antibodies in serum have been recorded (53) and recently a T-cell clone that reacted to the 65-kDa antigen was isolated from the synovial fluid of a patient with rheumatoid arthritis (18). Indeed, in adjuvant arthritis in rats induced by the mycobacterial antigen, T-cell clones can passively transfer the disease (19). However, one of the proliferative T-cell epitopes was found within residues 180 to 188 of the 65-kDa peptide of *M. bovis* (55), which is some distance from the *S. sanguis* B-cell epitopes in residues 88 to 123, 107 to 122, and 276 to 297. The nearest MAb to residues 180 to 188 is TB 78 (residues 170 to 236), but this failed to react with any of the *S. sanguis* strains tested. However, this does not rule out the possibility that residues 180 to 188 are shared at the T-cell level. The proliferative responses of human T cells from patients with BS are significantly raised when stimulated with the 65-kDa recombinant protein (4a), but the short peptides have not been investigated. A very recent report on group A streptococcal cell wall-induced arthritis in rats also presented evidence of T-cell-based cross-reactivity with *M. tuberculosis* antigen (43) and showed that pretreatment with the 65-kDa protein prevented the arthritis (54).

Earlier investigations of BS reported autoimmune responses to oral mucosal homogenates (8, 29, 41, 44). In view of the high degree of homology between microbial and human cellular HSPs (24), we examined the possibility that the findings of autoimmunity can be interpreted on the basis of a cross-reacting determinant. Indeed, a 65-kDa band was found with polyclonal anti-65-kDa protein serum and among the MAbs, Y1.2, C1.1, ML30, and IV D8 each showed one or two discrete bands of about 100 or 190 kDa with the fetal mucosa (Fig. 1 and 2). It is noteworthy that MAb Y1.2 shows about 60% cross-reactivity between the human mitochondrial P1 antigen and the 65-kDa stress proteins (10) and MAb ML30 recognizes human mitochondria (14). These findings raise the possibility that molecular cross-reactivity between microbial stress proteins and fetal oral mucosal antigens may account for the early reports in the literature of autoimmune responses in this disease. Although the function of stress proteins in BS, as in other diseases, needs further investigation, the homology between stress proteins and chaperonins (17) suggests that they facilitate posttranslational assembly of oligomeric proteins.

It should be emphasized that while MAbs to the mycobacterial HSP reacted with selected *S. sanguis* strains, suggesting that they contain cross-reactive determinants of HSPs, this has not been formally established. Similarly, finding 65- to 70-kDa bands by blotting *S. sanguis* extracts with sera from patients with BS does not indicate that the patients had antibodies to *S. sanguis* heat HSPs. Similar-sized antigens

with conserved specificities but differences in structure and function may exist. We will now attempt to isolate HSPs from the streptococci and oral mucosa to show that these are cross-reactive with the mycobacterial HSPs.

ACKNOWLEDGMENTS

Some of this investigation was carried out under a grant from the Arthritis and Rheumatism Council. Financial support was also received from the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases.

REFERENCES

1. **Anderson, D. C., M. E. Barry, and T. M. Buchanan.** 1988. Exact definition of species-specific and cross-reactive epitopes of the 65-kilodalton protein of *Mycobacterium leprae* using synthetic peptides. J. Immunol. **141**:607–613.
2. **Bonas, W. A., J. A. Bird-Stewart, M. A. Chamberlain, and I. W. Halliburton.** 1986. Molecular studies in Behcet's syndrome, p. 37–41. *In* T. Lehner and C. G. Barnes (ed.), Recent advances in Behcet's disease. Royal Society of Medicine Services, London.
3. **Britton, W. J., L. Hellqvist, A. Basten, and R. L. Raison.** 1985. *Mycobacterium leprae* antigens involved in human immune responses. I. Identification of four antigens by monoclonal antibodies. J. Immunol. **135**:4171–4177.
4. **Buchanan, T. M., H. Nomaguchi, D. C. Anderson, R. A. Young, T. P. Gillis, W. J. Britton, J. Ivanyi, A. H. Kolk, O. Closs, B. R. Bloom, and V. Mehra.** 1987. Characterization of antibody-reactive epitopes on the 65-kilodalton protein of *Mycobacterium leprae*. Infect. Immun. **55**:1000–1003.

4a. **Childerstone, A., and T. Lehner.** Unpublished data.

5. **Coates, A. R. M., B. W. Allen, J. Hewitt, J. Ivanyi, and D. A. Mitchison.** 1981. Antigenic diversity of *Mycobacterium tuberculosis* and *Mycobacterium bovis* detected by means of monoclonal antibodies. Lancet **ii**:167–169.
6. **De Bruyn, J., R. Bosmans, M. Turner, M. Weckx, J. Nyabenda, J.-P. van Vooren, P. Falmagne, H. G. Wiker, and M. Harboe.** 1987. Purification, partial characterization, and identification of a skin-reactive protein antigen of *Mycobacterium bovis* BCG. Infect. Immun. **55**:245–252.
7. **Denman, A. M., P. J. Fialkow, B. K. Pelton, A. C. Salo, D. J. Appleford, and C. Gilchrist.** 1980. Lymphocyte abnormalities in Behcet's syndrome. Clin. Exp. Immunol. **42**:175–185.
8. **Dolby, A. I.** 1969. Recurrent aphthous ulceration: effect of sera and peripheral blood upon epithelial tissue culture cells. Immunology **17**:709–713.
9. **Dubois, P.** 1989. Heat shock proteins and immunity. Res. Immunol. **140**:653–659.
10. **Dudani, A. K., and R. S. Gupta.** 1989. Immunological characterization of a human homolog of the 65-kilodalton mycobacterial antigen. Infect. Immun. **57**:2786–2793.
11. **Eglin, R. P., T. Lehner, and J. H. Subak-Sharpe.** 1982. Detection of RNA complementary to herpes simplex virus in mononuclear cells from patients with Behcet's syndrome and recurrent oral ulcers. Lancet **ii**:1356–1361.
12. **Emmrich, F., J. Thole, J. van Embden, and S. H. E. Kaufmann.** 1986. A recombinant 64 kilodalton protein of *Mycobacterium bovis* bacillus Calmette-Guerin specifically stimulates human T4 clones reactive to mycobacterial antigens. J. Exp. Med. **163**: 1024–1029.
13. **Engers, H. D., M. Abe, B. R. Bloom, V. Mehra, W. Britton, T. M. Buchanan, S. K. Khanolkar, D. B. Young, O. Closs, T. Gillis, M. Herboe, J. Ivanyi, A. H. J. Kolk, and C. C. Shepard.** 1985. Results of a World Health Organization-sponsored workshop on monoclonal antibodies to *Mycobacterium leprae*. Infect. Immun. **48**:603–605. (Letter.)
14. **Evans, D., P. Norton, and J. Ivanyi**, submitted for publication.
15. **Gillis, T. P., R. A. Miller, D. B. Young, S. F. Khanolkar, and T. M. Buchanan.** 1985. Immunochemical characterization of a protein associated with *Mycobacterium leprae* cell wall. Infect. Immun. **49**:371–377.
16. **Hamada, S., M. Torii, S. Kotani, N. Masuda, T. Ooshima, K. Yokogawa, and S. Kawata.** 1978. Lysis of *Streptococcus mutans* cells with mutanolysin, a lytic enzyme prepared from a culture liquor of *Streptomyces globisporus* 1829. Arch. Oral Biol. **23**:543–549.
17. **Hemmingsen, S. M., C. Woolford, S. M. van der Vies, K. Tilly, D. T. Dennis, C. P. Georgopoulos, R. W. Hendrix, and R. J. Ellis.** 1988. Homologous plant and bacterial proteins chaperone oligomeric protein assembly. Nature (London) **33**:330–334.
18. **Holoshitz, J., F. Koning, J. E. Coligan, J. De Bruyn, and S. Strobert.** 1989. Isolation of $CD4^-$ $CD8^-$ mycobacteria-reactive T lymphocyte clones from rheumatoid arthritis synovial fluid. Nature (London) **339**:226–229.
19. **Holoshitz, J., A. Matitiau, and I. R. Cohen.** 1984. Arthritis induced in rats by cloned T lymphocytes responsive to mycobacteria but not to collagen type II. J. Clin. Invest. **73**:211–215.
20. **Holoshitz, J., Y. Naparstek, A. Ben-Nun, and I. R. Cohen.** 1983. Lines of T lymphocytes induce or vaccinate against autimmune arthritis. Science **219**:56–58.
21. **Isogai, E., et al.** 1990. Close association of Streptococcus sanguis uncommon serotypes with Behcet's disease. Bifidobact. Microflora **9**:27–41.
22. **Ivanyi, J., K. Sharp, P. Jackett, and G. Bothamley.** 1988. Immunological study of the defined constituents of mycobacteria. Springer Semin. Immunopathol. **10**:279–300.
23. **Ivanyi, J., S. Sinha, R. Aston, D. Cussell, M. Keen, and U. Sengupta.** 1983. Definition of species specific and cross-reactive antigenic determinants of *Mycobacterium leprae* using monoclonal antibodies. Clin. Exp. Immunol. **52**:528–536.
24. **Jindal, S., A. K. Dudani, B. Singh, C. B. Harley, and R. S. Gupta.** 1989. Primary structure of a human mitochondrial protein homologous to the bacterial and plant chaperonins and to the 65-kilodalton mycobacterial antigen. J. Immunol. **9**:2279–2283.
25. **Kaneko, F., Y. Takahashi, Y. Muramatsu, and Y. Miura.** 1985. Immunological studies on aphthous ulcer and erythema nodosum-like eruptions in Behcet's disease. Br. J. Dermatol. **113**:303–312.
26. **Kaufmann, S. H. E., U. Vath, J. E. R. Thole, J. D. A. van Embden, and F. Emmrich.** 1987. Enumeration of T cells reactive with *Mycobacterium tuberculosis* organisms and specific for the recombinant mycobacterial 64-kDa protein. Eur. J. Immunol. **17**:351–357.
27. **Lamb, J. R., V. Bal, P. Mendez-Sampirio, A. Mehlert, A. So, J. Rothbard, S. Jindal, and D. B. Young.** 1989. Stress proteins may provide a link between the immune response to infection and autoimmunity. Int. Immunol. **1**:191–196.
28. **Lamb, J. R., J. Ivanyi, A. D. M. Rees, R. A. Young, and D. Young.** 1986. The identification of T-cell epitopes in *Mycobacterium tuberculosis* using human T lymphocyte clones. Lepr. Rev. **57**(Suppl. 2):131–137.
29. **Lehner, T.** 1964. Recurrent aphthous ulceration and autoimmunity. Lancet **ii**:1154–1155.
30. **Lehner, T.** 1967. Behcet's syndrome and autoimmunity. Br. Med. J. **i**:465–467.
31. **Lehner, T.** 1968. Autoimmunity in oral diseases with special reference to recurrent oral ulceration. Proc. R. Soc. Med. **61**:515–519.
32. **Lehner, T.** 1979. Immunopathology of Behcet's syndrome, p. 127–139. *In* T. Lehner and C. G. Barnes (ed.), Behcet's syndrome. Academic Press, Inc. (London), Ltd., London.
33. **Lehner, T.** 1986. The role of a disorder in immunoregulation, associated with herpes simplex virus type 1 in Behcet's disease, p. 31–36. *In* T. Lehner and C. G. Barnes (ed.), Recent advances in Behcet's disease. Royal Society of Medicine Services, London.
34. **Lehner, T., et al.** Recent advances in T cell immunoregulation and in the microbial etiology of Behcet's disease, *In* J. D. O'Duffy and R. Rogers, ed., *5th International Conference on Behcet's Disease, Mayo Clinic*, in press.
35. **Lehner, T., and J. R. Batchelor.** 1979. Classification and an immuno-genetic basis of Behcet's syndrome, p. 13–32. *In* T. Lehner and C. G. Barnes (ed.), Behcet's syndrome; clinical and

immunological features. Academic Press, Inc. (London), Ltd., London.
36. **Levis, W. R., H. C. Meeker, G. B. Schuller-Levis, T. P. Gillis, L. J. Marino, Jr., and J. Zabriskie.** 1986. Serodiagnosis of leprosy: relationships between antibodies to *Mycobacterium leprae* phenolglycolipid I and protein antigens. J. Clin. Microbiol. **24**:917–921.
37. **Lindquist, S., and E. A. Craig.** 1988. The heat-shock proteins. Annu. Rev. Genet. **22**:631–677.
38. **Mehra, V., D. Sweetser, and R. A. Young.** 1986. Efficient mapping of protein antigenic determinants. Proc. Natl. Acad. Sci. USA **83**:7013–7017.
39. **Mizushima, Y.** 1988. Recent research into Behcet's disease in Japan. Int. J. Tissue React. **X(2)**:59–65.
40. **Mizushima, Y.** 1989. Skin hypersensitivity to streptococcal antigens and the induction of systemic symptoms by the antigens in Behcet's disease—a multicenter study. J. Rheumatol. **16**(4):506–511.
41. **Oshima, Y., T. Shimizu, R. Yokohari, T. Matsumoto, K. Kano, T. Kagami, and H. Nagaya.** 1963. Clinical studies in Behcet's syndrome. Ann. Rheum. Dis. **22**:36–45.
42. **Polla, B. S.** 1988. A role of heat shock proteins in inflammation? Immunology Today **9**:134–137.
43. **Quinn DeJoy, S., K. M. Ferguson, T. M. Sapp, J. B. Zabriskie, A. L. Oronsky, and S. S. Kerwar.** 1989. Streptococcal cell wall arthritis. J. Exp. Med. **170**:369–382.
44. **Rogers, R., W. Sams, and R. Shorter.** 1974. Lymphocytotoxicity in recurrent aphthous stomatitis. Arch. Dermatol. **109**:361–363.
45. **Shinnick, T. M., D. Sweetser, J. Thole, J. van Embden, and R. A. Young.** 1987. The etiologic agents of leprosy and tuberculosis share an immunoreactive protein antigen with the vaccine strain *Mycobacterium bovis* BCG. Infect. Immun. **55**:1932–1935.
46. **Shinnick, T. M., M. H. Vodkin, and J. C. Williams.** 1988. The *Mycobacterium tuberculosis* 65-kilodalton antigen is a heat shock protein which corresponds to common antigen and to the *Escherichia coli* GroEL protein. Infect. Immun. **56**:446–451.
47. **Smith, R., and T. Lehner.** 1981. A radioimmunoassay for serum and gingival crevicular fluid antibodies to a purified protein of *Streptococcus mutans*. Clin. Exp. Immunol. **43**:417–424.
48. **Thole, J. E. R., H. G. Dauwerse, P. K. Das, D. G. Groothuis, L. M. Schouls, and J. D. A. van Embden.** 1985. Cloning of *Mycobacterium bovis* BCG DNA and expression of antigens in *Escherichia coli*. Infect. Immun. **50**:800–806.
49. **Thole, J. E. R., P. Hindersson, J. de Bruyn, F. Cremers, J. van der Zee, H. de Cock, J. Tommassen, W. van Eden, and J. D. A. van Embden.** 1988. Antigenic relatedness of a strongly immunogenic 65kDa mycobacterial protein antigen with a similarly sized ubiquitous bacterial common antigen. Microb. Pathog. **4**:71–83.
50. **Thole, J. E. R., W. J. Keulen, A. H. J. Kolk, D. G. Groothuis, L. G. Berwald, R. H. Tiesjema, and J. D. A. van Embden.** 1987. Characterization, sequence determination, and immunogenicity of a 64-kilodalton protein of *Mycobacterium bovis* BCG expressed in *Escherichia coli* K-12. Infect. Immun. **55**:1466–1475.
51. **Thole, J. E. R., W. C. A. van Schooten, W. J. Keulen, P. W. M. Hermans, A. A. M. Janson, R. R. P. de Vries, A. H. J. Kolk, and J. D. A. van Embden.** 1988. Use of recombinant antigens expressed in *Escherichia coli* K-12 to map B-cell and T-cell epitopes on the immunodominant 65-kilodalton protein of *Mycobacterium bovis* BCG. Infect. Immun. **56**:1633–1640.
52. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76**:4350–4354.
53. **Tsoulfa, F., G. A. W. Rook, J. D. A. van Embden, D. B. Young, A. Mehlert, D. A. Isenberg, F. C. Hay, and P. M. Lydyard.** 1989. Raised serum IgG and IgA antibodies to mycobacterial antigens in rheumatoid arthritis. Ann. Rheum. Dis. **48**:118–123.
54. **van den Brock, M. F., J. M. Hogervorst, M. J. C. van Bruggen, W. van Eden, R. van der Zee, and W. B. van den Berg.** 1989. Protection against streptococcal cell wall-induced arthritis by pretreatment with the 65kD mycobacterial heat shock protein. J. Exp. Med. **170**:446–449.
55. **van Eden, W., J. E. R. Thole, R. van der Zee, A. Noordzij, J. D. A. van Embden, E. J. Hensen, and I. R. Cohen.** 1988. Cloning of the mycobacterial epitope recognized by T lymphocytes in adjuvant arthritis. Nature (London) **331**:171–173.
56. **Winfield, J. B.** 1989. Stress proteins, arthritis and autoimmunity. Arthritis Rheum. **32**:1497–1504.
57. **Young, D. B., R. Lathigra, R. Hendrix, D. Sweetser, and R. A. Young.** 1988. Stress proteins are immune targets in leprosy and tuberculosis. Proc. Natl. Acad. Sci. USA **85**:4267–4270.