

Humoral Immune Responses of Type 1 Diabetes Patients to *Mycobacterium avium* subsp. *paratuberculosis* Lend Support to the Infectious Trigger Hypothesis[∇]

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***Mycobacterium avium* subsp. *paratuberculosis* is a zoonotic pathogen whose association with Crohn's disease in humans is under scrutiny. The objective of this work was to investigate its association with other chronic diseases such as type 1 diabetes mellitus (T1DM), where the involvement of a persistent pathogen such as *M. avium* subsp. *paratuberculosis* could be the trigger. For this purpose, 59 diabetic patients and 59 healthy controls were investigated for the presence of antibodies against two recombinant proteins of *M. avium* subsp. *paratuberculosis* and the whole-cell lysate. Extremely significant humoral immune responses to recombinant heparin binding hemagglutinin and glycosyl transferase proteins and the whole-cell lysates of *M. avium* subsp. *paratuberculosis* bacilli were observed in T1DM patients and compared to those of healthy controls. Finding evidence of *M. avium* subsp. *paratuberculosis* involvement in T1DM is perhaps a novel finding that might serve as a foundation stone in establishing an infectious etiology for T1DM.**

Immune-related disorders are frequently rampant in both developed and developing countries. It is speculated that such diseases probably reflect and connect to long-term effects of a change in lifestyle and thereby a reduced exposure to certain bacteria that have been inherently associated with human societies during most of mammalian evolution (3, 17). A very important group of bacteria among these organisms is saprophytic mycobacteria, which trigger regulatory immune cell populations (3, 17) such as cytokine-secreting and antigen-presenting cells. These immune cell populations are probably the deterrent to some autoimmune diseases such as type 1 (insulin-dependent) diabetes mellitus (T1DM).

T1DM constitutes interactions of polygenic traits with not-well-known environmental factors, and it is not known what triggers autoimmunity to self-antigens such as those expressed in the pancreatic islets of Langerhans cells (5, 11). Drinking of cow's milk in childhood is assumed to be a risk factor for the development of this disease (17). The role of mycobacterial proteins that cross-react with epitopes of human cell surface molecules has been explored (3, 5).

Human populations that lived hygienic lifestyles and therefore remained "sanitized" for decades might react aggressively to exposure to certain microbial communities such as *Mycobacterium avium* subsp. *paratuberculosis*. Due to the expansion of the dairy industry in developed countries as a result of modern animal breeding, the exposure of human populations to *M. avium* subsp. *paratuberculosis* has increased. Sardinia, Italy, is one such example, where intensive sheep farming is

practiced and the sheep population constitutes more than four times the existing human population of this Mediterranean island.

M. avium subsp. *paratuberculosis* bacilli have been notoriously known to trigger molecular mimicry (15, 23). It has long been a belief that genetic susceptibilities, epitope homologies, and endemic bacterial load in the environment might support the case for an infectious trigger, such as *M. avium* subsp. *paratuberculosis*, to be a causative agent of T1DM in genetically susceptible individuals (3, 8, 11, 17). However, their direct association with T1DM has remained largely elusive. Recent attempts (21) have been directed at the demonstration of clinically significant loads of *M. avium* subsp. *paratuberculosis* DNA in the blood of diabetes patients. However, it is essential to unravel the interaction of *M. avium* subsp. *paratuberculosis* bacilli with the host immune system to know if they are directly involved in the disease process.

We attempted to test the association of *M. avium* subsp. *paratuberculosis* with T1DM in an endemic setting like Sardinia and demonstrate for the first time the presence of clinically significant humoral responses of T1DM patients to recombinant *M. avium* subsp. *paratuberculosis* antigens and whole-cell lysates.

MATERIALS AND METHODS

A total of 118 participants, comprised of 59 patients with T1DM and 59 healthy controls, were previously tested for the presence of the *M. avium* subsp. *paratuberculosis*-specific IS900 signature using total DNA extracted from peripheral blood mononuclear cells (21). Informed consents from patients, including other necessary clearances, were obtained before blood samples were drawn. Patient details are shown in Table 1.

Briefly, blood from patients was centrifuged, and serum supernatants were used for enzyme-linked immunosorbent assay (ELISA); the remaining sera were aliquoted and stored frozen at -20°C for short-term storage (<6 months) and -80°C for long-term storage (>6 months).

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TABLE 1. Clinical characteristics and results of IS900 PCR testing for *M. avium* subsp. *paratuberculosis* in participants^a

Group and patient	Sex	Age (yr)	Yr of diagnosis	Family history of diabetes (type)	Seropositivity for:			PCR LIZ/AV (294 bp)
					<i>M. avium</i> subsp. <i>paratuberculosis</i> lysate (>0.6)	<i>M. avium</i> subsp. <i>paratuberculosis</i> HBHA (>0.5)	<i>M. avium</i> subsp. <i>paratuberculosis</i> GSD (>0.4)	
Diabetic								
01	M	21	1999		++	+++	-	+
02	F	31	1996		+++	++	+	-
03	F	28	1985		++	++	-	+
04	M	36	1982	I	++	-	+	+
05	M	41	1989	II	+	-	-	-
06	F	36	1997		+++	+	-	+
07	M	37	1996		+	+++	-	+
08	M	26	2005	II	+++	+++	+	+
09	M	30	1996	I/II	+	++	-	+
10	F	37	1998	I	+	++	-	+
11	M	35	1985	I	++	+++	-	+
12	F	37	1970	I	+	+++	-	-
13	F	27	1989	I	+++	+++	+	-
14	M	31	1979	I	+++	+++	+	+
15	M	40	2005	I	+	-	-	-
16	M	18	2003	I	-	-	-	-
17	M	38	1980		+	-	-	+
18	F	37	2005	I	+	-	-	+
19	F	28			+	+	-	+
20	M	26	2002	I	+++	+++	+	+
21	F	35			++	+	-	+
22	F	40	1996	II	++	-	-	+
23	F	28	1997	II	+	+++	+	+
24	F	34			-	-	-	-
25	M	41	1996	I	+	++	+	+
26	F	36	1994	I	-	-	-	+
27	M	38			-	+	-	-
28	F	39	2002	I	++	-	-	-
29	F	37	1988		+	++	+	-
30	F	32	1989		-	+	-	-
31	M	43	1996		-	-	-	-
32	F	33	1994	I	-	-	-	-
33	F	33			+++	+++	++	-
34	M	38	2002		-	-	-	-
35	M	33	1988		-	-	-	-
36	M	32	1976		-	-	-	+
37	M	26	1994	II	-	-	-	+
38	F	32	1998	I	+	++	+	+
39	F	38			-	++	+	+
40	M	34	1989		-	+	+	+
41	M	22	1989	I	+	+	+	+
42	M	41	1978	I	-	-	++	-
43	M	94			+	+++	+++	+
44	M	36			-	++	++	+
45	M	27	2004	I	-	+++	+++	+
46	M	33	1995	I	-	-	+	+
47 FR	M	28	2000	I	-	+	+++	+
48 FR	F	33	1983		-	-	+	+
49 FR	F	23	1984	I	-	++	+	+
50 FR	M	44	1989		-	-	-	+
51 FR	M	43	1986	II	-	++	++	+
52 FR	M	34			+	+++	+++	+
53 FR	F	59	1979	I	-	-	+	+
54 FR	F	39			-	-	-	+
55 FR	F	42	1967	I	-	-	-	+
56 FR	F	39	1981	I	+	++	+	+
57 FR	F	47	1966	II	-	-	-	+
58 FR	M	57	1995	I	-	-	-	-
59 FR	M	38	1973	I	+	-	+++	-
Control								
01C	F	33			-	-	-	+
02C	F	43			-	-	-	-

Continued on following page

TABLE 1—Continued

Group and patient	Sex	Age (yr)	Yr of diagnosis	Family history of diabetes (type)	Seropositivity for:			
					<i>M. avium</i> subsp. <i>paratuberculosis</i> lysate (>0.6)	<i>M. avium</i> subsp. <i>paratuberculosis</i> HBHA (>0.5)	<i>M. avium</i> subsp. <i>paratuberculosis</i> GSD (>0.4)	PCR LIZ/AV (294 bp)
03C	M	25			—	—	—	—
04C	F	50			—	—	—	+
05C	M	30			—	—	—	—
06C	F	29			—	—	—	+
07C	F	36			—	—	—	+
08C	M	57			—	—	—	—
09C	F	67			—	—	—	+
10C	M	45			—	—	—	—
11C	M	45			—	—	—	—
12C	M	53			—	—	—	—
13C	M	37			—	—	—	—
14C	M	33			—	—	—	—
15C	M	63			—	—	—	—
16C	M	63			—	—	—	—
17C	M	45			—	—	—	+
18C	F	60			—	—	—	—
19C	F	43			—	—	—	—
20C	F	34			—	—	—	—
21C	F	25			—	—	—	—
22C	M	57			—	—	—	—
23C	M	45			—	—	—	—
24C	F	26			—	—	—	—
25C	M	45			—	—	—	—
26C	F	41			—	—	—	+
27C	M	37			—	—	—	+
28C	M	48			—	—	—	—
29C	F	57			—	—	—	—
30C	M	31			—	—	—	—
31C	M	37			+	+	+	—
32C	M	39			—	—	—	—
33C	M	28			—	—	—	—
34C	F	35			—	—	—	—
35C	F	21			—	—	—	—
36C	F	21			—	—	—	—
37C	M	45			—	—	—	—
38C	M	39			—	—	+	—
39C	M	46			—	—	+	—
40C	F	19			—	—	—	—
41C	M	35			—	—	—	—
42C	M	49			—	—	—	—
43C	F	25			—	—	+	—
44C	M	42			—	—	—	+
45C	F	61			—	—	+	+
46C	M	31			—	—	—	—
47C	F	29			—	—	—	—
48C	M	53			—	—	—	—
49C	M	25			—	—	—	—
50C	M	23			—	—	—	—
51C	M	28			—	—	—	+
52C	F	35			—	—	+	—
53C	M	21			—	—	+	—
54C	M	28			—	—	—	—
55C	M	29			—	—	—	—
56C	M	23			—	—	—	—
57C	F	42			+	—	—	+
58C	F	36			—	—	—	—
59C	M	33			—	—	—	—

^a M, male; F, female. Type I diabetes is indicated as I, and type II diabetes is indicated as II. ELISA arbitrary values depending on the reading values at OD₄₀₅ are indicated as follows: for *M. avium* subsp. *paratuberculosis* lysate, + indicates a value of 0.6 to 0.8, ++ indicates a value of 0.8 to 1, and +++ indicates a value of >1; for the *M. avium* subsp. *paratuberculosis* GSD protein, + indicates a value of 0.4 to 0.6, ++ indicates a value of 0.6 to 0.8, and +++ indicates a value of >0.8; and for *M. avium* subsp. *paratuberculosis* HBHA protein, + indicates a value of 0.5 to 0.7, ++ indicates a value of 0.7 to 0.9, and +++ indicates a value of >0.9.

M. avium subsp. *paratuberculosis* ATCC cells were subjected to disruption on ice by using Ultrasonic homogenizers (Bandelin Sonopuls). Six bursts of 1 min each were achieved at 50% power intensity, with a 5-min cooling period between each burst. The lysate was centrifuged at $12,000 \times g$ for 20 min to remove unbroken cells and cellular debris. The supernatant was decanted and transferred into a fresh tube. The total protein concentration was determined by the spectrophotometric estimation of the optical density at 280 nm (OD_{280}). Forward primer EcoRI-*gsd*-F (GCGCGAATTCATGACTGCGC CAGTGTCTCG), containing the EcoRI restriction site (underlined sequence), and reverse primer HindIII-*gsd*-R (GCGCAAGCTTCTACGGTCTGCGCTTCG), containing the HindIII restriction site (underlined sequence), were used to amplify the complete *M. avium* subsp. *paratuberculosis* *gsd* gene as previously described (15). Expression vector pMAL-c2 (New England Biolabs) and the PCR product were double digested with EcoRI and HindIII enzymes and purified with the Qiaquick PCR purification kit. The ligation of restricted fragments resulted in an "in-frame" fusion between the *malE* gene of the vector and the *gsd* gene. The construct was electroporated into *Escherichia coli* BL21(DE3) cells (Invitrogen Life Technologies), and positive clones were selected on LB agar plates supplemented with 100 μ g/ml of ampicillin (Sigma). Recombinant colonies were confirmed by both EcoRI/HindIII restriction analysis and DNA sequencing. A culture of a selected clone grown overnight was used to inoculate 200 ml of rich broth (10 g/liter tryptone, 5 g/liter yeast extract, 5 g/liter NaCl) containing 100 μ g/ml ampicillin and 2 g/liter glucose. Cells were grown at 37°C with shaking until they reached an OD_{600} of approximately 0.5. Protein expression was induced for 3 h by the addition of 0.3 mM isopropyl- β -D-thiogalactopyranoside (IPTG) (Sigma-Aldrich). Cells were harvested by centrifugation at $4,000 \times g$ for 20 min, resuspended in 20 ml of column buffer (20 mM Tris-HCl, 200 mM NaCl, 1 mM EDTA), and frozen at -20°C. The next day, the sample was placed into an ice-water slurry and sonicated in short pulses of 15 s (for about 2 to 3 min), with 15 s of cooling between each sonication. The lysate was then clarified at $9,000 \times g$ for 30 min, and the supernatant (crude extract) was decanted and diluted 1:5 with column buffer. Since the recombinant fusion protein contained the maltose binding protein (MBP) as the tag, glycosyl transferase (GSD)-MBP was purified from crude extracts by using an amylose resin (New England Biolabs) according to the manufacturer's protocol. Eluted fractions were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Coomassie brilliant blue (Sigma-Aldrich) staining to assess protein yield, purity, and size. The GSD-MBP fusion migrated at the expected molecular mass of 73 kDa. Fractions containing the purified GSD protein were pooled and concentrated by using Centricon centrifugal filter devices (Millipore). Purified protein from the control strain consisted of an MBP fusion of the LacZ alpha peptide.

Recombinant HBHA was previously cloned and expressed in our laboratory (19). High-quality, endotoxin-free purification of the protein of high homogeneity was carried out as described above.

Optimal working dilutions for antigens, serum samples, and conjugated secondary antibody were determined after checkerboard titration. ELISA was performed in 96-well microplates (Nunc-Immuno plate). Purified GSD-MBP, purified HBHA protein, MBP-LacZ control peptide, and *M. avium* subsp. *paratuberculosis* crude lysate were diluted in carbonate bicarbonate buffer (Sigma-Aldrich) at a final concentration of 5 μ g/ml and used as antigens. Each well was coated with 50 μ l of diluted antigen overnight at 4°C. The next day, the unabsorbed antigen was discarded, and wells were blocked with 5% nonfat dried milk (Sigma-Aldrich) at 37°C for 1 h. Plates were washed three times with 200 μ l phosphate-buffered saline-Tween (PBS-T) (PBS-0.05% Tween 20) before 100 μ l of diluted serum (1:100 in PBS-T) was added to each well.

After 2 h, plates were washed five times with PBS-T and incubated for 1 h with 100 μ l of anti-human immunoglobulin G alkaline phosphatase antibody (Sigma-Aldrich) diluted 1:1,000 in PBS-T. Five rounds of washing were performed, and 200 μ l of Sigma Fast *p*-nitrophenyl phosphatase substrate was added to each well. As the color developed, plates were read at 405 nm on a VERSA Tunable Max microplate reader (Molecular Devices).

Glucose levels in the blood of the type 1 diabetic patients ranged between 50 and 400 mg/dl. These variable levels did not affect the ELISA performed (confirmed by the fact that sera with low and high glucose levels correlated very well with positivity to IS900 PCR). Moreover, we tested two T1DM sera (one positive and one negative for *M. avium* subsp. *paratuberculosis*) and PBS as a control by ELISA against *M. avium* subsp. *paratuberculosis* HBHA protein at different concentrations of glucose (50, 100, 200, 300, and 400 mg/dl). Positivity was not affected by the glucose concentrations (data not shown).

RESULTS

Among the diabetic patients, a total of 29 blood samples out of 46 were previously found to be positive for *M. avium* subsp. *paratuberculosis* (63%), whereas only 8 out of the 50 healthy control samples (16%) generated a positive signal as previously reported (21). While a majority of *M. avium* subsp. *paratuberculosis* PCR-positive diabetics carried a family history of diabetes or other genetic/autoimmune disorders, 16 PCR-positive individuals with diabetes did not have any history of diabetes or other autoimmune diseases in their family (Table 1).

Cloning, expression, and purification of *M. avium* subsp. *paratuberculosis* antigens was achieved up to a very high standard and homogeneity. The purified protein fractions were used for ELISA. We observed humoral responses of the diabetics to HBHA, whole-cell lysates, and the *M. avium* subsp. *paratuberculosis* GSD protein as an unequivocal signature of the presence of *M. avium* subsp. *paratuberculosis* bacilli within these patients (Fig. 1). The HBHA antigen gave strong ELISA titers (cutoff titer value of 0.5) in 55.9% of the diabetic patients and only 1.6% of the controls ($\chi^2 = 39.7$; $P < 0.0001$). Also, the GSD protein revealed significant differences in ELISA titers of the diabetic (45.7% positivity) and control (11.8% positivity) individuals at the cutoff titer value of 0.4 ($\chi^2 = 14.9$; $P < 0.01$). The overall humoral responses to the whole-cell lysates of the *M. avium* subsp. *paratuberculosis* bacilli were also as significant and are supportive of the infectious evidence as the HBHA and GSD antigens that we analyzed. The lysates revealed significantly high titers in 32 of the 59 patients (54%) compared to controls (3.3%) at a cutoff titer of 0.5 ($\chi^2 = 34.7$; $P < 0.0001$).

The fact that two out of the three BCG-vaccinated diabetic patients were positive by all the three ELISAs may indicate that a cross-reaction among *M. avium* subsp. *paratuberculosis* antigens and BCG antigens may occur. Note that one of the patients was negative by IS900 PCR. None of the patients and controls suffered from inflammatory bowel diseases.

A correlation between the ages of the patients and the presence of antibodies against *M. avium* subsp. *paratuberculosis* was found (according to the Student *t* test), as shown in Table 2. In particular, a stronger antibody response against *M. avium* subsp. *paratuberculosis* lysate was found in the first group of T1DM patients (18 to 28 years of age) than in third group (39 to 59 years of age) (Student *t* = 2.168788; $P = 0.039075$) and in the second group of T1D patients (29 to 38 years of age) than in the third group (39 to 59 years) (Student's *t* = 2.373435; $P = 0.022274$). The same situation was observed for the two antigens tested (HBHA and GSD) (Fig. 2 and Table 2).

A significant difference among the humoral antibody responses to specific *M. avium* subsp. *paratuberculosis* antigens and whole-cell lysates, as shown by diabetic patients and the nondiabetic controls (Fig. 1), might strongly signify the involvement of *M. avium* subsp. *paratuberculosis* in T1DM.

DISCUSSION

M. avium subsp. *paratuberculosis* is a pathogen with a broad host range, and it can persistently infect the intestinal tracts of many animals including primates (7, 18). It has been found to persist within the gut in a Ziehl-Neelsen-negative "cell wall-

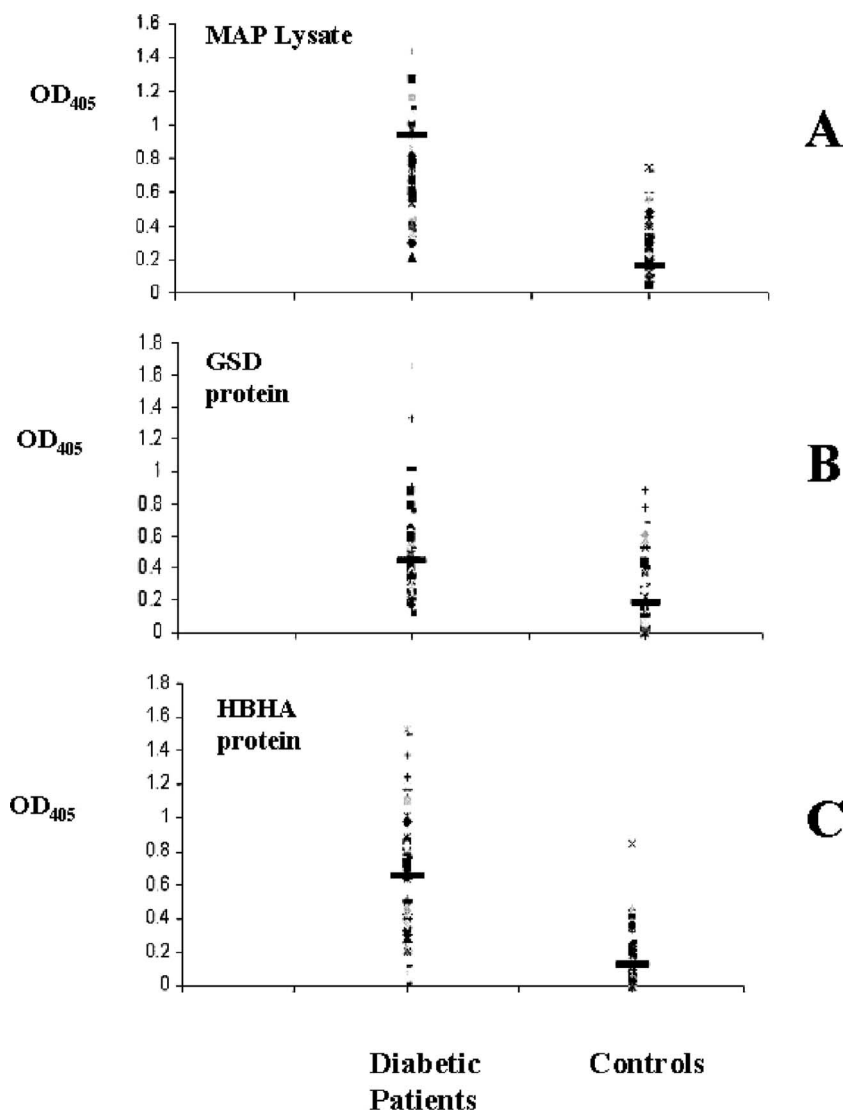


FIG. 1. Evaluation of serum samples from diabetic patients (left column) and healthy donors (right column) against *M. avium* subsp. *paratuberculosis* (MAP) lysate (A), GSD recombinant protein (B), and HBHA recombinant protein (C). Data are presented as values of the OD₄₀₅ observed following ELISA, as described in the text. Data from a representative experiment out of three are shown. The median value for each group is indicated by a dark solid horizontal line.

TABLE 2. Correlation between ages of the patients and the presence of antibodies against *M. avium* subsp. *paratuberculosis*

Protein and age group	Correlation for age (yr):			Student's <i>t</i> value	<i>P</i> value
	18–28	29–38	39–59		
<i>M. avium</i> subsp. <i>paratuberculosis</i> lysate					
18–28 yr	0.728586	0.71191		0.187714	0.852027
29–38 yr	0.728586		0.517673	2.168788	0.039075
39–59 yr		0.71191	0.517673	2.373435	0.022274
HBHA					
18–28 yr	0.879871	0.675797		1.463017	0.151087
29–38 yr	0.879871		0.419484	4.440892	0.000128
39–59 yr		0.675797	0.419484	2.101854	0.041461
GSD					
18–28 yr	0.53445	0.443817		0.886397	0.380574
29–38 yr	0.53445		0.371363	2.215312	0.035044
39–59 yr		0.443817	0.371363	0.800364	0.4279

deficient” form (22). These forms can potentially be the source of inflammatory antigens in the host that may direct autoimmune responses. T1DM is thought to develop as a consequence of such autoimmune responses that lead to the destruction of insulin-producing beta cells of the pancreas (11). There has long been speculation on the involvement of an infectious trigger underlying such autoimmune responses; however, no concrete evidence for the same was presented (17). Genetic evidences point to the existence of immune dysfunctions that promote both T1DM and mycobacterial infection (11, 17). Susceptibility factors such as *Nramp1* gene polymorphisms (10, 20) have also been linked to such diseases. Vitamin D deficiency has been implicated as being a risk factor for T1DM (3, 11). Interestingly, vitamin D is also implicated in limiting mycobacterial infections by upregulating the expression of an antimicrobial peptide (12). Such studies help link the two dis-

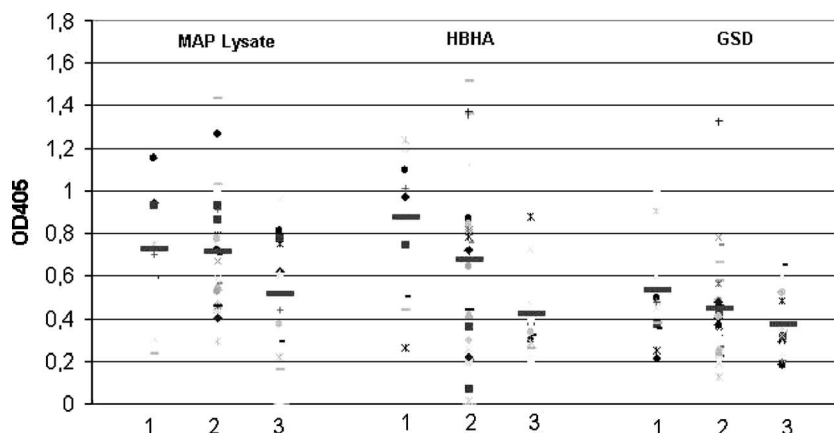


FIG. 2. Values of T1DM serum antibody titers against the different *M. avium* subsp. *paratuberculosis* (MAP) antigens of patients divided by age: 18 to 28 years of age (group 1), 29 to 38 years of age (group 2), and 39 to 59 years of age (group 3). Data are presented as values of OD₄₀₅ observed following ELISA, as described in the text. Data from a representative experiment out of three are shown. The median value for each group is indicated by a dark solid horizontal line.

eases, diabetes and Crohn's disease, where *M. avium* subsp. *paratuberculosis* could be the common agent, putatively behaving as an environmental trigger of autoimmunity. Our results do not rule out this possibility by demonstrating significant immune responses to *M. avium* subsp. *paratuberculosis* antigens. These observations therefore support the infectious trigger hypothesis described previously by Dow (5, 11), although it will certainly be important to dissect out the direct mechanism of the autoimmune responses mediated by the infectious triggers.

T1DM is characterized by elevated levels of T-helper 1 (Th1) responses targeted against several autoantigens including Hsp60, glutamic acid decarboxylase, and insulin. Given this, it becomes conceivable that some molecular mimicry has a role to play (4), especially for epitope homologies between the mycobacterial proteins like Hsp65 and the diabetes antigen glutamic acid decarboxylase (2). Such cross-reactive antigens in a genetically susceptible host might pave the way for the destruction of the islet cells. More recent studies actually firm up this hypothesis by proving that DNA vaccines involving mycobacterial Hsp65 protected NOD mice against diabetes (16). Moreover, seroreactivity against mycobacterial heat shock proteins has also been implicated in host tissue damage due to antibody cross-reactivity against self-antigens. Autoantibodies have been identified in patients with tuberculosis (due to infection with *Mycobacterium tuberculosis*) (6). Serum reactivity against mycobacterial antigens has also been correlated with human autoimmune diseases including Crohn's disease (13). Moreover, pancreatic antibodies are associated with Crohn's disease (9).

Shared genetic susceptibilities among mycobacterioses and T1DM could be another explanation of our results. This is because the island of Sardinia has the highest incidence of Crohn's disease and other autoimmune diseases such as T1DM, with a very high prevalence of *M. avium* subsp. *paratuberculosis* in Sardinian Crohn's disease patients (7, 11, 22). Since *M. avium* subsp. *paratuberculosis* is present in almost half of the sheep herds tested in Sardinia, it is supposed to be endemically contaminating water, milk, and animal feed, as

reported previously in the United Kingdom (14, 18, 23). High levels of exposure might thus cause enhanced infection rates. Therefore, our setting of Sardinia for such a clinical association study appears to be a legitimate choice. The fact that antibody titers against *M. avium* subsp. *paratuberculosis* were higher in young T1DM patients than in older patients may reflect that *M. avium* subsp. *paratuberculosis* infection occurs at an early age.

In conclusion, finding immune responses to *M. avium* subsp. *paratuberculosis* bacteria in T1DM should indeed be a novel observation that strengthens our thinking regarding an infectious cause for T1DM. These results also have implications for countries like India and United States, which respectively have the highest livestock populations and high incidences of *M. avium* subsp. *paratuberculosis* simultaneously with a high incidence of T1DM.

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