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

| Group and patient | Sex   | Age (yr) | Yr of diagnosis | Family history of diabetes (type) | Seropositivity for: |
|-------------------|-------|----------|-----------------|-----------------------------------|--------------------|
|                   |       |          |                 |                                   | *M. avium* subsp. *paratuberculosis* lysate (>0.6) | *M. avium* subsp. *paratuberculosis* HBHA (>0.5) | *M. avium* subsp. *paratuberculosis* GSD (>0.4) | PCR LIZ/AV (294 bp) |
| 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            | I                                 | +                  | –                  | –                  | –                    |
| 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            | I                                 | +                  | ++                 | +                  | –                    |
| 30                | F     | 32       | 1989            | I                                 | –                  | +                  | –                  | –                    |
| 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
| Group and patient | Sex | Age (yr) | Yr of diagnosis | Family history of diabetes (type) | Seropositivity for: |
|-------------------|-----|----------|-----------------|----------------------------------|-------------------|
|                   |     |          |                 |                                  | *M. avium* subsp. *paratuberculosis* lysate (≥0.6) | *M. avium* subsp. *paratuberculosis* HBHA (≥0.5) | *M. avium* subsp. *paratuberculosis* 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       |                 |                                  |                   |                   |                   |                   |

* M, male; F, female. Type 1 diabetes is indicated as I, and type II diabetes is indicated as II. ELISA arbitrary values depending on the reading values at OD₄₅₀ 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.
**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 diabetes 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 (chi² = 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 (chi² = 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 (chi² = 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* lysose 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-
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-

| Protein and age group | Correlation for age (yr): 18–28 | 29–38 | 39–59 | Student’s t value | P value |
|-----------------------|----------------------------------|-------|-------|--------------------|--------|
| M. avium subsp. paratuberculosis 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 | 0.878971 | 0.675797 | 1.463017 | 0.151087 |
| | 29–38 yr | 0.878971 | 0.419484 | 4.440892 | 0.000028 |
| | 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 |

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.
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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