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Immune Response to Infection with *Mycobacterium ulcerans*

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Mycobacterium ulcerans **is a slow-growing, acid-fast bacillus that causes chronic necrotizing skin ulcers known as Buruli ulcers. Previously reported information on immunity to this mycobacterium is limited. We examined immune responses to *M. ulcerans* and *M. bovis* BCG in patients with *M. ulcerans* disease and in 20 healthy control subjects (10 tuberculin test positive and 10 tuberculin test negative). Cell-mediated immunity was assessed by stimulating peripheral blood mononuclear cells (PBMC) with whole mycobacteria and then measuring PBMC proliferation and the production of gamma interferon (IFN-γ). Humoral immunity was assessed by immunoblotting. PBMC from all subjects showed significantly greater proliferation and IFN-γ production in response to stimulation with living mycobacteria compared with killed cells. However, PBMC from subjects with past or current *M. ulcerans* disease showed significantly reduced proliferation and production of IFN-γ in response to stimulation with live *M. ulcerans* or *M. bovis* than PBMC from healthy, tuberculin test-positive subjects ($P < 0.001$) and showed results in these assays comparable to those of tuberculin test-negative subjects ($P > 0.2$). Serum from 9 of 11 patients with *M. ulcerans* disease, but no control subject, contained antibodies to *M. ulcerans*. The results indicate that patients with *M. ulcerans* infection mount an immune response to *M. ulcerans* as evidenced by antibody production, but they demonstrate profound systemic T-cell anergy to mycobacterial antigens. These findings may explain some of the distinct clinical and pathological features of *M. ulcerans*-induced disease.**

Mycobacterium ulcerans is the third most frequent cause of mycobacterial infections in immunocompetent individuals, after *M. tuberculosis* and *M. leprae* (18). *M. ulcerans*-induced disease generally manifests itself as indolent cutaneous ulcers, known as Buruli ulcers, accompanied by minimal systemic symptoms. Unlike other mycobacterial diseases, infections with *M. ulcerans* are characterized by extracellular bacteria, a lack of inflammatory cells, and extensive tissue necrosis at the site of infection (18). The indolent course, prominent extracellular bacteria, paucity of mononuclear cell infiltrate, and lack of systemic symptoms suggest a depressed or absent T-cell immune response.

There are no published studies on the in vitro immune response of subjects with *M. ulcerans* infection. Although some individuals demonstrate a delayed-type hypersensitivity response to an extract of *M. ulcerans* (burulin) on skin testing, indicating a degree of T-cell sensitization, the close correlation between reactors to burulin and purified protein derivative suggests that sensitization is due to cross-reactivity with other mycobacterial species (17).

A soluble lipid product, called mycolactone, produced by *M. ulcerans* produces cutaneous histological lesions in guinea pigs similar to those observed in patients with *M. ulcerans* disease (5). Mycolactone also exhibits immunosuppressive properties in vitro, manifested by suppression of interleukin-2 production by T cells and tumor necrosis factor alpha production by monocytes, and the induction of macrophage apoptosis (6, 15) These findings have prompted the suggestion that the clinical features of *M. ulcerans* infection result from localized toxin-mediated immunosuppression (6, 15). In this study, we show that patients with active or resolved *M. ulcerans* infection exhibit profound systemic anergy to *M. ulcerans* and *M. bovis* BCG, as evidenced by a lack of significant lymphocyte proliferation or gamma interferon (IFN-γ) production in response to stimulation with living or heat-killed mycobacteria. This anergy is not due to a lack of recognition of *M. ulcerans*, since antibodies to *M. ulcerans* are present in subjects with unresponsive T cells. The findings suggest that systemic T-cell anergy to mycobacterial antigens contributes to the pathogenesis of *M. ulcerans*-induced disease.

MATERIALS AND METHODS

Patients and control subjects. Fourteen patients (all from Victoria, Australia) with culture-confirmed *M. ulcerans* disease were studied. Their ages ranged from 10 to 83 years (median, 59 years). Four patients had active disease, and 10 had recovered following surgical excision of the ulcer. The elapsed time from healing to immunological testing ranged from 6 months to 12 years (median, 1 year). Twenty control subjects were selected from healthy adults employed at the Royal Children's Hospital (ages ranged from 21 to 65 [median, 30] years) who had no history of *M. ulcerans* disease and did not reside in an area where the disease is endemic. Ten of these individuals were sensitive to tuberculin, and 10 were not, as determined by Mantoux testing with 10 U of tuberculin purified protein derivative. A positive result was indicated by 10 mm of induration at 48 h. The tuberculin sensitivity of the patients was not known. Informed consent was obtained from all patients and control subjects before they were enrolled in the study.

Cell cultures. Peripheral blood mononuclear cells (PBMC) were separated from heparinized blood by Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) density gradient centrifugation at 2,000 × *g* for 15 min and washed three times in sterile phosphate-buffered saline (PBS), pH 7.2. PBMC were cultured at a concentration of 10^6/ml in AIM-V medium (GIBCO, Grand Island, N.Y.) with

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β-mercaptoethanol (ICN, Costa Mesa, Calif.) at 37°C in a humidified atmosphere containing 5% CO_2. To analyze the kinetics of the response and determine the optimal number of bacteria for maximum stimulation, cultures were stimulated for 2 to 8 days with phytohemagglutinin (PHA) at 10 μg/ml or with living or heat-killed *M. ulcerans* or *M. bovis* BCG at 3×10^4, 3×10^6, 3×10^8, and 3×10^{10} cells/ml. Subsequently, 3×10^8 cells/ml were used in the stimulation assays. Control cultures were left unstimulated for the same period.

Preparation of *M. ulcerans* and *M. bovis*. *M. ulcerans* (WICH, human isolate) was grown in supplemented Middlebrook 7H9 broth (Organon Teknika Corp., Durham, N.C.) for 10 weeks at 32°C. A portion of the culture was killed by heating at 80°C for 2 h, washed three times in sterile PBS, and resuspended at 3×10^8 cells/ml (McFarland standard no. 1). Heat-killed bacteria were aliquoted and stored at −70°C. Whole *M. bovis* BCG cells (CSL Limited, Parkville, Victoria, Australia) were prepared in the same way, except that they were grown at 37°C for 14 days. Live bacteria were prepared immediately before use.

Proliferation assay. Triplicate cultures were harvested at 2, 4, 6, or 8 days onto glass fiber filter paper (Cambridge Technologies, Watertown, Mass.). Sixteen hours before harvesting, cultures were pulsed with 0.5 μCi of [^{3}H]thymidine (Amersham International, Buckinghamshire, England) per 10^5 cells. Radioactivity was measured by liquid scintillation counting using a Tri-Carb Liquid Scintillation Analyzer (Packard Instrument International, Zurich, Switzerland). The stimulation index (SI) was calculated as the ratio of mean counts per minute of the stimulated sample to the mean counts per minute of the unstimulated sample.

Interferon production. Tissue culture supernatants were collected at 2, 4, 6, or 8 days after stimulation with bacteria or PHA and assayed for IFN-γ by sandwich enzyme-linked immunosorbent assay. High-binding enzyme-linked immunosorbent assay plates (Costar, Corning, N.Y.) were coated with purified monoclonal antibody (anti-human IFN-γ monoclonal antibody; BD Pharmingen, San Diego, Calif.) at a concentration of 1 μg/ml. They were then blocked with PBS–Tween–10% fetal calf serum for 2 h, washed, and incubated with 100 μl of sample for 4 h. After washing, 0.5 μg of biotinylated monoclonal antibody per ml was added and the mixture was incubated for 45 min, after which avidin-peroxidase (2.5 ng/ml; Sigma Chemical Co., St. Louis, Mo.) was added. After further washing, the substrate (3,3′,5,5′-tetramethylbenzidine; Kirkegaard & Perry Laboratories; Inc., Gaithersburg, Md.) was added and the reaction was stopped by adding H_2SO_4 when sufficient color had developed. Plates were read at 450 nm on a spectrophotometer. All assays were performed in duplicate on at least two separate occasions, and the data were analyzed using Biomek 1000 data reduction software (Beckman Coulter Inc., Fullerton, Calif.).

Immunoblotting. Suspensions (100 μl) containing 10^9 cells of *M. ulcerans* or *M. bovis* BCG were centrifuged for 3 min at 10,000 × *g* and then resuspended in 100 μl of 2× sodium dodecyl sulfate (SDS) reducing buffer (9). Samples were boiled for 5 min, after which 20 μl was loaded into each well of an SDS–12% polyacrylamide gel. SDS-polyacrylamide gel electrophoresis was performed at 150 V for 1 h in a Mini-PROTEAN II gel apparatus (Bio-Rad, Hercules, Calif.). For immunoblot analysis, the separated material was transferred to nitrocellulose filters (Micron Separations, Westborough, Mass.) and then probed using serum (diluted 1 in 500 in 5% skim milk) from 11 of the 14 *M. ulcerans* patients (2 active, 9 inactive) and the 20 control subjects. Sheep anti-human immunoglobulin conjugated to horseradish peroxidase (Silenus, Melbourne, Victoria, Australia) was used as the secondary antibody. Filters were washed (PBS–0.1% Tween 20), after which membrane-bound, peroxidase-labeled immunoglobulin was detected using enhanced chemiluminescence (ECL Western blotting reagents, RPN 2109; Amersham International).

Statistical analysis. All analyses were performed using Student's *t* test. A value of $P < 0.05$ was taken to indicate statistical significance.

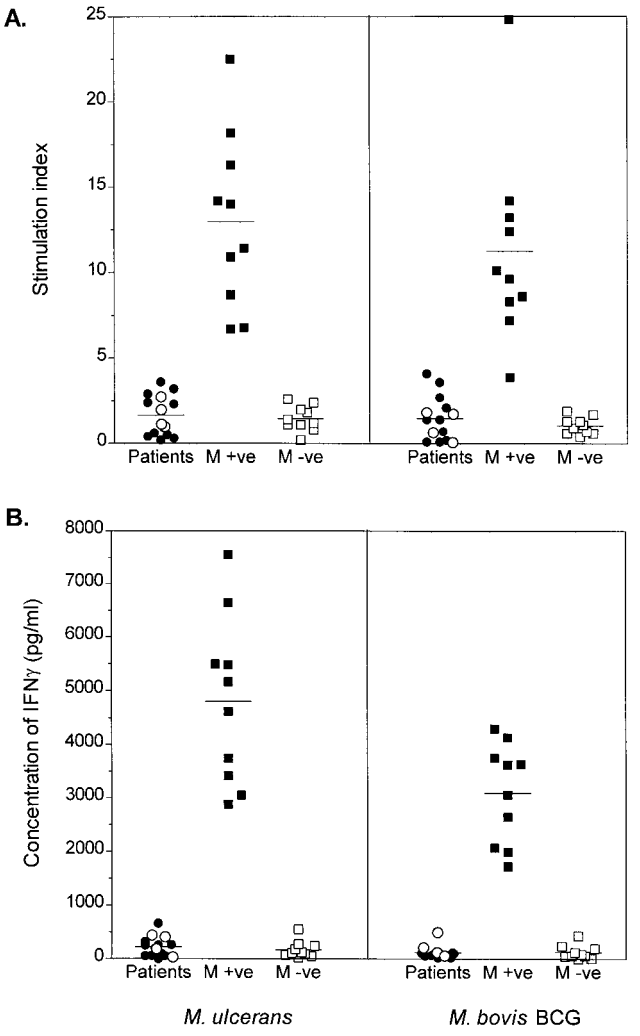

FIG. 1. (A) Proliferation of PBMC from patients with acute (○) or healed (●) *M. ulcerans* disease and from Mantoux test-positive (M +ve; ■) and Mantoux test-negative (M −ve; □) control subjects in response to 4 days of stimulation with living *M. ulcerans* or *M. bovis* BCG. The SI is the ratio of the amount of [^{3}H]thymidine incorporated by stimulated cells to that incorporated by unstimulated cells. Each point is the mean of at least three separate determinations. The mean value for each study group is shown. (B) IFN-γ production by PBMC from the same patient and control groups in response to 6 days of stimulation with living *M. ulcerans* or *M. bovis* BCG. Each point is the mean of two separate determinations. The mean value for each study group is shown.

RESULTS

Live versus killed bacteria. Stimulation of PBMC from Mantoux test-positive subjects, Mantoux test-negative subjects, and *M. ulcerans* patients with 10^8 living *M. ulcerans* organisms per ml induced significantly greater lymphocyte proliferation and IFN-γ production than did stimulation with the same number of killed cells. For example, the SI for the 10 Mantoux test-positive normal subjects was 13.3 ± 5.5 (mean ± standard deviation) in response to live *M. ulcerans*, compared to 8.3 ± 4.6 in response to killed mycobacteria ($P = 0.04$). IFN-γ production paralleled lymphocyte proliferation, and for Mantoux test-positive subjects it was 4,802 ± 1,560 pg/ml after stimulation with live *M. ulcerans*, compared to 862 ± 316 pg/ml after stimulation with killed cells ($P < 0.0001$). For the 10 Mantoux test-negative subjects, these values were 172 ± 156 and 56 ± 50 pg/ml, respectively ($P = 0.04$), while for the 14 *M. ulcerans* patients, they were 221 ± 189 and 105 ± 72 pg/ml ($P = 0.04$).

Proliferative responses. The proliferative response of PBMC from Mantoux test-positive control subjects in response to stimulation with living *M. ulcerans* was significantly greater (SI = 13.3 ± 5.5) than that of PBMC from Mantoux test-negative subjects (1.7 ± 0.8; $P < 0.0001$) (Fig. 1A). However, the SI of subjects with *M. ulcerans* disease (1.6 ± 1.2) did not differ from that of Mantoux test-negative subjects (1.7 ± 0.8; $P > 0.2$). There was no difference in SI between Mantoux test-

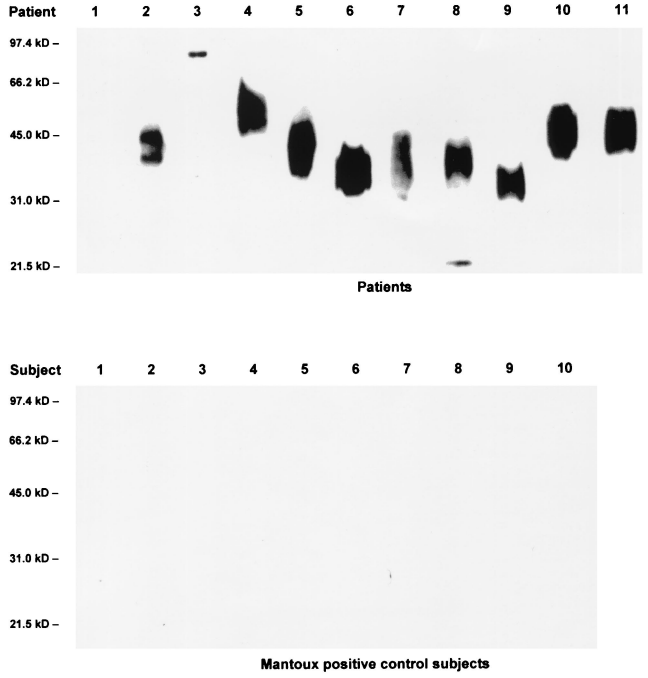

FIG. 2. Immunoblots of *M. ulcerans* preparations with serum from patients with *M. ulcerans* disease and from healthy Mantoux test-positive control subjects. The smeared appearance of the target antigens suggests that the antibodies reacted with partially degraded proteins, glycolipids, or both of these.

positive subjects (84.5 ± 26.3) and Mantoux test-negative control subjects (102.2 ± 16.8; $P = 0.08$) or *M. ulcerans* patients (102.4 ± 26.9; $P = 0.12$) in response to stimulation with PHA at 10 μg/ml.

IFN-γ production. The production of IFN-γ by PBMC from Mantoux test-positive controls, Mantoux test-negative controls, and *M. ulcerans* patients showed the same pattern as the proliferation data (Fig. 1B) and was significantly higher in Mantoux test-positive than Mantoux test-negative subjects in response to stimulation with *M. ulcerans* (4,802 ± 1,560 versus 172 ± 156 pg/ml; $P < 0.0001$) and BCG (3,091 ± 935 versus 126 ± 132 pg/ml; $P < 0.0001$). By contrast, IFN-γ production by PBMC from subjects with *M. ulcerans* disease did not differ from that by PBMC from Mantoux test-negative subjects in response to stimulation with either *M. ulcerans* (221 ± 189 versus 172 ± 156; $P > 0.2$) or *M. bovis* (122 ± 120 versus 126 ± 132; $P > 0.2$). There was no difference in IFN-γ production between Mantoux test-positive subjects (4,873 ± 1,745) and Mantoux test-negative (4,615 ± 1,920) controls or patients (3,014 ± 1,495) in response to stimulation with PHA at 10 μg/ml.

Serum antibodies to *M. ulcerans*. Immunoblotting of extracts of whole *M. ulcerans* mycobacteria with sera from patients with *M. ulcerans* disease revealed an antibody response in 9 of 11 subjects (Fig. 2). No antibodies to *M. ulcerans* were detected in any of the Mantoux test-positive or Mantoux test-negative control subjects.

DISCUSSION

M. ulcerans causes a unique mycobacterial disease characterized pathologically by a predominance of extracellular bacteria and a paucity of a mononuclear cell inflammatory response (18). These features, combined with an observed lack of systemic symptoms and regional lymphadenopathy (18), suggest that immunosuppression or anergy contributes to the pathogenesis of *M. ulcerans*-induced disease.

In this paper, we report marked anergy to *M. ulcerans* in patients with current or past *M. ulcerans*-induced disease, comparable to that to *M. bovis* BCG observed in Mantoux test-negative subjects. This anergy was not generalized, as evidenced by a normal proliferative response and IFN-γ production following stimulation with PHA. Moreover, the anergy was not due to failure to recognize *M. ulcerans*, as antibodies to these bacteria were demonstrated in 9 of 11 patients with unreactive PBMC but in none of the control subjects. Our findings of a specific serological response in patients with *M. ulcerans*-induced disease confirms a recent report by Dobos et al. (1), who found that 70% of 61 patients with acute or healed Buruli ulcers had antibodies to culture filtrate antigens of *M. ulcerans*.

Various mycobacterial species possess cross-reacting T-cell epitopes which can stimulate the proliferation of sensitized peripheral blood lymphocytes or T-cell clones (4, 14). *M. tuberculosis*-reactive T cells, for example, exhibit a broad spectrum of cross-reactivity with pathogenic and environmental *Mycobacterium* species (14). Similar cross-reactivity probably accounts for the finding that PBMC from Mantoux test-positive individuals (which included several "natural reactors" and others who had received BCG vaccine years earlier) demonstrated significantly greater lymphocyte proliferation and IFN-γ production in response to stimulation with *M. ulcerans* and *M. bovis* BCG than did Mantoux test-negative individuals drawn from the same population. In Victoria, Australia, *M. ulcerans* infections are restricted to a narrow geographical area (7). Hence, it is unlikely that all of our Mantoux test-positive control subjects, who resided outside this area, had experienced subclinical infection with *M. ulcerans* which accounted for the lymphocyte proliferative response and IFN-γ production by PBMC from these individuals. Negative *M. ulcerans* serology in these subjects provided further evidence of a lack of previous exposure to this mycobacterium.

George et al. (5) have identified a soluble factor (mycolactone) in *M. ulcerans* which produces toxic effects after intradermal injection into guinea pigs. The same researchers have demonstrated that mycolactone induces macrophage apoptosis and inhibits mitogen-or antigen-induced proliferation of T cells and the production of interleukin-2 by T cells and tumor necrosis factor alpha by monocytes (6, 15). In the light of these findings, they postulated that localized immunosuppression by mycolactone could contribute to the pathogenesis of cutaneous ulcers. While this is an attractive hypothesis, the question of whether mycolactone is released in sufficient amounts in vivo to cause immunosuppression is unresolved.

In the present study, we found no evidence of immunosuppression by whole living or heat-killed *M. ulcerans*. Indeed, we showed that live mycobacteria stimulated significantly greater lymphocyte proliferation and IFN-γ production by PBMC from Mantoux test-positive individuals than did the same number of killed mycobacteria. This finding is consistent with a previous demonstration of increased proliferative responses to live, compared to killed, cells of *M. tuberculosis* and *M. avium* (4).

The large number of extracellular bacteria and poor inflam-

matory responses in the tissues of patients infected with *M. ulcerans* stand in stark contrast to other mycobacterial diseases (18). This observation suggests that in individuals who develop overt disease, the bacteria are ineffectively phagocytosed or escape from phagocytes before being killed, processed, and presented to T lymphocytes. IFN-γ is a major factor in macrophage activation and plays a critical role in protection against infection with mycobacteria (3, 8, 13). T-cell anergy and lack of IFN-γ production in patients with *M. ulcerans* disease may account for the persistence of extracellular mycobacteria, the indolent nature of the disease, and its failure to respond to conventional antimycobacterial chemotherapy. In addition, inappropriate cytokine production may divert the immune systems of patients toward a predominantly Th2-type response (2), thus accounting for the enhanced antibody responses of our patients. The nature of the bacterial antigens to which these antibodies are directed is uncertain, but the range of molecular weights, together with the smeared appearance of the antigens, suggests that they are partially degraded proteins, cell surface glycolipids, or both of these (12, 16). The heterogeneity of the antibody response observed in our patients is in keeping with several reports of antibody responses in various mycobacterial infections, including Buruli ulcer (1, 10, 11).

We conclude that many of the distinctive clinical and pathological features of *M. ulcerans* infection are likely to be due to anergy to this organism and the failure to develop a significant Th1-type response. Our finding that Mantoux test-positive individuals demonstrate an in vitro response to live *M. ulcerans* equivalent to that evoked by *M. bovis* BCG indicates that a significant T-cell response to *M. ulcerans* can occur once sensitization has taken place and that the immunological defect in patients who develop *M. ulcerans* disease is likely to lie in the induction of an appropriate T-cell immune response.

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