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# Phenolic Glycolipid-1 (PGL-1) in Buruli Ulcer Lesions. First Demonstration by Immuno-Histochemistry[1]

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Buruli ulcer is caused by *Mycobacterium ulcerans.* The disease has now emerged as the third most common mycobacterial disease after tuberculosis and leprosy ([9]). The disease affects primarily subcutaneous fat tissue and, clinically, it may present as papules, plaques, nodules, edema or ulcers. Ulcers, which are seen at the most advanced stage of the clinical spectrum of the disease, have given the name to the disease. They may be very small and discrete in size or occupy the whole length and surface of a limb with disabling and fatal consequences ([1]). Chemotherapy has proven unsuccessful in the treatment of the disease, leaving surgery as the unique therapeutic alternative to date. A toxic pathogenic theory, involves a polyketide named mycolactone ([4]), secreted by the mycobacteria. Currently, one of the major goals of the Buruli disease's research is the establishment of an early detection test to permit timely treatment of the disease. Glycolipids of mycobacterial walls, suspected to be the first contact between the bacteria and the host defense barriers, may be among suitable candidates. The present study is an extension of a previous report ([8]) where phenolic glycolipid-1 (PGL-1) was observed in a small series of two clinical forms of the disease, a nodule and an ulcerated nodule of Buruli ulcer lesions. This carbohydrate-based antigen, has a unique trisaccharide that makes it specific for *M. leprae.* First found by Hunter and Brennan ([6]) in tissue infected with *Mycobacterium leprae,* the antigen triggers some oxygen radical scavenging properties, suspected to act in the long survival of *M. leprae* in a macrophage's lysosome. PGL-1 has also been shown to interfere with the action of subsets of lymphocytes *in vitro* ([7]). It is believed, thus, to play a central role in the pathogenesis of leprosy. Available specimens of lesions encompassing almost the whole clinical spectrum of the Buruli's disease, were histopathologically confirmed as lesions of infection by *Mycobacterium ulcerans* by hematoxylin and eosin (H&E) and acid-fast bacilli (AFB) stains (Fite-Faraco and Harada stains). By immunohistochemical methods, using very stable, synthetic monoclonal antibodies ([10]), targeting specific antigenic domains of *M. leprae* PGL-1, the immunoreactivity of the antigen was detected in all the AFB-positive specimens of the collection. This finding may suggest that *Mycobacterium ulcerans* may produce a substance similar to *M. leprae*'s PGL-1. The aim of this study, was to confirm the presence of this antigen in a larger series of Buruli ulcer lesions after the previous observation ([8]).

## MATERIALS AND METHODS

**Tissue specimens.** Specimens of skin lesions, clinically diagnosed as Buruli ulcer, were obtained in the process of reparative surgery from 30 patients admitted at the Agroyesum St Martin Hospital, in the Ashanti district of Ghana. Portions of the surgical sections were immediately fixed in 10% neutral formaldehyde for H&E AFB-staining (Fite-Faraco and Harada) and for

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THE TABLE. *Summary of the clinico-histopathological findings of the cases of Buruli ulcers (acid-fast bacilli: Fite-Faraco stain/PGL-1: Phenolic glycolipid-1).*

| Clinical form | Patients | Histopathology | |
|---|---|---|---|
| | | Acid-fast bacilli stain(+) | PGL-1(+) by immuno-stain |
| Plaques | 3 | 1 | 1 |
| Nodules | 10 | 5 | 5 |
| Ulcerated nodules | 1 | 0 | 0 |
| Deep ulcer bed | 7 | 4 | 4 |
| Healing ulcer | 9 | 2 | 2 |

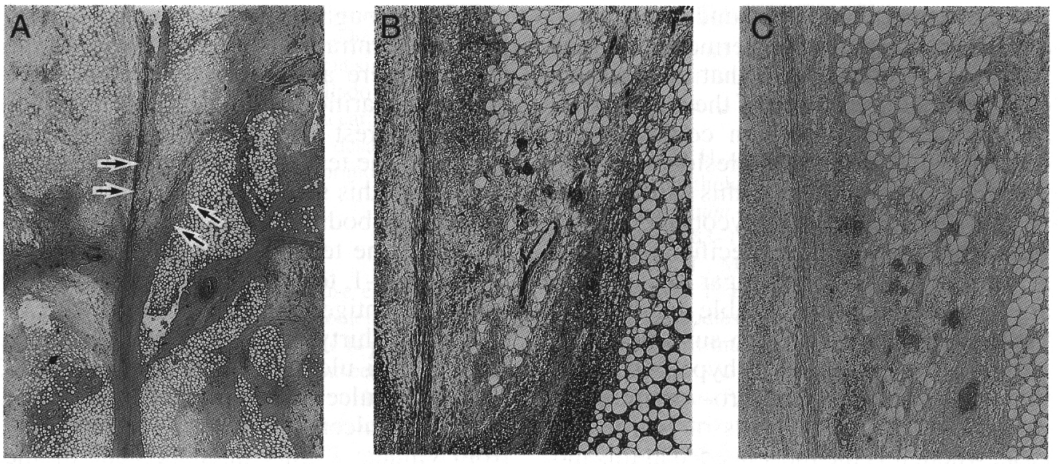
immunohistochemistry. The Table gives a summary of the clinical and histopathological data of the specimens used.

**Immunohistological staining.** Two primary mouse monoclonal antibodies, directed to two sugar parts of PGL-1, were used. The first monoclonal antibody recognizes the inner disaccharide, [2,3-di-*O*-methyl-α-L-rhamnopyranosyl-(1 → 2)-3-*O*-methyl-α-L-rhamnopyranose] of PGL-1 (diluted × 2.500), obtained by the National Institute for Leprosy Research, Tama, Japan, through Dr. T. Douglas, University of Hawaii, U.S.A. ([10]). The second monoclonal antibody recognizes the outer monosaccharide (3,6-di-*O*-methyl-β-D-glucopyranosyl) of PGL-1 (diluted × 40). The second antibody was kindly provided by Fuji-Rebio Japan ([10]). Antibody bridging techniques were performed according to the "Envision two steps visualization technique" (EnVision+, Dako), a timesaving and simplified technique, recently designed by Dako, Copenhagen, Denmark ([3]). In this technique, the molecules of the secondary antibodies (goat anti-rabbit or goat anti-mouse) are coupled to a dextran polymer backbone on which molecules of the enzyme horseradish peroxidase are already fixed (EV+/HRP). Briefly, following formalin fixation, all the specimens were embedded in paraffin. Paraffin blocks were then cut into 4 μm sections, deparaffinized with xylene and rehydrated in graded ethanol concentrations to distilled water. To block endogenous peroxidase activity, the slides were treated with 0.45% hydrogen peroxide in absolute methanol for 30 minutes, and further washed in distilled water and phosphate-buffered saline (PBS), pH 7.4, to which was added 0.25% Triton-X 100. Epitope retrieval for the sections in citrate buffer, was induced by heat using a domestic microwave at 600 W for 10 min. The sections were allowed 15 min for cooling in the buffer solution at room temperature. After washing, sections were incubated with the primary antibodies for 2 hr at room temperature. The sections were washed three times (5 min each) in PBS. The sections were then incubated for 30 min at room temperature with the dextran polymer conjugate (dextran polymer backbone, goat anti-mouse antiserum and horseradish peroxidase, Dako K4001 for monoclonal antibodies) strictly following the instructions of the manufacturer. For the visualization of the antigen, sections were developed for 5 min with diaminobenzidine tetrahydrochloride (Dako Liquid DAB+, K 3468). Finally, Harris' hematoxylin was used for the counterstaining, after which they were mounted in aqueous media. Controls included specimens of *M. leprae*-infected tissues, that previously stained positive for the antigen, and that had been kept in the tissue collection bank of the pathology laboratory of the National leprosarium, Tama, Tokyo.

## RESULTS

The 30 specimens were clinically diagnosed as Buruli ulcer, as shown on H&E preparations, with histopathological features responding to the criteria documented as specific for Buruli ulcer ([2, 5]). Briefly, the major criteria were septate panniculitis, subcutaneous fat necrosis and the presence of acid-fast bacilli by either Fite-Faraco or Harada stains. The septate panniculitis and fatty tissue necrosis were mild to moderate in the plaques and nodules, but severe in the ulcerated nodules, the deep ulcer beds and the ulcers in the healing stage, indicating that the two first lesions are among the earliest manifestations of the clinical spectrum of Buruli ulcer. Perivascular infiltration by small, round cells and coagulative necrosis of dermal collagen, variable in their degree of severity and extension, were variably found in the lesions, from the earliest ones to the advanced ones. Fibrous change with granulomatous formation in some cases, were findings of ulcers in the healing stage.

**AFB.** Acid-fast bacilli were detected by both Fite-Faraco and Harada stains in one




THE FIGURE. **A** = Typical panoramic view of the histopathology of Buruli ulcer in a 4-month-old nodule. Septate panniculitis and the aggregates of ghost fatty cells are the main features (Hematoxylin and eosin × 40). **B** = A higher magnification of the same micrograph as in **A** depicting both clusters of acid-fast bacilli and individually disseminated bacilli in a triangular configuration delimited by subcutaneous septa (Fite-Faraco × 100). **C** = In the same triangular configuration as in **B,** the clearly-defined immunostaining of PGL-1 around the clusters of acid-fast bacilli (immunohistochemical staining of anti-PGL-1 antibody × 200).

plaque (33%), five nodules (50%), four deep ulcer beds (65%), and two healing ulcers (25%). The only ulcerated nodule was devoid of AFB. As has been described ([2, 5]), in all lesions, *Mycobacterium ulcerans,* in clumps or small clusters, were located by predilection in the center of necrotic sloughs, but more proximally-located, small clusters of AFB were identified up to the lower part of the papillary dermis in some cases. Scarcely distributed, solitary, very thin clusters of AFB could be seen in adipocytes in some sections.

**Immunostained sections.** Three pathologists independently scored the distribution and intensity of PGL-1 in positively stained sections of Buruli ulcer. They concluded that the two monoclonal antibodies had almost similar, strong reactivity in all AFB positive lesions. Regardless of the clinical form of the lesion, PGL-1 was uniformly detected in all AFB positive specimens, in a diffuse, confluent pattern, embracing the contour of bacterial clusters, but sometimes extending over the outline of the bacterial clumps. The antigen was detected around solitary small clusters of AFB as well as in clumps of AFB and distributed in necrotic subcutaneous tissue, in fragmented collagen bundles, in fatty lobules, in subcutaneous septa and occasionally in walls of blood vessels. The Fig. A, B, and C, depict some of the main histopathological features of Buruli ulcer and the immunolocalization of PGL-1 in the lesions. No immunostaining was found in peripheral nerve tissues or in epithelioid cell granuloma. All AFB-negative specimens did not show the immunoreactivity to anti-PGL-1 antibodies.

## DISCUSSION

Features of the distinct and abundant staining elicited by the tested antibodies in lesions of Buruli ulcers, consistent with an apparent spillover of the immunostain, over the outline of the AFB clusters, is suggestive of a strong interaction between these antibodies and the antigenic determinant of PGL-1. In that regard, the two monoclonal antibodies used in our study react with well-defined epitope domains of *M. leprae* PGL-1 ([10]). The observation made in this study was not unexpected. In a previous study, the immunoreactivity of the same antigen was observed, using a smaller number of specimens of Buruli ulcer ([8]). These subsequent amplification experiments confirmed that, so far as immunohistochemistry is concerned, *Mycobacterium ulcerans* is producing a substance closely related to *M. leprae* PGL-1. It was first demonstrated in these studies that the immunoreactivity of the inner disaccharide of *M. leprae* PGL-1, shared to some extent by other mycobacte-

rial agents ([6]), could be found in *M. ulcerans*-infected lesions. Furthermore, and most important in our view, is that the presence of the immunoreactivity of the outer monosaccharide of this antigen could also be demonstrated in the same lesions. To date, the immunoreactivity of this latter sugar, has not been detected in mycobacteria other than *M. leprae* ([6]). The specificity given to *M. leprae* PGL-1 by this sugar has made the antigen eligible for a reliable candidate in the serodiagnosis and sero-surveillance of leprosy. It is premature to hypothesize that this may apply to the sero- or immunohistopathological diagnosis of Buruli disease, where it is the most needed at this moment. A first step should be the demonstration of the presence of the antigen in sera of Buruli ulcer patients. A second step should probably involve the biochemical demonstration of the extent to which the substance being demonstrated here bears resemblance to *M. leprae* PGL-1. If close biochemical associations are demonstrated, then further investigation would clarify if the hypothetical *M. ulcerans* PGL-1 share other, already established, functions of the *M. leprae* PGL-1.

The issue of early detection is among priorities, as far as the current dramatic state of the disease is concerned, if not the priority. The immunoreactivity of the PGL-1 was observed in early forms of the lesions of Buruli ulcer. This finding should deserve more scrutiny, if credit is given to previous reports ([6]), suggesting that the carbohydrate-based antigens of the mycobacterial wall might be the primary contacts between the bacteria and the host-defense barriers. Among these carbohydrate-based antigens, phenolic glycolipids are chemically well-characterized and available in commerce. If these products demonstrate serologically acceptable levels of specificity, in combination with histopathology, they might prove useful in the early detection of the disease. This study should stimulate more investigation for the biochemical characterization of the phenolic glycolipids of *M. ulcerans.*

## SUMMARY

Buruli ulcer, caused by *Mycobacterium ulcerans,* is emerging as the third most common mycobacterial disease after leprosy and tuberculosis in some tropical regions. Although a toxin of the polyketide family is central to the pathogenesis of the disease, there are still several parameters that need clarification. Among them and of crucial interest are the curative drug treatment and the test for early detection of the disease. In this study, we used mouse monoclonal antibodies, raised against synthetic sugars of the terminal trisaccharide of *M. leprae* PGL-1, to detect the immunoreactivity of this antigen in tissue infected with *M. ulcerans.* Thirty specimens of skin tissue from Buruli ulcer patients (3 plaques, 10 nodules, 1 ulcerated nodule, 7 deep ulcer beds and 9 ulcers in healing) were obtained from Ghana. Eighty-three percent of the submitted cases were compatible with the lesions of Buruli ulcer. AFB were positive in 33% of plaques, 40% of nodules, 44% of actives ulcers and 22% of the ulcer in healing stage. Immunohistochemically, phenolic glycolipid-1 (PGL-1) was detected in all AFB-positive cases. This observation implies that *Mycobacterium ulcerans* may express an *M. leprae* PGL-1-like substance and should tentatively emulate research to further characterize such a substance. The search for an early diagnostic tool for the Buruli disease may benefit from such investigations.

## RESUMEN

La úlcera Buruli, causada por *Mycobacterium ulcerans,* está emergiendo como la tercera enfermedad micobacteriana más común, después de la lepra y la tuberculosis, en algunas regiones tropicales. Aunque se sabe que una toxina de la familia de los policétidos participa en la patogénesis de la enfermedad, todavía se desconocen muchos aspectos de esta micobacteriosis. Entre los aspectos que requieren atención están el desarrollo de una quimioterapia eficaz, y el diseño de pruebas para el diagnóstico temprano de la enfermedad. En este estudio utilizamos anticuerpos monoclonales de ratón dirigidos contra azúcares sintéticos correspondientes a los encontrados en el trisacárido terminal del glicolípido fenólico-I (PGL-I) de *M. leprae* para determinar la reactividad de estos anticuerpos con los antígenos presentes en las lesiones causadas por *M. ulcerans* en la piel de pacientes de Ghana. Ochenta y tres por ciento de las muestras recibidas correspondieron a úlcera Buruli (30 especímenes) dentro de las cuales hubieron 3 placas, 10 nódulos, 1 nódulo ulcerado, 7 fondos de úlcera profunda y 9 úlceras en curación. Se encontraron bacilos ácido-resistentes (BAAR) en el 33% de las placas, 40% de los nódulos, 44% de las úlceras activas y 22% de las úlceras en curación. Todos los especimenes que

fueron BAAR-positivos fueron positivos para PGL-I. Esta observación implica que *Mycobacterium ulcerans* podría expresar alguna sustancia similar al PGL-I de *M. leprae.* El estudio más detallado de esta observación podría conducir no solo a la caracterización de dicha sustancia sino también al desarrollo de una prueba para el diagnóstico temprano de la enfermedad.

## RÉSUMÉ

L'ulcère de Buruli, dont la cause est *Mycobacterium ulcerans,* émerge actuellement comme la troisième maladie mycobactérienne la plus commune après la lèpre et la tuberculose, dans certaines régions tropicales. Bien qu'une toxine de la famille des polykétides soit critique pour la pathogenèse de cette maladie, de nombreux points restent à clarifier. Parmi ces derniers, un traitement médicamenteux curatif et un test pour une détection précoce de la maladie sont d'une importance cruciale. Dans cette étude, nous avons utilisé des anticorps monoclonaux d'origine murine dirigés contre des glucides synthétiques identiques au tri-saccharide terminal de PGL-1 de *M. leprae,* afin de détecter l'immunoréactivité de cet antigène au sein d'un tissu infecté par *M. ulcerans.* Trente spécimens de peau provenant de patients souffrant d'ulcères de Buruli (3 plaques, 10 nodules, 1 nodule ulcéré, 7 ulcères profonds et 9 ulcères en voie de cicatrisation) furent obtenus du Ghana. Quatre vingt trois pour-cent des cas soumis étaient compatibles avec des lésions d'ulcères de Buruli. Des bacilles AAR étaient présents dans 33% des plaques, 40% des nodules, 44% des ulcères actifs et 22% des ulcères au stade de cicatrisation. Immunohistochimiquement, le glycolipide phénolique de type 1 (PGL-1) fut détecté à partir de tous les cas contenant des bacilles AAR. Cette observation implique que *Mycobacterium ulcerans* pourrait exprimer une substance similaire au PGL-1 de *M. leprae* et que cette trouvaille devrait motiver une recherche afin de mieux caractériser une telle substance. La quête d'un outils pour un diagnostic précoce de la maladie de Buruli pourrait bénéficier d'une telle investigation.

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