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Efficacy of the Combination Rifampin-Streptomycin in Preventing Growth of *Mycobacterium ulcerans* in Early Lesions of Buruli Ulcer in Humans

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***Mycobacterium ulcerans* disease is common in some humid tropical areas, particularly in parts of West Africa, and current management is by surgical excision of skin lesions ranging from early nodules to extensive ulcers (Buruli ulcer). Antibiotic therapy would be more accessible to patients in areas of Buruli ulcer endemicity. We report a study of the efficacy of antibiotics in converting early lesions (nodules and plaques) from culture positive to culture negative. Lesions were excised either immediately or after treatment with rifampin orally at 10 mg/kg of body weight and streptomycin intramuscularly at 15 mg/kg of body weight daily for 2, 4, 8, or 12 weeks and examined by quantitative bacterial culture, PCR, and histopathology for *M. ulcerans*. Lesions were measured during treatment. Five lesions excised without antibiotic treatment and five lesions treated with antibiotics for 2 weeks were culture positive, whereas three lesions treated for 4 weeks, five treated for 8 weeks, and three treated for 12 weeks were culture negative. No lesions became enlarged during antibiotic treatment, and most became smaller. Treatment with rifampin and streptomycin for 4 weeks or more inhibited growth of *M. ulcerans* in human tissue, and it provides a basis for proceeding to a trial of antibiotic therapy as an alternative to surgery for early *M. ulcerans* disease.**

Mycobacterium ulcerans disease is common in some parts of the tropics, particularly in West Africa (31). The disease has been reported from over 30 countries in Africa, Southeast Asia, Australia, and South America (6). It is often found in swampy humid areas or near lakes and rivers, but the exact mode of transmission is not known. Most patients are children aged 15 years or younger in remote rural areas who have little or no access to health services. The disease often manifests itself as a painless nodule, a firm plaque, or an edematous lesion which soon ulcerates (6). Standard treatment is surgical excision with grafting, but surgery is often not accessible to poor patients in rural areas of Africa. Early detection and excision can prevent development of the large, disfiguring ulcers often associated with persistent deformity after healing (14). However, many patients present late (1), with extensive ulcers that require wide surgical excision followed by skin grafting, which is costly (5). Finding an effective drug treatment is one of the research priorities of the World Health Organization (WHO).

The only published controlled trials in humans suggest that both clofazamine (26) and cotrimoxazole (15) are ineffective for ulcers and that rifampin and dapsone combined have limited efficacy for ulcers (13). Anecdotal reports of antibiotic administration have been discouraging, and it has been postulated that antibiotics fail to penetrate *M. ulcerans* lesions because of the extensive necrosis caused by mycolactone, although there is no evidence to support this.

M. ulcerans has been shown in vitro to be susceptible to rifampin (17), aminoglycosides, macrolides (25), and quinolones (30). It was susceptible to the same drugs in the mouse footpad model (7, 11, 29), but clarithromycin and quinolones were bacteriostatic whereas rifampin, amikacin, and streptomycin appeared to be bactericidal (10); the size of mouse footpad lesions treated with rifampin and amikacin together for 12 weeks decreased progressively, the mean CFU counts of *M. ulcerans* were reduced, and there were no relapses (10, 21).

In 2000, the WHO Advisory Group on Buruli ulcer recommended a study to examine the possible benefit of antibiotic treatment in human subjects. The aim of the present study, conducted under the aegis of the WHO, was to establish

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TABLE 1. Age, sex, site, and type of lesion for 21 subjects with *M. ulcerans* disease

Group	No. of patients	Mean age in yr (range)	Sex		Site of lesion			Type of lesion	
			Male	Female	Upper limb	Lower limb	Head and neck, trunk	Nodule	Plaque
I	5	26 (16–54)	1	4	3	2	0	3	2
V	5	19 (15–33)	3	2	3	1	1	3	2
II	3	26 (15–32)	0	3	2	0	1	2	1
III	5	27 (15–32)	2	3	1	2	2	3	2
IV	3	30 (20–40)	1	2	2	1	0	3	0
Total	21	26	7	14	11	6	4	14	7

whether rifampin and streptomycin treatment for 2, 4, 8, or 12 weeks of humans with early nonulcerative *M. ulcerans* disease would convert culture-positive nodules and plaques to culture negative and to observe any change in the lesions.

MATERIALS AND METHODS

Study population. Patients with a clinical diagnosis of *M. ulcerans* disease were recruited from the Amansie West and Upper Denkyira districts in Ghana, where the disease is highly prevalent (3, 4). Communities and health facilities were informed about the study, and ethical approval was obtained from the Ministry of Health, Accra, Ghana, and the Secretariat Committee on Research Involving Human Subjects of the World Health Organization, Geneva, Switzerland. Patients were included if they were aged 15 years or older; had a single nodule or plaque less than 10 cm in maximum diameter, meeting the WHO clinical case definition for *M. ulcerans* disease (6); were not pregnant; were not under treatment with antibiotics; had no history of leprosy, tuberculosis, liver, kidney, or hearing problems; and gave informed written consent. Those who met the clinical case definition and had unremarkable results after an initial hearing test, hepatic and renal function tests, and a pregnancy test were included in the study.

Study design. Patients were randomized using computer-generated numbers initially to one of four groups. In group I, patients were managed conventionally by immediate excision of the lesion and closure of the wound. In groups II to IV, rifampin orally at 10 mg/kg of body weight and streptomycin intramuscularly at 15 mg/kg of body weight were administered daily for 4, 8, or 12 weeks, after which the lesions were excised not less than 24 h after the last dose of antibiotics. With a sample size of three per group, if 100% of the lesions in group I (no antibiotic) and none of those in one of the antibiotic treatment groups had yielded a positive culture, the result would be significant at the 5% level with 80% power (8). Therefore, we aimed to include five subjects with a firm retrospective laboratory diagnosis of *M. ulcerans* disease in each group (see diagnostic inclusion criteria). Up to seven patients were recruited to each group with the aim that each would contain five patients whose lesion met the laboratory criteria for diagnosis of *M. ulcerans* disease when the results were analyzed. During therapy, lesions were measured and the average diameter was used to calculate crude surface area by approximation to a circle. All patients were admitted to St. Martin's Hospital, Agroyesum, Ghana, for directly observed therapy. The principle investigator (S.E.) and the medical team saw each of the study patients during daily clinical rounds, and inquiries from patients included the common side effects. After preliminary analysis of the above-mentioned groups, five more patients were recruited sequentially to receive antibiotic therapy for 2 weeks before excision of the lesion (group V). Monitoring, tissue sampling, and follow-up were exactly as for the other groups. Patients treated with the antibiotic combination were followed up after discharge for 1 year to detect recurrent disease.

Renal function was monitored by weekly blood investigations for substances including creatinine and urea (Public Health Reference Laboratory, Kumasi, Ghana). Pregnancy test was done using a urinary latex agglutination pregnancy test kit ("Direct," Randox; Crumlin Co., Antrim, Ghana). Two-weekly hearing tests were conducted by pure-tone audiometry using a Kamplex AD Audiometer calibrated to the ANSI standard (standard 36, 1969) at frequencies of 250 through 8,000 Hz (Speech and Hearing Assessment Center, Kumasi, Ghana).

Excised tissue was divided for histopathology and microbiology examination. At the Angers laboratory, 1 g of tissue was minced and ground in a Potter-Elvehjem homogenizer (Kimble/Kontes, Vineland, New Jersey) with 10 ml phosphate-buffered saline at pH 7.0. A 10-fold dilution of the tissue homogenate was used for the enumeration of acid-fast bacilli (AFB) (28), culturing in Löwenstein Jensen medium after decontamination using the *N*-acetyl cystein-sodium hydroxide method (18), inoculation into the tails of mice (19), and amplification of the insertion sequence IS*2404* by the PCR (27). For AFB counts, 10 microliters of diluted homogenate was smeared on the 1.32-cm^2 central circular area of a cytoslide (Thermo Shandon Inc., Pittsburgh, Pennsylvania) and stained with Ziehl-Neelsen stain. Enumeration of AFB was performed in duplicate, scanning 20 fields with oil immersion objective (×1,000). Average AFB count was expressed per gram of tissue. Three milliliters of diluted homogenate was centrifuged and the pellet resuspended in 3 ml buffer solution. The suspension was diluted up to 100-fold if no AFB were seen on the smear and up to 10^6 fold if the AFB smear was positive. From the undiluted decontaminated suspension and each 10-fold dilution, 0.2 ml was inoculated onto three Löwenstein Jensen slopes. All inoculated culture media were incubated at 29°C and checked weekly for 6 months. In positive cultures, colonies were enumerated. PCR was performed on nondecontaminated undiluted homogenate. Dilutions of nondecontaminated homogenate were used for inoculation into mice; 0.1 ml of each dilution was inoculated subcutaneously into the tails of 10 BALB/c mice in order to calculate the most probable number of live *M. ulcerans* cells per gram of tissue (20). If lesions developed on any inoculated mice, a single representative mouse was sacrificed; inflammatory tail tissue was homogenized and used for the enumeration of AFB, PCR assay, and culture on Löewenstein Jensen medium. Inflammatory lesions typically develop between 8 and 13 weeks after inoculation, and mice were considered culture negative if no lesions developed within 6 months.

Histological examination was made after hematoxylin and eosin staining and Ziehl-Neelsen staining, silver methenamine staining for fungi, and examination under polarized light (24).

Diagnostic inclusion criteria. Final diagnosis was made retrospectively on laboratory based criteria. Normally, laboratory diagnosis is based on one definite laboratory criterion or visible AFB together with possible histopathology. Definite laboratory criteria are culture positive for *M. ulcerans* or definite histopathology, which is the presence of Buruli-type coagulative necrosis of the dermis or subcuticular tissue, with or without panniculitis, with or without granulomas, and with or without AFB (clumps of AFB are irregularly distributed in Buruli lesions). It was not known how antibiotics would influence culture or histopathological features, so in this study, final laboratory diagnosis was based on one definite criterion or positive PCR together with possible histopathology or visible AFB. Possible histopathology was the presence of panniculitis, with or without granulomas but without Buruli-type coagulative necrosis.

TABLE 2. Summary of results of all laboratory methods on specimens from 21 patients with *M. ulcerans* disease

Group	No. of specimens analyzed	No. with a positive result				No. where histology showed typical necrosis
		Culture	Mice	PCR	AFB	
I	5	5	5	5	4	4
V	5	5	5	5	5	5
II	3	0	0	3	3	2
III	5	0	0	5	5	5
IV	3	0	0	3	3	1
Total	21	10	10	21	20	17

TABLE 3. Histopathological findings in 21 cases with a PCR positive for *M. ulcerans*

Group	No. of lesions with positive PCR	Histopathology				
		Necrosis	Chronic inflammation	Acute inflammation	Granulomas	AFB positive
I	5	4	5	4	2	3 (4)[a]
V	5	5	5	5	0	5
II	3	2	3	3	2	3
III	5	5	5	4	2	5 (4)[a]
IV	3	1	2	2	1	3
Total	21	17	20	18	7	19

[a] Values in brackets are the number of specimens in which AFB were observed in the microbiology specimen.

RESULTS

Laboratory diagnosis of tissue samples and demography of patients. Between September 2001 and December 2002, 28 patients were recruited and randomized into each of four groups. After preliminary analysis of results, five more patients were recruited sequentially into group V. All 33 patients recruited had a nodule or a plaque (Table 1). Two patients in group III withdrew; one was found to be pregnant, and another withdrew himself. The lesion in one male patient in group II resolved completely during treatment, and no biopsy was taken. Therefore, 30 out of 33 patients completed treatment and their excised lesions were analyzed. A retrospective laboratory diagnosis of *M. ulcerans* disease was established in 21 patients, and Table 1 shows the age, sex, type, and location of lesions. PCR for *M. ulcerans* was positive in all of the lesions, and the histopathological changes observed were compatible with *M. ulcerans* disease (Tables 2 and 3). Of the 21 lesions, 10 were culture positive with compatible histopathology, 8 were culture negative but had definite histopathology and positive AFB, and 3 had possible histopathology, positive AFB, and positive PCR but culture was negative. The nine lesions excluded from further analysis were all PCR, culture, and AFB negative; on histology, one had lipoma, one had phycomycosis, and seven had nonspecific inflammation.

Microbiological response to antibiotic treatment. Of the 21 patients defined as having *M. ulcerans* disease, five lesions in group I patients who did not receive antibiotics and five lesions from patients in group V who received antibiotics for 2 weeks were culture positive and positive on mouse inoculation (Table 2). The number of organisms cultured from group V lesions (mean 0.2×10^4 CFU/gm) was similar to that from lesions from group I (mean 0.3×10^4 CFU/gm), but it ranged from 10 to 10^4 CFU/g in both groups. None of the lesions excised after antibiotic treatment for 4 weeks or more from 11 patients in group II, III, or IV yielded positive cultures for *M. ulcerans* either in vitro or after inoculation into mice.

AFB were detectable in all lesions with a positive diagnosis of *M. ulcerans* disease by other criteria except for one in group I. Thus, AFB persisted in the tissues, even when culture was negative, up to at least 12 weeks after antibiotic treatment was started. The mean numbers of AFB per gram of tissue were 2.0×10^8 in group I, 0.7×10^8 in group V, 1.8×10^8 in group II, 0.7×10^8 in group III, and 0.4×10^8 in group IV.

Clinical response to antibiotics. Most lesions became smaller during antibiotic treatment, and none of them became enlarged (Table 4). The average reduction in surface area at the end of treatment was 38%, ranging from 29% in those treated for 2 weeks to 52% after treatment for 4 weeks. No side effects were reported during antibiotic treatment, and all laboratory and hearing tests were normal throughout treatment.

During 12 months' follow-up after surgery, there was only one case of recurrence, in a subject in group I who had not received antibiotics. A nodule recurred at the same site 4 months later; antibiotics were administered for 2 weeks before and 2 weeks after surgery. The tissue excised was PCR and culture positive for *M. ulcerans*.

DISCUSSION

This study showed for the first time that antibiotic treatment of patients with nodules or plaques of early *M. ulcerans* disease can render the diseased tissue culture negative within 4 weeks. It demonstrated clearly that antibiotics can penetrate the necrotic subcutaneous fatty tissue in which *M. ulcerans* organisms are seen on tissue sections. Patients in this study did not have ulcers, but unpublished results of a subsequent prospective study in Bénin showed that small ulcers reduced in size during treatment for 4 weeks with rifampin and streptomycin and that many were healed after 8 weeks (9). Preliminary evidence of a response to treatment of edematous disease has also been presented (33).

Although the number of subjects in each group was small in the present study, the result was significant when group I or group V was compared with each of the other groups (see Materials and Methods). It was important to establish the minimum duration of treatment necessary to render lesions culture negative, and the protocol allowed for the inclusion of a 2-week treatment group. Patients were recruited sequentially to group V after preliminary analysis of results from the other groups had shown that there were no positive cultures after 4 or more weeks of antibiotic treatment. Culture was positive in

TABLE 4. Change in mean surface areas of lesions before and after treatment with antibiotics for 2, 4, 8, or 12 weeks

Duration of antibiotic therapy (wk)	Group	No. of patients	Mean surface area in cm^2 (range)		% Reduction in surface area
			Before treatment	After treatment	
0	I	5	15.5 (9.6–23.7)		
2	V	5	21.7 (8.3–43)	15.4 (4.3–27.3)	29
4	II	3	13.1 (12.6–19.6)	6.3 (4.2–15.5)	52
8	III	5	24.8 (5.5–76.9)	17.1 (3.1–45.3)	31
12	IV	3	15.1 (10.5–23.7)	8.9 (1.8–17)	41

all five cases recruited to group V, which suggests that treatment must be for at least 4 weeks.

Preliminary analysis showed that diagnosis was more difficult after antibiotic treatment because histopathology became less specific; three patients recruited to group IV who received antibiotics for 12 weeks could not be included in the final analysis because histopathology was nonspecific and culture, AFB, and PCR were all negative. Only 21 patients were analyzed because diagnosis was made retrospectively. At the time, prospective diagnosis by punch biopsy had not been shown to be a valid method and there were fears that biopsy might precipitate ulceration. Use of punch biopsies for diagnosis has now been studied in detail (23) and, since antibiotics have been shown in the present study to kill *M. ulcerans*, these concerns can be put aside in future studies. PCR emerged as the most useful test when culture was negative and, in other studies, it was 98% sensitive compared to only 40 to 42% for AFB (23, 24). There was no evidence of false-positive PCR results in the present study when the histopathology was taken into account. The possibility of false-negative results cannot be excluded, but if there were any, they would not alter the conclusions from the study.

The fact that there were no recurrences after 12 months among the patients who received antibiotic therapy in this study is encouraging, but the number of patients was small. Recurrence rates after surgical treatment are variable and depend upon the experience of the surgeon in defining the disease-free margin of the lesion and on the severity of disease. In 1-year follow-up after the excision of early lesions in the Amansie West district of Ghana, there was a 16% recurrence rate (2). Taking this rate, three or more recurrences would be expected among our 21 patients. The one recurrence in the present study was in a patient who had not received antibiotics initially. Antibiotics were given for 2 weeks before excision of the recurrent lesion, but the excised tissue was still culture positive for *M. ulcerans*, further indicating that 2 weeks of antibiotic treatment is insufficient.

If a successful antibiotic regime can be developed, this might obviate the need for surgery in some cases and reduce the extent of surgery in others, as well as probably reduce recurrence rates after surgery. Access to surgery is very limited in developing countries where the disease is endemic and the cost of surgery is beyond the means of most of those severely affected (5). In addition, hospitalization, for 3 months on average limits hospital bed capacity and further reduces the number of patients who can be treated. The cost of antibiotic treatment has not been fully analyzed, but rifampin and streptomycin together with needles and syringes for injection of streptomycin in ambulatory patients for 8 weeks is less than $10 whereas inpatient surgical excision of early lesions costs about $30 (5). The WHO is currently supplying rifampin and streptomycin to centers where patients can be monitored and followed up adequately. The importance of early detection is highlighted by the cost of treating a large ulcer, which is about $780, taking account of direct medical expenses and loss of income by adult patients and family caregivers over a 3-month period.

For ethical reasons, only adults were recruited in this study although most patients with the disease are children aged 5 to 15 years. Antibiotic treatment active in adults is likely to be active in children, but intramuscular injections of streptomycin might be less tolerable for children. There were no side effects despite careful monitoring, but an oral treatment regime would be preferable. The rifampin and streptomycin combination was chosen because it was the most effective in the mouse model of *M. ulcerans* disease and it is widely used in the management of human tuberculosis (32). The only two oral regimens with bactericidal activity in mice were rifampin, clarithromycin with sparfloxacin (10), and rifampin with sitafloxacin (12). Sparfloxacin was withdrawn because of phototoxicity, and sitafloxacin is not yet available for use in humans. Further investigation of oral antibiotic treatment for humans with *M. ulcerans* disease is needed.

Quantitative bacteriological methods were used in the present study for assessing the number of AFB in smears, the number of CFU in culture, and the number of viable units in mice. Results among untreated patients and in those treated for only 2 weeks were consistent, and as observed in mice (10), AFB counts were higher than both CFU and viable unit counts, suggesting that a proportion of bacilli seen in lesions were dead. This is analogous to the persistence of dead *M. leprae* in patients with lepromatous leprosy (16). Mouse inoculation and in vitro culture yielded similar results, so in vitro culture will be the method of choice to isolate *M. ulcerans* in future clinical trials.

We used various methods to document lesion size during antibiotic treatment: recording diameters, tracing lesions (22), and photography. It was difficult to make accurate measurements of nodular lesions, and photographs were not easy to read and interpret. We calculated surface areas using the mean of two diameters to approximate to a circle. The results showed that lesions did not enlarge during antibiotic therapy and some became smaller, but clinical efficacy can be studied only by conducting a clinical trial in which lesions are not excised after antibiotics.

This proof-of-principle study has shown for the first time that antibiotic treatment of patients with early *M. ulcerans* disease (nodules or plaques) for 4 weeks or more renders the diseased tissue culture negative. Although only early lesions were included, the results provide a basis for further studies to evaluate antibiotic treatment in all forms of *M. ulcerans* disease. Surgery will remain necessary in extensive ulcerative disease, but from a public health point of view, detection of early lesions and treatment with antibiotics would have a greater impact on the control of this devastating disease. Further studies are needed to investigate whether antibiotics alone can heal lesions and to determine the optimal duration of therapy.

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