RIFAMPICIN IN EXPERIMENTAL *MYCOBACTERIUM ULCERANS* INFECTION

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MYCOBACTERIUM ULCERANS infection was first reported in Australia (MacCallum *et al.*, 1948), but has since been recognised in many other parts of the world, including the Congo, Malaya, Mexico, New Guinea, Nigeria and Uganda. The initial lesion is a small, subcutaneous nodule, which extends at a variable rate and may eventually involve large areas of subcutaneous tissue (Uganda Buruli Group, 1970). The skin over the centre of the lesion usually breaks down to form an ulcer with deeply undermined edges. Healing may begin at any stage of the disease, but usually takes several months and often leaves extensive fibrosis and scarring.

The present treatment of *Myco. ulcerans* infection is surgical. Early excision of small nodules is usually immediately curative, but larger lesions often need extensive and repeated excisions of infected tissue, followed by skin grafting. The search for a chemotherapeutic alternative has so far proved disappointing, although some success has been reported in the treatment of early experimental lesions of mice with clofazimine (B663) or streptomycin (Leach and Fenner, 1954; Lunn and Rees, 1964; Pattyn and Royackers, 1965).

We have investigated the effects on experimental *Myco. ulcerans* infections of mouse foot-pads, of rifampicin, sulphadoxine plus pyrimethamine, and clofazimine alone and in combination.

MATERIALS AND METHODS

Organisms. The 32 strains of *Mycobacterium ulcerans* used came from Uganda (16, including NCTC 10445); Congo (11); Australia (4, including NCTC10013 and NCTC 10417) and Mexico (1).

In-vitro sensitivity testing. Löwenstein-Jensen medium containing rifampicin in a series of doubling concentrations over the range 1–32 µg per ml was obtained from a commercial source. Inoculation followed the procedure recommended by Cruickshank and Stewart (1961) and *Mycobacterium tuberculosis* H37Rv was included in each batch of tests. Results were read after incubation at 32°C for 3 wk, and recorded as resistance ratios.

In-vivo experiments. Suspensions of organisms grown for 4–6 wk on Löwenstein-Jensen medium were washed once in saline and treated for 1 min. in a M.S.E. 100-watt ultrasonic disintegrator to disperse clumps. The suspensions were then diluted to correspond approximately in opacity with Brown's tube no. 4. Male white Schneider mice, aged 4–5 wk, received 0.04 ml (± 0.01 ml) of inoculum in the right hind foot-pad. In preliminary experiments in which groups of five mice were inoculated with four strains from Uganda, three from the Congo, three from Australia and one from Mexico, only the Ugandan strains were sufficiently virulent, and one of these was used in all subsequent experiments, except expt 5.

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The animals were examined weekly and records kept of their general health and the state of the local lesion as assessed by the presence and extent of erythema, oedema, ulceration, or loss of digits and distal parts of the limb. They were also examined for the development of generalised oedema and lesions other than those in the inoculated limb. Mice that appeared to be suffering were killed, but some, particularly those with generalised oedema, died spontaneously.

The severity of disease was scored for individual mice as grade I: swelling of up to 50 per cent. in the size of the foot-pad, with mild erythema or, later, residual deformity after clinical recovery from the disease; grade II: more marked erythema and swelling up to twice the size of the healthy foot-pad; grade III: gross swelling with incipient ulceration, or an ulcerated foot in which oedema had subsided; grade IV: loss of the foot with persistent signs of disease in the stump, involvement of the thigh or scrotum, or death. The sum of the grades for individual mice gave the morbidity index for the group.

Drug regimens. The following regimens were used in courses of 1 mth, except where otherwise noted: (A) rifampicin 40 mg per kg on 6 days of the week; (B) rifampicin 40 mg per kg three times weekly; (C) rifampicin 160 mg per kg once weekly (five doses in all); (D) sulphadoxine 350 mg per kg twice weekly plus pyrimethamine (c. 14 mg per kg per week); (E) clofazimine 15 mg per kg on 6 days of the week; (F) C plus D; (G) C plus E. All drugs were given by gastric tube with the exception of pyrimethamine, which was added to drinking water. In each experiment a group of untreated mice given an injection of the same organism at the same time was used as a control.

Regimens A, B and C were each applied to groups of ten mice, starting 2 wk (expt 1) or 5 wk (expt 2) after inoculation. In *expt 3*, regimen C was used for 10 wk to treat mice with established severe (grade-III) infections, and in *expt 4* groups of ten mice began regimens D, E, F or G 2 wk, or regimens D, E or F 5 wk, after inoculation. In *expt 5*, groups of five mice began regimen C 2 wk after inoculation with the other three strains found to be suitably virulent but not used in the preceding experiments.

RESULTS

In-vitro tests. Of the 32 strains tested for sensitivity to rifampicin, 22 (69 per cent.) had a resistance ratio of 1 or less; seven (22 per cent.) had a ratio of 2; two (6 per cent.) had a ratio of 4, and only a single strain (3 per cent.) had a ratio of 8.

In-vivo tests. Among the untreated animals, clinical signs of disease first appeared between 10 and 21 days after inoculation. The time of onset varied between different batches of mice, but was remarkably constant among the individuals of any one batch. Within 3 wk of the first visible signs, morbidity increased from grade I to grade III. Thereafter the disease either progressed to grade IV and death, or appeared to halt at grade III for several weeks without noticeable change. In some of these mice, towards the end of the 20th wk the lesions showed signs of fibrosis and healing. Mortality (natural and by killing) varied from 50 to 95 per cent. in different batches of mice, except for expt 5 (which ran for only 10 wk) where mortality ranged from 0 to 20 per cent. with the three different organisms.

Experiment 1. Treatment of early lesions with three different regimens of rifampicin either delayed the onset of all signs of disease or markedly improved morbidity as compared with the control groups. In two of the groups (those receiving 40 mg per kg three times weekly, or 160 mg per kg once weekly), in which signs of disease were already present at the beginning of chemotherapy, a reduction in the severity of lesions was discernible within 3 wk. When drug

administration was stopped 6 wk after inoculation, lesions remained the same for approximately 8 wk, and then slowly progressed until at the end of the experiment (20 wk) the mice had grade-I or grade-II disease as compared with grade III or IV of the surviving control animals. None of the treated animals died or had to be killed during the course of the whole experiment.

Experiment 2. Treatment of more advanced lesions with the same three-dosage regimens of rifampicin produced improvement which continued for 6 wk after cessation of chemotherapy (9 wk after inoculation). At the point of maximum improvement (15 wk after inoculation) each treatment group had a morbidity index between half and two-thirds that of the untreated controls.

FIG. 1.—The effect on mouse foot-pad lesions of three different regimens of rifampicin, each administered for 1 mth, starting 2 wk and 5 wk after infection. The stippled area represents the range of morbidity of three untreated control groups shown as continuous lines. Changes in morbidity index in mice treated 2 wk after infection. ----- Changes in morbidity index in mice treated 5 wk after infection. Rifampicin treatment regimens: A. 40 mg per kg 6 days of the week; B. 40 mg per kg three times weekly; C. 160 mg per kg once weekly.

Between 15 and 20 wk there was some increase in the size of the lesions, particularly in animals that had received the 160 mg per kg per week regimen. At the end of the experiment mortality was 20 per cent. (two out of ten) of mice receiving 40 mg per kg per day; 10 per cent. of those receiving 40 mg per kg three times weekly; and 30 per cent. of those receiving 160 mg per kg per week. The results of experiments 1 and 2 are shown graphically in fig. 1.

Experiment 3. Treatment of mice with advanced (grade-III) lesions with rifampicin 160 mg per kg per week for 10 wk produced marked improvement over this period. Within a few days of the first dose, lesions regressed, until after 7 weeks' treatment, the infected feet had returned almost to normal size and

appearance, except for those ulcerated at the beginning of treatment, which showed a variable amount of residual scarring. For the 10 wk following completion of chemotherapy the mice continued without relapse. Sixty per cent. of untreated animals with similar lesions were dead at the end of the experiment and the remaining 40 per cent. had grade-III or grade-IV lesions.

Experiment 4. One month's treatment with clofazimine (15 mg per kg per day) or with sulphadoxine (350 mg per kg twice weekly) plus pyrimethamine in the drinking water failed to produce any improvement in the course of infection, whether instituted 2 or 5 wk after inoculation. The course of the disease

FIG. 2.—The effect on mouse foot-pad lesions of sulphadoxine plus pyrimethamine, clofazimine, and combinations of these drugs with rifampicin, each administered for 1 mth, starting 2 wk after infection. The stippled area represents the range of morbidity of three control groups of untreated mice. Diagonal dotted lines show the range of morbidity of three groups treated with different regimens of rifampicin alone. Treatment regimens: D. Sulphadoxine 350 mg per kg twice weekly, plus pyrimethamine; E. Clofazimine 15 mg per kg 6 days of the week; F. Rifampicin 160 mg per kg once weekly plus D; G. Rifampicin 160 mg per kg once weekly plus E.

in treated animals closely followed that in the controls throughout the full 20 wk of the experiment, at the end of which all surviving animals had grade-III or grade-IV lesions. The treated groups in this experiment had final mortalities of 60, 60, 50 and 40 per cent. This last figure, although 10 per cent. less than the mortality recorded for any of the control groups, represents the survival of one mouse and the difference cannot therefore be considered significant.

When rifampicin was combined with clofazimine in the treatment of early infections, the results during chemotherapy and in the ensuing 3 wk closely paralleled those obtained by the use of rifampicin alone. Later, however, the lesions progressed until at the end of the experiment one of the ten mice had died, one had a grade-II lesion, and eight had grade-III lesions. Similarly,

when rifampicin was combined with sulphadoxine and pyrimethamine in the treatment of early infections, the results paralleled those for treatment with rifampicin alone until 5 wk after the cessation of chemotherapy. Thereafter the lesions rapidly progressed until at 20 wk the mortality was 30 per cent. and the surviving mice had grade-III lesions.

Treatment of more advanced infections with the combination of rifampicin, sulphadoxine and pyrimethamine failed to produce any improvement, the treated group closely following the course of the untreated controls. At the end of the experiment only 40 per cent. of the animals survived, and these had grade-III lesions. The results of this experiment are shown graphically in fig. 2.

Experiment 5. Treatment with rifampicin of the animals infected with the other three virulent strains produced rapid improvement in each case. The lesions showed no sign of relapse at the end of the experiment, 4 wk after completion of chemotherapy, and none of the animals died.

DISCUSSION

The results show that rifampicin administered over a 1-mth period produced significant clinical improvement in experimental *Mycobacterium ulcerans* infection of the mouse foot-pad. Although the majority of in-vivo experiments were carried out with a single strain of Ugandan origin, in-vitro tests on other strains from Uganda, Congo and Mexico indicate them to be equally sensitive to the drug, and this was supported by the effectiveness of the drug against infection with three other virulent strains of Ugandan origin.

The investigation of three rifampicin dosage regimens ran consecutively rather than concurrently, and for this reason the size of the inoculum, the age of the mice and the temperature of the animal house may all have been subject to minor variation resulting in different times of onset of disease. With the daily dosage regimen, all mice had superficially normal foot-pads 2 wk after inoculation, and disease did not become manifest in the control group until 20 days after inoculation. In the mice receiving the intermittent regimens, however, disease was already apparent at the beginning of chemotherapy. When these considerations are taken into account the three dosage regimens are seen to produce very similar results in the treatment of early lesions.

In the treatment of later lesions (expt 2), where time of onset of clinical signs is of much less significance, the results of treatment with the three regimens are more comparable. The daily and twice-weekly treatment regimens both produced better results than once-weekly dosage. However, improvement was achieved with all three regimens and, when the weekly dosage regimen was employed in the treatment of selected grade-III lesions and administered over a 10-wk period (expt 3), excellent results were obtained.

Clofazimine and the sulphadoxine-pyrimethamine combinations were begun at the same time and given for the same period as rifampicin, so that a direct comparison between courses could be made. When given over a 1-mth period these drugs had almost no therapeutic effect, neither improving morbidity nor significantly reducing mortality. The dosage of clofazimine was the same as

that successfully employed by Lunn and Rees (1964) and the dosage of sulphadoxine and pyrimethamine was based on our unpublished observations of in-vitro sensitivity. However, it should be stressed that under different conditions, such as those employed by Lunn and Rees, who began treatment at the time of infection, they might prove effective. When other drugs were used in combination with adequate doses of rifampicin, the salutary effect on the disease was less than that achieved by the same dose of rifampicin alone. An interesting finding was that the stationary phase of 6–8 wk that followed rifampicin therapy before the lesions again progressed, was reduced by combining rifampicin with either clofazimine or sulphadoxine plus pyrimethamine.

Our experiments were not designed to produce a complete cure for the infection in mice, but to demonstrate the action of rifampicin on *Myc. ulcerans* *in vivo*. One month's treatment was almost invariably followed by relapse several weeks after its completion. However, the 10-wk course of treatment used in experiment 3 produced virtually complete clinical cure of the disease, although the follow-up of 10 wk was not sufficient to ensure that relapse would not occur.

These results suggest that it would be reasonable to undertake a clinical trial of rifampicin in the treatment of human *Myc. ulcerans* infection, and such a trial is in progress.

SUMMARY

The in-vitro sensitivity of 32 strains of *Mycobacterium ulcerans* to rifampicin was found to be similar to that of *Myc. tuberculosis*. A 1-mth course of rifampicin in three different dosage regimens was used to treat experimental *Myc. ulcerans* infection of mouse foot-pads starting 2 and 5 wk after infection. Each regimen led to clinical improvement, but the disease relapsed 6–8 wk after completion of therapy. Treatment of severe lesions for 10 wk produced apparent cure. A 1-mth course of sulphadoxine plus pyrimethamine or of clofazimine proved ineffective, and combination of these with rifampicin were no more effective than rifampicin alone.

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