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The Action of Dapsone on a Susceptible Strain of *Mycobacterium kansasii*

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Because studies of the action of dapsone on *Mycobacterium leprae* have been obstructed by the need to conduct the studies in infected animals, a study of the action of the drug has been carried out on a strain of *M. kansasii* shown to be inhibited by 0.3 μg dapsone per ml, a concentration 100 times larger than the minimal inhibitory concentration of the drug for *M. leprae*. In stationary broth cultures, dapsone was bactericidal in concentrations of 1.0 μg per ml or larger; populations of *M. kansasii* as small as 1.7×10^6 organisms appeared to contain individuals resistant to 1.0 and 10 μg dapsone per ml. The organisms were shown to bind the drug against a concentration gradient. The action of the drug was antagonized by 4-aminobenzoic acid (PABA) in a mole ratio (PABA: dapsone) of 2:1. PABA itself, in a concentration of 10 μg per ml, inhibited multiplication of this strain of *M. kansasii*.

Introduction

Dapsone (4,4′diaminodiphenylsulfone, DDS) is widely used in the treatment of leprosy, and *Mycobacterium leprae* has been shown to be exquisitely susceptible to the drug (Peters *et al.*, 1975). However, because *M. leprae* has not yet been cultivated in a cell-free medium, many aspects of the action of DDS on the organism have been little studied. Earlier workers studied the susceptibility to DDS of a variety of cultivable mycobacterial species in a search for chemotherapeutic agents active in various mycobacterial diseases, and also in an effort to find a cultivable mycobacterial species that could serve as a surrogate for *M. leprae* in studies of drug action. The description by Pattyn and van Ermengem (1968) of strains of *M. kansasii* more susceptible to dapsone than any cultivable mycobacterial strain heretofore described stimulated us to attempt to characterize the antimycobacterial action of this drug.

In the work to be described, we have measured the minimal inhibitory concentration (MIC) of dapsone for one rather suceptible strain of *M. kansasii* both on solid and in liquid media, and demonstrated that dapsone is

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bactericidal in concentrations not much greater than the MIC. In addition, we have adduced evidence that the drug is bound by the organisms. Finally, we have studied the combined effects of dapsone and 4-aminobenzoic acid (PABA) on this strain of *M. kansasii*.

Materials and Methods

Dapsone was purchased from K & K Laboratories, Hollywood, California; PABA was purchased from General Biochemicals, Chagrin Falls, Ohio. The strain of *M. kansasii* was supplied by S. R. Pattyn, Prince Leopold Institute for Tropical Medicine, Antwerp, Belgium (Pattyn and van Ermengem, 1968).

Cultures of *M. kansasii* were performed in Dubos broth supplemented with albumin (Difco Laboratories, Detroit, Michigan), Dubos agar to which oleic acid-albumin supplement had been added (Difco), or glycerol-urea agar (Tsukamura, 1967). To prepare inocula, the surface growth of a culture of the organism was carefully scraped from a Lowenstein-Jensen slant and weighed in a tared vial. A measured quantity of Dubos broth was added and the bacterial mass suspended with the aid of a variable-speed mixer. Single-cell inocula of *M. kansasii* were prepared by filtering portions of the bacterial suspension through a membrane filter with a mean pore size of 5 μm.

Direct counts of the organisms were performed on Reich counting slides (Bellco Glass, Vineland, New Jersey) according to the method of Shepard and McRae (1968). A typical preparation was found to contain 86% of the organisms as individuals and 14% in clumps of 2 organisms; no larger clumps were encountered. The plating efficiency of these suspensions was 92% on the average.

All cultures were incubated at 37°C. Growth of the organisms in broth was measured as the absorbance at 580 nm in a Coleman Sr. spectrophotometer. A comparison of direct counts with turbidimetric measurements of suspensions of *M. kansasii* in Dubos broth demonstrated that the concentration of organisms was linearly related to the absorbance of the suspension according to the regression equation: number of organisms $\times 10^{-7}$ per ml $= 7.40 + 23.6$ (absorbance $- 0.282$).

The concentration of dapsone was measured by fluorescence assay (Peters *et al.*, 1970). The drug was found to be stable in Dubos broth incubated at 37°C for 2 months.

Results

The first studies of the inhibition by dapsone of the multiplication of *M. kansasii* were performed on solid media. Glycerol-urea and Dubos agar plates containing no drug or 0.1, 0.3, 0.5, or 1.0 μg dapsone per ml were inoculated with about 60 organisms per plate from a suitably diluted single-cell suspension and incubated: colonies were enumerated at intervals for 2 months. The results of this experiment, presented in Table 1, show that colonies of *M. kansasii* generally appeared earlier on Dubos than on glycerol-urea agar. Dapsone in a concentration of 0.1 μg per ml slowed the appearance of colonies in relation to the time of appearance of colonies on drug-free medium. No

colonies appeared on glycerol-urea agar containing 0.5 μg dapsone per ml or on either medium containing 1.0 μg dapsone per ml. Only about three-quarters of the expected number of colonies appeared on the plates containing 0.3 μg dapsone per ml. Thus, slowing of the rate of appearance of colonies—i.e. of the rate of growth of the organisms—appears to be characteristic of the inhibition by dapsone of the growth of *M. kansasii* on solid media. The MIC of dapsone for this strain of *M. kansasii*, measured on solid media, appeared to lie in the range 0.3–0.5 μg per ml.

TABLE 1

Rate of appearance of colonies of M. kansasii *on minimal and complex solid media in presence of dapsone*

Duration of incubation (days)	Number of CFU per plate								
	Dapsone concentration (μg/ml)								
	0		0.1		0.3		0.5	1.0	
	GU*	Du†	GU	Du	GU	Du	GU	GU	Du
13	0	62 (46–78)‡	0	0	0	0	0	0	0
17	48 (34–62)	64 (48–80)	0	52 (38–66)	0	0	0	0	0
20	52 (38–66)	64 (48–80)	0	60 (45–75)	0	0	0	0	0
29	58 (43–73)	64 (48–80)	86 (68–104)	60 (45–75)	0	0	0	0	0
44	58 (43–73)	64 (48–80)	86 (68–104)	60 (45–75)	39 (27–51)	53 (39–67)	0	0	0

* Glycerol-urea agar
† Dubos agar
‡ 95% confidence limits.

Next, the effect of inoculum size on dapsone inhibition in broth cultures of *M. kansasii* was examined. Replicate tubes of Dubos broth containing a variety of concentrations of dapsone were inoculated with serial 10-fold dilutions of a suspension of *M. kansasii*, and the number of days of incubation before the appearance of turbidity was recorded (Table 2).

The MIC of dapsone for this strain of *M. kansasii*, measured in broth, appeared to lie in the range 0.1–1.0 μg per ml. The turbidity of the cultures inoculated with 1.7×10^7 organisms per ml, which had been rendered turbid by the inoculum, increased markedly after incubation for 1 day. All of the tubes inoculated with 1.7×10^6 organisms per ml were turbid after 2 days of incubation. Those cultures receiving smaller inocula became turbid later. The appearance of turbidity was further delayed in the tubes containing 0.1 μg dapsone per ml.

Turbidity appeared in 3 sets of cultures only after incubation for 22 days;

aliquots from these cultures were plated on dapsone-containing Dubos agar. The cultures inoculated with 1.7×10^5 *M. kansasii* per ml yielded growth on agar containing 1.0 or 10 μg dapsone per ml. Those inoculated with 1.7×10^4 organisms per ml yielded growth on agar containing 1.0 μg dapsone per ml, but not on agar containing 10 μg dapsone per ml. Thus, populations of 1.7×10^5 *M. kansasii* (10 ml, 1.7×10^4 organisms per ml) did not include individual organisms resistant to 10 μg dapsone per ml.

TABLE 2

Effect of inoculum size on inhibition of M. kansasii *by dapsone*

Number of *M. kansasii* inoculated per ml	Number of days from inoculation to appearance of turbidity				
	Dapsone concentration (μg/ml)				
	0	0.1	1.0	10	100
1.7×10^7	1	1	1	1	1
1.7×10^6	2	2	2	2	2
1.7×10^5	4	6	22	22	>24
1.7×10^4	7	8	22	>24	>24

The cultures containing 100 μg dapsone per ml that had been inoculated with 1.7×10^4 *M. kansasii* per ml, all of which failed to become turbid, were filtered onto membrane filters with a mean pore size of 0.15 μm. The filters were washed with sterile Dubos broth, transferred to the surfaces of drug-free Dubos agar plates, and incubated. No colonies appeared on the filters after incubation for 6 weeks, whereas colonies appeared after incubation for only $2\frac{1}{2}$ weeks on filters that had been used to filter drug-free cultures, demonstrating that dapsone in a concentration of 100 μg per ml had been bactericidal.

To obtain further evidence of the bactericidal activity of dapsone on *M. kansasii*, replicate sets of tubes of Dubos broth into which dapsone had been incorporated in concentrations of 1, 2, 4 or 8 μg per ml were inoculated with 2×10^3 *M. kansasii* per ml and incubated. After 1, 4 and 8 days of incubation, 0.1 ml aliquots were removed from each culture and spread on the surface of drug-free Dubos agar plates, which were incubated and observed at intervals during 8 weeks for the appearance of colonies. All 4 concentrations of dapsone were found to be bactericidal to *M. kansasii*. After incubation for 8 days, the mean number of colonies per ml of the cultures containing 1 μg dapsone per ml had been reduced by 80%, and no colonies were seen on the plates inoculated with the aliquots from the cultures containing 4 and 8 μg dapsone per ml. As shown in Fig. 1, the plots of the common logarithm of the mean number of CFU per ml of culture as a function of time of incubation are fairly linear, and give half-times of killing of *M. kansasii* of 2.8 days for 1 μg dapsone per ml, 1.4 days for 2 μg dapsone per ml, and 0.73 days for 4 and 8 μg dapsone per ml. Only the slopes of the regression lines corresponding to 1 and 8 μg dapsone

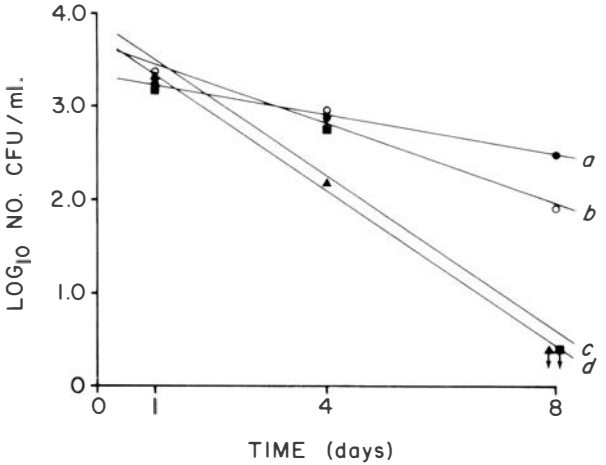

Fig. 1. Regression of the $\log_{10}$ number of CFU of *M. kansasii* per ml culture on the duration of incubation in the presence of several concentrations of dapsone; line *a* (●—●), 1 μg dapsone per ml; line *b* (○—○), 2 μg dapsone per ml; line *c* (■—■), 4 μg dapsone per ml; line *d* (▲—▲), 8 μg dapsone per ml.

per ml are significantly different from 0; they are also significantly different from each other (Goldstein, 1964).

To examine the possibility of binding of dapsone by *M. kansasii*, replicate sets of tubes of Dubos broth containing 0, 0.1, 1.0, 10 and 100 μg dapsone per ml were inoculated with 2.5×10^7 organisms per ml and incubated. Aliquots were removed for filtration on membrane filters with a mean pore size of 0.15 μm at intervals during 8 days. The concentration of dapsone was determined in both the culture filtrates and the filtered organisms (see Table 3). No dapsone was detected in the organisms filtered from the cultures containing

TABLE 3

Binding of dapsone by M. kansasii

Time of incubation (h)	Concentration of dapsone in medium (μg/ml)					
	0.1	1.0	10		100	
	DDS_C* (μg/mg)	DDS_C (μg/mg)	DDS_C (μg/mg)	Ratio†	DDS_C (μg/mg)	Ratio
54	<0.01	<0.01	0.08	8.1	0.71	7.1
198	<0.01	<0.01	0.14	14.0	1.5	15.0

* Dapsone concentration of filtered *M. kansasii*. Bacterial mass calculated from absorbance measured immediately before filtration, according to the experimentally-derived relationship: Absorbance = 0.282 + 0.627 (bacterial mass in mg − 0.5).

† $DDS_C \times 1000$/ concentration of dapsone in medium.

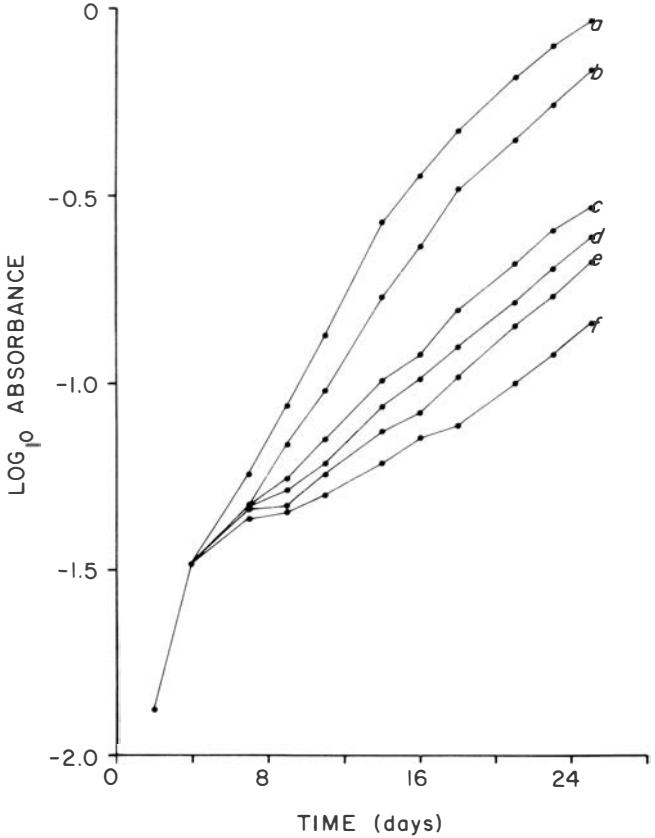

Fig. 2. Log_{10} absorbance of broth cultures of *M. kansasii* as a function of duration of incubation. Curve *a* represents multiplication of the organisms in drug-free broth, in broth containing 0.01, 0.1 or 1.0 μg PABA per ml, and in broth containing 0.1 μg dapsone *plus* 0.1 μg PABA or 0.8 μg dapsone *plus* 1.0 μg PABA per ml. Curve *b* represents the growth curve of *M. kansasii* in medium containing 10 μg PABA per ml or 0.8 μg dapsone *plus* 0.1 μg PABA per ml. Curves *c, e,* and *f* are respectively the growth curves of *M. kansasii* in the presence of 0.1, 0.4, and 0.8 μg dapsone per ml. Curve *d* represents the multiplication of the organisms in broth containing 0.2 μg dapsone per ml or 0.8 μg dapsone *plus* 0.01 μg PABA per ml.

0.1 and 1.0 μg dapsone per ml. Analysis of the filtrates from the cultures containing 1 and 10 μg dapsone per ml revealed that there had been no loss of dapsone nor production of monoacetyldapsone during the incubation. The organisms filtered from the cultures containing 10 and 100 μg dapsone per ml were found to contain dapsone in concentrations 7- to 8-fold larger than that of the medium after a little more than 2 days' incubation, and 14- to 15-fold that of the medium after incubation for a little more than 8 days.

To measure the effect of PABA on inhibition by dapsone of the multiplication of *M. kansasii* in liquid medium, replicate sets of tubes containing drug-free Dubos broth or broth into which had been incorporated dapsone, PABA,

or both in a variety of concentrations were inoculated with a single cell suspension of *M. kansasii* providing a concentration of 6.7×10^5 organisms per ml and incubated. The turbidity of the cultures was measured 3 times weekly. The results of this experiment are shown in Fig. 2 as plots of the common logarithm of the absorbance as a function of the duration of incubation. For the sake of clarity, similar growth curves of *M. kansasii* have been combined. Taken together, the curves suggest that the absorbance increases logarithmically with time over the absorbance range 0.050–0.300, whereas the increase is arithmetic above an absorbance value of 0.400. During the period of logarithmic multiplication in the drug-free cultures (curve *a*), the organisms doubled at the rate of once every 3.3 days. All 4 concentrations of dapsone exerted an inhibitory effect on multiplication of *M. kansasii* (curves *c–f*). The absorbance of the dapsone-containing cultures increased at the same rate as that of the drug-free cultures until the fourth day of incubation, by which time the number of organisms had increased about 22-fold (about 4.5 doublings). Thereafter, the organisms multiplied more slowly in the dapsone-containing media, with generation times calculated from the slopes of the regression lines as follows: 6.7 days in the cultures containing 0.1 μg dapsone per ml; 7.3 days in those containing 0.2 μg dapsone per ml; 7.1 days in the presence of 0.4 μg dapsone per ml; and 8.6 days in the presence of 0.8 μg dapsone per ml. The generation times of 6.7 and 7.3 days are not significantly different from each other, although they are significantly longer than those in drug-free cultures, and significantly shorter than the generation time of 8.6 days for growth in the presence of the largest concentration of dapsone (Goldstein, 1964).

The growth of *M. kansasii* in the presence of PABA was like that in the control cultures, except for the cultures containing 10 μg PABA per ml. This largest concentration of PABA inhibited bacterial multiplication somewhat (curve *b*); the doubling time of the organisms in the cultures containing 10 μg PABA per ml was 3.9 days, a value significantly larger than that for the control cultures. The inclusion of PABA in the dapsone-containing cultures blocked the inhibitory effects of dapsone. PABA in a concentration of 1 μg per ml completely antagonized the inhibition produced by 0.8 μg dapsone per ml; and 0.1 μg PABA per ml completely antagonized the inhibitory effect of 0.1 μg dapsone per ml. The effects of 0.8 μg dapsone per ml were incompletely blocked by 0.1 μg PABA per ml.

Discussion

The purpose of this research was to study some characteristics of the action of the antileprosy agent, dapsone, on a susceptible cultivable *Mycobacterium*. *M. leprae* is inhibited from multiplying in mice or rats by administration of dapsone to the animals in dosages yielding plasma concentrations in the range 1–5 ng per ml (Ellard *et al.*, 1971; Ozawa *et al.*, 1971; Peters *et al.*, 1975). However, because these organisms multiply only in the living host, it is impossible to study directly the actions of drugs on the organism.

It is apparent that treatment of patients with lepromatous leprosy with dapsone in effective dosage is accompanied by death of *M. leprae* (Shepard *et*

al., 1968), but it is impossible to state with certainty whether bacterial killing results from a bactericidal action of the drug, or whether, on the other hand, the drug exerts only a bacteriostatic action, and the organisms are killed by the host defense mechanisms, known to be deficient in patients with lepromatous leprosy (Shepard, 1968). Dapsone treatment of mice infected with *M. leprae* followed by withdrawal of the drug results in delay of resumption of bacterial multiplication longer than can be explained by the persistence of effective levels of the drug in the mouse (Levy *et al.,* 1972). But one cannot readily distinguish between the killing of a fraction of the population of *M. leprae* followed by multiplication of the survivors on the one hand and survival of the entire population in a state of prolonged bacteriostatis on the other (Levy, 1970, 1972).

A cultivable *Mycobacterium* that is susceptible to dapsone might represent a model of *M. leprae* suitable for studies of the action of the drug. Pattyn demonstrated that the MIC of dapsone for several strains of *M. kansasii* was 0.3 μg per ml (Pattyn and van Ermengem, 1968), 100 times that for *M. leprae.* Morrison (1968) has studied extensively a strain of *M. smegmatis* 607 as a model of *M. leprae*, but the MIC of dapsone for this organism is 2 μg per ml, 1000 times that for *M. leprae.* It appeared to us that the more susceptible organism might be more suitable as a model of *M. leprae.*

Our studies showed that dapsone was equally effective in inhibiting multiplication of *M. kansasii* on solid media, whether the medium was simple (glycerol-urea) or complex (Dubos), and confirmed Pattyn's estimate of the MIC, measured on Lowenstein-Jensen medium, a complex solid medium (Pattyn and van Ermengem, 1968). The number of colonies appearing on media containing 0.3 μg dapsone per ml was smaller than that appearing on drug-free medium, and no colonies appeared on media containing 0.5 or 1.0 μg dapsone per ml. Moreover, colonies appeared more slowly on dapsone-containing than on drug-free media.

Studies in stationary broth culture demonstrated slowing of multiplication of *M. kansasii* by dapsone in a concentration of 0.1 μg per ml, and suggested that the drug was bactericidal for smaller inocula in 10-fold greater concentration. Populations of 1.7×10^5 and 1.7×10^6 *M. kansasii* were found to contain individuals resistant to 1 and 10 μg dapsone per ml. In an additional study, the drug was found to be bactericidal for *M. kansasii* in concentrations of 1–8 μg per ml; the rate of killing appeared to increase with increasing dapsone concentration. Thus, dapsone is bactericidal at least for this strain of *M. kansasii* in concentrations only slightly larger than the MIC.

The experiments described in this report also suggest a basis for the prolonged bacteriostatic action of dapsone. In the course of incubation in stationary broth cultures containing dapsone, *M. kansasii* was found to accumulate the drug against a concentration gradient. In the presence of 10 and 100 μg dapsone per ml, the organisms were shown to accumulate dapsone to a concentration 7–15 times that of the medium. It is not possible to conclude whether the binding resulted from an active or a passive process. The apparent increase of binding during the period between 54 and 198 h favours an active process, because one would expect a passive binding process to reach

equilibrium well within 54 h (Riley and Levy, 1973). On the other hand, the demonstration of binding of dapsone *in vivo* and *in vitro* by the plasma proteins of a variety of mamalian species (Biggs *et al.*, 1971), and the demonstration here that these concentrations of dapsone are bactericidal suggest that the process was passive.

In additional studies in stationary broth culture, the activity of dapsone in the concentration range 0.1–0.8 μg per ml was quantitatively antagonized by PABA in a mole ratio (PABA: dapsone) of 2:1, a result similar to that reported by Pattyn and van Ermengem, 1968). Shepard reported (Shepard, 1967) that PABA only partially antagonizes the action of dapsone on *M. leprae* when the compounds were administered to mice in a mole ratio (PABA:dapsone) of about 200:1. The observation that PABA itself inhibited multiplication of *M. kansasii* is of some interest. The inhibition of a mycobacterium by PABA was first reported by Steenken and Heise (1943), who described inhibition of multiplication of *M. tuberculosis* H37Rv and H37Ra by PABA. Nitti subsequently reported (1951) inhibition of a strain of *M. smegmatis* by PABA in a concentration of 500 μg per ml. The Trudeau Mycobacterial Culture Collection (1972) lists 5 strains of *M. kansasii*, of which 3 do not grow in the presence of 2 mg PABA per ml. Thus, although other investigators have described inhibition of mycobacterial multiplication by PABA, we could find no report of a mycobacterial strain inhibited, as was our strain of *M. kansasii*, by PABA in a concentration as small as 10 μg per ml.

Although the results described in this present report may help to elucidate the action of dapsone against *M. leprae*, the 100-fold greater MIC of dapsone for *M. kansasii* than for *M. leprae* suggests that the actions of the drug on the 2 organisms may differ qualitatively as well as quantitatively.

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