INTERNATIONAL JOURNAL OF LEPROSY

Volume 50, Number 1
Printed in the U.S.A.

Bacterial Growth Kinetics of "*M. lufu*" in the Presence and Absence of Various Drugs Alone and in Combination. A Model for the Development of Combined Chemotherapy Against *M. leprae*?[1]

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Tremendous progress has been achieved in the chemotherapy of infectious diseases during the last 40 years. Today, in general, every infection caused by bacteria can be treated successfully with chemotherapy. One of the important preconditions for this development was the successful cultivation of the various microorganisms, thus providing a simple *in vitro* test system for the development of new drugs. The lack of such a system is the major drawback in a rational development of the chemotherapy of leprosy. The only established test system available is the mouse foot pad technique ([9, 22, 23, 24]). However, this is a complicated and time consuming test system and includes factors which are difficult to control, particularly influences of the host organism on drug uptake, metabolism, and excretion and therefore on the effective drug concentration for therapy. Besides this, these pharmacokinetic influences in mice are, in general, different from the influence of the human organism on the drug molecules. This limits the conclusions which can be drawn for the treatment of leprosy in man. The described circumstances have led to the situation that only a small number of drugs are available for the therapy of leprosy. These drugs are all derived from drugs which have been used in the treatment of tuberculosis. Surprisingly, diaminodiphenylsulfone (dapsone, DDS), which is not a strong inhibitor of *M. tuberculosis*, shows extremely high inhibitory potency against *M. leprae* in mouse foot pad experiments ([3]).

In contrast to the practice in the therapy of tuberculosis, monotherapy, not combined treatment, has generally been applied in the therapy of leprosy during the last decades. This has created the problem of less effective therapy and the risk of development of resistant mutants, especially against DDS ([10, 11, 12, 26]). In the early 1970s, Freerksen and Rosenfeld ([4]) tried to overcome these difficulties. They used a series of different mycobacterial strains as a model to test the effectivity of various combinations of tuberculostatic drugs. From the screening Isoprodian®, a combination of DDS, isonicotinic acid hydrazide (INH) and 2-propylisonicotinic acid thioamide (PTH), together with rifampin (RAMP), was derived and was found to be most effective against a large number of atypical mycobacterial strains. This drug combination was then applied very successfully in an eradication program for leprosy on the Island of Malta. There, for the first time, therapy was interrupted after 1–2 years of treatment. During the first 5 years of follow-up, no relapses were reported ([5, 6]).

MATERIALS AND METHODS

Materials. "*M. lufu*" ([13]) maintained on Gottsacker medium was used as the test organism. The culture broth was a modified Dubos-Davis medium with 0.25% w/v bovine serum albumin fraction V. The authors are indebted to Dr. F. Portaels, Head, Laboratory of Mycobacteriology, Institute for Tropical Medicine, Antwerpen, Belgium, for making this strain available to us.

[1] Received for publication on 11 May 1981; accepted for publication on 21 September 1981.

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Growth. A broth culture was inoculated from 4–6 weeks old Gottsacker slants into fresh modified Dubos-Davis medium. To obtain a uniform suspension, the bacteria were homogenized in 5 ml of medium (Potter[S] homogenator); the suspension was then diluted with 15 ml of medium and centrifuged for 4 min at 150 × *g*. Part of the supernatant was taken for electronic counting, and the remainder used to set up the cultures. Part of the supernatant was taken for electronic counting and the remainder used to set up the cultures. The suspension was diluted to contain ~10^5 cells/ml and 50 ml portions were transferred to 300 ml Erlenmeyer culture flasks containing a 2 cm magnetic stirring bar. The inhibitor was added and the cultures were kept at 31°C. Before taking samples for counting, the cultures were vigorously stirred magnetically for about 1 min.

Total count (Coulter Counter). Samples of the experimental cultures were diluted with particle free saline (0.85%)-formaldehyde (0.2%) solutions, so that a count of 500–20,000 organisms was obtained. Diluted samples were counted with a Coulter Counter model ZB equipped with a 30 μm orifice. Counts per 50 μl were obtained. Instrument settings were: 1/aperture current 1; 1/amplification 1/2; matching switch 40 K; gain 10; lower threshold 7, and upper threshold maximum.

Viable count (Coulter Counter). Samples (0.5 ml) from the inhibited culture were taken as a function of time, diluted into 50 ml of fresh broth (1/101), and incubated at 31°C. Samples were taken after 8 days and counted with the Coulter Counter as described above.

Sensitivity test. Twelve days after first exposure to the drugs, the cultures were diluted to 10^5 counts/ml with fresh broth, drugs were added again, and the growth rate determined. To make sure that the cultures were not contaminated, the identity and purity of the bacilli were checked according to the usual procedures.

Chemicals (drugs). Clofazimine (CLF) was obtained from Ciba Geigy, Switzerland; rifampin from Lepetit, Italy; and isonicotinic acid hydrazide, 4,4′-diaminodiphenylsulfone and 2-propyl-isonicotinic acid thioamide from Bayer AG, Germany. Trimethoprim (TMP) and pyrimethamine (PMA) were obtained from Deutsche Wellcome GmbH, Germany. The authors are indebted to these companies. 4-Cyano-N′-phenylsulfanilamide (CNSA) and 4-amino-N′-phenylsulfanilamide (ASA) were synthesized according to standard methods which have been described elsewhere ([15]).

RESULTS AND DISCUSSION

Determination of single drug activity and development of resistance, using the bacterial growth kinetic technique. In this paper a bacterial growth kinetic method is applied by which synergistic, additive, or antagonistic effects of various drugs and drug combinations including Isoprodian[30] can be quantified using a mycobacterial strain as a model. For these studies "*Mycobacterium lufu*" ([14]), which is not yet completely classified, was selected. The reason for this selection was its extremely high sensitivity to DDS, its sensitivity to RAMP and PTH, and its low sensitivity to INH, which is ineffective in the mouse foot pad model (quantitative data are not available). Thus the sensitivity pattern of *M. leprae* resembles more that of "*M. lufu*" than *M. tuberculosis* (Table 1). Other similarities are the slow generation rate (~25 hr) and the maximal growth temperature at ~31°C. An additional practical advantage of "*M. lufu*" is that it has less pronounced clumping properties than, for example, *M. tuberculosis*. This is a neces-

TABLE 1. *Minimum inhibitory concentrations (MIC) or minimum effective dose (MED from mouse foot pad) for various mycobacteria.*

	MIC *M. tubercul.* μg/ml	MED[a] *M. leprae* μg/ml	MIC "*M. lufu*" μg/ml
DDS[b]	32.0	0.003	0.05
INH[c]	0.07	—	5–10
PTH[d]	0.5	0.05	0.60
RAMP[e]	0.25	0.3	0.25

[a] From Ref. 2.
[b] dapsone.
[c] isoniazid.
[d] prothionamide.
[e] rifampin.

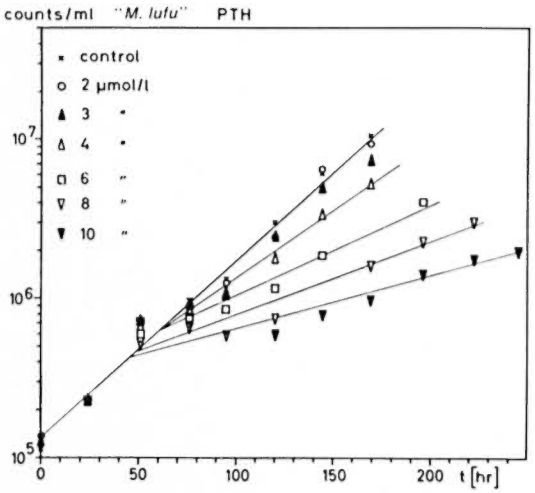

FIG. 1. Typical generation rate curves of "*M. lufu*" at 31°C in the presence of various concentrations of prothionamide (PTH) (electronic counts, total counts). The curves and generation rate constants k_{app} (in $sec^{-1} \times 10^{-6}$) were as follows: (×) control 7.49; (○) 7.47; (▲) 6.39; (△) 5.73; (□) 4.99; (▽) 2.78; (▼) 2.26.

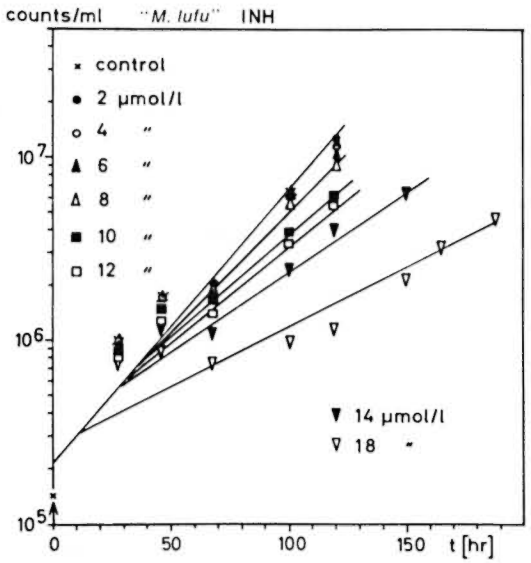

FIG. 2. Typical generation rate curves of "*M. lufu*" at 31°C in the presence of various concentrations of isonicotinic acid hydrazide (INH) (electronic counts, total counts). The curves and generation rate constants k_{app} (in $sec^{-1} \times 10^{-6}$) were as follows: (×) control 9.29; (●) 9.29; (○) 9.10; (▲) 8.9; (△) 8.52; (■) 7.46; (□) 7.11; (▼) 6.09; (▽) 4.42.

sary pre-condition using the Coulter Counter technique.

In previous papers we have reported on bacterial growth kinetic studies to quantify the synergistic action of dihydrofolate inhibitors like trimethoprim in combination with sulfonamides and sulfones using *E. coli* as a test organism ([16, 17, 18, 19]). Other than the application of special treatment to overcome the "clumping" of the bacteria, which would prevent reproducible counting and changes in the nutrient medium, the same technique as described for *E. coli* was used. The number of bacteria in a certain culture volume is counted electronically (Coulter Counter) during the logarithmic growth phase as a function of time. This is described by the following exponential equation:

$$N = N_0 e^{kt} \qquad (1)$$

where N is the number of bacteria per ml at time t, e is the base of the system of natural logarithms, N_0 is the number of bacteria per ml at the beginning of the experiment, k the generation rate constant, and t is time. A semilogarithmic plot of the number of bacteria against time results in straight lines.

$$\log N = \log N_0 + \frac{k}{2.303} t \qquad (2)$$

the slope of which is proportional to the generation rate constant k. Under the assumption that every bacterial cell is multiplying at the same rate, any decrease in generation rate in the presence of inhibitors is directly related to the potency of the inhibitor. That means the decrease in gener-

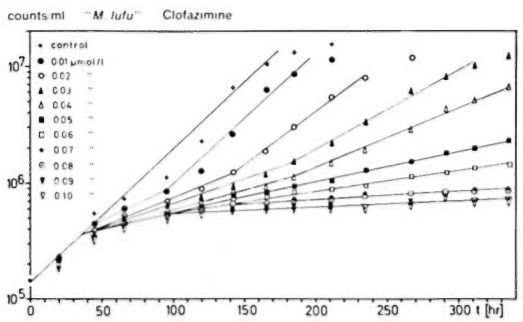

FIG. 3. Typical generation rate curves of "*M. lufu*" at 31°C in the presence of various concentrations of clofazimine (Lamprene®, CLF) (electronic counts, total counts). The curves and generation rate constants k_{app} (in $sec^{-1} \times 10^{-6}$) were as follows for the first inhibited phase: (×) control 7.02; (●) 3.81; (○) 3.19; (▲) 2.72; (△) 2.19; (■) 1.65; (□) 1.13; and for the second inhibited phase: (•) 0.0; (⊙) 0.0; (▼) 0.0; (▽) 0.0.

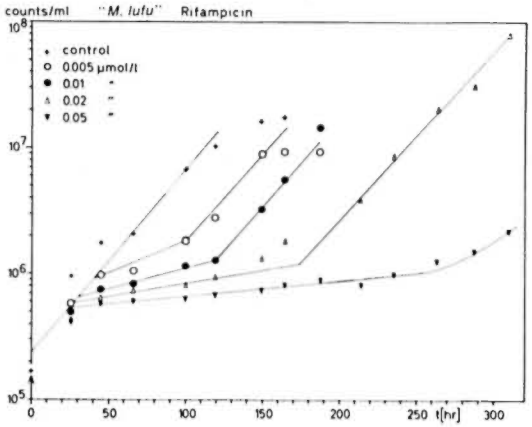

FIG. 4. Typical generation rate curves of "*M. lufu*" at 31°C in the presence of various concentrations of rifampin (RAMP) (electronic counts, total counts). The curves and generation rate constants k_{app} (in $sec^{-1} \times 10^{-6}$) were as follows for the first inhibited phase: (×) control 8.04; (○) 3.31; (●) 2.20; (△) 1.84; (▼) 0.081.

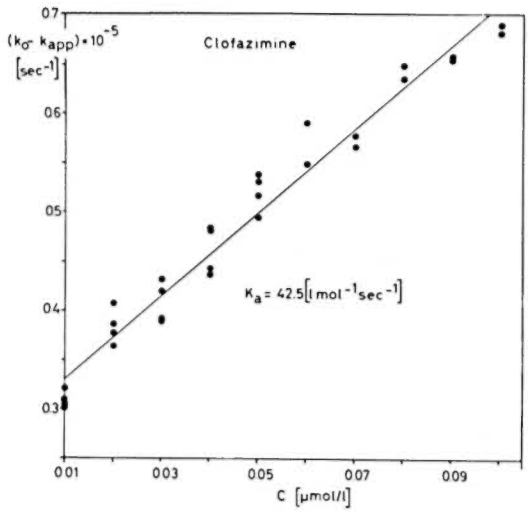

FIG. 5. Example of quantitative relations between apparent "*M. lufu*" growth rate constant k_{app} (total counts) and clofazimine (CLF) concentrations. The curve is plotted in accordance with equation 3.

ation rate as a function of inhibitor concentration (single and in combination) can be determined and the fractional inhibition compared to the control can be calculated. The inhibitory power of a certain drug can then be determined by the relation between its concentration and the related decrease in generation rate.

Before evaluating the effect of drug combinations to discriminate between their possible additive, synergistic or antagonistic action, the inhibitory power of the single drugs was determined. This was done for DDS, PTH, INH, RAMP, CLF, TMP, PMA, ASA, and CNSA.

Typical examples for the dependence of decrease in generation rate on inhibitor concentration are shown in Figs. 1–4 for PTH, INH, CLF, and RAMP, respectively. A range of drug concentration is used where the growth curves maintain a positive slope. From such plots the activity constant K_a is calculated by linearizing the dependence of the decrease in generation rate on drug concentrations. Two types of functions are observed: 1) a direct relation between the observed decrease in generation rate and the inhibitor concentration described by equation 3,

$$k_o - k_{app} = CK_a + K_b \qquad (3)$$

and 2) a reciprocal relation (Lineweaver-Burk type plot) described by equation 4,

$$\frac{1}{k_o - k_{app}} = \frac{1}{C}K_a + K_b \qquad (4)$$

where k_o is the generation rate constant in the absence of antibacterials, k_{app} in their presence, and K_a and K_b are constants. K_a is a measure of the activity of a particular drug tested and C is its concentration. Figures 5 and 6 show examples of the two types.

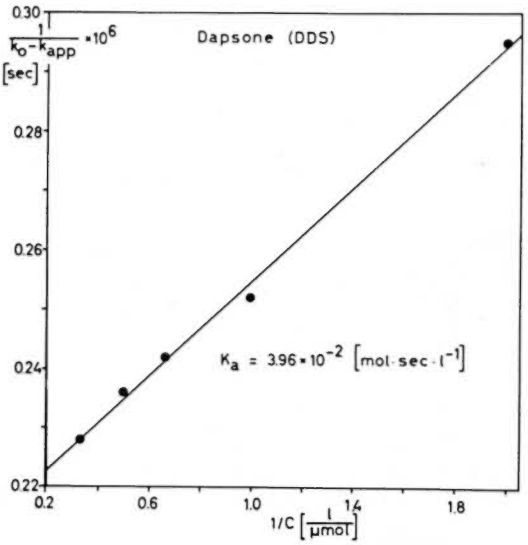

FIG. 6. Example of a quantitative relation between apparent "*M. lufu*" growth rate constants (total counts) k_{app} and dapsone (DDS) concentrations. The curve is plotted in accordance with equation 4.

TABLE 2. *Activity constant of some inhibitors of* "M. lufu" *derived from bacterial growth kinetic experiments.*

Inhibitor	K_a mol·sec·l^{-1}	K_a l·mol^{-1}·sec^{-1}	Conc. range studied (μmol/l)	i_{50} (μmol/l)
4-ASAB	0.21	—	2–50	6.35 ± 2.25
4-CNSAB	2.12	—	10–50	42.0 ± 12.4
DDS[a]	0.039	—	0.5–3.0	0.45 ± 0.038
INH[b]	—	0.38	8–20	15.65 ± 10.4
Clofazimine	—	42.5	0.01–0.1	0.022 ± 0.004
PTH[c]	—	0.58	3–10	9.68 ± 4.9
RAMP[d]	0.00027	292	0.003–0.009	0.0024 ± 0.0002

[a] dapsone.
[b] isoniazid.
[c] prothionamide.
[d] rifampin.

Type 1 plots (CLF, INH, PTH, RAMP) seem to apply to bactericidal drugs, type 2 to merely bacteriostatic acting compounds (DDS, sulfonamides (SA)). The list of K_a values obtained is given in Table 2. For K_a values derived by equation 3 a larger value means stronger inhibitory power; for K_a values derived by equation 4, a smaller number indicates stronger inhibitory efficiency. In the case of RAMP, it was not possible to differentiate on statistical reasoning between the two equations. For comparison purposes, 50% inhibitory concentrations (i_{50}) of each drug were calculated and are also given in Table 2. The reproducibility within days for the bacterial growth kinetic experiments is demonstrated on the example of "*M. lufu*" cultures in the presence of 0.2 μmol/l DDS (Table 3).

There is, however, another interesting observation in Figs. 1–4. In contrast to the results presented in Figs. 1–2 for INH, PTH, and also DDS, where the new steady state growth rates obtained after drug addition are maintained throughout the total observation time, the growth rates return to the control rate in the presence of low concentrations of CLF and RAMP despite a very strong inhibition for the first generations. If these cultures are exposed again to these drugs at the same concentration, no decrease in growth rate is observed. This excludes instability or metabolic inactivation of the drug as the reason, rather it indicates the selection of bacteria with lower sensitivity against these drugs, i.e., the development of resistant bacteria under the experimental conditions.

TABLE 3. *Control rate constants k_o and apparent first-order rate constants k_{app} for the growth of* "M. lufu" *in the presence of a constant concentration of dapsone (DDS) in repeated experiments on different days. The variance in $k_o - k_{app}$ is 0.022, standard deviation 0.149; with respect to the mean (2.078) this is ±7%.*

DDS [μmol/l]	$k_o \times 10^{-6}$ [sec^{-1}]	$k_{app} \times 10^{-6}$ [sec^{-1}]	$k_o - k_{app} \times 10^{-6}$ [sec^{-1}]	Inhibition %
0.2	8.17	5.91	2.26	27.7
0.2	7.86	5.86	2.26	25.5
0.2	7.69	5.76	1.93	25.1
0.2	8.13	5.97	2.16	26.6

Examples for such experiments, where the bacteria with decreased sensitivity have been inoculated into new broth and have again been exposed to the same concentration of inhibitor or inhibitor combinations as in the first exposure, are summarized in Figure 7 (see Materials and Methods, sensitivity test). Whereas in the case of RAMP alone and in the combination of RAMP and PTH no inhibition of the recultivated bacteria occurs, the cultures remain sensitive in all other examples of single drugs or drug combinations. In the case of INH (5 μM) + RAMP (0.01 μM) the inhibitory effect is only observable for about 150 hr. After this the generation rate approaches the control rate, but the bacteria remain sensitive if exposed again to the same drug concentration. The reason for this is not known. The reproducibility of these results

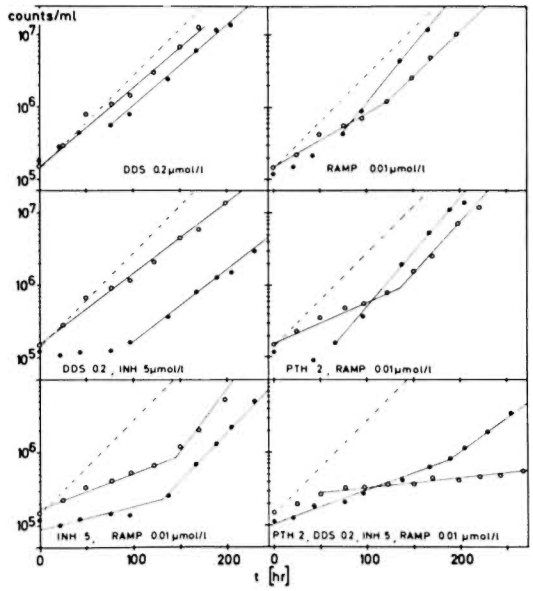

FIG. 7. Generation rates of "*M. lufu*" in the presence of different drugs at various concentrations as indicated. (- - -) control; (○) generation rate during first exposure; (●) generation rate during second exposure. DDS = dapsone; INH = isoniazid; RAMP = rifampin; PTH = prothionamide.

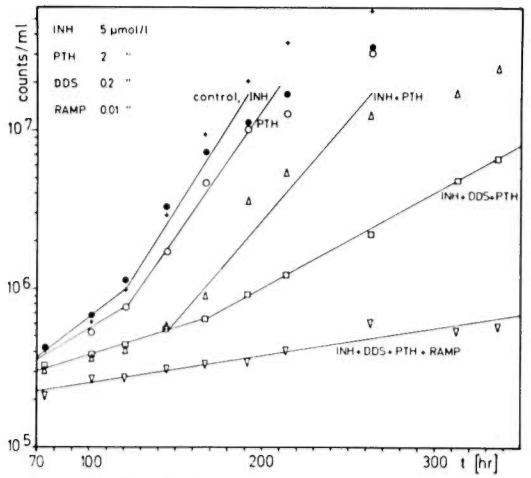

FIG. 8. Typical generation rate curves of "*M. lufu*" at 31°C in the presence of isoniazid (INH), prothionamide (PTH), dapsone (DDS) and rifampin (RAMP) alone and/or in different combinations at the concentrations indicated.

is satisfactory. The calculation of the fraction of less sensitive bacteria present in the original culture might be misleading because of the fact that we do not deal with single counts, despite the homogenization of the bacteria. By the homogenization procedure, only a homogeneous distribution of bacterial aggregates is obtained, allowing a reproducible counting.

Determination of combined drug activity. After the evaluation of the single drug activities, the inhibitory power of various drug combinations was studied. Figures 8–9 show the results with various combinations of DDS, PTH, INH, and RAMP. Some of these combinations, at the chosen concentrations, show synergistic effects, i.e., more than additive inhibitory effects (INH + PTH), others only additive or almost antagonistic behavior (PTH + DDS). The most pronounced potentiation is observed on combining PTH and INH. INH alone does not show a significant effect on the generation rate with the concentration used. It significantly potentiates, however, the effect of PTH alone (see slopes in Figure 8 for INH and PTH and also the % inhibition expressed as decrease in generation rate compared to the control in Table 4). More detailed results will be published elsewhere.

The development of resistance can again be seen, especially against RAMP, in Figures 4 and 9. The strong inhibitory effect is only operative for 150 hr; after that the genera-

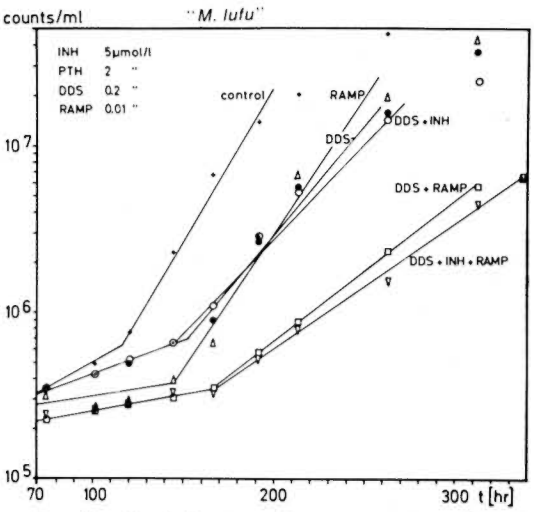

FIG. 9. Typical generation rate curves of "*M. lufu*" at 31°C in the presence of isoniazid (INH), prothionamide (PTH), dapsone (DDS) and rifampin (RAMP) alone and/or in different combinations at the concentrations indicated.

TABLE 4. *Single and combined action of dapsone (DDS), isoniazid (INH), prothionamide (PTH) and rifampin (RAMP) on* "M. lufu." *Concentrations in μmol/l; bacterial growth kinetics technique, Coulter Counter.*

DDS	INH	PTH	RAMP	k(h^{-1})	% inhibition
0	0	0	0	0.026	0
0.2	0	0	0	0.0204	21.5
0	5	0	0	0.026	0
0	0	2	0	0.0238	8.5
0	0	0	0.01	0.016	38.8
0.2	5	0	0	0.0198	23.8
0.2	0	2	0	0.0201	22.5
0.2	0	0	0.01	0.0121	53.5
0	5	2	0	0.0168	35.4
0	0	2	0.01	0.0116	55.4
0.2	5	2	0	0.0121	53.5
0.2	0	2	0.01	0.092	64.6
0	5	2	0.01	0.083	68.1
0.2	5	0	0.01	0.0147	43.5
0.2	5	2	0.01	0.058	77.7

TABLE 5. *Bacterial growth kinetic results (Coulter Counter,* "M. lufu") *for isoniazid (INH), PTH (prothionamide (PTH), dapsone (DDS), rifampin (RAMP) alone and in combinations.*

			% inhibition
a) after 145 hr of inhibited growth			
0 control		$22.9 \cdot 10^5$ counts/ml	0
1 INH	0.68 μg/ml	$23.4 \cdot 10^5$ counts/ml	0
2 PTH	0.36 μg/ml	$10.0 \cdot 10^5$ counts/ml	55
3 DDS	0.05 μg/ml	$6.5 \cdot 10^5$ counts/ml	70.7
4 RAMP	0.008 μg/ml	$3.9 \cdot 10^5$ counts/ml	82.6
5 INH + PTH		$4.3 \cdot 10^5$ counts/ml	80.0
6 INH + PTH + DDS		$3.5 \cdot 10^5$ counts/ml	84.4
7 INH + PTH + DDS + RAMP		$2.1 \cdot 10^5$ counts/ml	90.8
b) after 265 hr of inhibited growth (developm. of resistance)			
0 control		$462 \cdot 10^5$ counts/ml	
1 INH	0.68 μg/ml	$299 \cdot 10^5$ counts/ml	35
2 PTH	0.36 μg/ml	$264 \cdot 10^5$ counts/ml	43
3 DDS	0.05 μg/ml	$158 \cdot 10^5$ counts/ml	66
4 RAMP	0.008 μg/ml	$192 \cdot 10^5$ counts/ml	58.5
5 INH + PTH		$94 \cdot 10^5$ counts/ml	80
6 INH + PT + DDS		$17.8 \cdot 10^5$ counts/ml	96
7 INH + PTH + DDS + RAMP		$2.8 \cdot 10^5$ counts/ml	99

tion rate returns to the generation rate of the control. The different degrees of development of resistance are also demonstrated in Table 5, where the fractional inhibition (in % of the control) is given after 145 hr and 265 hr. If these values are compared to each other, it becomes obvious that, especially in the case of single drugs, a significant drop in inhibition is observed after 265 hr. The exception is INH, but this is probably due to its delayed onset of inhibition. In general, only the drug combinations maintain their inhibitory index or become even better. The most effective combination with constant inhibitory power is the combination DDS, INH, PTH, RAMP which, as already mentioned, has successfully been used as Isoprodian® + RAMP in the eradication program on the Island of Malta ([5]). The results presented here on "*M. lufu*" are additional support for the selection of this drug combination in leprosy therapy. The concentrations used in the present study are, however, not identical with the concentrations achieved in plasma under Isoprodian® + RAMP therapy, which, in general, are higher. The concentrations applied in this *in vitro* study are smaller in order to maintain positive slopes in the bacterial growth curves, a necessary condition to discriminate and quantify the effects of the single drugs and their various combinations by the Coulter Counter technique.

Other possible drug combinations in leprosy therapy. The combination of sulfonamides (SA) with TMP or TMP analogs is the classical combination where a strong synergistic effect has been found ([1, 7]) and this was quantitatively determined using bacterial growth kinetic techniques ([16]). Sulfonamides, however, are weak inhibitors of mycobacterial growth and are therefore replaced in our studies by sulfones which inhibit the same enzyme as SA in the *de novo* synthesis of dihydrofolic acid. Sulfones are strong inhibitors of this enzyme ([21]) especially in *M. leprae* and "*M. lufu*" ([8]). TMP has already been tested using the mouse foot pad technique. No inhibitory effect was reported ([25]). Because of the discussed limitations of this test system, we evaluated the inhibitory activity of TMP and PMA against the model strain of "*M.*

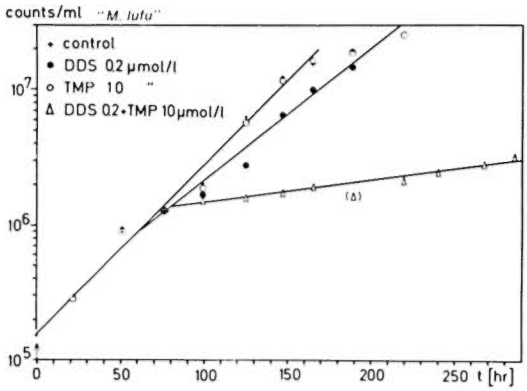

FIG. 10. Typical generation rate curves of "*M. lufu*" at 31°C in the presence of 0.2 µmol/l dapsone (DDS) and 10 µmol/l trimethoprim (TMP) alone and in combination (total counts). The curves and rate constants k_{app} (in $sec^{-1} \times 10^{-6}$) were as follows: (×) control 7.64; (○) 7.64; (●) 5.78; (△) 0.67.

lufu." No inhibitory effect could be detected even at high inhibitor concentrations (≥20 µM). It was therefore surprising that, in combination with small concentrations of DDS, very pronounced synergistic effects were observed. If DDS concentrations of 0.2 or 0.5 µM are used, decreases in growth rates of ~25% and ~70%, respectively, are obtained. With the addition of 10 or 5 µM TMP or PMA, an almost total bacteriostatic effect is achieved, whereas these TMP or PMA concentrations alone do not cause a significant deviation in generation rate compared to the drug free control (Fig. 10, Table 6). The effect of DDS/TMP and DDS/PMA combinations is maintained for several generation times. No development of resistant mutants (less sensitive bacteria) is observed. The concentration needed to achieve this effect can readily be obtained in plasma. Therefore these combinations could very well be candidates to be tested in the therapy of leprosy. Further evaluation is necessary.

Differentiation between bacteriostatic and bactericidal effects. Under conditions where the drug not only reduces the rate of bacterial growth but where simultaneous killing or kill without inhibition occurs, the applied Coulter Counter technique does not allow one to differentiate. In addition to, or instead of, total counts using the Coulter Counter technique, plate counts have to be performed. This is especially difficult with "*M. lufu*," where we have not succeeded in performing reproducible plate counts.

In a previous paper we have reported on a kinetic approach, using a modified Coulter Counter technique, to discriminate between total and viable counts ([20]), thus avoiding the performance of plate counts. As already pointed out, the generation rate of bacterial cells in the logarithmic growth phase can be described by equation 2. If a sufficiently high concentration of an antibacterial drug is applied to the bacterial culture, a constant number of counts is ob-

TABLE 6. *Inhibitory action of dapsone (DDS), trimethoprim (TMP), pyrimethamine (PMA) alone and in combination (inhibitor concentration in µmol/l) on* "M. lufu."

DDS	TMP	PMA	$k \times 10^{-6}$ [sec^{-1}]	% inhibition	Experiment no.
0	0	0	6.78	0	161
0	0	2	6.78	0	
0.4	0	0	3.89	41.7	
0.4	0	2	1.55	77.0	
0	0	0	7.52	0	M174
0	0	5	7.50	0	
0.1	0	0	7.77	0	
0.1	0	5	5.28	30.0	
0	0	0	7.22	0	M173
0.1	0	0	7.20	0	
0	0	5	7.22	0	
0.1	0	5	4.44	38.5	
0	0	0	7.64	0	M169
0	10	0	7.64	0	
0.2	0	0	5.78	24.4	
0.2	10	0	0.67	91.3	
0	0	0	9.90	0	M255
0	5	0	9.89	0	
0.5	0	0	3.11	68.6	
0.5	5	0	0.63	93.6	
0	0	0	9.37	0	M252
0	10	0	10.2	0	
0.5	0	0	2.22	76.3	
0.5	10	0	0.33	96.5	
0	0	0	7.69	0	M2
0	10	0	7.69	0	
0.2	0	0	5.76	25.1	
0.2	10	0	0.029	96.2	

tained (bacteriostasis) by the Coulter Counter in the sample volume (see Fig. 3 for example). Under these conditions, a differentiation between viable and killed bacteria is not possible, however, if the samples taken from such an inhibited culture at certain time intervals are diluted with fresh broth in such a way that the concentration of inhibitor becomes negligible, the resting bacteria start to multiply again. Only those bacteria which have not been killed by the drug start to grow again. The number of viable bacteria at time t'_0, however, is very small and not countable. In addition, the relative error caused by the number of killed bacteria compared to the viable bacteria present in the experimental volume would be large. After a certain time interval of new incubation, however, the number of viable bacteria has increased significantly and can be counted by Coulter Counter technique. If the condition holds that the culture in the "absence" of the inhibitor (dilution 1:101) is growing at the same rate as the control culture, the generation rate constant of the control (k_0) can be inserted into equation 2 together with the number of viable bacteria N, determined after a certain time interval t'. The number of viable bacteria N_0 at time t'_0, when the sample volume was taken from the culture and diluted into fresh broth, can thus be calculated. This has been shown to accurately describe bactericidal drug action in the case of fast growing bacteria ([20]). Difficulties which arise with "*M. lufu*" are a possible lag phase after dilution and incubation with fresh broth (i.e., the living bacteria do not multiply immediately) and the observed dependence of the generation time on the inoculum size. We have applied the described method therefore in a slightly modified way which still may be suitable to discriminate between bactericidal and bacteriostatic drug action even if the quantitative aspect of the kill rates observed is less accurate. Since only a constant error may be involved, the relative ranking of the effects of different drugs and drug concentrations remains correct nevertheless.

In the case of "*M. lufu*" we have taken samples from inhibited cultures as a function of time (diluted 1:101) and after a new incubation for a fixed time interval (8 days) the viable bacteria have been counted. In

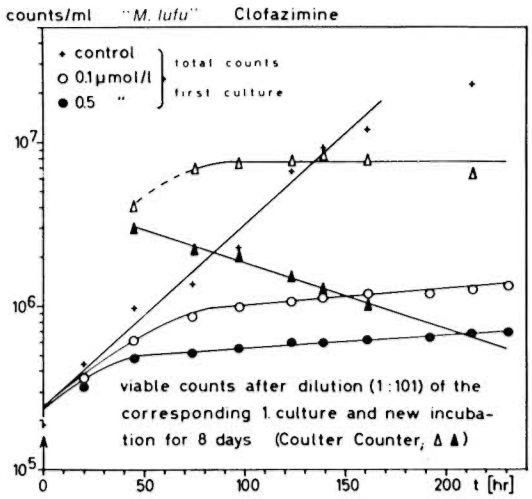

FIG. 11. Typical generation rate curves of "*M. lufu*" at 31°C in the presence of 0.1 and 0.5 µmol/l clofazimine. The curves and rate constants k_{app} (in $sec^{-1} \times 10^{-6}$) were as follows (total counts): (×) control 7.06; (○) 0.56; (●) 0.44; and after dilution (1:101) of the corresponding 1st culture with fresh broth at various time intervals and new incubation for 8 days the curves and rate constants k_{app} (in $sec^{-1} \times 10^{-6}$) were as follows (viable counts): (△) 0.0; (▲) −5.06.

the case of merely bacteriostatic acting drugs a constant number should be observed, whereas a bactericidal effect should produce decreasing numbers. Results for two clofazimine (CLF) concentrations are given in Figure 11 and are compared with the direct Coulter Counter approach (total counts—see also Figure 3). It is obvious that CLF acts bactericidally on "*M. lufu*" at a 0.5 µM concentration and bacteriostatically for the lower concentration (0.1 µM).

When this paper was in the publishing process, a paper by S. R. Pattyn, *et al.* ([27]) (1981) appeared, which seems to come to other conclusions on the value of INH, PTH, and DDS combinations. This is due to totally different experimental conditions: DDS concentrations are used in mouse foot pad experiments where the maximal inhibitory effect is already achieved by this drug alone.

SUMMARY

Bacterial growth kinetic studies were performed in a series of potential inhibitors of *M. leprae* using "*M. lufu*" as a model strain. Reasons why "*M. lufu*" is considered to be a better model than *M. tuber-*

culosis are presented. The inhibitory power of the single drugs has been quantified, the activity constants are calculated, and the synergistic, additive, or antagonistic behavior of the combinations is evaluated. It is demonstrated that a combination consisting of dapsone (DDS), prothionamide (PTH), isoniazid (INH), and rifampin (RAMP) is a very powerful inhibitor of "*M. lufu*" and prevents or delays the development of resistance under the experimental conditions described. This finding is in agreement with the therapeutic effect of this combination (Isoprodian® + rifampin) achieved in a leprosy eradication program on the Island of Malta. Whereas there is no direct proof that "*M. lufu*" is the best suitable model for drug evaluation against *M. leprae*, there is, however, nothing in the presented results which is against this model, especially as the actions of DDS and PTH or RAMP is concerned. A new combination of DDS with trimethoprim (TMP) or TMP derivatives has also been studied and seems to be a promising candidate. In addition, a technique is described to differentiate between bacteriostatic and bactericidal action of the tested inhibitors against "*M. lufu.*"

RESUMEN

Se hicieron estudios cinéticos sobre el crecimiento bacteriano usando una serie de inhibidores potenciales del *M. leprae*, y al "*M. lufu*" como cepa de prueba. Se presentan las razones de porqué el "*M. lufu*" se considera un mejor modelo que el *M. tuberculosis*. Se cuantificó el poder inhibitorio de drogas individuales, se calcularon las constantes de actividad y se evaluó el poder sinergístico, aditivo, o antagónico de las combinaciones. Se demostró que una combinación de dapsona (DDS), protionamida (PTH), isoniacida (INH) y rifampina (RAMP), es un muy poderoso inhibidor del "*M. lufu*" que además retarda el desarrollo de resistencia bajo las condiciones experimentales descritas. Este hallazgo esta de acuerdo con el efecto terapéutico de esta combinación (Isoprodian® + rifampina) alcanzado en un programa de erradicación de la lepra en la Isla de Malta. Aunque no hay prueba directa de que el "*M. lufu*" sea el mejor modelo para la evaluación de drogas contra el *M. leprae*, en los resultados presentados no hay nada en contra del modelo, especialmente en lo que concierne a la acción de la DDS y la PTH, o de RAMP. También se estudió una nueva combinación de DDS con trimetoprim (TMP) o con derivados del TMP que parece ser una alternativa promisoria. Además se describe una técnica para diferenciar entre la acción bacteriostática o bactericida de los inhibidores probados contra el "*M. lufu.*"

RÉSUMÉ

On a procédé à des études de la cinétique, de la croissance bactérienne chez une série d'inhibiteurs éventuels de *M. leprae*, en utilisant "*M. lufu*" comme souche modèle. Les raisons pour lesquelle "*M. lufu*" a été considéré comme un meilleur modèle que *M. tuberculosis*, sont exposées. La capacité inhibitrice de médicaments isolés a été quantifiée, les constantes d'activités ont été calculées, et le comportement synergétique, additif, ou antagoniste des combinaisons a été évalué. On a pu démontrer qu'une combinaison consistant en dapsone (DDS), prothionamide (PTH), isoniazide (INH), et rifampicine (RAMP), constituait un inhibiteur puissant de "*M. lufu,*" et prévenait ou retardait le développement de résistance dans les conditions expérimentales décrites. Cette constatation est en accord avec l'observation d'un effet thérapeutique de cette combinaison (Isoprodian® + rifampicine) telle qu'on l'a constatée dans le programme d'éradication de la lèpre à Malte. Alors qu'il n'existe pas de preuves directes que "*M. lufu*" est le meilleur modèle disponible pour l'évaluation des médicaments contre *M. leprae*, rien cependant dans les résultats présentés ne milite contre ce modèle, et particulièrement pour ce qui concerne l'action de la DDS, de la PTH et de la RAMP. On a également étudié une nouvelle combinaison de la DDS avec le trimethoprime (TMP) ou des dérivés de celui-ci; ce médicament semble être prometteur. De plus, on décrit une technique qui permet de différencier l'action bactéricide de l'action bacteriostatique des inhibiteurs étudiés contre "*M. lufu.*"

Acknowledgments. This investigation received financial support from the Chemotherapy of Leprosy (THELEP) component of the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases and from the German Leprosy Relief Association, D-8700, Würzburg, Germany.

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