Minimum Inhibitory and Bactericidal Dosages of Rifampicin Against *Mycobacterium leprae* in the Mouse Foot Pad: Relationship to Serum Rifampicin Concentrations[1]

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With the development of the mouse foot pad technic for assessment of the antileprous activity of compounds ([18, 19]), it has become possible to estimate the minimum inhibitory concentration (MIC) of such drugs. For dapsone (DDS), establishment of a linear relationship between dietary dosage and resultant serum DDS concentrations has led to estimates of the MIC of this drug for *Mycobacterium leprae*, by correlation with the minimum drug dosage suppressing *M. leprae* growth ([11, 13, 20]). For sulphadoxine and sulphadimethoxine, Ellard *et al* ([5]), although unable to determine a linear relationship between dosage and serum concentration, were able to estimate the respective serum MIC values which lay within the sensitivity of the technic and were measurable directly.

Rifampicin (RMP), when administered continuously to mice, inhibits the growth of *M. leprae* in the foot pad ([10, 12]). Its bactericidal action, as measured by the kinetic method of Shepard ([15, 16]), is considerably superior to that of DDS ([10, 21]). An approximate value for the MIC of RMP in mouse serum for *M. leprae* in the mouse foot pad was reported by Holmes and Hilson ([10]).

This paper describes a more precise determination of the minimum inhibitory and bactericidal dosages of RMP for *M. leprae* in the foot pad and estimation of the corresponding serum RMP concentrations.

MATERIALS AND METHODS

The general method employed has been described previously ([10]).

Strains of *M. leprae*. Eight strains of *M. leprae* were used, all derived from previously untreated lepromatous leprosy cases. They had undergone between one and four mouse passages, except strains SBL 13682 and SBL 16282, which were transferred directly from a patient to mice.

Mouse inoculation and assessment of bacillary growth. P-strain mice were used in all studies. Inocula of between 5.0×10^3 and 1.0×10^4 in the left hind foot pad were employed. For each of the strains, 20 mice were used as untreated controls, receiving pelleted diet 41B (Oxoid). Groups of 15 treated animals received the same diet in powdered form containing the appropriate concentrations of RMP, incorporated as previously described ([10]). Drug administration, when continuous, for determination of minimum inhibitory dosages (MIDs), was started on the day of inoculation (day 0), except in the case of strain SBL 16282 (day 75 post-inoculation). When of limited duration, for determination of bactericidal action, RMP administration was started on day 31 or 33 post-inoculation, and continued for varying periods of time. Calculations of drug dosage in terms of milligrams per kilogram body weight were made on the basis of a daily consumption of 5 gm diet by mice of 25 gm average weight. In a complete titration, drug dosage was at approximately 0.5 $\log_{10}$ intervals (e.g., 0.01, 0.003, 0.001 and 0.0003 percent).

Harvests of acid-fast bacilli (AFB) were made by killing several mice (usually three) from the control group, 90 to 120 days post-inoculation, and dissection of the inoculated foot pads. Treated groups were not sampled until the AFB counts in control animals had reached at least 10^5 per foot pad. Counts of AFB in foot pad homogenates ([10]) were made by the method of Hilson and Elek ([8]).

Serum rifampicin concentrations. The relationship between dietary RMP dosage and

resultant serum RMP concentrations was determined in mice receiving graded dosages of the drug. Sixty mice were starved for 24 hours before random allocation to four groups, receiving 0.001, 0.003, 0.01 and 0.03 percent RMP in the diet respectively for 16 days continuously. At intervals of 2, 8 and 16 days, groups of five or six mice were exsanguinated by cardiac puncture and the RMP concentration in individual serum samples determined. Estimations were made by a microbiological assay procedure using *Sarcina lutea* ([10]).

RESULTS

Minimum inhibitory rifampicin dosage. The sensitivity of eight strains of *M. leprae* to RMP was determined by continuous administration of graded dosages of the drug (Table 1). Growth in control animals was monitored as described above. Harvests from three to five mice per treated group were made at the time when growth in control animals had reached approximately 10^6 per foot pad and one to two months subsequently.

Table 1 records the fraction of RMP-treated mice from both harvests, for each RMP dosage, in which growth of *M. leprae* had occurred; AFB counts greater than 2.0 × 10^4 per foot pad were taken to indicate bacillary growth. The minimal inhibitory dose (MID) of RMP for each strain, listed in column six of Table 1, was taken as the lowest dosage suppressing *M. leprae* in the majority or all of the animals. Four of the eight strains of *M. leprae* investigated were inhibited by administration of 0.001% RMP (2.0 mg/kg). The growth of two further

strains (SBL 13682 and 8865) was suppressed by 0.001% RMP in 5/6 and 4/6 mice respectively, and by 0.003% (6.0 mg/kg) in all six mice harvested. In the animals in which positive AFB counts were obtained, the degree of growth was considerably less than in control animals. Thus, in view of the fact that the lower dosage inhibited these two strains in the majority of mice, it is likely that this dosage represents a value approximating to the MID. The MID of RMP for the remaining two strains showed some variation from the general trend; strain SBL 16282 was suppressed by 0.0003% (0.6 mg/kg) and strain 9593 by 0.003% (6.0 mg/kg).

Minimum bactericidal RMP dosage. The bactericidal action of RMP on three strains of *M. leprae* was assessed by the kinetic technic ([15, 16]) as previously described ([10]). For strains SBL 16237 and SBL 16325, mice were treated with graded dietary doses of RMP from day 33 to 89 post-inoculation. Mice inoculated with strain SBL 16263 were similarly treated from day 31. Harvests of three control mice were made 90 to 120 days after inoculation and at intervals subsequently, and the pattern of growth in treated mice determined by harvests of two to three mice before early growth was expected and three mice during logarithmic growth.

Figure 1 records mean foot pad AFB counts obtained with strain SBL 16237; similar graphs were obtained for the other strains. It shows that administration of RMP 0.01% led to complete failure of *M. leprae* growth during the 550 day observation period. Administration of lower drug dosages led to varying periods of *M. leprae*

TABLE 1. *The pattern of bacillary growth in mice inoculated with eight strains of* M. leprae *and treated with graded dietary dosages of rifampicin: estimation of the minimum inhibitory dosage.*

M. leprae strain	No. mice from two harvests with bacillary growth at the given RMP dosage				MID (%)
	0.0001%	0.0003%	0.001%	0.003%	
SBL 13682		3/3	1/6	0/6	0.001–0.003
SBL 16220		5/6	0/6	0/6	0.001
SBL 16237	6/6	4/6	0/6		0.001
SBL 16263		6/6	0/6	0/6	0.001
SBL 16282	7/8	0/8	0/8		0.0003
SBL 16325		6/6	0/6	0/6	0.001
8865		6/6	2/6	0/6	0.001–0.003
9593		6/6	5/6	0/6	0.003

TABLE 2. *Delayed growth of* M. leprae *following temporary administration of rifampicin in graded dosages: interpretation in terms of bactericidal action.*

M. leprae strain	RMP dosage (%)	Administration period (days)	Effective exposure to RMP (days)	Bacterial growth delay (days)	Delay due to[c] bactericidal action (days)	Fraction[a] surviving (%)
SBL 16237	0.0003	56	57	24	0	100
	0.001	56	57	115	58	1.17
	0.003	56	57	249	192	<0.01[b]
	0.01	56	58	>489	>431	<0.01
SBL 16325	0.0006	56	57	6	0	100
	0.001	56	57	35	0	100
	0.003	56	57	>287	>190	<0.01
SBL 16263	0.001	60	61	89	28	13.90
	0.01	60	62	>323	>261	<0.02

[a]On the assumption of 100% viable inocula, the figures 0.01% and 0.02% represent failure of a single bacillus to survive (actual inocula solid ratios: SBL 16237: 20%; SBL 16263: 15%; SBL 16282: 16%).

[b]Estimate based on 1 positive AFB count from a total of only 2 on day 344 post-inoculation.

[c]Column 5—column 4.

growth delay as compared with controls.

Table 2 lists the growth delay periods obtained with each drug regimen for the three *M. leprae* strains, under the heading "Bacterial growth delay." In the case of each treated group growth curve, the extent of displacement from the control of the point corresponding to a count of $10^{5.3}$ AFB per foot pad was measured from the graphs. The considerations on which calculations of growth delay are based have been previously discussed ([10, 16]). Their significance in relation to the bactericidal action of *M. leprae* will be discussed below.

Mouse serum RMP concentrations. Table 3 records the group mean serum RMP concentrations obtained at each sampling time. The regression functions of the grouped sample values at each harvest were tested for linearity. For the two latter samples, the calculated test quotients did not exceed the statistical significance limit, and the regressions were assumed to be linear ($p < 0.01$). There was thus a linear relationship between RMP dosage and serum RMP concentration after the initial period of stabilization of tissue and serum distribution of RMP; before this (two days) such a relationship could not be established ($p > 0.05$).

DISCUSSION

Minimum inhibitory rifampicin dosage. Since the growth of four of the eight strains of *M. leprae* investigated was suppressed by 0.001% RMP in the diet, and only one and two mice respectively showed growth of two further strains at this drug dosage, 0.001% may be accepted as the average MID of RMP for the strains tested. A similar value for the MID of RMP, based on work with three strains of *M. leprae*, has previously been reported ([10]). Furthermore, Rees *et al* ([12]) reported that the growth of five strains of *M. leprae* was suppressed by 0.0025% RMP; the MID was not determined.

Based on the present results, RMP is weight/weight about ten times less potent in suppressive activity than DDS ([10, 20]). The only other compound which has been found more active weight/weight than RMP in the foot pad model is the riminophenazine compound B663, which although not titrated for the degree of activity of continuous administration, was active by the kinetic technic at 0.0001% ([17]). The MID of a further riminophenazine, B1912, for one strain of *M. leprae* (0.001%) was equivalent to the present determined value for RMP ([7]). The importance of the estimates of MID values for antileprous compounds is in relation to the concentration of each drug which is attained in the serum and tissues of animals receiving equivalent dietary drug dosages. This relationship in the case of RMP will be discussed later.

Minimum bactericidal rifampicin dosage. In the use of the kinetic technic, growth delay in excess of that attributable to bacteri-
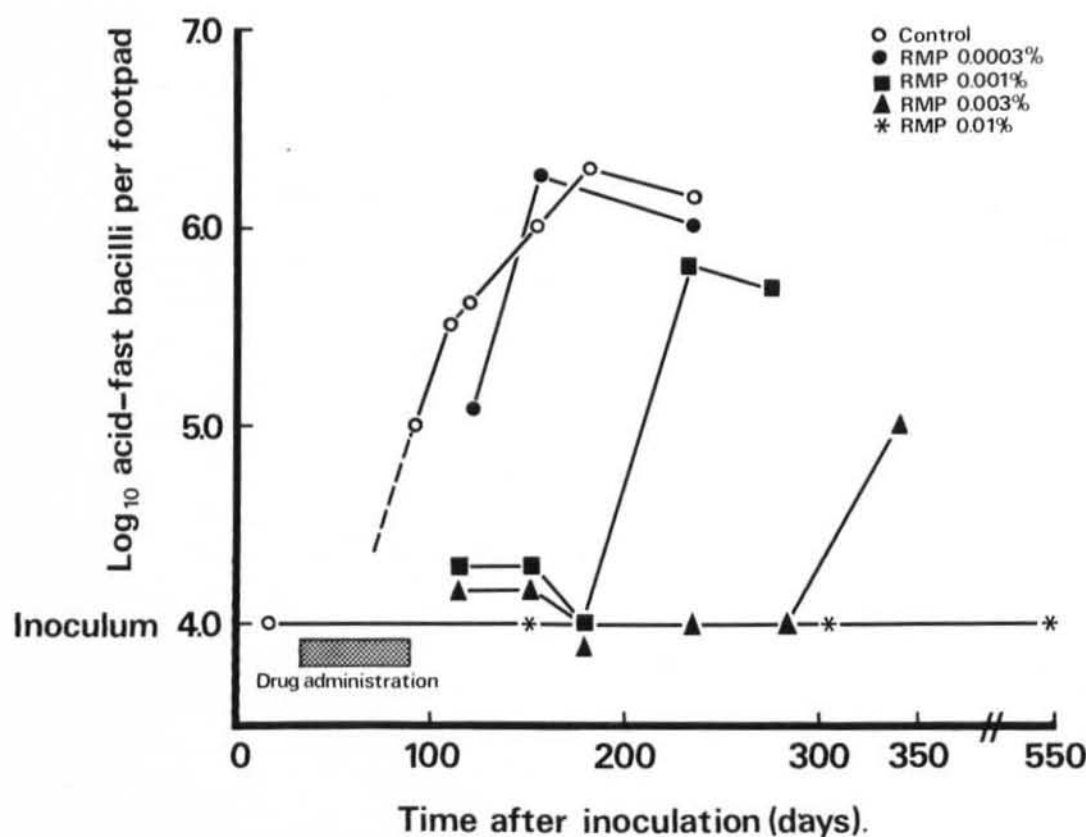
FIG. 1. Assessment of the bactericidal action of rifampicin on *M. leprae* (strain SBL 16237) in the mouse foot pad. Four dosages of the drug were given in unpelleted diet for a 56 day period beginning 31 days after infection. Control mice received pelleted diet. Control harvests were usually of three mice; harvests of treated mice were of two mice before and two or three mice during logarithmic growth. The lower limit of sensitivity of the counting procedure was 1.0×10^4 AFB per foot pad.

ostasis during the maintenance of effective serum drug levels is considered to be due to bactericidal drug action. This interpretation involves the adoption of a value for drug excretion rate, and the assumption that after drug withdrawal there is no period of prolonged bacteriostasis before resumption of multiplication of surviving bacilli ([16]). A value for the excretion rate of RMP has been taken from published data on the rate of disappearance of RMP from mouse serum following withdrawal of dietary drug administration ([9, 10]). For a dosage of 0.01%, serum levels below those equivalent to the MIC of RMP for *M. leprae* are reached two days after cessation of drug administration. For lower dosages it is assumed that inactive RMP concentrations would be reached within one day (Table 2).

Prolonged bacteriostasis, which would contribute in part to observed growth de-

lays, must be considered as a possibility in view of the work of Dickinson and Mitchison ([4]) and Holmes ([9]) with cultivable mycobacteria *in vitro;* extensive prolonged bacteriostatic effects were observed. Such effects, if applied to *M. leprae*, would constitute a substantial part of the growth delays hitherto considered to be due to bactericidal action alone. Proof of the existence of prolonged bacteriostasis in the case of *M. leprae* can be established by the kinetic technic only if the growth curve of bacilli surviving drug action is found, by downward extrapolation, to originate apparently from less than one viable bacillus. In the present work, in only one case was this observed (SBL 16237; 0.003% RMP), and the evidence of growth was based on one positive AFB count from a total of only two. Moreover, other published data has failed to reveal a similar finding. It appears, there-

TABLE 3. *Serum rifampicin concentrations in mice receiving graded dietary dosages of the drug: mean ± S.E.M.*

RMP dosage	Mean serum RMP concentration (μg/ml) at the given sample day		
(% in diet)	3	8	16
0.001	0.12±0.07	0.15±0.03	0.20±0.10
0.003	0.91±0.18	0.86±0.29	0.90±0.56
0.01	3.55±1.56	2.47±0.64	2.59±0.57
0.03	12.01±3.92	6.64±1.24	8.00±0.55
Regression function	Y = −0.332 + 409.091X	Y = 0.089 + 220.270X	Y = −0.012 + 266.865X
Linearity	p= >0.05	p= <0.01	p= <0.01

fore, that the kinetic technic provides a means of assessing drug bactericidal action, even though a strict quantitative determination may not be afforded. Column seven of Table 2 records the percentage survival of *M. leprae* exposed to RMP, as calculated from the "delay due to bactericidal action" by reference to the bacillary growth rate in control animals ([10, 16]). For each strain, RMP was found to have a powerful bactericidal action when administered in dietary dosage greater than the estimated MID (0.001% for all three strains). In the case of strain SBL 16237, the MID administered for 56 days killed 99% of the inoculum. On the basis of an inoculum of 1.0×10^4 AFB per foot pad and a solid ratio (viability) of 20% (Table 2), this represents a survival of about 20 bacilli. For strain SBL 16263, the action of 0.001% RMP was less pronounced (14% survival), and against strain SBL 16325 the effect was purely bacteriostatic (100% survival). In view of the variability of the degree of bactericidal action of 0.001% RMP on *M. leprae*, the minimum bactericidal dosage (MBD) of RMP for the three strains of *M. leprae* is taken as 0.003% (6.0 mg/kg), administration of which for 56 days resulted in the failure or late appearance of growth of the *M. leprae* strains tested at this dosage level.

Minimum inhibitory and bactericidal serum rifampicin concentrations. The derived values for MID and MBD of RMP may be interpreted in terms of serum RMP concentrations by reference to the data recorded in Table 3. Serum RMP levels equivalent to the MID for six strains of *M. leprae* are thus of the order of 0.2 μg/ml. For the two remaining strains the MID is equivalent to RMP concentrations of 0.9 and 0.06 to 0.09 μg/ml (by extrapolation) respectively. The

estimated values for MIC compare with the previously reported estimate of 0.3 μg/ml which was derived from serum level determinations using a tube-dilution microbiological assay technic ([7]). The minimum bactericidal concentration (MBC) of RMP in the serum, corresponding to a dietary dosage of 0.003% is 0.9 μg/ml: no previous determinations of the MBC of RMP for *M. leprae* have been reported.

The present values for MIC and MBC may be as much as five to ten times greater than the absolute degree of sensitivity of *M. leprae* to RMP in view of the fact that a large proportion of the total drug estimated is bound to serum protein ([14]). The amount of "free" drug available for diffusion into the tissues and for antimicrobial activity is thus considerably reduced. Furthermore, RMP metabolites in mouse serum, by which *S. lutea* growth is inhibited and which are therefore detected by the microbiological assay technic, may not be active against *M. leprae*. Nevertheless, for relating the RMP sensitivity of *M. leprae* in the mouse foot pad to serum RMP levels achieved in man and clinical efficacy of the drug, the present determinations provide a basis.

Correlation between the estimated MIC and serum RMP levels achieved in man receiving acceptable doses cannot be made directly. In mice receiving the drug in the diet, serum RMP concentrations remain relatively constant during long periods of administration ([9, 10]). In man, where doses are spaced and drug absorption is not continuous, serum concentrations fluctuate between individual doses. Induction of liver microsomal enzymes results in decreasing peak serum RMP levels during long periods of administration ([3]). With single doses of 450–

600 mg RMP, corresponding to the daily doses used by Rees *et al* ([12]) in a clinical trial of RMP in the treatment of lepromatous leprosy, peak serum concentrations of 7–16 μg/ml during early stages of therapy have been reported ([1,6]). Moreover, concentrations in excess of 1.0 μg/ml were present for 12 hours or more following each dose ([6]). Even after prolonged administration, serum levels of 0.2 μg/ml are present 24 hours after each 450 mg dose ([2]). It appears, therefore, that RMP concentrations equivalent to the estimated MIC and MBC for *M. leprae* would be present in the serum of patients receiving normal daily doses for long periods after each dose.

SUMMARY

The minimum dietary dosage of rifampicin (RMP) suppressing the growth of eight strains of *Mycobacterium leprae* in the mouse foot pad has been determined. Graded dosages of the drug were administered continuously to mice infected with *M. leprae* from the day of inoculation. The growth of six strains was suppressed by 0.001% RMP in the diet; the remaining two strains were suppressed by 0.0003% and 0.003% RMP respectively.

By use of the kinetic technic of Shepard, the bactericidal effect on three strains of *M. leprae* of graded dietary dosage of RMP administered for 56 days has been determined. Considerable bactericidal activity was observed with dosages greater than the minimum inhibitory dosage (MID). The MID (0.001%) was bactericidal against strain SBL 16237 (1.17% survival), bacteriostatic against strain SBL 16325 (100% survival) and weakly bactericidal against strain SBL 16263 (13.9% survival).

Serum RMP concentrations in mice receiving graded dietary dosages of the drug were estimated by a microbiological assay technic using *Sarcina lutea*. A linear relationship between dosage and resultant serum RMP concentrations was found. The MID of RMP for six *M. leprae* strains (0.001%) was equivalent to a serum RMP concentration of 0.2 μg/ml. For the two remaining strains the MID was equivalent to a serum concentration of 0.06–0.09 and 0.9 μg/ml respectively. The minimum bactericidal dosage of RMP (0.003%) gave serum levels of approximately 0.9 μg/ml. Serum RMP concentrations equivalent to the minimum inhibitory and bactericidal dosages for *M. leprae* are maintained for long periods in patients receiving a daily RMP dosage of 600 mg which has been used in recent clinical trials of the drug in the treatment of leprosy.

RESUMEN

Se determinó la dosis mínima de rifampicina (RMP) que, agregada a la dieta, detiene el crecimiento de ocho cepas de *Mycobacterium leprae* en la almohadilla de la pata del ratón. Se administraron dosis fijas de la droga en forma continua, a ratones infectados con *M. leprae*, desde el mismo día de la inoculación. En seis cepas el crecimiento se detuvo con 0,001% de RMP en la dieta; en las otras dos el crecimiento se detuvo con dosis de 0,0003% y 0,003% respectivamente.

Utilizando la técnica quinética de Shepard, se determinó el efecto bactericida de la administración durante 56 días de dosis medidas de RMP en la dieta sobre tres cepas de *M. leprae*. Se observó una actividad bactericida considerable con dosis superiores a la dosis inhibitoria mínima (DIM). La DIM (0,001%) fué bactericida contra la cepa SBL 16237 (1,17% de sobrevida), bacteriostática contra la cepa SBL 16325 (100% de sobrevida) y débilmente bactericida contra la cepa SBL 16263 (13,9% de sobrevida).

Las concentraciones de RMP en el suero de los ratones que estaban recibiendo las dosis medidas de la droga fueron evaluadas por medio de una técnica de medición biológica, utilizando *Sarcinia lutea*. Se halló una relación lineal entre la dosis y la concentración de RMP resultante en el suero. La DIM de RMP para seis cepas de *M. leprae* (0,001%) fue equivalente a una concentración en el suero de RMP de 0.2 μg/ml. Para el resto de las cepas la MID fue equivalente a una concentracion sérica de 0.06–0.09 y 0.9 μg/ml. La dosis bactericida mínima de RMP (0,003%) dió niveles séricos de aproximadamente 0.9 μg/ml. Las concentraciones de RMP en el suero equivalentes a las dosis inhibitoria mínima y bactericida para el *M. leprae* se mantienen durante largos períodos en los pacientes que reciben una dosis diaria de RMP de 600 mg, que ha sido usada en estudios clínicos recientes de esta droga para el tratamiento de la lepra.

RÉSUMÉ

On a déterminé la dose minimale de rifampicine qui doit être présente dans la ration (RMP) pour supprimer la croissance de huit souches de *Mycobacterium leprae* dans le coussinet plantaire de la souris. On a administré à des souris des doses progressives du médicament, de facon continue, à partir du jour d'inoculation. La croissance de six souches a été supprimée par 0,001 pour cent RMP dans la ration. Les deux autres

souches ont été inhibées par 0,0003 pour cent et 0,003 pour cent RMP respectivement.

On a eu recours à la technique cinétique de Shepard pour déterminer l'action bactéricide qu'entraîne sur trois souches de *M. leprae* l'administration pendant 56 jours de doses progressives de RMP dans la ration. Un effet bactéricide considérable a été observé avec des doses dépassant la dose minimale d'inhibition (MID). Le MID (0,001 pour cent) était bactéricide contre la souche SBL 16237 (1,17 pour cent de survie), bactériostatique contre la souche SBL 16325 (100 pour cent de survie) et faiblement bactéricide contre la souche SBL 16263 (13,9 pour cent de survie).

On a utilisé une technique microbiologique avec *Sarcina lutea* pour estimer des concentrations sériques du RMP chez des souris recevant le médicament dans la ration en doses progressives. On a constaté une relation linéaire entre la dose administrée et les concentrations sériques qui en résultaient. La MID de RMP pour six souches de *M. leprae* (0,001 pour cent) était équivalente à une concentration sérique de RMP de 0,2 μg/ml. Le deux souches restantes ont démontré respectivement une MID de 0,06–0,09 et 0,9 μg/ml. La dose bactéricide minimale de RMP (0,003 pour cent) a livré des niveaux sériques avoisinant 0,9 μg/ml. Des concentrations sériques de RMP équivalentes aux doses minimales d'inhibition et aux doses bactéricides pour *M. leprae* ont été maintenues durant de longues périodes chez des malades recevant une dose journalière de RMP s'élevant à 600 mg, au cours d'un essai clinique récent de ce médicament pour le traitement de la lèpre.

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