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Minimum inhibitory concentration of drugs against *Mycobacterium leprae* as determined by an *in vitro* assay

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Abstract. The observations that live *Mycobacterium leprae* after entry into cultured peritoneal macrophages from mice, reduced the EA rosetting macrophages, have been exploited to determine the minimum inhibitory concentration of diamino diphenyl sulphone and rifampicin. Diamino diphenyl sulphone showed a minimum inhibitory concentration of 0·028 μg/ml and rifampicin 0·11 μg/ml when given externally. However, there was accumulation of diamino diphenyl sulphone inside the macrophages. At an external concentration of 0·028 μg/ml the concentration inside the macrophage was 0·5 μg/ml. The minimum inhibitory concentration for diamino diphenyl sulphone in this assay system is higher by several folds and that for rifampicin is slightly lower, than what is reported earlier with mice foot pad experiments. The minimum inhibitory concentration reported in this assay system is quite close to what is observed for *in vitro* inhibition of *Mycobacterium lufu* with both the drugs.

Keywords. *Mycobacterium leprae;* minimum inhibition concentration; drugs; F_C receptor assay.

Introduction

Among the drugs that are in clinical use against leprosy the diamino diphenyl sulphone (DDS) and rifampicin are most widely used, either individually or as components of recently introduced multidrug therapy (WHO, 1982).

Due to nonavailability of an *in vitro* drug assay using *Mycobacterium leprae*, the minimum inhibitory concentration (MIC) has been reported using experimentally infected mice. The MIC for DDS has been determined as 0·003 μg/ml (Peters *et al.*, 1975) and for rifampicin as 0·3 μg/ml (Holmes and Hilson, 1972) based on the serum level. While this is an interesting and useful piece of information, less confidence is placed on these values in determining the dose of the drug given to the patients. The conventionally administered dose of 100 mg/day and 600 mg at a time, for DDS and rifampicin respectively, according to Allard (1980), provides a peak serum level of 500 and 30 times the MIC. This is given perhaps, with the idea of avoiding development of drug resistant *M. leprae* in the patients. Thus the heavy dose of drug has no relationship to the MIC as determined in mice. It is thus useful to have a much more direct *in vitro* method of determining susceptibility of *M. leprae* to these drugs and the MIC.

Abbreviations used: DDS, Diamino diphenyl sulphone; MIC, minimum inhibitory concentration; SRBC, sheep red blood cells.

It has been demonstrated that the presence of live *M. leprae* inside the macrophages from human or mice, reduced the level of F_c receptor expressing cells. This was not obtained with killed *M. leprae* (Birdi *et al.*, 1983; Birdi and Antia, 1984; Mankar *et al.*, 1984). The basic conclusion derived from this study was that live *M. leprae* but not inactive *M. leprae* were able to alter the surface structure of macrophages. This surface structure alteration resulted in the reduction of F_c receptor expressing macrophages only in the presence of live *M. leprae.* Thus if drugs are active on *M. leprae,* then in the presence of the drug and *M. leprae,* F_c receptor expressing macrophages will not be reduced and the level will be as good as the control (Birdi and Antia, 1984; Mankar *et al.*, 1984).

In this report we present data indicating the MIC of DDS and rifampicin that would inactivate *M. leprae,* such an inactivation being determined using the above *in vitro* test system.

Materials and methods

DDS uptake by macrophages

Macrophages from the peritoneal cavity of Swiss white mice were obtained and cultured in Leighton tubes as described previously (Mankar *et al.*, 1984). Seventy two hours after distribution of macrophages, the drug DDS was added to the medium. After 3 days of exposure to the drug, the cells were harvested by scraping them with a rubber policeman. They were washed 3 times with saline (0·85 %) to remove extracellular DDS. After suspending the macrophages in 1 ml saline, the cell count was taken in a haemocytometer. The macrophages were then lysed by subjecting them to 8 cycles of freeze-thawing. Distilled ethyl acetate (AR grade) was added to extract DDS from the macrophages. The extraction was done thrice by adding 2 ml ethyl acetate each time. To keep the pH basic, 0·5 ml of 10 N NaOH was added during the first extraction. The mixture was vortexed each time to facilitate the extraction of DDS from the lysate to the ethyl acetate layer (Allard and Gammon, 1969; Ozawa *et al.*, 1971; Peters *et al.*, 1981).

After separation of the solvent and aqueous phase in 20 min, the ethyl acetate phase was transferred to another tube with a pasteur pippette. To the ethyl acetate phase 0·5 gm of NaCl (AR grade) was added to remove water molecules carried during the extraction. The ethyl acetate phase was then allowed to separate from the NaCl which sedimented at the bottom. The ethyl acetate phase was removed and checked for fluorescence on a spectrofluorimeter (SFM23-Kontron). This assay method provided a sensitivity upto 2 ng/ml for DDS at wavelength settings of 285 nm for excitation and 350 nm for emission. A culture of macrophage, which had not been exposed to the drug otherwise treated exactly the same as the experimental served as control.

DDS (in ng)/10^6 macrophages is equal to a fluorescence level of 1×10^6 experimental macrophages, from which the fluorescence level of 1×10^6 control macrophages was subtracted. The quantitation was done using a standard curve (experimentally determined) of fluorescence units against increasing concentrations of DDS added to macrophage cultures.

Volume measurement

The macrophages exposed to DDS as described earlier were suspended in osmolar saline and a drop was placed on a wax coated slide. The macrophages which had settled appeared rounded and uniformly circular under microscopic observation. The diameter of macrophages was measured using previously calibrated ocular micrometer. One hundred macrophages were scanned and the diameter was measured, to determine the average diameter. The volume of a macrophage was calculated using the formula, $V = 4/3\ \pi r^3$ where r is radius, which was determined as 10 μm (table 1). Thus $V = 4/3 \times 3{\cdot}17 \times (10 \times 10^{-4})$· This worked out to be $4{\cdot}2 \times 10^{-9}$ cc for a single macrophage.

Determination of minimum inhibitory dose of DDS for M· leprae

The determination of minimum external dose necessary to inhibit the *M. leprae* inside the macrophage was done by the EA rosetting technique· Mouse peritoneal macrophage cultures were set and 72 h later the drug sulphone (Burroughs Welcome India) was added in known concentrations· After exposure of the culture to the drug for 72 h, they were washed to remove the external DDS and were infected with *M. leprae* (armadillo derived)· The extracellular drug was removed so as to avoid killing of *M. leprae* outside the cells· After the removal of extracellular DDS, there was very little diffusion of (5 % of internal level) cellular DDS to outside during the 72 h of incubation. *M. leprae* were phagocytosed by macrophages and were allowed to interact with the drug inside macrophages for 72 h (5×10^6 *M. leprae/tube* was the dose)· On the day of termination all the cultures were washed to remove the excess bacilli and subjected to EA rosetting with antibody coated sheep red blood cells (SRBC) (Mankar *et al.,* 1984)· *M. leprae* was prepared from infected armadilllo tissue adopting the procedure of Ambrose *et al.* (1978)· In all our experiments there was very little or no phagocytosis of SRBC by the macrophages.

Results

The radius of cultured macrophages was found to be 10 μm as an average value determined from several experiments using different concentrations of DDS and 100 cells in each experiment (table 1). This radius was used to calculate the volume of the macrophages and the volume was determined to be $4{\cdot}2 \times 10^{-9}$ cc for a single macrophage· The macrophages mostly retained a uniform circular shape· To avoid experimental variation several experiments were done as stated above and the average value has been used.

The percentage of macrophages expressing F_c receptors was reduced in the presence of dive *M. leprae*. In the presence of appropriate concentration of DDS in the incubation fluid, this F_c reduction was counteracted (table 2)·At DDS concentrations of 14·2ng/ml and 21·4ng/ml, the level of F_c receptor expressing macrophages in the presence of live *M. leprae* was 24 ± 6 and 46 ± 7 % as compared to the control values of 49 ± 14 and 70 ± 1 % respectively· However concentrations of DDS at 28·5 ng/ml and above completely reversed the lowered level of F_c receptor containing macrophages

Table 1. Average radius of macrophage in culture while exposed to varying concentrations of DDS.

Conc. of DDS	No. of cells with radius of 9 μ	10 μ	11 μ	Average value (in μm)	
10 ng	21	69	9	9·78	
	16	55	29	10·13	
15 ng	17	61	22	10·05	
	18	62	20	10·02	
20 ng	19	65	16	9·97	Common
	16	65	19	10·03	Average
50 ng	12	52	36	10·20	value
	15	68	17	10·07	10 μ
100 ng	24	63	13	9·87	
	26	51	23	9·97	
1000 ng	22	35	43	10·21	
	19	40	41	10·22	

Total no. of cells counted was 100 in each of the experiment and two experiments were conducted at each concentration of DDS.

Table 2. Percentage of macrophage exhibiting EA rosetting in the presence of *M, leprae* and various concentrations of DDS.

DDS conc. (ng).		% EA rosetting (mean +S.D.)*				
Per tube	Per ml medium	Control A	+M.L. B	+DDS C	+DDS +M.L. D	+M.L. (H.K.) E
10	14·2	49±14†	24±6	30±8	31±9	55±13
15	21·4	70±1	46±7	55±3	59±3	70±1
20	28·5	75±5	48±6	56±5	76±5	75±5
50	71·4	72±2	46±8	60±2	71±2	72±2
100	142·8	68±3	42±3	56±9	70±3	69±5

*The value for each concentration of DDS is the mean of 4 experiments. *P* value is for data calculated for each concentration of the drug separately.
A-B, $P < 0{\cdot}05$; A-C, $P < 0{\cdot}05$; A-D, $P < 0{\cdot}05$ upto 15ng;A-E, $P > 0{\cdot}05$; B-D, $P > 0{\cdot}05$ upto 15 ng. Significance calculated by Student's *t* distribution test.
† A different sample of amboceptor (antibody) for SRBC was used in this set of experiment and so the values are lower for this concentration.

caused by live *M. leprae.* This indicated the drug, induced inactivation of *M. leprae* at this concentration· The cultures using heat killed *M. leprae* showed the same rosetting ability as the control where no live *M. leprae* was added, the results being similar to those cultures treated with live *M, leprae* and concentration of DDS at 28·5 ng/ml or higher· The drug by itself, in the absence of *M. leprae* lowered the F_c receptor containing macrophages· This was noteworthy, but as we were looking for a reversal of the lowered F_c receptor level to higher level in the presence of the drug and *M. leprae* the effect of drug by itself is of little significance in this connection.

Table 3 presents data that were calculated to determine the DDS concentration of macrophages (μg/ml of the cells) using various concentrations of the drug in the culture· It is clearly seen that the concentration of the drug inside the macrophages increases with increasing concentration of the drug that was provided outside the cells.

From table 2 it is clear that at 28·5 ng/ml (external concentration) DDS inactivates *M. leprae,* so as to block its effect on the membrane of the macrophages and thereby prevent the reduction of F_c receptor bearing macrophages· At this concentration of 28·5 ng/ml, the level of DDS inside the macrophages is 0·50 μg/ml, the range varying from 0·3 μg–0·6 μg/ml.

Data presented in table 4 show that a minimum concentration of 114 ng/ml of rifampicin (Sigma, USA) is needed to inactivate the *M. leprae* inside the macrophages· This was shown by the restoration of the normal level of F_c receptor macrophages when this concentration of the drug was used in the presence of live M. *leprae.*

Table 3. Level of DDS inside macrophages (as μg/ml) on exposure to various amounts of the drug.

External concentration ng/tube	ng/ml	DDS/10^6 macrophage* (ng)	DDS/one* macrophage (fg)	DDS in macrophage* (vol.) (μg/ml)
10	14·2	1·5	1·5	0·37 ± 0.02
20	28·5	2·15	2·15	0·50 ± 0·09
50	71·4	6·1	6·1	1·44 ± 0·24
100	142·8	8·0	8·0	1·89 ± 0·21
200	285·6	25·3	25·3	6·02 ± 1·09
500	714·0	35	35	8·36 ± 1·6

* Value determined as mean ± S.D. from 4 experiments at each concentration of DDS. The volume of macrophage used for calculation was $4{\cdot}2 \times 10^{-3}$ cc/10^6 macrophage.

Table 4. Percentage of macrophages exhibiting EA rosetting in the presence of *M. leprae* and at various concentration of rifampicin.

RFP conc. (ng) Per tube	(per ml) (ng)	%EA rosetting (Mean ± S.D.)* Control A	+ *M. leprae* B	+ RFP C	+ RFP + *M. leprae* D	*M. leprae* (H.K) E
20	28·5	59 ± 3	31 ± 3	43 ± 7	49 ± 7	59 ± 3
50	71·4	65 ± 3	38 ± 4	51 ± 6	52 ± 5	66 ± 4
80	114·2	65 ± 6	34 ± 3	51 ± 6	63 ± 4	64 ± 5
100	142·8	62 ± 4	36 ± 5	53 ± 4	62 ± 5	62 ± 1

* 3 experiments were carried out for each concentration and the mean ± S.D. is presented. 5 × 106/Leighton tube of *M. leprae* was used.

Significance of difference (Student's *t* distribution test). *P* value is for data under each concentration of rifampicin used.

A-B, $P < 0{\cdot}05$; A-C, $P < 0{\cdot}05$; A-D, $P < 0{\cdot}05$ upto 50ng only; A-E, $P > 0{\cdot}05$; B-D, $P > 0{\cdot}05$ upto 50 ng only.

Discussion

The MIC for DDS using the F_c receptor assay system appears to be 28 ng/ml of the medium. Satish and Nath (1981) and Nath *et al.* (1982) in their experiments had shown that at 10 ng/ml level of DDS, [^{3}H]-thymidine incorporation by *M. leprae* could be considerably reduced and even 3 ng/ml was also shown to be active to some extent· This was explained as being in agreement with the MIC for DDS reported from mice experiments· However the basic fact that the drug accumulated inside the macrophages was not taken into consideration, which is an important feature in any assay using host cells·At an outside concentration of 10 ng/ml the expected concentration inside the macrophages is 0·30-0·38 μg/ml as seen in our experiments.

When the concentration of DDS in the culture was varied from 10-500 ng there was an increasing level of DDS inside the macrophages, although a linear relationship was seen only in the lower concentration range of 10–100 ng levels (table 3)· This lower range is quite satisfactory for our experimental purpose; since at these levels DDS is active.

Seydel and Wempe (1982) had shown that *M. lufu* which appears as the best model organism to study the action of DDS, the inactivation and growth inhibition occurred at an MIC of 0·03-0·05 μg/ml and this is quite close to the external DDS concentration observed in our assay system (0·028 μg/ml).

DDS has the ability to bind to proteins and some of the internal DDS could be protein bound· Since we extract DDS with ethyl acetate it is possible that the protein-bound fraction is also included in the estimation and therefore the active free DDS concentration inside *M. leprae* may be even lower.

In mice foot pad experiments the MIC determined on the basis of the serum level of DDS can give only part of the information, since the bacteria that are to be killed are in the foot pad and therefore the accumulated level of DDS in the local tissue is the most relevant parameter· The accumulated level will depend on storage and degradation of the drug in the tissues· It has been reported that the tissue level is likely to be 2-fold that of the serum level. Gordon *et al.* (1974) have reported that the serum level of DDS, when mice were given 0·002% DDS in the diet, was 180 ng/ml and in the tissue it was 1·6 times higher. Shepard (1967) calculated an MIC of 0·01-0·03 μg/ml and Ozawa *et al.* (1971) reported it to be 2·5-10 ng/ml· Allard *et al.* (1971) concluded that the MIC of DDS to *M. leprae* is less than 10 ng/ml, based on the serum level· Holmes and Hilson (1972) have shown that the serum value at 0·0001 % of DDS in diet was between 0·01- 0·03 μg/ml. This is similar to the value recorded by us. The often quoted report of Peters *et al.* (1975) indicating an MIC value of 0·003 μg/ml in rats and by Levy and Peters (1976) in mice, is in close agreement with the value obtained in the present study.

The level of an MIC of 28 ng/ml needed in our test system may probably be due to the fact that our experiments are limited to only 72 h exposure of the bacteria to the drug compared to the exposure of *M. leprae* in mouse foot pad for several weeks to the lowest reported level of 6 ng/ml (or perhaps higher) of the drug in the tissue before any viability is checked· Seydel and Wempe (1982) using the cell free enzyme system showed that the concentration required to lower the folate synthesizing enzyme activity from *M. lufu* by 50 % with DDS was 0·084 μg/ml (0·42 μM). They also pointed out that the DDS concentration needed for *in vitro* inactivation of the enzyme is definitely much

higher than 0·003 μg/ml reported as the MIC· Thus it is quite probable that the accumulated tissue level of DDS acts on *M. leprae* in mice foot pad experiments.

In patients receiving 100 mg/day, DDS reached peak serum levels of 2·5-6 μg/ml (Modderman *et al.,* 1983)· Thus the serum level is at least 5-12 times more than the MIC determined by the present method. So it can be argued that a level even lower than 100 mg/day should be quite sufficient as a therapeutic dose· It could also be mentioned that sulpha drugs are always given at a dose level capable of completely inactivating the folate synthesizing enzyme.

In comparison to DDS, rifampicin directly blocks the multiplication of bacteria and it is bactericidal· The MIC determined in the mice was 0·3 μg/ml (Holmes and Hilson, 1972). In our system we needed a minimum external dose of 0·114 μg/ml (80 ng/Leighton tube) to inactivate *M. leprae* inside the macrophages.

It has not been possible to estimate the concentration of rifampicin inside the macrophages when a concentration of 0·114 μg/ml (80 ng/Leighton tube) is given· The sensitivity of the bioassay is only up to a level above 0·1 μg/ml. Thus the level of accumulation cannot be more than 0·1 μg/ml and this needs further investigation· It is possible that there is accumulation of rifampicin inside the macrophages and the actual concentration of the drug to which *M. leprae* inside the cells is exposed could be higher than the external concentration· Using *M. lufu* as test organism, the MIC of rifampicin is reported to be 0·25 μg/ml by Seydel *et al.* (1982).

The potentiality of determining the MIC of a drug using the F_c receptor assay, can also help us to determine the synergistic effect of one drug in the presence of another. Such studies have been completed using this assay system with two drugs DDS and Brodimoprim (Seydel *et al.,* 1983; Jagannathan and Mahadevan, 1985).

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