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THE PENETRATION OF DAPSONE, RIFAMPICIN, ISONIAZID AND PYRAZINAMIDE INTO PERIPHERAL NERVES

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1 Dapsone, rifampicin, isoniazid and pyrazinamide were shown to penetrate readily into the sciatic nerves of the dog and sheep.

2 These findings suggest that the continued persistence of viable drug-sensitive leprosy bacilli in the peripheral nerves of patients treated for long periods with either dapsone or rifampicin is not due to inadequate intraneural drug penetration.

Introduction

Dapsone (DDS) and rifampicin are two of the most important drugs used for the treatment of leprosy (Ellard, 1974). Daily treatment with 50 mg DDS reduces the number of viable *Mycobacterium leprae* in skin biopsies or nasal washings to less than 0.1% of their original total within about 3 months (Shepard, Levy & Fasal, 1968. 1972a) while a similar effect can be achieved by giving 600 mg rifampicin daily for as short a time as 1-3 weeks (Rees, Pearson & Waters, 1970; Shepard, Levy & Fasal, 1972b, 1974). Nevertheless, small numbers of viable *M. leprae* persist, often in preferred sites such as peripheral nerve and striated muscle (Pearson, Rees & Weddell, 1970) and, despite up to 10 years of continuous DDS treatment, these persisting bacilli can retain their sensitivity to DDS (Waters, Rees, McDougall & Weddell, 1974). Similar findings have also been encountered after up to 2 years daily treatment with rifampicin (Rees, 1975). These observations raised the possibility that both DDS and rifampicin might penetrate poorly into peripheral nerves.

To examine this hypothesis we investigated the penetration of DDS, rifampicin, isoniazid and pyrazinamide into the sciatic nerves of the dog and sheep. Isoniazid and pyrazinamide were chosen as suitable drugs for comparison since previous studies had indicated that they readily penetrate most body tissues. Thus both drugs are excellently absorbed in man, appear to be distributed throughout the total body water, are almost completely reabsorbed by the kidney, readily penetrate into the cerebrospinal fluid (CSF) and are highly effective against intracellular infections of *Mycobacterium tuberculosis* (Peters, Miller & Brown, 1965; Ellard, 1969; Gelber, Jacobsen & Levy, 1969; Weiner & Tinker, 1972; Ellard, Gammon & Tiitinen, 1973; Forgan-Smith, Ellard, Newton & Mitchison, 1973; Fox & Mitchison, 1975; Ellard & Gammon, unpublished observations). However, there appears to be very little

knowledge concerning the penetration of these drugs, or indeed of other chemotherapeutic agents, into peripheral nerves.

Methods

The oral dosages of DDS, rifampicin, isoniazid and pyrazinamide given to the dog and the sheep are shown in Table 1. Each of the 4 drugs was given in the same animal on 2 occasions separated by an interval of 12 h so that their relative penetration into the nerves could be compared directly and so that moderately prolonged plasma concentrations would be maintained before removal of the nerves. Eight hours after the second dose, the animals were stunned with a humane killer, killed by exsanguination and the sciatic nerves immediately removed by meticulous exposure of the area concerned. After slitting the epineurium and taking care to avoid contact with other tissues or tissue fluids, segments of sciatic nerve were removed and placed directly in liquid nitrogen in which they were stored until analysis.

Plasma and ultrafiltrate (Toribara, Terepka & Dewey, 1957) concentrations of pyrazinamide were determined colorimetrically (Ellard, 1969); DDS, isoniazid and their potential metabolites monoacetyl-DDS and acetylisoniazid were determined fluorimetrically (Ellard & Gammon, 1969; Ellard, Gammon & Wallace, 1972), and rifampicin was assayed microbiologically by a plate-diffusion method with *Staphylococcus aureus* (Dickinson, Aber, Allen, Ellard & Mitchison, 1974).

Portions of the nerves weighing approximately 1 g were cut into small pieces, finely ground in a glass pestle and mortar with sand and then successively extracted by vigorously shaking on a vortex mixer with 2 x 10 ml chloroform/methanol (2 : 1 by volume) and 2 x 10 ml chloroform/methanol (1 : 2 by volume). The extracts were filtered and pooled.

For the determination of DDS and isoniazid, portions (10 ml) of the pooled extracts and of suitable aqueous standards diluted with chloroform/methanol (1 : 1) were evaporated to dryness on a rotary evaporator and the residues extracted by shaking with 2 ml 0.1N HCl plus 3 ml ethyl acetate. Two ml of the ethyl acetate extract was then diluted to 8 ml with ethyl acetate, extracted into 1.2N HCl and thence into ethyl acetate and the DDS determined. Isoniazid was determined by reacting portions of the 0.1N HCl extracts by the procedure used for its fluorimetric determination and correcting for the presence of fluorescent compounds derived from normal nerve by subtracting the fluorescence obtained when duplicate portions were pretreated with nitrous acid prior to reaction. For the microbiological determination of rifampicin, portions of the chloroform/methanol extracts of the nerve were taken through a solvent extraction procedure very similar to that described previously (Dickinson *et al.*, 1974) and the dried residues of the extracts dissolved in 0.3 ml portions of normal human serum. In the range of rifampicin concentrations assayed, the results obtained with aqueous standards and standards prepared by addition of rifampicin to extracts of normal sheep nerve did not differ significantly. Pyrazinamide was determined colorimetrically by drying a 10 ml portion of the chloroform/methanol dog nerve extract, extracting the residue with a mixture of 1 ml 0.1N H_2SO_4, 4 ml benzene/butan-1-ol (4 : 1 by volume) and 4 ml *n*-heptane, reacting 0.5 ml of the aqueous extract with alkaline nitroprusside and scanning the absorbance of the reaction product against an extract of normal (sheep) nerve treated in the same way.

Results

Preliminary experiments, in which single doses of the drugs were given to separate animals and plasma concentrations determined 1, 2, 4, 6 and 24 h later, revealed that in the dog the plasma half-life of isoniazid was about 4.5 h, pyrazinamide 5 h, rifampicin 10 h and DDS 18 hours. These values may be compared with estimates based on previously published data of 2.5, 5, 7-8 and 11 h, respectively (Peters, 1960; Cohn, 1969; Biggs, Gordon & Peters, 1971; Finkel, Pittillo & Mellett, 1971; Weiner & Tinker, 1972). Both DDS and isoniazid were acetylated by the sheep but neither compound was acetylated by the dog. Equivalent doses of DDS, rifampicin and isoniazid in the sheep resulted in lower but considerably more prolonged plasma concentrations than were attained in the dog. However, since measurable plasma concentrations of pyrazinamide were only achieved in the sheep for about 2 h after giving a 50 mg/kg dose of the drug, its penetration into sheep nerve could not be investigated. These preliminary studies, together with analyses on normal sheep nerve, confirmed the specificity of the methods used for the determination of all four drugs in both plasma and nerve. Furthermore, the fluorescence characteristics of the DDS and isoniazid extracts derived from the nerves of the treated animals were identical to those of aqueous standards and the rifampicin extracts failed to inhibit the growth of a rifampicin-resistant variant of the same *St. aureus* strain (Mitchison, Allen & Miller, 1970).

The concentrations of DDS, rifampicin,

Table 1 Concentrations of dapsone, rifampicin, isoniazid and pyrazinamide in the plasma, ultrafiltrate and sciatic nerve of the dog and sheep (μg/ml or μg/g)

Drug	*Species*	*Dose*	*Plasma (hours after second dose)*					*Plasma ultrafiltrate*	*Sciatic nerve*
		(mg/kg)	*0*	*2*	*4*	*6*	*8*	*at 8 h*	*at 8 h*
Dapsone	Dog	15	3.6	9.2	15.1	13.7	12.3	3.6	16.8
	Sheep	20	7.4	12.5	15.4	16.2	16.6	4.1	10.7
Rifampicin	Dog	20	7.2	11.3	31.0	28.3	24.6	1.5	4.4
	Sheep	50	8.3	9.5	10.9	11.7	13.3	1.1	1.3
Isoniazid	Dog	15	1.2	3.2	9.3	8.4	5.0	2.9	6.2
	Sheep	30	5.2	14.2	11.7	13.9	11.6	6.8	6.4
Pyrazinamide	Dog	50	7.7	47.7	35.7	29.2	20.8	20.8	20.0

isoniazid and pyrazinamide found in the plasma and nerve of the treated dog and sheep are summarized in Table 1. All four drugs readily penetrated into the sciatic nerve and in every case the concentrations found in the nerves equalled or exceeded the concomitant concentrations of the unbound drugs in the plasma. Since the vasculature of peripheral nerve is very slight, the contribution of any traces of blood present in the nerves after exsanguination to the estimated drug concentrations in the nerves would have been negligible. The highest nerve to ultrafiltrate drug ratios (3-5 fold) were encountered with the most lipid-soluble drug studied (DDS).

Discussion

The finding that pyrazinamide was not significantly bound by dog plasma proteins confirms previous evidence (Weiner & Tinker, 1972). By contrast isoniazid was approximately 40% protein-bound, DDS 70-75% protein-bound and rifampicin over 90% protein-bound by both dog and sheep plasma. The extent of protein binding of DDS by dog and sheep plasma was similar to that found by other workers for dog, human, rabbit, rat and mouse plasma (Biggs *et al.*, 1971). Several studies have demonstrated that rifampicin is highly bound to plasma proteins (Boman, 1973; Boman and Ringberger, 1974), but previous evidence for isoniazid protein-binding has been conflicting (Jenne, McDonald & Mendoza, 1961; Claunch, Castro & Barnes, 1963; Lloyd & Mitchison, 1964; Mattila & Takki, 1969). We found that whereas the acetylisoniazid formed *in vivo* by the sheep was not significantly protein-bound, the protein-binding of the monacetyl-DDS formed was virtually complete (unpublished observations). The extensive protein-binding of monoacetyl-DDS by plasma from other species has already been noted by other investigators (Biggs *et al.*, 1971).

The demonstration that DDS and rifampicin penetrated into sciatic nerve as readily as did isoniazid and pyrazinamide accords with other evidence concerning their tissue penetration. Thus DDS is well absorbed in man (Israili, Cucinell, Vaught, Davis, Lesser & Dayton, 1973) and evidence from the limited excretion of unchanged DDS when the pH of the urine is controlled to prevent the breakdown of the acid-labile DDS-*N*-glucuronide, and its relatively long half-life in man (Ellard & Gammon, 1969), suggests that some 97-98% of the protein-free drug is probably reabsorbed from the kidney. DDS has also been shown to penetrate into the muscle and brain of mice and rats (Francis, 1953; Murray, Gordon & Peters, 1974). Studies in the dog have demonstrated the excellent absorption of orally-administered rifampicin (Keberle, Schmid & Meyer-Brunot, 1968). Rifampicin also penetrates into human CSF (Forgan-Smith *et al.*, 1973), and leukocytes (Mandell, 1973), and whole body autoradiographic studies have shown it to enter the sciatic nerves of the mouse (Keberle *et al.*, 1968).

This experimental study, which has demonstrated the excellent penetration into the sciatic nerve of DDS, rifampicin and isoniazid in the dog and sheep and of pyrazinamide in the dog strongly suggests that they would also penetrate into human peripheral nerves. In the dog and sheep the concentrations of DDS found in nerve were actually 3-5 times higher than the concomitant concentrations of the unbound drug in the plasma and similar to the total plasma DDS concentrations. It must therefore be concluded that the persistence of small numbers of viable DDS-sensitive *M. leprae* in the peripheral nerves despite many years of treatment with DDS doses giving

blood levels far in excess of its minimal inhibitory concentration against *M. leprae* (Ellard, Gammon, Rees & Waters, 1971; Waters *et al.*, 1974) cannot be due to inadequate tissue penetration of the drug.

It is suggested that unbound rifampicin readily penetrated into the sciatic nerves of both the dog and sheep but that the extensive protein-binding of the drug was responsible for the rifampicin concentrations in the nerve being only 10-20% of the total plasma concentrations of the drug. In view of the similar high degree of protein-binding of rifampicin by human plasma (Boman & Ringberger, 1974), analogous findings might also be expected in man. At present there is insufficient experimental evidence to indicate the possible influence of protein-binding on the antimycobacterial activity of rifampicin. If the antileprosy activity of rifampicin were determined solely by the concentrations of the unbound drug, one would expect it to be fully effective against intraneural *M. leprae*. If, however, its activity were a function of the total rifampicin concentrations achieved, reduced activity might be anticipated against intraneural bacilli. However, the results of a recent clinical trial (Rees & Waters, unpublished observations), in which the initial rates with which various daily dose sizes of rifampicin killed *M. leprae* were compared, suggest that even if the latter hypothesis were corect, a daily dose of 600 mg rifampicin should still exert a powerful antibacterial effect against intraneural *M. leprae*.

It must therefore be concluded that the persistence of viable drug-sensitive leprosy bacilli despite continued treatment with DDS or rifampicin cannot be due to inadequate tissue penetration of either drug. A more probable explanation is that a significant proportion of leprosy bacilli in tissue sites such as the nerves are dormant and as a consequence physiologically resistant to killing by either drug. DDS is in any case primarily a bacteriostatic drug, while it is known that rifampicin has very little bactericidal activity against non-growing *M. tuberculosis* (Dickinson, Jackett & Mitchison, 1972). This aspect of the chemotherapy of leprosy may therefore be similar to that encountered in the chemotherapy of tuberculosis where it is apparent that drugs that are highly bactericidal against acitvely growing *M. tuberculosis* are unable to kill dormant bacilli (Fox & Mitchison, 1975).

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