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Letter to the Editor

Suppression of an Ascitic Rat Hepatoma with Cord Factor and Nocardia Cell Wall Skeleton in Squalene Emulsions*

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BCG HAS been widely used in experimental and clinical immunotherapy [1–3], but in view of the problems associated with standardisation of vaccines, and hazards associated with the use of viable organisms [4, 5] more defined, non-living, Mycobacterial products need to be examined.

Previous studies in this department [6] demonstrated suppression of an ascitic rat hepatoma with cord factor (trehalose-6, 6′-dimycolate), and some of its synthetic analogues, given in vegetable or mineral oil in detergent emulsion. It is well recognised, however, that the adjuvant and tumour suppressive effects of Mycobacterial cell wall preparations, and their glycolipids and synthetic analogues, depend upon the type and concentration of oil used in the vehicle of administration [6–9]. In this context, Yarkoni and Rapp [9] have reported that squalene (2, 6, 10, 15, 19, 23-hexamethyl-2, 6, 10, 14, 18, 22-tetracosahexaene), an intermediate in the biosynthesis of cholesterol, and squalane (2, 6, 10, 15, 19, 23-hexamethyltetracosane) are highly efficient vehicles for intradermal injection of cord factor into a mouse sarcoma. In addition, *Nocardia rubra* cell wall preparations show many of the antitumour effects of Mycobacterial preparations without the toxicity associated with BCG [10–12] and this too is effective in squalene emulsions [13]. The objective of the present studies, with the rat ascitic hepatoma (D23As) screening system previously developed [6], was to determine the efficiency of squalene emulsions as vehicles for administration of cord factor and its synthetic analogues, and to assess the response to *Nocardia* cell wall skeleton.

Extraction of trehalose-6, 6′-dimycolate (TDM) from *Mycobacterium bovis*, and preparation of synthetic trehalose-6, 6′-dipalmitate (TDP) and dibehenate (TDB) and the β isomer of 6, 6′-di-O-2-eicosyl-3-hydroxytetracosanoyl-α, α-trehalose (designated C100) have been described previously [6]. They were dissolved in squalene (Sigma Chemical Company, London, U.K.) and the solution emulsified in 0.1% Triton WR1339 in pH 7.2 phosphate buffered saline by grinding in a Potter homogeniser. Final concentration of glycolipid was 1 mg/ml, and of oil 10% (v/v).

Nocardia rubra cell wall skeleton lyophilised in a medium containing 10% squalene, 5% mannitol and 0.2% Tween 80 [13] was supplied by the Fujisawa Pharmaceutical Co. Ltd., Osaka, Japan (Lot 11929SK) and reconstituted with water before injection.

Figure 1 illustrates the influence of cord

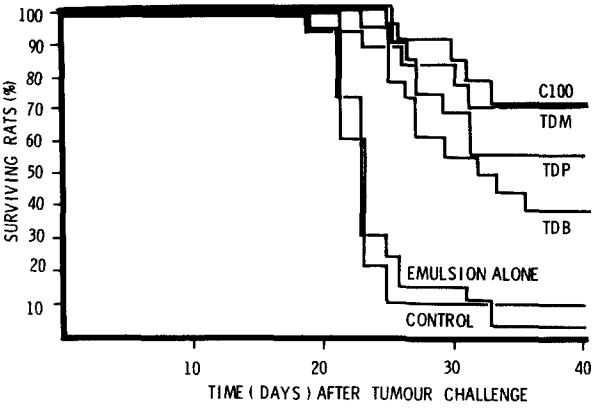

Fig. 1. Influence of trehalose diesters on growth of ascitic rat hepatoma D23As (10^4 *cell challenge). Rats were treated intraperitoneally with* 1 mg *material on day of challenge.* P <0.005, *all treated groups compared to emulsion alone control.* 18–19 *rats/group.* TDM—*trehalose dimycolate;* TDB—*trehalose dibehenate;* TDP—*trehalose dipalmitate.*

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factor and synthetic glycolipids on growth of inocula of 10^4 D23As cells. Control rats were killed 18–25 days after tumour challenge. Animals treated only with squalene:Triton emulsion showed comparable survivals. However, treatment with all agents (trehalose dipalmitate, dimycolate and dibehenate and the C100 preparation) significantly prolonged the overall survival, and reduced the final tumour incidence. This is in contrast to previous studies [6] with arachis oil emulsions, where only the dimycolate and dibehenate were markedly effective, even with challenge inocula as low as 10^3 D23As cells.

In a further experiment (Fig. 2) the tumour challenge inoculum was increased to 10^5 cells and here differences in potencies between the diesters are more obvious, treatment with only trehalose dimycolate giving a statistically significant prolongation of survival and reduced tumour incidence, although animals treated with the C100 preparation showed a proportion of long term survivors. In previous studies with arachis oil emulsions [6], there was no clear relationship between the size of the acid esterified to trechalose and the tumour-suppressive property of the product. With squalene vehicle there is an indication that increasing the size of the acyl moiety results in increased tumour-suppressive potency (Fig. 2).

Rats in which ascites tumour development from a challenge of 10^5 cells had been suppressed by trehalose dimycolate were subsequently given a further challenge with 10^4 D23As cells alone. This grew out in only 1 of 10 rats compared with growth in 7 of 10 control animals.

Figure 3 illustrates the effect of a single injection of *Nocardia* cell wall skeleton (NCWS) on the development of the ascitic hepatoma. A small, but statistically significant, prolongation of survival was seen in the treated rats compared with untreated controls. In a further test (Fig. 4), rats received either single or repeated injections and here all treated groups showed statistically significant prolongation of survivals and reduced tumour incidence, although the best response was achieved with two or three injections. Although the present tests were not designed to compare quantitatively the response to NCWS and cord factor, the NCWS, at least on repeated administration, was as efficient as cord factor in prolonging survival and preventing growth of tumour in a large proportion of rats. The response to both preparations was also comparable to that previously seen with live BCG vaccine [6].

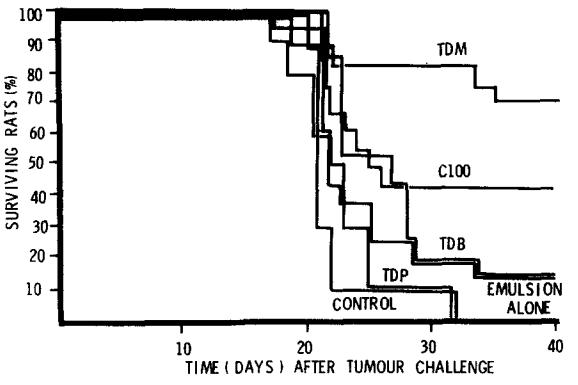

Fig. 2. Influence of trehalose diesters (1 mg) *on growth of ascitic rat hepatoma* D23As (10^5 *cell challenge*). $P<0.005$ *for trehalose dimycolate-treated rats; other groups not significantly different from emulsion alone control.* 10–19 *rats/group.*

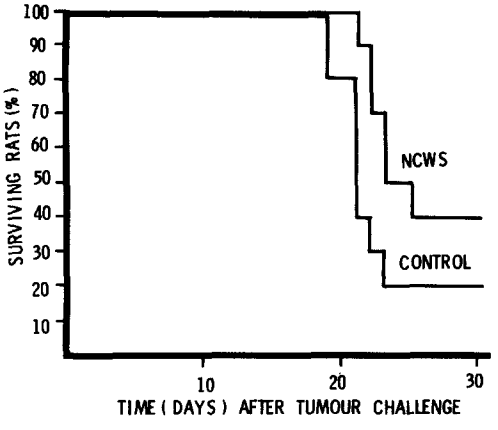

Fig. 3. Influence of Nocardia *cell wall skeleton (NCWS) on growth of ascitic rat hepatoma* D23As (10^4 *cell challenge*). *NCWS* 0.5 mg *was given intraperitoneally on day of challenge.* $P<0.025$, *treated rats compared with untreated controls.* 10 *rats/group.*

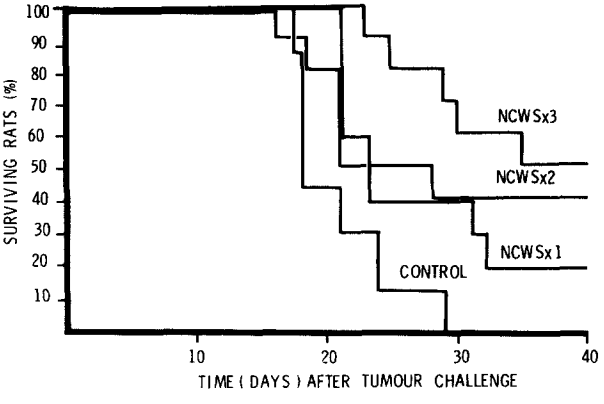

Fig. 4. Influence of single or repeated injections of Nocardia cell wall skeleton on growth of ascitic rat hepatoma D23As (10^4 *cell challenge*). *NCWS* (0.5 mg) *was given* X1 (*day of challenge*) ; X2 (*day of challenge and day* 4); *or* X3 (*day of challenge and days* 4 *and* 7). 10 *rats/group.*

In conclusion, these studies, with a relatively simple screening system, indicate that squalene emulsions are efficient vehicles for administration of cord factor and synthetic analogues, and that *Nocardia* cell wall skeleton is similarly effective. The efficiency of these agents and vehicles is now to be assessed against other more clinically relevant tumour models, particularly metastasising rat mammary carcinomas of spontaneous origin.

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