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THE EFFECTS OF METHOTREXATE AND MELPHALAN IN SHEEP

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1 Sheep were injected with either methotrexate or melphalan in an attempt to lower the numbers of neutrophil granulocytes in peripheral blood.

2 Methotrexate, 5.0 mg/kg intravenously, on three consecutive days, produced no noticeable clinical or haematological changes.

3 A single dose of melphalan, 1.0 mg/kg intravenously, depleted lymphocytes and granulocytes, but the numbers of the latter then rose to values exceeding the levels before melphalan treatment. Doubling the dose of melphalan proved fatal.

4 The failure of methotrexate to produce changes can be explained in terms of ruminant metabolism.

Introduction

Sheep leucocytes produce prostaglandins and such compounds have been demonstrated in wound fluid from a mild inflammatory lesion in sheep (Greenwood & Kerry, 1975). In order to study the contribution which emigrated blood granulocytes might be making to this prostaglandin production, an attempt was made to reduce the granulocyte count in sheep by the use of two bone marrow depressants, methotrexate (amethopterin) and melphalan.

The observed effects of these two drugs in ruminants may be of wider interest.

Methods

Methotrexate (Lederle Laboratories) was suspended in 0.9% w/v NaCl solution (saline) and just sufficient sodium bicarbonate was added to dissolve the drug (insoluble at neutral and acid pH), giving a solution of 25 mg/ml at approximately pH 8. The methotrexate was sterilized by membrane filtration.

Methotrexate was injected into two Clun Forest wethers (35 to 40 kg in weight) through an intravenous catheter on three consecutive days, in a dose of either 2.5 or 5.0 mg/kg body weight. A 2.5 ml blood sample was taken from the jugular vein before the first injection, and every day for up to 16 days, and haemocytometer white cell counts and differential leucocyte counts carried out.

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Melphalan ('Alkeran', Burroughs Wellcome & Co.) 100 mg was dissolved by the addition of 1.0 ml sterile 92% v/v ethanol containing 2.0% w/v HCl, and then thoroughly mixed with 9.0 ml of a sterile solution containing 1.2% w/v dipotassium phosphate (K_2HPO_4) and 60% v/v propylene glycol B.P. in water. The drug solution was injected within 15 min of preparation.

Two Clun Forest wethers received single intravenous doses of melphalan at 1.0 mg/kg and 2.0 mg/kg body weight. As with methotrexate, the total and differential white blood cell counts were monitored daily.

Results

The two sheep injected with methotrexate remained clinically healthy and devoid of any side effects. There was no reduction in the counts of total white cells or specific cell types.

Melphalan 1.0 mg/kg body weight produced in one sheep a temporary loss of appetite and diarrhoea on days 3 and 4 after injection. The fleece was loose on day 15 after injection and was shed completely by day 22. This was gradually replaced, new fleece being detected on day 30 after injection. The sheep then regained full health and has continued to thrive for over two years. A single sheep injected with melphalan 2.0 mg/kg body weight rapidly became ill, refused to take food and died on the fourth day after injection. A *post mortem* examination was not carried out.

The results of melphalan treatment on the blood picture of both sheep are shown in Table 1. Melphalan 1.0 mg/kg body weight produced leucopenia. The total leucocyte count was lowest on days 4 and 5 after injection, then gradually recovered, and by day 25 had reached pre-injection levels. Differential counts showed that lymphocyte numbers fell until day 5 when the maximum effect was seen. From day 6 onwards, the population slowly increased. Circulating granulocytes fell on day 4 to the very low level of 20/μl, but from day 5 the numbers gradually recovered and by day 25 attained double the pre-injection level.

A dose of melphalan of 2.0 mg/kg produced severe leucopenia within three days after injection. The depression of lymphocyte numbers was greater than of cells of the myeloid series. This sheep died.

Discussion

Methotrexate and melphalan interfere with leucopoiesis by different mechanisms. Their different effectiveness in the sheep is probably due to ruminant metabolism.

Methotrexate antagonizes the reduction of dihydrofolate to tetrahydrofolate, an effect which relies upon the structural resemblance of the drug to dihydrofolic acid and the affinity for it of the enzyme, dihydrofolic acid reductase. The active compound tetrahydrofolate assists in the introduction of a one carbon moiety into the purine ring.

The ruminant may overcome this metabolic antagonism by at least three likely mechanisms but it is not yet possible to assess the part played by each, or any:

(1) The content of nucleic acids in the diet is high, as, for example, in hay (McAllan & Smith, 1973) and large quantities of microbial ribonucleic acid (RNA) are supplied from organisms passing from the rumen to the abomasum. The ruminant pancreas has, moreover, a high ribonuclease content (Barnard, 1969). Infusions of labelled RNA, purine and pyrimidine bases into the abomasum suggest that exogenous nucleic acid precursors can be absorbed and utilized by the sheep (Condon, Hall & Hatfield, 1970; Condon & Hatfield, 1970), perhaps making the animal less dependent upon nucleic acid synthesis *de novo*. In these circumstances purine and pyrimidine synthesis may always have less importance, or the suppression of this synthesis by methotrexate may cause an increased absorption and utilization of exogenous purines and pyrimidines.

(2) The bacteria of the rumen produce folic acid. It is possible that they also possess the enzyme system, described in some micro-organisms, for producing formyltetrahydrofolate. If this is produced in the rumen and absorbed as such in the gut, antagonism of dihydrofolic acid reduction by parenterally administered methotrexate would be useless. Even if methotrexate is effective in its antagonism, the quantity of dihydrofolate produced by rumen bacteria could be so large that the high affinity of the reducing enzyme for methotrexate could be overcome. In addition, the sheep may use a completely different metabolic pathway for the transfer of the one carbon moiety into purines and pyrimidines, so that the block of dihydrofolate reduction may be irrelevant.

(3) The diverse flora of the ruminant intestinal tract may be able to metabolize methotrexate to a less effective compound. Valerino, Johns, Zaharko & Oliverio (1972) showed that bacteria from the mouse caecum could metabolize methotrexate producing, as the major metabolite in the gut, 4-amino-4-deoxy-N^{10}-

Table 1 Effect of melphalan on total and differential leucocyte counts in sheep blood

	Day	Total leucocyte count/μl	Differential absolute leucocyte count/μl			
			Lymphocytes	Neutrophils	Monocytes	Eosinophils
Sheep 1						
	0	7500	5550	1920	30	0
	4	1000	956	20	24	0
	25	7000	2555	4123	98	224
Sheep 2						
	0	8500	6426	1930	60	84
	3	500	105 (animal died)	368	27	0

Sheep 1 was given melphalan 1.0 mg/kg body weight, i.v.; Sheep 2 was given melphalan 2.0 mg/kg body weight, i.v.

The haematological data on Day 0 are normal for sheep.

methyl pteroic acid. However, Chabner, Myers, Coleman & Johns (1975) stress that only a small fraction of the drug is converted in this way in the mouse. There is no information on such bacterial action in the sheep.

Melphalan is an alkylating agent and prevents normal cell division by damaging DNA and RNA. Its actions should therefore be independent of the peculiarities of ruminant metabolism, provided it survived systemically in an effective form. In the rat, melphalan produced similar haematological results as in the sheep, i.e. a persistent depression of lymphocyte numbers and an initial fall in neutrophils followed by an increase (up to five times the normal figure). This rebound was attributed to intense bone marrow activity following the brief myeloid depression (Elson, 1958). Bone marrow activity was at a peak 5 to 6 days after drug treatment, and peripheral neutrophil numbers highest at 8 to 10 days. Nitrogen mustards act in the rat predominantly on the lymphatic tissues (Elson, 1958). Alkylating agents of the busulphan group appear to be more effective upon neutrophil production in several species (e.g. man, rat) and it would be of interest to examine the effect of these compounds on the blood picture of the sheep.

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