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CHANGES IN THE LIPID COMPOSITION OF TUMOUR AND HEPATIC TISSUES OF RATS BEARING GUÉRIN CARCINOMA FOLLOWING TREATMENT WITH THE NITROGEN MUSTARD DEGRANOL*

PRISCA L. HÁGONY and M. JÁKY

Research Institute of Oncopathology
and Research Institute of the Vegetable Oil Industry, Budapest

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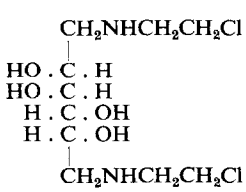
Abstract—Degranol had no effect on the total lipid content of the whole tumour if referred to dry tissue. In the florid tumour part it reduced the glycerides and increased the phospholipids, while in the necrotic part it increased the former and reduced the latter in amount. In the hepatic tissues it caused a fall of the glycerides and produced a smaller rise of the phospholipids. It also caused a rise in the fatty acids in all three types of tissue, reaching peaks at 96 hr after injection. No changes were observed in total cholesterol after giving the drug. At 24 and 48 hr following treatment, the polyunsaturated spots in the fatty acid components of the phospholipids were found to have decreased. The fatty acid components of the glycerides in the nectrotic tumour tissues contained stearin, palmitin, linoleic acid, and in addition, polyunsaturated fatty acids. The spot of the latter was found to have disappeared at 24 hr after Degranol. At 72 hr it reappeared and by that time it was also seen in the florid tumour tissues.

MALIGNANT tumours show excessive accumulation of fat which is not seen in normal tissue.[1] Their necrotic parts reveal characteristic changes in total lipid composition: glycerides decrease in amount while steroids and fatty acids increase.[2] In the view of some investigators these alterations characterize every type of tumour,[3] in that of others they are primary in certain tumour types but secondary in the rest.[4] Cytotoxic agents materially influence the histology of transplanatable rat tumours.[5]

The aim of the present work was to study the effect of Degranol (BCM) on the lipid composition of tumours; more precisely, of the tissues of the three different zones distinguishable in every tumour, namely the florid, the necrotic, and the intermediate zone. The study was at the same time extended to the hepatic tissues of the tumour bearing animal.

* Degranol is the nitrogen mustard derivative of D-mannitol, and its biological and chemical pro-



perties have been described by Keller and Németh.[26]

Accordingly, we determined the amount of total lipid extracted from the tissues; the ratio in it of the acid-soluble to the acid-insoluble lipids; the free fatty acid content of the acid-soluble fraction; and the amount of the total, the free and the esterified cholesterol, respectively. In addition, both the composition and amounts of the fatty acids released on saponification of the glycerides and phospholipids were determined.

METHODS

Wistar rats, 3 months old and all fed the same diet throughout the experiments, were used. Subcutaneous implants of bits of Guérin tumour, the size of a pea, were made in the left interscapular region, and when the tumours reached $n = 4$ size[6] each rat was given a 50 mg/kg intravenous dose of Degranol. Nine animals at a time were killed at 24, 48, 72, 96, 120, 148 and 220 hr after injection, and their tumours and livers were pooled in sets of three for further work. The tumours were cleaned from their connective-tissue sheath and cooled to from 0° to +3 °C, after which they were divided into three parts corresponding to the macroscopically distinguishable florid, necrotic and intermediate zones.

Wet weights of the separate tumour parts were established gravimetrically, and the liver was weighed. Water was removed from the tissues by lyophylization according to the method of Hack.[7] Water content was computed from difference in weight. Total lipid was extracted with hot petroleum ether at a temperature not exceeding 80 °C, and the extract was evaporated to dryness. From the residue the glycerides, the free fatty acids, and the cholesterol were dissolved in acetone, leaving over the acetone-insoluble phospholipids. All these lipid constitutents were determined gravimetrically.

Free fatty acid content was determined with Jáky's[8] micromethod. For the purposes of evaluation a series of known fatty acid contents was prepared from 50 μg upwards. Evaluation was done by colorimetry.

Total and esterified cholesterol was determined by the Liebermann–Burchardt reaction, and free cholesterol was computed from the difference between the two.[9]

Glycerides and phospholipids were saponified in the warm in a stream of N_2. The fatty acid components were separated by paper chromatography, in a hydrophobic phase, using the paraffin impregnation method of Spiteri and Michales,[11] slightly modified. In some preliminary experiments Kaufmann's[10] petroleum impregnation procedure had been tried, but was discarded because it involved substantial loss of time and material to prepare a fraction that would distil through at temperatures between 190 and 220 °C from the commercial petroleum we had to use for the lack of "Undekan" or some similar preparation, and because it had proved very difficult to attain and maintain in the strips the optimal petroleum concentration of from 180 to 200 mg/g. Our modification of the Spiteri–Michales method was to dissolve the paraffin 15% benzene instead of 10% ether, because the form evaporates more slowly: which is an advantage when great many strips have to be impregnated. Kaufmann's[12] solvent system (75% acetic acid and 25% acetonitrile) was used in the preliminary exploratory experiments, but on paper impregnated with paraffin it yielded more than usually high R_f values and imperfectly separated spots. According to Kaufmann[13] the separation of fatty acids is brought about partly by the action of the more powerful acetic acid which represses the tendency of fatty acids to dissociate at different rates. This involves several days before adequate separation develops. Kaufmann[12] shortened separation time by including acetonitrile in the solvent system without interfering with

the part played by the acetic acid. His system is ideal when petroleum is used for impregnation, but requires modification for the use of paraffin. The solvent system used by us contained 75% acetic acid, 5% water and 20% acetonitrile, whereby we increased the dissociative capacity of the acetic acid and decreased the polarity of the acetonitrile. With its use adequate separation was achieved in 24 hr. Chromatography was carried out by ascending flow at between 16 and 24 °C on Schleicher–Schull no. 2043/b paper of 30 mm width and 300 mm length. This permitted adequate separation of from 300 to 400 μg of substance. The finished chromatograms were dried and the spots developed in copper acetate and rubeanic acid. Model fatty acids were used to identify stearin, palmitin, oil and linol. The alkaline potassium permanganate solution of Kaufmann[14] was employed in demonstrating the presence of unsaturated fatty acids, but in twice as high a dilution as he recommended. Lower concentrations sufficed to oxidize these acids, and the pale pink colour of the permanganate in no way hindered the clear distinction of spots. Qualitative determination of the chromatographically separated fatty acid spots was followed by quantitative estimation. A Juan-type photodensitometer was used, which in addition to the extinction curve draws its integral, and thus permits direct reading of the percentage of spots on the individual strips. A similar method of evaluation has been described by Seher.[15]

RESULTS

The average water content of the tissues was 75% in the liver, and 85% in the florid and necrotic tumour parts, respectively. Treatment with Degranol had no effect on it in the hepatic and the necrotic tumour tissues, but decreased it to 80 per cent in those of the florid tumour part at 96 hr after the drug. According to the data in the literature the water content is generally higher in tumours than in normal tissues.[16]

The total lipid content of the tumours of the untreated controls ranged between 8·1 and 8·9 per cent and remained largely within these limits after treatment with Degranol. For hepatic tissues the figures varied from 11 to 15 per cent in the controls, but after Degranol decreased to from 8 to 9 per cent between 24 and 148 hr, to return to the 10 to 15 per cent level after 180 hr. Lustig[17] found that the total lipid content of the normal human lung, referred to dry matter, was 8·7 per cent, and that of the tumour-bearing lung 11·26 per cent. According to Fujiwara *et al.*,[18] the total lipid amounts to 9·1 per cent of the dry matter in the tissues of the normal rat liver and 8·2 per cent of the hepatoma-bearing animal.

In the controls, the acetone-soluble lipid fraction of the whole tumour, referred to dry weight, varied from 30 to 50 mg, and the insoluble fraction from 30 to 60 mg. Treatment with Degranol had no effect on either of these levels. The acetone-soluble lipid fraction of the hepatic tissues of the untreated animals ranged between 55 and 80 mg, with a tendency to decline 24 hr after the drug. By 120 hr it sank to levels from 5 to 10 mg, thereafter to rise again but not above 80 mg. The acetone-insoluble lipid fraction of the control livers was between 30 and 60 mg, and remained unaffected by the treatment.

As regards the total lipid content of the florid and the necrotic tumour tissues, the data in the literature are at variance. Christol and Monier[19] found the former was 2·6 per cent and the latter only 1·7 per cent of the dry weight. Bullock and Cramer,[20] on the other hand, showed that in Walker carcinoma the lipid content of the necrotic tumour tissues exceed that of the florid tissues. Boyd and McEwen,[21] however, found

for the Walker carcinoma 256 no difference in the total lipid content of the florid and the necrotic tissues.

The lipid content of the acetone-soluble fraction in the florid tumour parts varied from 35 to 85 mg in the untreated controls, but in the treated animals it gradually decreased to fall to between 10 and 25 mg by 220 hr. The acetone-soluble lipid content in the necrotic parts rose from 8–25 mg in the controls to 75–90 mg in the treated animals.

The acetone-insoluble lipid fraction of the florid tumour tissues was 30—60 mg in the controls and attained between 68 and 80 mg in the treated animals. The same fraction of the necrotic tissue was 25—55 mg in the controls, and gradually decreased in the treated rats, sinking by 220 hr to levels of from 8 to 30 mg.

Table 1 shows that, expressed in μg/mg, the fatty acid content of the florid tumour parts was 80 to 90 μg in the untreated animals, and that between 96 and 120 hr after

TABLE 1. CHANGES IN FREE FATTY ACID CONTENT FOLLOWING TREATMENT WITH DEGRANOL
(μg of free fatty acid per mg of acetone-soluble fat)

Number of hours after Degranol		Florid tumour part	Necrotic tumour part	Liver
Untreated controls	1	90	300	50
	2	80	200	50
	3	80	200	50
Untreated controls	1	80	100	50
	2	80	150	50
	3	80	150	50
Untreated controls	1	80	100	50
	2	80	100	50
	3	—	—	—
24	1	50	300	80
	2	50	300	80
	3	80	300	80
48	1	50	90	80
	2	50	90	80
	3	70	90	80
72	1	80	120	100
	2	100	120	100
	3	100	120	100
96	1	200	250	200
	2	250	250	200
	3	250	250	200
120	1	200	280	—
	2	250	280	50
	3	250	280	200
148	1	200	250	200
	2	—	250	200
	3	200	250	200
220	1	90	90	50
	2	50	90	50
	3	90	90	50

treatment it was 250 μg, but fell to 50 μg by 220 hr. That of the necrotic tumour parts varied between 100 and 300 μg in the controls; in the treated rats it consistently reached a peak at 24 hr, fell to from 90 to 120 μg between 48 and 72 hr, and climbed to a second peak of from 250 to 280 μg between 96 and 120 hr with a terminal drop to 90 μg. The free fatty acid content of the hepatic tissues was 80 μg in the untreated rats; in the treated ones a peak of 200 μg at 96 hr was followed by a rather rapid decline to 50 μg by 220 hr.

As regards cholesterol, the abundant data in the literature agree in that the total amount of this constituent is significantly greater in tumours than in normal tissues,[22] and in necrotic than in florid tumour parts. Lang (cited by Haven and Bloor[22]) found that a decrease in tumour-cell activity meant an increase in cholesterol content.

The values for total cholesterol were found to vary very widely. Referred to grammes of dry tissue, they ranged between 15,000 and 20,000 μg for the florid, between 30,000 and 40,000 μg for the necrotic tumour parts, and between 5000 and 10,000 μg for the tissues of the liver. Degranol had no effect on either of them.

Of the tumour lipids, mainly the phospholipids have been subjected to more minute analyses by earlier investigators, and generally for changes in amount and the degree of unsaturation only; these changes were established by determinations of iodine numbers. Haven and associates[13] found that the ratio of the saturated to the unsaturated fatty acids of the tumour phospholipids was 30 : 70. On evidence furnished by the same authors, in animals fed the same diet the degree of unsaturation of the phospholipids in the tumours remains unchanged indpendently of the location of the tumour.[24] Kellner and Lustig[2] showed that by feeding diets rich in oil it was possible to increase the iodine number of the total fatty acids of transplanted mouse tumour. Haven found no essential difference in iodine numbers between tumour and muscle tissues: with Walker carcinoma the iodine numbers rose from 85 on a fat-poor diet, to 95 on coco-nut oil, and to 106 on cod liver oil diet, in tumour and muscle alike. This author also showed that by force-feeding of a diet high in fat it was possible to increase the amount of the total lipids of the tumour, without, however, obtaining a corresponding increase in tumour size.

Fig. 1 illustrates the fatty acid composition of the phospholipids in the florid and necrotic tumour parts and the hepatic tissues of rats bearing Guérin carcinoma. Fig. 2 shows the changes in the composition observed at 24 and 48 hr following treatment with Degranol. The components are stearin, palmitin and oil, linol, and polyunsaturated fatty acid. The hepatic tissues of the untreated animals show an additional spot of the last-mentioned constituent. At 24 and 48 hr after Degranol a marked decrease in the amount of the polyunsaturated fatty acids was observed in the florid and the necrotic tumour tissues, but at 72 hr they were present in these tissues in higher concentrations than in those of the untreated controls. From the hepatic tissues the spot due to the least saturated fatty acid disappeared at 24 hr, but reappeared at 72 hr after treatment.

Figs. 3 and 4 show the fatty acid composition of the glycerides in the florid and necrotic tumour parts and in the hepatic tissues in untreated animals, respectively, in animals at 72 hr after Degranol treatment. The components are stearin, palmitin and oil, linol, and polyunsaturated fatty acid. The necrotic tumour tissues reveal one additional spot of polyunsaturated fatty acid. At 24 and 48 hr following treatment a marked decrease in the amount of the polyunsaturated fatty acids was noted in the

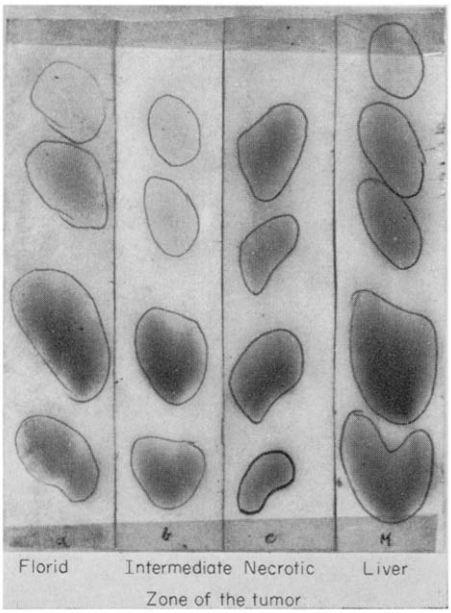


FIG. 1. Fatty acid composition of the phospholipids in tumour and liver in tumour-bearing rats.

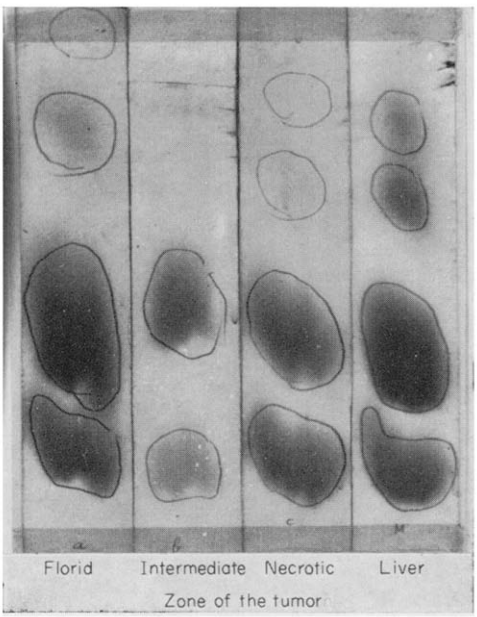


FIG. 2. Changes in the fatty acid composition of tumour and liver phospholipids 24 hr after Degranol treatment.

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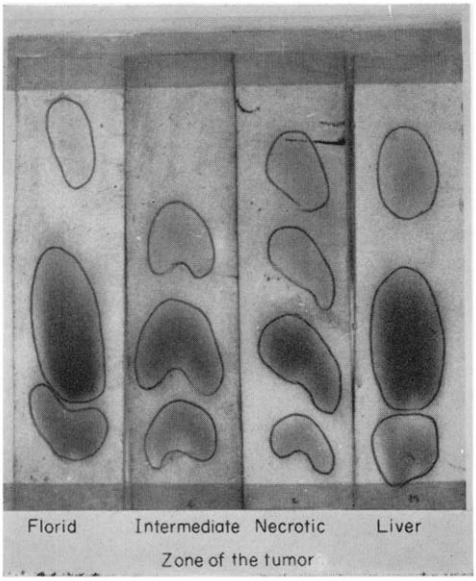

FIG. 3. Fatty acid composition of tumour and liver glycerides in tumour-bearing rats.

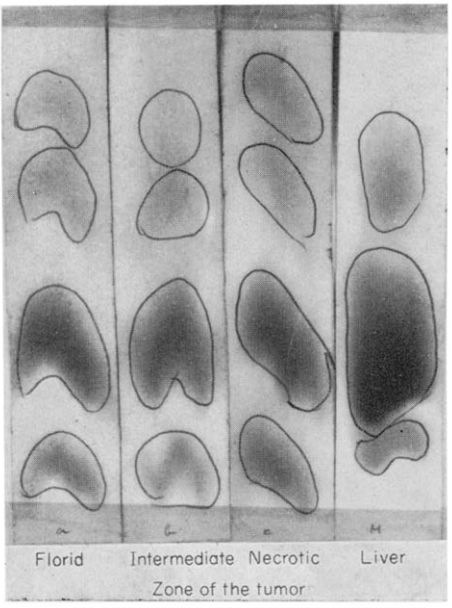

FIG. 4. Changes in the fatty acid composition of tumour and liver glycerides 72 hr after Degranol treatment.

necrotic tumour tissues, but at 72 hr the polyunsaturated fatty acid spot was again present in the florid and necrotic tissues alike.

DISCUSSION

Degranol has been found to have no effect on the lipid content of the whole tumour, but to give rise to characteristic changes in that of each the florid and necrotic parts separated on gross inspection. Of course, in Guérin tumours, where necrotic parts sporadically infiltrate florid parts, complete separation is extremely difficult, if at all possible; this means, the values for controls and treated animals alike will be widely scattered and will render the results problematical.

The presence of fatty acid in necrotic tumour tissues was also observed by Kellner and Lustig.[2] The increase in its amount following treatment with Degranol is presumably due to a change in the activity of some kind of lipase.

The changes in the fatty acid composition of the phospholipids, which we observed to follow treatment with Degranol, undoubtedly appear to be the results of the drug's action. Support is afforded for this view by the finding that unsaturated fatty acid components decrease in amount as the morphological changes increase in severity. One of the polyunsaturated spots proved to be lineolic acid. This is an essential fatty acid, which has been shown by Deuel *et al.*[25] to be an indispensable requisite for the action of the growth hormone. The presence of polyunsaturated fatty acids in tumours, particularly in the phospholipid fraction, has been established unequivocally. The fact that a toxic dose of Degranol reduces the amount of these particular fatty acids, permits the conclusion that the drug is not without effect on tumour metabolism.

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