Biochimica et Biophysica Acta 902 (1987) 149–153
Elsevier

BBA 70521

BBA Report

Free fatty acid uptake is increased in doxorubicin-resistant rat glioblastoma cells

P. Vrignaud and J. Robert

Fondation Bergonié et Université de Bordeaux II, Bordeaux (France)

(Received 20 February 1987)

Key words: Fatty acid uptake; Doxorubicin; (Rat glioblastoma cell)

We have investigated the mechanism by which the rat glioblastoma C6 cell line resistant to doxorubicin presented an increase of free fatty acid utilization from the nutrient source as compared to the wild sensitive strain. We have shown that this was not due to an accelerated turnover of the phospholipid hydrophobic or hydrophilic moieties, but to an important increase of the uptake of the free fatty acids. This was demonstrated by studies performed with linoleic and linolenic acids during very short incubations, at low temperatures, and in the absence of albumin in the medium. This enhancement of free fatty acid uptake may explain the differences which exist in the acyl group composition of membrane lipids between doxorubicin-sensitive and -resistant C6 cells, because of the suppression of the essential fatty acid-deficient status of the cells.

Resistance to doxorubicin is a genetic phenomenon which has been shown to have one or several membrane targets. The most constant modification encountered in plasma membranes of resistant cells is the amplification of a high molecular weight glycoprotein [1,2] but several other membrane modifications have been described in different cell strains. In particular, membrane fluidity changes are acquired by some cell types upon development of doxorubicin resistance [3–7]. The mechanism by which this change in membrane fluidity occurs has not yet been identified in every cell strain. In a doxorubicin-resistant rat glioblastoma cell line, we have observed that the increase of membrane fluidity was correlated with an increase of the proportion of polyunsaturated fatty acids in membrane phospholipids [8]. This observation has raised the question of the mechanism by which such an increase of some acyl groups could occur. We were able to show that no different activities of acyl group unsaturation and elongation were exhibited by resistant cells as compared to sensitive ones, and that the membrane gross transport of fatty acids was strikingly increased in resistant cells. However, several different steps of fatty acid transport could be involved and could explain the modifications observed in 'transport' measured during a 24 h period: the insertion of free fatty acyl groups in the plasma membrane, the translocation of the chains through this membrane, their conjugation with coenzyme A, their incorporation in phospholipids through various acyltransferases, their turnover within the phospholipid molecule, etc... In this study, we have explored in detail two of these possibilities: the uptake of acyl groups through the plasma membrane and the turnover of the acyl group moiety of the cell phospholipids. Our results show unequivocally that the uptake step is responsible for the global transport increase we had previously observed. It is not possible, however, to identify the rate-limiting step of

Correspondence: J. Robert, Fondation Bergonié & Université de Bordeaux II, 180, rue de Saint-Genès, 33076 Bordeaux, France.

this uptake as the association-insertion step into the plasma membrane, or as the translocation it self.

The C6 clone, originating from a rat gioblastoma induced by *N*-nitrosomethylurea, was routinely cultivated in plastic 64 cm^2 Petri dishes (Nunc) with Dulbecco's modified Eagle's medium (Seromed) supplemented with 10% fetal calf serum (Seromed) in a CO_2 incubator. Doxorubicin-resistant sublines were obtained as already described [8,9] and routinely cultivated in the presence of 0.5 or 1 μg/ml of doxorubicin, provided by Roger-Bellon Laboratories. For turnover and translocation experiments, the cells were grown in smaller Petri dishes. Since the cell density was lower for resistant cells than for sensitive cells at the plateau phase, we used 10 cm^2 Petri dishes for sensitive cells and 20 cm^2 Petri dishes for resistant cells, both with 3 ml culture medium, in order to have similar cell amounts at the time of the experiments and similar specific activities and concentration of lipid precursors. All measurements were made with cells in plateau phase of growth.

The turnover of various moieties of the phospholipid molecules was studied as already described [10]. The radioactive precursors were purchased from Amersham: [1-^{14}C]linoleic acid (59.0 μCi/mmol), sodium [U-^{14}C]acetate (58 mCi/mmol) and carrier-free [^{32}P]orthophosphate. They were added to cell cultures in 64 cm^2 Petri dishes (1 μCi/Petri dish for linoleate and acetate and 20 μCi/Petri dish for ^{32}P) and maintained for 24 h at the contact of the cells. The medium was then removed, the cell layer washed with cold medium and the cells further cultivated in usual medium for 48 h. At selected times during the radioactive labelling and the chase, Petri dishes were removed from the incubator, the cell layer was recovered, and the lipids were extracted [11]. Counting of an aliquot of the lipid extract was done and several lipid analyses were performed: assay of phosphorus [12], thin-layer chromatography of phospholipids [13] and counting of the individual phospholipids. The half-life of the precursor in the total lipid fraction or in a phospholipid class was performed by linear regression of the natural logarithm of the radioactive count vs. the time elapsed since the removal of the radioactive medium.

With linoleic acid as a precursor, the amount of radioactivity incorporated in 24 h was three times higher for resistant cells than for sensitive cells (Fig. 1A). All the radioactivity extracted from the cells belonged to esterified acyl groups, mostly in phospholipids. During the chase period, no significant change in radioactivity was observed, indi-

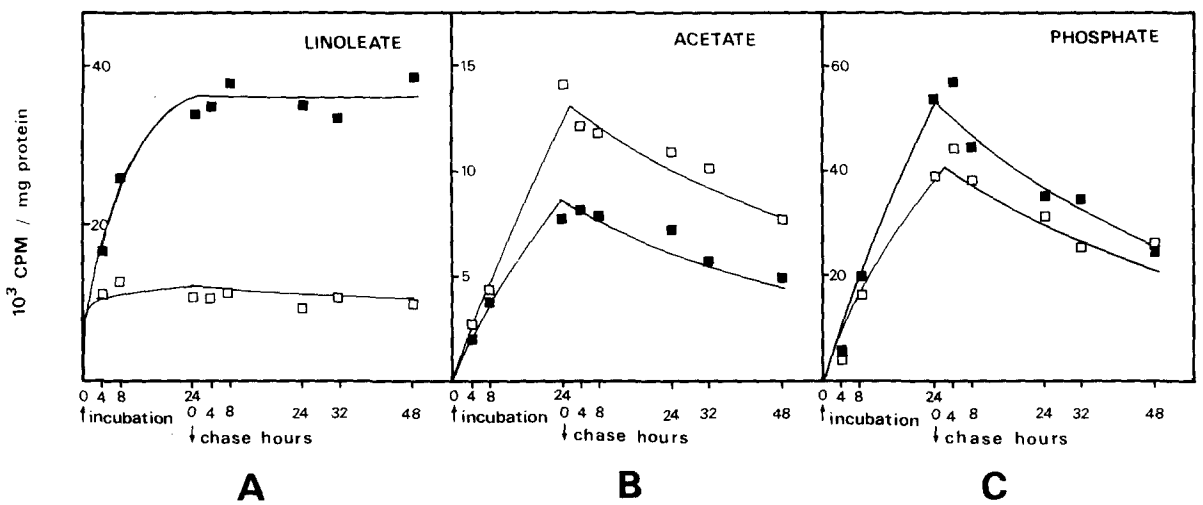

Fig. 1. Incorporation and chase of three phospholipid precursors in doxorubicin-sensitive (□) and -resistant (■) C6 cells. 1 μCi of linoleate (A) or acetate (B) or 20 μCi of phosphate (C) was added to each Petri dish. Cells were harvested at 4, 8 and 24 h, and the lipids extracted and counted. The medium was then changed back to normal medium in the remaining Petri dishes and the chase performed; cells were harvested 4 h, 8 h, 24 h, 32 h and 48 h after the removal of the label, and the lipids were extracted and counted.

TABLE I

DISTRIBUTION OF THE RADIOACTIVITY OF VARIOUS PRECURSORS IN INDIVIDUAL PHOSPHOLIPIDS AT THE BEGINNING AND AT THE END OF THE CHASE PERIOD

The percentage of the radioactivity found in each phospholipid is presented both for sensitive C6 cells and resistant cells (C6 R) at the beginning of the chase period (0 time, corresponds to 24 h incubation with the labelled precursor) and at the end of the chase period (48 h after removal of the precursor). An increase of the percentage of radioactivity in a phospholipid during the chase indicates that no or little renewal of the phospholipid moiety considered occurred; a decrease of this percentage indicates that the moiety considered was renewed in the phospholipid. PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol.

Precursor	PC		PE		Sphingomyelin		PS		PI		Cardiolipin	
	0	+48 h	0	+48 h	0	+48 h	0	+48 h	0	+48 h	0	+48 h
Linoleate												
C6	50	39	20	24	0.7	0.5	5.4	6.0	3.0	3.7	20	25
C6R	57	44	14	19	0.7	0.6	2.2	2.7	5.6	5.5	18	27
Acetate												
C6	63	49	15	23	8.0	11	4.8	6.7	7.2	5.8	2.4	4.6
C6R	63	54	16	19	7.1	11	3.9	5.4	6.1	4.3	6.1	4.3
Phosphate												
C6	56	44	28	30	3.6	9.8	3.1	6.0	8.3	5.4	1.3	4.5
C6R	48	44	30	30	2.4	9.4	2.6	6.3	15	6.0	2.1	3.9

cating that no renewal of this moiety occurred in phospholipids of either sensitive or resistant cells. Only small changes occurred in the distribution of the radioactivity in individual phospholipids during the chase period (table I).

Acetate is considered to reflect the whole hy-

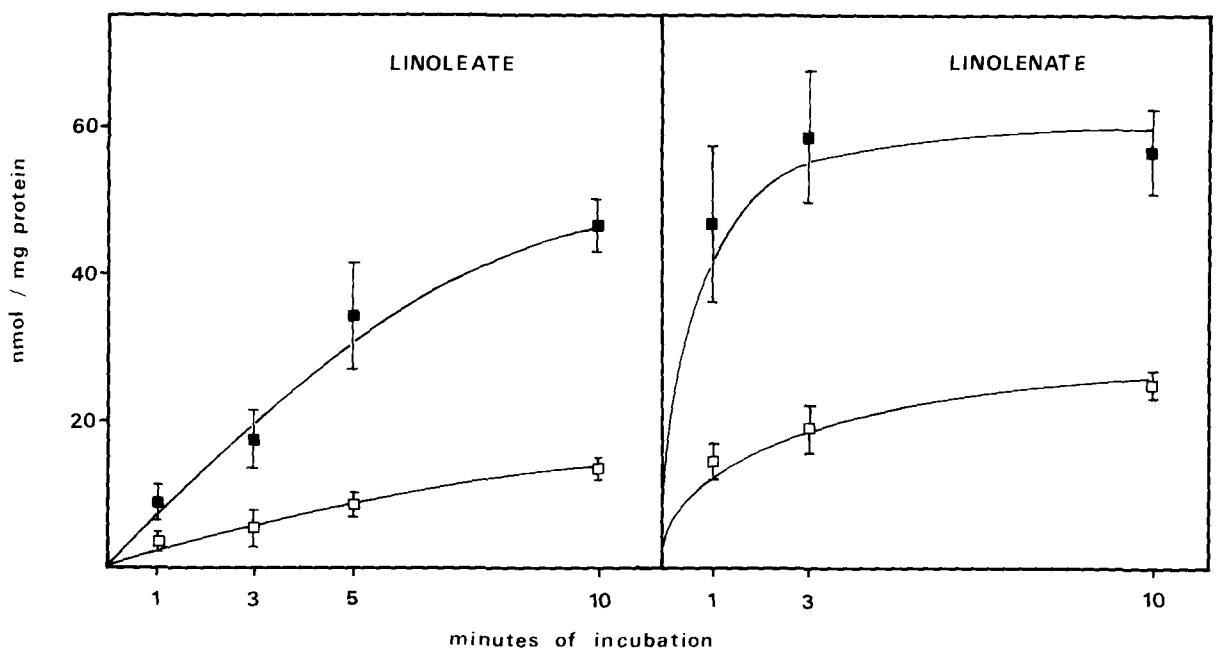

Fig. 2. Uptake of linoleic acid (A) and linolenic acid (B) by doxorubicin-sensitive (□) and -resistant (■) C6 cells. Incubations were performed in triplicate with 1 μCi of each fatty acid (1 μCi/μmol) in a cold room (4±1°C). At selected times, the medium was removed and the cells were harvested, and the lipids were extracted and counted. Bars represent S.D. on both sides of the mean.

drophobic moieties of the phospholipids (saturated + monounsaturated fatty acids). The incorporation of this precursor was somewhat higher in sensitive cells than in résistant ones, but the slope of the chase was quite similar, with half-lives of 58 and 53 h, respectively (not significantly different) (Fig. 1B). The renewal mainly concerned phosphatidylcholine, since the percentage of radioactivity in this lipid fell from 63% to 49–54% during the 48 h of the chase (Table I).

With inorganic phosphate as a precursor, the renewal of the polar môiety of the phospholipid molecule was investigated. Incorporation of this precursor in both cell types was not significantly different over 24 h and the chase period provided also similar radioactive decay with half-lives of 40 and 50 h (not statistically different) (Fig. 1C). The distribution of the radioactivity in individual phospholipids revealed that phosphatidylcholine and phosphatidylinositol were the only phospholipids to have their phosphorus renewed during the chase period (Table I), both in sensitive and resistant cells. Those results were consistent with the turnover rates of the phospholipid moieties already determined in C6 cells [10].

Uptake experiments were performed in a cold room ($4 \pm 1°C$) during short periods of time (1, 3, 5 and 10 min) in order to avoid any metabolic transformation of the acyl groups. Radioactive free fatty acids were purchased from Amersham: [1-^{14}C]linoleic acid (spec. act. 59.0 mCi/mmol) and [1-^{14}C]linoleic acid (spec. act. 56.2 mCi/mmol). They were diluted with non-labelled free fatty acids purchased from Sigma, and dissolved in dimethylsulfoxide (Merck) at a specific activity of 1 μCi/μmol. 1 μCi was added per Petri dish of cultured cells after the usual medium had been replaced by fresh medium devoid of fetal calf serum and equilibration at 4°C. The final concentration of dimethylsulfoxide in culture medium was always lower than 0.5% and had no effect upon cell viability for the duration of the incubations. Incubations were performed in triplicate. The medium was removed at the end of the incubations, and the cell layer was recovered after washing with medium supplemented with fetal calf serum. The lipids were extracted [11] and an aliquot was used for radioactive counting. A TLC of the phospholipids of these extracts was always performed and revealed that no incorporation of the fatty acids in the phospholipids or neutral lipids had occurred during these incubations at low temperature.

When studied over 10 minutes, the uptake of linoleic acid was linear both for sensitive and resistant cells (Fig. 2). Resistant cells incorporated however three times mores of the free fatty acid than sensitive cells did (17 vs. 5.3 nmol/mg protein for 3 min incubation). Linolenic acid was in contrast incorporated more rapidly in the cells, but the same difference between resistant and sensitive cells was observed (58 vs. 19 nmol/mg protein for 3 min incubation) (Fig. 2).

The real uptake of the fatty acids through plasma membrane, which is supposed to be a passive or a facilitated diffusion [14,15] must be measured over short periods of time (< 10 min), at low temperatures in order to avoid any metabolic transformation, and in the absence of any protein carrier such as albumin [16]. Our results show that, either the insertion of the acyl groups into the plasma membrane, or the step of translocation, is altered in our doxorubicin-resistant cells and is responsible for the modifications of gross acyl group transport which had been already observed. It is not known whether this alteration of the uptake of acyl groups through the plasma membrane is necessary for the expression of cell resistance to doxorubicin or is only a side-effect accompanying the development of the resistance. It should be emphasized, however, that several independent selections of resistant cells from the C6 wild strain have lead to the same membrane alterations, both with doxorubicin and with another anthracycline as selective pressure for resistance. In view of the fact that the membrane transport of doxorubicin is altered in most resistant cells, the association of this alteration with a modification of fatty acid transport by the same membrane system may not be a fortuitous phenomenon but could be an obligate phenomenon for the expression of resistance by C6 cells.

This work was possible thanks to grants from the Fédération Nationale des Centres de Lutte contre le Cancer and the Association pour la Recherche sur le Cancer.

References

1 Kartner, N., Shales, M., Riordan, J.R. and Ling, V. (1983) Cancer Res. 43, 4413–4419
2 Bell, D.R., Gerlach, J.H., Kartner, N., Buick, R.N. and Ling, V. (1985) J. Clin. Oncol. 3, 311–315
3 Wheeler, C., Rader, R. and Kessel, D. (1982) Biochem. Pharmacol. 31, 2691–2693
4 Ramu, A., Glaubiger, D., Magrath, I.T. and Joshi, A. (1983) Cancer Res. 43, 5533–5537
5 Siegfried, J.A., Kennedy, K.A., Sartorelli, A.C. and Tritton, T.R. (1983) J. Biol. Chem. 258, 339–343
6 Rintoul, D.A. and Center, M.S. (1984) Cancer Res. 44, 4978–4980
7 Montaudon, D., Vrignaud, P., Londos-Gagliardi, D. and Robert, J. (1986) Cancer Res. 46, 5602–5605
8 Vrignaud, P., Montaudon, D., Londos-Gagliardi, D. and Robert, J. (1986) Cancer Res. 46, 3258–3261
9 Vrignaud, P., Londos-Gagliardi, D. and Robert, J. (1986) Oncology 43, 60–66
10 Hugues, P., Montaudon, D. and Robert, J. (1985) Int. J. Biochem. 17, 611–617
11 Folch, J., Lees, M. and Sloane-Stanley, G.H. (1957) J. Biol. Chem. 226, 497–509
12 Macheboeuf, M. and Delsal, J. (1943) Bull. Soc. Chim. Biol. 25, 116–120
13 Yavin, E. and Zutra, A. (1977) Anal. Biochem. 80, 430–437
14 Abumrad, N.A., Park, J.H. and Park, C.R. (1984) J. Biochem. 259, 8945–8953
15 Stremmel, W. and Berk, P.D. (1986) Proc. Natl. Acad. Sci. USA 83, 3086–3090
16 Morand, O., Fibach, E. and Gatt, S. (1982) Biochim. Biophys. Acta 693, 143–150