LACTATE-MEDIATED CHANGES IN GROWTH MORPHOLOGY AND TRANSFORMATION FREQUENCY OF IRRADIATED C3H 10T½ CELLS.

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ABSTRACT

Treatment of mammalian cells with lactate or inhibitors of glycolysis alters their radiation response, particularly in the low dose region of the dose response curve. The occurrence of both high lactate levels and high glycolytic metabolism in tumours is well known and therefore the effect of lactate on a cell line sensitive to radiation induced transformation was examined using a single exposure to Cobalt 60 gamma rays as the carcinogen challenge. The results indicate that cells treated with 5mM lactate before irradiation exhibit changes in morphology and growth rate and that the transformation frequency is increased by three to ten fold following 24 hours lactate treatment just prior to irradiation.

Examination of radiation survival curves showed a positive correlation between transformation frequency and size of the shoulder, but increasing transformation frequency was associated with a decrease in Do. A mechanism involving altered Redox potential in lactate treated cells is suggested.

The results are discussed in terms of their possible significance for radiotherapy.

INTRODUCTION

For many years it has been recognised that an abnormally high glycolytic metabolism with an associated accumulation of lactate occurs in tumours, but the significance of this is not clear (Aisenberg, 1961; Weinhouse, 1972). We have recently shown that lactate has a role in the differentiation of primary thyroid cultures (Mothersill *et al.*, 1981) and that it can modify the response of mammalian cells in culture to both single and split doses of radiation (Seymour and Mothersill, 1981; Mothersill *et al.* 1982). These effects occured at levels which occur naturally in cell culture (Morgan and Faik, 1979; Seymour and Mothersill, 1981). Split dose irradiation and other treatments which stimulate radiation recovery or repair are associated with altered levels of neoplastic transformation (Borek, 1977; Little, 1977; Miller and

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Hall, 1978).

Because of these reports and the obscure relationship between lactate and both neoplasia and differentiation, it was decided to examine the effect of lactate on growth, morphology and radiation response of the C3H 10T½ mouse embryo system developed by Reznikoff (1973 a and b). This system has been extensively used in the study of radiation induced transformation (Terzaghi and Little, 1976; Kennedy et al., 1980; Miller et al., 1978; Borek, 1977; Ham et al., 1980; Miller and Hall, 1978; Little, 1979; Borek et al., 1979). The transformed foci have been shown to develop into tumour nodules when injected into suitable hosts (Terzaghi and Little, 1976).

MATERIALS AND METHODS

Cell and Culture Condition.

The C3H mouse embryo cell line (10T½, clone 8) was kindly provided by C. Heidelberger (University of Southern California). The cell system was developed by Reznikoff et al. (1973a) and was found suitable for transformation assay following treatment of cell cultures with chemical carcinogens. Cells used for experiments described in this report were between the 8th and 13th passages. No spontaneously transformed foci were found in untreated control cultures in any of the experiments described in this report.

Cells were grown in Eagle's basal medium supplemented with 10% heat inactivated foetal calf serum, gentamicin (4 iu/ml) was added to help prevent bacterial contamination. Serum, antibiotics and culture medium were obtained from Gibco Biocult Ltd., Paisley, Scotland. Cells were grown in 40 ml Nunc plastic culture flasks. Stocks were passaged in accordance with the protocol outlined by Reznikoff et al., 1973 a and b).

Irradiation.

Gamma irradiation was carried out on a standard cobalt teletherapy unit at 60 cms SSD which delivered at a dose rate of 2.0 Gy/min. Experimental flasks were irradiated 48 hours after seeding unless otherwise specified.

Cell Survival following Irradiation.

This was assayed during the technique developed by Puck and Marcus (1956). Cells surviving radiation treatment develop into macroscopic colonies which are stained and counted 10 - 12 days later.

Transformation Assay

The method followed was similar to that described by Reznikoff et al. (1973a). Cells were seeded in 40 ml culture flasks at a density such that about 50-100 viable cells resulted per flask; 6 to 50 replicate flasks were seeded for each date point depending upon the expected transformation frequency. Culture medium was changed twice a week until confluence was reached and once weekly thereafter until termination of the experiment six weeks after seeding. Control and experimental dishes were handled in the same manner. Upon termination of the experiment, dishes were fixed with 10% buffered formalin, then stained with dilute carbol fuschin (Ziehl-Nielsson 1:15 V:V).

Transformed foci were scored according to the criteria outlined by Reznikoff et al. (1973 a and b). Because of the controversy that surrounds the relationship between the number of transformed foci and the initial cell number subjected to treatment (Kennedy et al., 1980), the transformation frequency was determined by counting the number of transformed foci per flask and per surviving cell.

Lactate Treatment

L-(+)-Lactate (hemi calcium salt; Sigma Lond.) was added in 0.5 ml of medium 24 hours after plating the cells and 24 hours before exposure of the cells to radiation. Solutions were sterilised by membrane filtration before addition to the cultures. No change in pH could be detected following the addition of the lactate salt. Control flasks received 0.5 ml of medium or 0.5 ml medium containing equinolar amounts of calcium chloride at the same time.

Statistical Analysis

Means and standard errors were determined on pooled data from at least three experiments in each case. Significance was determined using the paired t-test. Survival curve parameters were obtained from linear regression analysis of the experimental portions of the curves.

RESULTS

Plate 1 shows the effect of 24 hours pre-treatment with 5 mM calcium L-(+)-Lactate on the macro- and microscopic appearance of irradiated (10 Gy) and unirradiated C3H 10T½ cells stained six weeks later. It is apparent that the ordered fibroblast structure of the cell monolayer is lost when 24 hours lactate treatment is given soon after plating. This change is not dependent on irradiation of the cells. Experiments using calcium or sodium chloride instead of calcium lactate confirmed that the effects

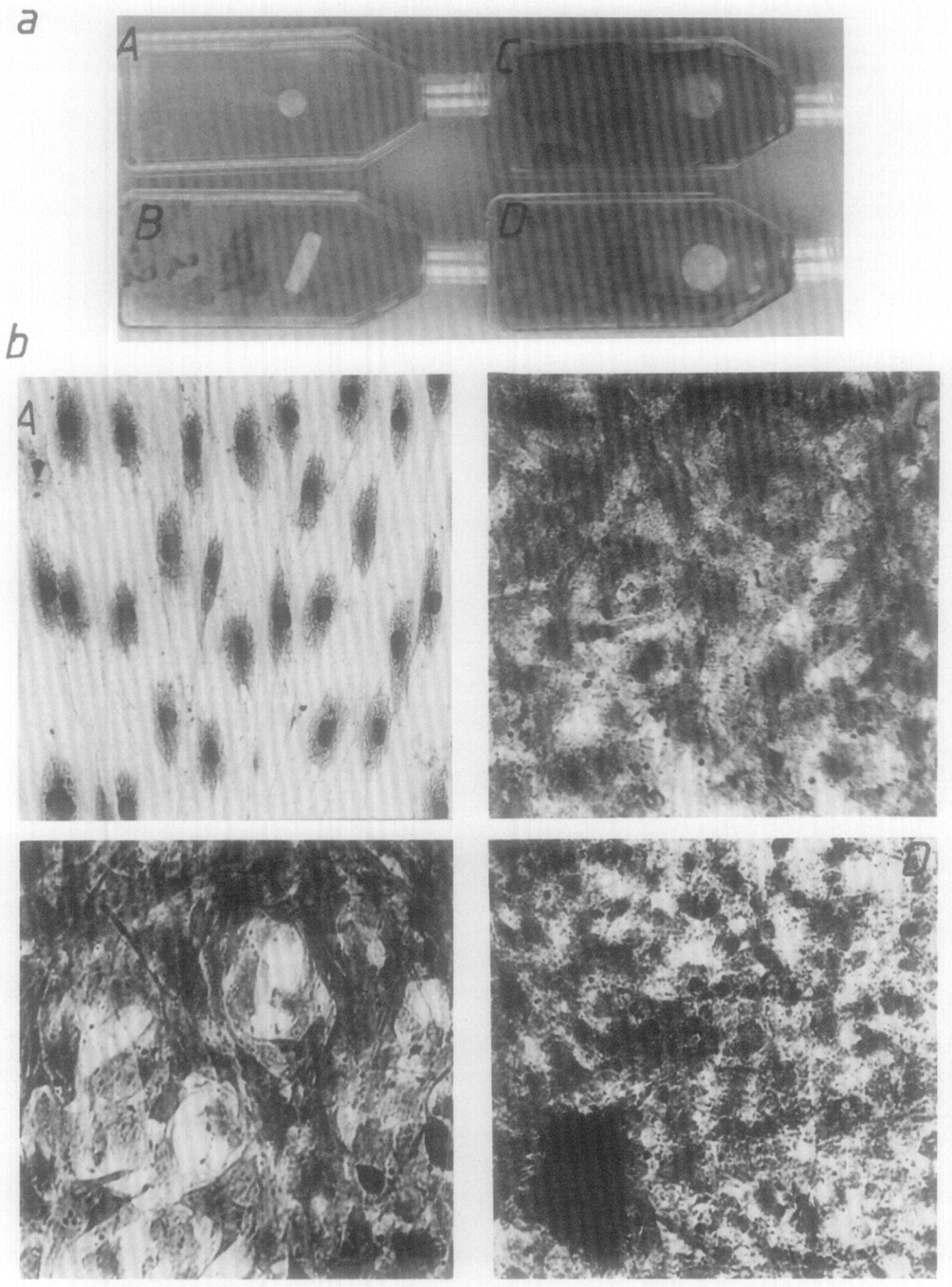

Plate 1: The macroscopic (a) and microscopic (b) appearance of irradiated and unirradiated C3H 10T½ cultures.
A = Control; B = Control + 7.5 Gy;
C = 5 mM Lactate; D = 5 mM Lactate + 7.5 Gy.

observed were not due to the calcium component of the lactate salt (data not shown).

Fig. 1 shows the effect of 5 mM lactate on the growth curve of C3H 10T½ cells. It can be seen that the growth rate is faster and the plateau phase density is higher following lactate treatment. The cells were maintained in a CO_2 incubator and measurements of medium pH exclude the possibility that this was a factor since it remained constant over the seven day period.

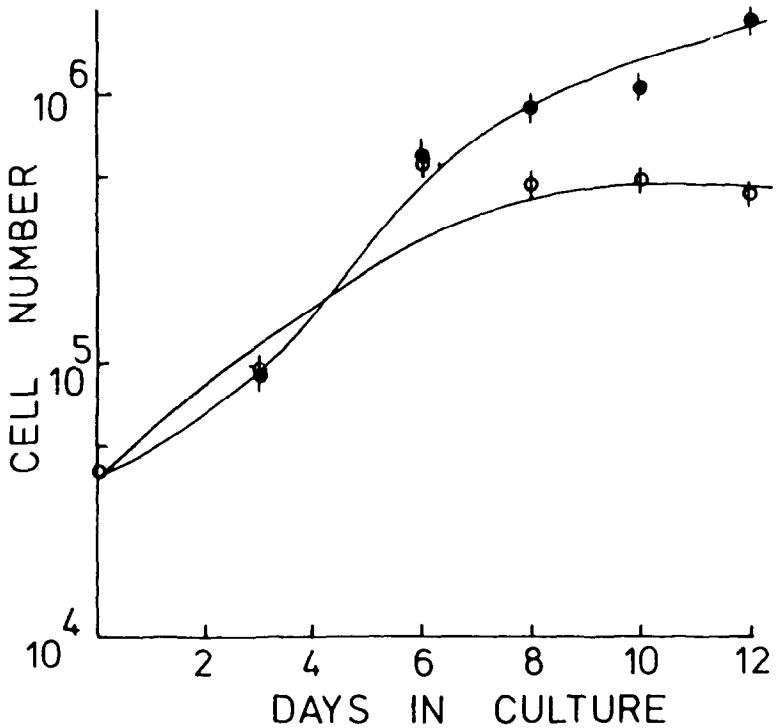

Fig. 1: The growth of C3H 10T½ cells cultured for 7 days in control medium (o) or medium containing 5mM lactate (•).

Figs. 2a and 2b show the effect of 5 mM lactate treatment for 24 hours prior to irradiation on the frequency of transformed foci detected six weeks later. It can be seen that there is a logarithmic increase in transformation frequency with increasing radiation dose up to a dose of 12.5 Gy in the controls. With lactate treatment the same pattern of increase occurs but the transformation frequency is higher and the plateau at 12.5 Gy and 15 Gy is not apparent. These results were not qualitatively different whether they were expressed as foci per surviving cell (Fig. 2a) or foci per flask (Fig. 2b).

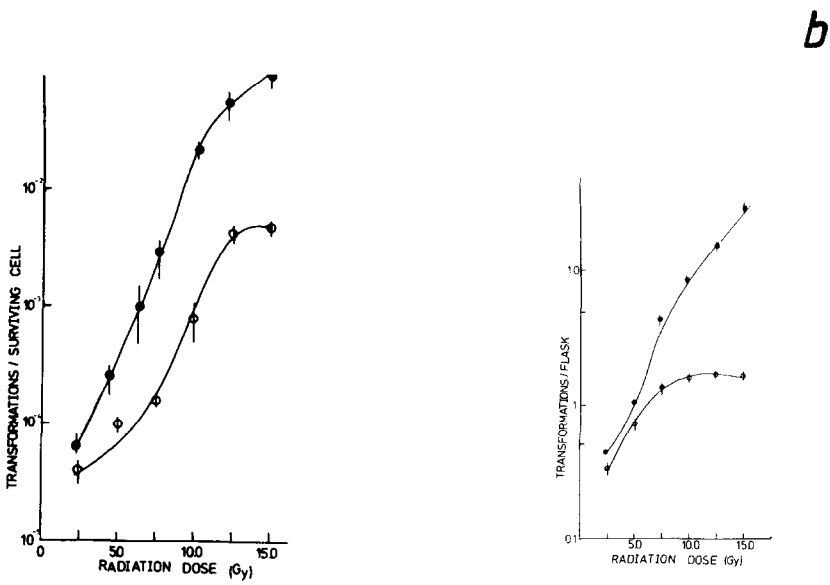

Fig. 2: The frequency of transformed foci detected following exposure of C3H 10T½ cells to increasing doses of radiation in the presence (●) and absence (o) of 5 mM Lactate. 2a: Results expressed per surviving cell, 2b: Results expressed per flask.

The effect of lactate pre-treatment on the survival of irradiated C3H 10T½ cells is shown in Fig. 3 (a) - (d). Lactate changes the shape of the survival curve and the effects are dependent on the lactate concentration. The survival curve parameters n - the extrapolation number, Do - the dose required to reduce the survival to 37% of the original fraction (calculated on the exponential part of the graph) and Dq. the quasi-threshold dose (the intercept between the extrapolation of the exponential part of the curve and a line drawn from the 100% level parallel to the axis) are presented on Table I. When these results are correlated with the transformation frequency obtained at this same lactate concentration they show a direct relationship between extrapolation number, Dq and transformation frequency, while there is an inverse relationship between Do and transformation frequency.

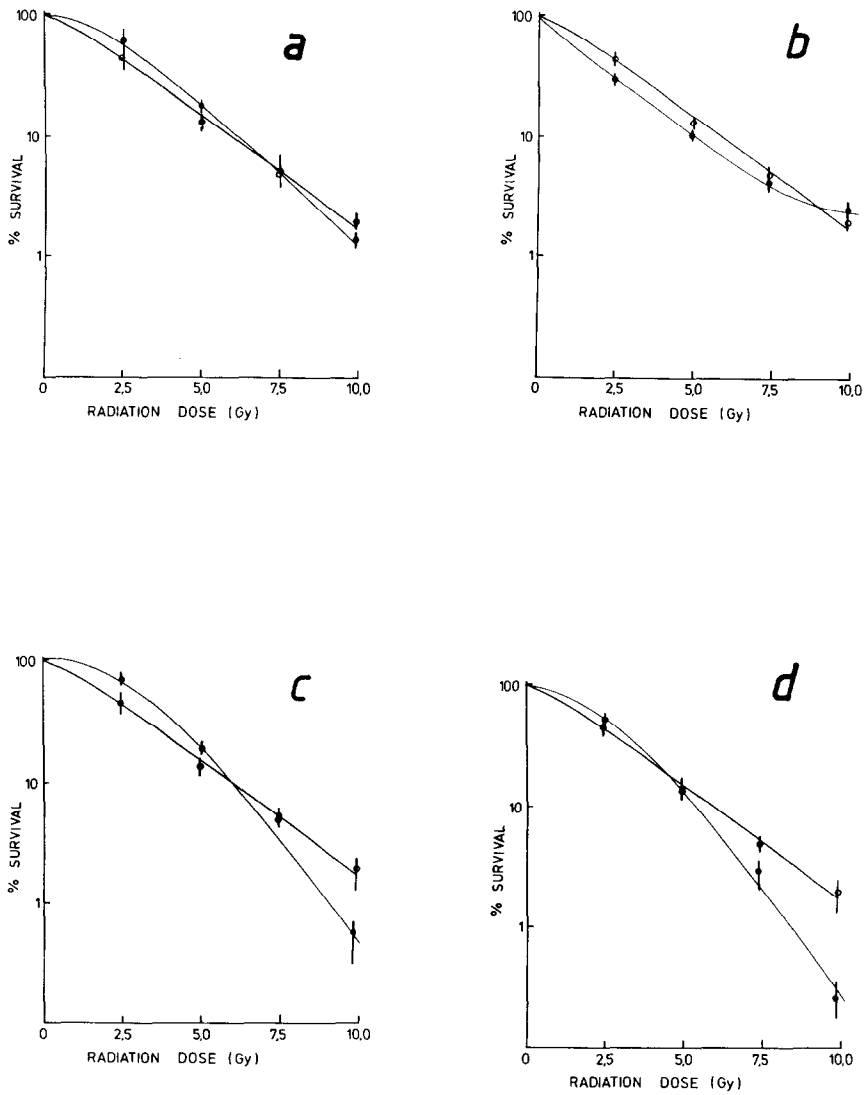

Fig. 3: The effect of pretreatment with increasing concentrations of Lactate on the radiation survival of C3H 10T½ cells. (a) 5 mM Lactate; (b) 10 mM Lactate; (c) 25 mM Lactate and (d) 50 mM Lactate (•) Control (o).

TABLE I

Survival curve parameters, Dq ('quasi-threshold dose'), 'n' (The extrapolation number) and Do for cells treated with L-(+)-Lactate (0-50mM) for 24 hours prior to irradiation. Mean values ± S.E. for 60 flasks per point pooled from three experiments.

Lactate Conc. (mM)	Dq (Gy)	'n' value	Do (Gy)	Transformation Frequency	
0	4 ±0.4	1.2±0.08	2.9±0.16	1.4 ±0.6	
5	2.4 ±0.3	3.8±0.27	1.5±0.2	15.2 ±1.2	p 0.005
10	3.0 ±0.5	8.8±1.3	1.3±0.1	10.4 ±1.2	p 0.005
25	1.75±0.09	1.9±0.2	2.2±0.2	4.4 ±0.5	p 0.05
50	0	1	2.5±0.4	1.58±0.08	N.S.

DISCUSSION

It would appear from the results presented in this paper that lactate can significantly alter the morphology, growth rate and radiation induced transformation frequency of C3H 10T½ cells.

The changes in morphology and growth suggest that lactate treatment reduced the contact inhibition characteristic of the cell line (Reznikoff, 1973a). This combined with the development of many processes on the cell surface could indicate that lactate is affecting the cell membrane.

The control radiation transformation data (Figs. 2 and 3) are qualitatively similar to those obtained by other workers (Little, 1977; Terzaghi and Little, 1976) but the dose at which the plateau occurs is higher. This is probably due to a smaller culture area (25 cm^2 instead of 78 cm^2) since the area available for cell growth is known to be an important factor in focus development (Hall, 1982, Columbia University New York, personal communication).

The effect of lactate treatment on the transformation dose response curve is interesting since it shows that the number of foci obtained continues to rise as the radiation dose increases and no plateau is reached.

The number of foci obtained per flask following lactate treatment is very high. In some cases, the entire flask was composed of transformed foci. This is unusual since the number of foci is normally limited and it has been suggested (Hall, 1982) that the appearance of one focus inhibits their further production. Lactate may therefore be inhibiting the inhibition mechanism.

Lactate changes both the shoulder and the slope of the radiation survival curve for C3h 10T½ cells. This effect is different to that obtained with CHO-KI cells (Seymour and Mothersill, 1981) where only the shoulder region was affected.

The correlation between survival curve parameters and transformation frequency (Table I) could indicate that common mechanisms underly the killing and transforming effects of radiation, or that lactate affects both mechanisms in a similar way. The observation (Seymour, 1983) that lactate can inhibit glucose breakdown and its effect on the redox balance of the cell (Lehningher, 1976) could implicate a mechanism(s) involving energy metabolism.

If lactate can also increase radiation induced transformation *in vivo*, there could be important implications for radiotherapy, particularly where longterm survival of the patient is expected.

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