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THE RELATIONS BETWEEN LACTATE PRODUCTION, RESPIRATION AND NUCLEAR DAMAGE IN IRRADIATED RAT THYMOCYTES

by

J.F. WHITFIELD, H. BROHEE and T. YOUDALE

1965

Joint Nuclear Research Center
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A possible mechanism which can fully explain these observations is discussed.

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Summary

Respiratory inhibitors prevent the postirradiation disappearance of nuclear structure in rat thymocytes. This effect cannot be ascribed to the compensatory stimulation of lactate production since high concentrations of nicotinamide which prevent nuclear changes inhibit both respiration and the radiation-induced burst of pyruvate and lactate production.

The effect cannot be ascribed to the inhibition of respiration *per se* since certain concentrations of 2,4 dinitrophenol stimulate respiration, but strongly inhibit the disappearance of nuclear structure.

A possible mechanism which can fully explain these observations is discussed.

Compounds which inhibit respiration also strongly reduce the rate of appearance of cells with structurally homogeneous (pycnotic) nuclei in irradiated populations of rat thymocytes (11,18). Since most of these agents also stimulate lactate production in both normal and irradiated cultures, Myers (11) has concluded that they prevent the development of nuclear damage by causing an excessive intranuclear accumulation of lactic acid. The resulting excess acidity in the nucleus would stabilize the nucleoproteins and prevent the disaggregation of the chromatin granules and threads which constitute the normal nuclear structure.

On the other hand, Whitfield et al (17) have shown that the main postirradiation burst of lactate production by thymocytes follows the loss of nuclear structure and the appearance of histones in the cytoplasm. In fact, this burst of lactate production is most probably caused by the nuclear changes and therefore cannot affect them. Prevention of the nuclear changes by concentrations of nicotinamide which inhibit respiration is associated with a very strong reduction in the postirradiation lactate production (15,17,18).

These observations would seem to contradict Myers' hypothesis. However, in our previous work we only studied the postirradiation excretion of lactate by the cells into the medium using the less specific colorimetric method of Barker and Summerson (2) to estimate the lactate concentration. Therefore, in the present study, we will show, by using a highly specific enzymatic technique, that nicotinamide does indeed prevent a radiation-induced stimulation of

total lactate production as well as inhibiting respiration. The data we will present here will show that reduction of nuclear damage by nicotinamide or 2,4 dinitrophenol cannot be attributed to either the reduction of respiration per se or to a stimulation of the production of lactic and pyruvic acids.

Materials and Methods Thymocytes (from one month-old Sprague Dawley rats) were suspended in a glucose-phosphate medium buffered at pH 7.2 with tris (hydroxymethyl) aminomethane. Phosphate was added in the form of Na_2HPO_4 and its concentration was 15 mM. The method of isolation of thymocytes and the medium have been described in full detail previously (16).

Thymocyte suspensions were irradiated at 37°C with 1000 r (at a rate of 100 r $min.^{-1}$) of 250 kV x-rays from a Seifert Isovolt x-ray machine. Nicotinamide, or 2,4 dinitrophenol, was added immediately after irradiation.

At various times after irradiation, 3 ml of undiluted cell suspension (containing about 1 x 10^8 cells per ml) were mixed with 1.5 ml of cold, 1.8 M $HClO_4$. The mixture was then centrifuged and the lactate and pyruvate contents of the supernatant were determined. The supernatant contained the lactate and pyruvate which had been extracted from the cells by the $HClO_4$ as well as the amount which had already been excreted by the cells into the medium. The lactate concentration was determined by the extent of reduction of nicotinamide adenine dinucleotide (NAD) in the presence of lactic dehydrogenase.

The pyruvate concentration was measured by the extent of oxidation of NADH in the presence of lactic dehydrogenase. The reagents and the detailed procedure were included in the lactate and pyruvate estimation kits supplied by C.F. Boehringer and Sons, Mannheim (Germany).

To measure the respiration rate, 2 ml samples were removed from the cell suspensionsand put into respirometer flasks. Oxygen consumption was measured for 2 hours after irradiation with a Warburg constant volume respirometer.

The rate of appearance of cells with structurally homogeneous (pycnotic) nuclei was determined by removing a few drops from the cultures and fixing the cells in neutral formalin and staining them with Delafield's haematoxylin according to the procedure of Whitfield et al (16).

Results Exposure of thymocyte cultures to high concentrations of nicotinamide inhibited their respiration. At a concentration of 0.10 M, nicotinamide reduced the respiration rate of both normal and irradiated thymocytes from 21 ± 1.52 to 14.5 ± 0.68 $\mu l\ O_2$ consumed per 10^8 cells per hour; this difference is highly significant ($p < 0.001$). It has already been amply demonstrated that this concentration of nicotinamide can very strongly reduce the postirradiation rate of development of nuclear structural homogeneity (15,18).

It would be expected that since high concentrations of nicotinamide are respiration inhibitors they would stimulate lactic acid production. However, we have already shown that they reduce the excessive excretion of lactic acid into the medium by irradiated cells. From Table 1, it can be seen that nicotinamide treatment also reduced the total lactate content of the irradiated culture to the level in unirradiated cultures.

However, nicotinamide may simply block the production of lactate from pyruvate in which case there could be a large accumulation of pyruvate. While irradiation caused a small increase in the pyruvate production this increase was also entirely prevented by nicotinamide (Table 1).

It is therefore quite clear that the strong inhibition of nuclear damage by nicotinamide cannot be attributed to an intranuclear accumulation of either lactic or pyruvic acids. On the other hand, it cannot be explained on the basis of a maintenance of the normal cellular level of nicotinamide adenine dinucleotide (NAD) since the radiation-induced NAD loss is simultaneous with, or more often follows, nuclear structural homogenisation (4).

Another possibility was that a reduction of respiration would itself inhibit nuclear changes. This was disproven by exposing irradiated cultures to various concentrations of 2,4 dinitrophenol immediately after irradiation. High concentrations of

dinitrophenol did indeed inhibit both respiration and radiation-induced nuclear changes, but lower concentrations increased the respiration rate to levels well above that of the untreated, irradiated culture while still inhibiting the nuclear changes (Table 2).

It could still be argued that since dinitrophenol is a strong stimulant of lactic acid production, this is the reason for its effect on nuclear changes. However, lowering the concentration of dinitrophenol from 1×10^{-4} to 5×10^{-5} M did not reduce the lactic acid production but it did markedly increase the rate of nuclear homogenisation (Tables 2 and 3). A further decrease of the dinitrophenol concentration to 1×10^{-5} M completely eliminated the excessive lactic acid production, but a definite reduction in the rate of nuclear homogenisation was still obtained (Table 3). Therefore, the correlation between lactate production and the rate of nuclear changes is not sufficiently close to warrant the hypothesis that intranuclear accumulation of lactate inhibits nuclear structural homogenisation.

Discussion These observations show that the reduction of nuclear damage by respiratory inhibitors cannot be due either to accumulation of lactate as suggested by Myers (11) or to a reduction of respiration *per se*. The data strongly suggest that there is a third process normally linked to respiration which operates to affect the nuclear changes.

We have attributed the homogenisation of nuclear structure in irradiated thymocytes to a phosphate-induced dissociation of histones from the desoxyribonucleic acid of the nucleoproteins which constitute the chromatin structures of the interphase nucleus (16, 18). This suggestion was based on several lines of evidence. Ernst (6,7) has observed a very early dissociation of histones from DNA in irradiated thymocytes and this has been directly observed cytologically by Whitfield et al (16). The rate of nuclear homogenisation in irradiated (but not unirradiated) cultures is directly proportional to the concentration of inorganic phosphate in the culture medium (14,16). Finally, it has been shown that exposure of isolated nuclei to organic and inorganic phosphate compounds causes a complete loss of nuclear structure (12,13).

An hypothesis to explain the various observations made by ourselves and Myers (11) could be formulated in the following way. During the first hour after irradiation, respiration and inorganic phosphate uptake by the cell are normal (8,18). However, the ability to combine the accumulating phosphate with adenosine diphosphate to form adenosine triphosphate is impaired (5,8). Therefore, the accumulating phosphate would be free to attack the already weakened histone-DNA linkages (9) in the irradiated nucleoproteins of the chromatin granules. Since the maintenance of the interphase aggregates of nucleoproteins depends on the chromatin-condensing capacities of the histones (1,16), separation of the histones from the DNA must result in a dissolution of the prominent reticulo-granular structure of the nucleus.

Phosphate uptake by the whole cell and isolated mitochondria is driven by respiration (3,10,19) and any reduction in respiration rate will reduce the amount of phosphate accumulated by the cell. Therefore, according our hypothesis, respiratory inhibition should inhibit postirradiation nuclear changes. Dinitrophenol uncouples oxidative phosphorylation from respiration as does radiation. However, unlike radiation, it also inhibits phosphate uptake (3). Therefore, exposure to dinitrophenol (at lower concentrations) would be expected to have no effect on, or to stimulate, respiration, but it will inhibit phosphate uptake (3) and nuclear changes.

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TABLE 1

The effect of nicotinamide on postirradiation lactate and pyruvate production.

Condition	Pyruvate /ug per ml culture at 6 hours	Lactate /ug per ml at 6 hours
Unirradiated	8.57	64.7
Irradiated (1000r)	12.85	266.0
Irradiated (1000r) + 0.10 M nicotinamide	8.57	56.1

TABLE 2

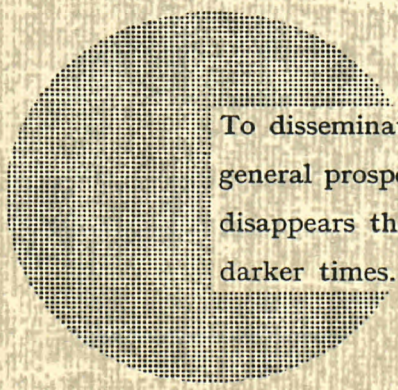
The effects of 2,4 dinitrophenol on respiration and nuclear structural homogenisation (pycnosis) in irradiated (1000 r) rat thymocytes.

Dinitrophenol	Respiration rate ($\mu l\ O_2$ per 10^8 cells per hour)	Percent cells with homogeneous nuclei at 2 hrs postirradiation
0	20.4	30.0
1×10^{-3}	9.8	0.3
5×10^{-4}	10.4	0.7
1×10^{-4}	23.5	1.8
5×10^{-5}	35.6	11.1
1×10^{-5}	27.8	20.1

TABLE 3

The effects of 2.4 dinitrophenol on postirradiation (1000 r) lactate production and nuclear changes.

Dinitrophenol Concentration	μg lactate produced by 10^8 cells during 4 hours postirradiation	Percent cells with homogeneous (pycnotic) nuclei 4 hours after irradiation
0	85.2	62.2
1×10^{-4}	436.0	4.5
5×10^{-5}	439.0	14.1
1×10^{-5}	85.2	47.2



To disseminate knowledge is to disseminate prosperity — I mean general prosperity and not individual riches — and with prosperity disappears the greater part of the evil which is our heritage from darker times.

Alfred Nobel

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