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NUCLEAR PYKNOSIS AND THE DEVELOPMENT OF RADIATION DAMAGE IN PERIPHERAL LYMPHOCYTES

by

J.F. SCAIFE

1968

Joint Nuclear Research Center
Ispra Establishment - Italy

Biology Division

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SUMMARY

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KEYWORDS

LYMPHOCYTES
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DOSIMETRY
RADIOSENSITIVITY
TESTING
TRITIUM
LABELLED COMPOUNDS
CYTIDINE
BLOOD CIRCULATION
SENSITIVITY
RATS
RABBITS
MAN

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INTRODUCTION

The extreme radiosensitivity of mammalian lymphocytes is well established and a precipitous decline in the number of circulating lymphocytes is characteristic of radiation exposure in animals and humans (1). In vitro studies have shown that irradiated lymphocytes undergo a characteristic form of interphase cell death designated as nuclear pyknosis, although this is not unique to ionizing radiations and can be produced by a variety of cytotoxic agents (8). Extensive studies of this mechanism of cell death have been made, mostly with rat thymocytes, and the obligatory need for aerobic respiration established (4, 5, 6, 10). In addition the important observations have been made, that the rate of nuclear pyknotic development in vitro is greatly accelerated by inorganic phosphate and serum in the medium (6, 10).

Nuclear pyknosis is demonstrated by all lymphatic cells in the body, though possibly in a quantitatively varying degree, after radiation exposure. In lymphatic organs-lymph nodes, thymus, bone marrow, appendix, Peyer's patches etc., this degeneration is readily visible in cytological preparations. The number of pyknotic cells in vivo reaches a maximum after a few hours due to phagocytic activity, and eventually all are removed. In blood, the reticuloendothelial system is particularly efficient in this respect and pyknotic lymphocytes are very rarely seen in the peripheral circulation. This has precluded this sensitive phenomenon from being used as a biological dosimeter for radiation exposure. However, it has been shown (11) that in rats the development of nuclear pyknosis in vitro is identical whether the whole animal is irradiated and the blood then immediately removed, or the

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isolated blood itself is irradiated with the same dose. Although preliminary investigations were unpromising, this biological method of dosimetry has been proposed for assessing radiation exposure in man (11). From what is known of the rapid exchange of peripheral lymphocytes with other lymphatic tissues, however, (2, 3) it is evident that a blood sample would have to be removed very rapidly following exposure of an individual to be of significant validity.

This work was undertaken to evaluate the response of human peripheral lymphocytes to X-irradiation with regard to the feasibility of using them in biological dosimetry, and also if possible to determine whether the RES can recognize an irradiated circulating lymphocyte and if so, how soon after irradiation, or whether the RES itself must also be irradiated to initiate its scavenging activity.

METHODS

The preparation of cell suspensions, media, cytological examinations, and conditions of irradiation, were as previously described (6), except that blood samples were taken using the anticoagulant EDTA (0.15 ml of a solution of the disodium salt per 5 ml of blood at a concentration of 1 gm per 15 ml in physiological saline). Care was taken to ensure that all blood samples were fully aerated prior to and during irradiation.

The yield of lymphocytes with EDTA was higher than when heparin was used and there was less tendency to clump and adhere to glass surfaces. All glassware was siliconized. Syringes were of sterile disposable plastic.

Rat blood samples (5-6 ml) were removed by cardiac puncture under ether anaesthesia from male albino animals weighing 150-170 gm.

Human blood (20 ml) was removed by veni puncture of the arm from healthy volunteers. All operations including the final suspension and incubation of the lymphocytes were carried out under sterile conditions due to the need to maintain the cultures for 24 hours.

Rabbit blood (20 ml) was taken from the central ear artery of one ear. Mouse blood (1 ml) was taken by cardiac puncture and pooled from 3 animals in order to obtain sufficient quantities for an efficient separation of lymphocytes.

For radioactive labelling experiments the separated "buffy coat" from 20 ml of blood was resuspended in 2 ml of plasma containing 100 uCi of H^3-labelled precursor. After one hour at 30°C with gentle agitation the leucocytes were sedimented and resuspended in fresh plasma. A cell count was made by haemacytometer and 0.2-0.5 ml taken for H^3 estimation while 1 ml of the remaining suspension was reinjected into the ear vein of the same rabbit. The distribution of the reinjected labelled lymphocytes was followed by removing 5 ml aliquots of blood by cardiac puncture from ether-anaesthetized rabbits and separating the leucocyte buffy layer in the same manner as above, removing aliquots for cell counting and H^3-assay.

Aliquots for H^3-assay were diluted with fresh plasma and physiological saline and centrifuged to sediment the cells. The cell pellet was washed twice with cold 5% trichloracetic acid (TCA) at 0°C. In the case of cytidine labelling the pellet was hydrolysed with 1.5 ml of 5% TCA at 90°C for 15 min, centrifuged and the extract neutralized and made up to 2.0 ml. For H^3-histidine labelling hydrolysis was done directly in 0.3 N KOH solution. A 0.5 ml aliquot was counted by liquid scintillation counting for H^3-content.

Autoradiography was done with Kodak AR10 stripping film with an exposure time of 12 weeks. Cytidine 5-T, 23.6 Ci/mM, 2-deoxycytidine 5-T, 14.7 Ci/mM and L-histidine 2-5-T, 7.5 Ci/mM were obtained from the Radiochemical Center, Amersham.

RESULTS

In order to facilitate the development of nuclear pyknosis and produce a linear dose-response curve, lymphocytes were incubated in a modified Krebs-Ringer medium containing 30 mM phosphate and 10% added bovine foetal serum (6). Using these methods with commercially available male albino rats, con-

sistently reproducible results have been obtained for in vitro irradiation as is shown in Fig. 1. The sensitivity of the method is such that a dose of 5R can be easily detected in any one experiment carried out by the same investigator. The time of incubation is only 7 hours and the response is linear under these conditions up to 100 R. By contrast examination of the blood of rats which had received 200 R whole body X-irradiation never showed more than 1% of pyknotic cells in the circulating blood up to 8 hours following irradiation. The damaged cells are therefore rapidly removed from the circulation by the RES.

The response of rabbit lymphocytes is also shown in Fig. 1 and is somewhat less than that obtained with rats. In the case of mice it was not found possible to obtain satisfactory results since a clumping reaction was observed in which large numbers of platelets were involved, resulting in the loss of cells from the suspensions. This reaction was independent of the presence or absence of serum in the medium and is possibly associated with specific immune responses due to the pooling of the blood samples from several mice. In suspensions of rat or rabbit lymphocytes there was never more than a 1-2% loss of cells over the 7 hour incubation period.

For human lymphocytes in suspension it was confirmed here that the development of nuclear pyknosis proceeds at a much slower rate than for rat or rabbit lymphocytes. Cell death (7), nuclear pyknosis (9, 11) and time to death of humans (1) appears to be prolonged in comparison with other species. This process was not accelerated by resuspending the lymphocytes in fresh medium after 7 hours incubation, by incubating in the presence of 100% oxygen in place of air, by eliminating calcium from the medium, by incubating in complete culture media such as T. C. 199 with or without additional phosphate, or by incubating in the presence of rat thymus or spleen cell suspensions irradiated with 500 R X-rays. It is hence considered to be a species-specific response. The overall rate of pyknosis, including controls, can be accelerated by incubation at elevated temperatures (12). The results obtained with human lymphocyte suspensions prepared from aliquots of blood given different radiation doses and incubated for 24 hours in sterile

media are shown in Fig. 2. Two individuals with widely differing responses were retested after a period of 30 days and gave almost identical dose-response curves to those initially obtained. These results are thus contradictory to previously reported ones (11) in that variability is clearly correlated with individual blood samples and not with the experimental procedure. A similar wide dose-response relation has been previously reported for human lymphocytes (7). It would thus seem that individual variability would preclude this method as a reliable system of biological dosimetry, unless individual control values could be previously determined and calibrated, and their stability and reproducibility established.

Autoradiographic and liquid scintillation investigations with H^3-cytidine have shown that labelling is much more intense if the cells are tagged as a concentrated lymphocyte suspension rather than as whole blood. Practically no labelling was observed with H^3-deoxycytidine in confirmation of the fact that peripheral lymphocytes are essentially non-dividing cells and are hence not synthesizing DNA. In smears made from cells labelled with H^3-cytidine nearly all the labelled cells were lymphocytes, although not all lymphocytes were labelled, the isotope tending to be concentrated in the larger cells. Hence the reinjection of H^3-cytidine-labelled leucocyte suspensions into rabbits can be considered as a valid measure of circulating lymphocytes. In the case of H^3-histidine labelling, however, a considerable number of polymorphs were also labelled.

The rate of disappearance of labelled normal and irradiated (200 R) lymphocytes from the peripheral blood of normal rabbits is shown in Fig. 3. Since, for experimental reasons, no attempt was made to control the absolute number of cells injected in relation to the original lymphocyte counts of the rabbits some variability in the initial "zero time" values would be expected. However, it is evident that there is a very rapid disappearance of the labelled cells from the circulating blood, especially during the first 10-15 min after injection. Following this time the rate of disappearance is roughly exponential. There was no evident difference in the rate of disappearance of unirradiated lymphocytes and lymphocytes which had received 200 R at the end of their labelling period i. e. approximately 20 min prior

to reinjection into the same rabbit. It is thus not possible to answer the original question posed in this work, whether the RES can recognize an irradiated cell without itself being activated by irradiation. Whether this system is a measure of a true physiological phenomenon in that normal lymphocytes are in rapid dynamic equilibrium with other lymphatic tissues, or whether it merely indicates that the reinjected cells were damaged by the isolation and labelling procedures, would require further investigations.

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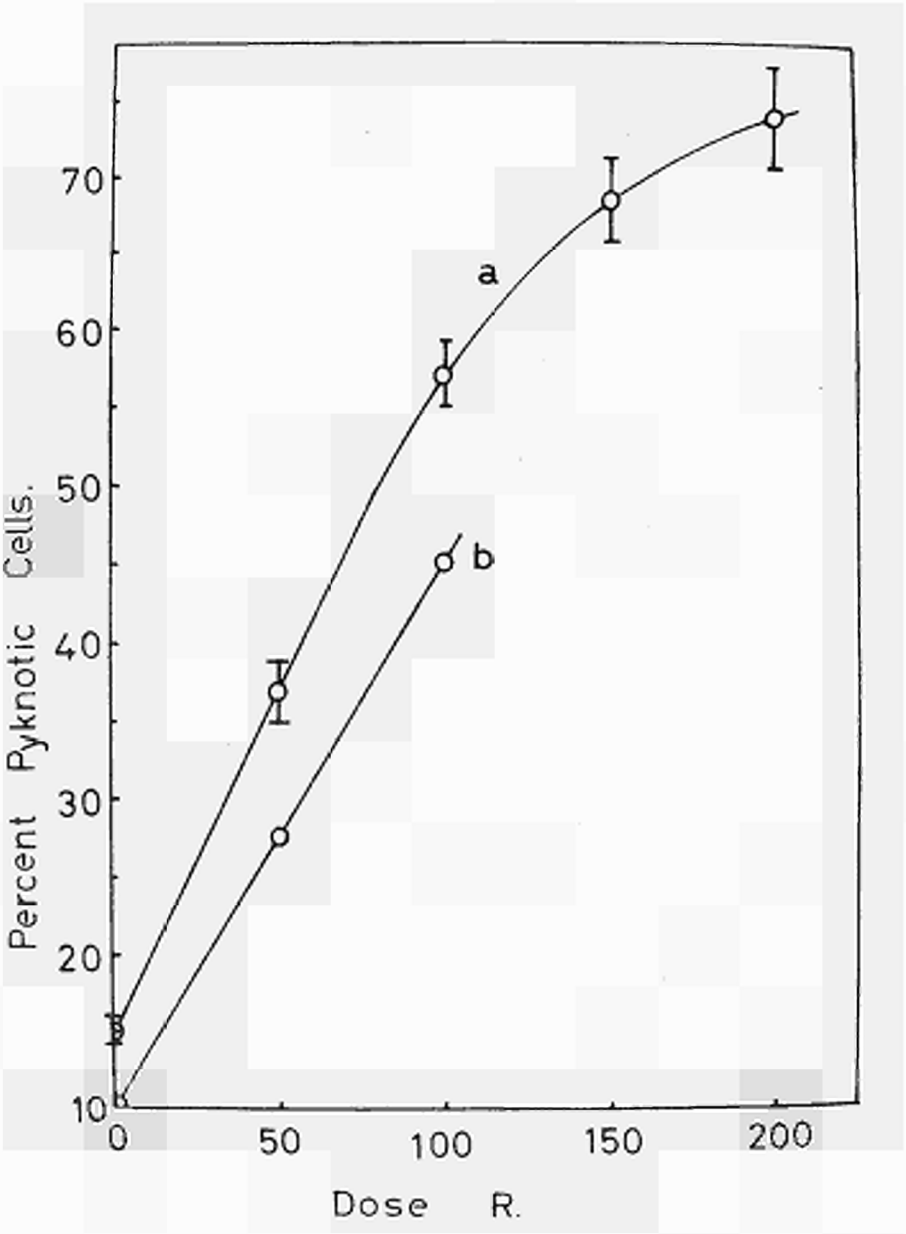

Fig. 1 The development of nuclear pyknosis in X-irradiated peripheral lymphocytes after 7 hours incubation. a, rat, with mean deviation of 8 experiments. Each point represents an individual animal. b, rabbit, the mean of 3 animals, each point represents an aliquot of blood from the same animal.

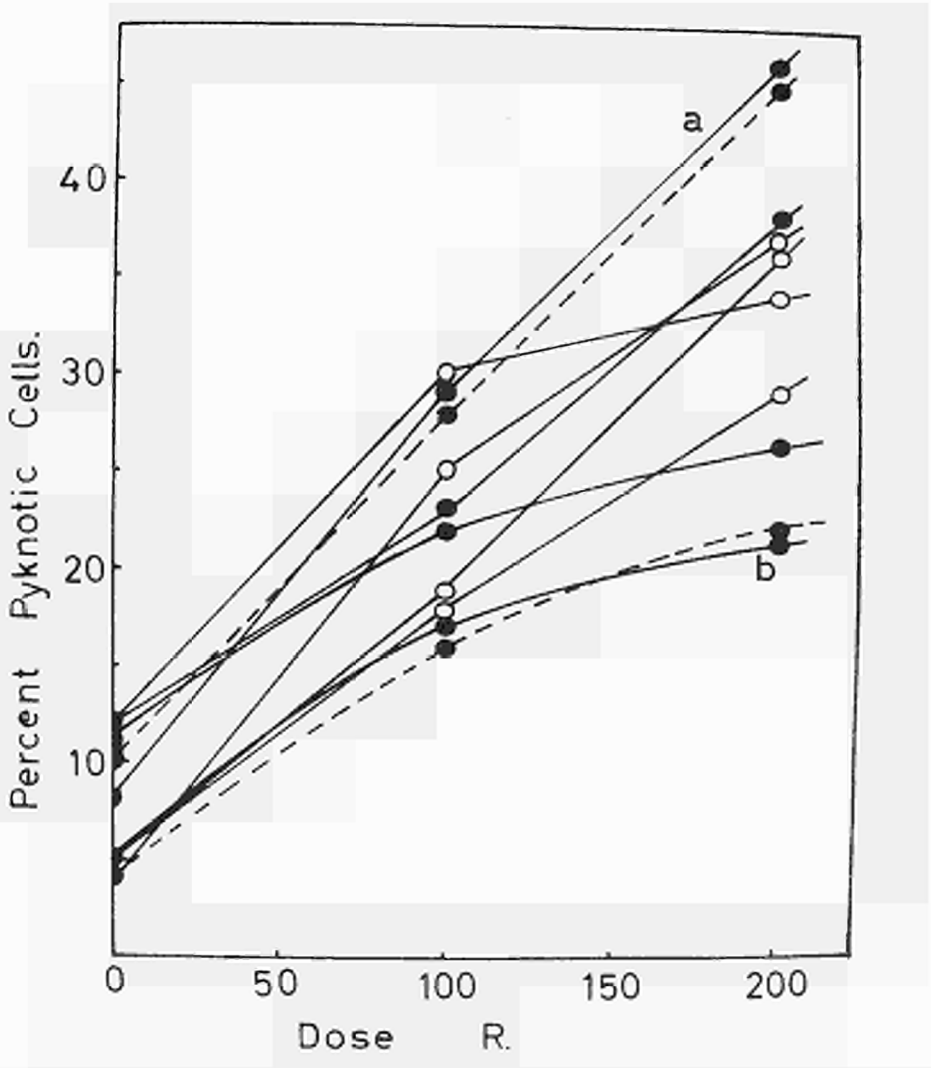

Fig. 2 The development of nuclear pyknosis in X-irradiated peripheral human lymphocytes after 24 hours incubation.
The dose-response curves are typical of those obtained with numerous donors. Four donors were bled in each experiment ● —— ●, first four; 0 —— 0, second four; ● - - - - ●, are repeat curves obtained with individuals a and b for blood samples taken 30 days later.

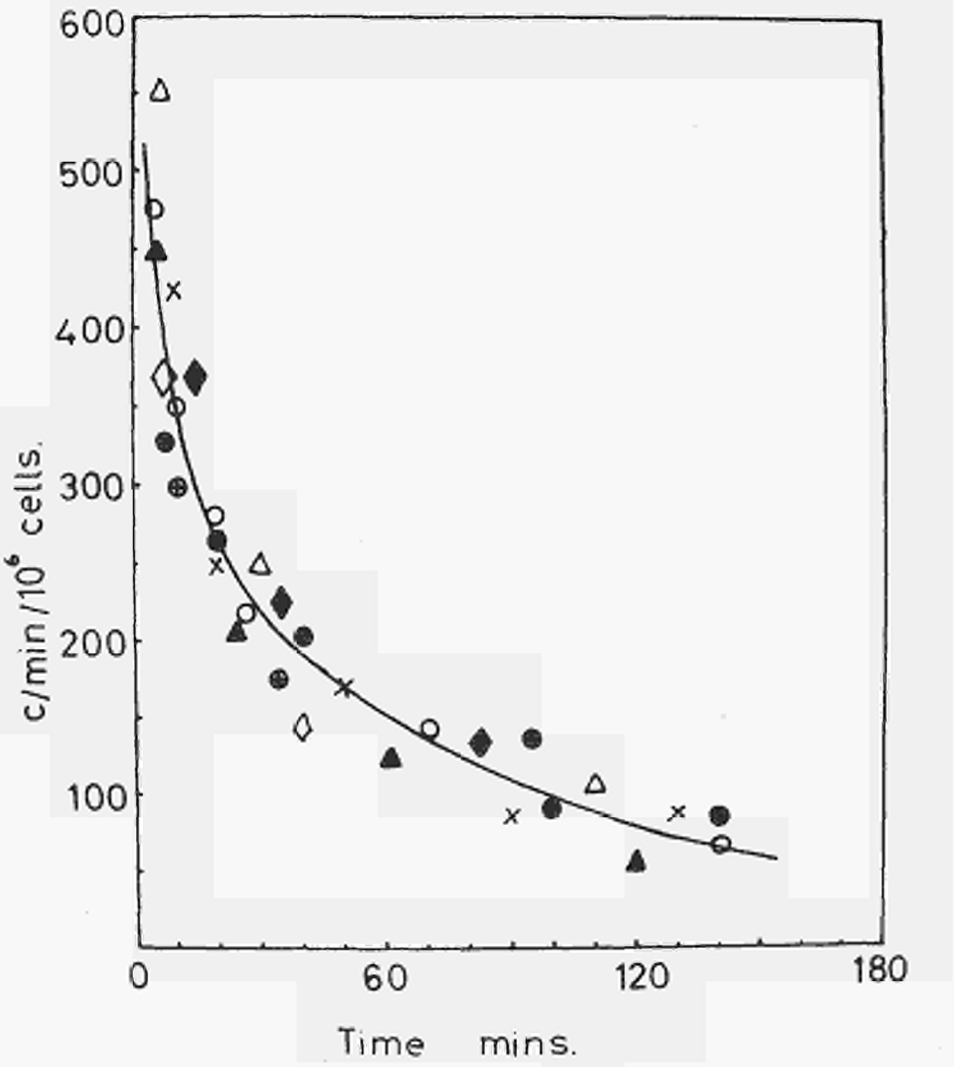

Fig. 3 The rate of disappearance of H^3-labelled rabbit lymphocytes from the circulating blood after reinjection into the same animal at zero time. Activity is scored as $c/m/10^6$ total leukocytes isolated as measured by liquid scintillation counting. Open symbols represent unirradiated lymphocytes. Closed symbols represent lymphocytes which had received 200 R X-rays approximately 20 min before reinjection. Each type of symbol represents an individual animal.

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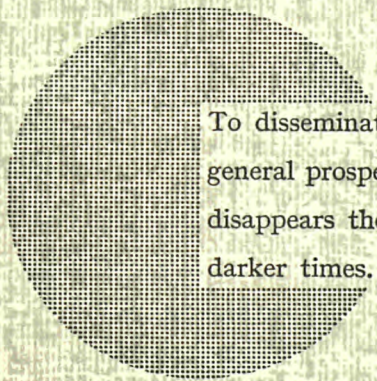

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Alfred Nobel

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