Effect of X-rays on Thymocytes and Its Modification by Cysteine. (19534)

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That many of the biological effects of X-irradiation are diminished by lack of oxygen or the addition of chemicals such as cysteine seems firmly established(1). The mechanism of the protection is poorly understood, however. It has been inferred from the radiochemistry of water that short-lived, reactive intermediates, primarily oxidants, form in the aqueous environment of the cell and of the intracellular elements. Anoxia may act by decreasing the formation of such intermediates and cysteine by competing for them. On the other hand, there is the possibility that these factors may also protect by altering the quality or quantity of the biological targets, *e.g.*, enzymes or genes(2). In the former instance, the biological effectiveness of the radiation would be decreased, while in the latter case, the radiosensitivity of the biological system would be diminished. There is, of course, no compelling reason to believe that the mode of action is identical in all living systems or indeed in a specific system under different conditions.

As part of a study of protective mechanisms, we are investigating the effects of cysteine, anoxia and related factors on irradiated suspensions of thymic cells. It was believed that this simple system would make possible a more precise evaluation of the protective phenomena. The suitability of thymic cells for this purpose was apparent from the work of Schrek(3). The thymic cell response to X-radiation and certain of the characteristics of its modification by cysteine will be described in the present report.

Methods. Inbred rabbits of either sex weighing about 1.5 kg were anesthetized with nembutal. The thymus was removed aseptically, dissected free of fat, and minced with scissors on a sterile watch glass. The mince was then placed on a monel metal screen (No. 80 mesh) in a Seitz filter and washed with 10 cc Ringer solution buffered at pH 7.4 with M/15 Na_2HPO_4 and NaH_2PO_4. The filtrate was centrifuged for 5 minutes at 600 g, the supernate discarded and the packed cells resuspended in a solution containing equal volumes of homologous serum and phosphate buffered Ringer solution. Similar cell concentrations were readily achieved from one experiment to another by making a 1:20 dilution by volume of the packed cells with the serum-Ringer solution. The stock suspension, consisting for the most part of small cells with large nuclei and scanty cytoplasm, was divided immediately into 2 cc aliquots placed in 15 cc conical test tubes. Three cc of the serum-Ringer diluent containing cysteine HCl neutralized with sodium hydroxide or the equivalent of sodium chloride were then added. Varying amounts of cysteine were used, the concentrations ranging from 3.2 to 19.2 mM. These samples contained approximately 100 mg of cells with a concentration of 40000 to 60000 cells per cu mm. About 75% of the cells were viable as determined by eosin staining. In some experiments, the serum-Ringer solution containing cysteine or sodium chloride was added after irradiation. Although the cell concentration during irradiation was more than doubled by this procedure, as will be shown later radiosensitivity was not affected. At least 4 aliquots were prepared in each experiment, 2 containing cysteine and 2 sodium chloride. Only one of each pair was exposed to the X-rays. All of the tubes were incubated with frequent shaking in a water bath at 37°C for varying periods of time prior to irradiation. The X-radiation factors were 200 kv, 15 ma, 0.25-mm Cu and 3-mm Bakelite filters, 32.5 cm target distance, 0.76-mm Cu half value layer and 100 r per minute dose rate. The total radiation dose ranged from 50 to 2000 r. In a few experiments the effects of irradiation at 100 and 500 r per minute were compared. After the exposure, two 0.2 cc aliquots were pipetted from each of the test tubes into 10 cc sterile culture tubes, tightly stoppered, placed at a 25° angle in a water bath at 37°C and shaken at a rate of 125 times per minute. Cell counts were

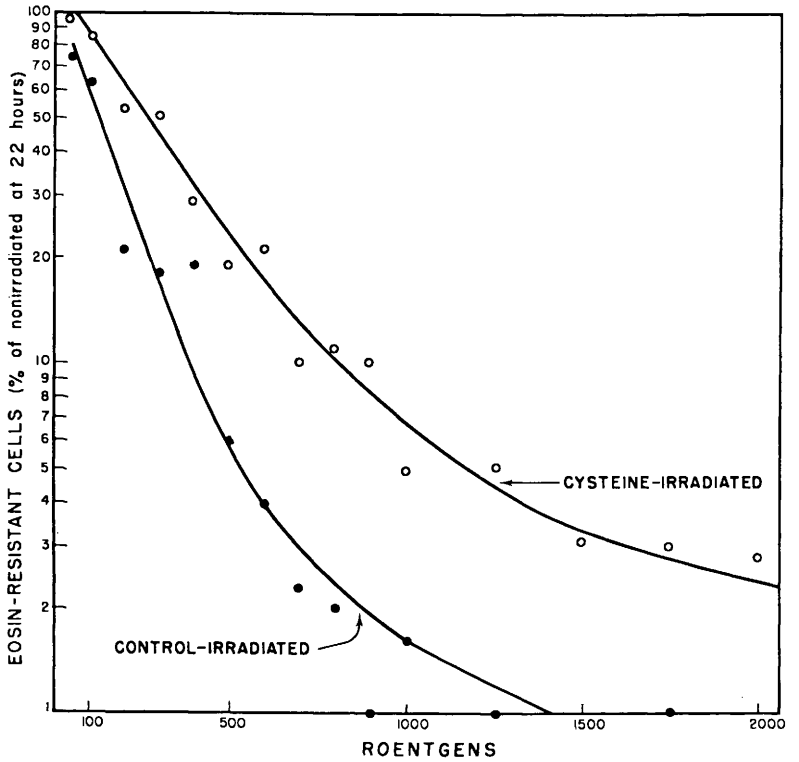

FIG. 1. Dose-response relationship for control and cysteine protected thymic cells. (Cysteine added to cell suspensions 15 min before X-irradiation, final conc. 19.2 mM).

generally made 22 hours after irradiation. Tyrode's solution (3.8 cc) containing 1% Eosin Y (National Aniline) was added to the 0.2 cc aliquot of the cell suspension. The mixture was shaken for 2 minutes, a drop was placed in a Spencer Bright Line hemacytometer and a count was made of the number of unstained cells in the central square of the chamber (0.1 cu mm) using a binocular microscope and a magnification of 270. Two hundred to 300 unstained cells, exclusive of erythrocytes, were counted. Occasional tests were made to rule out the presence of bacteria.

Unstained cells are assumed to be viable since they exhibit motility, protoplasmic streaming and pseudopod extension when cultured(4). On the other hand, quiescent cells, which resemble indistinctly granular spheres and manifest varying degrees of molecular vacuolization stain with eosin. Since thymic cells prepared in this manner almost never divide, population reduction due to cellular senescence is a constant factor, 50 ± 9% of the control cells staining with eosin at 22 hours. Cysteine in the concentrations employed did not alter the viability of the nonirradiated cells. The number of eosin-resistant cells in the irradiated suspensions was expressed as a percentage of the nonirradiated population for each thymic preparation at 22 hours. In a few experiments only the serum-Ringer medium was irradiated with and without cysteine and the thymic cells were added immediately afterward. In other studies cell preparations were centrifuged at 600 g for one minute immediately before irradiation. The packed cells with the supernate present were then exposed to X-rays, after which they were resuspended in the same fluid.

Results. X-radiation decreases the life span of rabbit thymocytes *in vitro,* the percentage of surviving cells (eosin resistant) decreasing exponentially with increasing radiation dosage. As may be seen in Fig. 1, the 22-hour LD_{50} is 125 r and definite effects appear with 50 r, a dosage which is similar to the threshold

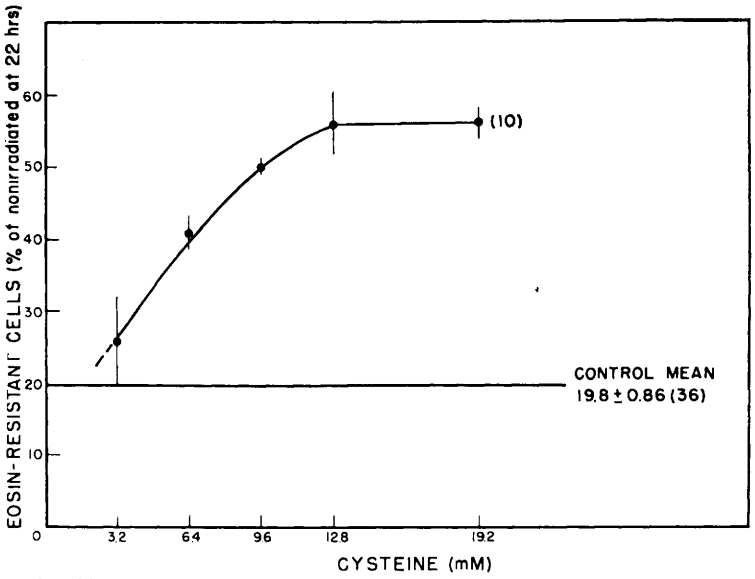

FIG. 2. Relationship between cysteine concentration and radiation effect (200 r). (Each value represents the mean of 3 experiments unless designated otherwise. Vertical lines refer to standard error of mean.)

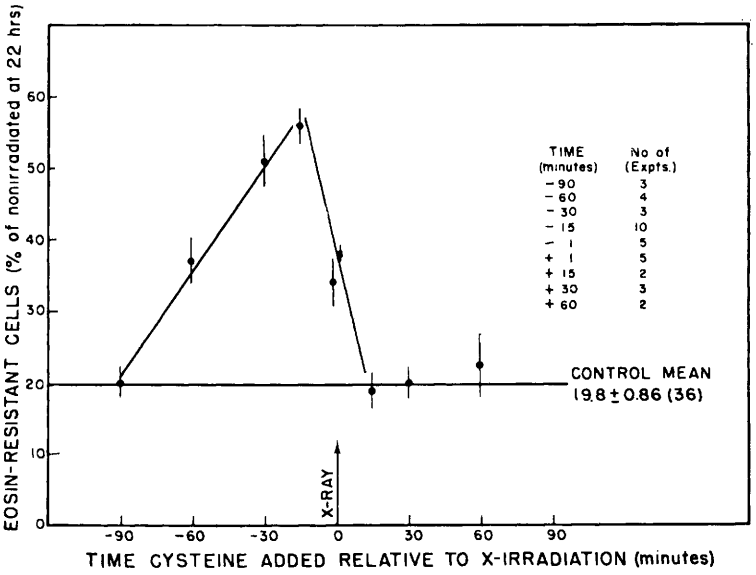

FIG. 3. Time course of the cysteine protection against 200 r. (Cysteine added at designated times to make final concentration 19.2 mM. Vertical lines refer to standard error of mean.)

for lymphopenia following whole body irradiation. Comparable results were reported previously(3). The thymic cell response is identical with dosage rates of 100 and 500 r per minute and cell concentrations of 40000 and 80000 per cu mm. Cysteine displaces the radiation dose-response curve by a constant percentage over a wide dosage range (Fig. 1). In effect, cysteine appears to cancel 0.53 r (σ 0.019 r) for each roentgen that is delivered to the cell suspension.

The degree of protection is dependent upon the concentration of cysteine as well as upon the time of its addition relative to the period

TABLE I. Cell Survival Following Irradiation of the Medium or of Packed Cells.

Group	Roentgens	No. of exp.	Eosin-resistant cells at 22 hr, % of nonirradiated
A. Medium irradiated			
Control	500	1	65
"	1000	1	58
Cysteine*	1000	1	95
Control	5000†	1	49
Cysteine	5000	1	97
B. Packed cells			
Air	200	3	39 ± 3.6‡
Air-cysteine	200	3	50 ± 3.9
Oxygen	200	4	24 ± 1.4
" -cysteine	200	2	50 ± 2.6

* Final conc. 19.2 mM.

† Dose rate 500 r/min; other exposures at 100 r/min.

‡ Estimated standard error of the mean.

of irradiation. The minimal effective concentration is approximately 6.4 mM, and maximal protection is noted with 12.8 and 19.2 mM, the latter being the maximally tolerated dose. Since our analyses showed that cysteine is equally distributed between cells and fluid, these concentrations correspond to doses ranging from 0.4 to 2.3 mg of cysteine per g of cells. The relationship between the cysteine dose and the radiation effect is presented in Fig. 2. The time course of cysteine protection is given in Fig. 3 where it will be seen that the survival of irradiated thymocytes is increased to the same degree whether cysteine is added one minute before or after exposure to 200 r. Addition 15 to 30 minutes before irradiation is optimal, however. Significant protection is also obtained when the amino acid is introduced into the suspension 60 minutes before the exposure, while its addition 90 minutes before or 15, 30, or 60 minutes after the exposure does not enhance cell survival.

Irradiated samples of the serum-Ringer solution are slightly toxic to thymic cells. Toxicity of the irradiated medium is prevented by the addition of cysteine. The factors responsible must persist for only a brief interval since the effect changes only slightly with increasing radiation dosage. Of interest is the finding that thymocytes packed by centrifugation are considerably less sensitive than cells suspended in serum-Ringer solution, even though the supernate is present during the exposure. The survival of such packed cells is not increased significantly by cysteine. On the other hand, cells equilibrated with oxygen before centrifugation appear to be as sensitive as cells in suspension and, in contrast to the results noted above, are readily protected (Table I).

A few nonirradiated and irradiated thymic cell preparations were incubated at 37°C on a warm stage microscope and studied cinematographically. Control cells and cells to which cysteine was added were essentially similar in their appearance and activity. Although there was no immediate difference between the irradiated controls and the cysteine treated cells, preparations of the latter contained many motile forms at 24 hours, while those of the former were predominantly quiescent. Such quiescent cells stained with eosin.

Discussion. A given amount of cysteine accounts for a rather constant percentage of the biological effect of irradiation over a wide dose range. This can be explained equally well by postulating an interaction with either the radiation or its toxic intermediates or with the biological system. A similar proportionality and degree of protection have been observed in mice for lymphopenia, granulocytopenia, splenic involution and lethality(5). The relatively uniform protection against a number of radiation sequelae points to a true dose reduction and suggests that cysteine alters a common pathway. The report(6) that glutathione pretreatment does not affect splenic and thymic involution following X-irradiation is not at variance with these findings since the anticipated difference in organ weights between 800 and 600 r, which represents the degree of protection by glutathione from survival data(7), is only about 5%(8).

Although there is a clear relationship between cysteine dosage and its effectiveness at a given time, protection of thymic cells *in vitro* is not a simple function of the sulfhydryl level during exposure. Equivalent sulfhydryl concentrations are found, within 15 minutes, in cells and ambient fluid; the levels decline at a constant rate (30% in 90 minutes). Addi-

tion of 9.6 mM of cysteine 15 minutes before irradiation confers definite protection, while the addition of twice this amount 90 minutes before is not effective, although the sulfhydryl concentrations at the time of exposure are comparable. This difference is probably not a result of the decreased ratio of reduced to oxidized sulfhydryl with time. It may be a consequence of exhaustion of essential precursors, secondary reactions, or redistribution of cysteine within the cell. Under these conditions, the relative effectiveness with time of administration (Fig. 3) might depend upon cysteine dosage. An optimal reaction time is also indicated since protection decreases as the irradiation period is approached. The postirradiation effect, as well as that of pretreatment, may be a result of the reversal of a chain of radiation action, reconstitution or replacement of an injured target, or substitution of an alternative metabolic pathway. Persistence of toxic substances in the medium can account for only part of the postirradiation effect.

It is well to recall that the time course of protection against acute radiation lethality follows a somewhat different pattern in the intact animal(9). The optimal time of administration in rats and mice is immediately before irradiation; there is no after effect. The apparent discrepancy may be a consequence of differences in the kinetics of reactions with cysteine and in the time constants for development of irreversible injury. The thymic cell suspension is a relatively sluggish, non-dividing system, in which oxygen tension is rate limiting for the disappearance of cysteine, presumably by oxidation(10). On the other hand, the tissues that appear to be causally related to lethality of the animal are those with the most rapid cell turnover.

The apparently anomalous finding that packed cells are relatively radio-resistant and not affected significantly by cysteine may be attributed to their hypoxic state. Results of other experiments now in progress are consistent with these observations and indicate that removal of oxygen from the thymic cell suspension is as effective as cysteine but that the two are not additive. Although cysteine has been shown to increase the survival of aerobically-grown *E. coli* irradiated in an oxygen-free phosphate buffer, anaerobically-grown bacteria resemble thymic cells in the lack of additivity(11,12). These considerations suggest perhaps that cysteine may act by diminishing availability of oxygen in the biological system. If this is true, less complete oxygen deprivation and minimal amounts of the amino acid may be additive in their effects on thymic cells. This does not imply, however, that oxygen diminution *per se* is the decisive event in the protection of thymic cells by cysteine. There is, in fact, reason to believe that other events must ensue, since protection appears to be dependent upon temperature during the early postirradiation period.

Summary. Sensitivity of rabbit thymocytes to X-irradiation *in vitro* is decreased by a constant factor over a wide dosage range when cysteine is added to the suspension. Protection depends upon cysteine concentration and time of administration but is not a simple function of the sulfhydryl level. There is a definite postirradiation effect of cysteine which can be accounted for only in part by the persistence of toxic substances in the medium. The relative resistance of packed cells and the failure of cysteine to protect them may be a consequence of hypoxia. It is suggested that the effect of cysteine on irradiated thymic cell suspensions may be a result of competition with cell substrates for oxygen, but that the binding of oxygen *per se* is not necessarily the decisive event in the protection. The results are discussed in connection with related observations in the intact animal.

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Effect on Joint Permeability of Adrenal Cortex Extract,* ACTH, Cortisone Hydroxy-phenyl Cinchoninic Acid and Hyaluronidase. (19535)

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Recent experimental and clinical studies have indicated a functional relationship between the mesenchymal tissues, the anterior pituitary gland and the adrenal cortex. The sequence of events in this relationship indicates that under conditions of stress, augmented adrenocorticotropic hormone (ACTH) production stimulates the adrenal cortex to release corticoid hormones. The corticoid hormones influence, in some way, the integrity of the connective tissues or ground substance. However, the changes in function and structure of the mesenchymal tissues which occur under the influence of these hormones are still not completely understood. It has been postulated that the mechanism of action of ACTH and cortisone is related in part to inhibition of the enzyme hyaluronidase and to a resulting decrease of membrane permeability(1-4). Furthermore, it has been hypothesized that the above mentioned hormones may exert their beneficial effects in the collagen diseases in part at least through this action on permeability(5,6).

In an attempt to determine whether joint permeability of rabbits is altered by injection of these hormones and related substances, a method was used which is similar to that reported by Seifter *et al.*(4). It consists of injecting phenolsulfonephthalein solution (PSP) into the knee joint of a rabbit under nembutal anesthesia and determining the rate of its excretion in the urine. The following procedure was carried out:

The bladder of the anesthetized rabbit was washed with fresh 10 cc portions of tap water until the sample withdrawn was clear. After waiting 10 minutes the bladder was washed again; a 10 cc portion of water was then introduced and withdrawn from the bladder to be used as the blank specimen in the photocolorimetric determination of PSP. Twenty-five hundredths cc (1500 μg) of PSP was then injected into the knee joint of the rabbit. Care was taken to ensure that the PSP was injected into the joint cavity and that hemorrhage and undue trauma were not produced. After 5 minutes the bladder was washed and a sample taken in the described manner. Similar samples were obtained at 10-minute intervals throughout the remainder of the experiment, which lasted 135 minutes. The time of appearance of PSP in the urine was noted, and the concentration of dye measured in each sample, using a Lumetron photoelectric colorimeter. After the above control studies were performed separate series

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