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Original Contributions

EFFECT OF PURINE NUCLEOSIDES AND NUCLEOTIDES ON THE *IN VIVO* RADIATION RESPONSE OF NORMAL TISSUE IN THE RAT

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WAG/Rij rats were exposed to single doses of radiation following intraperitoneal injection of inosine, pyruvate and inorganic phosphate (IPP). When skin reactions and structural damage were scored in the hind legs of irradiated rats, IPP was observed to decrease radiation sensitivity. Radioprotection was also observed in rats that were pretreated with inosine alone and with the purine nucleosides adenosine and guanosine. The purine nucleotides 5′-inosine monophosphate (IMP), 5′-adenosine monophosphate (AMP), 5′-guanosine monophosphate (GMP) and cyclic adenosine 3′,5′-monophosphate (cyclic AMP) were also protective. The purine bases hypoxanthine, adenine and guanine were not protective. A decline in blood pressure was observed following administration of either IPP or inosine alone but not following AMP or cyclic AMP. The mechanism(s) whereby the purine nucleosides and nucleotides result in radioprotection are currently being investigated.

Radioprotection, Purine nucleosides and nucleotides.

INTRODUCTION

2,3-Diphosphoglycerate (DPG) is a red cell metabolite that lowers the affinity of hemoglobin for oxygen[1,3] and thereby serves a physiological role in the regulation of oxygen delivery to tissues. Compensatory elevations in DPG have been observed in the adaptation to various conditions associated with lowered arterial oxygen tension[11,13] and result in increased tissue oxygenation. Hypoxic foci within tumors are known to limit the effectiveness of low linear-energy-transfer ionizing radiation, and extensive efforts have been directed toward the development of methods that increase the radiosensitivity of hypoxic tumor cells. The present studies were undertaken to investigate the possibility of increasing tumor oxygenation and thereby enhancing radiosensitivity in the rat through elevation of red cell DPG.

Treatment of stored blood with the combination of inosine, pyruvate and inorganic phosphate (IPP) has been shown to raise red cell DPG.[14] *In vivo* studies in Rhesus monkeys[19] and in rabbits[7] have also demonstrated elevation in red cell DPG following infusion of IPP. The administration of inosine and phosphate has been shown to elevate DPG in human subjects.[4] When IPP is given to the WAG/Rij rat, DPG is maximally elevated four hours following drug administration.[20]

We have investigated the effect of IPP on normal tissue tolerance and tumor control in irradiated WAG/Rij rats in order to determine whether elevations in red cell DPG increase the therapeutic effectiveness of irradiation. Tumor control data in animals bearing the BA1112 rhabdomyosarcoma show no enhancement of radiosensitivity by IPP and are described elsewhere.[20] The unexpected observation that IPP protects normal tissue against radiation damage prompted an investigation of the effect of various purine nucleosides and nucleotides on the radiation response of normal tissue in the WAG/Rij rat and constitutes the basis for this report.

METHODS AND MATERIALS

Six to eight-week-old male and female WAG/Rij rats were anesthetized with intraperitoneal sodium pentobarbital (50 mg/kg in females and 70 mg/kg in males) approximately 30 minutes prior to irradiation. The left hind legs of anesthetized animals were treated with single fractions of 250 KVp X rays with a half value layer of 0.55 mm copper. Further details of the radiation procedure are described elsewhere.[12] Skin reactions and structural damage were scored on arbitrary scales (Tables 1 and 2) adapted from the work of Field and Thomlinson.[6] The skin reactions were recorded twice a week for the first 50 days post-irradiation and once a week from 50 to 100 days post-irradiation. Structural damage was scored by two observers at 50 and 100 days. Statistical compari-

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Table 1. Stages of skin reaction

Score	Skin reaction
0.5	No apparent difference from normal
0.75	Slight reddening, scaling or crusting
1.00	Definite reddening, scaling or crusting
1.25	Severe reddening, scaling or crusting
1.50	Severe reddening and scaling and possible breakdown
1.75	Small areas of breakdown
2.00	10–25% breakdown
2.25	25–50% breakdown
2.50	50–75% breakdown
2.75	75 90% breakdown
3.00	More than 90% breakdown

Table 2. Stages of structural damage

Score	Structural damage
0.5	No apparent difference from normal
1.00	Slight damage to toes
1.50	Definite damage and/or slight fusion of toes
1.75	Definite fusion of some toes
2.00	Most toes fused, but general shape unchanged
2.50	Foot almost shapeless, only stubs of toes present
2.75	Foot shapeless, no toes
3.00	Only stub of foot remaining

sons of the scores among different groups of rats were made using pairwise Mann-Whitney U tests.[18] Rats were given the purine nucleoside inosine, pyruvate and bisodium phosphate (IPP) in a 0.1 M concentration. The purine bases hypoxanthine, adenine and guanine, the purine nucleosides adenosine and guanosine, and the purine nucleotides 5′-inosine monophosphate (IMP), 5′-adenosine monophosphate (AMP), cyclic adenosine 3′,5′-monophosphate (cyclic AMP) and 5′-guanosine monophosphate (GMP) were also prepared in 0.1 M concentration. All solutions were administered intraperitoneally in a volume of 0.02 ml/gm of body weight to yield a final concentration in the animal of 2 mM.* Rat tail vein pressures were measured with an electrosphygmomanometer.†

RESULTS

Figure 1 shows mean skin reactions observed on the hind legs of WAG/Rij rats as a function of time following a single dose of radiation in animals given saline, IPP immediately prior to irradiation (IPP-0) and IPP four hours prior to irradiation (IPP-4). At day 25 following 2250 rad, scores of 1.8 ± .1 (breakdown of skin), 0.5 + 0 (no effect) and 1.3 + .3 (severe reddening and scaling) were observed for saline, IPP-0 and IPP-4 groups of rats, respectively. The observed differences between the IPP-0 and saline groups and between the IPP-0 and IPP-4

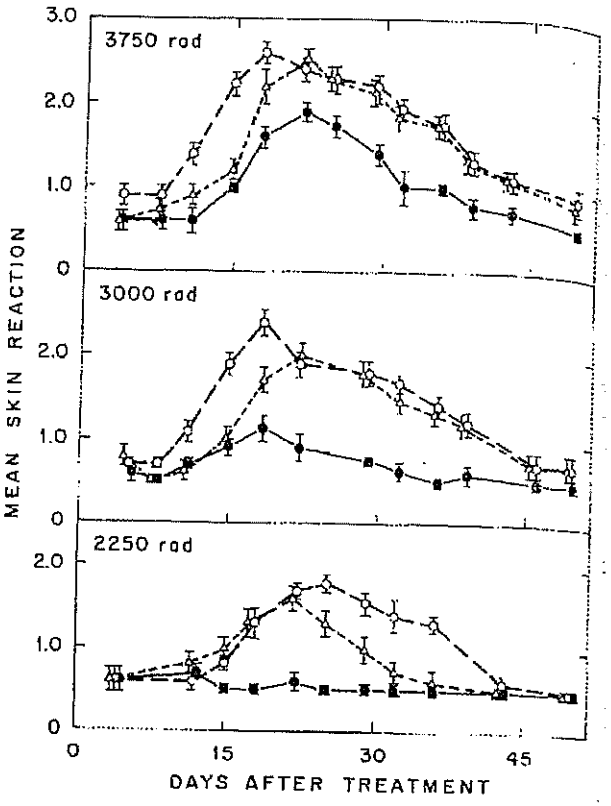

Fig. 1. Mean skin reactions following single-dose radiation to the hind legs of saline-injected WAG/Rij rats (○) and rats injected with IPP immediately prior (●) and four hours prior (Δ) to irradiation. Four animals were treated in each group at the doses shown. Skin reactions were scored on an arbitrary scale, and each point represents mean ± 1 S.E.

groups were statistically significant (p = .01). Similarly, following a single dose of 3000 rad, rats administered IPP immediately prior to irradiation showed less skin reaction at 25 days (p = .02) than rats that were injected with either saline or IPP administered four hours prior to irradiation. While skin reaction was lowest in the IPP-0 group, the differences were not statistically significant at 3750 rad.

Table 3 records the structural damage observed following single doses of radiation in saline, IPP-0 and IPP-4 groups of rats. Within each group, comparable levels of damage were noted at both 50 and 100 days. IPP protected against structural damage following a single dose of 2250 rad whether administered four hours or immediately prior to radiation. At 100 days following irradiation, scores of 0.5 ± 0 (no change) were observed in both groups of IPP-treated rats, compared to 1.2 ± .3 (toe damage) in the saline-injected animals. The difference was statistically significant (p = .01). At 100 days following a dose of 3000 rad, no change was seen in the

*The purine bases, nucleosides and nucleotides were obtained from Sigma Chemical Company, St. Louis, Missouri.

†NARCO Biosystem Programmed Electrosphygmomanometer.

Table 3. Average structural damage following irradiation of rat hind legs*

Dose (rad)	Saline		IPP-0		IPP-4	
	50 d	100 d	50 d	100 d	50 d	100 d
2,250	0.9 ± .2†	1.2 ± .3	0.5 ± 0	0.5 ± 0	0.5 ± 0	0.5 ± 0
3,000	1.8 ± .1	1.8 ± .2	0.5 ± 0	0.5 ± 0	1.4 ± .2	1.3 ± .2
3,750	1.9 ± .1	2.0 ± .2	1.1 ± .3	1.2 ± .3	1.8 ± .1	1.9 ± .2

*Four animals irradiated in each group; damage scored according to arbitrary scale after 50 and 100 days; rats injected either with saline, IPP immediately prior to irradiation (IPP-0) or IPP 4 hours prior to irradiation (IPP-4).
†Mean ± 1 S.E.

IPP-0 rats, compared to marked damage (definite fusion of the toes) in saline-injected animals; the scores were statistically significantly different ($p = .01$). At this dose level, while less damage was observed in the IPP-4 rats compared to the saline group, the differences were not significant. At a dose of 3750 rad, there was no difference between the IPP-4 and the saline groups, but again the IPP-0 rats showed significantly less structural damage ($p = .02$).

To investigate further the protective effect of IPP against radiation-induced skin reactions, IPP administration and irradiation were carried out at varying intervals. When IPP was administered either 15 minutes or immediately prior to a single dose of 2250 rad, minimal or no skin changes were noted, compared to marked levels of skin reaction in saline-injected rats ($p = .01$ at day 25). Skin reactions in rats injected with IPP 30 minutes prior to irradiation were also significantly less than those observed in the saline group ($p = .02$). Protection was maximal when IPP was administered at closer intervals prior to irradiation. When IPP was administered immediately following irradiation, skin reaction was also considerably less than that observed in saline-injected rats ($p = .02$).

Skin reactions were observed in rats following irradiation with a single dose of 2250 rad when inosine, pyruvate and phosphate were administered individually immediately prior to irradiation. Animals injected with pyruvate or phosphate underwent radiation-induced skin changes that did not differ from those of saline-injected controls. Skin reactions were significantly reduced in rats treated with inosine alone ($p = .02$ at day 25).

Inosine is a purine nucleoside formed from the purine base hypoxanthine. Figure 2 depicts mean skin reactions as a function of time following 2250 rad in a single dose when saline, hypoxanthine, inosine and the purine

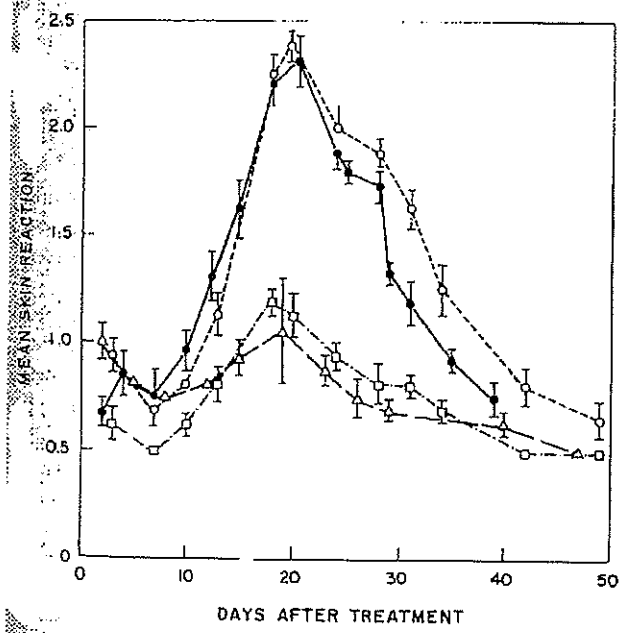

Fig. 2. Mean skin reactions following 2250 rad in a single dose to the hind legs of saline-injected WAG/Rij rats (●) and rats injected immediately prior to irradiation with hypoxanthine (○), inosine (△) and IMP (□). Four animals were treated in each group, and skin reactions were scored on an arbitrary scale. Each point represents mean ± 1 S.E.

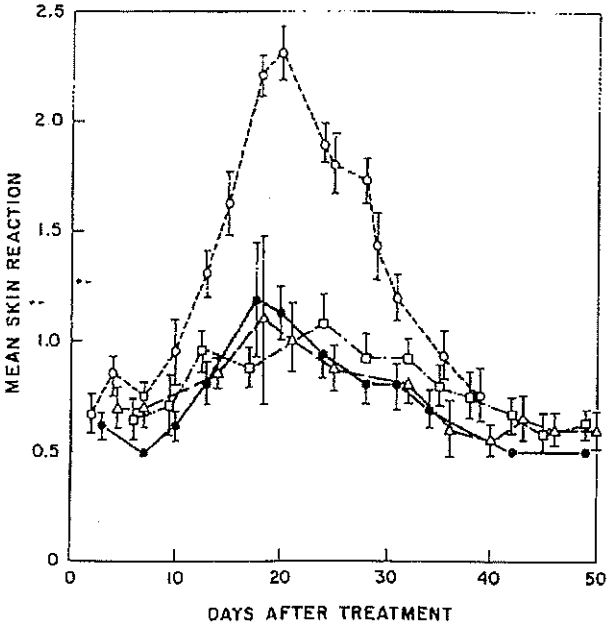

Fig. 3. Mean skin reactions following 2250 rad in a single dose to the hind legs of WAG/Rij rats treated with saline (○), AMP (●), GMP (△) and cyclic AMP (□) prior to irradiation. Four animals were treated in each group, and skin reactions were scored on an arbitrary scale. Each point represents mean ± 1 S.E.

Table 4. Blood pressure measurements from the tail veins of rats following administration of purine nucleosides and nucleotides*

Drug	Control†	IPP	Inosine	Cyclic AMP	Hypoxanthine
No. Animals	7	8	6	7	6
Blood pressure (mm Hg)					
Pre-drug	99 ± 4‡	98 ± 4	100 ± 2	103 ± 3	98 ± 3
1 min post drug	115 ± 6	106 ± 9	81 ± 11	98 ± 8	122 ± 10
5 min post drug	113 ± 6	53 ± 10	60 ± 18	93 ± 16	121 ± 7
10 min post drug	113 ± 7	43 ± 7	60 ± 18	100 ± 15	111 ± 14
15 min post drug	115 ± 7	52 ± 11	89 ± 15	103 ± 16	114 ± 16

*All drugs administered intraperitoneally in 0.1 M concentration at .02 ml/gm body weight.
†Controls injected with either bacteriostatic water or pyruvate plus Na_2HPO_4.
‡Mean ± 1 S.E.

nucleotide IMP were administered to rats immediately prior to irradiation. Radiation effects were equivalent in the saline-injected controls and the hypoxanthine-treated animals. Inosine and IMP were both protective, resulting in lowered mean skin reactions ($p = .01$) than were observed in the saline and hypoxanthine groups. In addition to hypoxanthine, we tested the purine bases adenine and guanine. Neither modified skin reaction when administered to the WAG/Rij rat prior to a single dose of 2250 rad.

Mean skin reactions were observed following 2250 rad when various purine nucleosides were administered to the rat prior to irradiation. Animals that were given adenosine and guanosine demonstrated a reduced skin reaction when compared to saline-injected controls ($p = .02$ at day 25). However, the protective effect did not appear as marked as that observed following the administration of inosine. The purine nucleotides AMP and GMP showed a protective effect comparable to that of IMP. Cyclic AMP was also protective in this system (Figure 3).

Blood pressure measurements were obtained from rat tail veins following drug administration to investigate the possibility that a physiological mechanism such as hypoxia could explain the radioresistance observed following administration of purine nucleosides and nucleotides (Table 4). A rapid and sustained fall in blood pressure was observed in IPP-treated rats. Inosine lowered the blood pressure in most animals (4 of 6). Neither cyclic AMP nor hypoxanthine caused a blood pressure decline. The effect of AMP was studied in two rats, and no change in blood pressure was observed.

DISCUSSION

The present studies were undertaken with the expectation that increased red cell DPG would provide a method for oxygenating previously hypoxic cells and thereby increase tumor radiation sensitivity. On the contrary, we have observed that the combination of inosine, pyruvate and inorganic phosphate, which elevates DPG, exerts a protective effect against radiation-induced skin reactions and structural damage in the WAG/Rij rat. The protective effect of IPP is maximal when the drugs are administered immediately prior to radiation. However, structural damage is significantly reduced in IPP-treated rats when the drugs are administered four hours prior to irradiation, at which time DPG elevations are maximal. Skin damage is reduced when the drugs are administered immediately following irradiation. Inosine alone has a protective effect comparable to that of the IPP combination. The purine nucleosides adenosine and guanosine are protective to a lesser degree. The purine bases do not protect at all, and the purine nucleotides AMP, GMP, IMP and cyclic AMP are nearly fully protective.

The observed decline in blood pressure following administration of either IPP or inosine alone suggests that the radioprotective action of these agents may be explained by a physiological mechanism whereby hypoxia is induced. However, there is a small but measurable protective effect of IPP administered in the post-irradiation period, at which time alteration in tissue oxygenation would not be expected to modify radiation damage. This suggests that some component of the protective effect of IPP is independent of hypoxia. Clearly, another mechanism must be invoked to account for the radioprotective effect of the nucleotides cyclic AMP and AMP, neither of which was observed to lower blood pressure.

Previous studies have shown that agents which elevate intracellular cyclic AMP either by stimulation of adenyl cyclase or by inhibition of cyclic AMP phosphodiesterase can modify the radiation response of numerous cultured mammalian cell lines including human kidney T cells,[17] HeLa cells,[15] Chinese hamster ovarian cells,[16] mouse ascites tumor cells[2] and V-79 cells.[9] A direct radioprotective effect of cyclic AMP *in vitro* has not been observed.[9] *In vivo* studies have shown increased radioresistance following both elevation of intracellular cyclic AMP and administration of exogenous cyclic AMP. The mean lethal dose for mice that were given whole body irradiation increases from 1150 to 1500 rad when animals were pretreated with diethylaminoreserpine, an inhibitor of cyclic AMP phosphodiesterase.[10] Numerous agents that act to increase intracellular concentration of cyclic AMP have also been shown to increase the survival of clonogenic cells of the epithelium of mouse small intestine following irradiation.[10] Increased survival following

whole body irradiation has been demonstrated for mice pretreated with a combination of cyclic AMP and ATP and with AMP administered singly.[8] Dibutyryl cyclic AMP administered to the mouse prior to irradiation has been shown to increase the survival of hair follicles and intestinal stem cells.[5]

The present studies extend the list of radioprotectors to include not only AMP and cyclic AMP but also the purine nucleosides and the nucleotides of bases other than adenine. The mechanism whereby these compounds exert a radioprotective effect is now under study. *In vitro*[9] and *in vivo*[10] investigations of intracellular cyclic AMP have suggested that protection is related to enhancement of the size of the shoulder on the cell survival curve with increased capacity of cells to accumulate sublethal damage. *In vitro* studies also suggest that intracellular cyclic AMP increases the slope of the exponential region of the cell survival curve, possibly on the basis of failure to repair potentially lethal damage.[9] The radioprotective action of intracellular cyclic AMP may differ from that of exogenous cyclic AMP, which may be metabolized extracellularly to base analogues that mediate its effect. It is to be hoped that continued study of the various base analogues shown to be radioprotective in the present investigations will enhance understanding of the mechanism of radiation injury.

REFERENCES

1. Benesch, R., Benesch, R.E.: The effect of organic phosphates from the human erythrocyte on the allosteric properties of hemoglobin. *Biochem. Biophys. Res. Commun.* **26:** 162–167, 1967.
2. Boynton, A.L., Evans, T.C.: Investigation of effects of cyclic AMP and related drugs on recovery of radiation induced mitotic delay in mouse ascites tumor cells (Abst.). *Radiat. Res.* **55:** 595–596, 1973.
3. Chanutin, A., Curnish, R.R.: Effect of organic and inorganic phosphates on the oxygen equilibrium of human erythrocytes. *Arch. Biochem. Biophys.* **121:** 96–102, 1967.
4. deVerdier, C.H., Westman, M.: Intravenous infusion of inosine in man: effect on erythrocyte 2,3-diphosphoglycerate concentration and on blood oxygen affinity. *Scand. J. Clin. Lab. Invest.* **32:** 205–210, 1973.
5. Dubrazsky, N.B., Hunter, N., Mason, K., Withers, H.R.: Dibutyryl cyclic adenosine monophosphate: effect on radiosensitivity of tumors and normal tissues in mice. *Radiology* **126:** 799–802, 1978.
6. Field, S.B., Thomlinson, R.H.: The relative effects of fast neutrons and x-rays on tumor and normal tissue in the rat. *Brit. J. Radiol.* **40:** 834–843, 1967.
7. Fry, J.L., Proctor, H.J.: Rapid increase in red cell 2,3-diphosphoglycerate in hypoxic rabbits following infusion of inosine, pyruvate and inorganic phosphate. *Surg. Forum* **23:** 220–221, 1972.
8. Langendorff, H., Langendorff, M.: Chemical radiation protection and the cyclic AMP mechanism. *Int. J. Radiat. Biol.* **19:** 493–495, 1971.
9. Lehnert, S.: Modification of post irradiation survival of mammalian cells by intracellular cyclic AMP. *Radiat. Res.* **62:** 107–116, 1975.
10. Lehnert, S.: Radioprotection of mouse intestine by inhibitors of cyclic AMP phosphodiesterase. *Int. J. Radiat. Oncol. Biol. Phys.* **5:** 825–833, 1979.
11. Lenfant, C., Torrance, J., English, E., Finch, C.A., Reynafarje, C., Ramos, J., Faura, J.: Effect of altitude on oxygen binding by hemoglobin and on organic phosphate levels. *J. Clin. Invest.* **47:** 2652–2656, 1968.
12. Moulder, J.E., Fischer, J.J., Milardo, R.: Time-dose relationships for the cure of an experimental rat tumor with fractionated radiation. *Int. J. Radiat. Oncol. Biol. Phys.* **1:** 431–438, 1975.
13. Oski, F.A., Gottlieb, A.J., Delivoria-Papadopoulos, M., Miller, W.W.: Red cell 2,3-diphosphyglycerate levels in subjects with chronic hypoxemia. *New Eng. J. Med.* **280:** 1165–1166, 1969.
14. Oski, F.A., Travis, S.F., Miller, L.D., Delivoria-Papadopoulos, M., Cannon, E.: The *in vitro* restoration of red cell 2,3-diphosphoglycerate levels in banked blood. *Blood* **37:** 52–58, 1971.
15. Prasad, K.N.: Cyclic AMP and the radiosensitivity of HeLa cells in culture. *Radiat. Res.* **51:** 520–521, 1972.
16. Prasad, K.N.: Radioprotective effect of prostaglandin and an inhibitor of cyclic nucleotide phosphodiesterase on mammalian cells in culture. *Int. J. Radiat. Biol.* **22:** 187–189, 1973.
17. Scaife, J.F.: Cyclic 3',5'-adenosine monophosphate: its possible role in mammalian cell mitosis and radiation-induced mitotic G2 delay. *Int. J. Radiat. Biol.* **19:** 191–195, 1971.
18. Siegal, S.: *Nonparametric Statistics.* New York, McGraw-Hill, 1956, pp. 116–126.
19. Sugerman, H.J., Pollock, T.W., Rosato, E.F., Delivoria-Papadopoulos, M., Miller, L.D., Oski, F.A.: Experimentally induced alterations in affinity of hemoglobin for oxygen. II. *In vivo* effect of inosine, pyruvate and phosphate on oxygen hemoglobin affinity. *Blood* **39:** 525–529, 1972.
20. Weissberg, J.B., Fischer, J.J.: Effect of red cell 2,3-diphosphoglycerate elevation on tumor control in irradiated rats. In preparation.