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Acute γ-Irradiation-Induced Mortality in Chickens

I. Mechanism of Resistance Afforded by Pretreatment with Cyclophosphamide or Sublethal Irradiation[1] (39775)

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Generalized vascular collapse is responsible for the acute radiation death seen in chickens within 24 hr after whole body γ-irradiation (1). This syndrome appears rapidly after irradiation and includes an increase in vascular permeability, plasma leakage, hemoconcentration, and degenerative changes in the endothelium (2). This drastic increase in vascular permeability may represent an exaggerated form of similar permeability changes seen after irradiation in rabbits, rodents, and dogs (3, 4). With the use of vital dyes in irradiated rabbit skin, Jolles and Harrison (5) showed that this leakage is abolished by pretreating the animals with alkylating agents, irradiation, or a protease inhibitor such as soy bean trypsin inhibitor (SBTI). This latter inhibitor also prevents early irradiation mortality in chickens (6-8), as do a number of other proteolytic enzyme inhibitors (Palladino, M. A., Troll, W., and Thorbecke, G. J., unpublished observations), which suggests that the vascular collapse is mediated by release of one or more proteolytic enzymes within hours after irradiation. This phenomenon is worthy of study, even if a similar acute mortality is only rarely induced in man (9), since less dramatic increases in vascular permeability, particularly in the brain, may well contribute to radiation "sickness" in man.

Alkylating agents are known to decrease the peripheral blood level of both polymorphonuclear leukocytes and lymphocytes. Thus the increased resistance caused by these agents might be due to the leukopenia of these animals. The observation that chickens "bursectomized" by cyclophosphamide treatment have an increased ability to withstand irradiation led us to examine the possibility that enzyme release from bursa cells might mediate the irradiation effects in normal chickens. Thus in the present studies the effect on subsequent irradiation sensitivity of another method of bursectomy, testosterone treatment of the embryo, was investigated. In addition, studies were performed to determine whether reconstitution of cyclophosphamide-treated chickens with various hematopoietic cells would reestablish their sensitivity to irradiation.

Materials and methods. FP (B15B21) and SC (B2B2) fertile eggs were obtained from Hy-Line Poultry Farms (Johnston, Iowa). East Lansing line 6 subline 3 (EL 6) and line 7 subline 2 (EL 7) were supplied as day-old chicks by the Regional Poultry Research Laboratory, U.S. Department of Agriculture, East Lansing, Mich. Whole body γ-irradiation was administered with a ^{137}Cs Gammator M (Radiation Machinery Corp., Parsippany, N.J.) at a dose rate of 630 R/min. Chickens were generally 7-10 days old at the time of irradiation. Chemical bursectomy was performed either by *in ovo* injection of 3.8 mg of testosterone propionate (Elkins-Sinn, Cherry Hill, N.J.) on the 11th day of incubation or by injection of 3 mg of cyclophosphamide (Cytoxan, Mead Johnson, Evansville, Ind.) ip daily for 3 days after hatching (10).

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Lymphoid cells for reconstitution, obtained from 6-week-old, histocompatible donors, were suspended in 0.165 *M* Dulbecco's phosphate-buffered saline (pH 7.2) containing 0.2% bovine serum albumin (Miles Laboratories, Research Division, Elkhart, Ind.) and injected iv 1 to 4 days after the last cyclophosphamide injection. Toxicity of iv-injected bursa cells was prevented by addition of heparin (200 U/ml).

SBTI (Worthington Biochemical Corp., Freehold, N.J.) was injected iv 30 min prior to whole body γ-irradiation. In some animals, Evans blue in a dose of 5 mg/20 g body weight was injected iv 2 to 3 hr after irradiation; chicks were sacrificed 15 min later for observation of internal organs and for photography.

Neomycin (mycifradin sulfate, Upjohn, Kalamazoo, Mich.) at a dose of 0.4 to 1 mg, was injected im daily for 6 days prior to irradiation. *E. coli* endotoxin 0 111:B4 (Difco Labs., Detroit, Mich.) was given as a single 100-μg dose iv 24 hr before irradiation. Trypsin (Worthington Biochemical Corp., Freehold, N.J.; 185 U/mg) was injected iv at a dose of 6 mg/kg body weight divided over two iv injections given 24 and 22 hr prior to irradiation. Vitamin A (Aquasol A, U.S.V. Pharmaceutical Corp., Tuckahoe, N.Y.), in two 4-mg injections, and cortisone (Cortone acetate, Merck Sharp and Dohme, West Point, Pa.), in two 7.5-mg injections, were given ip 24 and 4 hr before irradiation.

Results. Comparison of acute irradiation mortality indexes after different methods of bursectomy. In preliminary experiments, 95% of SC chickens (59 out of 62) died within 24 hr (the majority even within 6 hr) after 820-R total-body γ-irradiation (Table I). Pretreatment with SBTI 0.5 hr before irradiation protected nearly all the animals (only 4% mortality or 1 of 27 birds; data not shown). The enzyme inhibitor still protected approximately 40% (or 8/20) of the animals when given 24 to 72 hr before irradiation; this is in agreement with observations of Stearner and Azuma (6).

The protective effects of the two methods of chemical bursectomy were compared. Following cyclophosphamide treatment during the first 3 days of life, 820-R γ-irradiation given 4 to 7 days after the last cyclophosphamide injection induced very low mortality (13%, Table I), whereas the mortality in birds of the same age bursectomized *in ovo* by testosterone treatment was similar to that in untreated birds (89%, Table I). At an irradiation dosage of 700 R, which gave 65% mortality in normal chicks (cf. 0% mortality after 500 R, Table I), cyclophosphamide clearly protected all animals (0 vs 65%, Table I), whereas testosterone-pretreated birds were only partially protected (30 vs 65%, $P < 0.02$, Table I).

TABLE I. COMPARISON BETWEEN TWO METHODS OF BURSECTOMY WITH RESPECT TO THEIR PROTECTIVE EFFECT AGAINST ACUTE[a] IRRADIATION MORTALITY.

γ-Irradiation dose (R)	Method of bursectomy	Dead/total	Mortality (%)
500		0/20	0
700		13/20	65
700	Cyclophosphamide[b]	0/25	0
700	Testosterone	17/56	30
820		59/62	95
820	Cyclophosphamide[b]	8/64	13
820	Testosterone	25/28	89

[a] Acute, within 24 hr and usually within 6 hr after irradiation.

[b] Last injection 4–7 days prior to γ-irradiation.

Attempts to render cyclophosphamide-treated chicks more sensitive to irradiation by cell transfer. Beginning 1 to 4 days after the last cyclophosphamide injection, birds received iv cell transfers, usually three daily injections of 5×10^7 cells each. In some experiments a single injection was given. Prior to irradiation, the peripheral blood leukocyte levels in reconstituted animals, where examined, were two to three times higher than those in cyclophosphamide-treated controls. The birds were irradiated 1–2 days after the last cell transfer and their mortality rates were compared with those of cyclophosphamide-treated controls (Table II). Although the mortality rates for bursa and bone marrow recipients were slightly higher than for controls, the differences were insignificant ($P > 0.1$), and it could not be concluded that lymphoid cells mediated the irradiation effect.

Effect of irradiation on vascular permeability. It was considered of interest to deter-

mine whether the vascular collapse and loss of circulating plasma described by others (1, 2) was associated with enhanced vascular permeability. In view of the rapidity with which hemoconcentration and plasma volume changes occur (1, 2), the birds were injected with Evans blue within 2 to 3 hr after irradiation. Enhanced permeability was evident from the color of the leg muscles, liver, and intestines (Fig. 1). The intensely blue liver and intestines of unprotected, irradiated chickens contrasted with the relatively normal color of cyclophosphamide- or SBTI-treated chicks, both of which had also been irradiated. Thus, there was an excellent correlation between the absence of the development of enhanced permeability, as judged by a lack of Evans blue staining of the organs, and the resistance to the acute irradiation effect in the pretreated animals.

TABLE II. EFFECT OF LYMPHOID CELL RECONSTITUTION IN CYCLOPHOSPHAMIDE-PRETREATED SC CHICKS ON ACUTE γ-IRRADIATION (820 R) MORTALITY.

Transferred[a] cell type	Number of cells $\times 10^7$	Peripheral blood leukocytes/ml $\times 10^{-7}$ [b]	Dead/total	Mortality (%)
	None	0.72	3/24	13
Thymus	5-15	1.8	4/26	15
Bursa	5	2.3	5/15	33
Spleen	12	1.3	0/10	0
Bone marrow	15	2.6	4/17	24

[a] Injected intravenously 1 to 4 days after the last cyclophosphamide injection and 1 to 2 days prior to irradiation. Most animals received three daily injections of cells.

[b] Determined prior to irradiation. Normal chickens of this age had 2×10^7 mononuclear leukocytes per milliliter of peripheral blood.

Some birds were irradiated with a leg-shield in place. Since shielding was not readily effected when irradiation was given with the ^{137}C source, animals in these experiments were X-irradiated (820 R) with a Varian Clinac 4-linear accelerator (4 meV, 350 R/min) and irradiation of one of the legs was totally blocked with a lead shield. Evans blue was injected 2 hr after irradiation and the animals were killed 10 min later. There was a marked difference between the muscles of the shielded and the unshielded legs: Whereas the shielded leg displayed its normal reddish-brown color, the muscles of the other leg were intensely blue, clearly demonstrating a local rather

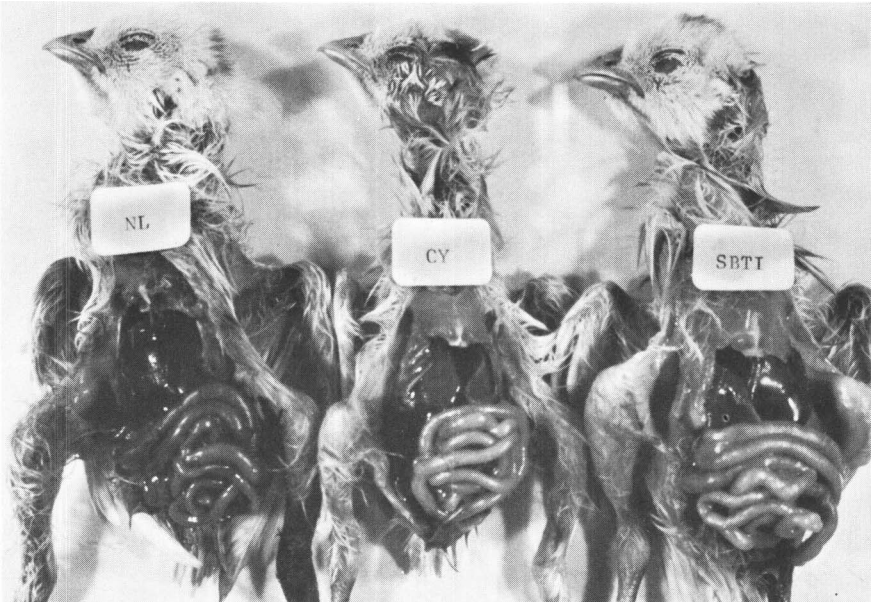

FIG. 1. Abdominal organs of chickens injected with Evans blue 2 hr after γ-irradiation (820 R) and killed 15 min later. Color of intestines and liver of unprotected irradiated (NL) animal was intensely blue, as compared to the usual appearance in cyclophosphamide (CY)-pretreated and soybean trypsin inhibitor (SBTI)-pretreated animals.

than a systemic effect of the irradiation on permeability.

Protective effect of a lower-dose (500-R) irradiation on acute mortality from a higher dose. Since all chickens survived 500-R γ-irradiation, the effect of pretreatment with this dose on subsequent high-dose γ-irradiation mortality was investigated. Groups of 8 to 10 chickens from two different strains were irradiated with 820 R (SC strain) or 930 R (FP strain) at 1 to 10 days after pretreatment with 500 R. The results are illustrated in Fig. 2. An impressive protective effect, which lasted a minimum of 7 days, was demonstrated. Such a resistance to a second irradiation after both alkylating agents or X-irradiation has also been noted with respect to permeability changes in the skin of the rabbit (5). Thus, both in the chicken and in the rabbit, treatment with alkylating agents or γ-irradiation may result in a prolonged state of resistance to the acute irradiation shock effect.

Since vascular permeability changes possibly due to release of a trypsin-like enzyme appeared to be mediating this phenomenon, it was concluded that the protection was caused by depletion of this hypothetical enzyme by the earlier treatment with cyclophosphamide or with 500-R irradiation. This "depleted" state apparently persisted for at least 7 days and the results indicated an accelerated trend to recovery of the sensitivity to irradiation between 7 and 10 days after 500 R in both strains (Fig. 2).

In additional chickens, a higher (820-R) irradiation pretreatment dose (given after injection of SBTI and thus not resulting in acute mortality) was followed 3 days later by another dose of 820 R without any mortality (0/41). In fact chickens which had survived a combined dose of 1320 or 1640 R showed surprisingly good survival rates over the first 2–3 weeks after irradiation. Similarly, the majority of the chickens which had received cyclophosphamide after hatching and a dose of 820 R 1 week later survived for 2 weeks, and 30% survived for even more than 6 weeks after the irradiation (Table III). A similar combination of cyclophosphamide and 630 R caused only 35% mortality within 6 weeks. Thus, long-term irradiation mortality in chickens that have

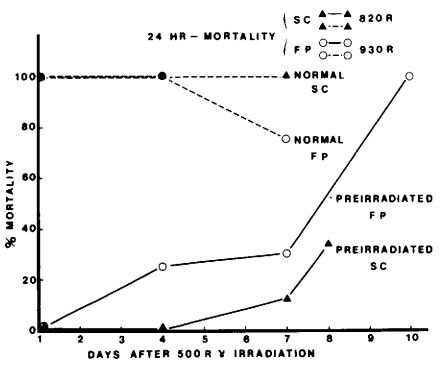

FIG. 2. Mortality, within 24 hr, after whole-body γ-irradiation (820 R in SC and 930 R in FP chickens) in normal (O-----O; ▲-----▲) and in 500-R preirradiated birds (O——O; ▲——▲). Note the return of sensitivity to γ-irradiation between Days 7 and 10 after the preirradiation.

TABLE III. LONG-TERM SURVIVAL AFTER γ-IRRADIATION OF PRETREATED CHICKENS.

Pretreatment (R)	Mortality after 2 weeks (%)	Mortality after 6 weeks (%)
500 + 820	50	Not done
Cyclo + 820	35	71
Cyclo + 630	20	35

survived the first 24 hr is as low or lower than it is in mammals.

Sensitivity of different chicken strains to γ-irradiation and to intravenously injected trypsin. A difference in sensitivity to γ-irradiation between SC and FP chickens had been noted in the previous experiments (Fig. 2). In view of the fact that protection by SBTI implied release of a trypsin-like enzyme as a factor in the chain of events leading to acute irradiation mortality, four different chicken strains were compared for sensitivity to a single iv injection of trypsin or to 820-R irradiation. A good correlation appeared to exist between the percentage of mortality induced by 820 R or by iv injection of 6 mg/kg of trypsin (Table IV). The SC chickens had extremely high mortality, whereas the EL6 and EL7 strains (which have the same major histocompatibility genotype as SC) had very low mortality after both treatments. The FP strain was intermediate, but nearer to the two EL strains.

In all strains, the effects of a trypsin injection were immediately apparent: The chickens sat very still, but there followed a period

of hyperactive wing flapping in those chickens that succumbed within a few minutes after the injection. Those that survived the first 5 to 10 min showed a quick recovery to normal behavior. There was no evidence that the trypsin-induced mortality was due to a vascular collapse similar to the one caused by irradiation, but trypsin injection did induce enhanced permeability as judged by Evans blue extravasation.

Effect of pretreatment with various agents on acute irradiation mortality. The possible release after irradiation of endotoxin from intestinal bacteria could have been important in the development of the fatal shock. To investigate this possibility, attempts were made to sterilize the intestinal tract by pretreatment for 6 days with neomycin. This pretreatment provided very slight, if any, protection against the subsequent irradiation (Table V). Further, were endotoxin to activate the same enzyme(s) that causes the vascular collapse after irradiation, the endotoxin might also be expected to deplete it and thus protect against irradiation. However, such was not the case: 91% of chicks succumbed to irradiation given 24 hr after an iv injection of 100 μg of *E. coli* endotoxin (Table V). A similar argument could be applied to trypsin. In this case, however, a definite protective effect of pretreatment with a sublethal dose given 24 hr prior to irradiation was seen in both the SC and FP strains (Table V).

Lysosomal membrane damage might be involved in the complex changes seen after irradiation of chickens (6); thus, lysosome stabilizing agents were examined for their ability to protect against irradiation. In Table V it is shown that a definite protective effect against 820 R in SC chickens was obtained by pretreatment with either vitamin A (38 vs 95%, $P < 0.001$) or cortisone (44 vs 95%, $P < 0.001$),

Discussion. The present results show clearly that pretreatment of chickens with cyclophosphamide or with a sublethal dose of γ-irradiation protects against the acute mortality induced by a single high dose of irradiation. Injection of high doses of hemopoietic or lymphoid cells did not reestablish the sensitivity to irradiation, an observation in agreement with those of Stearner and Christian (11) that 3-day chick embryos already show enhanced vascular permeability after irradiation and that they can also be protected against the irradiation effect by SBTI. Thus, circulating leukocytes do not appear of importance in mediating this effect.

TABLE IV. COMPARATIVE RESISTANCE TO TRYPSIN AND γ-IRRADIATION IN CHICKS OF VARIOUS STRAINS.

		Sensitivity to			
		Trypsin, 6 mg/kg		820-R γ-Irradiation	
Chicken strain	Age (days)	Dead/total	Mortality (%)	Dead/total	Mortality (%)
SC	7–11	14/14	100	59/62	95
FP	7–11	3/10	30	4/15	27
EL6	7–14	1/10	10	2/18	11
EL7	7–14	0/11	0	1/17	6

TABLE V. EFFECT OF PRETREATMENT WITH VARIOUS AGENTS ON SUBSEQUENT ACUTE γ-IRRADIATION-INDUCED MORTALITY.

Pretreatment	Chicken strain[a]	Interval before irradiation (hr)	Dead/total	Mortality (%)
Vitamin A (4 mg ip × 2)	SC (820 R)	24, 4	6/16	38
Cortisone (7.5 mg ip × 2)	SC (820 R)	24, 4	7/16	44
E. coli endotoxin (100 μg iv)	SC (820 R)	24	21/23	91
Neomycin (0.4–1 mg/day im)	SC (820 R)	144, 120, 96, 72, 48, 24	12/15	80
Trypsin (3 mg/kg iv × 2)	SC (820 R)	24, 22	4/10	40
None	SC (820 R)		59/62	95
Trypsin (6 mg/kg iv × 2)	FP (930 R)	24	2/7	29
None	FP (930 R)		40/45	89

[a] Dose of γ-irradiation is given in parentheses.

The slow spontaneous recovery after pretreatment with irradiation or cyclophosphamide suggests that 7–10 days are needed for the return to normal of tissue levels of the enzyme which mediates the radiation-induced changes. Stearner *et al.* (12, 13) have suggested previously that the enhanced resistance to irradiation in chickens, which appeared maximal within 4 hr after the first of a split-dose exposure, may be due to elimination of some subcellular factor essential for the mortality. Thus, it should be stressed that the protective effect exerted by the first exposure involves induction of a higher than normal resistance to irradiation due to depletion of a factor rather than to a repair mechanism. Although Stearner *et al.* (12, 13) found less resistance at 24 than at 4 hr after the first dose, the present studies show that the recovery of normal sensitivity to irradiation takes at least 1 week. It should be noted that the formation of an inhibitor of the enzyme released in response to the irradiation, or to the cyclophosphamide treatment, which slowly disappears during the following week would also explain this phenomenon.

The effect of local shielding during irradiation suggests that the enzyme is in sessile stores rather than in the circulation. A direct effect of irradiation in the chicken causing vascular damage and hemorrhage was also previously demonstrated (14, 15). A local effect of irradiation in inducing enhanced permeability in the rabbit skin was found to be linked to activation of a protease system (3). The nature of inhibitors (Palladino, M. A., Troll, W., and Thorbecke, G. J., unpublished observations) found to protect against acute irradiation mortality in the chicken suggests the involvement of a kallikrein- or plasmin-like serine protease (16–18) in mediating the vascular changes. Lysosomal damage, as also seen in endotoxin shock (19), has been implied by Stearner and Azuma (6), who showed increased levels of acid phosphatase in plasma from irradiated chickens. However, they concluded that any lysosomal changes were probably secondary in nature since they were prevented by injection of SBTI prior to irradiation. While the protective effect of vitamin A and of cortisone noted in the present studies is compatible with a role of lysosomal damage in mortality, it does not imply that the primary event is at the level of the lysosomes.

The only species which show this peculiar acute irradiation mortality are chickens and ducks (20). The other species of birds examined, including pigeons, parakeets, and canaries, do not exhibit this phenomenon (21). In the present studies, it was found that the SC strain is much more sensitive than are three other chicken strains studied, although an increase in the irradiation dose also induces acute mortality in the other strains. It seems of interest that the SC strain also was killed by doses of intravenously injected trypsin lower than those needed to kill the other three strains. It seems possible that the high sensitivity to the trypsin-like enzyme that mediates the irradiation-induced vascular collapse in the chicken is related to the relative lack of plasminogen inactivator in chicken plasma (22). SC chickens might have even less of this inactivator than the other strains have and might, therefore, be more sensitive to enzymes causing plasminogen activation, such as those possibly involved in the acute irradiation effect. Further studies are needed to define this possibility. The partially protective effect of a sublethal dose of trypsin given 24 hr prior to irradiation suggests that activation of a trypsin-like enzyme causes a depletion of enzyme system(s) responsible for the acute radiation mortality similar to that seen after cyclophosphamide injection or sublethal irradiation.

Summary. Chickens given a lethal dose of γ-irradiation die of generalized vascular collapse within 24 hr after irradiation. This syndrome can be prevented by pretreatment with soybean trypsin inhibitor, cyclophosphamide, or sublethal doses of irradiation; injection of trypsin, vitamin A, or cortisone gives partial protection. Hormonal bursectomy by testosterone, without cyclophosphamide treatment does not protect nearly as effectively. Reconstitution of cyclophosphamide-treated birds with bone marrow or lymphoid cells does not reinstate sensitivity to irradiation. Spontaneous reconstitution of this sensitivity after a sublethal dose of γ-irradiation takes 7 to 10 days.

Variations in the sensitivities of four different chicken strains to the lethal effects of γ-irradiation and of iv-injected trypsin ran in parallel. The possible activation by irradiation of a kinin system to which chickens are particularly sensitive is discussed as the cause of death. It is also pointed out that parameters of irradiation-induced increased permeability in chickens strongly resemble those of the less dramatic permeability changes seen in mammals.

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