THE CONTRASTING EFFECTS OF CYCLOPHOSPHAMIDE AND RADIATION ON THE IMMUNE RESPONSES OF THE MOUSE*

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(Received for publication 24 November 1970)

While it is customary to stress the similarity of X-irradiation and the cytotoxic alkylating agents, we have been impressed by the contrast in the effect of X-ray and these drugs on the immune system. The contrast in action of the two modalities was observed in the course of earlier studies of two phenomena in CBA mice: immunological tolerance induced with the immunosuppressant cyclophosphamide (1), and the role of lethal irradiation in unmasking the immune deficit of thymectomized adult animals (2). Thus, radiation could not substitute for cyclophosphamide in the drug-tolerance system, and cyclophosphamide was of no use in bringing out the thymectomy defect. Other experiments indicated that bone marrow cells, which, as is well known, protected animals from lethal irradiation, were of no value against lethal drug toxicity. This dichotomy between the immune action of drug and X-ray appeared to us to be of sufficient significance that additional experiments were undertaken to outline the phenomenon. The present manuscript describes the new experiments and discusses the results in terms of the different cellular and kinetic mechanisms involved in the action of the two modalities.

Materials and Methods

The various techniques employed have been described in detail in earlier publications (1–3). Female CBA mice 10–14 wk of age, obtained from the Jackson Laboratory, Bar Harbor, Maine, were used. Cyclophosphamide (Cytoxan, Mead Johnson Laboratories, Evansville, Ind.) was injected as a freshly made solution containing 5 mg/ml and, unless otherwise stated, the drug was given in four divided daily doses.

Lethally irradiated mice received 875 R (280 kv, 1.4 mm Cu, 67 R/min) and bone marrow cells prepared from the femurs of 10 to 20-wk-old mice of the same sex and strain. Sublethally irradiated animals received 525 R without bone marrow.

Hemolysin-forming spleen cells were determined by the method of Jerne (4) as previously modified (1). Animals were challenged intravenously with 0.2 ml of 10% sheep cells (5×10^8 cells) 4 days before sacrifice. An appropriate portion of sieved spleen was incubated with sheep erythrocytes in Gey's solution which was gelled by the addition of agarose. The gelled

* Supported by research grant CA-07179 from the National Cancer Institute, U.S. Public Health Service. This is publication No. 1401 of the Cancer Commission of Harvard University.

suspension was incubated at 37°C for 2 hr, 1 ml of 1:10 guinea pig serum was added, and the suspension incubated for an additional hour to develop the direct (19S) plaques. Duplicate plates were incubated with rabbit anti-mouse gamma globulin serum (5) before complement addition to determine the indirect (7S) hemolysin-forming cells.

TABLE I

The Effect of Bone Marrow from Normal and Cyclophosphamide-Treated Animals on Survival after Lethal Irradiation or Cyclophosphamide Administration

Expt. No.	Cyclophosphamide*	Radiation	Bone Marrow		Animals	Survivors‡
			Amount	Source		
	(*mg/kg*)	(*R*)	(× 10^5)			
1	300	0	0		14	10
	400	0	0		8	2
	500	0	0		6	0
	600	0	0		8	0
	900	0	0		2	0
2	400	0	0		7	0
	400	0	5.0	Normal	9	0
3 a	0	875	4.0	Normal	24	24
3 b	0	875	2.0	Normal	5	2
	0	875	0.5	Normal	5	3
	0	875	0.05	Normal	5	3
	0	875	0.02	Normal	5	0
4 a	400	0	0		6	5
	500	0	0		12	0
	0	875	0		6	0
	0	875	0.6	Cytox§	4	0
4 b	400	0	0		6	0
	0	875	0		6	0
	0	875	1.5	Cytox§	6	0

* Injected as a single dose.

‡ Alive at 30 days.

§ Marrow derived from animals (from the same experiment) that had received 400 mg/kg of cyclophosphamide 48 hr earlier.

Adult mice were thymectomized by the previously described method (2), and animals were examined at postmortem for gross thymic remnants. Skin grafts were evaluated visually after conventional full-thickness skin grafting (3). Tolerance-inducing injections of sheep erythrocytes consisted of the intravenous injection of 0.8 ml of a 30% suspension of once saline-washed fresh sheep cells immediately before the initial cyclophosphamide injection or as specified in the text in relation to irradiation.

RESULTS

The Influence of Bone Marrow from Normal and Cyclophosphamide-Treated Animals upon Survival after Lethal Irradiation or Cyclophosphamide Injection.—

Table I presents data from experiments designed to contrast the effectiveness of bone marrow in protecting animals after lethal irradiation with the ineffectiveness of these cells after lethal doses of cyclophosphamide. Thus CBA mice uniformly die after 875 R if not given bone marrow (Table I, experiments 4 a and b) while their survival is good if they receive 4×10^6 marrow cells (Table I, experiment 3 a). The data in experiment 3 b suggest that the marrow requirement is satisfied by as little as 5×10^4 cells.

TABLE II

Sheep Cell Response in Normal and Thymectomized Mice after Cyclophosphomide Administration and Lethal Irradiation

Expt. No.	Thymectomy	Cyclophosphamide*	Sheep cells	Radiation‡	Time plaqued§	Plaque-forming spleen cells‖	
						19S	7S
		(mg/kg)		(R)	(wk)		
1	−	0	0	0	2½	131,000	
	−	200	0	0	2½	89,000	
	+	200	0	0	2½	110,000	
	−	200	6×10^9	0	2½	85	
2	−	0	0	0	3	84,000	53,000
	−	190	0	0	3	67,000	60,000
	+	190	0	0	3	90,000	81,000
	−	190	6×10^9	0	3	16	0
3	−	0	0	875	5	19,600	
	+	0	0	875	5	150	

* Cyclophosphamide was given in divided doses for 4 successive days. When a tolerance-inducing injection of sheep cells was employed, it was given on the same day as the initial cyclophosphamide injection.

‡ Followed by 5×10^6 bone marrow cells.

§ From time of drug or irradiation.

‖ Arithmetic mean of the plaque-forming cells from the individually plagued spleens of four to six animals.

Data in Table I indicate that single doses of cyclophosphamide of 500 mg/kg are uniformly fatal, doses of 300 mg/kg are usually tolerated, and doses of 400 mg/kg result in mortality which varies widely from experiment to experiment. However, normal bone marrow affords no protection from cyclophosphamide mortality even when the drug dose is in the low-lethal range (Table I, experiment 2).

Since bone marrow fails to protect animals from fatal cyclophosphamide toxicity, one may inquire whether these animals are dying with adequate numbers of hematopoietic stem cells in their marrow. To answer this question, mice that had received 400 mg/kg of cyclophosphamide 48 hr earlier were

used as marrow donors for lethally irradiated recipients. Other (cyclophosphamide control) animals received the drug and were observed for drug toxicity. The two experiments differed in that in one (Table I, experiment 4 a) five of six drug control animals survived 30 days while in the other experiment (Table I, experiment 4 b) they all died. However, in neither instance was bone marrow from cyclophosphamide-treated donors able to salvage animals after lethal irradiation.

Cyclophosphamide Administration in Normal and Thymectomized Mice.—The experiments illustrated in Tables II and III explore the ability of cyclophosphamide to expose the immunological deficit of thymectomized adult

TABLE III

Skin Graft Survival in Normal and Thymectomized CBA Mice after Cyclophosphamide and Lethal Irradiation

Group No.	Thymectomy	Cyclophosphamide*	Radiation	Bone marrow	Skin graft survival‡	
					C57BL	AKR
		(*mg/kg*)	(*R*)	(× 10^6)		
1	−	0	0	0	13 (13, 13, 13, 13)	13 (13, 13, 13, 15)
2	−	150	0	0	13 (13, 13, 13)	13 (13, 13, 13, 13)
3	−	250	0	0	13 (13, 13, 13)	13 (13, 13, 17)
4	+	150	0	0	13 (13, 13, 13)	13 (13, 13, 15)
5	+	250	0	0	13 (13, 13, 13)	15 (15, 15, 15)
6	+	0	0	0	13 (13, 13, 13, 13)	13 (13, 13, 13, 13)
7	−	0	850	5	14 (13, 15)	17 (17, 17)
8	+	0	850	5	61 (19, 61, 61, 61, 91)	200+ (61, 200+, 200+, 200+, 200+)

* Cyclophosphamide injected in divided dosage on day of grafting and 3 successive days.
‡ Median survival. Survival of individual grafts in parentheses.

mice. The data in Table II indicate that the drug was ineffective in depleting hemolysin-forming spleen cells, while from Table III it is clear that the chemical does not prolong the survival of either H-2–compatible (AKR) or H-2–incompatible (C57BL) skin homografts in thymectomized animals. In contrast, thymectomized mice after lethal irradiation show a marked deficiency of hemolysin-forming spleen cells (Table II) and prolongation of skin graft survival (Table III).

The Inability of Lethal and Sublethal Irradiation to Induce Tolerance to Sheep Erythrocytes.—Table IV depicts two experiments in which an attempt was made to induce tolerance to sheep cells by lethal and sublethal irradiation. The dose of sheep erythrocytes (6×10^9) employed here regularly produces complete immunological tolerance when given with cyclophosphamide (Table II, experiments 1 and 2). In contrast to drug treatment, irradiated animals

that have received sheep cells show only immunization. Such immunization follows both lethal irradiation with bone marrow and sublethal irradiation, and occurs regardless of whether the sheep erythrocytes are given together with, precede, or follow, the irradiation (Table IV, experiment 2).

TABLE IV

Failure to Induce Tolerance to Sheep Cells with Lethal and Sublethal Irradiation

Experiment	Sheep Cells		Radiation	Bone marrow	Time§	Plaque-forming cells	
	Amount	Day*				19S	7S
			(*R*)		(*wk*)		
1	0		875	5×10^6	5	22,000	15,000
	6×10^9	0	875	5×10^6	5	22,000	>40,000
	0		525	0	5	1,100	650
	6×10^9	0	525	0	5	9,800	>30,000
	0		875	5×10^6	10	30,100	20,000
	6×10^9	0	875	5×10^6	10	5,100	>100,000
	0		525	0	10	12,200	5,300
	6×10^9	0	525	0	10	5,100	100,000
2	0		875	5×10^6	5	6,100	6,900
	6×10^9	−2	875	5×10^6	5	77,000	38,000
	6×10^9	−1	875	5×10^6	5	98,000	123,000
	6×10^9	0	875	5×10^6	5	83,000	89,000
	6×10^9	1	875	5×10^6	5	88,000	128,000
	6×10^9	+2	875	5×10^6	5	107,000	115,000

* Day 0 is the day of irradiation. There were five animals in each group in experiment 1 and four animals in each group in experiment 2.

§ Weeks after irradiation that spleens were plaqued. Animals that received the tolerance-inducing injection of sheep cell also received twice weekly intraperitoneal injections of 5×10^8 cells.

DISCUSSION

Our observations on the effects of lethal irradiation and cyclophosphamide injection on immunological reactivity of CBA mice are summarized in Table V. The data that have been presented indicate that lethal irradiation (but not cyclophosphamide) leads to profound immune impairment in the thymectomized animal whereas cyclophosphamide (but not radiation) easily induces immunological tolerance. Furthermore, bone marrow cells protect against the lethal effects of irradiation (875 R) but not against the drug, and earlier studies have established that lethal irradiation (2) depletes the spleen more profoundly than tolerated doses of cyclophosphamide (6). While no one of these observations taken by itself is sufficient, taken together they represent convincing evidence of a fundamental difference in the immunosuppressive action

of this drug and radiation. (Both cyclophosphamide and X-ray produce a temporary nonspecific depression of immunological reactivity [7]).

A critical analysis of the mode of action of X-irradiation and cyclophosphamide has been made by Bruce, Meeker, and Valeriote (8). Their essential observation is that nondividing normal hematopoietic stem cells from bone marrow display the same radiation sensitivity as do rapidly dividing lymphoma cells, while the lymphoma cells are much more sensitive to cyclophosphamide. From this observation they conclude that cyclophosphamide action depends markedly on the rate of cell proliferation while irradiation does not. (In addition, the shape of the dose:survival curves leads these authors to

TABLE V

Comparison of Effects of Lethal Irradiation and Cyclophosphamide Injection in CBA Mice

	Lethal irradiation	Cyclophosphamide
Protection by bone marrow	+	−
Protection of immune impairment in thymectomized animal	+	−
Induction of immune tolerance	−	+
Nonspecific immune impairment	+ (7)	+ (7)
Reduction in number of spleen cells*	6/221 (2)	40/180 (6)
Dependence of lethal effect on rate of cell proliferation (8)	−	+

* Expressed as millions of spleen cell at maximum drug or radiation effect over initial value.

conclude that both irradiation and cyclophosphamide kill cells in all portions of the generation cycle [8]).

The conclusions of Bruce et al. are useful in analyzing the effects of radiation and cyclophosphamide on immune parameters. The tolerance-inducing capacity of the drug can be related to destruction of a rapidly proliferating cell (presumably replicating in response to antigenic stimulation) which is destroyed by the same mechanism that destroys the rapidly dividing lymphoma cell. The radiation-sensitivity and cyclophosphamide-insensitivity of the immune responses of the thymectomized animal implies that the cell depleted here, like the marrow stem cell, is not actively proliferating. Since bone marrow does not relieve the immune defect of the thymectomized irradiated animal, it seems clear that the cell depleted by irradiation is not the marrow stem cell itself, but rather a lymphoid cell (perhaps derived from it by thymus processing) that shares a slow mitotic rate.

Our inability to sustain lethally irradiated animals with marrow from mice

that received cyclophosphamide (Table I, experiment 4) is not conclusive evidence that drug-treated animals lack sufficient hematopoietic stem cells to survive. Thus, in earlier work (3) we found that marrow from mice that received 525 R (a sublethal dose) was unable to sustain lethally irradiated animals, and even the modest dose of 262 R impaired the resuscitative quality of marrow. Clearly, the answer is to be found in a quantitative consideration of the number of hematopoietic stem cells needed for survival. Data from Bruce et al. (8) indicates that 400 mg/kg of cyclophosphamide (8 mg/mouse) reduces the number of marrow stem cells to approximately 1% that of untreated animals. A survival figure very close to 1% is also obtained from the same reference for stem cells subjected to 525 R, a dose which is well tolerated by the CBA mouse. (The LD_{50} for female CBA mice is 689 ± 8 R (9), a radiation dose which reduces stem cell survival to 0.1% [8]). Presumably, the 1% survival reduces the viable stem cells in the transplanted marrow below the 5×10^4 (approximately) that is needed for survival. In any event, it seems clear that the cyclophosphamide-treated mouse dies of causes in addition to lack of hematopoietic stem cells, and it seems likely (from consideration of the radiation data above) that such animals have sufficient marrow stem cells to survive.

We recognize that the reasoning of the two previous paragraphs, though plausible, is based on indirect evidence, and the conclusion derived cannot be proposed with certainty. It is also possible that additional permutations in the fractionation and dose of drug and radiation might lead to somewhat different results (10). Nevertheless, it seems to us quite clear that the drug cyclophosphamide and X-irradiation suppress immunity by quite separate and distinct mechanisms. Other investigators have reached a similar conclusion from different data (11, 12).

SUMMARY

The immunosuppressant cyclophosphamide easily induces specific immunological tolerance in CBA mice, but is unable to produce an immunological defect in adult thymectomized animals. In contrast, lethal (and sublethal) irradiation does not induce tolerance but readily brings out the deficit of thymectomy. Furthermore, bone marrow cells which protect lethally irradiated animals do not prevent drug deaths.

This sharp dichotomy indicates that the drug and radiation influence the lymphoid system by different mechanisms. It seems likely from the work of others that cyclophosphamide action is markedly dependent on rapid cell proliferation, while radiation is not. From this it follows that the cell which must be depleted to expose the immune defect of the thymectomized animal is a nonproliferating lymphoid element with the slow mitotic rate of the marrow stem cell.

BIBLIOGRAPHY

1. Aisenberg, A. C. 1967. Studies on cyclophosphamide-induced tolerance to sheep erythrocytes. *J. Exp. Med.* **125:**833.
2. Aisenberg, A. C., and C. Davis. 1968. The thymus and recovery of sheep erythrocyte response in irradiated mice. *J. Exp. Med.* **129:**1327.
3. Aisenberg, A. C. 1970. Allogenic thymus grafts and the restoration of immune function in irradiated thymectomized mice. *J. Exp. Med.* **131:**275.
4. Jerne, N. K., A. A. Nordin, and C. Henry. 1963. The agar plaque technique for recognizing antibody-producing cells. *In* Cell-Bound Antibodies. B. Amos and H. Koprowski, editors. The Wistar Institute Press, Philadelphia, Pa. 109.
5. Dresser, D. W., and H. H. Wortes. 1965. Use of an antiglobulin serum to detect cells producing antibody with low haemolytic efficiency. *Nature (London).* **208:**859.
6. Aisenberg, A. C., and B. Wilkes. 1967. Immunological tolerance induced by cyclophosphamide assayed by plaque spleen cell method. *Nature (London).* **213:**498.
7. Aisenberg, A. C., and C. Davis. 1968. The thymus and recovery from cyclophosphamide-induced tolerance to sheep erythrocytes. *J. Exp. Med.* **128:**35.
8. Bruce, W. R., B. E. Meeker, and F. A. Valeriote. 1966. Comparison of the sensitivity of normal hematopoietic and transplanted lymphoma colony-forming cells to chemotherapeutic agents administered *in vivo*. *J. Nat. Cancer. Inst.* **37:**233.
9. Storer, J. B. 1966. Acute responses to ionizing radiation. *In* Biology of the Laboratory Mouse. C. L. Breen, editor, McGraw-Hill Book Company. New York. 2nd edition. 427.
10. Frisch, A. W., and G. H. Davies. 1965. Inhibition of hemogglutinin synthesis by cytoxan. *Cancer Res.* **25:**5.
11. Berenbaum, M. C. 1962. The effect of cytotoxic agents on the production of antibody to T. A. B. vaccine in the mouse. *Biochem. Pharmacol.* **2:**29.
12. Taliaferro, W. H., and L. G. Taliaferro. 1948. Reduction in immunity in chicken malaria following treatment with nitrogen mustard. *J. Infec. Dis.* **82:**5.