The Function of Irradiated Blood Elements
I. Limitations on the Response to Phytohemagglutinin as an Indicator of Immunocompetence in Irradiated Lymphocytes.[1] (35534)

ROBERT E. COIFMAN, ROBERT A. GOOD, AND HILAIRE J. MEUWISSEN
University of Minnesota, Research Laboratories of the Variety Club Heart Hospital, Department of Pediatrics, Minneapolis, Minnesota 55455

Patients with either congenital thymic or dual system immunologic deficiency disease (1) or, as in one published case, an immature fetus (2) may develop fatal graft-versus-host reactions (GVH) if transfused with whole blood or blood fractions containing viable immunocompetent lymphocytes. In view of the known high radiosensitivity of lymphoid cells (3–6) which must proliferate in order to endanger the recipient, and the relative radioresistance of the nonreplicating cell types or plasma proteins which are the objects of most transfusions (7, 8)[2,3] it has been suggested that the delivery of 3000 rads *in vitro* under appropriate conditions of oxygenation (9) be used to eliminate the risk of GVH as a complication of transfusion (1).[4] However, McCullough *et al.* (10) have suggested on the basis of persisting uptake of isotope-labeled thymidine after stimulation with phytohemagglutinin (PHA) that lymphocytes in whole blood remain immunocompetent after radiation doses as high as 6000 R. The object of this communication is to attempt to resolve this discrepancy.

Methods. In each experiment, lymphocyte-rich buffy coat was prepared in minimal essential medium for use as responding cells to stimulation by either Difco phytohemagglutinin-M (PHA) or in mixed lymphocyte culture (MLC) by allogeneic leukocytes similarly prepared and then treated with mytomycin. Cell suspensions to be irradiated under conditions of high (atmospheric) oxygen tension were placed in flat-sided tissue culture bottles in which liquid depth was less than 5 mm. The 0-radiation control was processed in a similar fashion. The suspension of cells to be irradiated under low oxygen tension was placed in a single gas-tight syringe with a length of narrow bore tubing connected to the hub also filled with cell suspension to serve as a gas diffusion barrier. The syringe was placed in a 37° incubator until the cells had metabolized sufficient oxygen to reduce the oxygen tension in the suspension to near zero as measured on an Instrumentation Laboratories Model 113 blood gas analyzer. The syringe was then irradiated in steps, with withdrawal of cells after each desired dose had been delivered.

All radiation was performed with a G.E. Maximar, 220 kVp, without iometer, delivering an air dose of 462 R/min through a 1-mm Al filter. Lymphocytes were cultured as described previously (11). The measured parameter of lymphocyte stimulation was the uptake of ^{14}C-thymidine present in the medium for 5 hr prior to termination. Activity was measured by liquid scintillation as counts per minute (cpm) of the trichloracetic acid-insoluble cell pellet. Each experimental data point represents the mean of triplicate cultures.

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[2] Coifman, R. E., and Good, R. A., The function of irradiated blood elements. III. The metabolic, bactericidal, and chemotactic properties of granulocytes irradiated *in vitro*, in preparation.

[3] Pabst, H. F., Meuwissen, H. J., Edson, J. R., and Good, R. A., Correlation of platelet function and lymphocyte survival after irradiation, submitted for publication.

[4] Coifman, R. E., and Good, R. A., The function of irradiated blood elements. V. Radiation as a means to prevent graft-versus-host disease in the clinical use of blood fractions, in preparation.

TABLE I. PHA and Mixed Leukocyte Culture Responses of Irradiated Cells.

Radiation [in 1000 R (kR)]	(cpm) after stimulation with: PHA	fx of control	MLC Donor: 1	2
Expt. 1, donor R.G.				
0 (control)	2208	1.0	219	254
$-O_2$[a]				
1.5	762	.345	0	0
3.0	515	.233	0	0
4.5	282	.128	0	0
6.0	50	.0226	0	0
$+O_2$[b]				
1.5	690	.313	0	0
3.0	33	.0149	0	0
4.5	5	.00226	0	0
6.0	0	0	0	0

	(cpm) after stimulation with PHA: Expt. 2	fx of control	Expt. 3	fx of control
Expt. 2, donor Q.O.; and Expt. 3, donor R.C.				
0 (control)	1536	1.0	2147[c]	1.0
$-O_2$[a]				
1.5	1251[c]	.818	725	.338
3.0	814[c]	.519	618	.278
4.5	797[c]	.508	387	.1803
6.0	532[c]	.34	400	.186
$+O_2$[b]				
1.5	977[c]	.638	717[c]	.335
3.0	795[c]	.507	468	.218
4.5	281[c]	.169	106[c]	.0494
6.0	59[c]	.0375	8	.00373

[a] Anoxic cells: pO_2 measured <4 mm Hg/in. prior to irradiation in Expts. 1 and 3; assumed comparably low in Expt. 2 as incubation time to drop pO_2 from initial mixing in air to 22 mm Hg was doubled when cell volume was insufficient to permit an additional reading.

[b] Oxygenated: liquid layer <5 mm thick in equilibrium with air in tissue culture bottle during irradiation.

[c] Corrected for unstimulated cpm which were $\neq 0$ in these experiments only; range of values, 1–18.

Results. Results are listed in Table I, which also includes normalized counts, expressed as fractions of control, following PHA stimulation. In every case the uptake of labeled thymidine fell when the cells were irradiated prior to stimulation. Cells stimulated with PHA after 6000 R in the absence of oxygen still were able to incorporate nucleotide at from 2 to 34% of control rates, while the uptake of nucleotide in cells irradiated in the presence of oxygen fell off much more rapidly. Uptake by cells stimulated by allogeneic leukocytes was below the threshold of measurement for all nonzero radiation values tested. The normalized counts are plotted in Fig. 1, along with the irradiated whole-blood PHA response data of McCollough (10) and the band enclosing the range of oxygenated radiation sensitivity curves reported by numerous investigators for a variety of human

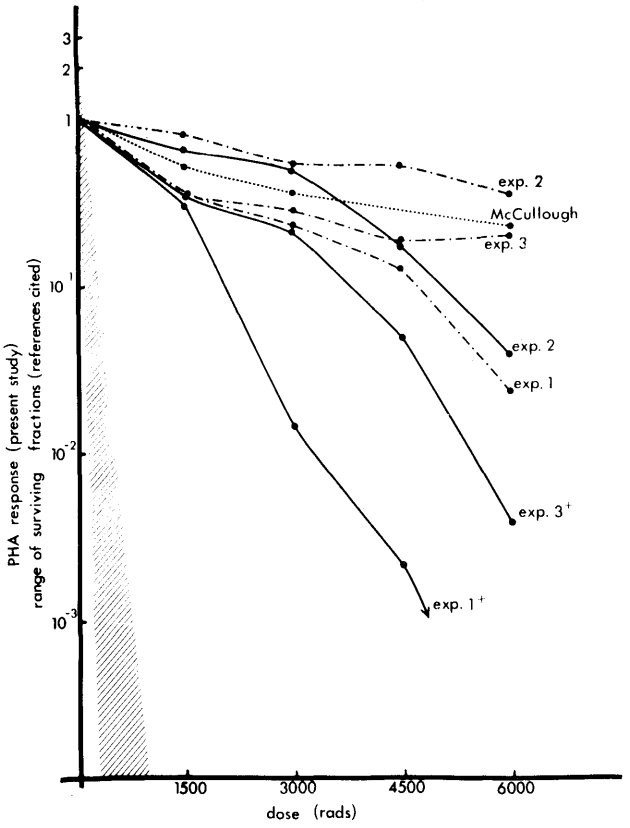

FIG. 1. Observed and reported PHA data and band of reported replication data for irradiated cells.

normal and tumor-derived cell strains and for both lymphoid and hematopoietic function in the mouse (3–5, 12–16). In each of the radiation sensitivity studies, the parameter measured is not physical or physiologic destruction of the cells in question but the loss of their ability to replicate. As such, we are in a strict sense talking about "radiation sterilization" rather than "radiation killing." The dose–response parameters of these studies are listed in Table II. In every case the data were consistent with multiple hit first-order kinetics, with extrapolation numbers (x-axis intercepts, interpreted as the number of "hits" needed to inactivate a cell) between 1.5 and 3 and LD_{37} values varying from a most radiosensitive 47 R to a maximum radioresistance of 166 R. The LD_{37} values in the six experiments in which lymphoid cell function was measured varied from 47 to 78 R, the most radiosensitive portion of the total range. The band of radiation sensitivity data measured as actual immunologic competence or cell replication as shown in Fig. 1 extends from the lowest to the highest radiosensitivity parameters listed in Table II.

Discussion. It seems likely from our data that McCollough's irradiation experiment was conducted under conditions of hypoxia, as his curve closely approximates those of our "$-O_2$" experiments.

Figure 1 shows that our PHA data and those of McCollough lie far outside of the range of other experiments measuring the reproductive sterilization of mouse lymphoid or hematopoietic or human normal or tumor cells as a function of radiation dose under conditions of oxygenation ($+O_2$). As a group, the PHA data reveal absence of the first-order dose–response characteristics seen in direct measurements of radiation killing or sterilization, and the curves obtained in our

TABLE II. Published Radiation Sensitivity Data for the Replicating Ability of Rodent Lymphoid and Hematopoietic Cells and Human Cells.

Author	Ref.	Cell type	Function measured	Extrapolation no.	LD_{37}
Celada and Carter	(3)	Mouse spleen	Antibody synthesis	1.78 and 2.17	47 and 57.7
Makinodan *et al.*	(4)	Mouse spleen	Antibody synthesis	2.72	70
Smith and Voss	(5)	Mouse lymph node	GVH	2.7	74
Blackett	(6)	Mouse spleen	GVH	3	75
Celada and Carter	(3)	Mouse spleen	Graft vs graft (GVG)	3.2	78
Puck and Marcus	(12)	HeLa	Colony formation *in vitro*	2	96
Puck *et al.*	(13)	Clonal cell strains of human conjunctiva, liver and appendix	Colony formation *in vitro*	2	96
Puck *et al.*	(13)	Clonal cell strains of human spleen, skin, and ovary	Colony formation *in vitro*	1.5	100
McCulloch and Till	(14)	Mouse bone marrow	Survival of reconstituted recipients	—[a]	105
Till and McCulloch	(15)	Mouse bone marrow	Formation of hemopoietic colonies in recipient spleens	2	115
Dewey	(16)	Clonal cell strain of human liver origin	Colony formation *in vitro*	2	119
Puck *et al.*	(13)	Clonal cell strain from human embryonic lung	Colony formation *in vitro*	2	166

[a] Not derived by technique used.

three experiments under identical ($+O_2$) conditions vary more among themselves than do those of 13 different experiments by seven different groups of investigators—all of which fall within the band shown. By contrast, our data with allogeneic cell stimulation, in which no response could be elicited at any of the radiation doses studies, are consistent with the known dose–response characteristics of mammalian and particularly lymphoid cell radiation sterilization.

Puck and co-workers observed that, for all of a wide variety of tissues studied (12, 13), cells which had received radiation doses as high as 10,000 R and could no longer replicate were still capable of growth and prolonged survival and could perform many complex metabolic processes. Giant cells produced by radiation-sterilization could be kept alive and active in culture for periods as long as 3 weeks. The occasional presence of early prophase chromatin configuration combined with absence of later stages of mitosis in Puck and co-workers' studies suggests that DNA synthesis, measured in our experiments by the ability to incorporate labeled thymidine into acid-insoluble material, is a relatively radioresistant metabolic process.

While the ability to recognize and initiate a response to stimulation with allogeneic cells may in itself be a similar metabolic function to recognition and initiation of a response to stimulation with PHA, the ability to produce a positive response in our MLC system requires an additional and, as we conclude, more radiosensitive step.

Unlike cells responding to PHA, which must only be alive and able to incorporate thymidine 3 days after stimulation in order to be counted, the the approximately 2% of lymphocytes able to respond to any single allogeneic cell stimulus must have undergone a week-long process of proliferation, amplifying an initially small biologic response, and then still be actively incorporating thymidine.

We do not question the expediency of PHA stimulation as a simple laboratory test

of immunologic competence in lymphoid cells, but we feel that the foregoing presentation points out some of its limitations. Immunologic competence implies an ability first to recognize and be turned on by an antigenic stimulus and then as a second step, to amplify the original stimulus by proliferation. Later this enlarged cell population must be able to perform additional functions, *e.g.*, synthesis of antibody, elaboration of migration inhibitory factor, and release of lymphotoxins on contact with the stimulating antigen. Stimulation *in vitro* with PHA measures only Step 1. If the cells being tested have not been modified since removal from a location reflecting prior ability to proliferate, *e.g.*, the peripheral blood, a positive response indicates that clones of cells able to respond to stimulation with PHA have demonstrated their ability to proliferate sufficiently to populate the blood of the individual being tested. A negative response suggests that cells capable of response to the mitogen are either absent or else have not proliferated sufficiently to be present in measurable numbers. Not even a positive PHA response reflects Step 3, the ability of the cell to function once proliferation has taken place. If a factor modifying the ability of the cells to proliferate, such as radiation, is introduced between collection and stimulation, we can no longer assume that cells able to be stimulated to synthesize DNA can also proliferate in a normal fashion. Thus responsiveness to PHA is not a valid indicator of immunocompetence for irradiated cells.

Our present findings do not resolve the cogent clinical question of whether a given dose of radiation delivered to blood or potentially lymphocyte-contaminated cell fractions under appropriate conditions of oxygenation can eliminate completely the hazard of GVH. They do, however, point out the inadequacy of the PHA response as a parameter for addressing this question. Our observations indicate that responsiveness of lymphocytes to stimulation by allogeneic cells reflects more directly the ability of radiation to produce replicative sterilization, but the limited range of sensitivity of this test renders it unsuitable for quantitation of radiation doses $\geqq$1500 R.

It is of interest that in a considerably more complex experimental system—measurement of spleen enlargement as per Blackett (6) in F_1 hybrid rats 9 days after a 1-hr period of cross-circulation with parental strain donors whose blood had been subjected to extracorporeal irradiation (ECI) 1 hr previously—Cauchi and Field reported an LD_{37} value of 85 R with an extrapolation number (our extrapolation from their published graph) of about 5 (17). When parental strain donors received their radiation as a total-body dose, or alternatively, when the radiation was delivered to blood passing at controlled flow rate between donor artery and recipient vein during cross-circulation, however, these authors reported the much higher LD_{37} value of 450 R, with an extrapolation number (again our extrapolation) of about 1.5. We tried to fit first-order decay curves with this slope to our "O+" PHA curves, which could conceivably be approaching linearity at approximately this slope as they drop below the threshold of measurement at high radiation values, and find that for the three experiments extrapolation numbers would be 15, 1100, and $>$20,000. In view of the extremely close agreement in extrapolation number between the numerous other studies cited it is difficult to conclude that we are measuring the same thing. We suggest that manipulations of the donor animal, by total-body radiation, ECI, and exposure to recipient antigens by cross-circulation may interact to cause complex variations in the number and character of immunologically competent cells present in the circulation of the donor during cross-circulation.

The question remains open as to whether there might be a radiation-resistant information-carrying cell which is mobilized into the circulation under some of the conditions explored by these authors and can transmit its information to other cells, possibly of host origin, which then proliferate and act upon it. While one would not cross-circulate or otherwise deliberately expose a blood donor to cell-surface antigens of his intended recipient, the question also remains open as to whether such cells, if they do in fact exist, might be driven into the circulation by such stimuli as inapparent infection and thus con-

taminate blood components administered to a GVH-susceptible recipient. We are presently unaware of other experimental evidence suggesting that such cell populations might exist, but we propose to devote more attention to this question elsewhere in the course of our study of the function of irradiated blood elements. We do not find our PHA data indicative of the presence of cells of this type in the model we have studied.

In the present experiments, as in both the intended clinical application and the references we have cited for directly measured radiation sensitivity data for lymphoid cells, the cells to be irradiated have not yet been exposed to recipient tissue antigens and are in a mitotically inactive G_0 state. We have thus not felt it necessary to explore the area of possible variations in radiation sensitivity as a function of stage in the cycle of cell replication.

Summary. The ability of a lymphoid cell population to be stimulated to incorporate radio-labeled thymidine by phytohemagglutinin (PHA) has been widely accepted as a ready measure of cellular immune competence. Persisting PHA responsiveness in lymphocytes from irradiated blood, in particular, has been taken as evidence that radiation does not protect against the hazard of graft-versus-host reaction (GVH) in blood transfusion. Our independent conclusion that radiation of blood under appropriate conditions does protect immunologically incompetent recipients against GVH made necessary an effort to resolve this apparent contradiction. Irradiated cells stimulated with either PHA or allogeneic leukocytes have been studied in terms of known radiation sensitivity curves for cellular proliferation. These studies permit the conclusion that PHA responsiveness is not a valid measure of the immunocompetence of irradiated cells. Since the ability to replicate seems much more radiosensitive than the ability to respond, tests such as PHA responsiveness which measure only the ability to recognize and react to a mitogenic stimulus lose their validity in this context.

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