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Mouse Spleen Lymphocyte Bactericidal and Peroxidase Activities: Enhancement by Whole Body X-Irradiation[1] (39349)

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Leukocytes collected from x-irradiated subjects generally indicate depression in phagocytosis-associated activities (1-3). In our efforts to investigate the effect of x-irradiation on host-defense, the principal cells studied have been the guinea pig peritoneal exudate PMN (1-3) and human peripheral leukocytes (4, 5). For comparative purposes, and in order to broaden our understanding of various aspects of host-defense, we have extended our investigation to include the bactericidal and metabolic activities of spleen cells (predominantly lymphocytes) collected from nonirradiated and total body x-irradiated mice (6).

It is realized that spleen cells, mainly lymphocytes, as judged by light microscopy, are not generally considered as conventional phagocytic cells. However, in previous studies we have noted that these cells are able to kill bacteria and, when challenged with particles, they respond metabolically similar to conventional phagocytic cells (6, 7). Electron microscope studies have indicated (unpublished results) that actual particle entry does not occur. It would appear that the metabolic and bactericidal changes noted are not due to conventional phagocytosis but due to cell surface-particle interaction. On the basis of histochemical data, our preliminary results indicate that peroxidase is present in these cells. Generally others have reported little or no peroxidase activity in these cells. However, by the guaiacol peroxidase assay (7), we have observed here that CD1 mouse spleen lymphocytes have significant peroxidase activity. Many peroxidases (i.e., myelo, lacto, horseradish, and macrophage peroxidase) and H_2O_2, in the presence of a halide ion, kill a variety of microorganisms (8-11). Some of the biochemical and bactericidal activities of CD1 mouse spleen lymphocyte peroxidase (SLPO) and H_2O_2 and the effect of whole body x-irradiation and particle addition on these functions are reported here.

Methods and materials. CD1 mice obtained from the Charles River Laboratory, Boston, Massachusetts, and subsequently bred in St. Margaret's Hospital animal quarters were used. Animals of either sex, weighing approximately 20-30 g (8-12 weeks) were x-irradiated in a Portable Andrex X-Ray unit, as previously reported (1-3).

Total and differential white blood cell counts were determined on blood collected from the tail vein immediately before and also generally at 24 hr after x-irradiation. The animals were sacrificed at 1, 3, 5, 10, and 15 days after x-irradiation by cervical dislocation. Spleen cells from nonirradiated mice (8-12 weeks old) served as controls. The spleens were freed from fatty tissues, washed with Krebs-Ringer phosphate medium (KRPM, pH 7.4), and placed on filter paper. The wet weight of each spleen was recorded. Spleen cells were then prepared and counted, as previously reported (12, 13). Total leukocyte and differential counts were noted. The cells were suspended in KRPM for use in metabolic and bactericidal studies.

The oxidation of [1-^{14}C]glucose and [6-^{14}C]glucose was studied in single-arm Warburg flasks. The reaction mixture in the main compartment contained 30 μmol of glucose (sp act 0.01 μCi/μmol of glucose) and 20-25 $\times$ 10^6 spleen cells in a total volume of 3 ml of KRPM, pH 7.4. To study the effect of particle addition on glucose oxidation, polysyrene latex spherules of

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0.81-μm diameter were added at a cell to particle ratio of 1:100. The $^{14}CO_2$ resulting from the oxidation G-1-^{14}C or G-6-^{14}C was counted in a Packard liquid scintillation spectrometer equipped with an external standard. The details on the procedure have been reported earlier (10, 12, 13).

Preparation of peroxidase-containing 20,000 *g* granules and estimation of peroxidase activity: Spleen cells collected from x-irradiated and nonirradiated mice were freed of red blood cells by cold water lysis (7, 12, 13). The cells were then suspended in a desired volume of 0.25 *M* sucrose and homogenized for 5 min in a motor-driven Teflon-tipped Potter-Elvejhem glass homogenizer. The homogenate was centrifuged at 20,000 *g* for 30 min at 4° in a refrigerated Sorvall RC 2 centrifuge. The 20,000 *g* pellet was resuspended in a 0.25 *M* sucrose. The peroxidase activity of the 20,000 *g* granules was estimated by both guaiacol oxidation (at pH 7.0 in the presence of 0.25 *M* sucrose) and decarboxylation activity (KRPM, pH 5.5). Both methods have been previously described (7, 10, 12, 13).

Bactericidal Activity. Both intact cells and 20,000 *g* granules were assayed for bactericidal activity. The test organism, *Escherichia coli,* was grown overnight, harvested in the logarithmic growth phase, washed, and resuspended in KRPM of pH 5.5. The Reaction mixture contained 2×10^4 to 2×10^8 *E. coli* and the following supplements: Granules equivalent to 0.003–0.03 guaiacol unit, H_2O_2, 0.1 μmol, and KRPM, pH 5.5, to a final volume of 2.0 ml. (For assaying intact spleen cells, 5×10^6 spleen cells and 5×10^6 *E. coli* were incubated for 60 min at 37° in KRPM of pH 7.4.) After a 30-min incubation period at 37°, aliquots of the reaction mixture were plated in trypticase soy agar and colonies counted at 24 hr (10, 12, 13).

All chemicals were of reagent grade. [1-^{14}C]Glucose, [6-^{14}C]glucose, and L-[1-^{14}C]alanine were obtained from the New England Nuclear Corporation, Boston, Massachusetts. Polystyrene latex particles of 0.81-μm diameter were obtained from the Difco Laboratories, Detroit, Michigan.

Results. The peroxidase activity of the 20,000 *g* granules isolated from mouse spleen cells collected 24 hr after whole body x-irradiation is presented in Table I. It can be seen that on a per cell basis, the guaiacol oxidation by granules from 400 R x-irradiated mice spleen is approximately fivefold more than that of control. This guaiacol oxidation activity, assayed in the presence of 0.25 *M* sucrose is approximately 40% of that in the absence of sucrose. However, sucrose has been used in order to protect the peroxidase-containing granules from lysis. The decarboxylation of L-[1-^{14}C]alanine by this fraction is also presented in Table I. This reaction also measures peroxidative activity. A dramatic increase in $^{14}CO_2$ formation by granules collected from x-irradiated mice is noted when compared to the decarboxylation activities of the corresponding nonirradiated controls.

The 20,000 *g* granules isolated from spleen cells of x-irradiated and non-irradiated mice were tested for their bactericidal activity (Table I). The granules collected from mouse spleens 1 day after 400 R x-irradiation had significantly higher bactericidal activity when compared to spleen cell granules collected from nonirradiated controls.

Intact spleen cells collected 1 day after 400 R whole body x-irradiation (at a cell to bacterium ratio of 1:1) killed approximately 2 logarithms of *E. coli* as compared to approximately 1 log by spleen cells from nonirradiated controls.

TABLE I. PEROXIDASE AND BACTERICIDAL ACTIVITIES OF THE 20,000 *g* PELLET ISOLATED FROM SPLEEN CELLS OF NON AND X-IRRADIATED MICE.

	Control	X-irradiated[a]
Guaiacol oxidation[b]	0.15	0.69
Decarboxylation[c]	78.3	2193
Bactericidal activity[d]	2–5	4–7

[a] Results (three or more experiments) of cells collected 1-day post 400 R x-irradiation are presented. The activities of preparations from x-irradiated mice are significantly higher than those from control mice.

[b] Guaiacol unit per 10^8 cells. The assay procedure has been published (7, 10, 12, 13).

[c] Nanomoles of L-[1-^{14}C]alanine decarboxylated to $^{14}CO_2$ in 30 min at 37° by 20,000 *g* granules containing 0.03 guaiacol unit of peroxidase.

[d] Logarithms of *E. coli* killed by freshly prepared 20,000 *g* granules containing 0.003–0.03 guaiacol unit of peroxidase in 30 min at 37°.

The amount of $^{14}CO_2$ formation from the oxidation of [1-^{14}C]glucose is an approximate measure of the amount of glucose metabolized by the hexose monophosphate shunt (HMS) pathway. One hundred million spleen cells collected from nonirradiated mice oxidized 27 nmol of glucose through the HMS in 30 min at 37°. The addition of polystyrene latex particles resulted in a fourfold increase in HMS activity. Spleen cells, in the absence of added particles, collected 1 day after 400 R x-irradiation oxidized 69 nmol of [1-^{14}C]glucose and this result is significantly higher than that of nonirradiated controls. If polystyrene latex particles are added to cells collected from x-irradiated mice, 240 nmol of [1-^{14}C]glucose are oxidized. These results can be seen in Table II. The amount of $^{14}CO_2$ oxidized from [1-^{14}C]glucose due to particle addition to spleen cells from nonirradiated as well as 100 to 800 R x-irradiated mice are always significantly higher than the corresponding $^{14}CO_2$ formed in the absence of particles. The extent of oxidation of [6-^{14}C]glucose is less than that of [1-^{14}C]glucose. X-irradiation and particle addition have a stimulatory effect though the overall oxidation is less than that of [1-^{14}C]glucose. These results also can be seen in Table II.

The effects of various doses of whole body x-irradiation on peripheral white blood and spleen cells are presented in Table III. It can be seen that with increasing doses of x-irradiation, the weight of the spleen, the total number of spleen cells and the total white blood cells of the peripheral circulation are decreased significantly in 24 hr. A correlation appears to exist between the doses and the changes in these parameters within 1 day after x-irradiation. In general, in the peripheral blood and spleen, the percentage of lymphocytes decreases while the percentage of segmented cells increases with increasing x-irradiation doses.

The results presented in Tables I and II are from 1 day post 400 R x-irradiation. Effects of 100, 200, or 800 R have also been investigated and are, in general, proportional to the increasing doses of x-irradiation. The extent of stimulation of these activities (glucose, alanine and guaiacol oxidations, antimicrobial and cell differentials) generally increases with increasing days post x-irradiation up to 5 days. Ten to fifteen days post 100 or 200 R x-irradiation all of

TABLE II. EFFECT OF WHOLE BODY X-IRRADIATION (400 R) AND PARTICLE ADDITION ON THE OXIDATION OF GLUCOSE BY MOUSE SPLEEN CELLS.

	Control		X-irradiated	
	Particles		Particles	
	–	+	–	+
G-1-^{14}C	27[b]	103	69	240
G-6-^{14}C	6[c]	15	16	76

[a] The ratio of spleen cell: Polystyrene latex particles (0.81 μm diameter) was 1:100.
Please see text and Table I for additional details.
[b] Nanomoles of $^{14}CO_2$ oxidized from [1-^{14}C]glucose by 10^8 cells in 30 min at 37°.
[c] Nanomoles of $^{14}CO_2$ oxidized from [6-^{14}C]glucose by 10^8 cells in 30 min at 37°.

TABLE III. EFFECT OF WHOLE BODY X-IRRADIATION ON MOUSE SPLEEN AND PERIPHERAL BLOOD LEUKOCYTES.[a]

	Spleen wet weight (mg)	Total spleen cells × 10^6	Total WBC peripheral × 10^6	Cell differentials (%)[b]			
				Peripheral		Spleen Cells	
				L	S	L	S
Nonirradiated	134	62	16	66	27	95	4
1-day post x-irradiation and dose (R)							
100	101	43	12	60	35	84	15
200	75	28	5	42	54	73	21
400	58	14	6	28	66	65	34
800	54	7	4	17	78	66	34

[a] Average from 10 or more mice is presented.
[b] L, lymphocytes; S, segmented cells. Young immature myelocytic cells, monocytes, and eosinophils are not tabulated but never amount to greater than 1% in spleen except in 200 R.

these activities generally returned to normal levels.

Discussion. It is apparent from the results of this study that whole body x-irradiation stimulates the *in vitro* HMS, peroxidative and bactericidal activities of mouse spleen cells. Increased HMS could supply some of the H_2O_2 required for the stimulated decarboxylation and bactericidal activities.

Recently it has been reported that the *in vivo* survival of *Brucella abortus* in the spleens of previously irradiated (700 R) CBA mice is significantly decreased as compared to normal (14). A possible explanation for this observation is our present finding of increased peroxidase activity in spleen lymphocytes from x-irradiated animals.

In attempting to explain the stimulated activities following *in vivo* x-irradiation, one must consider the significant change in lymphocyte/phagocyte ratio in the spleen. The percentage of lymphocytes has decreased while the percentage of polymorphonuclears has increased in both the spleen and the peripheral blood (Table III). Although myeloperoxidase of PMN has been reported to be approximately three times more potent on equal guaiacol unit bases than spleen cell peroxidase (SPO) from AKR mice (12, 13), the dramatic stimulations and increased bactericidal activities noted could not be fully accounted for by the percentage decrease in lymphocytes and a simultaneous percentage increase of PMN. For example, an MPO preparation from guinea pig peritoneal cells (over 90% PMN) at concentrations (i.e., 0.003–0.03 guaiacol unit in 2-ml reaction medium) identical to that used in this study killed approximately 1×10^3–1×10^5 bacteria. In this present study the granular (SLPO) preparation from the spleen cells of x-irradiated mice killed approximately 1×10^4–1×10^7 bacteria (Table I). The increased percentage of phagocytes in x-irradiated spleens may account for some of the increase in activities. However, lymphocytes are in the majority, and it seems reasonable to assume that lymphocyte peroxidase plays a major role in this increased bactericidal activity.

To examine further whether the increased percentage of granulocytes from x-irradiated mice spleen could contribute to the stimulated activity, we have attempted to remove the granulocytes from spleen cell preparations by allowing them to adhere to Falcon plastic tissue culture flasks. Complete removal of granulocytes, especially from x-irradiated mice spleen, has not yet been achieved. However, removal of 50% of the granulocytes from an irradiated spleen cell population had no effect on the bactericidal activity of the 20,000 *g* fraction. This occurred with different concentrations of peroxidase. For example, with 0.003 guaiacol units approximately 10^5 bacteria out of a total of 10^8 bacteria are killed. Removal of 50% PMN from this preparation and then testing 0.003 units, as previously reported, resulted in identical killing. Therefore, the major factor in the dramatic increase in decarboxylation and bactericidal functions by peroxidase from x-irradiated spleen cells must be attributed to enhanced activity of the lymphocytic peroxidase.

The decarboxylation and bactericidal activities of the 20,000 *g* granules collected fron non- and x-irradiated mouse spleen may be dependent on the rate of peroxidase-substrate interaction. For example, we have observed that freshly prepared granules from x-irradiated mice have significantly higher bactericidal activity than that of nonirradiated controls. If the preparation from nonirradiated control is frozen and thawed, the bactericidal potency of this preparation is increased. It appears that release of peroxidase from the granules is necessary for optimal utilization of the enzyme. The stress of *in vivo* x-irradiation may in general be compared to the stress of addition of phagocytizable particles. In the latter event, lysosomal enzymes are released from the granules to the phagocytic vacuoles (15). This stress could favor the utilization of the substrates by the released enzymes. It may be mentioned here that the viability (as judged by eosin dye exclusion) of spleen cells 1 day after 400 R x-irradiation is approximately 50% as compared to that of 85–90% for spleen cells from nonirradiated controls. X-irradiation perhaps alters cellular permeability and, therefore, favors the interaction of the peroxidase and its substrates (H_2O_2, guaiacol, bacteria, etc.) and this may account for the enhanced activities

observed. It appears that the spleen cell enzyme is made easily available to the substrates (H_2O_2, amino acids, chloride, bacteria, etc.) by x-irradiation, by lysis (freezing and thawing), or by isolation and purification. It is pertinent to mention here our observation that the bactericidal activity of a peroxidase isolated and partially purified from normal mouse spleen cell is equivalent to that of 20,000 *g* granules from x-irradiated mouse spleen (0.03 guaiacol unit of the partially purified peroxidase kills 10^7 *E. coli* in 30 min at 37°) (16).

Optimal activity of SLPO, as determined by guaiacol oxidation, occurs at a pH different than that routinely used for MPO. Concentration of substrates (H_2O_2 and guaiacol) required for SLPO is also different than those used for MPO. However, both SLPO and MPO have strong affinities for H_2O_2 and chloride. Both chlorinate and decarboxylate amino acid and both enzymes can participate in peroxidase-mediated bactericidal activities (6, 8, 10). Recently, we have isolated a peroxidase from a preparation of human peripheral blood containing 100% lymphocytes. These results will be reported in a later publication.

Our results indicate that a lymphocyte peroxidase, not MPO, is responsible for the antimicrobial and biochemical activities of spleen cell preparations. It would be of interest to investigate whether the lymphocyte enzyme is organ-specific or species-specific or universally present in all lymphocytic cells and to determine its role in lymphocyte function.

Summary. A 20,000 *g* pellet obtained from the homogenate of CD1 mouse spleen cells has measurable peroxidase and bactericidal activities. Whole body x-irradiation stimulates the *in vitro* peroxidative and bactericidal activities of the spleen cells. These cells do not phagocytize. However, addition of polystyrene latex particles to spleen cells from non- or x-irradiated mice, increases glucose oxidation. The extent of this stimulation (three- to fourfold) is less than that of PMN (six- to tenfold). Interaction between particles and lymphocyte surface may be the cause of this stimulation. The increased bactericidal activity due to x-ray is attributed mainly to an increase in the peroxidase activity of the lymphocytes. Surface (membrane) action rather than phagocytosis may be involved in the bactericidal process. The spleen lymphocyte peroxidase is distinct from myeloperoxidase in several respects.

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