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Studies on *In Vitro* Models of Cellular Immunity: The Role of T and B Cells in the Secretion of Lymphotoxin[1]

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When normal murine spleen cells were treated with anti-theta serum and complement, they failed to produce LT or synthesize cellular DNA when stimulated *in vitro* with PHA. Theta-positive cells were responsible for LT production in spleens removed from X-irradiated and bone marrow- or thymus-reconstituted animals. Finally, spleens from congenitally athymic Nu/Nu mice failed to produce LT when stimulated with pokeweed mitogen or phytohemagglutinin.

INTRODUCTION

Understanding the contribution of a specific lymphoid cell or cell type in the generation of an immune response is of central importance. Attempts to provide a functional characterization of lymphoid cells have been in large measure restricted to immediate-type responses in mice (1, 2). Reconstituting immunologically deficient animals with immunocompetent transplants has revealed that an interaction is required between thymus-dependent (T cell), and thymus-independent (B cell) lymphocyte subpopulations before stimulation of antibody synthesis to certain antigens can occur (3). Additional studies are required to determine the role of B and T cells in delayed-type hypersensitivity reactions.

The development of *in vitro* methods for the study of cell-mediated immune (CMI) reactions has increased our understanding of the mechanisms involved in direct lymphocyte cytotoxicity (4), tumor immunity (5), and delayed hypersensitivity states (6). The work of numerous investigators, using these same methods, have led to the concept of the lymphokine system (7), a group of cell-free substances secreted by the activated lymphoid cell during CMI reactions (8). These materials, once released, may be the effector molecules which mediate cellular hypersensitivity. The *in vitro* release of effector molecules can be induced by many treatments; several of which are (a) sensitive lymphoid cells cultured in the presence of the sensitizing antigen (9), or (b) normal cells cultured with mitogenic

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agents, e.g., phytohemagglutinin (PHA), concanavalin A (Con-A), (1, 15). While physically unrelated, these treatments all cause lymphocyte transformation. In the cell-free medium from a single activated lymphocyte culture, multiple activities can be detected, which can be assigned to purportedly different molecules (10). The present *in vitro* studies were designed to reveal the role of T and B lymphocyte subpopulations in the induction of DNA synthesis and lymphotoxin (LT) secretion, two parameters considered to be *in vitro* measures of cellular immunity by mitogen-stimulated murine lymphoid cells.

MATERIALS AND METHODS

Animals. A/J mice were purchased for these experiments from the laboratories of R. B. Jackson, Bar Harbor, ME. Congenitally athymic mice (Nu/Nu), and their normal litter mates (Nu/+, or +/+) were secured from the animal colonies of Dr. Norman B. Reed, Montana State U., Bozeman, MT.

Cell cultures. Media employed in these studies consisted of RPMI 1640 (Flow Laboratory, Los Angeles, CA) supplemented with streptomycin (100 μg/ml), penicillin (100 U/ml), mycostatin (50 μg/ml), and 5% fetal bovine serum (Flow Laboratories, Los Angeles, CA). The serum was heat inactivated at 56°C, for 30 min before use. Cell cultures of mouse lymphoreticular tissues were established as previously described (9). Each culture contained 5×10^6 lymphoid cells in a 1-ml volume, in 16 × 125-mm disposable glass screw-capped culture tubes. The tubes were maintained under an atmosphere of 5% CO_2 in air, and incubated upright at 37°C.

Stock L cell cultures were maintained by serial passage in 16-oz prescription bottles, as described previously (15). These stock cultures were treated with trypsin to free the attached monolayer cells. Trypsin was diluted by the addition of culture medium, the cells were washed once by sedimentation at 300*g* and the pellet was resuspended in medium to a final volume of 10 ml. Vital cells counts were performed in a Neubauer chamber in 0.1% Eosin Y, and the original cell suspension adjusted to a density of 50,000 L cells/ml. One milliliter of this cell suspension was added to each individual culture tube, then the tube was gassed with a mixture of 5% CO_2 in air, capped, placed at a 5-degree angle, and incubated at 37°C. After 18–24 hr, each tube culture was examined and only those having uniform monolayers were used.

Preparation of splenic lymphocyte suspensions. A-strain mice were sacrificed by cervical dislocation, the spleens removed aseptically, and placed in sterile petri plates containing culture medium. The spleens were teased apart with sterile forceps in order to liberate the cells. This treatment produced a mixture of single cells, cellular aggregates, and tissue fragments. To obtain a single cell suspension the mixture was removed from the petri by pipet, and placed in conical centrifuge tubes at 4°C. The suspension was then permitted to stand for 10 min, after which the single cell-rich supernatant fluid was decanted into a second conical centrifuge tube and spun at 300*g* for 10 min. The cell pellets obtained by this procedure were resuspended in 1640 + 5% FBS. Aliquots of the preparation were taken and diluted in 0.1% Eosin Y, placed in a hemocytometer, and both the differential and total cell counts determined. The majority of these cells were small lymphocytes, and were 85–95% viable.

Mitogens. Phytohemagglutinin-P (PHA) was obtained from Difco Labs, Detroit, MI (Lot 533815), and pokeweed mitogen (PWM) (control No. 29094 H) was obtained from Grand Island Biological Company, Oakland, CA 94609.

Preparation of anti-theta serum. Anti-theta sera were induced by repetitive injections of C3H/St mouse (L. Strono Res. Found., San Diego, CA) thymocytes into AKR mice according to the method originally described by Reif and Allen (11). Inasmuch as immunized animals varied considerably in their individual antiserum titers against the theta antigen, they were bled individually and each serum sample assayed for anti-theta activity, using release of ^{51}Cr from labeled thymus cells as a measure of cytotoxicity (12). Only sera with a reciprocal titer greater than 300 were pooled and used in the present studies.

Antiserum treatment of lymphoid cells. Eight million spleen cellls were suspended in 1 ml RPMI 1640, containing 13% immune or control antiserum. After 45 min at 4°C, the cells were washed once in 1640 by sedimentation and resuspension. The cell pellet was then resuspended in 1 ml of an 8% solution of normal guinea pig serum in saline and further incubated at 37°C for 30 min. The cells were washed once in 5 ml 1640 and total viable cell counts performed in 0.1% Eosin Y.

Thymectomized, X-irradiated, bone marrow-reconstituted mice. Male A/J mice were thymectomized as adults (6–7 weeks of age) according to the method of Miller (13). Two weeks after surgery, the animals were given 1000 R whole-body X-irradiation using opposing 220 kV Picker machines set 160 cm apart. Operations took place at 210 kV and 15 mA with an inherent filtration equivalent to 0125 cm Cu and 1 mm Al. Within 3–4 hr after irradiation, the A/Jax mice were injected intravenously with syngeneic 15×10^6 bone marrow transfer. At the time of sacrifice (12 weeks postgrafting), each animal was checked for the presence of thymus remnants and mice with remnants were not employed in these studies.

Lymphotoxin assay. The amount of LT in test and control media was determined by ascertaining its cytolytic activity on target L cell monolayers. Duplicate tube cultures were exposed to cell-free supernatant fluids from the different representative cultures. Test and control media were in continuous contact with assay monolayers for 48–72 hr, and thereafter replaced with 0.5 μCi/ml ^{14}C-amino acid (Schwartz Bioresearch, Van Nuys, CA) labeling medium. This assay is based on the ability of the remaining viable cells to incorporate ^{14}C-labeled materials into TCA-precipitable protein over an interval of 30 min as previously described (9). Toxicity is expressed in direct counts or as the percentage value of cpm test/cpm control $\times$ 100. Both diluted and undiluted supernatant fluids were tested for activity. In kinetic experiments, it was required that a number of the first supernatant fluids collected be stored at 4°C until samples of all the various points were collected.

Assay for DNA synthesis. The incorporation of tritiated thymidine (^3H-TdR) into DNA was assayed as previously described (15). Two tenths-milliliter volume of a 20-μCi ^3H-TdR/ml solution (Schwartz Bioresearch, Van Nuys, CA) was added to the different cell cultures 4 hr before their termination. Elsewhere, it has been demonstrated that this level of the radioisotope does not adversely affect normal cell functions (16). Nucleic acids were extracted by the hot phenol method of Sherrer and Darnell (17), and the amount of radioactive material present was determined in a Beckman Scintillation Counter, as has been previously described (9).

RESULTS

The first experiments were designed to determine the levels of PHA which induce maximum *in vitro* LT production and lymphocyte DNA synthesis. The spleen cells from untreated A-strain mice were cultured at a density of 5×10^6 cells in 1-ml cultures in the presence of various doses of PHA. Nucleic acid synthesis was measured by the addition of 2 μCi/ml tritiated thymidine (^{3}H-TdR) directly to the tube cultures 68 hr after their initiation. The amount of label present in the phenol-extracted cell pellet was determined after 4 hr of incorporation. The supernatant media was assayed for LT activity. The data shown in Fig. 1, A and B, are the results of one of five separate tests. In each experiment replicate tubes were included at each dosage. From these data, it is clear that DNA synthesis and LT production reached maximal levels in cultures stimulated with 10–20 μg PHA/ml.

The next experiments were designed to attempt to determine the type of lymphoid cell responsible for the initiation and/or production of LT in mitogen-stimulated cultures. The spleen cells taken from A-strain mice were treated with antitheta serum and guinea pig complement in order to prepare a B cell-rich cell suspension. These cells were placed in culture with 20 μg/ml PHA, using the above conditions, and were later assayed for both DNA synthesis and LT production. The results of three separate experiments, including replicate tubes at each point have been

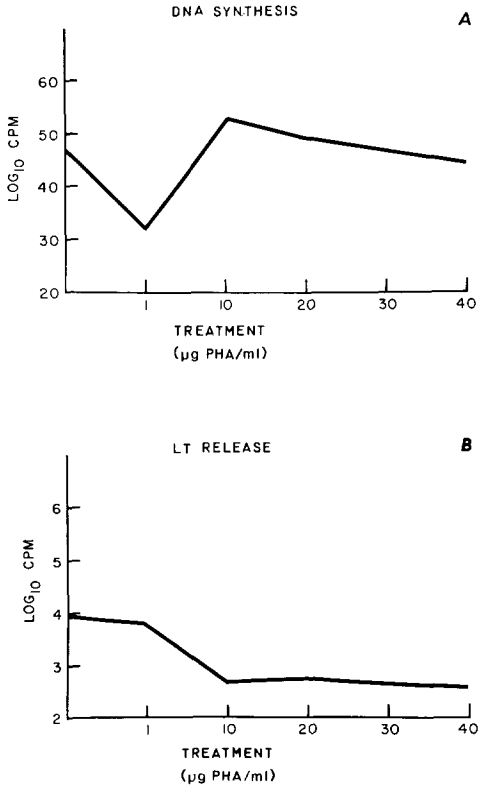

FIG. 1. The effect of different doses of PHA on cellular DNA synthesis and *in vitro* secretion of LT by nonimmune A/Jax lymphoid cells: (A) DNA synthesis was assayed at 72 hr as described in the text, (B) LT secretion, the amount of LT in the medium as determined by measuring the capacity of L cell monolayers to incorporate ^{14}C-AA, as described in the text.

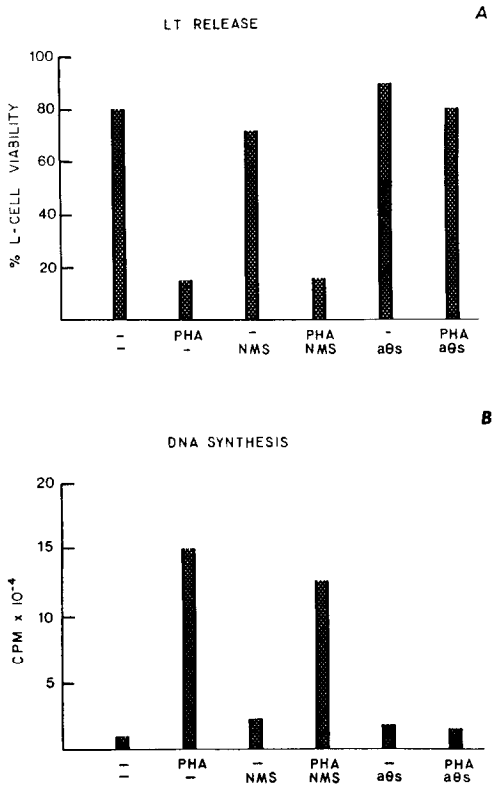

FIG. 2. The effect of antitheta serum on DNA synthesis and LT secretion in cultures of PHA-stimulated normal A/Jax spleen cells. A. DNA synthesis. Cells were treated with various control and test antisera, stimulated with PHA and tested for their capacity to incorporate ^{3}H-TdR after 72-hr incubation. "-" indicates no treatment. PHA = 20 μg PHA added/tube, NMS = normal mouse serum and complement treated, aθs = antitheta and complement treatment. B. LT secretion. Cell-free media from test and control cultures labeled above were tested for LT activity on target L cell cultures as described in the text.

averaged and are shown in Fig. 2, A and B. The results indicate that antitheta serum and complement treatment removed or inhibited the cells required for PHA-induced *in vitro* DNA synthesis and LT secretion.

The previous data indicated that PHA-induced DNA synthesis and LT secretion required the presence of theta-positive cells. However, it seemed appropriate to employ another test system to further substantiate this observation. Cell suspensions rich in T or B cells were obtained from normal or immunologically reconstituted irradiated A/Jax animals. As controls, bone marrow, spleen, and thymus cells acquired from normal mice were cultured with various levels of PHA. These experimental findings were then compared with the results obtained with spleen cells from the reconstituted animals. The data shown in Fig. 3, reveal that mitogen-stimulated spleen cells obtained from animals which were reconstituted with B cell-rich bone marrow transplants, synthesized less cellular DNA than did their normal controls. However, the levels of LT production appeared to be similar in PHA-stimulated cultures obtained from either bone marrow reconstituted or normal mice. In contrast, the spleen cells from mice restored with transplants of syngeneic thymocytes did not respond to PHA stimulation. When bone marrow cells and thymo-

cytes taken from untreated donors were placed directly in culture with different amounts of PHA, there was no evidence of the activation of either DNA synthesis or LT secretion.

The observation that spleen cells from bone marrow-restored animals were able to produce LT as normal spleen cells was further substantiated by a series of kinetic experiments. The experiments were designed as follows: 10-ml cultures were initiated with mitogen in Falcon plastic flasks, then 2-ml aliquots were removed at 12-hr intervals, cleared of both cells and debris by centrifugation, and stored at 4°C until all samples were collected. The cytotoxicity assays were performed as usual with the exception that both test and control media were serially diluted by 2-fold steps with fresh media to determine the level of LT production. The end point was the dilution which resulted in reduction of cell numbers below 50% of the untreated control. While these data are not shown, spleen cell cultures from either normal or bone marrow-reconstituted animals, when cultured with PHA secreted identical levels of LT. To eliminate any possible contaminating T cells from the spleens of B cell-reconstituted animals in the next experiment, the spleen cell suspensions were first exposed to antitheta serum and complement, then cultured with PHA. The

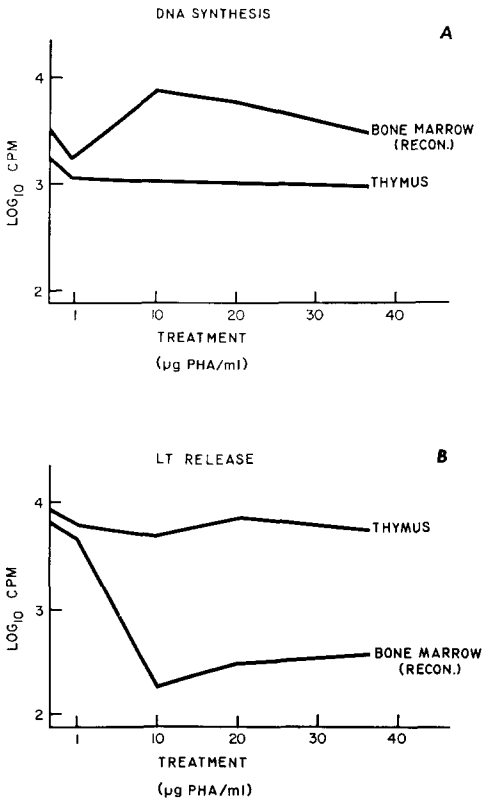

FIG. 3. Comparison of the capacity of normal thymus cells or spleen cells from bone marrow-reconstituted mice to synthesize DNA and secrete LT after stimulation *in vitro* with PHA. A. DNA synthesis. PHA and control cells were assayed for capacity to incorporate ^{3}H-TdR into cellular DNA after 72 hr. B. LT secretion. The cell-free media from stimulated and non-stimulated controls were tested for activity on target L cell monolayer cultures as described in Methods.

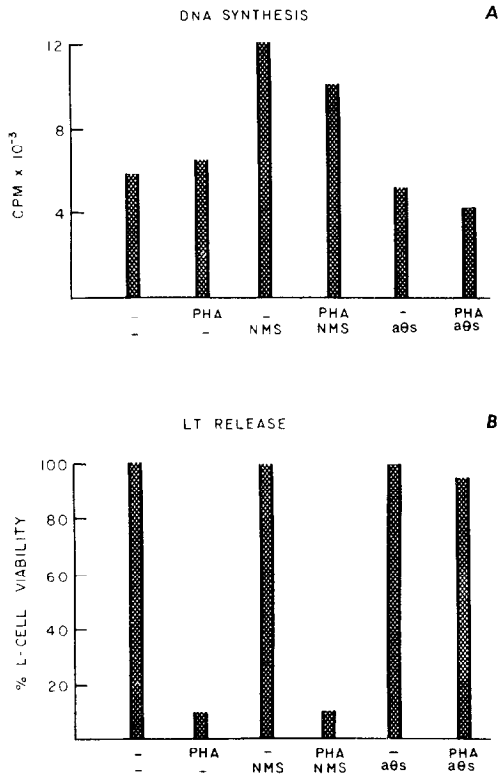

FIG. 4. Synthesis of DNA and secretion of LT by PHA-stimulated spleen cells from bone marrow-reconstituted A/Jax mice after treatment with control and antitheta serum. A. LT secretion. B. DNA synthesis.

data shown in Fig. 4, reveals that a reduction in DNA synthesis with the virtual elimination of the capacity of the cells to secrete LT resulted from the antiserum treatment.

Because of the antiserum treatment used in the above experiments to eliminate theta-positive cells, the possibility remained that surviving cells failed to secrete LT, because of nonspecific alterations in their inducibility. Alternatively, the unrespon-

TABLE 1

MITOGEN-INDUCED BIOCHEMICAL RESPONSES IN SPLEEN CELLS FROM NUDE MICE AND THEIR NORMAL SIBLINGS[a]

Animals	Mitogen	(^{3}H-DNA cpm) lymphocyte		^{14}C-Protein lymphocyte		% L cell viability	
		Control	Test	Control	Test	Control	Test
Normal	PHA	3,326	10,792	3,352	3,033	52	6
Nude	PHA	4,404	1,958	2,858	2,275	82	100
Normal	PWM	1.150	40,902	4,062	6,605	100	6
Nude	PWM	2,804	1,267	N.D.	N.D.	85	100

[a] The data expressed are averages of three to five cultures at each point.

siveness could simply reflect the inefficacy of PHA to stimulate B cells (19). To attempt to clarify the above situation, we decided to examine the reactivity of spleen cells from naturally occurring thymus-deficient mice. Mice congenitally athymic (nude mice), and their normal (Nu/+ or +/+) siblings were employed in the following studies. The culture conditions and methods of culture were essentially identical to those when normal spleen cells were examined; however, in addition to PHA, pokeweed mitogen was used, because it has been reported to stimulate B cells (19), and it also stimulates LT secretion. The results shown in Table 1 represent the averages obtained in two separate tests. Each employed two nude mice and two of their normal siblings. In each experiment, as many as six replicate cultures were included to represent each test condition. These data reveal that spleen cells taken from nude mice failed to produce LT or undergo DNA synthesis when cultured in the presence of either mitogen. Whereas their normal siblings gave a vigorous response.

DISCUSSION

The optimal activating dose of PHA for normal murine spleen cells *in vitro* ranged between 10 and 20 μg/ml. However, thymus cells cultured with the same levels of mitogen did not respond with either DNA synthesis or LT secretion. The observation that thymus cells are refractory to mitogen or antigen activation is in agreement with the results of numerous investigators measuring the capacity of these cells to participate directly in other immunologic reactions (4, 20–22).

Removal of theta-positive cells by antiserum treatment completely ablated the capacity of PHA to induce murine spleen cells to LT secretion or DNA synthesis *in vitro*. However, spleen cells from thymus-reconstituted X-irradiated syngeneic C57BL/6 mice were refractory to PHA stimulation. This was surprising and can only be explained on the basis that the transplanted thymus cells were not at the level of differentiation to allow a response. However, these experiments were difficult to interpret, because of nonspecific toxicity in supernatant fluids from both the stimulated and unstimulated cultures. In contrast, spleen cells from irradiated animals reconstituted with syngeneic bone marrow were inducible with PHA to DNA synthesis and LT secretion. This response also required T cells, for when they were removed with antitheta serum, the response was completely ablated. This finding was interesting, for only 5% of the cells taken from spleens of the marrow-reconstituted animals were destroyed by the antitheta serum treatment. In comparison, 50% of the cells obtained from the spleens of a normal animal are sensitive. Yet, even though there were only 10% of the reactive T cells present in the marrow-reconstituted animal, the actual amount of LT released was the same as that found in stimulated cultures from the normal animal. If release is due solely to activated T cells, they must only be a small percentage of the total T cell population present in the normal spleen. The other alternative is that a very small number of T cells are required as helper cells to initiate the B cell, which then secretes LT.

In order to rule out any nonspecific effects of antiserum and complement treatment, we tested the reactivity of congenitally athymic (Nu/Nu) mice. The spleen cells obtained from these animals were completely unresponsive to treatment with PHA or PWM. In contrast. both DNA synthesis and LT secretion occurred at normal levels in PWM- and PHA-stimulated cultures from the normal sibling heterozygous (Nu/+) mice.

The present results obtained using both *in vivo* and *in vitro* techniques unequivocally demonstrate that the release of LT by mitogen-stimulated spleen cells requires T cells. Moreover, it seems that only a small percentage of the T cell population are required for the response. These cells may be the LT-secreting cell. However, we cannot as yet rule out the possibility that they act as helper cells to initiate B cells, which then secrete LT. T cells have been shown to be central as helper cells in the initiation of antibody formation *in vitro* (3). It appears that this helper function involves synthesis and secretion of soluble effector molecules, which in some way permit induction of the B cell. It has been suggested that killer lymphocytes in mice are T-type cells (22). The finding that LT secretion requires T cells suggests that the killer T cell and the LT-secreting cell may be one and the same, and that the mechanism(s) of destruction may, indeed, involve local secretion of a soluble cell toxin(s).

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