Failure of Lymphocytes from Guinea Pig Bone Marrow to Respond to Phytohemagglutinin (35315)

ALAN WINKELSTEIN
(Introduced by Dane R. Boggs)
Department of Medicine, University of Pittsburgh School of Medicine, and Montefiore Hospital, Pittsburgh, Pennsylvania 15213

The bone marrow appears to be an important source of cells classified morphologically as lymphocytes. However, there is accumulating data suggesting that these cells are functionally distinct from the bulk of peripheral lymphocytes. The latter, comprising cells in the lymph nodes, spleen, blood, and thoracic duct are characterized *in vivo* by; (i) a slow turnover rate with a prolonged intermitotic interval; (ii) proliferation in response to antigen stimulation; (iii) immunologic compentency; (iv) recirculation (1, 2). *In vitro*, these cells characteristically respond to the nonspecific mitogen, phytohemagglutinin (PHA) by transforming into actively proliferating blasts; changes thought to simulate those occurring in the regional lymph nodes of intact animals after antigen challenge (3).

In contradistinction, bone marrow lymphocytes, *in vivo*, are believed to be rapidly replicating cells (4, 5); they are weak immunologic effectors (6) and the normal flow of cells is primarily to the thymus rather than to the recirculating pool of peripheral lymphocytes (7). Evidence has been presented suggesting that these cells differentiate into antibody-producing lymphocytes; this maturation process is directed by the thymus (8).

To further delineate the functional capacity of marrow lymphocytes, guinea pig bone marrow cultures were assayed for increased DNA synthesis following PHA stimulation. These data indicate that, in this species, PHA stimulation does not induce an increase in proliferative activity. This suggests that marrow lymphoid cells may not be responsive to the same *in vitro* stimulus as peripheral lymphocytes.

Material and Method. Male, Hartley strain guinea pigs, weighing between 300 and 400 g, were used in these experiments. Animals were killed by ether asphyxiation. Using sterile techniques, the spleen, thymus, and all visible lymph nodes from the mesenteric, periaortic, cervical, and axillary areas were removed, minced in media TC-199, enumerated, centrifuged, and diluted to appropriate volumes with fetal calf serum. No animal with demonstrable infection was included in this study.

Bone marrow cells were obtained by removing both femurs, cutting both the proximal and distal epiphyseal plates, inserting the bone into a polyethylene tube and expelling the marrow contents into media TC-199. These cells were enumerated, centrifuged at 1200 rpm, and appropriately diluted in fetal calf serum. All determinations were performed in triplicate in sterile, sealed serum vials. Each culture contained 4 ml of TC-199 and 1 ml of fetal calf serum, with 100 units/ml of penicillin and 100 μg/ml of streptomycin. Stimulated cultures received either 0.1 ml of a 2:1 or 10:1 dilution of phytohemagglutinin (Burroughs-Welcome). For convenience, the concentration of PHA is expressed below as either 0.05 or 0.01 ml. Each culture contained 10 to 15 $\times$ 10^6 cells. Cultures were incubated at 37° for 2 days except in those cases that were serially harvested. Two hr prior to harvesting, tritiated thymidine (^{3}HTdr) (1.0 μCi/5 $\times$ 10^6 cells; New England Nuclear; sp act 6.7 Ci/mmole) was added.

Cultures were harvested in the following manner. The cells were separated from supernate by centrifugation, washed twice in phosphate buffered saline (pH 7.4), three times in 5% cold trichloroacetic acid, and once in methanol. The insoluble residue was dis-

TABLE I.

	^{3}H Tdr uptake							
	Lymph nodes		Spleen		Thymus		Marrow	
PHA conc	No.	(dpm/10^6 cells)	No.	(dpm/10^6 cells)	No.	(dpm/10^6 cells)	No.	(dpm/10^6 cells)
0.05	25	547 ± 152	9	119 ± 70	6	4 ± 2	24	669 ± 109
0.01	27	355 ± 109	6	124 ± 52	3	10 ± 3	27	754 ± 112
Unstimulated	32	5.3 ± 1.0	9	14 ± 4	5	1.0 ± 0.3	29	715 ± 114

[a] Values represent the mean ± 1 SE for the indicated number of animals. All assays were performed after 2 days in culture. ^{3}H Tdr, 1.0 μc per 5 × 10^6 cells was added two hours prior to harvesting.

solved in 1 ml of Soluene and added to Liquiflor-toluene scintillation fluid. Each sample was counted for 5 min in a Packard tricarb liquid scintillation counter and results were corrected for background and quenching. The final values [counts per minute per million cells (dpm/10^6 cells)], based on initial cell count, represented the average of the three separate determinations. In general, replicative cultures agreed within 10% of each other.

Serial studies of bone marrow proliferative activity were performed to assess progressive changes in the rate of cell replication. An initial ^{3}HTdr uptake was determined by incubating marrows from seven animals for 2 hr; these specimens were then harvested in the usual manner. Cultures from nine animals were harvested after 1 day and four after 3 days. A total of 24 stimulated and 32 unstimulated marrow cultures were evaluated on day 2. For comparison, serial lymph node cultures were harvested on days 1, 2, and 3. The mean uptake on the second day represents the average of 25 animals. Five specimens were cultured for 1 day, and three for 3 days. In all studies, the isotope was added 2 hr prior to harvesting.

An additional series of mixed cell cultures, containing lymph node and marrow cells were evaluated to estimate the relative sensitivity of the assay. In each of five experiments three dilutions of lymph node cells were employed; the final lymph node cell concentrations were 10, 25, and 50%. Activity was expressed as the percentage of the unstimulated replicate control.

Results. The response of lymph node, spleen, thymus, and marrow cell cultures of phytohemagglutinin (PHA) are summarized in Table I. To minimize any inhibitory effect of high PHA concentrations while maximizing its stimulatory activity, each tissue was separately evaluated with two concentrations of this mitogen. Thus, for each cell suspension, triplicate cultures were stimulated with 0.05 ml of standard PHA solution, three other cultures with 0.01 ml. Three additional cultures served as unstimulated controls.

There was a significant increase in the uptake of ^{3}HTdr in lymph node cultures at both concentrations of PHA. Very low activity was noted in the unstimulated controls. The average increase in ^{3}HTdr incorporation was approximately 100-fold with either concentration of PHA. No stimulated cultures showed less than a 10-fold increase over the control value. Lesser amounts of PHA resulted in consistently suboptimal stimulation; whereas higher concentrations of this mitogen appeared to be toxic to the cultures. Maximum stimulatory activity was demonstrated after 2 days in culture; increasing the duration of culture to 3 days appeared to result in significant cell death.

Although not extensively evaluated, cultures of spleen cells also showed a significant increase in proliferative activity following PHA stimulation.

Bone marrow cultures showed *in vitro* proliferative activity significantly different from either lymph node or spleen cells. In contrast to the negligible ^{3}HTdr incorporation observed in unstimulated lymph node cultures, marrow cells exhibited a high rate of DNA synthesis. This activity may reflect the rapid

TABLE II. Percentage ^{3}H Tdr Uptake in Mixed Lymph Node–Marrow Cultures.

Lymph node cells (%)[a]	^{3}H Tdr uptake (%)[b]
0	100 ± 9
10	128 ± 11
25	137 ± 13
50	167 ± 12

[a] Lymph node cells expressed as percentage of the total cells initially added to culture.

[b] Percentage uptake of PHA-stimulated culture compared to unstimulated cohorts ± 1 SE. Each value represents the average of five experiments.

turnover of hematopoietic cells. Significantly, the rate of replication was not increased in PHA-stimulated cultures, suggesting that the majority of lymphoid cells in the marrow do not proliferate in response to the mitogen.

Failure to observe increased ^{3}HTdr uptake in PHA-stimulated cultures could not be attributed to a deficiency of lymphoid cells in the marrow. By morphologic criteria, 38% of marrow cells were small lymphocytes; and additional 2% were medium or large lymphoid cells. These results are consistent with other investigations which report 37–44% of guinea pig marrow cells to be lymphocytes (9).

The high rate of cell replication in unstimulated cultures might obscure a minor population of PHA response of cells. Nevertheless, experiments using mixed lymph node–marrow cells demonstrate the relative sensitivity of the assay system. These data, summarized in Table II, indicate that in PHA-stimulated cultures the addition of lymph node cells resulted in a measurable increase in ^{3}HTdr incorporation.

Failure of marrow cells to respond to PHA was further substantiated by comparing individual stimulated cultures with their unstimulated cohorts. Considering the ^{3}HTdr incorporation of each unstimulated culture to represent a 100% activity, the average uptake of the stimulated replicates was 95.3 ± 3.9%. Grouped by quartiles, in 36 of 51 determinations, the activity ranged from 76 to 125%. Only four values were greater than the 125% while 11 were less than 75%. These results further demonstrate the lack of increased proliferative activity of marrow cells in response to PHA.

Serial measurements of ^{3}HTdr uptake (Fig. 1) indicate that the maximum bone marrow proliferative activity occurs during the first 2 hr of culture. There is a progressive decline of similar magnitude in isotope uptake over a 3-day period in both the PHA-stimulated and unstimulated cultures. At no point was the activity in the unstimulated cultures significantly different than the controls.

In these animals, the thymus was involuted and a sufficient number of cells was obtained in only six experiments. These data showed, however, that thymic lymphocytes respond poorly to PHA. Maximum ^{3}HTdr incorporation occurred during the first 2 hr of incu-

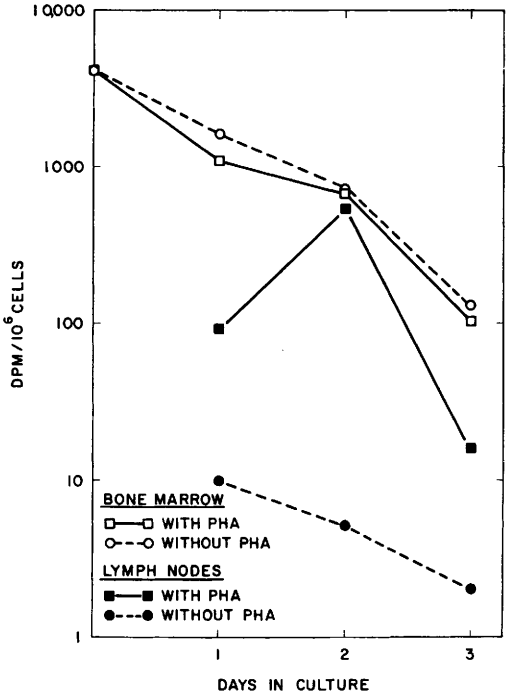

FIG. 1. Serial proliferative activity in PHA-stimulated and unstimulated cultures: Maximum uptake of ^{3}HTdr occurs in the first 2 hr of culture; there is a progressive decline in activity over the 3-day period. No significant difference between the PHA-stimulated and unstimulated was demonstrated. For comparison, the ^{3}HTdr uptake of serial lymph node culture is shown; ^{3}HTdr was added 2 hr prior to harvesting.

bation. After 2 days in culture, there was slightly greater radioactivity in the PHA-stimulated cultures than the unstimulated controls.

Discussion. These data indicate a marked difference in the *in vitro* replicative activity of guinea pig lymph node and bone marrow cells. Lymph node cells clearly responded to phytohemagglutinin (PHA) stimulation; after 2 days the incorporation of a pulse of ^{3}HTdr was approximately 100 times greater than that of the unstimulated controls. In contrast, no difference was observed in isotope incorporation between stimulated and unstimulated marrow cultures. This suggests that marrow cells do not proliferate in response to this nonspecific mitogen.

Failure to demonstrate increased proliferative activity cannot be attributed to a lack of lymphoid cells. In our series, the guinea pig marrow contains abundant lymphoid cells; data that are in agreement with the finding of others (9). Thus, the failure to demonstrate an increase in proliferative activity by marrow cultures following PHA stimulation is not due to either a deficiency in the number of lymphoid cells or overall unresponsiveness of guinea pig lymphocytes to this mitogen. These observations suggest a majority of lymphoid cells in the marrow are not responsive to PHA.

Eurenius *et al.* (10) recently demonstrated that high concentrations of PHA are inhibitory to lymphoid cell replication in the guinea pig, a finding confirmed by our observations. Because of this, stimulating activity was routinely evaluated at two concentrations of PHA. As shown by the data, no significant difference in the degree of stimulation was noted with either concentration. Lesser or greater amounts of PHA produced submaximal stimuation. Two-day cultures were optimal; at 3 days, considerably lower proliferactive activity was observed.

In serial marrow cultures, maximal ^{3}HTdr incorporation occurs during the first 2 hr of incubation; thereafter decreasing levels of isotope incorporation are noted. This activity can be attributed primarily to hematopoiesis. However, as marrow lymphocytes *in vivo* constitute an actively replicating cell population (4, 5), a proportion of this initial activity may represent lymphopoiesis. By 2 days, 80 to 90% of the total activity has been dissipated. In the serial cultures, no difference between ^{3}HTdr incorporation in PHA-stimulated and unstimulated cultures could be demonstrated at any time.

The assay system is not sufficiently discriminating to detect a minor population of response of lymphocytes in the marrow. However, as shown in the mixed lymph node–marrow cultures, an increase in ^{3}HTdr uptake can be demonstrated following PHA stimulation if a significant number of responsive cells is present. Based on the number of marrow lymphocytes and the response of lymph node cells to PHA, a minimal increment of approximately 25% in ^{3}HTdr incorporation would be anticipated if the bulk of these cells were stimulated. As these data demonstrate, no increase could be detected either when results were averaged or individual stimulated cultures were compared to their unstimulated cohorts.

The failure of marrow lymphocytes to respond to PHA may be attributed to either lymphoid cell heterogeneity or difference in replicative activity at the onset of culture. Whereas peripheral lymphocytes are in an intermitotic phase, marrow cells are actively replicating (4, 5). This proliferative activity may render marrow lymphocytes incapable of responding to exogenous stimuli *in vitro*. Alternatively, marrow lymphocytes may represent a population of cells distinct from the bulk of peripheral lymphocytes and thus not be responsive to the same exogenous stimuli that promote *in vitro* lymphopoiesis.

Recently, Levine and Claman reported that in mice, PHA stimulation resulted in no increase of proliferative activity (11). In another series, Tridente *et al.* (12) found that PHA induced minimal and probably insignificant stimulation in mouse marrow cultures.

These data suggest that marrow lymphoid cells exhibit replicative patterns similar to that observed in the normal thymus *in vitro* (13, 14). Thymus cell cultures show an initial high rate of cell replication; only a minor population appears to be responsive to PHA. The preliminary results of thymus cultures in this study are concordant with these data.

These *in vitro* findings are consistent with

data regarding the physiological role of marrow lymphocytes. Some of these cells probably subserve functions other than as components of the immune system. For example, they may be stem cells for erythropoiesis (15). Some of the marrow lymphocytes are believed to be precursors of immunologically competent peripheral lymphocytes (16, 17). Davies *et al.* (18) have shown that the marrow is the primary source of cells destined to ultimately form antibody-producing cells. However, in adult, lethally-irradiated and thymectomized mice, marrow lymphoid cells are incapable of effecting an immune response in the absence of thymic influences (19). These cells require one or more thymic factors to develop into immunologically competent cells.

Furthermore, the participation of cells originated in the thymus may be essential for some immunological responses (8). *In vitro*, neither thymic nor marrow cells alone are capable of generating an antibody response to sheep red blood cells, but a combination of the two cell types is capable of responding to this antigen.

Current concepts depict the marrow lymphoid cells *in vivo* as a rapidly replicating cell line. The normal migration of these cells is to the thymus where replicating continues and the cells are acted upon by thymic factors to mature into immunologically competent cells (7). These cells then seed peripheral lymphatic tissues where combination with both macrophages and cells primarily of thymic origin forms an "antigen sensitive unit" capable of responding to immunologic challenges. The macrophage and thymic-derived lymphocytes are primarily involved in antigen recognition; the marrow-derived cell ultimately forms the immune effector cell (8).

Since the lymphoid cells of the marrow have not achieved immunologic competency, it is not surprising that these cells are incapable of responding to PHA. This mitogen is believed to stimulate only those cells which have differentiated into long-lived peripheral lymphocytes. The *in vivo* replication of precursor cells, either in the marrow or thymus, appears to be controlled by regulatory mechanisms quite different from peripheral lymphocytes. These differences between proliferative stimuli are reflected *in vitro* by a failure of either thymic or marrow cells to replicate in response to a potent stimulus for *in vitro* peripheral lymphopoiesis, phytohemagglutinin.

Summary. The nonspecific mitogen, phytohemagglutinin (PHA) did not induce increased cell proliferation in guinea pig bone marrow cell cultures. PHA was demonstrated to be a potent stimulus for lymph node cell replication. Unstimulated marrow cultures showed a high rate of ^{3}HTdr incorporation, activity attributed primarily to hematopoiesis. This activity was not increased following PHA stimulation. Guinea pig bone marrow contained approximately 40% lymphoid cells; thus, failure to demonstrate a response could not be attributed to a deficiency of these cells. Furthermore, in mixed lymph node–bone marrow cell cultures, the addition of responsive lymphocytes resulted in measurable augmentation of isotope incorporation. These results are consistent with concepts regarding the heterogeneity of lymphoid populations; the majority of marrow lymphocytes appear to be unresponsive to this stimulus of *in vitro* peripheral lymphopoiesis.

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