PHYTOHEMAGGLUTININ-INDUCED DIFFERENTIATION
AND BLASTOGENESIS OF PRECURSOR
T CELLS FROM MOUSE BONE MARROW*

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The existence of precursor T cells in the bone marrow (1-3) is the one of the
more unexpected recent findings in cellular immunology. Their residence in this
tissue raises the possibility of in situ T-cell generation in the marrow by
circulating thymic hormone and by a variety of nonspecific factors (3-5). Recent
findings from our laboratory concerning the mouse bone marrow phytohemag-
glutinin (PHA)1 response strongly support this contention. The kinetics of the
response of bone marrow lymphocytes to PHA differ markedly from those
observed in stimulated cultures of thymus (Thy), lymph node (LN), and spleen
(Spl) cells (6) where mature T lymphocytes are primarily responsible for blasto-
genesis (7). Furthermore, we showed that PHA responsive cells in the bone
marrow of nude mice are lymphocytes which are independent of thymic influ-
ence (8). In this communication we (a) characterize the PHA responsive cell in
the marrow in terms of surface markers, (b) test the identity of stimulated blast
cells, and (c) determine whether PHA can induce maturation of precursor T
cells into blast cells with T-cell surface markers.

Materials and Methods

Animals. 10 to 14-wk-old female CBA/J mice were obtained from The Jackson Laboratory, Bar
Harbor, Maine. Dr. Karl-Erik Hellstrom (Department of Pathology, University of Washington)
supplied 19 8 to 28-wk-old nu/nu CBA mice of both sexes and five normal littermates. Nude mice
used in the experiments were all large, healthy, and free of infection.

Cell Suspensions. Mice were killed by cervical dislocation, and suspensions of LN, Spl, and
bone marrow (BM) were prepared in citrated Hank’s balanced salt solution (HBSS, Microbiologi-
cal Associates, Bethesda, Md.) as previously described (6). A lymphocyte enriched (82.1 ± 4.8%
lymphoid cells) low density fraction (BML) of bone marrow was obtained by sucrose gradient

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1 Abbreviations used in this paper: ARB, anti-rat brain antiserum; ATS, anti-mouse thymocyte
serum; BM, bone marrow; BML, bone marrow lymphocytes; C, complement; FITC, fluorescein
isothiocyanate; GAMIG, goat anti-mouse gamma globulin; GVH, graft versus host; HBSS, Hank’s
balanced salt solution; LN, lymph node; LPS, lipopolysaccharides; MLR, mixed lymphocyte reac-
tion; NMS, normal mouse serum; NRS, normal rabbit serum; PHA, phytohemagglutinin; RAGIG, rabbit
anti-goat IgG; Spl, spleen; Thy, thymus.

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sedimentation (6) based on the method of Yoshida and Osmond (9). Spl and BML suspensions were rendered free of erythrocytes by hypotonic lysis (6).

Cell Cultures. Cell suspensions were washed three times in HBSS before counting in a hemocytometer. Cells were plated in duplicate or triplicate (10⁶ cells/well, 0.2 ml total volume) in Microtest II Plates (Falcon no. 3040 Falcon Plastics, Division of BioQuest, Oxnard, Calif.) in tissue culture medium RPMI 1640 supplemented with 100 U/ml penicillin G, 100 μg/ml streptomycin, 4 mM L-glutamine (all from Microbiological Associates), and 1% sterile CBA mouse serum. Mitogen-stimulated wells contained PHA (Burroughs Corp., Detroit, Mich.) at a concentration of 4 μg/ml (for LN and Spl) or 20 μg/ml (for BML) or *Escherichia coli* lipopolysaccharide (LPS, Difco Laboratories, Detroit, Mich.) at a concentration of 50 μg/ml. Cultures were incubated at 37°C with 5% CO₂ in a Forma Scientific incubator, Marietta, Ohio for periods up to 5 days. Stimulation was assessed by [³H]TdR incorporation (1 μCi per well, 6.7 Ci/mmol, ICN Pharmaceuticals Inc., Life Sciences Group, Cleveland, Ohio) during the terminal 18 h of incubation. [³H]TdR activity of the cells was determined by scintillation counting as previously described (6).

Cytotoxic Antisera. (a) T-cell specific antisera used in these experiments were as follows (dilutions in parentheses): AKR anti-C3H anti-δ serum (1:10 αδ, Litton Bionetics, Inc., Kensington, Md.), rabbit anti-mouse thymocyte antiserum (1:5 ATS, Microbiological Associates, absorbed three times with CBA erythrocytes, two times with CBA liver, one time with CBA kidney), and goat anti-rat brain antiserum (1:16 ARB) prepared as previously described (10, 11). The ARB reagent has been shown to react with T cells of both the rat and the mouse (10, 11).

(b) B-cell-specific antisera employed were commercial goat anti-mouse gamma globulin (1:5 αγ) and goat anti-mouse IgM (1:5 αμM) from Cappel Laboratories, Inc., Downingtown, Pa.

(c) Control sera were normal mouse serum (1:5 NMS), normal rabbit serum (1:5 NRS), and normal goat serum (1:5 NGS, Grand Island Biological Co.). All antisera and control sera were inactivated at 56°C for 30 min and diluted in RPMI.

Complement-mediated cytotoxic antibody lysis was performed before cell culture (12) by using the above antisera together with complement (C) derived from guinea pig serum absorbed with agar (13). 10 × 10⁶ lymphoid cells in 0.5 ml RPMI were incubated on ice for 45 min with 0.5 ml of one of the above antisera, control sera, or with RPMI. Subsequently, 0.5 ml of 1:3 C was added and the tubes were incubated another 30 min at 37°C in a 5% CO₂ incubator. After incubation, all cells were washed three times with HBSS, resuspended in RPMI, and plated as previously described.

Immunofluorescence. Cells were examined at days 0, 2, 3, and 5 of culture for staining with fluorescent T and B reagents in both wet mount preparations and in fixed radioautographs as previously described (14). After culture 10⁶ cells were dispersed by repeated aspiration with a Pasteur pipet and washed in HBSS. They were then exposed to fluoresceinated antiserum at 0°C in the presence of 0.1% NaN₃ for 45 min. T cells were identified by staining with F(ab')₂ fragments of a goat ARB antiserum conjugated to fluorescein isothiocyanate (FITC-ARB). Fluorescence of T cells was augmented by subsequent staining with F(ab')₂ fragments of a fluoresceinated rabbit anti-goat IgG antiserum (FITC-RAGIG). B cells were stained separately with fluoresceinated F(ab')₂ goat anti-mouse gamma globulin (FITC-GAMIG). Double negative or null cells were defined as unstained cells in preparations exposed to all three reagents. Stained cells were washed 3 times in HBSS and resuspended in 0.1 ml of RPMI. A 25-μl Oxford Sampler (Oxford Labs., San Mateo, Calif.) was used to deposit stained cells on glass microscope slides. Some slides were immediately examined as wet mounts in a Zeiss fluorescence microscope (Carl Zeiss, Inc., New York) by using a Kp 500 exciter filter and a 530 barrier filter, while others were air dried, fixed in ethanol, and processed for radioautography. Circumferential, bright green ring fluorescence clearly demarcated FITC-positive cells from negative cells (black) and dead cells (dull, uniform, yellow-green fluorescence). In wet mounts the incidence of PHA blasts (cells ≥10 μm in diameter) was determined by counting 1,000 consecutive, viable cells. Then the percentage of FITC-positive blasts was evaluated by scoring 500 consecutive blasts for fluorescence.

Radioautographs were prepared by using Kodak NTB2 emulsion, and were exposed in the dark for 6 h. After developing in Dektol and fixing in Kodak Acid Fixer (Eastman Kodak Co., Rochester, N. Y.), the radioautographs were examined in the fluorescence microscope. In such preparations, cells exhibiting [³H]TdR uptake (≥10 grains/cell) were considered to be PHA blasts. The incidence of PHA blasts per 1,000 cells was determined also by using this criterion, and the proportion of FITC-labeled blasts was defined by scoring 500 consecutive [³H]TdR-labeled cells for fluorescence.
TABLE I

|          | PHA   | PHA   | PHA   |
|----------|-------|-------|-------|
| LN (Day 3) | Unstimulated | RPMI + C | 326 ± 69 | 1,182 ± 207 |
| Spl (Day 3) | RPMI + C | 62,134* | 12,198 ± 727 |
| BML (Day 5) | RPMI + C | 1,283 ± 36 | 16,610 ± 256 |

* Single value.

Absorption of FITC-GAMIG with Sepharose beads (Pharmacia Fine Chemicals, Div. of Pharmacia, Inc., Piscataway, N. J.) coated with mouse gamma globulin (Cappel Laboratories Inc.) abolished all staining by this reagent in LN, Spl, and BML. FITC-RAGIG alone did not stain any mouse cells in any of the organs studied. Absorption of FITC-ARB with mouse thymocytes abrogated its staining of Thy, LN, and Spl cells, but did not abolish its staining of BML. This reagent also reacted with a proportion of cells (~10%) in nude mouse BML. These findings are consistent with the known reactivity of anti-brain sera with bone marrow cells other than T lymphocytes (15).

Results

Identity of the PHA Responsive Cells in BML. Complement-mediated cytotoxic antibody lysis was used to specifically eliminate T or B cells before stimulation of lymphoid cell suspensions with PHA. Two concordant experiments demonstrated that lysis of T cells by any one of 3 anti-T-cell antisera (α8, ATS, ARB) completely obliterated the PHA response of LN and Spl (Table I, Fig. 1). On the other hand, none of these antisera caused a significant diminu-
differentiation in the BML PHA response. Specific lysis of B cells with \( \alpha \gamma + C \) (Table I, Fig. 1) or \( \alpha \text{IgM} + C \) (Fig. 1) showed that this lymphocyte subpopulation was also not responsible for the PHA response of BML. In the same experiments, the selective abolition of the LPS response of Spl and BML by \( \alpha \gamma + C \) and \( \alpha \text{IgM} + C \) (Fig. 2) established the effectiveness of these antisera in killing B cells. Interestingly, LN did not possess a sizeable LPS-responsive B-cell subpopulation—Fig. 2). These results show that PHA-responsive BM lymphocytes do not possess mature T- or B-cell surface antigens in sufficient quantities to permit their lysis by cytotoxic antibodies and complement, and therefore the responsive cells must reside in the heterogeneous double negative or null lymphocyte compartment of the marrow (14).

Identity of PHA Blasts. Fluoresceinated T- and B-cell antisera were employed to determine the identity of blast cells generated by PHA in cultures of LN and BML. The analyses were performed at the peaks of the blastogenic responses, which occurred on day 3 for LN and day 5 for BML. Two independent criteria were utilized for identifying blasts, namely: size (\( \geq 10 \mu m \) in wet mount preparations) and \[^{3}H\]TdR incorporation (\( \geq 10 \) grains in radioautographs). Excellent agreement was observed between results obtained by using the different criteria to define blasts (Table II). LN PHA blasts were predominantly (90-99\%) T cells, although a small, but significant percentage of B blasts (1-13\%) was also observed. Similarly, the majority of BML PHA blasts (60-80\%) stained with FITC-ARB, 15-20\% stained with the B-cell reagent, and 10-20\% of blasts failed to stain when exposed to both antisera. Some of the double-negative BML PHA blasts may actually have been large nonlymphoid cells (e.g., macrophages) since the BML fraction initially consisted of 10-20\% nonlymphoid cells. Furthermore, because considerable spontaneous B blastogenesis was observed in BML cultured without PHA (Table III), the percentages of PHA B blasts shown in Table II may be overestimates. Since spontaneous T blastogenesis did not occur, the true incidence of PHA-induced blasts in BML bearing T-cell surface markers may be significantly greater than that suggested by Table II.
### TABLE II

**Identity of PHA Blasts in LN and BML Cultures Determined by Immunofluorescence**

|       | Exp A | Exp B | Exp A | Exp B |
|-------|-------|-------|-------|-------|
|       | Diameter criterion | Diameter criterion | Diameter criterion | Diameter criterion |
| TN | (%) | (%) | (%) | (%) |
| LN (Day 3) | 99.0 | 92.0 | 97.0 | 76.8 |
| [3H]TdR criterion | 92.0 | 12.9 | 95.0 | 16.8 |
| BML (Day 5) | 78.8 | 15.8 | 72.8 | 19.0 |
| [3H]TdR criterion | 72.8 | 15.5 | 60.4 | 19.5 |

|                | [3H]TdR cpm | [3H]TdR cpm |
|----------------|-------------|-------------|
| Unstimulated   | 115         | 133         |
| Stimulated     | 177,679     | 333,078     |

### TABLE III

**Blastogenesis in Control Cultures Lacking PHA**

|       | [%] | [%] | [%] | [%] |
|-------|-----|-----|-----|-----|
| TN   |     |     |     |     |
| LN (Day 3) | Diameter criterion | 0.2, 0.0 | 0.2 |
| [3H]TdR criterion | 0.2 |
| BML (Day 5) | Diameter criterion | 16.6 |
| [3H]TdR criterion | 3.8 |

### TABLE IV

**Specificity of FITC-ARB Labeling of T Blasts Generated in BML by PHA**

| Percent of cells ≥ 10 μm staining with | Day 0 | Day 5 |
|--------------------------------------|-------|-------|
| FITC-ARB                             | 8.0   | 62.0  |
| FITC-ARB absorbed with thymocytes four times | 9.8   | 5.4   |

Because FITC-ARB is known to stain other hemopoietic cells in the marrow besides mature T lymphocytes (15), it was necessary to show that the staining of BML PHA blasts with this reagent was a result of the antiserum's T-cell specificities. This was accomplished by showing that fluorescent blasts were not present in day 5 BML PHA cultures stained with FITC-ARB which had previously been extensively absorbed with thymocytes (Table IV).

These results unequivocally demonstrate that although PHA responsiveness in the marrow is not dependent on the presence of T cells, the majority of the BML PHA responsive cells exhibit T-cell-specific surface antigens at the peak of blastogenesis.

**Acquisition of T-Cell Surface Markers by BML PHA Blasts.** The temporal pattern of appearance of T-cell surface markers on BML null lymphocytes...
stimulated with PHA was demonstrated in the experiment depicted in Figure 3A. As seen in this graph and as reported previously (6), mitogen-stimulated BML cultures approach maximal levels of DNA synthesis ([3H]TdR cpm) and blastogenesis (percent blasts) on day 5 of culture. As previously noted (6), this time-course is considerably delayed and protracted compared to that of other lymphoid organs whose mitogenic responses peak on day 3 (Fig. 3B). During the culture period, the incidence of FITC-ARB-positive BML blasts increased from 8% on day 0 to >60% on day 5 (Fig. 3A). Concomitantly, there was a rapid decline in the percent of null blasts from 80% on day 0 to 20% on day 5. The results obtained with LN (Fig. 3B) differ markedly from those related for BML. Roughly 90% of LN blasts bore T-surface markers (FITC-ARB positive) at all time periods from day 0 to day 5, and at no time were null blasts prevalent. The findings obtained with LN support the contention that mature T cells are primarily responsible for the PHA response of this tissue, whereas the results obtained with BML suggest the generation of T-cell surface markers on BM null lymphocytes under the influence of PHA. Such newly induced BML T cells are presumably responsible for the observed blastogenic response. However, the possibility that the FITC-ARB positive blasts observed at day 5 were derived by the proliferation or selective survival of the ARB positive cells present at day 0 remained to be tested.

Induction of T Lymphocytes in Cultures of Nude BML Pretreated with ARB + C. To investigate the forementioned possibility, the PHA response of BML which was obtained from nude mice and pretreated with ARB + C to abolish any errant T cells was studied. Blastogenesis in such cultures was undiminished compared to stimulation seen in cultures of normal BML (Table V). On the other hand, neither nude LN nor normal LN pretreated with ARB + C mounted significant responses to this lectin (Table V). Immunofluorescent analysis of cultures of mitogen-stimulated nude BML pretreated with ARB + C revealed that even under these conditions, FITC-ARB positive blasts increased from 0 to 68% during a 5-day culture period with PHA (Table VI). Concomitantly, there was a diminution from 85 to 25% in the frequency of null blasts. These results are identical with those obtained by using normal BML (Fig. 3A). This experiment, therefore, rules out the possibility that the increase in FITC-ARB-positive BML blasts observed during incubation with PHA (Fig. 3A) was the result of selective survival or proliferation of a small population of T cells initially present in the marrow.

Discussion

The experiments reported in this communication unequivocally identify the PHA responsive cell of bone marrow as a null lymphocyte which in the course of the mitogenic response develops T-cell-specific antigenic surface markers. In functional terms, the responding cell can be regarded as a precursor T cell. In contrast to the marrow, the cells which respond to PHA in the Spl and LN are mature T cells, and pre T cells capable of induction by PHA are absent or undetectable in these tissues (8). These conclusions confirm and explain a number of controversial observations extant in the literature concerning the thymic independence of the PHA and graft versus host (GVH) responses me-
Fig. 3. T-cells markers on PHA blasts from BML and LN. Total blasts = percent of cells with diameter ≥ 10 μm. T blasts = percent of total blasts which stained with FITC-ARB. Null blasts = percent of total blasts which did not stain in preparations exposed to both FITC-ARB and FITC-RAMIG. A. BML PHA response. B. LN PHA response.
DISTRIBUTION OF MARROW PRE T CELLS

Table V

| Cell type  | Treatment | Unstimulated | Stimulated  |
|------------|-----------|--------------|-------------|
| Nude LN    | None      | 326 ± 49     | 94 ± 21     |
| Normal LN  | None      | 165 ± 17     | 245,125 ± 10,535 |
| Normal LN  | ARB + C   | 29 ± 5       | 293 ± 44    |
| Nude BML   | None      | 6,495 ± 518  | 51,356*     |
| Normal BML | None      | 3,478 ± 540  | 42,818 ± 2,446 |
| Nude BML   | ARB + C   | 2,137 ± 183  | 54,960 ± 1,004 |

* Single value.

Table VI

| Day       | ARB+ | αγ+ | ARB−αγ− |
|-----------|------|-----|---------|
| Day 0     | 0    | 11.4| 85.0    |
| Day 3     | 39.8 | 8.0 | 50.8    |
| Day 5     | 66.0 | 17.2| 24.6    |

HD differentiated by BM (16-18) in contrast to the thymic dependence of these responses exhibited by LN and Spl.

These conclusions are justified by the following compelling evidence: (a) Radioautographic studies have demonstrated that the PHA responsive cells in BML are morphologically classifiable as lymphocytes (6). (b) The kinetics of the BML PHA response differ from the kinetics exhibited by mature T cells in response to this mitogen (6). (c) Experiments with nude mice have verified the thymic independence of the BML PHA response (8). (d) Selective lysis of T or B cells with specific cytotoxic antisera plus complement showed that the responsive cells in the marrow lacked T or B differentiation markers. (e) Immunofluorescent analyses have demonstrated the transformation of null BML lymphocytes into T blasts as a result of exposure to PHA.

Previous contrary reports purporting to show that the BM PHA response is at least partially dependent on thymic influence (19, 20) may be due to differences in the mouse strains used. It is conceivable that some strains may possess greater numbers of recirculating mature T cells in the marrow than are found in CBA mice (21). In such strains the response to PHA may be a heterogeneous one, partially reliant on pre T cells (i.e., thymic independent) and partially dependent on mature T lymphocytes (thymic dependent).

The findings in this communication confirm the widely held contention that the PHA responses of peripheral lymphoid organs (LN, Spl, etc.) are primarily due to mature T lymphocytes (7). However, these studies also demonstrate that small numbers of B cells from these tissues also undergo blastogenesis in response to this mitogen as has previously been reported (22-24). A plausible model illustrating the postulated sequence of stimulation of pre T, T, and B lymphocytes by PHA is presented in Fig. 4.
FIG. 4. Hypothetical sequence of stimulation of pre T cells, mature T cells, and B cells by PHA. In LN and Spl where mature T cells are plentiful and pre T cells are scarce, the bulk of the mitogenic response to PHA is due to stimulation of thymus-derived cells, although some recruitment of B cells into the blastogenic process does occur. In BML pre T cells are numerous and mature T cells are rare. PHA induces differentiation of these marrow pre T cells to mature T cells which subsequently undergo blastogenesis and recruit B cells as previously described.

The mode of action of PHA in eliciting differentiation of marrow precursor T cells is not known. Convincing arguments have been propounded suggesting a common mechanism mediated by cyclic AMP responsible for inducing differentiation of BM pre T cells by such diverse agents as thymic hormone (1-3), murine hepatitis virus (4), and Concanavalin A (5). Whether PHA exerts its effects on marrow pre T cells via cAMP remains speculative since the effects of mitogens on cyclic nucleotide levels in BM lymphocytes have not been studied. Even in peripheral lymphocytes where lectin-induced alterations in cyclic nucleotide levels have been extensively studied, their role in mitogenesis remains obscure and controversial (25-28).

Previous studies demonstrating induction of pre T cells to mature T cells by various agents have shown that this process can occur within a few hours (1, 2, 5). In our experiments, however, no attempt was made to ascertain the rapidity of the induction process mediated by PHA. Our immunofluorescent assay employed T blasts, not T small lymphocytes, as the end point (Fig. 3), and the generation of such stimulated cells entails two processes: namely, maturation and blastogenesis, which together require several days for completion.

The findings of the present paper have significant implications for other cell-mediated immune responses (GVH reaction and mixed lymphocyte reaction, MLR) exhibited by marrow cells. Precursor T cells prevalent in the marrow and readily inducible to functional maturity by a variety of stimuli, including pathogenic agents (4), presumably play an appreciable role in the expression of the BM GVH and MLR responses. The appearance of chronic GVH disease in BM transplant recipients despite prior elimination of mature T cells from the BM inocula supports such a hypothesis (29). Prospective BM transplants could be rendered free of pre T and mature T cells by exposure to PHA or cAMP followed by cytolysis with anti-T cell antisera and complement, thereby eliminating the mediators of GVH disease. However, it must be recognized that intact pluripo-
tent stem cells must obviously persist unharmed. Such stem cells would presumably serve as progenitors for new precursor T cells, which, in turn, could be rapidly induced to mature T cells by a variety of stimuli. Such a scenario might ultimately lead to establishment of significant chronic GVH disease, unless intermittent efforts were continually made to suppress or eliminate T cells in transplant recipients.

Summary

The cells in mouse bone marrow (BM) capable of responding to phytohemagglutinin (PHA) were shown to be precursor T cells in experiments employing athymic mice, immunofluorescence, and specific lysis of T or B cells with cytotoxic antisera + complement. In contrast, the responses of lymph node (LN) and spleen (Spl) cells to this mitogen were shown by the same techniques to rely upon resident populations of mature T lymphocytes in these peripheral lymphoid organs. Cytolysis of T cells with anti-θ (anti-Thy 1), anti-thymocyte, or antibrain antisera abolished the PHA responses of LN and Spl, but had no appreciable effect on the BM PHA response. Lysis of B cells with anti-mouse gamma globulin or anti-mouse IgM antisera had no significant effect on either Spl or BM blastogenesis in response to this lectin. Immunofluorescent studies with fluoresceinated anti-brain sera demonstrated acquisition of T-cell surface antigens by BM null lymphocytes during the blastogenic response of this tissue to PHA. The results of these immunofluorescence experiments were reproducible even when marrow obtained from nude mice and pretreated with anti-brain serum plus complement was employed. The implications of these findings with regard to prophylaxis against graft versus host disease in BM transplant recipients are discussed.

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