Human Erythroid Burst-Promoting Activity Produced by Phytohemagglutinin-Stimulated, Radioresistant Peripheral Blood Mononuclear Cells

By Dina Meytes, Andrew Ma, Jorge A. Ortega, Nomie A. Shore, and Peter P. Dukes

The regulation of erythroid burst-colony formation was studied in cultures of human peripheral blood mononuclear cells. Numbers of erythropoietin-stimulated colonies obtainable from the cells in response to various treatments were compared. One-day preincubation of the cells with phytohemagglutinin (PHA) doubled the yield of colonies. Irradiation of the cells with 3000 rad eliminated their ability to form erythroid bursts, but did not impair the ability of PHA-treated cells to enhance burst formation when added to a fresh batch of cells. This was due to a humoral factor, since media conditioned by PHA-treated washed cells were as effective as the cells themselves. When cells were separated into subpopulations by an adherence procedure and according to their ability to form rosettes with sheep red blood cells, it was found that the PHA-dependent burst-promoting activity released into the medium originated in a nonadherent, nonrosetting (T-cell depleted) cell population.

It has recently been reported that human peripheral blood erythroid burst-forming units (BFU-E) are mononuclear null cells and that the formation of recognizable erythroid colonies from these progenitors in plasma clots depends not only on the provision of erythropoietin (Ep) but also on the presence of adequate numbers of T cells in the clots. It was also found that the null cells (BFU-E) responded equally as well to the addition to the cultures of media conditioned by T cells stimulated with tetanus toxoid as to the addition of T cells themselves. This T-cell requirement has recently been disputed. It was therefore of interest to study the effect of preincubation with phytohemagglutinin M (PHA) on subsequent erythroid colony formation by peripheral blood mononuclear cells. These studies led to the discovery that another subset of mononuclear cells, which does not have T-cell or monocyte-like characteristics, is capable of increasing the number of BFU-E derived from unfractionated human peripheral mononuclear cells.

MATERIALS AND METHODS

BFU-E Culture

Cells were cultured for BFU-E quantitation by the method of Iscove as modified by Ogawa. Mononuclear cells were obtained from heparinized peripheral venous blood of healthy adults by centrifugation on Ficoll-Hypaque (Pharmacia Fine Chemicals, Piscataway, N.J.) at 400 g for 30 min;

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3-9 × 10⁶ cells in 1 ml final volume were plated in tissue culture dishes (35 × 10 mm, 2-mm grid, Lux Scientific Corporation, Thousand Oaks, Calif.). The standard medium employed was α-medium (Flow Laboratories, Inglewood, Calif.), which contained 0.8% methylcellulose (Dow Chemical Co., Midland, Mich.), 30% fetal calf serum (Grand Island Biologicals, New York, N.Y.), 0.1 μmole/ml of α-thioglycerol (Sigma Chemical Co., St. Louis, Mo.), 50 U/ml of penicillin, and 50 μg/ml of streptomycin (Flow Laboratories); 1.3 U/ml of human urinary Ep (preparation OPR, 148.1 U/mg protein) was added when indicated. Erythropoietin was kindly provided by the National Heart, Lung and Blood Institute, Bethesda, Md.; it has been collected and concentrated by the Department of Physiology, University of the Northeast, Corrientes, Argentina, and was further processed in our laboratory under NIH Grant HL 10880. BFU-E were scored after 10–11 days of incubation in a fully humidified atmosphere of 95% air and 5% CO₂ at 37°C. Colonies consisting of three or more subcolonies of erythroid cells or large single accumulations of erythroid cells (>500 cells) were defined as BFU-E-derived colonies. Hemoglobinization of colonies was routinely verified using ortho-tolidine (J. T. Baker, Philipsburg, N.J.) as a heme-specific staining agent. A 4% (w/v) stock solution of ortho-tolidine (3,3′-dimethylbenzidine) in glacial acetic acid was prepared. Immediately before staining, 1 part of stock solution was mixed with 2 parts of distilled water and 1 part of 3% H₂O₂; 20 drops of the staining solution were layered over the cultures. Colonies were enumerated 1 mm after staining under 40× magnification and direct illumination. A number of colonies were picked at random from extra culture dishes, washed, cytacentrifuged, and stained with Wright stain to facilitate their morphological identification.

Preincubation Experiments

Aliquots of peripheral blood mononuclear cells containing 3–8 × 10⁶ cells in 6-ml volumes were preincubated in suspension culture in tissue culture dishes (60 × 15 mm, Falcon). The culture medium consisted of 3 parts α-medium and 1 part autologous plasma, containing penicillin and streptomycin as described above. The medium was made 1.2% with respect to PHA-M (Difco, Detroit, Mich.) unless otherwise specified. Following preincubation, cells were collected (adherent cells were detached using a rubber policeman), washed with 12 ml α-medium, suspended in 9 ml of the standard methylcellulose medium, and cultured in 1-ml aliquots for an interval of time sufficient to complete a 10-day total incubation period. Controls consisting of cells preincubated without PHA, as well as cells that had not been preincubated at all, were always included in these experiments. In one experiment, the medium was made 50 mM with respect to N-acetylgalactosamine during the final hour of preincubation in order to dissociate bound PHA from the cell surface, according to the method of Whiteside and Rabin.⁵

Subpopulations of Peripheral Blood Mononuclear Cells

Separation of mononuclear cells based on their adherence properties was carried out as follows: 5 × 10⁶ cells in a 4-ml volume of α-medium containing 20% fetal calf serum were incubated in 60 × 15 mm tissue culture dishes for 3 hr (37°C, 95% air-5% CO₂), then the nonadherent and the adherent cells were collected. Nonadherent cells obtained by this procedure contained only 1% α-naphtyl esterase positive cells.

T-lymphocyte-enriched and -depleted subpopulations were obtained from the nonadherent cells using sedimentation on Ficoll-Hypaque of spontaneous rosettes formed by T lymphocytes and sheep red blood cells (SRBC) that had been pretreated with 2-aminoethylisothiouronium bromide hydrobromide (AET, Sigma).⁶ The T-cell-depleted subpopulation contained 4.4% ± 1.9% (5) rosetting cells (mean ± SE, number of determinations in parenthesis), the T-cell-enriched subpopulation contained 85.9% ± 3.2% (5) rosetting cells.

Irradiation of Cells

Peripheral blood mononuclear cells suspended in α-medium containing 2% autologous plasma (5–10 × 10⁶/ml) were irradiated with a 60Co-teletherapy unit at a dose rate of 108 rad/min to a total of 3000 rad. After irradiation, the cells were washed in the above medium.

Conditioned Media

In order to prepare conditioned media, mononuclear cells (2 × 10⁶ cells suspended in 4-ml volumes) were first incubated for 1 day in snap-cap tissue culture tubes (17 × 100 mm, Falcon) in the same
PH A-containing medium as was used for the preincubation experiments described above. Then the cells
were washed and recultured in the lectin-free medium, which contained either autologous plasma or
fetal calf serum, for an additional day. This medium was freed of cells by centrifugation, concentrated
to half its original volume by lyophilization, and reconstituted to the composition of the standard
methylcellulose-containing medium; 2 volumes of conditioned medium were always combined with 1
volume of standard medium for subsequent use in experiments. By this procedure, each aliquot of $5 \times 10^5$
fresh cells was exposed to the products of approximately $3 \times 10^5$ conditioning cells. Control media
that were derived from media that had been incubated in the absence of cells, or from media conditioned
by cells that had not been pretreated with PHA, were also prepared in a similar way.

**Statistical Evaluation**

Numbers of BFU-E derived colonies reported for individual experiments are always means of
quadruplicate determinations. When results from several experiments were pooled, data were normal-
ized relative to appropriate controls, and the means of the mean BFU-E numbers of individual
experiments (expressed as percent of control) are reported. In either case, the significance of differences
between means was examined with the Student's t test.

**RESULTS**

Preincubation of peripheral blood mononuclear cells in liquid suspension culture with PHA increased the number of BFU-E-derived colonies obtained from these
cells on subsequent culture in the methylcellulose system. Preliminary experiments
showed that a culture medium containing 1.2% of reconstituted PHA was optimally
stimulatory. When N-acetylgalactosamine was used during the last hour of the
preincubation period to dissociate PHA from the cell surface, the increment in
erthroid burst formation remained unchanged.

The effects of 1- and 2-day preincubation, with and without PHA, are compared
in Table 1. It is evident that 1-day preincubation of cells with PHA caused a
significant increase in numbers of BFU-E-derived colonies ($p < 0.01$), both relative
to the unpreincubated controls and relative to cultures that had been preincubated
without PHA. It should be noted that preincubation with PHA increased not only
the number but also the size of the erythroid colonies that were obtained on
subsequent culture. The presence of PHA during a 2-day preincubation period
failed to increase BFU-E numbers above the unpreincubated control levels, but
caused a marked diffuse proliferation of lymphocytes in the dishes. Figure 1
demonstrates that the enhancing effect of 1-day preincubation in PHA may be
observed with cells from normal individuals with a low and a high baseline level of
BFU-E, respectively.

In order to elucidate whether PHA enhanced colony formation by acting directly
on the BFU-E or whether an interaction with other cells was involved, cell mixing
experiments were performed. The effect of PHA-treated irradiated cells on a fresh

| Table 1. Effect of Preincubation of Peripheral Blood Mononuclear Cells With PHA
| on BFU-E-Derived Colony Formation |
|---------------------|---------------------|---------------------|
| Days of Preincubation | 1 Day | 2 Days |
| Treatment of Cells | BFU-E (Percent of Control) | BFU-E (Percent of Control) |
| Preincubation without PHA | 62.2 ± 5.4 (6)† | 28.3 ± 10.1 (4) |
| Preincubation with PHA | 221.3 ± 3.0 (7) | 68.5 ± 28.3 (4) |

*Number of BFU-E obtained without preincubation equals 100%.
†Mean ± SE; number of experiments is in parenthesis.
batch of mononuclear cells was investigated. Peripheral blood mononuclear cells were irradiated with 3000 rad, washed, and cultured suspended in liquid medium containing PHA. Appropriate controls were treated similarly and cultured in the absence of PHA. After 1 day of culture, the irradiated cells were washed again and mixed with fresh batches of autologous mononuclear cells. Incubation mixtures contained $4.0 \times 10^5$ irradiated cells and $6.5 \times 10^5$ fresh cells in methylcellulose medium; results after 10-day incubation are presented in Table 2. Controls, consisting of irradiated cells by themselves, contained no BFU-E. Addition of autologous irradiated cells to fresh cells did not bring about a change in BFU-E number relative to that observed when fresh cells alone were cultured. Addition of PHA-treated irradiated cells, however, caused a twofold increase in BFU-E response.

Experiments were performed to determine whether the PHA-responsive cells acted by direct cell-to-cell contact or through a BFU-E-enhancing secretion product. Media were conditioned by either untreated or irradiated cells. Incubation for 1 day with PHA was followed by 1 day incubation without PHA. Media collected from the second incubation period were tested for their effects on BFU-E-derived colony formation by fresh allogeneic peripheral blood mononuclear cells. This incubation scheme for the production of conditioned media was adopted because in preliminary experiments it was found that, whenever 1.2% PHA was included for the whole 10-day incubation period in the methylcellulose medium, lymphocytes proliferated and BFU-E were suppressed. Table 3 shows that the addition of media conditioned by PHA-treated unirradiated or irradiated cells to the incubation mixture caused significant increases in numbers of BFU-E-derived colonies ($p < 0.05$) over those observed in controls. Addition of media conditioned

<table>
<thead>
<tr>
<th>Group</th>
<th>Input</th>
<th>Experiment 1</th>
<th>Experiment 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>$4.0 \times 10^5$ irradiated cells</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>$6.5 \times 10^5$ fresh cells</td>
<td>92 ± 8*</td>
<td>40 ± 6</td>
</tr>
<tr>
<td>3</td>
<td>$6.5 \times 10^5$ fresh cells + $4.0 \times 10^5$ irradiated cells</td>
<td>89 ± 10</td>
<td>55 ± 8</td>
</tr>
<tr>
<td>4</td>
<td>$6.5 \times 10^5$ fresh cells + $4.0 \times 10^5$ irradiated PHA-treated cells</td>
<td>189 ± 14</td>
<td>90 ± 9</td>
</tr>
</tbody>
</table>

$p$ Value for difference between group 3 and group 4

*BFU-E (mean ± SE).
In order to identify the subpopulation of peripheral blood mononuclear cells that is responsive to PHA by releasing a BFU-E-enhancing product, mononuclear cells were separated into enriched populations by utilizing adherence to plastic dishes followed by sedimentation of spontaneous rosettes formed with SRBC. The subpopulations thus obtained were used to produce conditioned media following preincubation in the presence and absence of PHA. Figure 2 depicts the pooled normalized results of five experiments comparing the effects of the various conditioned media. Without previous exposure to PHA, none of the subpopulations tested were able to produce conditioned media with BFU-E-enhancing properties. In response to PHA, however, the nonadherent cells and, to an even greater extent the nonadherent, nonrosetting cells, produced active conditioned media. The ratios of BFU-E observed in response to media conditioned by PHA-treated and -nontreated cells, respectively, were calculated for each individual experiment. The means and SE of these ratios were as follows: adherent cells, 1.54 ± 0.27; nonadherent cells, 1.73 ± 0.14; nonadherent rosetting cells, 1.11 ± 0.26; nonadherent nonrosetting cells, 2.48 ± 0.48 (p < 0.05 for difference between the last two

**Table 3. Effect of Conditioned Media on Peripheral Blood BFU-E**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Control Medium</th>
<th>Medium Conditioned by Control Cells</th>
<th>Medium Conditioned by PHA-Treated Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unirradiated conditioning cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>80 ± 8*</td>
<td>88 ± 15 (109%)†</td>
<td>Not done</td>
</tr>
<tr>
<td>2</td>
<td>326 ± 27</td>
<td>354 ± 18 (109%)</td>
<td>426 ± 28 (131%)</td>
</tr>
<tr>
<td>3</td>
<td>158 ± 22</td>
<td>168 ± 34 (106%)</td>
<td>332 ± 42 (210%)</td>
</tr>
<tr>
<td>Irradiated conditioning cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>73 ± 25</td>
<td>Not done</td>
<td>210 ± 36 (289%)</td>
</tr>
<tr>
<td>2</td>
<td>314 ± 6</td>
<td>312 ± 6 (99%)</td>
<td>562 ± 31 (180%)</td>
</tr>
<tr>
<td>3</td>
<td>182 ± 23</td>
<td>153 ± 27 (84%)</td>
<td>430 ± 22 (236%)</td>
</tr>
</tbody>
</table>

*BFU-E/10⁶ cells plated (mean ± SE).
†Number of BFU-E obtained in control medium equals 100%.

by cells that had not been exposed to PHA had no such effect. Reconstitution of media preincubated in the absence of cells with small quantities of PHA, comparable to amounts that were contained in the liquid adhering to the washed cells after PHA treatment (final 0.0003% of the medium), was also without effect (not shown).
HUMAN ERYTHROID BURST-PROMOTING ACTIVITY

means). It is evident that the nonadherent nonrosetting (T-cell depleted) subpopulation was highly enriched in cells capable of producing a BFU-E-enhancing activity after exposure to PHA.

DISCUSSION

The development of erythroid colonies from murine and human hematopoietic cells in semisolid media requires the addition of Ep to the system. The production of colonies from all but the most mature members of the progenitor cell sequence seems to depend additionally on the provision of other factors, collectively termed burst-promoting activities (BPA).

Iscove found pokeweed-mitogen-stimulated mouse spleen cell conditioned medium to be a source of BPA. Wagemaker described a mouse marrow-cell-associated principle required for BFU-E response (BFA), the production of which was relatively radiation resistant. A factor from human urine (RP), active on mouse and human progenitor cells, with similarities to in vitro produced BPA, has been studied in our laboratory. Preincubation of mouse marrow cells with RP increased their subsequent burst response to Ep. Aye found that human leukocyte-conditioned medium (LCM) greatly enhanced erythroid burst formation in response to Ep by nonadherent human bone marrow cells. Nathan et al. reported that tetanus-toxoid-stimulated human T lymphocytes produced a BPA active on null cells. Fauser and Messner described an assay for a bipotent human progenitor cell CFU-G/E that required that the cells be first incubated with PHA-stimulated LCM followed later by the addition of Ep.

In this report we described our finding that preincubation of peripheral mononuclear cells for 24 hr with 1.2% PHA caused a twofold increase in the number of erythroid bursts obtainable from treated washed cells compared to untreated controls. That this effect was not mediated by a direct action of the lectin on the BFU-E from which the colonies originated was shown by the following. Continuous exposure of the cultures to 1.2% PHA suppressed erythroid colony formation; inclusion into the medium of amounts of PHA, which could have been carried along in the liquid adhering to washed cells, had no effect; the displacement of bound PHA from the surface of the mononuclear cells by N-acetylgalactosamine did not reduce the increased colony formation by the treated cells; and finally, one particular separated subpopulation of mononuclear cells was the principal producer of BPA in response to PHA, although all cell subpopulations had been exposed to the same PHA concentration and all conditioned media had been prepared identically.

Based on differential sensitivity to irradiation with 3000 rad, it was shown that the cells on which the PHA acted, causing an enhancement of burst formation, were not the BFU-E themselves. This amount of radiation totally eliminated BFU-E from cell suspensions, but left the BFU-E-enhancing cells active.

The ability of media conditioned by PHA-treated washed cells to bring about an increase in erythroid colonies demonstrated that the enhancement was mediated by a product released by the cells into the medium. Other workers have reported that media conditioned by peripheral leukocytes for 7 days with or without PHA (LCM and PHA-LCM) have burst-promoting activity. However, while Aye observed no potentiating effect of LCM on human marrow cells unless adherent...
cells had been removed, we found that the product of the conditioning cells from the peripheral blood caused a doubling of the maximal Ep-induced BFU-E response by blood mononuclear cells not subjected to any further separation. Moreover, the cells producing this activity were nonadherent. Another point of difference was that the activity produced by Ficoll-Hypaque separated mononuclear cells required a much shorter incubation period than 7 days and was PHA-dependent.

Nathan et al.1 have reported that T cells are required for erythroid burst-colony formation by peripheral blood null cells. They also demonstrated that isolated T cells, when cultured for 3 days with tetanus toxoid, released an erythroid burst-promoting factor active on null cells; 10⁶ null cells were stimulated either by 2.5 × 10⁶ T cells or by their BFU-E-enhancing products. Irradiation of the T cells rendered them incapable of producing this activity. In contrast to these findings, the PHA-dependent BPA in our system was produced by a T-cell-depleted subpopulation. Its production was resistant to 3000-rad irradiation. Much fewer conditioning cells per responding cell were used, thereby reducing the likelihood of contributions by cells contaminating the active subpopulation. This suggests that the erythroid burst-promoting factor described by Nathan et al.¹ and the factor described in this report are produced by different populations of cells. Whether this PHA-induced factor acts through an intermediary T cell remains to be investigated (Fig. 3).

Thus, in addition to bone-marrow-adherent cells and T cells, a population of relatively radioresistant nonadherent human peripheral blood mononuclear cells, which are incapable of rosette formation with SRBC, has been shown to increase erythropoiesis in culture. This adds to the complexity of the cellular interactions involved in the regulation of the erythroid cell clonal culture systems. Both the chemical nature and the relevance to disorders of hematopoiesis of the PHA-dependent factor will now be investigated.

![Fig. 3. Scheme for mechanisms of enhancement of burst formation by PHA.](image-url)
REFERENCES


Human erythroid burst-promoting activity produced by phytohemagglutinin- stimulated, radioresistant peripheral blood mononuclear cells

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