Neutralization of Erythroid Burst-Promoting Activity In Vitro With Antimembrane Antibodies

By Nicholas Dainiak, Laurie Feldman, and Carl M. Cohen

To investigate the relatedness of soluble and pelletable vesicular erythroid burst-promoting activity (BPA) present in lymphocyte-conditioned medium (LCM), we immunized rabbits with partially purified lymphocyte plasma membranes and tested the antisera for biological and immunologic crossreactivity with LCM and its component fractions. When preincubated with IgG purified from post-immune but not from preimmune serum, BPA expression by unseparated LCM, LCM-derived pellets, and supernatants was abolished in a dose-related fashion. As little as 0.001 mg/mL postimmune IgG reduced burst formation by 50%. Antimembrane IgG crossreacted on immunoblots with multiple components of both supernatants and pellets of LCM. Crossreactivity was also seen in LCM-derived supernatants that were subjected to ultracentrifugation. Soluble BPA was adsorbed from LCM supernatants incubated with antimembrane IgG-coated *Staphylococcus aureus*. Conversely, incubation of purified antimembrane IgG with intact circulating lymphocytes removed BPA-neutralizing effects from the antibody preparation. Antimembrane IgG incompletely suppressed erythroid colony-forming unit (CFU-E)-derived colony formation, an effect that could not be explained by alteration in erythropoietin sensitivity or action. There was no effect of the antibody preparation on erythroid differentiation of K562 cells or on CFU granulocyte/macrophage-derived colony growth, (CFU-G/M) by human or murine bone marrow. Taken together, our findings suggest that antibodies directed against lymphocyte plasma membranes react with both soluble and vesicular BPA, and that these physically separable erythroid growth factors may share antigenic determinants.

**Materials and Methods**

**Preparation of LCM**

LCM was prepared from 100 to 150 mL of peripheral blood from 15 hematologically normal donors, as described previously. Informed consent was obtained before performance of phlebotomy, in accordance with the Human Investigation Committee at St. Elizabeth's Hospital of Boston.

In brief, blood was aspirated into *α*-medium (Flow Laboratories, Inglewood, Calif.) plus 20 U/mL preservative-free heparin and separated over Ficoll-Paque (Pharmacia Fine Chemicals, Piscataway, NJ). Mononuclear cells at the interface were removed, mixed with a carbonyl iron suspension (Technicon Instruments Corporation, Tarrytown, NY), and separated over Ficoll-Paque again. Interface cells were washed three times in *α*-medium and resuspended to a density of 5 x 10⁶/mL in *α*-medium supplemented with L-glutamine, streptomycin, and penicillin (GIBCO, Grand Island, NY). After 24-hour incubation at 37 °C in 4% CO₂, medium was harvested and separated into supernatants and pellets by centrifugation at 40,000 g for 30 minutes. Vesicle-containing pellets were suspended in 5 mmol/L Na-phosphate, pH 7.6, and resuspended in NCTC-109 (Microbiological Associates, Bethesda, Md) before use in culture. In some cases, high-speed supernatants were prepared for immunologic analysis by centrifugation of LCM supernatants at 100,000 g for 60 minutes.

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Preparation of Antimembrane Antibodies

Plateletpheresis residues were obtained from the American Red Cross. Mononuclear cell concentrates were prepared as described previously and depleted of residual platelets by the method of Perper et al. The cells were suspended at a density of 5 x 10^6/mL in α-medium and incubated in plastic culture flasks for 90 minutes. Nonadherent cells were removed, washed once in α-medium and three times in PBS. Approximately 85% to 90% of cells at this point were morphologically and cytochemically identifiable as lymphocytes. Plasma membranes were isolated according to a modification of the method of Jett et al. and as described previously.

Approximately 100 to 350 μg of partially purified plasma membrane protein emulsified in Freund's complete adjuvant was injected intradermally at multiple sites into each of four rabbits. Animals were rehydrated with protein emulsified in Freund's incomplete adjuvant at three and seven weeks after the primary injection. One week after the last immunization, antisera were collected and immunoglobulin G (IgG) was precipitated with ammonium sulfate and purified on diethylaminoethanol (DEAE) cellulose. To test for cross-reactivity with membrane components, purified membrane proteins were electrophoresed on 3.5% to 18.0% acrylamide gels according to Laemmli, and transferred to nitrocellulose paper by the electroblot technique. The blots were then incubated with IgG purified from preimmune or postimmune sera, followed by peroxidase-conjugated goat anti-rabbit IgG, and developed with chloro-naphthol. Multiple reactive proteins were observed with postimmune serum but not with preimmune serum from each of the rabbits.

Assays for BPA and Granulocyte/Macrophage Colony-Stimulating Activity (GM-CSA)

Approximately 0.5 mL of human marrow was aspirated from the posterior iliac crests of 20 hematologically normal donors. The cells were collected in Eagle's MEM (GIBCO) containing 20 U/mL preservative-free heparin, and were separated over Ficoll-Paque. Mononuclear cells at the interface were washed three times in α-medium passed over polyester and cultured as described previously. For BPA assays, 1 to 10 x 10^6 nonadherent marrow cells were cultured by a modification of the method of Tepperman et al. Cultures were prepared with 4.5% or 27.0% (vol/vol) human type A, platelet-poor plasma-derived serum (PDS) prepared as described previously. PDS was mixed with activated charcoal for 12 hours at 4 °C before addition to culture. Cultures contained 2 IU/mL sheep type III erythropoietin (Connaught Laboratories, Swiftwater). Clots of 125 μL volume were formed in microtiter wells with 0.1 mg/mL of highly purified fibrinogen (Kabi AG, Stockholm), 2 mmol/L CaCl2 and 5 μg/mL thrombin.

After 12 to 14 days of incubation at 37 °C, 4% CO2 in humidified air, clots were fixed to glass slides in gluteraldehyde and stained with benzidine and hematoxylin. Erythroid bursts appearing as single aggregates of ≥50 benzidine-positive cells or ≥3 aggregates of eight to 49 benzidine-positive cells were enumerated. The amount of BPA present in cultures established with 4.5% PDS plus 9% (vol/vol) unseparated LCM, LCM supernatants, or LCM pellets was determined as the percentage of maximum colony growth occurring in cultures established with 27.0% PDS plus 9% (vol/vol) NCTC-109. BPA neutralization was determined in preimmune and postimmune immunoglobulin samples by preincubation for 30 minutes. 37 °C with unseparated LCM or its fractions at the final protein concentrations indicated in figure legends. In other cases, 100-μL aliquots (0.9%, vol/vol) of IgG were added directly to cultures containing 2.0 IU/mL erythropoietin plus 9% unseparated LCM, LCM supernatant, LCM pellet, or NCTC-109.

In additional experiments, effects of preimmune and postimmune rabbit IgG on proliferation of erythroid colony-forming units (CFU-Es) was determined by adding 9% (vol/vol) of immunoglobulin solutions to human marrow cultures containing 0.5, 1.0, 2.0, or 4.0 IU/mL erythropoietin. Cultures were established with 9% (vol/vol) immunoglobulin or NCTC-109 at protein concentrations denoted in figure legends. CFU-E-derived colonies were enumerated by scoring those colonies containing 8 to 49 benzidine-positive cells after six to eight days of incubation.

Because antimembrane immunoglobulin may possibly neutralize other hematopoietic cell proliferation factors such as GM-CSA, we tested postimmune IgG in double-layer soft agar cultures of CF, marrow cells and human marrow cells. For murine marrow cultures, the method of Pike and Robinson was followed, using lung-conditioned medium as a source of GM-CSA that was prepared in the following manner. Whole lungs were sterilized by washing with Salmonella typhosa B endotoxin (Difco, Detroit), gently sliced into four or five sections in 2 mL of Hanks' balanced salt solution (without calcium or magnesium) and incubated at 37 °C, 4% CO2 in humidified air for 72 hours. Supernatant freed of cells by centrifugation at 650 × g for 20 minutes was millipore filtered (0.45 μm) and stored at −20 °C until use. Lung-conditioned medium plus 100 μL of preimmune or postimmune IgG was added to underlayers, and marrow cells were added to agar overlayers. For human marrow cultures, 106 Ficoll-Paque–separated marrow cells were added to agar overlayers and 106 peripheral blood mononuclear cells were added to underlayers as a source of human GM-CSA in the presence and absence of IgG as above. Colonies containing ≥50 cells per clone and clusters containing three to 49 cells per clone were scored, and the effect of rabbit IgG on CFU-GM proliferation was determined relative to murine and human marrow cultures containing 100 μL NCTC-109.

Testing for Effects on K562 Cell Erythroid Differentiation

Because antimembrane antibody may exert an effect on human erythroid progenitor differentiation independent of BPA, we tested postimmune IgG in secondary cultures of K562 cells (kindly provided by Dr James Meir). Erythroid differentiation was induced in fibrin clots with Na butyrate (Sigma Chemical Co, St Louis) as previously described. In the presence and absence of 0.1 mg/mL IgG purified from postimmune rabbit serum, 105 cells per milliliter were subcultured. Cell proliferation was continued for seven to ten days after addition of the inducing agent, at which time fibrin clots were removed, fixed to glass slides, and stained with benzidine. The percentages of positively staining cells and cell clusters consisting of ≥8 cells were determined and compared to results in cultures containing NCTC-109.

Testing for Cross-reactivity of Antimembrane IgG With LCM and Intact Leukocytes

Five milliliters of unseparated LCM and low- and high-speed LCM supernatants from 5 mL of starting LCM were dialyzed overnight against distilled water, lyophilized and resuspended to 50 μL, and electrophoresed in 3.5% to 18.0% acrylamide gels according to the method of Laemmli. Vesicles from LCM-derived pellets were washed in 5 mol/L Na-phosphate, pH 7.6 and electrophoresed in the same way. Proteins from unseparated LCM and LCM fractions were transferred electroblotically onto nitrocellulose paper and incubated with postimmune immunoglobulin; reactive proteins were identified by staining with peroxidase-conjugated second antibody.
as described above. In addition studies, immunoaffinity columns were prepared with IgG purified from postimmune serum. Purified IgG was covalently coupled to Affigel 10 (BioRad Laboratories, Richmond, Calif), and the resultant affinity resin was incubated batchwise with LCM supernatant (one hour at 25 °C). After incubation, the resin was poured into a column and washed with buffer to remove nonspecifically bound proteins. Specific elution was achieved by flushing with one column vol 0.2 mol/L glycine, pH 2.3. Both the flow-through and specifically eluted fractions were dialyzed against distilled water and lyophilized and resuspended as described above.

In additional studies, immunoaffinity columns were prepared with IgG purified from postimmune serum. Purified IgG was covalently coupled to Affigel 10 (BioRad Laboratories, Richmond, Calif), and the resultant affinity resin was incubated batchwise with LCM supernatant (one hour at 25 °C). After incubation, the resin was poured into a column and washed with buffer to remove nonspecifically bound proteins. Specific elution was achieved by flushing with one column vol 0.2 mol/L glycine, pH 2.3. Both the flow-through and specifically eluted fractions were dialyzed against distilled water and lyophilized and resuspended for gel electrophoresis as above.

To determine whether the same antibody molecules that inhibit BPA also cross-react with leukocyte cell surfaces, circulating intact mononuclear cells depleted of macrophages by mixture with carbonyl iron were incubated at a density of 10^9/ml at 25 °C for 30 minutes in medium with and without 100 μg/ml preimmune or postimmune rabbit IgG. The cells were pelleted at 650 g and 100 μl resultant adsorbed immunoglobulin supernatants or NCTC-109 were tested in mouse cultures containing unseparated LCM supernatants plus 2 IU/ml erythropoietin. Effects on burst formation were compared to cultures established concurrently with 100 μl unadsorbed antimembrane immunoglobulin.

Adsorption of Soluble BPA From LCM

To determine whether rabbit antimembrane immunoglobulin can be used to remove soluble BPA, various amounts of postimmune IgG (0, 50, 100, or 200 μg) were incubated with a 10^9 Staphylococcal protein A solution (100 μl, capable of binding 200 μg IgG) (Miles-Yeda Ltd, Israel) at 37 °C for 45 minutes. Mixtures were centrifuged at 1,200 g, washed twice, and incubated with LCM supernatant at 37 °C for 30 minutes. The solid phase was pelleted, and 100 μl adsorbed supernatants were tested in culture. Results were compared to cultures concurrently established with 100 μl unadsorbed LCM supernatants or NCTC-109.

Erythropoietin Bioassay

Hypertransfused, polycythemic CF, female mice (Cartworth Labs, Woburn, Mass) with hematocrits greater than 55% were used to determine the effect of antimembrane IgG on erythropoietin activity in vivo. On two consecutive days, 0.25 ml (0.50 ml total) normal saline or sheep Step III erythropoietin at final concentrations of 0.125 and 0.250 IU/ml with and without exposure to Staphylococcal protein A coated with 100 μg postimmune IgG as described above were subcutaneously injected into five animals. In other tests, 0.5 and 2.0 IU/ml erythropoietin were incubated with 0.3 mg/ml postimmune immunoglobulin before injection. 5Fe was injected into the tail veins 24 hours later, and its incorporation into red blood cells after 24 hours was determined according to the method of Shadduck et al. 17

Statistical Methods

The number of colonies formed was determined in each of four 125-μl clots for erythroid cultures and in three 1-mL agar plates for GM cultures. The means ± SEM of quadruplicate determinations for each test point in cultures for erythroid colony formation and of triplicate determinations for each test point in cultures for granulocyte-macrophage colony formation were calculated. Data sets were compared by the two-sample ranks test of Wilcoxon and White. 18

RESULTS

Cross-reactivity of Antimembrane IgG With LCM and Its Component Fractions

Previous work from our laboratory has shown that rabbits immunized with human leukocyte plasma membrane protein produce antibodies that cross-react with multiple membrane components. 19 To determine whether rabbit antimembrane antibody reacts with components of LCM, we incubated unseparated LCM and LCM supernatants and pellets with IgG from postimmune sera, using the electroblot technique. Figure 1 shows that multiple cross-reactive proteins are present not only in blots with membrane vesicle-containing pellets, but also in those containing vesicle-free LCM supernatants. In contrast, little or no cross-reactivity was observed when IgG from preimmune sera was tested (data not shown).

Because it is possible that LCM supernatants contain residual membrane-derived vesicles in suspension, they were recentrifuged at 100,000 g for 60 minutes. The resultant high-speed supernatants were incubated with rabbit antimembrane IgG and results were compared to incubations with unseparated LCM and low-speed supernatant obtained from the same LCM preparation. We observed that both low- and high-speed LCM supernatants contain similar cross-reactive proteins (Fig 1).

Effects of Antimembrane IgG on Erythroid Colony Formation

To assess the effects of antimembrane IgG on the expression of BPA in vitro, various amounts of rabbit

![Fig 1. Cross-reactivity of antimembrane IgG with LCM and its component fractions. (a) Silver-stained gels of (A) unseparated LCM, (B) supernatant after centrifugation at 40,000 g for 30 minutes, (C) supernatant after centrifugation at 40,000 g, then at 100,000 g for 60 minutes, and (D) vesicle-containing pellet from 40,000 g centrifugation. Gels contain approximately 50 to 75 μg of protein. (b) Immunoblots of corresponding LCM fractions (A', B', C', D') prepared from the same LCM batch as in (a), following incubation with 1.5 mg per lane antimembrane IgG. Incubation with second antibody and staining were performed as described in Materials and Methods. Large arrows are directed at major cross-reactive proteins.](www.bloodjournal.org)
immunoglobulin were preincubated with unseparated LCM or its fractions before their addition to marrow culture. We observed that while IgG from preimmune serum had no effect on the support of erythroid burst formation by LCM or its component fractions ($P > .05$ at all concentrations tested), IgG from postimmune serum from the same rabbit neutralized BPA in a dose-related fashion ($P < .05$ at 0.0001 mg/mL and $P < .01$ at all other concentrations) (Fig 2A). Similar results were obtained when immunoglobulin was added directly to marrow cultures (data not shown). Inhibition of BPA in LCM was observed at all seeding concentrations tested (Fig 3).

Because antimembrane IgG cross-reacts immunologically with both pellets and supernatants, we attempted to determine whether it could neutralize the erythroid growth-supporting effects of these fractions of LCM in culture. Figure 2B shows that antimembrane IgG neutralizes BPA expression not only by unseparated LCM and LCM-derived vesicles, but also by vesicle-free supernatant ($P < .05$ or less). We observed that 0.001 mg/mL IgG reduced burst formation by approximately 50%. No erythroid bursts formed in cultures containing greater than 0.1 mg/mL antimembrane IgG. In other experiments, 0.01 mg/mL antimembrane IgG abolished all burst formation. Furthermore, there was no change in IgG effect on BFU-E proliferation when other concentrations of erythropoietin (0.5, 1.0, or 4.0 IU/mL) were added to culture (data not shown).

We next determined whether antimembrane IgG altered CFU-E-derived colony formation. Figure 4A displays our results indicating incomplete but significant ($P < .05$) suppression of CFU-E proliferation at high concentrations of antibody ($\geq 0.1$ mg/mL). No suppression of CFU-E-derived colony growth was observed at an antimembrane antibody concentration of 0.001 mg/mL ($P > .10$). Because suppression at high antibody concentrations might be mediated by an inhibitory action directed against erythropoietin activity, we tested for suppressive effects of 0.1 mg/mL antimembrane IgG at increasing erythropoietin concentrations. Figure 4B shows that addition of higher amounts of erythropoietin to tissue culture does not overwhelm inhibitory action of the antibody molecule. This observation suggests that antimembrane IgG inhibits CFU-E proliferation by a mechanism exclusive of an effect on erythropoietin action.

Because individual clones of K562 cells may be homogeneous, we asked whether antimembrane IgG alters surface membranes of these neoplastic hematopoietic progenitors and results in altered erythroid differentiation. Faintly positive cells were present in fibrin clots containing Na butyrate upon reaction with benzidine and peroxide. In contrast, cells from cultures containing NCTC-109 alone were nonreactive. When added to subcultures of K562 cells, the antibody did not alter relative percentages of benzidine-positive cells or numbers of benzidine-positive cell clusters (Table 1). Furthermore, no benzidine positivity was observed in fibrin clots containing 100 µL unseparated
If antimembrane IgG cross-reacts immunologically with and neutralizes the bioactivity of soluble BPA, it should be useful in removal of soluble BPA from solution. Therefore, we incubated LCM supernatant with Staphylococcal protein A coated by various amounts of antimembrane IgG. Incubation of uncoated Staphylococcal protein A with supernatant had no effect on expression of BPA (Fig 5). However, as the amount of antimembrane IgG was increased from 50 to 200 \( \mu \text{g} \), the expression of soluble BPA by adsorbed supernatant was linearly reduced \((P > .05, \ P = .05, \text{and } P < .01 \text{ at } 50, 100, \text{and } 200 \ \mu \text{g IgG, respectively). Incubation of supernatant with Staphylococcal protein A coated with similar amounts of IgG from preimmune serum had no effect on the expression of soluble BPA in culture (data not shown).}

**Adsorption of Soluble BPA From LCM**

We next attempted to remove soluble BPA molecules by affinity chromatography using antimembrane IgG coupled to AffiGel 10. Figure 6 shows that the column had affinity for specific components of LCM supernatant. Studies aimed at identifying these components are now in progress.

**Adsorption of BPA Neutralizing Activity to Leukocyte Surfaces**

To determine whether IgG molecules that inhibit BPA in culture also recognize and bind to circulating LCM and/or 2.0 IU/mL erythropoietin in the absence of added inducing agent (data not shown).

**Table 1. Antimembrane IgG Effects on Erythroid Differentiation by K562 Cells**

<table>
<thead>
<tr>
<th>Addition to Culture</th>
<th>Benzidine-Positive Cells (%)</th>
<th>No. of Benzidine-Positive Clusters per 10^6 Cells*</th>
</tr>
</thead>
<tbody>
<tr>
<td>NCTC-109</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>Na butyrate (10^{-4} \text{ mol/L})</td>
<td>32 ± 6</td>
<td>11 ± 2</td>
</tr>
<tr>
<td>Antimembrane IgG + NCTC-109</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>Na butyrate (10^{-4} \text{ mol/L})</td>
<td>27 ± 4</td>
<td>9 ± 1</td>
</tr>
</tbody>
</table>

*The means ± SEM are shown for faintly positive clusters formed in four fibrin clots.
leukocyte membranes, IgG purified from preimmune or postimmune serum was preadsorbed with circulating leukocytes. Mononuclear cells were incubated in α-medium with and without IgG purified from preimmune and postimmune serum from the same rabbit. The cells were pelleted and the supernatants were tested in marrow cultures containing unseparated LCM. Figure 7 shows that LCM preincubated with adsorbed IgG from either preimmune or postimmune serum express BPA in culture normally. In contrast, very few erythroid bursts form in cultures established with LCM plus unadsorbed IgG from postimmune serum ($P < .01$).

Because it is possible that the lack of inhibition of BPA expression in cultures prepared with leukocyte adsorbed antimembrane IgG may be caused by release of erythroid growth-promoting factors rather than removal of inhibitory substances, we added α-medium supernatants that were prepared by pelleting mononuclear cells after short-term incubation (30 minutes) to marrow cultures in the presence and absence of unseparated LCM. Relative to cultures containing NCTC-109, no enhancement of burst proliferation was observed ($P > .05$) unless LCM prepared in the usual fashion was also added to culture (Fig 7). Last, to exclude the possibility that short-term incubation of mononuclear cells prompted the release of soluble molecules that interfere with the anti-BPA action of

Fig 5. Adsorption of LCM supernatants with antimembrane IgG-coated Staphylococcal protein A. Cultures were established with 2.0 IU/ml erythropoietin, 100 μL of unadsorbed LCM supernatant plus either antimembrane IgG or NCTC-109, or LCM supernatant adsorbed with Staphylococcal protein A preincubated with 0, 50, 100, or 200 μg antimembrane IgG, as described in Materials and Methods. The means ± 1 SEM are shown for bursts formed in each of four clots. Cultures with 100% maximum proliferation contained a mean of 68 bursts per clot at a marrow cell density of $6 \times 10^5$ per ml.

Fig 6. Adsorption of specific LCM components by affinity chromatography. LCM supernatant was adsorbed with Affigel 10 covalently linked to antimembrane IgG. SDS gels (3.5% to 18.0% acrylamide) of supernatant before (A) and after (B) chromatography are shown. Gels contain 50 μg protein and were silver stained.

Fig 7. Removal of anti-BPA from antimembrane IgG. Cultures contained 2 IU/ml of erythropoietin plus 100 μL of NCTC-109. LCM, LCM preincubated with antimembrane IgG, LCM preincubated with either adsorbed preimmune IgG or adsorbed antimembrane IgG from the same rabbit, cell supernatant (obtained after 30 minutes of incubation, 25 °C), LCM plus cell supernatant, or LCM preincubated with cell supernatant. Unseparated LCM from the same lot was used in each case. The means ± SEM are shown for bursts formed in each of four fibrin clots containing $6 \times 10^5$ human marrow cells per milliliter in concurrent cultures.
antimembrane IgG, α-medium supernatants were incubated with antimembrane IgG before addition to marrow culture. No alteration in BPA neutralizing activity was observed relative to clots containing antimembrane IgG alone (Fig 7).

Effects of Antimembrane IgG on Other Hematopoietic Growth Factors

We next addressed the question of antimembrane antibody specificity by determining whether it could neutralize erythropoietin activity in vivo. Table 2 shows that neither adsorption of known amounts of erythropoietin with antimembrane-coated Staphylococcal protein A nor preincubation of erythropoietin with antimembrane IgG affected 59Fe incorporation into newly formed mouse erythrocytes. Furthermore, when the antibody was added to murine marrow cultures with a source of GM-CSA, there was no reduction (P > .05) in formation of CFU-GM–derived colonies or clusters (Fig 8). These findings suggest that antibodies directed against circulating leukocyte membranes do not neutralize the activities of erythropoietin in vivo or colony-stimulating activity in vitro.

DISCUSSION

It is well known that normal and malignant cells shed cell surface components when incubated in liquid culture medium. Evidence from ongoing studies in a number of tissue culture systems suggests that cell surface-derived molecules may physically interact with neighboring cells, and that such interaction results in transfer of information regarding cellular differentiation. Erythropoietic differentiation and proliferation appear to be influenced by both soluble and pelletable plasma membrane–derived molecules. Moreover, studies using time lapse microcinematography suggest that more mature bone marrow precursor cell division is regulated by surface contact with lymphocytes. Our results presented here suggest that although they are physically distinct, cell surface and soluble regulators of erythropoiesis may be related by virtue of a common cellular site of origin: the plasma membrane.

This hypothesis is supported by our findings that (1) antimembrane IgG cross-reacts on immunoblots with components of low- and high-speed LCM supernatants, (2) incubation of antibody-coated Staphylococcal protein A with LCM fractions removes BPA from both pellets and supernatants, and (3) antimembrane IgG neutralizes BPA and possibly other erythroid growth factors in serum as well as in LCM fractions (Fig 3). Moreover, our observation that antimembrane IgG preadsorbed onto intact leukocytes expresses no antiBPA action (Fig 7) suggests that soluble and vesicular BPA are antigenically related to leukocyte cell surface components. Whether a similar relationship holds true with other sources of BPA such as media incubated with human spleen cells, bone marrow cells, or neoplastic T-lymphoblasts has not yet been determined. However, our results do not exclude the possibility that active soluble molecules may be adherent to vesicle surfaces. As more biochemical data become available regarding the structure of BPA, the relatedness of purified vesicular and soluble components of media conditioned by these cells will be better appreciated. Use of the well-known immunogenic properties of cell membranes to generate antiserum with anti-BPA activity and application of immu-

Table 2. Effects of Antimembrane IgG on Erythropoietin Bioactivity

<table>
<thead>
<tr>
<th>Study</th>
<th>Test Material</th>
<th>Antimembrane IgG</th>
<th>Mean ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>NCTC-109</td>
<td>Absent</td>
<td>0.07 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>Ep, 0.05 IU/mL</td>
<td>Absent</td>
<td>0.04 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>Ep, 0.125 IU/mL</td>
<td>Absent</td>
<td>3.30 ± 0.90</td>
</tr>
<tr>
<td></td>
<td>Ep, 0.125 IU/mL</td>
<td>Present</td>
<td>3.38 ± 1.05</td>
</tr>
<tr>
<td></td>
<td>Ep, 0.25 IU/mL</td>
<td>Absent</td>
<td>7.05 ± 0.84</td>
</tr>
<tr>
<td></td>
<td>Ep, 0.25 IU/mL</td>
<td>Present</td>
<td>6.49 ± 1.22</td>
</tr>
<tr>
<td></td>
<td>Ep, 2.00 IU/mL</td>
<td>Absent</td>
<td>11.41 ± 1.41</td>
</tr>
<tr>
<td></td>
<td>Ep, 4.00 IU/mL</td>
<td>Absent</td>
<td>16.12 ± 1.80</td>
</tr>
<tr>
<td>B</td>
<td>NCTC-109</td>
<td>Absent</td>
<td>0.16 ± 0.07</td>
</tr>
<tr>
<td></td>
<td>Ep, 0.50 IU/mL</td>
<td>Absent</td>
<td>3.81 ± 1.24</td>
</tr>
<tr>
<td></td>
<td>Ep, 0.50 IU/mL</td>
<td>Present</td>
<td>4.72 ± 0.88</td>
</tr>
<tr>
<td></td>
<td>Ep, 1.00 IU/mL</td>
<td>Absent</td>
<td>7.23 ± 0.70</td>
</tr>
<tr>
<td></td>
<td>Ep, 2.00 IU/mL</td>
<td>Absent</td>
<td>12.05 ± 0.81</td>
</tr>
<tr>
<td></td>
<td>Ep, 2.00 IU/mL</td>
<td>Present</td>
<td>11.37 ± 1.65</td>
</tr>
<tr>
<td></td>
<td>Ep, 4.00 IU/mL</td>
<td>Absent</td>
<td>13.57 ± 1.14</td>
</tr>
</tbody>
</table>

In study A, erythropoietin at the indicated concentrations was incubated with and without antibody-coated Staphylococcal protein A, 37 °C, 45 minutes. In study B, erythropoietin at the indicated concentrations was incubated in NCTC-109 directly with or without antibody prior to injection. Mean ± SEM of the percentage of 59Fe incorporation was observed in five animals.
noadsorption techniques may facilitate such purification efforts.

The mechanism by which antimembrane IgG exerts antiproliferative activity is difficult to define because of molecular and cellular complexities of in vitro stem cell assays currently in use. One possibility is that antibody molecules interact directly with progenitor and/or accessory cell surface components to exert a biological effect by physical blockade of BPA receptors and/or other uncharacterized growth factor receptors that mediate cellular interactions. Alone, this is an unlikely explanation for loss of BPA expression in marrow culture, since preincubation of vesicle-free LCM supernatants with IgG immobilized on Staphylococcal protein A beads and spun out before addition to culture successfully removed soluble BPA (Fig 5). In addition, our studies with a population of K562 cells support the argument that IgG operates via BPA. However, it is probable that antimembrane IgG recognizes antigenic sites not only on BPA molecules but also on accessory (and possibly progenitor) cell surfaces as well, since anti-BPA action can be removed from postimmune IgG by incubation with intact leukocytes. Whether such interactions take place with one or several subpopulations of mononuclear cells or nucleated cells in general is not known.

The plasma membrane components that are critical in generation of anti-BPA activity are not yet characterized. Because an immune stimulus was not used in our LCM preparations, it is possible that antimembrane antibodies recognize exfoliated antigenic structures that are expressed constitutively. In addition, it is also possible that biologically active membrane-derived vesicles may be released from cultured leukocytes as part of an allogeneic reaction during bone marrow incubation. Studies with antibodies generated by circulating leukocytes that are autologous to cultured bone marrow cells will be useful in exploring these possibilities.

We were surprised to find that antimembrane IgG suppressed CFU-E-derived colony growth, albeit incompletely and only at high protein concentrations. Our studies in bone marrow culture and in hypertransfused mice suggest that this effect may be mediated by BPA or BPA-like receptors, and are consistent with the two-stage model of erythropoiesis control of Iscove and Guilbert. Accordingly, BPA receptor expression is gradually lost during progenitor maturation so that partial neutralization of a serum growth factor (or factors) that supports but is not essential for CFU-E proliferation by anti-BPA antibody would be expected.

Although postimmune IgG does not inhibit CFU-GM proliferation, we are reluctant to conclude at this time that antimembrane IgG is erythroid specific, since conditioning cells used for GM-CSA production were prepared from whole murine lungs or from unseparated peripheral blood mononuclear cells. Additional studies with antibodies prepared against vesicles derived from specific cell populations will aid in determining whether such antibodies are lineage-specific and/or species-specific and whether other hematopoietic proliferation or growth suppressive factors are derived from the surface of conditioning cells.

From the present studies, it is apparent that BPA present in both shed membrane vesicles and soluble components of medium conditioned by circulating leukocytes can be neutralized by antibodies directed against partially purified leukocyte plasma membranes. Our data suggest that these two physically separable erythroid proliferation factors are immunologically related.

REFERENCES


Neutralization of erythroid burst-promoting activity in vitro with antimembrane antibodies

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