GROWTH OF B-LYMPHOCYTE CLONES IN SEMISOLID CULTURE IS MITOGEN DEPENDENT*

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In vivo cloning techniques have made it possible to estimate the frequency, triggering requirements, and proliferative potential of individual B lymphocytes, as well as the diversity of major class and specificity of their end products (1–3). Recently, an in vitro technique for cloning murine B lymphocytes in agar has been described (4). In this report, we present evidence that a polysaccharide B-cell mitogen/polyclonal activator is present in unrefined agar and it or another appropriate B-cell mitogen is essential to B-cell proliferation in semisolid cultures.

Materials and Methods

Animals. Conventional mice were either obtained from The Jackson Laboratories, Bar Harbor, Maine, (CBA/J) or Cumberland View Farms (CBA/Cum). nu/nu mice were bred at Sloan-Kettering Institute for Cancer Research, Rye, N. Y.

Semisolid Cultures. Single spleen cell suspensions were prepared, freed of coarse debris by settling and washed once with RPMI 1640 medium containing 10% fetal calf serum. Single strength McCoy's modified 5a medium plus 15% fetal calf serum, 2 mM L-glutamine, 16 μg/ml L-asparagine, 8 μg/ml L-serine, and 5 × 10^{-5} M 2-mercaptoethanol (2-ME) was warmed to 37°C and mixed with a 1/10 volume of boiled 3% Bacto agar in H2O. Spleen cells (2 × 10⁴) were added and 1-ml aliquots were allowed to gel in 35-mm tissue culture dishes. Alternatively, agarose at 0.2% final concentration or 1% methyl cellulose were used instead of agar. Granulocyte-macrophage progenitors (CFU-c) were stimulated with an optimal concentration of medium conditioned by murine myelomonocytic leukemia (WEHI 3) cells in cultures of 7.5 × 10⁴ bone marrow cells using the same medium without mercaptoethanol. Cultures were held at 37°C in a humidified atmosphere of 10% CO₂-90% air for 5–7 days before examination with a dissecting microscope.

Liquid Cultures. Duplicate cultures of spleen cells (2 × 10⁶/ml) were prepared in RPMI 1640 medium containing 5% fetal calf serum and 5 × 10^{-5} M 2-ME in 0.2-ml volumes plus 0.01 ml of mitogen or phosphate-buffered saline (PBS). After 3 days incubation at 37°C and 10% CO₂, the cultures were labeled by the addition of 0.2 μCi/well of 125I-iododeoxyuridine (125IUDR) (200 Ci/mm; New England Nuclear, Boston, Mass.). After an additional 3 h at 37°C, cultures were harvested on filter paper, washed with TCA using a semiautomatic cell harvester, and counted in a gamma counter. Lipopolysaccharide from Salmonella thyphosa W0901 (Difco Laboratories, Detroit, Mich.) and dextran sulfate (500,000 mol wt; Sigma Chemical Co., St. Louis, Mo.) were used as mitogens.

Extraction and Chemical Characterization of Agar Mitogen. Bacto agar (lot no. 504388 and 551975; Difco Laboratories) was extracted with 37°C H₂O for 1 h with occasional shaking, filtered through Whatman no. 1 paper on a scinttered glass funnel, and lyophilized. Up to 80 mg was recovered/d of agar. Before use the material was dissolved in 37°C H₂O and subjected to one of the

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**Table I**

| Supporting matrix         | Spleen cells + 2-ME | Bone marrow cells + CSF |
|---------------------------|---------------------|-------------------------|
| Calbiochem agarose        | 9 ± 4*              | 260 ± 62                |
| Indubiose agarose         | 0                   | 205 ± 34                |
| Seakem agarose            | 2 ± 2               | 264 ± 69                |
| Sigma 6277                | 0                   | 231 ± 37                |
| Sigma 6013                | 0                   | 246 ± 62                |
| Sigma 6138                | 0                   | 202 ± 35                |
| Noble Agar                | 532 ± 84            | 216 ± 46                |
| Bacto Agar                | 667 ± 34            | 275 ± 29                |

* Results of a single experiment employing the same medium and different types of agar or agarose. Triplicate cultures of 2 × 10⁴ spleen cells were examined after 5 days for foci of B-cell proliferation and tabulated as the mean number of aggregates (>4 cells) and colonies (>20 cells) per culture ± SD. Colony appearance and size distribution in agar supported cultures were as described (4), whereas, only very small aggregates were found in agarose cultures. Mean numbers of colonies ± SD present in triplicate cultures of 7.5 × 10⁴ bone marrow cells and colony-stimulating factor (CSF) were determined after 7 days incubation.

Results

**Importance of Supporting Matrix.** While attempting to optimize culture conditions for B-lymphocyte cloning, we found that only agar could be used as a supporting matrix (Table I). None of six brands of agarose at 0.15, 0.20, or 0.3% final concentration or methyl cellulose were suitable, whereas, these materials were approximately equivalent to agar in their ability to support granulocyte-macrophage progenitor (CFU-c) colony formation. B-lymphocyte colonies grew in agarose-agar mixtures in proportion to the percentage of agar present.

**Isolation of and Chemical Characterization of Agar Mitogen.** An aqueous extract was prepared from Bacto agar as described in the Materials and Methods. Addition of this material to agarose-semisolid cultures almost completely restored the ability of murine B cells to form colonies (Fig. 1). Furthermore, addition of as little as 1 μg/ml of this material to liquid cultures significantly stimulated the uptake of 125IUDR or [3H]thymidine in conventional 3-day liquid cultures of spleen cells. In both culture systems, 2-ME was an absolute require-
ment for cell proliferation. A variety of chemical treatments did not destroy this mitogenic activity, including boiling for 5 min; dialysis against H₂O, PBS, or 8 M urea; reduction and alkylation; alkaline hydrolysis; treatment with pronase; or concentration by ultrafiltration or lyophilization. Treatment with sodium m-periodate did, however, inactivate this substance (Fig. 2). Periodate-treated agar extract was not toxic or inhibitory for CFU-c colony formation but was antimitotic in cultures stimulated with untreated agar extract and to a lesser extent with lipopolysaccharide (LPS) (not shown). The extract did not gel at concentrations of 4% (40 mg/ml) after boiling in H₂O, but became turbid on standing at high concentration. Precipitates of presumably aggregated material which formed under such conditions were again H₂O soluble upon reboiling and mitogenic. Stimulating activity was present in at least two orcinol-positive peaks eluted from a Sepharose 6B column, including the excluded fraction, which had no detectable UV absorption.

Synergistic Effects of Mitogens on B-Cell Colony Formation. Addition of the B-cell mitogens, dextran sulfate (DS) or LPS, to agarose cultures of normal or nude spleen cells permitted the formation of a small number of colonies (Table II). More than additive numbers were observed when both mitogens were added at the same time. Similarly, the colony-stimulating ability of agar extract was greatly potentiated by the presence of LPS and to a lesser extent by DS. Cultures prepared in Bacto Agar could be further stimulated by mitogen addition. At maximum, approximately 10% of the B cells cultured proliferated to form colony size aggregates (assuming 45% of normal spleen cells are B lymphocytes).

Discussion

These studies indicate that the presence of a mitogen is obligate for murine B-lymphocyte proliferation in semisolid culture. Unrefined agar contains one or more mitogens with the chemical characteristics of a polysaccharide. This could be one of the sulfated polygalactans of agarpectin (5), but further analysis is
needed to determine if more than one mitogen is present. That this material is not of bacterial origin is suggested by the additive or greater than additive responses observed when agar mitogen was added to optimal amounts of DS or LPS. Additive effects of two mitogens indicate differences in the mitogens and possibly also in the responding cell populations, whereas, greater than additive colony stimulation suggests that some cells may require multiple mitogen encounters for proliferation. Both phenomena have previously been described for liquid culture systems (6-8). Resistance to alkali treatment, absolute dependence on the presence of mercaptoethanol, differential sensitivity of the
stimulated cells to anti-μ antibodies, and different patterns of Ig class expression by proliferating cells further distinguishes agar mitogen from LPS.¹

Agar has been used by others as a supporting matrix for studying cell-cell interactions in mitogen responses (9), to clone mitogen-activated cells (10-12), and in culture systems where agar was assumed to be inert (13). Our findings show that mitogenic stimulation by constituents of common agar can be avoided by either using agarose as a supporting material or by omitting 2-ME from the culture medium. It should be noted that these experiments were performed in the presence of fetal calf serum which can also contain weak mitogens (14), but stimulation also occurred with agar mitogen in serum-free liquid cultures. Several attempts to demonstrate mitogenic stimulation of human, avian, and porcine lymphoid cells by agar mitogen in either semisolid or liquid culture have been unsuccessful.

A more complete analysis of the responding cells and variables in culture conditions will be presented elsewhere.¹ Briefly, many strains of mice respond to agar mitogen, including C3H/HeN mice which are unresponsive to certain preparations of LPS (15). The responding cells in adult spleen are Ia+ and IgM+ and proliferation is sensitive to divalent antibodies directed to these. Sedimentation velocity separation of these cells suggests size heterogeneity. That the responding cells are B cells is evident from their insensitivity to anti-Thy-1 plus complement and their frequency in nu/nu mice.

Summary

A substance which was mitogenic for murine B lymphocytes in the presence of 2-mercaptoethanol was isolated from agar. Stimulating activity of this material was stable to proteolysis or protein denaturants but was destroyed by periodate treatment. Agar-derived mitogen stimulation was distinct from that obtained with dextran sulfate or lipopolysaccharide and may define different populations of B lymphocytes.

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