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Brief Definitive Report

LYMPHOCYTE CULTURE: INDUCTION OF COLONIES BY CONDITIONED MEDIUM FROM HUMAN LYMPHOID CELL LINES*

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It has been shown that macrophage and granulocyte colonies can be induced in semisolid agar (1–3) in the presence of substances termed colony-stimulating factors (CSF), which are released predominantly by monocytes (4). However, attempts to induce formation of lymphoid colonies with CSF have so far proved unsuccessful. In the mouse, B lymphoid colonies are formed in the presence of 2-mercaptoethanol (5), and T lymphoid colonies can be induced with the plant lectins phytohemagglutinin (PHA) and concanavalin A (6). T lymphoid colonies can also be established from human peripheral blood lymphocytes in the presence of PHA (7–9), whereas with pokeweed mitogen mixed T and B lymphoid colonies are formed (9). Established human lymphoid cell lines multiply spontaneously in the absence of plant lectins or mercaptoethanol, and it seemed possible that such cells might release growth-stimulating substances into the culture medium. We have therefore investigated the ability of conditioned medium (CM) obtained from lymphoid cell lines to induce normal human peripheral blood lymphocytes (PBL) to form lymphoid colonies in agar.

Materials and Methods

Three B-cell lines – 4061 (provided by Dr. J. Sinkovics, Houston, Tex.), PGLC 33H, and RPMI 1788 – were used as sources of CM. After 3–4 days of culture in serum-free medium at 37°C, cell-free supernates were obtained by centrifugation at 800 *g* for 10 min, filtration through 0.22-μm filters (Millipore Corp., Bedford, Mass.), and ultracentrifugation at 47,500 *g* for 1 h.

Peripheral blood was collected from adult healthy volunteers in pyrogen- and preservative-free heparin. Lymphocytes were obtained by centrifugation over Ficoll Isopaque gradients and washed three times in RPMI 1640 medium (Grand Island Biological Co., Grand Island, N.Y.). Contamination with monocytes varied between 3 and 20% as assessed by latex ingestion and staining for peroxidase (10). Cells were seeded at various concentrations in 2 ml 0.3% agar made by the addition of 1 vol of double-strength RPMI 1640 (containing 200 μg/ml streptomycin, 200 U/ml benzyl penicillin, and 20% fetal calf serum [Gibco]) to 1 vol of 0.6% agar (Difco Laboratories, Detroit, Mich.) made up in pyrogen-free distilled water. Various amounts of CM were added directly to the 0.3% agar mixture. After culture in 35-mm plastic Petri dishes at 37°C in 5% CO_2 in air for various times, the plates were counted under an inverted microscope. Colonies were defined as clumps containing more than 50 cells. Colony cells were tested for their ability to form E rosettes, by using the technique of Wybran et al. (11), and for the presence of surface

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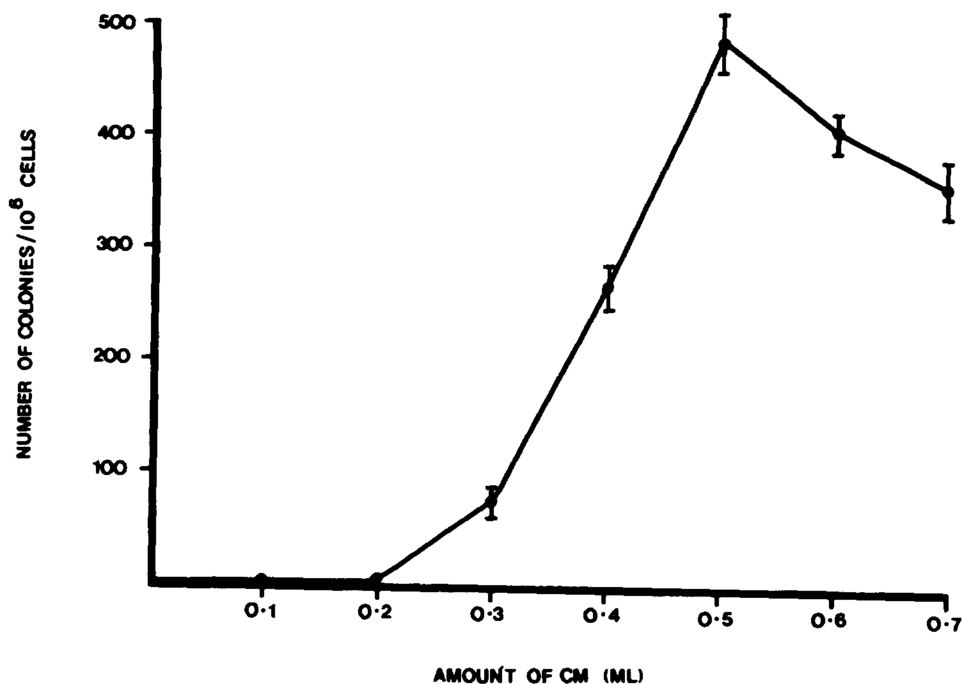

FIG. 1. Number of colonies induced by different amounts of CM (each point represents the mean of six replicate experiments).

immunoglobulin, by using fluorescence microscopy and $F(ab')_2$ goat polyvalent anti-human immunoglobulin as described by Winchester et al. (12).

Results

In all cultures, the number of lymphocytes decreased rapidly during the first 3-4 days. In control dishes without CM, few viable cells remained at 8 days, whereas CM at a sufficient concentration induced colonies containing more than 50 cells, which began to appear on days 4-5. To determine optimum conditions for colony formation in this system, 10^6 cells/ml were cultured with increasing amounts of CM (Fig. 1). Few colonies were obtained when 0.1 or 0.2 ml CM was added, but with 0.3, 0.4, and 0.5 ml the number of colonies increased proportionally. Larger amounts did not produce an appreciable increment and also caused excessive dilution of the agar. Thus, 0.5 ml CM (20%) was employed in subsequent experiments, and the final concentration of agar was 0.24%.

The effects of seeding different numbers of lymphocytes at the optimum CM concentration (20%) were then studied (Fig. 2). Few colonies were formed at an initial concentration of 4×10^5 cells/ml or less. At 6×10^5–10×10^5 cells/ml, the number of colonies increased progressively (Fig. 2); and at higher initial concentrations the plates in many cases became so crowded with colonies that accurate counting was not possible. Subsequent experiments were therefore carried out by using 10^6 cells/ml.

The morphology and number of colonies formed by PBL from 20 healthy subjects were investigated after culturing 10^6 cells/ml in 20% CM. Most of the

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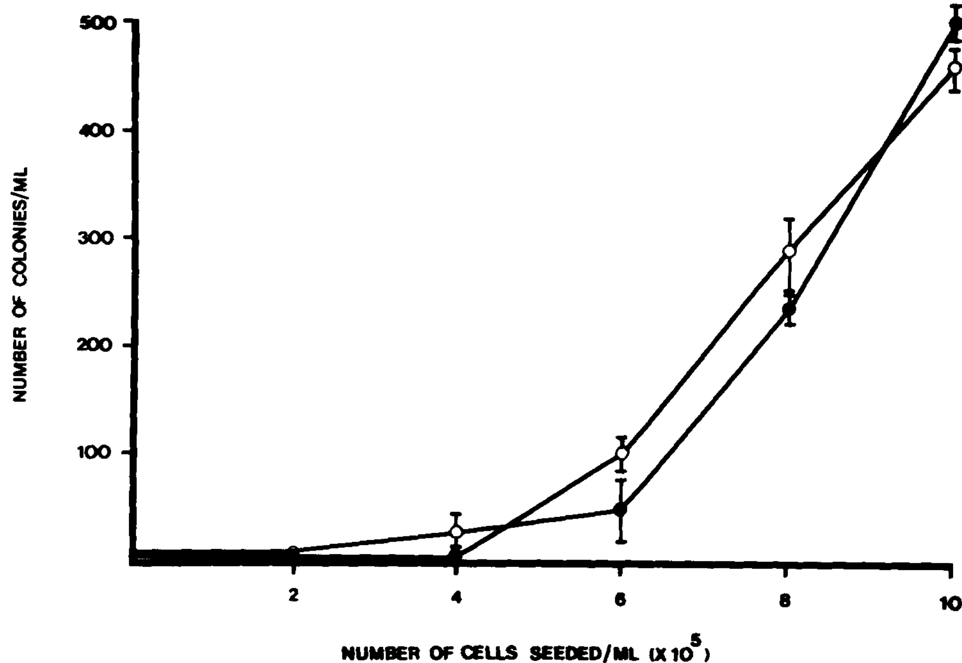

FIG. 2. Relationship between number of cells seeded and number of colonies induced (each point represents the mean of four replicate experiments). Two experiments performed on separate days by using cells from the same donor.

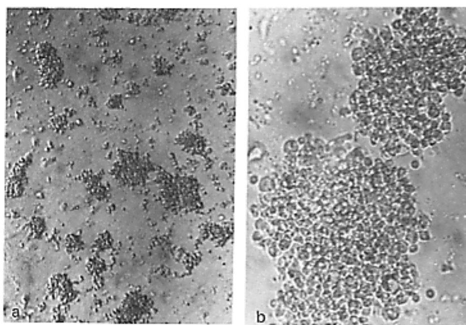

FIG. 3. Photomicrographs of unstained colonies induced by CM. (a) × 40, (b) × 250.

colonies consisted of tightly packed clumps of cells of uniform size (Fig. 3). Removal of intact colonies from Petri dishes with a drawn-out Pasteur pipette and treatment of dispersed cells with standard histochemical stains revealed lymphoid cells with scanty cytoplasm, and the appearances of individual cells were remarkably constant. No colonies with characteristics of either granulocyte or macrophage series were observed. Occasionally small colonies appeared in the form of loose monolayers of larger pleomorphic cells, but these had dispersed in many cases by the end of the culture period. Colonies were first visible on days 4-5, reached maximum size on days 8-10 when they contained up to 1,000 cells, and dispersed by day 14-16. The total number of colonies varied between 50 and 500 per 10^6 cells cultured for the 20 subjects tested. Some

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day-to-day variation was apparent within this range when studies were performed on several different days, but in individual subjects the results obtained in replicate cultures on a given day were relatively constant. The morphology and number of colonies formed were similar for the three CM tested.

To determine the number of colony cells behaving as T and B lymphocytes, colonies were removed from the plates, gently dispersed in RPMI 1640, and washed three times in medium to remove as much agar as possible. E rosette and surface immunoglobulin assays were then performed. Since removal of agar was difficult, control experiments were performed by using peripheral blood lymphocytes from the same subject both before and after immersion for 4 h in agar containing no CM. In six control experiments, the mean percentage of cells forming E rosettes fell from 65 to 50%, and the mean value for cells positive for surface immunoglobulin was reduced from 12 to 8%. When colony cells derived from the same six subjects were examined, 65–76% formed E rosettes. In contrast, cells displaying surface immunoglobulin were seen only rarely, although in three further experiments in which pooled cells obtained from the whole plate rather than individual colonies were examined, such cells were found to constitute 1, 2, and 4% of the total, respectively. Thus it appears that the majority of cells in colonies induced by CM obtained from these three B-cell lines are of T-cell type since they form E rosettes and are negative for surface immunoglobulin.

Discussion

This system for the induction of lymphoid colonies from normal peripheral blood with CM may be compared with three recent reports concerning the use of PHA for this purpose (7–9). The culture conditions employed are comparable, although a harder underlayer of 0.5% agar was not used in the present study. The similarity between the results reported in these three studies and the results of present experiments are particularly striking in terms of the relationship between the number of cells seeded and the final number of colonies and the total numbers of colonies obtained under optimal conditions. Furthermore, the morphology of cells derived from colonies and the percentages forming E rosettes and bearing surface immunoglobulin appear to be similar.

The nature of the mitogenic substances in CM is not clear. One possibility is that viral products related to the Epstein-Barr (EB) virus genome, which is known to be present in B-cell lymphoid lines (13), may be responsible; however, none of the B-cell lines employed are known producer cell lines although they are likely to be positive for the EB nuclear antigen (13, 14). Moreover, it is highly unlikely that viral DNA was present in the CM used in our experiments, since the cell-free supernates were obtained by centrifugation and filtration and subjected to ultracentrifugation. In addition, in preliminary experiments we have found that colony formation is also induced by CM from two T-cell lines, MOLT-4 and CEM, which are known to be negative for EB viral products (14). Another possibility is that lipopolysaccharide (endotoxin) may be present in CM and cause formation of colonies. This seems unlikely since the mitogenic effect apears to be exerted predominantly on B cells (15), and recent experiments have shown that although lipopolysaccharide potentiated the colony-inducing activity of mitogenic factors such as PHA, it did not induce colony formation *de*

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novo (16). Furthermore the cell lines used in this study were free from bacterial contamination, and the CM did not contain endotoxin as judged by the limulus assay (17).

Further work is necessary not only to characterize the factor(s) in CM responsible for induction of colony formation and to investigate cell supernates from other cell lines including fibroblasts for similar activity, but also to examine the ability of lymphocytes from patients with lymphoproliferative and other malignant conditions to form colonies. It is interesting in this regard that PHA-induced colony formation has been reported to be severely depressed in unselected patients with malignant disease (8, 18). Such studies will be facilitated by the ease with which growth of lymphoid cell lines and preparation of large pools of CM can be accomplished.

Summary

The presence of phytohemagglutinin or pokeweed mitogen in cultures of human peripheral blood mononuclear cells in agar is known to stimulate the formation of lymphoid colonies. We now report that similar colonies can be induced in the absence of plant lectins upon addition of filtered and ultracentrifuged conditioned medium (CM) obtained from certain human lymphoblastoid cell lines. Colony formation required at least 6×10^5 mononuclear cells per milliliter, and optimum results were obtained at concentrations of 1×10^6 cells/ml in the presence of 20% CM (50–500 colonies per 10^6 cells cultured). Individual cells within colonies displayed uniform morphological characteristics of lymphoid cells, and the majority formed rosettes with sheep erythrocytes, suggesting that they were of T-cell type.

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