Immunology, 1975, **29**, 1041.

Clonal Proliferation of PHA-stimulated Human Lymphocytes in Soft Agar Culture

L. A. ROZENSZAJN, D. SHOHAM AND I. KALECHMAN

Clinical Laboratories, Meir Hospital, Kfar Saba, Israel, and Department of Life Sciences, Bar-Ilan University, Ramat-Gan, Israel

(*Received* 30*th March* 1975; *accepted for publication* 19*th June* 1975)

Summary. The purpose of this investigation was the induction of clonal proliferation of PHA-stimulated normal human lymphocytes using a two-layer soft agar technique. Essential conditions for colony formation include preceding sensitization of lymphocytes with PHA, and continuous presence of PHA in the soft agar culture. Two types of colonies developed: large colonies which appeared 3–4 days after seeding and comprised, after 5–6 days, 200–500 cells, and small colonies which were seen after 6–7 days of culture, resulting in production of 50–150 cells. Morphological study showed that all cells were blast-like and the mitotic index exceeded that in liquid medium by a factor of 50.

Comparison between the number of colonies developing from cultured bone marrow and spleen cells with those from peripheral blood showed that, in proportion to the number of lymphocytes seeded, a larger number of colonies developed from bone marrow cells and a lower number of colonies developed from spleen cells.

The time required for sensitization of lymphocytes in liquid medium with PHA was found to be no less than 12 hours. The greatest number of colonies appeared when the optimal concentration of PHA was placed in the lower agar layer. A linear relation between the number of cells seeded and the number of resulting colonies was found. One out of 2×10^3 or 3×10^3 lymphocytes in peripheral blood has the potential to develop as colony.

The rosette-forming ability and morphological identification of the cells suggest that the colonies are composed of T lymphocytes.

INTRODUCTION

Several plant lectins, such as phytohaemagglutinin (PHA) (Nowell, 1960), concanavalin A (Douglas, Kamin, Davis and Fudenberg, 1967; Leon and Powell, 1968), and pokeweed mitogen (PWM) (Farnes, Barker, Brownhill and Fanger, 1964) stimulate, in culture conditions, small human resting lymphocytes to undergo transformation manifested by the appearance of blast-like cells and mitotic forms. Most blastoid cells present in cultures after the first 3 days are derived, by repeated division, from a small precursor pool, and they produce daughter cells of the same morphological type. After two or three generations of

Correspondence: Professor L. A. Rozenszajn, Clinical Laboratories, Meir Hospital, Kfar Saba, Israel.

new cells, within 10–14 days, the cultures begin to decline and become moribund (Mills, 1970; Bender and Prescott, 1962).

Different types of membrane markers have been demonstrated on normal human lymphocytes. Spontaneous rosette formation with sheep red blood cells seems to be a property of T cells (Jondal, Holm and Wigzell, 1972; Wybran, Carr and Fudenberg, 1972; Lay, Mender, Bianco and Nussenzweig, 1971), while lymphocytes bearing membrane-associated immunoglobulin are considered to be B cells (Raff, 1970; Unanue, Grey, Rabellino, Campbell and Schmidtke 1971; Pernis, Ferrarini, Forni and Amante, 1971). Rosette formation with mouse red blood cells may be another marker for B cells (Stathopoulos and Elliott, 1974).

Experiments with mice suggest that con A and PHA stimulate T lymphocytes, whereas PWM induces proliferation of both B and T cells (Raff, 1973).

In order to investigate the process of proliferation, differentiation, and maturation of haemopoietic cells, methods using soft agar were developed which permit growth of clones of haemopoietic cells from a single stem cell, such as macrophage–monocytes, granulocytes, and eosinophils in the presence of stimulating factors (Paran, Sachs, Barak and Resnitzky, 1970; Pike and Robinson, 1970; Iscove, Senn, Till and McCulloch, 1971; Chervernick and Boggs, 1971; Shoham, Ben David and Rozenszajn, 1974; Cline and Golde, 1974).

By means of the technique of time-lapse cinematography it was demonstrated that lymphocytes can also develop in clonal form after stimulation by antigens such as PPD (Marshall, Valentine and Lawrence, 1969).

Isolated lymphocytes were also induced to undergo cell division as a result of stimulation with PHA in a liquid culture medium. Development of lymphocyte clones was followed by using microtest plates (Chi and Bloom, 1970; Coulson, Turk, Glade and Chessin, 1968).

Successful culture of lymphocytes in soft agar has not been reported previously. The aim of the present investigation was the induction of clonal proliferation in soft agar of mitogen-stimulated normal human lymphocytes from peripheral blood, lymphoid tissues, and bone marrow. A further aim was to study the conditions of clone-forming ability of such cells as well as the identity of the individual clones which had developed from one particular precursor cell.

MATERIALS AND METHODS

Culture methods

Human lymphocytes taken from thirty healthy volunteers were cultured by the modified method of Moorhead (Moorhead, Nowell, Mellman, Battips and Hungerford, 1960; Mellman, 1965), using PHA to induce transformation and mitosis of lymphocytes. Venous blood samples (20–30 ml) were obtained by means of disposable plastic syringes containing 250–500 i.u. heparin (pyrogen-free, Evans). The lymphocytes were isolated from the other white blood cells on glass columns according to the technique of Rabinowitz (1964).

Each tissue culture contained about 8×10^6 lymphocytes, 2 ml of autologous plasma, 0·1 ml of Bactophytohaemagglutinin M (PHA) (Difco), and Eagle's medium (EM) (Dulbecco's modified, Gibco) up to 8 ml. The medium contained 1000 i.u./ml of penicillin and 2 mg/ml of streptomycin. Duplicate tubes of lymphocyte cultures were set up in each

experiment. One of them was used to culture the cells in soft agar, and the other one for estimation of the lymphocyte transformation after 72 hours of culture.

Soft agar culture

After 15–18 hours of incubation at 37° in a water-saturated atmosphere containing 5–7·5 per cent CO_2, the culture tubes were centrifuged at 150 ***g*** for 5 minutes, the supernatant was removed, and the sedimented cells were washed three times in EM. The sediments were then resuspended at a concentration of 10^6 lymphocytes in 0·2 ml of EM. Resuspended PHA-sensitized lymphocytes were seeded in amounts of 10^6 cells per each Petri dish.

Preparation of plates and seeding

The culture method used was the soft agar gel system for culturing bone marrow cells (Pluznik and Sachs, 1965; Bradley and Metcalf, 1966) using Petri dishes of 15 × 60 mm (Falcon). The lower agar layer in each Petri dish consisted of 5 ml of medium composed of: 50 per cent EM; 15 per cent 2 × EM; 20 per cent foetal calf serum (FCS) (Gibco); 15 per cent agar (stock agar solution of 3·3 per cent); 0·0625 ml of PHA were added to 5 ml of medium. The plates were allowed to gel at 24°, and overlayed with soft agar containing the seeded cells (10^6 cells).

This upper layer with a volume of 1·7 ml, was composed of: 36 per cent 2 × EM; 18 per cent FCS; 18 per cent water; 18 per cent agar (stock agar solution 1·8 per cent); 10 per cent EM containing 10^6 lymphocytes. The mixture was permitted to gel at 24° for 15 minutes.

The dishes were incubated at 37° in a water-saturated atmosphere with 5–7·5 per cent CO_2. As controls, cultures were set up in which the lower agar layer did not contain PHA or the lymphocytes present in the upper layer had not been exposed to PHA.

During 3–7 days of culturing, the development of clones and their morphology were observed under an inverted microscope using a magnification of × 50. To follow the development of clones, ten isolated blast-like cells from different cultures were selected and observed under an inverted microscope every 3–4 hours, for a period of 40 hours, beginning with the 3rd day after seeding. Each time the number of cells was determined.

Bone marrow cells

Bone marrow samples were obtained from patients who required this diagnostic procedure; material selected for this study was used only when bone marrow smears studied by means of light microscopy of May–Grünwald–Giemsa (MGG) stained smears were diagnosed as normal. Subsequently the number of lymphocytes was estimated by differential counting of 500 nucleated cells.

The bone marrow samples were stimulated with PHA during 15 hours in a liquid culture medium and the nucleated cells were seeded in amounts of 10^6 cells per Petri dish. Six out of twelve bone marrow samples cultured, were evaluated for colony formation.

Spleen cells

The specimens were prepared from five spleens obtained from patients splenectomized because of traumatic rupture of spleen. The spleens were cut into small pieces, crushed, and passed through stainless steel mesh in the presence of EM. The isolated cells were sensitized with PHA and 10^6 cells were seeded per plate.

Removal of small and large lymphocyte colonies from culture medium

Plates with colonies were eluted by addition of 1 ml of EM or PBS and allowed to stand for 1 hour in an incubator at 37°. The small and defined colonies float on the fluid permitting their collection. The large colonies were picked out with a capillary tube, 0·8 × 100 mm, and its contents were blown into a tube containing EM. Both types of colonies were gently and repeatedly mixed with EM by means of a Pasteur pipette in order to separate the cells of each colony. They were washed three times with phosphate-buffered saline (PBS) and resuspended in PBS.

Morphological studies

In each experiment the number and morphology of the colonies were evaluated from six plates of culture. Twelve colonies in each culture were punched out using capillary tubes, 0·8 × 100 mm, and their contents were blown onto clear slides, mixed with inactivated (56° for 30 minutes) FCS and allowed to dry in air for at least 3–6 hours.

After 3–4 days and after 6–7 days of culture the morphology of the cells was examined by phase contrast microscopy and by staining the specimens according to May–Grünwald–Giemsa. The percentage of transformed lymphocytes, i.e. large blast-like cells with basophilic cytoplasm was estimated and the mitotic index (number of mitosis per 1000 blasts) was determined.

The results obtained with cells cultured in soft agar were compared with those obtained with cells growing in the liquid culture tubes.

Cytochemical studies

In specimens obtained for morphological studies, cytochemical staining was performed. The staining methods used were peroxidase for general evaluation (Osgood and Ashworth, 1958). Non-specific esterase was determined by using alpha-naphthyl acetate and naphthol AS-D chloroacetate as substrates (Rosenszajn, Leibovich, Shoham and Epstein, 1968). Periodic acid Schiff (PAS) reaction was carried out with and without ptyaline digestion (Hayhoe, Quaglino and Elemans, 1960). Luxol fast blue stain for eosinophils (Shoham *et al.*, 1974) and alcoholic aqueous toluidine blue stains were also used (Undritz, 1952).

Electron microscopy

Cells isolated from the colonies were fixed in 2·5 per cent glutaraldehyde in 0·1 M cacodylate–HCl buffer (pH 7·2) and kept at 4° for 2 hours. They were then washed with cacodylate–HCl buffer, refixed in 1 per cent OsO_4 in cacodylate–HCl buffer, dehydrated and embedded in Epon 812 as described by Luft (1961). Sections were made with an LKB Ultratome and they were examined with a Philips 300 electron microscope.

Chromosome preparation

After 5 days of culture of the lymphocytes in agar, 1 ml of 20 μg/ml colcemide was added to each dish and incubated for a period of $2\frac{1}{2}$ hours.

The small defined colonies which floated in the colcemide solution, were centrifuged and washed three times with EM. The larger diffuse colonies were punched out, as described above, and treated in the same way. Chromosome preparations were made, and the karyotype analysed for fifty cells in metaphase, using phase contrast microscopy.

Representative cells were photographed on 35 mm Microfilm Kodak and karyotypes

were prepared from the enlarged prints. The Denver system of nomenclature was used (Denver Study Group, 1960).

Determination of immunoglobulin and rosette formation on lymphocytes from colonies

The separated and washed lymphocytes, in concentrations of about 2×10^6 cells/0·05 ml PBS, were incubated with 0·05 ml of fluoresceinated anti-immunoglobulin sera (Institute Pasteur), diluted 1:2 in PBS, for 1 hour at 37°. They were washed three times with PBS and resuspended in mixture of equal volumes of glycerine and PBS. One drop of the cell suspension was pipetted on a glass slide covered with a coverslip and examined under a u.v. microscope (Aisenberg and Bloch, 1972).

For estimation of rosette formation, separated and washed lymphocytes were concentrated to 10^7 cells/ml PBS for rosettes of sheep red blood cells (SRBC) (Wybran, Chantler and Fudenberg, 1973), and to 4×10^6 cells/ml PBS for rosettes of mouse red blood cells (MRBC) (C57Bl strain) (Stathopoulos and Elliott, 1974).

The number of rosettes was determined by phase contrast microscopy counting 500 cells with a magnification of ×320 and ×800. A rosette was defined as a lymphocyte surrounded by at least three red blood cells.

As control for rosette formation leucocytes from patients suffering from chronic lymphatic leukaemia (CLL) were used, as well as monocytes–macrophages and eosinophils obtained from colonies of normal human bone marrow cultured in soft agar, and blast-like cells obtained by culturing normal human lymphocytes in liquid media.

Experimental factors influencing development of colonies from lymphocyte cultures

Optimal time for sensitization of lymphocytes with PHA. The cells were incubated in the presence of PHA for the following time periods: 2, 4, 6, 8, 10, 12, 24, 48 and 72 hours. After incubation, cells were washed three times with EM in order to remove the PHA, and 10^6 cells were inoculated per agar plate. Six days later the numbers of colonies were counted.

Effect of varying concentrations of PHA on development of colonies. Various concentrations of PHA ranging from 0·00125 ml to 0·3 ml per ml medium were placed in agar plates. PHA was added either to the lower or the upper layer. Numbers of colonies were determined after 6 days of incubation.

Effect of PHA on development of colonies by lymphocytes undergoing cell division. After lymphocytes were sensitized with PHA for 72 hours they were washed three times in EM, resuspended, and seeded in amounts of 10^6 cells per culture dish in absence of PHA in the lower agar layer. As control, sensitized lymphocytes were seeded in the presence of PHA.

Relationship between numbers of colonies and numbers of lymphocytes seeded. Lymphocytes sensitized with PHA were seeded in culture plates in decreasing amounts ranging from 10^6 to 10^4 cells per dish. In additional experiments, to 2×10^5 and 5×10^5 and 10^6 PHA-sensitized lymphocytes, the appropriate number of unsensitized lymphocytes was added so that the total number of lymphocytes per plate equalled 10^6.

Test for cell aggregation simulating development of colonies. Two days after seeding, small groups of cells were encircled with pyrex rings of 2·5 mm diameter, penetrating the entire thickness of the upper agar layer and part of the lower layer.

After 6 days of incubation, the size of the colonies within the rings was compared with that of colonies outside the rings.

Effect on colony formation of lymphocytes sensitized and cultured with the same or different mitogens (con A, PWM, PHA)

Three sets of duplicate test tubes were used; each tube contained 8 ml of EM with 10^6 lymphocytes per ml. To each tube of these sets, one of the following was added: 0·1 ml PHA; 0·1 ml PWM (Gibco); 12·5 μg, 25 μg, or 50 μg of con A (Miles–Yeda).

The cells were seeded in soft agar for colony formation as described above. The lower agar layer contained one of three mitogens in 1 ml of culture medium: 0·0125 ml PHA; 0·0125 ml PWM, 1·25 μg, 2·5 μg or 5·0 μg of con A. The lymphocytes sensitized by each of these mitogens were seeded on medium containing the same or one of the other mitogens, and colony formation was determined.

The transformation of lymphocytes was evaluated from the duplicate tube after 72 hours of culture, and an additional examination was made after 168 hours of the culture containing PWM.

RESULTS

Human lymphocytes from peripheral blood, bone marrow and spleen which were stimulated with PHA in EM prior to being seeded in soft agar, developed into clones containing at least fifty cells each, 3–5 days after seeding.

Colonies of lymphocytes developed only in continuous presence of PHA, both in the preliminary suspension medium and in the soft agar layer. No colonies developed in control cultures.

Three phases were observed in the development of these colonies: (1) logarithmic phase of multiplication during the first 5 days; (2) stationary phase lasting 3 days; (3) gradual degeneration of colonies.

Two days after seeding, numerous single blast-like cells in the upper layer were noted, by means of the inverted microscope. Most of these cells disappeared during the succeeding 3 days, while the remainder developed into colonies through the 3rd to 5th day of culture. After 10 days, the colonies degenerated and the cells were lysed. No other types of colonies, such as monocytes–macrophages or granulocytes, were found during the development of lymphocyte colonies or after their degeneration.

Two types of colonies developed: large colonies which appeared 3–4 days after seeding on the bottom of the upper agar layer and comprised, after 5–6 days, 200–500 or more cells (Fig. 1), and small colonies which were seen in a higher plane in the upper agar layer after 6–7 days of culture resulting in the production of 50–150 cells (Fig. 2).

The number of colonies per 10^6 cells seeded ranged, for peripheral blood, from 250 to 500, and for spleen from 60 to 150 colonies. From 60 to 100 colonies were obtained from 10^6 nucleated bone marrow cells. It should be noted that, morphologically, 7–13 per cent of bone marrow cells were lymphocytes.

No differences were observed in the morphology, development, and decline of the colonies derived from cultured lymphocytes from peripheral blood, bone marrow, or spleen cells.

The morphological study of the colonies which appeared after 3–4 days and 6–7 days of culture showed that all cells were large pyroninophilic cells and blast-like, some of them in mitosis. The cells growing in the upper and lower layers showed similar morphology. The mitotic index determined in the colonies was 4–5 per cent; in the suspension culture it was 0·1 per cent. The karyotype of these mitoses was perfectly normal.

Under phase microscopy the cells had a characteristic morphology manifested by prominent nucleoli and cytoplasmic vacuoles, thus resembling transformed normal human lymphocytes stimulated by PHA in blood culture (Fig. 3).

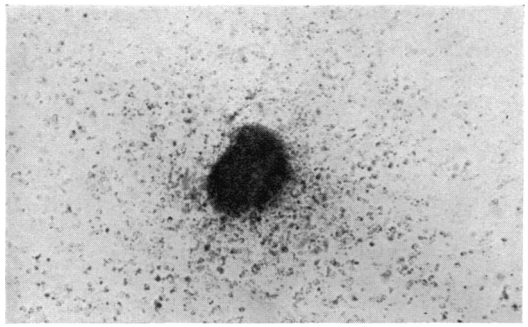

FIG. 1. Appearance under light microscope of unstained colony of human blood lymphocytes. Large colony which developed on the bottom of the upper agar layer. (Magnification ×20.)

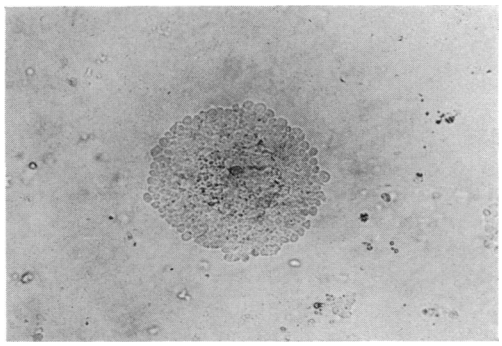

FIG. 2. Appearance under light microscope of unstained colony of human blood lymphocytes. Small colony which developed in the upper agar layer. (Magnification ×40.)

By testing esterase activity and using α-naphthyl acetate as substrate, many small granules were seen scattered throughout the cytoplasm. When naphthol AS-D chloroacetate was used as substrate, non-enzymatic activity was found; in some cytoplasmic vacuoles a single azo dye granule was seen. When peroxidase was tested no enzymatic activity was demonstrated. The PAS staining was moderately positive in a few cells, and Luxol staining and toluidine blue stains were negative.

By electron microscopy (Fig. 4a and b) the cells were characterized by large nuclei with prominent nucleoli. The cytoplasm contained abundant ribosomes, short and long filaments of endoplasmic reticulum, and mitochondria which were generally round or ovoid, but elongated forms were often found.

The Golgi apparatus opposite the nuclear indentation was usually well developed, consisting of small vesicles and a lamellar structure.

The cytoplasm contained a small number of lysosomes shown as dense particles, vacuoles and lipid material. Many cells showed pseudopodia.

We failed to get colonies when con A or PWM were used. The percentage of lymphocytes transformed by PWM was 30 per cent after 72 hours and 45 per cent after 168 hours of incubation. Using con A in concentrations of 1·25, 2·5 and 5 μg/ml, the percentage of transformed lymphocytes was 10, 20 and 30, respectively, after 72 hours of incubation. With PHA the percentage of transformed lymphocytes was 83.

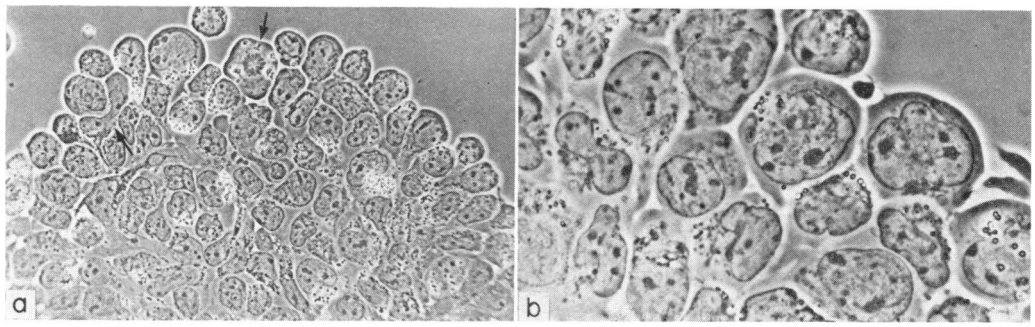

FIG. 3. Appearance in phase contrast microscope of cells derived from small colony after 5 days of culture. (a) Note mitosis (arrow) and cytoplasmic projections indicative of motility. (b) Note prominence of nucleoli and spherical refractile cytoplasmic inclusions.

ROSETTE FORMATION AND PRESENCE OF IMMUNOGLOBULINS

Technically, these procedures could not be done with cells from the lower colonies because these cells could not be separated from the agar.

Lymphocytes from the upper colonies formed 50–75 per cent of SRBC rosettes and 0–2 per cent of MRBC rosettes with red cells of the C57Bl strain (Fig. 5). In control tests for rosette formation, using monocytes–macrophages and eosinophils from soft agar culture, no rosette-forming ability was found. Lymphocytes from CLL showed 80–90 per cent of MRBC rosettes and 1–4 per cent of SRBC rosettes. Blast-like cells from lymphocyte cultures (liquid media) showed 65–70 per cent SRBC rosettes and 1–4 per cent MRBC rosettes.

No significant immunofluorescence of lymphocytes was detected when surface immunoglobulin receptors were sought.

EVALUATION OF FACTORS INFLUENCING THE CULTURE METHOD

The optimal sensitization time was defined as that period during which lymphocytes, when incubated in the presence of PHA, developed into the maximal number of colonies. This time was found to be no less than 12 hours. Incubation periods in liquid medium with PHA, exceeding 12 hours, did not lead to any further significant increase in the number of developing colonies (Fig. 6).

For the sake of convenience, the period of incubation of lymphocytes with PHA was 15–18 hours in all experiments, except where other times are specifically mentioned.

By contrast with development of 250–500 colonies in plates containing PHA, no colonies

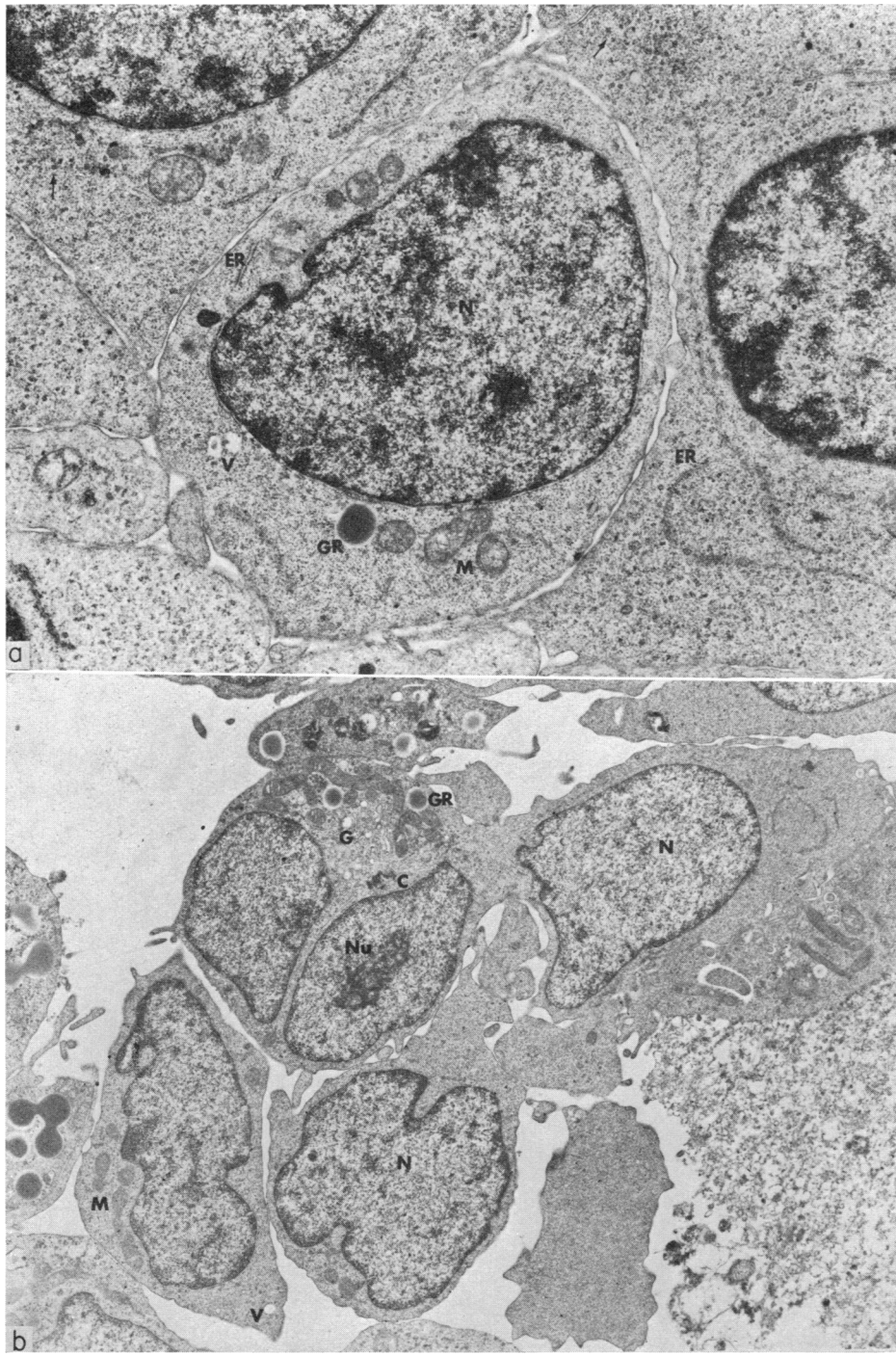

FIG. 4. Electron micrographs of cells from colonies developed from cultured normal human lymphocytes after 5 days of culture of soft agar. Note increase in euchromatin in the nucleus (N), polyribosomes (arrow), centriole (c), nucleolus (Nu), Golgi apparatus (G), granular inclusions (GR), small vacuoles (V), small and large mitochondria (M) and profiles of endoplasmic reticulum (ER). (a) Cells in contact with each other, probably from the more compact region of the colony. (Magnification ×8160.) (b) Many of the cells had pseudopodia, and cellular debris was observed. (Magnification ×3960.)

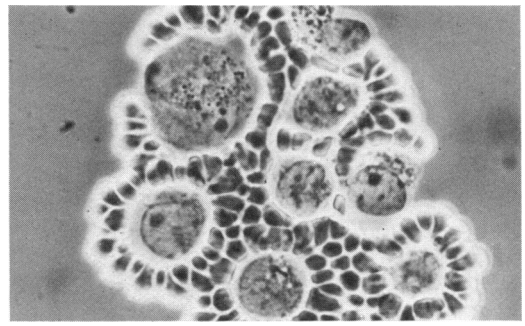

FIG. 5. Sheep red blood cells rosette formation with lymphocytes from colonies after 5 days of culture (phase contrast microscopy).

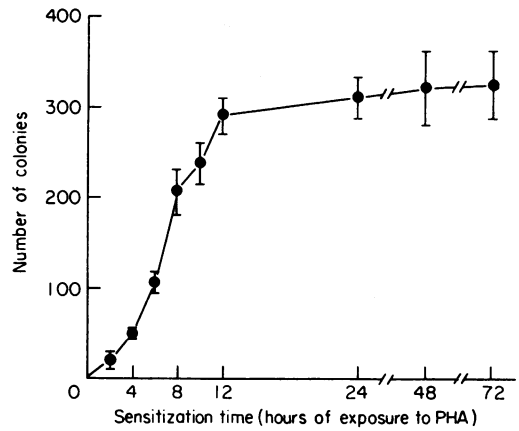

FIG. 6. Effect on number of colonies of sensitization time (exposure to PHA) of lymphocytes (10^6 cells). Each point represents the mean ± s.e. of seven peripheral blood lymphocyte cultures.

developed from PHA sensitized cells during 12 to 72 hours when PHA was absent from the agar layer.

As shown in Fig. 7, the greatest number of colonies appeared when PHA was placed in the lower agar layer. Optimal concentrations of PHA were 0·0125 ml/ml of medium for PHA present in the lower layer, and 0·06 ml/ml of medium for PHA present in the upper layer. Higher concentrations of PHA depressed colony formation, and no colonies developed when PHA concentration reached 0·3 ml/ml of medium, regardless of whether PHA was added to the lower or upper agar layer.

Fig. 8 shows a linear relation between the number of cells seeded and the number of resulting colonies. Thus, one out of 2×10^3 or 3×10^3 lymphocytes in peripheral blood has the potential to develop as colony.

Since colonies developing within areas enclosed by pyrex rings were indistinguishable from colonies developing in other areas, one can rule out the possibility that they resulted from aggregation of migrating cells. By means of repeated observations under the phase microscopy it was possible to follow the development of a colony starting from one single cell until it reached 50–60 cells.

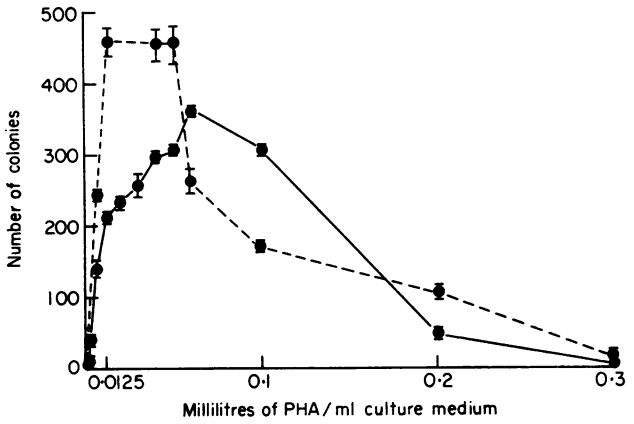

FIG. 7. Comparison of clone development in presence of PHA in upper (●—●) or lower (●- -●) agar layer. The mean ± s.e. for each point was based on four lymphocyte cultures.

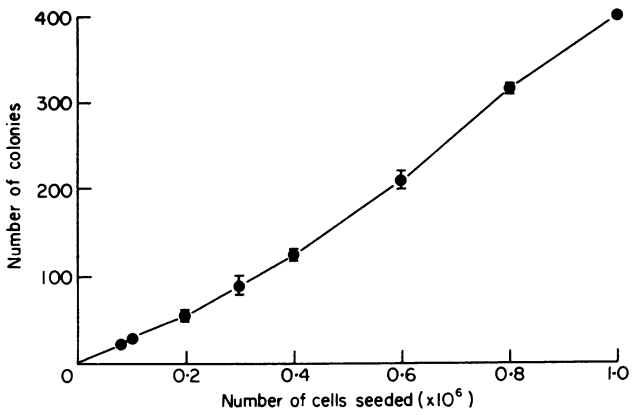

FIG. 8. Relationship between number of seeded lymphocytes and number of developing colonies, as demonstrated by six different lymphocyte cultures.

DISCUSSION

In the work described we demonstrate the ability of human lymphocytes to grow and develop as colonies in soft agar.

Essential conditions for colony formation are an initial sensitization of lymphocytes with PHA and the continuous presence of PHA in the soft agar culture. PHA in the agar is required for completion and resumption of the mitotic cycle, even when lymphocytes have been stimulated with PHA for 72 hours prior to seeding in agar culture.

The clonal origin of a colony was confirmed by repeated observations under inverted microscopy of development of colonies starting from a single cell and the development of colonies in the areas enclosed by pyrex rings.

A single transformed lymphocyte undergoes repeated divisions, yielding after 5–6 days a population of 200–500 or more cells. The mitotic index, under these conditions, exceeds that in liquid medium by a factor of fifty.

One advantage of the soft agar technique over liquid medium is the ability of locating colonies made up of cells originating from one blast-like cell.

With reference to the delayed appearance of the small colonies we assume that the earlier developing larger colonies act as feeder layer stimulating the development of the small colonies.

Cells from the small and large colonies are morphologically and cytochemically similar. The difference in the appearance of the two types of colonies is due to the depth of growth, with small colonies developing on the surface of the upper agar layer.

A comparison between the number of colonies developing from cultured bone marrow cells with those from peripheral blood show that, in proportion to the number of lymphocytes seeded, a larger number of colonies developed from bone marrow cells. A possible explanation may be that non-lymphocytic nucleated bone marrow cells act as feeder layer, thus stimulating clone development.

On the other hand, the lower number of colonies developing from seeding spleen cells as compared with peripheral blood, may reflect the higher proportion of B cells in the spleen (Rabellino, Colon, Grey and Unanue, 1971). This assumption is supported by our observation that about 20 per cent of human spleen cells form SRBC rosettes, by contrast with about 75 per cent of lymphocytes from peripheral blood.

The relationship between the number of colonies and the number of lymphocytes seeded (Fig. 8) shows that only a limited proportion of transformed lymphocytes has the potential to divide and grow as colony.

When less than 10^6 sensitized cells were seeded and supplemented with non-sensitized cells in order to make up the total number of cells to 10^6, the number of colonies was only related to the number of the presensitized cells.

This direct correlation between the number of colonies formed and the amount of sensitized cells seeded rules out competition among cells for essential growth factors in the medium as the reason for the small number of colonies.

When the cells were seeded in numbers greater than 10^6, the colonies overlapped during their development, and the number of colonies could not be accurately determined.

In our results the colony-forming lymphocytes represent lower values than those reported by others (Chi and Bloom, 1970; Marshall *et al.*, 1969). These differences may be explained by variations in techniques, and by our definition of a colony as requiring the presence of fifty cells or more.

The nature of the cells was determined by studying their rosette-forming ability, and by tests for surface immunoglobulin.

The two main lymphocyte populations, *viz*, thymus-derived (T) and bone marrow-derived (B) lymphocytes, can be identified by the ability of human T cells to form non-immune E rosettes with SRBC (Jondal *et al.*, 1972; Wybran *et al.*, 1972; Lay *et al.*, 1971). B cells can be identified by immunofluorescent demonstration of surface immunoglobulin (Raff, 1970; Unanue *et al.*, 1971; Pernis *et al.*, 1971) and the ability of the cells to form rosettes with MRBC (Stathopoulos and Elliott, 1974).

Our results suggest that the colonies are composed of T cells, in spite of the fact that not all of them formed rosettes with SRBC.

As judged by the SRBC rosette-forming ability, the fine structure observed under EM, their morphological identification by phase contrast microscopy, as well as the MGG staining, the colonies were composed of typical transformed cells similar to those obtained by culturing peripheral blood lymphocytes in the presence of PHA in liquid culture media

(Tanaka, Epstein, Brecher and Stohlmann, 1963; Cooper, Barham and Hale, 1963; Bessis, 1973; Zucker-Franklin, 1969).

A linear relationship was found between the concentration of PHA and the number of developing colonies, regardless of whether the PHA was placed in the upper or lower agar layer (Fig. 7). A decrease in the number of colonies was observed when the PHA concentration was higher than the amount necessary for optimal colony development.

Further investigations will be needed in order to explain the differences in the stimulatory effect of PHA when placed in the lower or upper agar layer, as well as the failure of lectins other than PHA to induce colony formation.

Using naphthol AS-D chloroacetate and α-naphthyl acetate as substrates, a strong esterase activity is considered to be characteristic of neutrophils and monocytes–macrophages respectively, while Luxol fast blue positive staining characterizes eosinophilic granulocytes (Shoham *et al.*, 1974).

Thus, as evaluated by cytochemical staining methods, including peroxidase stain, absence of phagocytosis of agar (negative staining with toluidine blue), and as based on the morphology of the cells, no evidence could be detected that the colony cells were either macrophages or granulocytes.

The low enzymatic activity found by using α-naphthyl acetate as substrate, was similar to that obtained with PHA-stimulated lymphocytes (Rosenszajn and Fischer, 1969).

There is a definite qualitative difference in the kinetics of development of colonies grown from lymphocytes originating from peripheral blood, bone marrow and spleen, as compared with colonies of monocytes–macrophages and granulocytes originating from bone marrow. Whereas the monocyte–macrophage and granulocyte colonies begin to appear only on the 6th to 7th day of culture, reach their maximum on the 14th day, and degenerate after 21 days (Shoham *et al.*, 1974), the lymphocyte colonies reach maximum growth after 5–6 days, and decline on the 10th day of culture.

PHA-sensitized lymphocytes produce a substance with colony-stimulating activity (CSA) having the ability to stimulate the development of granulopoietic and macrophage colonies during 8–10 days of culture in an agar culture medium (Cline and Golde, 1974).

In our system, lymphocyte colonies appeared well developed after 4 days of culture and degenerated after 10 days, and no other types of colonies were observed after this period of time. While in our system CSA may also have been produced, apparently its quantity was insufficient for stimulating development of macrophages and granulocyte colonies from the bone marrow cells.

The basic differences between the lymphocytic colonies and tissue cultures of human bone marrow are the preceding sensitization of the cells with PHA, continuous presence of PHA, and absence of human spleen-conditioned medium in the agar layer.

Experiments in progress in our laboratory indicate that the conditioned medium from human spleen which induced proliferation, in soft agar cultures of human bone marrow cells, of monocytes-macrophages and eosinophilic granulocytes (Shoham *et al.*, 1974) acts as inhibitor in development of lymphocyte colonies induced by PHA.

ACKNOWLEDGMENTS

We are grateful to E. Ben David, M.Sc., for expert technical assistance in the electron microscopy study.

The authors wish to thank Professor K. Stern for his helpful comments on this paper.

This work was supported in part by a grant from the Research Committee, Bar-Ilan University.

Presented in part at the 15th Congress, International Society of Hematology, Jerusalem, September 1974.

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