Brief Definitive Report

LONG-TERM GROWTH OF LINES OF MURINE DINITROPHENYL-SPECIFIC B LYMPHOCYTES IN VITRO*

By MARLENE ALDO-BENSON and LINDA SCHEIDERER

From the Department of Medicine and the Division of Rheumatology, Indiana University School of Medicine, Indianapolis, Indiana 46223

At the present time, studies of the factors associated with activation and differentiation of B lymphocytes are confined to systems in which B cell mitogens or anti-Immunoglobulin (Ig) are used to induce activation (1–3). Studies of antigen-induced B cell activation are hindered by the heterogeneity of lymphocyte populations. Although antigen-specific myelomas and leukemias have been advocated as model systems for studying factors associated with B cell differentiation and immunologic tolerance, these systems have their limitations (4–6). Myelomas cannot be used to study normal events associated with antigen induced activation of B cells, and because of their malignant state, extrapolation of data obtained with these systems to normal B cells is difficult.

The availability of long-term lines of antigen-specific B lymphocytes with which to study antigen-induced triggering and tolerance would greatly advance our knowledge in this area. Short-term growth of colonies of B lymphocytes has been achieved in vitro in a number of laboratories (7–10). Recently, Sredni and associates (11) have achieved the long-term growth of clones of human B lymphocytes in vitro. Howard et al. (12) have also been able to maintain growth of uncloned purified murine B lymphocytes for long periods in vitro. Although the antigen specificity of these B cell lines is unknown, these long-term cultures are a definite advance in the goal of developing lines of antigen-specific B cells.

In this paper we describe the technique and culture conditions that we have used to grow and maintain long-term cultures of murine B lymphocytes. Two of these lines have specificity for the hapten dinitrophenyl (DNP) and secrete anti-DNP antibody in response to antigen stimulation. Characteristics of both the DNP-specific lines and other B cell lines are described.

Materials and Methods

Animals. 8-wk-old BALB/c female mice (Harlan Industries, Indianapolis, IN) were used for cloning and production of growth factors. 8–14-wk-old (C57BL/6 × DBA_2)F_1 male mice (BDF_1) were used for production of growth factors. 8-wk-old C57BL/6 mice (Harlan Industries) were used to passage and maintain EL-4 lymphoma.

Hapten-Protein Conjugates and Antisera. Hapten protein conjugates were made as previously described (13, 14). Fluorescein-labeled anti-Ly-1 and anti-Ly-2,3 were obtained from Becton, Dickinson & Co., Orangeburg, NY. Monoclonal anti-Thy-1.2 was obtained from Cedarlane Laboratories (Westbury, NY). Fluorescein-labeled anti-mouse Ig was obtained from N. L. Cappel Laboratories (Cochranville, PA).

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Growth Factors. 2×10^6 cells from EL-4 lymphoma (gift of Dr. Jon Schmidtke, Eli Lilly and Co., Indianapolis, IN) were cultured in serum-free RPMI 1640 medium (Gibco Laboratories, Grand Island Biological Co., Grand Island, NY) containing 10 μg/ml lipopolysaccharide (LPS). Culture supernatants were harvested at 48 h, filter sterilized, and frozen at −70°C.

Peritoneal exudate cells from 8–14-wk-old C67BL/6 or BALB/c mice injected intraperitoneally with 1 ml sterile 4.25% Brewer's thioglycollate broth (Difco Laboratories, Detroit, MI) were cultured in serum-free RPMI medium containing 10 μg/ml LPS for 48 h.

Mitogen-induced growth factor (MIGF) was prepared by incubating spleen cells from BDF_1 mice or BALB/c mice in serum-free RPMI 1640 containing 2 μg/ml of either phytohemagglutinin (PHA) (Wellcome Laboratories, Beckham, England) or concanavalin A (Con A) (Pharmacia Fine Chemicals, Piscataway, NJ). Supernatants were harvested at 72 h.

Preparation of DNP-enriched Lymphocytes and Cloning in Soft Agar. BALB/c splenic lymphocytes were suspended in Dulbecco's Modified Eagle's Medium (DME) with 10% newborn calf serum (NCS), and enriched for DNP-specific antigen binding cells (ABC) by panning on DNP-gelatin plates using the method of Haas and Layton (14). The procedure was repeated a second time, and the degree of enrichment of DNP-ABC was determined by a rosette forming (RFC) assay using trinitrophenyl (TNP)-sheep erythrocytes (SRBC) as target cells (15). The cells were then cloned in soft agar using a modification of method of Pillai and Scott (10). LPS (LPS-*Escherichia coli* 055:B5; Difco Laboratories) was added to both agar layers. After 24 h incubation at 37°C, 5% CO_2, they were overlayered with 2 ml complete DME containing 40% MIGF. The plates were supplemented with fresh media at 3-d intervals.

Propagation of Clones. As the clones became macroscopic they were picked with the aid of a dissecting microscope from the soft agar using a finely drawn pasteur pipet. Each separate clone was placed in 1 well of a 96-well microtiter plate (Falcon Labware, Oxnard, CA) with 200 μl of complete DME with 40% MIGF (maintenance media). When cells grew to confluency they were harvested and the entire contents of that well was transferred in 2 ml of maintenance medium to one well of a 24-well culture plate. Each well was split when the cells had grown to confluency. Fresh maintenance medium was added to the wells every 3 d. The cell lines are now maintained in 25-cm^2 flasks and half the media changed every 3 d.

Staining procedure. Cell suspensions were passed through glass wool to remove dead or damaged cells (16). The cells were incubated with fluorescein-labeled protein for 30 min at 4°C, and examined under a Leitz Ortholux 2 phase contrast and fluorescent microscope (E. Leitz, Inc., Rockleigh, NJ). Similar staining procedures were followed for control normal spleen cells.

Radioimmunoassay. DNP-KLH was labeled with ^{125}I using the iodogen method (17). A standard curve was set up using a known amount of monoclonal IgM anti-DNP from the hybridoma cell line 35-12 (obtained from Dr. Julian Fleischmann, Washington University, St. Louis, MO). The known amounts of antibody or supernatants from the cell lines were incubated with ^{125}I-DNP bovine serum albumin for 2 h at 37°C and overnight at 4°C. The immune complexes were precipitated by a cold 30% solution of polyethylene glycol 6000 (Fisher Scientific Co., Pittsburgh, PA) and the pellet was counted in a Beckman gamma counter (Beckman Instruments, Fullerton, CA).

Antigen Stimulation of Cell Lines. 10^4 lymphocytes from an individual cell line in medium with 40% MIGF was placed in each well of a 96-well microtiter plate. In some cultures, 10^5 irradiated filler cells were added. The filler cells were obtained from spleens of 8-wk-old BALB/c mice that had been primed with 0.1 ml of a 0.01% SRBC suspension 6 d earlier. 5 ng of DNP-Ficoll or DNP-KLH was added to some of the wells. After 5 d incubation without supplementation the cultures were harvested. Contents of four wells were pooled and a plaque-forming cell (PFC) assay was performed with TNP-SRBC as target cells. Results are means of three to four samples and are expressed as PFC per 10^6 lymphocytes.

Results

Growth of Cells. Enrichment of spleen cells yielded cells containing 26% DNP-specific cells. After cloning, individual clones began to develop by day 5 and were visible macroscopically by day 7. A total of 142 clones were harvested. 72 clones grew to confluency within 24 d. Cells from each confluent well, which were then placed in

1 well of a 24-well tissue culture plate without LPS, grew to confluency in 1–3 wk. Subsequent subcultures from the same cell lines were cultured with combined (1:1) EL4 growth factor and peritoneal exudate growth factor (PEGF) and continued to grow at the same rate as cells grown in MIGF. Addition of all three growth factors together did not appear to increase rate of growth of the cell lines. As cells were in culture they became smaller than the original lymphocytes and were predominantly very small. In several weeks some of the cells increased in size, so that they were predominantly larger but there were still a few very small cells. These changes have continued to occur in a cyclic fashion.

Characteristics of Long-Term Cell Lines. After ~6 wk in culture, cells from each line were stained with fluorescein anti-IgG or fluorescein anti-Lyt antisera or stained for nonspecific esterase. Results on representative cell lines are shown in Table I. Most of the cell lines were pure B cells, but a few were a mixture of T and B cells. Some of the cell lines shown in Table I were subjected to anti-Thy-1 antiserum and complement, and percent cytotoxicity was noted. This confirmed the results of fluorescent staining. All 72 cell lines were tested for DNP specificity by rosetting with TNP-SRBC targets. Results of representative cell lines are shown on Table II. B_1 and C_3 consistently showed >70% TNP-specific rosetting cells. Radioimmunoassay of concentrated supernatants from some of the cell lines (Table II) indicates that anti-DNP is produced spontaneously in small amounts by cell lines containing >10% DNP specific lymphocytes.

Further studies were done to determine if DNP-specific cell lines could be stimulated to produce antibody by the addition of antigen. Cells from the cell lines B_1 and C_3, which are pure DNP-specific lines, were cultured with either 5 ng DNP-KLH or 5 ng

TABLE I

Characteristics of Cell Lines in Long-Term Culture

Cell lines	Percent staining F_1-anti-Ig	Percent staining F_1-anti-Lyt	Percent staining esterase
B1	99	<1	<1
C3	99	<1	<1
C9	50	50	ND*
D6	95	5	ND
F3	99	<1	ND
H7	99	<1	<1
I4	99	<1	<1

* Not determined.

TABLE II

Antigen Specificity of Continuous Lines of B Cells

Cell lines	Percent TNP-specified RFC*	Spontaneous anti-DNP secretion‡
		μg/ml
B1	84	58
C3	70	53
C9§	38	63
D6§	36	60
F3	36	63
H7	48	40
I4	12	42

* Background RFC with uncoated SRBC <2%.

‡ No antigen added to cultures; antibody detected using radioimmunoassay in culture supernatants concentrated four times.

§ Mixed T cell and B cell cultures.

TABLE III

Anti-DNP Secretion in B Cell Lines in Response to Antigen

Cell line	Anti-DNP PFC/10^6 lymphocytes		
	No antigen	DNP-KLH	DNP-Ficoll
C3*	10	50	0
B1	0	0	20
H7‡	0	20	30
Normal Spleen	10	67	60

* DNP-specific B cell line.
‡ Mixed DNP-specific B cells and nonspecific B cells.

TABLE IV

Anti-DNP Secretion in B Cell Lines in Response to Antigen and Filler Cells

Cell lines*	Antigen	Filler cells‡	Anti-DNP PFC per 10^6 lymphocytes
B_1	0	+	100
	DNP-Ficoll	+	290
C_3	0	+	50
	DNP-Ficoll	+	509
Normal spleen	0	+	67
	DNP-Ficoll	+	245

* All cultures contained MIGF.
‡ 10^6 irradiated normal spleen cells.

DNP-Ficoll. MIGF was added as a source of T cell factors. Control cultures were done with a heterogenous cell line (H_7) and normal spleen cells. The results (Table III) indicate that C_3 and B_1 respond to antigen in vitro. B_1 and C_3 were also cultured with antigen and MIGF in the presence of irradiated normal spleen filler cells (Table IV). The presence of filler cells increases the background PFC of the cells from these lines, but antigen stimulation produced a larger number of PFC.

Discussion

The data presented here describe the conditions in which lines of murine B lymphocytes can be propagated in vitro for prolonged periods of time. We have propagated two lines of murine B cells that are composed mainly if not entirely of DNP-specific B lymphocytes for >9 mo. Both of these cell lines can secrete small amounts of antibody spontaneously. However, antigen stimulation of the DNP-specific lines results in increased antibody production.

It is obvious from the analysis of the cell lines that each clone picked from the soft agar was not always the progeny of a single cell. Some of the cell lines even contain a percentage of T cells; most, though, are pure B cells. It is highly likely, however, that the two cell lines that show >70% DNP-specific rosetting are pure DNP-specific lines. The rosetting with TNP-SRBC is highly specific for DNP receptors on lymphocytes. Because cells are dividing and in some stages do not express surface receptors, it is not unusual for pure populations of cells to have <100% rosetting (4). We can not be sure, however, that these two cell lines each represent a single clone. The low frequency of pure clones in B cells grown in long term culture was also noted by Sredni et al. (11) in clones of human B cells.

The growth characteristics of the cells during cloning are similar to those noted by other investigators. When the clones are picked from the soft agar, presence of LPS is no longer necessary for growth, suggesting that B cells need to be activated for growth to occur, but maintenance of the activated state is not necessary for propagation. The

presence of media containing growth factors is necessary for growth in vitro. The factor necessary for B cell growth is present in supernatants of cells incubated for 72 h with PHA but not in supernatants of cells incubated for 48 h. Sredni et al. (11) also noted that 72-h supernatants of human PHA-stimulated cells contain a growth factor that supports human B cell growth in vitro. Howard et al. (18) have reported that a separate B cell growth factor (BCGF) distinct from interleukin 1 or 2 (IL-1, IL-2) is necessary for propagation of B cells. The MIGF used in the experiments reported here most likely contains IL-1 and IL-2 but also a B cell growth factor and perhaps other undefined factors. Since the B cell lines could be stimulated by antigen in vitro in the presence of MIGF without added T cells, it is likely that this supernatant also contains T cell helper activity similar to T cell-replacing factor (TRF) (19). It should also be noted that the action of the growth factors is not H-2 restricted, since the MIGF was prepared in BDF_1 mice and the cell lines are from BALB/c mice. This observation is in agreement with Howard et al. (12). Growth factors obtained from both EL-4 T cell lymphoma and from cultures of peritoneal exudate cells together are also able to support the growth of B cells. These factors are also likely to contain BCGF as well as IL-1, IL-2, and perhaps TRF (19).

The cyclic changes in the appearance of the cells during prolonged culture occurs with both MIGF and the EL-4 plus PEGF. Metcalf et al. (7) noted that many cells in their colonies were smaller than normal lymphocytes and size was heterogeneous. Howard et al. (12) noted that long-term cultures of B cells also showed very small cells as well as larger cells. It is possible that the very small lymphocytes represent early B cells that are less differentiated and divide in vitro. The large cells may be more differentiated cells with surface receptors. For this to occur, some differentiation factors may be necessary. B cell differentiation factors are secreted by T cells and may be present in MIGF (20).

The availability of these DNP-specific cell lines and techniques for further antigen-specific cell line development provide a powerful tool for the investigation of factors associated with antigen-induced B cell triggering. We have shown that these antigen-specific B cell lines can be induced to differentiate to antibody-secreting cells in vitro by antigen. Defining the factors associated with this process is now a feasible undertaking, and will be the subject of further studies.

Summary

Studies of cellular events associated with antigen-induced triggering and differentiation of B cells would be greatly facilitated by the availability of homogeneous cell lines of antigen-specific lymphocytes that can be maintained in long-term culture. By combining the techniques of enrichment of lymphocytes for antigen-specific cells, cloning in soft agar, and long-term propagation of B cells we have been able to isolate, propagate, and maintain two lines of dinitrophenyl (DNP) -specific B lymphocytes. These cell lines are B lymphocytes that have 70% and >80% DNP-specific rosette-forming cells, respectively. Both cell lines secrete small amounts of antibody spontaneously but can be stimulated by antigen in vitro in the presence of either supernatants from phytohemagglutinin-stimulated spleen cells or irradiated normal filler cells. Thus far these lines have been maintained in vitro for >9 mo. They will be useful in studying factors associated with B cell response.

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