USE OF EPSTEIN-BARR VIRUS-TRANSFORMED B CELL LINES FOR THE GENERATION OF IMMUNOGLOBULIN-PRODUCING HUMAN B CELL HYBRIDOMAS

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In the generation of murine B cell hybridomas secreting immunoglobulin (Ig) of defined specificity (1), activated B lymphocytes are fused with enzyme-deficient variants of terminally differentiated plasma cells. Myeloma plasma cells have been used in these experiments to facilitate the construction of hybrid cells with the characteristic of secreting large amounts of Ig. Fortunately, the available library of murine plasmacytomas is large and a number of appropriately adapted murine plasma cell lines exist. In the human, the available number of terminally differentiated plasma cells that grow well in vitro is more restricted, and only a few myeloma cell lines have been fused in the hypoxanthine-aminopterin-thymidine (HAT) selection system with limited success (2, 3). This paucity of human myeloma lines has prompted investigators to construct interspecies hybridomas between rodent myeloma cell lines and human B cells (4). However, the ability to transform human B cells with Epstein-Barr virus (EBV) has resulted in a large number of B cell lines (BCL) at various stages of B cell differentiation. In general, these lines are not terminally differentiated, express surface membrane Ig, and secrete small quantities of Ig. In the studies reported below, several chemically mutagenized HGPRTase-deficient variants of human EBV-transformed cell lines were selected and used for the construction of human B cell hybridomas producing Ig. These experiments show that such lines function effectively as parental tumor partners in fusion studies. The resultant hybridomas allow for both the synthesis and secretion of antigen-specific Ig.

Materials and Methods

Isolation and Stimulation of Normal B Lymphocytes. T and B cell fractions were obtained from mononuclear cell (MNC) suspensions of peripheral venous blood and tonsillar or splenic tissue by the sheep erythrocyte rosetting technique. 1 \times 10^6 B cells were cultured with 2 \times 10^6 irradiated (1,500 rad) T cells and pokeweed mitogen (PWM) (1:100; Gibco Laboratories, Grand Island, NY) in 2 cc of RPMI 1640 medium (M. A. Bioproducts, Walkersville, MD) supplemented with 10% fetal bovine serum (Reheis Chemical Co., Phoenix, AZ). After 5-7 days, cells were harvested, washed twice in RPMI 1640 medium, and fused.

HGPRTase-deficient Human B Cell Lines. The enzyme-deficient EBV-transformed line GM 0467 (5) was obtained from the Human Genetic Mutant Cell Repository, Institute for Medical Research, Camden, NJ. The 8-azaguanine-resistant subclone of WI-L2 (6), AG \textsuperscript{8} 35c1 (7), was a gift from Dr. A. S. Tung of the Merck Institute for Therapeutic Research. These lines were subcloned further in semisolid agarose in the presence of 20 \mu g/ml of 8-azaguanine over a monolayer of HGPRTase-deficient human fibroblasts (GM 152). Clones were picked under...

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microscopic visualization after 8–12 d of growth and maintained in RPMI 1640 with 10% fetal bovine serum (FBS) and 20 μg/ml of 8-azaguanine. Two selected subclones, WI-L2 AG³5sc1.1 (herein called H35.1.1) and 0467.3, were used in the fusion experiments reported below.

Cell Fusion Techniques and Conditions for Growth of Hybrid Cells. PWM-stimulated human B cell blasts were fused with HGPRTase-deficient human B cell line cells in the presence of polyethylene glycol (PEG) (~1,000 mol wt; J. T. Baker Chemical Co., Phillipsburg, NJ) by a modification of the technique of Kennett et al. (8). 1 × 10⁷ mitogen-stimulated B cell blasts and an equal number of B cell line cells were incubated at room temperature with 0.5 ml of 40% PEG for 2 min with continuous swirling and then centrifuged for 6 min at 1,800 rpm. After washing in RPMI 1640 medium, 1 × 10⁶ cells were dispensed in complete medium into tissue culture plates (Linbro Chemical Co., Hamden, CT). Complete medium consisted of RPMI 1640 (75%), NCTC-109 (10%), FBS (15%), penicillin (100 U/ml), streptomycin (100 μg/ml), kanamycin sulfate (50 μg/ml), hypoxanthine (10⁻⁴ M), thymidine (5 × 10⁻⁸ M), bovine insulin (0.2 U/ml), and oxaloacetate (10⁻³ M). 24 h later, 0.1 ml of aminopterin (10⁻⁷ M) was added to each well. All wells were fed every 3 d for 3–6 wk with a similar concentration of aminopterin in complete medium. Wells were then scored as to growth of cells, and supernatants were screened for Ig production as described below.

Detection of Ig Production. Synthesis of Ig was determined by indirect intracytoplasmic immunofluorescence on fixed hybrid cell preparations using monoclonal murine antibodies to human κ, λ, μ, and γ chains (provided by Dr. J. D. Capra, University of Texas, Dallas, TX) and rhodamine-labeled rabbit anti-mouse antibodies. A modification of the enzyme-linked immunosorbent assay (ELISA) reported by Giallongo et al. (9) was used to determine Ig secretion. Specific anti-tetanus toxoid antibodies were detected by a direct binding assay in which hybridoma culture supernatants or known quantities of affinity-purified, polyclonal, human anti-tetanus antibodies were incubated for 1 h at 37°C with plates (Nunc Immuno Plates, Vanguard International, Neptune, NJ) coated with 10 μg/ml of tetanus toxoid (or tetanus toxin), washed extensively and then exposed to a pool of affinity-purified, peroxidase-labeled goat antibodies to human κ and λ chains (Tago, Inc., Burlingame, CA) for 1 h at 37°C. After further washing, the assay was developed with an appropriate substrate (9), and the optical densities at 490 nm were determined. Quantitations of the amounts of Ig secreted were determined by constructing a standard curve using the results obtained with various dilutions of purified human anti-tetanus antibodies. Nonspecific Ig secretion was quantitated by an inhibition assay in which the affinity-purified, peroxidase-labeled goat antibodies specific for κ and λ were blocked from binding to human Ig-coated plates by either hybridoma culture supernatants or culture medium containing known amounts of human myeloma proteins of the appropriate light chain type.

Results

Construction of Ig-producing Human B Cell Hybridomas Using HGPRTase-deficient Human B Cell Lines. Two representative human BCL are described in these experiments. These are selected EBV-transformed progeny of a spleen cell from a patient with hereditary spherocytosis (H35.1.1) and a peripheral blood cell from a patient with mononucleosis (0467.3). The former is a mutagenized, HGPRTase-deficient variant (7) of the WI-L2 line (6), the latter an enzyme-deficient variant (5) of the PGLC33H line. Both of these lines have been cloned before and several times after chemical mutagenesis. The H35.1.1 clone expresses κ light chains on the surface membrane of 1–2% of the cells during log phase growth and contains only 1–2% μ producing plasmacytoid cells. 0467.3 expresses 1–3% surface IgM λ bearing cells with 13–21% μ λ containing plasma cells. H35.1.1 and 0467.3 produce ~1–50 ng/ml of Ig in a 48-h culture period as quantitated by the ELISA inhibition assay described above.

In a series of experiments, PWM-activated peripheral blood, tonsillar, or splenic B lymphocytes were fused with these BCL in the presence of PEG. After fusion, 10⁶ cells were dispensed into wells and cultured in HAT selection medium (10) for 3–6 wk. Table I lists the results of three representative fusions between H35.1.1 and mitogen-
activated B cells from three anatomic sources. In these experiments, from 14.9-22.1% of the wells initially cultured were positive for growth after HAT selection. Although fusions were obtained with B lymphocytes from all three sources, there is a suggestion that B cells from solid tissues (spleen and tonsil) fuse more efficiently. This observation has been true for B cell fusions with several other parental BCL. To determine the percent of growth-positive wells synthesizing Ig, aliquots of each culture were stained for the presence of large amounts of intracytoplasmic light or heavy chain production. Because the parental line H35.1.1 contains negligible numbers of plasma cells, all cultures with >10% plasma cells were scored as positive for Ig synthesis. As listed in Table I, between 21 and 70% of the growth-positive wells were found to contain cells with large amounts of intracellular light chain. Many cultures contained from 50 to 100% mature plasma cells. Immunofluorescent staining for heavy chains demonstrated that >90% of the cells were producing IgM, usually of only one light chain type. In addition, selected growth positive cultures negative for Ig secretion were proven to be hybrids by the coexpression of both parental HLA-Dr alleles on the same cells and by chromosomal hyperploidy.

Fig. 1 illustrates the amounts of Ig secreted by the hybridomas produced from a fusion between 0467.3 and PWM-stimulated tonsillar B lymphocytes. In this experiment, 21% of the wells were growth positive (21/100) and 91% of these (19/21) were found to contain cells with large quantities of human Ig. 13 Ig-synthesizing cultures were chosen at random and analyzed for Ig secretion by the ELISA inhibition assay. 12 of the 13 cultures secreted between 0.1 and 5.0 μg/ml of Ig as compared with ~50 ng/ml in the parental line 0467.3 grown under similar conditions. An evaluation of the light chains secreted by these cultures suggested that in several instances secretion of the parental IgM λ was lost. Furthermore, this implies that the cultures were clonal at this stage. Analysis of heavy chain production showed that 17 of the 19 cultures were synthesizing IgM and 2 of the 19 made IgG.

**Secretion of Specific Human Anti-Tetanus Toxoid Antibodies by a Human B Cell Hybridoma.**

Because the B cells used for the fusion shown in Fig. 1 came from the tonsils of a donor who had received a tetanus toxoid booster immunization 1 wk before tonsillectomy, the supernatants of the Ig-producing fusions were assayed for anti-tetanus toxoid binding activity. One culture was found that produced 400 ng/ml of specific antibody. This antibody was found to bind also to tetanus toxin but not to diphtheria toxoid or other irrelevant proteins. This culture (termed TT1), which has been stable...
Fig. 1. Concentrations of κ and λ Ig present in 13 randomly chosen growth-positive wells from fusion of PWM-activated human tonsillar B cells with 0467.3 line. Data were obtained using inhibition of anti-κ or anti-λ binding to human Ig-coated wells. Known concentrations of human Fr II were used to construct a standard inhibition curve against which culture supernatants were compared.

### Table II

| Marker Characteristics of Anti-Tetanus Toxoid Antibody-secreting Human Hybridomas |
|---------------------------------|-----------------|-----------------|-----------------|
|                                 | μ    | γ   | κ   | λ   | μ    | γ   | κ   | λ   | Specific anti-tetanus antibody secreted |
| 0467.3                          | 2    | —   | 1.3 | 3   | 13   | —   | 3   | 21  | <0.001 |
| TT1 (uncloned)                  | 78   | —   | 70  | 28  | 28   | —   | 32  | 840 | 1.6  |
| TT1.1                           | 35   | —   | 31  | 81  | 81   | —   | 83  | 1.6  |
| TT1.2                           | 21   | —   | 26  | >95 | >95  | —   | >95 | 2.8  |
| TT1.7                           | >95  | —   | >95 | >95 | >95  | —   | >95 | 1.8  |
| TT1.8                           | >95  | —   | >95 | >95 | >95  | —   | >95 | 1.3  |
| x4                              | 18   | 23  | —   | 50  | 54   | —   | —   | <0.001 |

in vitro for 9 mo, was cloned by limiting dilution technique in medium containing 50% human fibroblast culture supernatant. After 14 d, 38% of the wells plated were secreting detectable amounts of tetanus toxoid specific antibody. The fastest dividing of these clones were saved. Table II lists a comparison of the marker characteristics of four of the clones, the parental line 0467.3, the uncloned hybridoma TT1 and another hybridoma from the same fusion secreting an irrelevant IgG κ (x4). Clones TT1.1 and 1.2 appear similar, with 20-30% of the cells expressing IgM λ on their surface and 80-95% of the cells containing large amounts of intracellular Ig. Clones TT1.7 and 1.8 have a much higher percentage of surface staining cells (~95%) with a similar percentage of plasma cells. Whether these cells represent fusions of distinct cell types within the initial growth-positive well or rather variants with different growth characteristics is unclear. However, it is clear that the clones show a marked enrichment for plasma cells and anti-tetanus antibody production as compared to the parent TT1.

Because the TT1 clones secreted Ig specific for tetanus toxoid, attempts were made to demonstrate surface expression of these same antibodies. In cytofluorographic analysis, 37.9% of the hybrid cells bound fluorescein-labeled tetanus toxoid as compared with <1% of the parental line cells 0467.3. A 9-channel peak shift suggested that more cells were actually binding the fluorochrome-labeled tetanus toxoid antigen.
Discussion

These data demonstrate that EBV-transformed BCL function effectively as fusion partners for the construction of Ig-producing human B cell hybridomas. Using primarily two HGPRTase-deficient BCL (H35.1.1 and 0467.3), ~20% of the cultures plated were positive for growth after HAT selection. From 10 to 90% of these growth-positive wells synthesized new Ig. One culture produced human antibody specific for tetanus toxoid and tetanus toxin. This was cloned by limiting dilution into several subclones, shown to secrete large quantities of specific antibody and also to bind fluorochrome-labeled antigen, presumably by membrane-associated Ig. Proof of cell hybridization was provided by growth in selective media, the presence of intact Ig production, frequently of a light chain type not expressed by the parental line, the coexpression of both parental HLA-Dr antigens, and karyotypic analyses.

The enzyme-deficient EBV lines reported here were especially convenient for fusion studies since they could be cloned efficiently. Most EBV-transformed B cell cultures clone inefficiently in their initial state. However, during the chemical mutagenesis procedure selection for cloning ability is made. The variants obtained and the hybrids constructed retain this growth characteristic.

Synthesis and secretion of new Ig by the hybridomas were easily detected by comparing either the total number of intracellular Ig-positive cells or the amounts of Ig produced. The uncloned hybridomas constructed with these lines frequently contained 50–100% plasma cells as determined by intracytoplasmic immunofluorescence. In addition, these lines secreted from 0.01 to 5.0 µg/ml of antibody after 48 h culture. This suggests that the differentiation stage of the parental tumor line does not dictate the differentiation stage of the resultant hybridoma. Although several IgG-producing hybridomas have been found, the dominant isotype produced by these hybridomas is IgM. It is unclear at this stage whether this relates to the parental tumor lines or to the subset or differentiation state of the PWM-activated normal B cells. During this study, considerable work was carried out with two plasma cell lines, including one previously described (2), after eradication of mycoplasma. After fusion, many of these hybridomas actually showed a decrease in the number of plasma cells by fluorescence analyses with anti-Ig reagents, just the reverse of what was observed for the EBV lines. However, some successful Ig-producing fusion products were obtained and the relative merits of the two types of parental lines awaits further study.

In addition to the production of anti-tetanus antibody by fused cells from EBV lines, the synthesis of antibodies with rheumatoid factor activity have been found that express a major cross-reactive idiotypic determinant (11). Furthermore, a number of other antibody-secreting hybridoma systems are currently under detailed analysis, including those for anti-nuclear antibodies from cells of patients with systemic lupus erythematosus and for antibodies to allergens from cells of allergic patients.

Thus, the work with the two lines described above, as well as with others obtained by EBV transformation not discussed in this report, suggests that these are general properties of EBV lines. Indeed, one of the lines from a myeloma patient previously described as effective in human B cell hybridization (3) was found to show the presence of EBV nuclear antigen. Because EBV lines are easily generated and many are already available for chemical mutagenesis, it should not prove too difficult to obtain parental lines for human B cell hybridization studies.
Summary

HGPRTase-deficient EBV-transformed B cell lines were shown to be effective fusion partners with mitogen-activated human B cells for the construction of Ig-producing human B cell hybridomas. In a series of experiments using these lines and B cells from several tissue sources, ~20% of the cultures plated were consistently positive for growth after hypoxanthine-aminopterin-thymidine selection and ~30% of these synthesized significant new Ig. A marked increase in Ig secretion was observed after hybridization, which was due to new Ig; Ig from the parental line was shown to disappear in several instances. Special analyses were carried out on a human hybridoma secreting antibody specific for tetanus toxoid and tetanus toxin and stable subclones were derived. These studies suggest that EBV-transformed lines will prove useful in human hybridization studies, thus making a large library of B cell lines available for the generation of human monoclonal antibodies.

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