CELL GROWTH CYCLE BLOCK OF T CELL HYBRIDOMAS 
UPON ACTIVATION WITH ANTIGEN

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For many years the study of cellular immunology was impeded by the inability 
to propagate and expand homogeneous populations of antigen-specific lympho-
cytes in vitro. In the mid-1970’s, Köhler and Milstein (1, 2) developed a technique 
that allowed the immortalization of antigen-specific B cells by fusing them to 
transformed and spontaneously proliferating myeloma cells, thereby producing 
B cell hybridomas. Hybridoma technology was subsequently applied to T lympho-
cytes, providing a convenient source of T cells for in vitro study (3–5), and 
the establishment of antigen-specific T cell hybridomas made it possible to analyze 
the requirements for antigenic stimulation in model systems (6). Murine antigen-
specific T cell hybridomas are commonly prepared by using polyethylene glycol 
to fuse antigen-primed T cell blasts to the spontaneously proliferating AKR-
derived thymoma, BW5147. These T cell hybridomas can be tested for their 
ability to respond to the antigen used to prime the normal lymphocyte fusion 
partner, and the cells that exhibit the appropriate specificity can be cloned and 
expanded to large numbers.

For normal T lymphocytes, occupancy of the antigen receptor usually results 
in a complex series of activation events that include expression of new cell surface 
receptors, production of lymphokines, and entry of the cell into its growth cycle 
(7). This last response is not pertinent when using T cell hybridomas, because 
they are spontaneously proliferating cells. Therefore, the approach that has 
generally been used to assess the result of antigen receptor occupancy of T cell 
hybridomas is to quantitate their production of lymphokines, typically IL-2 (6).
In recent years it has become evident that the occupancy of a variety of receptors 
that typically promote growth in normal cells, e.g., occupancy of receptors for 
epidermal growth factor (8), transforming growth factor β (9), and even IL-2 
(10, 11), can inhibit the growth of certain transformed cells. We therefore 
undertook an analysis of the effect of antigen-mediated activation upon the 
growth of murine T cell hybridomas. We found that stimulation with antigen 
resulted in a dose-dependent decrease in both [³H]thymidine incorporation and 
cell growth. This phenomenon displayed the same ligand-specificity as did IL-2 
production, as demonstrated by its requirement for a specific allelic form of the 
Ia molecule, antyclonotypic (i.e., anti-antigen receptor) antibody blocking exper-
Immunology, and stimulation with antionotypic antibodies crosslinked to Sepharose beads. Flow cytometric analysis revealed that antigen activation produced a block in the cell growth cycle that was predominantly at the G1/S interface, although cells also appeared to be slowed or halted in S phase. Furthermore, with the use of correlated DNA and surface immunofluorescence flow cytometry, it was demonstrated that the addition of antigen to a mixture of T cells specific for different antigens resulted in a cell cycle block in only the stimulated T cell hybrid. The implications of this phenomenon are discussed, including its utility as a rapid and quantitative measure of T cell hybridoma activation, as a means of selecting T cell hybridomas that have functional mutations in the antigen-specific receptor or elsewhere in the activation pathway, and as a possible model for the induction of T cell tolerance.

Materials and Methods

Animals. B10.A/SgSn (B10.A) mice were obtained from Harlan Sprague-Dawley (Madison, WI) through a contract with the Animal Genetics Production Branch (Developmental Therapeutics Program, Division of Cancer Treatment, NCI, Frederick, MD). B10.D2 mice were derived from pedigreed pairs originally obtained from Dr. Jack Stimpfling, Great Falls, MT. Mice of either sex were used between the ages of 2–12 mo.

Antigens. Pigeon cytochrome c, chicken ovalbumin, and hen egg lysozyme (HEL)1 were purchased from Sigma Chemical Co. (St. Louis, MO). The random terpolymer poly-(Glu65-Ala30-Tyr40) (GAT) was purchased from Vega Biochemicals (Tucson, AZ). The COOH-terminal pigeon cytochrome c fragment 81–104 was prepared by CNBr cleavage as described (12).

Antibodies. 10-2.16 is a murine IgG2b antibody that binds the Aα:Kb molecule (13). 14.4.4 is a murine IgG2a antibody that recognizes the Eα:Kd molecule (14). Both antibodies were prepared as ascites and used at a final dilution of 1:500 (vol/vol). A2B4-2 is a murine IgG2a antibody that binds the antigen-specific receptor borne by the T cell hybridoma 2B4 (15). Ascites containing A2B4-2 was purified by sequential passage over columns of DEAE Affi-Gel Blue (Bio-Rad, Richmond, CA) and protein A–Sepharose (Pharmacia Fine Chemicals, Uppsala, Sweden). The purified antibody was coupled to activated Sepharose (Pharmacia Fine Chemicals) at a concentration of 2 mg/ml (determined spectrophotometrically), and was the generous gift of Drs. H. Narimatsu and R. Schwartz (National Institute of Allergy and Infectious Diseases, NIH). For the blocking experiments, the antionotypic antibody A2B4-2 was purified from ascites in a single step by passage over a protein A–Sepharose column, and was kindly provided by Dr. B. Fox (National Institute of Allergy and Infectious Diseases, NIH).

T Cell Hybridomas. All of the T cell hybridomas used in this study were obtained by polyethylene glycol–mediated fusion of antigen-primed lymph node T cells to the AKR-derived thymoma BW5147 as described (16). The strain and haplotype of the BW5147 fusion partners, the antigen/1a molecule specificity of the hybridomas, and a reference in which each hybridoma was described are given in Table I. The T cells 2B4.11 and C10.9 were obtained by subcloning the 2B4 and C10 lines, respectively, at limiting dilution, and were selected for use in these studies because of their stability as judged by their functional responses, and in the case of 2B4.11, the homogeneous expression of the antigen-specific receptor. The T cell hybridomas A4.A1.4 and A6.A2.7 were obtained by subcloning the A4.A1 and A6.A2 lines, respectively, at limiting dilution, and were selected for their superior ability to produce IL-2 compared to the parent cell line. All of the T cell hybridomas were maintained in exponential growth in a medium consisting of RPMI 1640 (Biofluids, Rockville, MD) supplemented with 10% heat-inactivated fetal calf serum,

1 Abbreviations used in this paper: CTLL, IL-2-dependent cytotoxic T cell line; EGF, epidermal growth factor; HEL, hen egg lysozyme.
4 mM glutamine, 100 U/ml penicillin, 150 µg/ml gentamicin, and 50 µM 2-ME (growth medium).

**Stimulation of T Cell Hybridomas.** 5–10 × 10^3 T hybridoma cells were cultured in duplicate or triplicate in 96-well, flat-bottomed microtiter plates (3596; Costar, Cambridge, MA) in a final volume of 50:50 (vol/vol) mixture of RPMI 1640 (Biofluids) and Eagle's-Hank's amino acid (EHAA) medium (MA Bioproducts, Walkersville, MD) supplemented as above (complete medium). In the case of antigen stimulation, 5 × 10^5 irradiated (3300 rad) splenocytes were added to each well along with the indicated concentrations of antigen. Stimulation with antibody-coupled beads was performed by resuspending a 50:50 (vol/vol) slurry of A2B4-2-coupled Sepharose beads and removing the volumes indicated. The beads were washed with PBS three times, resuspended in complete medium, and added to the T cells in the absence of any other cell type (i.e., no splenocytes were present).

**IL-2 Assay.** After 24 h of culture, 50 µl of supernatant was removed from each well and frozen to ensure that no viable cells were included. The IL-2 content was determined by incubating an IL-2-dependent T cell line (CTLL) at 3 × 10^5 cells/well with twofold serial dilutions of the supernatant. After ~20 h, the CTLL were pulsed with 1 µCi of [3H]thymidine per well (6.7 Ci/mmol; ICN, Irvine, CA) and, after another 8 h, were precipitated onto glass filter strips with the use of a semiautomated cell harvester (PHD Cell Harvester; Cambridge Technology, Cambridge, MA). The incorporation of [3H]thymidine was determined by liquid scintillation counting. One unit of IL-2 activity is defined as that dilution of supernatant capable of stimulating the IL-2-dependent T cell to half of its maximal proliferative response.

**[3H]Thymidine Incorporation by T Cell Hybridomas.** At 24 h of culture, after 50 µl of supernatant had been removed for the measurement of IL-2, 1 µCi of [3H]thymidine was added to each T cell hybridoma-containing well (5–10 × 10^3 cells/well in 150 µl). 2–3 h later the wells were harvested as above, and [3H]thymidine incorporation was assessed by liquid scintillation counting.

**Cell Counting.** 2B4.11 or C10.9 T cells were placed in 96-well flat-bottomed plates at a density of 5 × 10^4 cells/ml in complete medium. 5 × 10^5 irradiated (3,300 rad) B10.A syngeneic, or B10.D2 (allogeneic), splenocytes were added to each well as a source of APC, along with varying amounts of the appropriate antigen. At 24-h intervals, three wells were resuspended by gentle pipetting. 10–20-µl aliquots were removed, and viable cells were counted in the presence of 0.4% trypan blue stain (Gibco Laboratories, Grand Island, NY) by light microscopy. T hybridoma cells were easily differentiated from the irradiated splenocytes on the basis of morphology and the exclusion of trypan blue dye. The standard error of these determinations was always <10%.

**Flow Cytometry.** T cell hybridomas were cultured at 4 × 10^5 cells/ml in 5-ml plastic tubes (Becton Dickinson, Oxnard, CA) containing 5 × 10^5 irradiated (3,300 rad) B10.A splenocytes in the presence or absence of antigen. After 24 h, viable cells were isolated by recovery from a Lympholyte-M density gradient (Cederlane Laboratories, Ontario, Canada), washed in saline G (PBS containing 1 g/liter glucose without any calcium or magnesium, prepared by the NIH media unit), and the cell pellet was fixed by slowly adding chilled absolute ethanol while vigorously mixing the cells. After fixing for 2–24 h in ethanol, the cells were centrifuged, washed in saline G, and resuspended in saline G containing 1 mg/ml of RNase A (Worthington Biochemicals, Freehold, NJ) and 18 µg/ml of propidium iodide (Sigma Chemical Co.). The cells were allowed to equilibrate for at least 1 h before being analyzed for cellular DNA content with a Cytofluorograf System 50 (Ortho Diagnostic Systems, Westwood, MA) using 488-nm excitation at 200 mW. Doublets and higher aggregates were excluded from analysis by using correlated area/peak measurements of DNA content similar to that described previously (21). Cell cycle analyses were done by the polynomial model of Dean and Jett (22). Cell size was simultaneously measured by using axial extinction at 633 nm from a 0.8 mW helium-neon laser. For correlated DNA/surface immunofluorescence measurements, 1–2 × 10^6 2B4.11 or C10.9 cells (or a mixture of both) were incubated in HBSS (Biofluids) containing 0.2% NaN₃, 1% fetal calf serum; and with or without 100 µl of A2B4-2 culture supernatant (a
saturating concentration, data not shown) for 30 min at 4°C. The cells were washed three times and incubated with 5 μg of affinity-purified fluoresceinated goat anti-mouse Ig F(ab')_2 (Cappel Laboratories, West Chester, PA) for 45 min at 4°C. The cells were washed twice in saline G before fixation with ethanol. Analyses were performed using a Cytoflurograf System 50 at 200 mW laser power at 488 nm. Under these conditions, the red fluorescence associated with DNA could be easily separated from the green fluorescence associated with the cell surface A2B4-2 antigen by appropriate filtration. Contour plots were generated using software developed by Dr. Peter Rabinovitch (University of Washington) for the Ortho 2150 computer system.

Results

Stimulation with Antigen Inhibits Uptake of [³H]Thymidine by T Cell Hybridomas. In the course of studies concerning the effect of varying T cell number upon antigen-stimulated IL-2 production, we noted that T cell hybridomas appeared to grow more slowly in the presence of their antigen. To quantitatively assess this phenomenon, a variety of antigen-specific T cell hybridomas were cocultured with antigen-presenting cells (APC) and assayed for their ability to incorporate [³H]thymidine in the presence or absence of antigen (Table I). Eight T cell hybrids of varying antigen- and major histocompatibility complex (MHC)-specificity were tested; all displayed a marked inhibition of [³H]thymidine incorporation 24–26 h after the addition of the appropriate antigen. In all cases, the T cell hybridomas were found to produce measurable amounts of IL-2 at concentrations of antigen that caused substantial inhibition of [³H]thymidine incorporation. Two of the hybridomas, 2B4.11 and C10.9, were chosen for further study. 2B4.11 responds to the COOH-terminal portion of the antigen pigeon cytochrome c (pigeon fragment 81–104) in association with the E\textsubscript{b}:E\textsubscript{a} Ia molecule (17), and C10.9 recognizes HEL in the presence of the A\textsubscript{b}:A\textsubscript{a} Ia molecule (18). Antigen dose-response curves were performed in the presence of a constant number of B10.A splenic APC, which bear both of these Ia molecules (Fig. 1). Both of the T cell hybrids exhibited a decrease in [³H]thymidine incorporation and a corresponding increase in the amount of IL-2 produced as the concentration of antigen was increased. In fact, similar concentrations of antigen were required to achieve 50% inhibition of [³H]thymidine incorporation and 50% stimulation of IL-2 release. This inversely proportional relationship suggested that the inhibition of [³H]thymidine incorporation was a consequence of cellular activation, and demonstrated that, for these two T cell hybridomas, the sensitivity of this assay in detecting antigenic stimulation was comparable to one that measures the secretion of IL-2.

Inhibition of [³H]Thymidine Uptake Is a Consequence of Receptor Occupancy. A variety of approaches were used to examine the antigen-specificity of the inhibition of [³H]thymidine incorporation. First, titration of the “wrong” antigen (either pigeon fragment 81–104 with C10.9, or HEL with 2B4.11) in the presence of B10.A APC failed to cause either IL-2 release or inhibition of [³H]thymidine incorporation (data not shown). Second, since these antigens must be “corecognized” with a specific Ia molecule, monoclonal antibodies that bind to the two required Ia molecules were tested for their effect on [³H]thymidine incorporation (Table I). The monoclonal antibody 14.4.4 binds to the E\textsubscript{b}:E\textsubscript{a} Ia
Table I
T Cell Hybridoma Proliferation and IL-2 Production upon Stimulation with Antigen

| T cell  | Mouse strain (haplotype)* | Reference Ia moleculea | Antigen | Concentration | [³H]Thymidine uptake | IL-2 production |
|---------|---------------------------|------------------------|---------|---------------|---------------------|----------------|
|         |                           |                        |         |               | cpm                | U              |
|         |                           |                        |         | 0             | 41,900             | 1,100         |
|         |                           |                        |         | 3 μM          | 11,100             | 52,600         |
|         |                           |                        |         | 30 μM         | 5,000              | 55,400         |
| 2B4.11  | B10.A (H-2b)              | 17                     | E3ΔE2  | Cytochrome c | 84,000             | 1,300          |
|         |                           |                        |         | 3 μM          | 57,500             | 54,600         |
|         |                           |                        |         | 30 μM         | 18,700             | 55,400         |
| 2H10    | B10.A (H-2b)              | 17                     | E3ΔE2  | Cytochrome c | 89,600             | 800            |
|         |                           |                        |         | 3 μM          | 71,600             | 44,100         |
|         |                           |                        |         | 30 μM         | 51,100             | 45,900         |
| 2C2     | B10.A (H-2b)              | 17                     | E3ΔE2  | Cytochrome c | 65,000             | 200            |
|         |                           |                        |         | 30 μg/ml      | 27,900             | 51,000         |
|         |                           |                        |         | 500 μg/ml     | 11,500             | 35,700         |
| C10.9   | B10.A (H-2b)              | 18                     | A3ΔA2  | HEL           | 57,500             | 3,800          |
|         |                           |                        |         | 30 μg/ml      | 41,600             | 19,600         |
|         |                           |                        |         | 500 μg/ml     | 14,300             | 30,300         |
| A1.1.4  | B10.A (H-2b)              | 18                     | A3ΔA2  | HEL           | 27,500             | 4,500          |
|         |                           |                        |         | 30 μg/ml      | 14,600             | 27,900         |
|         |                           |                        |         | 500 μg/ml     | 2,500              | 40,500         |
| 3D0.54.8| BALB/c (H-2d)             | 19                     | A3ΔA2  | Ovalbumin     | 59,000             | 5,000          |
|         |                           |                        |         | 100 μg/ml     | 10,600             | 11,000         |
|         |                           |                        |         | 1,000 μg/ml   | 6,100              | 18,000         |
| F9.140  | BALB/c (H-2d)             | 20                     | A3ΔA2  | GAT           | 26,800             | 2,300          |
|         |                           |                        |         | 100 μg/ml     | 18,800             | 6,600          |
|         |                           |                        |         | 1,000 μg/ml   | 11,000             | 27,000         |

10⁴ T cell hybrids were assessed for [³H]thymidine incorporation and IL-2 production after 24 h of incubation, as detailed in Materials and Methods. The cells should not be compared on the basis of absolute [³H]thymidine incorporation because the data are taken from different experiments.

* Mouse strain and haplotype of the normal T cells used as the fusion partners for BW5147.
† The source of splenic APC for each of the specified allelic forms of the Ia molecule are E3ΔE2, B10.A; A3ΔA2, B10.D2.
‡ Pigeon cytochrome c fragment 81–104 was used in these experiments.
§ [³H]Thymidine incorporation by CTLL cells in the presence of a 1:4 (25%) dilution of supernatant.

molecule (corecognized by 2B4.11), and the monoclonal antibody 10-2.16 binds to the A3ΔA2 Ia molecule (corecognized by C10.9). The addition of 10-2.16 blocked the inhibition of [³H]thymidine incorporation observed when C10.9 was incubated with HEL, but had no effect when added to 2B4.11 plus pigeon fragment 81–104 (Table II). Conversely, the antibody 14.4.4 blocked the inhibitory effect of the pigeon fragment 81–104 on the [³H]thymidine incorporation of 2B4.11 by ~100-fold, but had no effect on the response of C10.9 to HEL.

Further evidence that it was occupancy of the T cell hybridomas' antigen-specific receptors that resulted in inhibition of [³H]thymidine incorporation was obtained by using the monoclonal antibody A2B4-2, a mouse IgG2a that recognizes an epitope on the antigen receptor of 2B4.11, but that is not on the antigen receptor of C10.9 (see reference 15 and Fig. 4, below). This antibody is an
Incorporation of [3H]thymidine and secretion of IL-2 as a function of antigenic stimulation. (A) $10^5$ 2B4.11 T cells or (B) $10^6$ C10.9 T cells were incubated in the presence of $5 \times 10^3$ irradiated B10.A splenocytes and varying concentrations of the appropriate antigen. After 24 h, an aliquot of supernatant was removed from each well for the quantitation of IL-2 content (circles), [3H]thymidine was added to each well, and [3H]thymidine incorporation by the T hybridoma cells (squares) was determined as described in Materials and Methods. In parallel culture wells the antibody A2B4-2 was added at a final concentration of 0.63 μg/ml (closed symbols).

extremely potent inhibitor of antigen-stimulated IL-2 release by 2B4.11 (15). A2B4-2 was added to an assay in which 2B4.11 was stimulated with antigen (Fig. 1). The anticonnlytotic antibody blocked both the release of IL-2 and the inhibition of [3H]thymidine incorporation. No effect was observed when similar concentrations of A2B4-2 were added to an assay performed with C10.9.

Although the soluble form of the antibody A2B4-2 exhibits no stimulatory effect upon 2B4.11, when it is crosslinked to Sepharose beads it will cause 2B4.11, but not other T cell hybridomas that bear unrelated antigen receptors, to release IL-2 in a dose-dependent fashion (H. Narimatsu and R. Schwartz, personal communication). Table III contains the results of a typical experiment in which A2B4-2 crosslinked to Sepharose was used to stimulate 2B4.11. Whereas soluble A2B4-2 had no stimulatory effect, the addition of A2B4-2 crosslinked to Sepharose beads resulted in both IL-2 release and inhibition of [3H]thymidine incorporation. The antigen-specificity of these responses was demonstrated by
TABLE II

Reversal of Antigen Inhibition with Anti-Ia Monoclonal Antibodies

| T cell | Antigen | 81-104 | HEL | [3H]Thymidine incorporation after culture with: |
|--------|---------|--------|-----|-----------------------------------------------|
|        | µM      | µg/ml  | cpm | No antibody | 10-2.16 | 14.4.4 |
| C10.9  | 0       | 0      | 47,700 | 43,200 | 46,200 |
|        | 0       | 3      | 30,600 | 42,800 | 20,400 |
|        | 0       | 30     | 12,700 | 58,600 | 13,400 |
|        | 0       | 300    | 7,700  | 50,400 | 7,700  |
| 2B4.11 | 0       | 0      | 55,100 | 52,700 | 68,300 |
|        | 0.3     | 0      | 37,500 | 40,000 | 49,700 |
|        | 3       | 0      | 24,400 | 32,200 | 66,500 |
|        | 30      | 0      | 15,600 | 13,800 | 32,500 |

10⁴ T cell hybridomas were cultured in triplicate with 5 × 10⁴ irradiated B10.A splenocytes as APC and varying concentrations of the indicated antigen, in the presence or absence of a 1:500 dilution of ascites containing 10-2.16 (anti-Aβ:Aβ) or 14.4.4 (anti-Eβ:Eβ). Incorporation of [3H]thymidine was determined after 24 h in culture as described in Materials and Methods.

TABLE III

Effect of A2B4-2-coupled Sepharose Beads on 2B4.11 and C10.9

| A2B4-2-coupled beads | Soluble A2B4-2 | [3H]Thymidine incorporation with T cell hybridoma: | IL-2 production by T cell hybridoma |
|-----------------------|---------------|-----------------------------------------------|----------------------------------|
| µl                    |               | 2B4.11 | C10.9 | 2B4.11 | C10.9 |
| -                     | -             | 119,900 | 145,700 | 0  | 0 |
| -                     | +             | 113,300 | 158,500 | 0  | 0 |
| 0.125                 | -             | 66,900  | 154,000 | 24 | 0 |
| 0.25                  | -             | 64,600  | 148,000 | 56 | 0 |
| 0.5                   | -             | 58,300  | 145,200 | 40 | 0 |
| 1.0                   | -             | 44,400  | 143,000 | 46 | 0 |
| 2.0                   | -             | 37,900  | 148,700 | 58 | 0 |
| 2.0                   | +             | 112,200 | 154,300 | 0  | 0 |

10⁴ 2B4.11 or C10.9 T cells were cultured in duplicate wells in the presence of either soluble A2B4-2 antibody (0.63 µg/ml of purified antibody), varying amounts of A2B4-2-coupled Sepharose beads, or both, in a total volume of 200 µl. After 24 h, 50 µl of supernatant was removed from each well for the determination of IL-2 units, and the cells were pulsed with 1 µCi of [3H]thymidine for 2 h, after which time the wells were harvested and the [3H]thymidine incorporation determined by scintillation counting.

the fact that both were reversed by the addition of soluble A2B4-2. Stimulation of the T cell 2B4.11 with pigeon fragment 81–104 in association with the Eβ:Eβ Ia molecule incorporated into lipid planar membranes also resulted in both IL-2 production and growth inhibition (J. D. Ashwell and Barbara Fox, unpublished results). Taken together, these data indicate that the occupancy (or crosslinking) of the antigen-specific T cell receptor in the absence of any signals provided by
FIGURE 2. Flow cytometric analysis of the effect of antigen stimulation upon T cell hybridoma DNA content and volume. C10.9 T cells were incubated at a concentration of 3 x 10^5 cells/ml with 5 x 10^5 irradiated B10.A splenocytes per milliliter in the absence (solid lines) or presence (dotted lines) of 300 μg/ml of HEL. After 24 h the T hybridoma cells were separated from the APC by density centrifugation and fixed with ethanol. (A) DNA content was assessed by staining with propidium iodide. The first large peak represents cells in G1, those cells with twice the amount of DNA are in G2 or M, and those cells that are synthesizing DNA are found between the two peaks, in S phase. The fraction of cells in each phase of the cell growth cycle in the absence of antigen was, G1, 36%; S, 62%; and G2 plus M, 2%. In the presence of HEL, it was G1, 78%; S, 20%; and G2 plus M, 2%. (B) Cell size was determined by axial extinction measurements and expressed as arbitrarily defined relative units.

other cells can lead to the inhibition of [3H]thymidine incorporation by T cell hybridomas.

**Antigen Stimulation Causes a G1/S Cell Growth Cycle Block.** The incorporation of [3H]thymidine reflects both the quantity and the rate of DNA synthesis, and its decrease could be due to a block anywhere in a cell's growth cycle. To determine the point(s) at which antigen stimulation blocked the cell cycle, C10.9 T cells were incubated with irradiated APC in the presence or absence of HEL. After 24 h the T cell hybridomas were separated from the APC by density centrifugation and analyzed by flow cytometry (Fig. 2). In the absence of specific antigen (Fig. 2A, solid line), the DNA profile for C10.9 T cells was typical of rapidly proliferating cells, with ~36% of the cells in G1, 62% in S, and 2% in G2 and M phases of the cell cycle. The addition of the antigen HEL to the 24-h culture caused a marked increase in mean cell size (Fig. 2B), accompanied by a change in the DNA profile (Fig. 2A, broken line), with the majority of the cells (78%) in G1, 20% in S, and 2% of the cells in G2 or M phase. The major effect after stimulation with antigen thus seemed to be a cell cycle block at the G1/S border. Similar data was obtained with the T cell 2B4.11 (data not shown; see
The inhibition of $^{3}\mathrm{H}\text{thymidine}$ incorporation is evident only in the stimulated T hybridoma cell. $5 \times 10^6$ 2B4.11 (□) or C10.9 (▲) T cells were assayed for their ability to incorporate the $^{3}\mathrm{H}\text{thymidine}$ after 24 h of culture in the presence of irradiated B10.A splenocytes and varying amounts of antigen. In parallel cultures, $5 \times 10^6$ of each of these cells were mixed together and stimulated with either pigeon fragment 81–104 (■), HEL (▲), or both antigens simultaneously (▲). A second effect of antigen stimulation was to cause some cells in S phase to either slow or cease their synthesis of DNA (Fig. 2A). This interpretation is supported by experiments in which cell cycle analysis of antigen-stimulated cells was performed after 24 h and also after 48 h, in the presence or absence of the mitotic inhibitor colcemid. It was observed that those cells found to be in S phase at 24 h had failed to accumulate in M phase by 48 h, suggesting that they were no longer cycling (data not shown).

*Inhibition of Growth Is the Result of a Direct Effect upon the Stimulated Cell.* The cell cycle block induced by stimulation with antigen could be a direct result of antigen receptor occupancy, or it could be secondary to the binding of soluble substances (such as lymphotoxin) produced by the activated T cell hybrids. To test these possibilities, equal numbers of C10.9 and 2B4.11 were assayed, both independently and when admixed, for the effect of antigen stimulation upon $^{3}\mathrm{H}\text{thymidine}$ incorporation (Fig. 3). When incubated separately, the growth of both T cell hybridomas was inhibited in an antigen dose-dependent fashion. When mixed together in the absence of antigen the amount of $^{3}\mathrm{H}\text{thymidine}$ incorporated was approximately equal to the sum of the individual populations. The addition of either antigen alone resulted in the loss of the contribution made by the appropriate antigen-specific T cell. At maximal stimulation the combined response was reduced by ~50%. Thus, at concentrations of pigeon fragment 81–104 that were shown to cause maximal growth inhibition of 2B4.11 (e.g., 30 μM), the mixed population incorporated ~26,000 cpm, and at the comparable concentrations of HEL (300 μg/ml) the mixed population incorporated 27,000 cpm. In the presence of both antigens, however, $^{3}\mathrm{H}\text{thymidine}$ incorporation by the mixed population was almost completely inhibited. These results suggested that the inhibitory effect of antigenic stimulation was direct, being manifested only in T cells hybridomas in which the antigen receptors were engaged.

To further analyze the potential for a secreted inhibitor of growth, we made
Figure 4. Cell growth cycle blockade after antigenic stimulation is evident only in the stimulated T hybridoma cell. (A and B) $3 \times 10^5$ 2B4.11 T cells/ml were incubated with $5 \times 10^8$ irradiated B10.A splenocytes in the absence (A) or presence (B) of 100 μM pigeon fragment 81–104. (C and D) $3 \times 10^5$ C10.9 T cells/ml were incubated with $5 \times 10^8$ irradiated B10.A splenocytes in the absence (C) or presence (D) of 500 μg/ml of HEL. (E–G) $1.5 \times 10^5$ 2B4.11 and $1.5 \times 10^5$ C10.9 T cells were incubated together (MIX) in the absence of antigen (E), in the presence of 300 μg/ml of HEL (F), or in the presence of 100 μM pigeon fragment 81–104 (G). After 24 h the cells were stained with A2B4-2 and FITC-goat anti-mouse Ig so that the two cell populations could be distinguished in mixed cultures.

Use of the fact that the monoclonal antibody A2B4-2 can distinguish between 2B4.11 and C10.9. Correlated DNA/immunofluorescence analysis was performed upon a mixture of these two T cells in the presence of no antigen, pigeon fragment 81–104, or HEL (Fig. 4). Each T cell divided rapidly when incubated alone or in the presence of the other T cell (Fig. 4, A, C, and E). Surface staining with A2B4-2 allowed the clear differentiation of the two cell populations, even when they were mixed together (Fig. 4E). The addition of pigeon fragment 81–104 to 2B4.11 and HEL to C10.9, resulted in predominantly a G1/S interface block (Fig. 4, A vs. B; C vs. D). The addition of these antigens to the inappropriate cell had no effect on the cell cycle (data not shown). Strikingly, in the mixed population, the addition of HEL blocked the entry into S phase of virtually all C10.9 T cells (A2B4-2-), while having almost no effect upon the cell cycle of 2B4.11 T cells (A2B4-2+) (Fig. 4F). The reciprocal experiment, in which pigeon fragment 81–104 was used to stimulate the mixed population, demonstrated a marked cell cycle block of the 2B4.11 T cells, with virtually no effect upon the
C10.9 T cells (Fig. 4G). This result demonstrates that the major effect of stimulation with antigen is upon the antigen-specific T cell, and suggests that soluble factors alone were not responsible for the cell cycle block.

**Effect of Antigen Stimulation on T Cell Growth.** To quantitate the effect of antigenic stimulation upon the growth of T cell hybridomas, C10.9 or 2B4.11 T cells were cultured in the presence of either B10.A APC, which bear both Ia molecules required for the presentation of soluble antigen to these two T cell hybrids, or B10.D2 APC, which do not bear the necessary allelic forms of the Ia molecule. The T cell hybridomas were cultured in the presence or absence of the appropriate antigens, and cell counts were performed at 24-h intervals (Fig. 5). C10.9 T cells grown in the presence of B10.A APC without antigen, or in the presence of B10.D2 APC plus HEL, grew with a doubling time of \( \approx 12 \) h (Fig. 5A). In contrast, the growth of C10.9 T cells cultured in the presence of B10.A APC and 300 \( \mu \)g/ml of HEL was markedly inhibited early in the course of the culture. After a lag of \( \approx 4-5 \) d, the number of viable cells began to increase exponentially, with a doubling time virtually identical to that displayed by the unstimulated C10.9 T cells. A similar result was obtained when this experiment was performed with 2B4.11 T cells (Fig. 5B). As with C10.9, the unstimulated 2B4.11 cells grew rapidly, with a doubling time of \( \approx 13.5 \) h. The growth of 2B4.11 T cells cultured in the presence of B10.A APC and 30 \( \mu \)M pigeon fragment 81–104 was markedly slowed early in the course of the culture. Again, after a lag of \( \approx 4 \) d, the number of viable cells in the wells that had been appropriately stimulated began to increase exponentially, with a doubling time similar to that of the unstimulated 2B4.11 population. In another experiment (Fig. 5C), the effect of different concentrations of antigen on cell growth was determined. Little growth of 2B4.11 was evident in the presence of 3 \( \mu \)M pigeon fragment 81–104 until day 3, whereas its growth was delayed \( \approx 1 \) d longer in the presence of 100 \( \mu \)M antigen (the growth curve for 30 \( \mu \)M antigen [not shown] was superimposable with that for 100 \( \mu \)M antigen).

Functional assays were performed to determine the responsiveness of those T cells that had expanded after a lag period, despite the presence of antigen. From the experiment displayed in Fig. 5A, 10 independent wells from which C10.9 cells had arisen after culture in the presence of B10.A APC and 300 \( \mu \)g/ml of HEL were selected at random and expanded. All 10 of these sublines were substantially less sensitive to antigenic stimulation than was the parent C10.9 clone, and on average appeared to be at least 100-fold more difficult to activate, as judged both by IL-2 production and growth inhibition (Table IV). The decreased sensitivity to antigen was a stable property. When these 10 C10.9 sublines were restested after 14 d (\( \approx 28 \) generations) with HEL concentrations of up to 300 \( \mu \)g/ml, their responses were very similar to those given in Table IV, with a mean decrease in [\( ^{3} \)H]thymidine incorporation of only 57% at the highest concentration of antigen (data not shown). Thus, it appeared that the original C10.9 line contained a subpopulation(s) that was a relatively poor responder to HEL, and whose growth was therefore not completely inhibited in the presence of antigen. Given a doubling time of 12 h (Fig. 5A), it can be determined that an antigen-resistant subpopulation of only 0.5% in the original C10.9 line could account for the growth that was observed in the antigen-stimulated wells after 5
d. Since both measurements of T cell activation (IL-2 production and growth inhibition) were diminished, these sublines might have consisted of clones that had lost a substantial portion of their antigen-specific receptors. It was not possible to test this directly, because no anticlonotypic antibody exists for this T cell. It was also possible that other molecules involved in the response to antigen might have been lost by the resistant sublines. Indeed, flow cytometric analysis performed with the anti-L3T4 antibody, GK1.5, revealed that, whereas the large majority (91%) of the parent C10.9 T cells stained intensely with this antibody,
TABLE IV
Responsiveness of C10.9 Sublines to Antigen/MHC

| Number of C10.9 sublines tested | Maximal [3H]thymidine incorporation (%) with HEL (μg/ml): |
|--------------------------------|------------------------------------------------------|
|                                | 0         | 1         | 3         | 10        | 30        | 100       |
| A.                             |           |           |           |           |           |           |
| 1 (no selection)*              | 100       | 50        | 34        | 41        | 21        | 8         |
| 10                             | 100       | 95 ± 5†   | 100 ± 5   | 97 ± 5    | 91 ± 6    | 72 ± 5    |
| B.                             |           |           |           |           |           | 3H-2-production (U)   |
| 1 (no selection)*              | 0         | 3.5       | 3.8       | 6         | 10        | 15        |
| 10                             | 0         | 0.05 ± 0.05† | 0.4 ± 0.27 | 1.1 ± 0.4 | 1.9 ± 0.37 | 5 ± 0.56 |

Ten of the antigen-containing microtiter wells from the cell growth experiment shown in Fig. 5A, in which C10.9 was cultured in the presence of HEL (500 μg/ml), were collected on day 6, and the cells were expanded in growth medium alone. 4 d later, each C10.9 subline was tested for its ability to produce IL-2 and to incorporate [3H]thymidine upon stimulation with HEL in the presence of irradiated B10.A APC.

* The original C10.9 clone, carried in vitro, from which the sublines were derived.
† Mean ± SEM.

TABLE V
Responsiveness of 2B4.11 Sublines to Antigen/MHC

| Number of 2B4.11 sublines tested | Selecting antigen concentration | Maximal [3H]thymidine incorporation (%) with pigeon fragment 81-104 (μM): |
|---------------------------------|---------------------------------|------------------------------------------------------|
|                                 |                                | 0         | 0.3       | 1         | 3         | 10        |
| A.                             |                                |           |           |           |           |           |
| 1*                             | None                            | 100       | 34        | 22        | 10        | 7         | 5         |
| 5†                             | None                            | 100       | 34 ± 3†   | 24 ± 2    | 12 ± 2    | 12 ± 2    | 5 ± 2     |
| 5                              | 3 μM                            | 100       | 43 ± 3    | 30 ± 4    | 24 ± 2    | 15 ± 2    | 15 ± 3    |
| 5                              | 100 μM                          | 100       | 40 ± 3    | 32 ± 2    | 27 ± 4    | 19 ± 2    | 18 ± 1    |
| B.                             |                                |           |           |           |           | 3H-2 production (U)   |
| 1*                             | None                            | 0         | 20        | 32        | 40        | 40        | 35        |
| 5†                             | None                            | 0         | 16 ± 8†   | 33 ± 10   | 57 ± 9    | 71 ± 11   | 69 ± 7    |
| 5                              | 3 μM                            | 0         | 17 ± 1    | 33 ± 4    | 36 ± 6    | 43 ± 4    | 43 ± 5    |
| 5                              | 100 μM                          | 0         | 10 ± 2    | 13 ± 2    | 17 ± 2    | 13 ± 2    | 15 ± 3    |

2B4.11 sublines were established from independent microtiter wells on day 4 of the cell growth experiment shown in Fig. 5C, in which 2B4.11 was cultured in the absence or presence of pigeon fragment 81-104 (3 or 100 μM). 7 d later each 2B4.11 subline was tested for its ability to produce IL-2 and to incorporate [3H]thymidine upon stimulation with pigeon fragment 81-104 in the presence of irradiated B10.A APC.

* The original 2B4.11 clone, carried in vitro, from which the sublines were derived.
† Sublines derived from microtiter wells in which no antigen was added.
‡ Mean ± SEM.

only 9% of the C10.9 cells that had grown in antigen-containing wells bore the L3T4 molecule.

An analysis performed with 2B4.11 gave a similar, but quantitatively different, result (Table V). Sublines of 2B4.11 were established from the experiment displayed in Fig. 5C. Five sublines that arose in the absence of antigen were as sensitive to antigenic stimulation, as was the original 2B4.11 clone carried in
vitro, both in terms of their production of IL-2 and the inhibition of growth. At their maximal response, sublines of 2B4.11 that had been grown in the presence of either 3 μM or 100 μM pigeon fragment 81–104 produced considerably less IL-2 than did the sublines that had not been selected with antigen. The ability of antigen to inhibit their incorporation of [³H]thymidine was also somewhat decreased, especially at high concentrations of antigen (i.e., the plateau inhibitory response was less). Flow cytometric analysis and cell surface staining with the anticonotypic antibody A2B4-2 showed that stimulation with increasing doses of antigen did indeed select for receptor-negative variants of 2B4.11. On average, the fraction of receptor-negative 2B4.11 T cells, as a function of the concentration of pigeon fragment 81–104 used to select them, was: 0 μM, 3%; 3 μM, 6%; 100 μM, 18%. The expression of L3T4 was not a useful parameter to follow for these cells, since the parent clone bears negligible amounts of this molecule (data not shown).

Failure to Reverse Cell Cycle Block 24 h after Removal of Antigen. These data suggest that selection of relatively antigen-resistant cells does occur in the presence of antigen, but also argue that in some cases a substantial number of antigen-responsive cells can survive. This result is compatible with the hypothesis that the cell cycle block is reversible, presumably allowing the inhibited cells to resume their growth as the APC in the cultures die and become nonfunctional. An alternative possibility is that, because of the requirement that a T cell must encounter an antigen-presenting cell for stimulation to occur, a small number of antigen-responsive 2B4.11 T cells may simply have escaped sufficient stimulation to be inhibited (with a doubling time of 13.5 h the survival of ~1.5% and 6% of the antigen-stimulated 2B4.11 T cells could account for the growth patterns depicted in Fig. 5B and C, respectively).

To directly test whether the cell cycle block was reversible, flow cytometric cell cycle analysis was again used (Fig. 6). The T cell C10.9 was incubated with irradiated B10.A APC in the presence or absence of HEL. After 24 h the T cells from both groups were separated from the APC by density centrifugation, washed thoroughly, and placed back in culture in the presence of the anti-la antibody 10-2.16 to ensure that any residual antigen-pulsed APC would not be stimulatory (see Table II). In a separate antigen-stimulated group, the medium was changed at 24 h and fresh antigen was added to maintain the stimulation. After another 24 h, which would be enough time for two doublings of untreated C10.9 T cells, cell growth cycle analysis was performed on each group (Fig. 6).

The profile of cells incubated for 48 h in the absence of antigen was found to contain ~58% of the cells in G₁, 40% in S, and 2% in G₂ plus M (Fig. 6A). Incubation in the presence of continuous antigen inhibited the movement of cells from G₁ to S phase (Fig. 6B). ~71% of the cells were found to be in G₁, 27% in S, and 2% in G₂ plus M. A significant number of cells remained in S phase, indicating either an incomplete G₁/S block or an S phase cell cycle block, as suggested above. The removal of the APC and antigen at 24 h had little if any effect upon the degree of the G₁/S cell cycle block measured at 48 h, because this group was found to have a cell cycle profile nearly identical to that of cells that were maintained in continuous antigen for 48 h (Fig. 6, B vs. C), with 86% in G₁, 13% in S, and 1% in G₂ plus M. It can also be seen that the characteristic
FIGURE 6. Lack of reversibility of the G₁/S block after 24 h. 10⁵ C10.9 T cells/ml were incubated with 5 × 10⁶ irradiated B10.A splenocytes in the presence (B and C) or absence (A) of 300 µg/ml HEL. (A) After 48 h the T cells were separated from the irradiated splenocytes by density centrifugation, fixed with ethanol, and analyzed for DNA content. (B) 24 h after the initiation of the cultures the medium was removed and replaced with fresh complete medium containing 300 µg/ml HEL. After another 24 h the T cells were isolated by density centrifugation and cell cycle analysis performed. (C) 24 h after the initiation of the cultures the T cells were isolated by density centrifugation, washed three times, and placed back in culture in fresh complete medium in the absence of antigen. A 1:500 final concentration of 10:2.16-containing ascites was added to each tube to ensure that if a small number of APC were carried over into the second culture they would be unable to stimulate the T cells (see Table II). After an additional 24 h of incubation, cell cycle analysis was performed. (D) Superimposed profiles of the relative DNA content of the three C10.9 T cell populations: no antigen (solid line), continuous antigen (dashed line), and antigen removed at 24 h (dotted line).

cell size increase persisted in these cells 24 h after the removal of antigen. Interestingly, the population of cells from which antigen was removed at 24 h had fewer cells in S and correspondingly more in G₁, suggesting that the S block was at least partially reversible, its release allowing some of these cells to resume cycling and to eventually be blocked at the G₁/S interface. Thus, for at least the large majority of the T cells, after 24 h of exposure to antigen the G₁/S cell cycle block appeared to be irreversible up to 24 h later. Similar results were obtained with the T cell hybridoma 2B4.11 (data not shown). Taken together, these results are compatible with the hypothesis that the eventual growth of T cell hybridomas after stimulation with antigen represents two phenomena: (a) the selection of T cell variants that are less sensitive to antigenic stimulation than the parent population (most evident for C10.9, Table IV), and (b) the growth of a small
population of typically responsive T cells that were insufficiently stimulated in the initial culture to develop a cell cycle block (e.g., 2B4.11, Table V).

Discussion

T cell hybridomas have been used extensively in the study of the requirements for, and the consequences of, T cell activation. One of the first, and still the most common, assays used to assess the response of a T cell hybridoma to a given stimulus is to measure the culture supernatants for the presence of IL-2 (23). Whereas most T cell hybrids do not produce IL-2 spontaneously, they secrete it upon stimulation with a variety of agents, including lectin, antigen/MHC, and crosslinked anticonnotypic antibodies (6, 23–25). In addition to IL-2, activated T cell hybrids can produce a large variety of soluble factors upon stimulation, including BSF-1 and other B cell proliferation and differentiation factors, IFN-γ, colony-stimulating factors, and lymphotoxin (26–30). In the present report we have demonstrated that T cell hybridomas activated via the antigen-specific receptor also develop a block in their cell growth cycle. The signals for growth inhibition appeared to share the same recognition requirements as the signals for lymphokine release. Further, the ability of anticonnotypic antibodies crosslinked to an insoluble matrix, or antigen plus Ia molecules incorporated into an artificial lipid planar membrane, to cause both IL-2 production and growth inhibition demonstrated that no cell other than the responding T cell hybrid was required to manifest this phenomenon.

The present report adds the antigen-specific T cell receptor to the growing list of receptors whose occupancy, while functioning to promote growth in normal cells, inhibits the growth of some transformed cell lines. For example, IL-2 is a well-described stimulus of proliferation in normal T cells (31). It was recently observed that some spontaneously dividing human T cell leukemia virus–transformed T cells ceased to grow in the presence of recombinant IL-2 (10). In addition, exposure of a murine T cell line, transfected with the human IL-2 receptor gene, to recombinant IL-2 resulted in inhibition of its growth (11). Another example is epidermal growth factor (EGF), which usually functions to stimulate anchorage-independent growth in a variety of nontransformed cells (32). Normally mitogenic concentrations of EGF inhibit the growth of the rat pituitary cell line GH4C1 (33), and the human epidermoid carcinoma A431, a cell that bears an unusually high level of EGF receptors (8). A closely related hormone, transforming growth factor type β (TGF-β), has also been found to inhibit the anchorage-independent growth of a variety of tumor cells (9, 34) as well as normal IL-2-stimulated human T cells (35).

There is also a striking parallel between the effect of antigen stimulation on normal T cells and their hybrid counterparts and the effect of another lymphocyte-specific stimulus, antiimmunoglobulin (anti-Ig), on normal B cells and some B cell tumors. In normal resting murine B lymphocytes, crosslinking of surface Ig with an appropriate anti-mouse Ig causes an increase in intracellular Ca²⁺, the breakdown of phosphatidylinositols, and the movement of resting cells into the cell growth cycle (36–38). Anti-Ig treatment of some spontaneously dividing B cell tumors, such as WEHI 231 (39, 40) and BCL₁ (41), on the other hand, results in growth inhibition. In the case of WEHI 231 this is associated with a
G₁/S cell cycle block that, after ~24–48 h, becomes irreversible and is followed by cell death (42). Since WEHI 231 bears the phenotype of an immature B cell, this phenomenon has been suggested as a model for the induction of tolerance in immature B lymphocytes (42, 43). Bearing in mind that the T cell hybridomas used in the present report were prepared by fusing antigen-primed peripheral T cells to a thymoma cell line, it is possible that the antigen-dependent cell cycle block of T cell hybridomas may be related to tolerance induction in immature T lymphocytes. This speculation obviously requires a considerably more thorough understanding of how cellular activation via the antigen receptor occurs before it can be directly examined.

In this regard it is worth considering the nature of the T cell growth cycle block induced by antigenic stimulation. The major block was found to be at the G₁/S interface, with a second block in S phase itself. Removing the stimulus after 24 h and waiting another 24 h before analysis allowed some of the cells that were trapped in S to resume cycling and to be subsequently blocked at G₁/S. The data presented in this study cannot exclude the possibility that the G₁/S block was reversible, but its release required more than 24 h in the absence of antigen. It is also formally possible that the eventual growth of some antigen-responsive 2B4.11 T cells (Table V) was due to a leaky G₁/S block, although this would seem unlikely in view of the fact that the T cells that eventually grew in the presence of continuous antigen did so with a doubling time identical to that of unstimulated cells (Fig. 5). The simplest interpretation of the data is that the cell cycle block is irreversible, and that the T cells that were found to grow after a lag period represented either a small fraction that had escaped stimulation, or those cells that, for any of a variety of reasons, were difficult to stimulate with antigen/MHC. It is interesting to note that exposure of a variety of murine T cell neoplasms, including BW5147 (44, 45) and a murine T cell hybridoma (23), to lectins such as concanavalin A and phytohemagglutinin, has been shown to lead to a decrease in DNA and RNA synthesis and eventually cell death. The growth of both 2B4.11 and C10.9 was also profoundly diminished after 24 h of coculture with concentrations of concanavalin A that induced maximal IL-2 production from these cells (unpublished observation). It is possible that lectin-mediated cellular activation, perhaps by signalling through the antigen receptor/T3 complex (46), delivers the same growth inhibitory signals as does antigen/MHC, and may provide a means of studying this phenomenon in T cell neoplasms for which the appropriate antigen/MHC combination is unknown.

A question of particular interest is how does antigen receptor occupancy result in cell cycle blockade of T cell hybridomas? Experiments in which two T cell hybridomas of different antigen specificities were cultured together in the presence of a single antigen demonstrated that the cell cycle block occurred in only the T cell specific for that antigen (Figs. 3 and 4). It would therefore seem unlikely that the T cell hybridomas produced, and were in turn inhibited by, toxic lymphokines. However, these experiments do not rule out the possibility that antigen activation resulted in two distinct events: the secretion of an inhibitory lymphokine, and the expression of the receptor for that lymphokine. One candidate for such a lymphokine/receptor pair would be the IL-2/IL-2 receptor combination (10, 11). We have ruled out this particular possibility by
the demonstration that the T cell hybridomas used in this study have few if any IL-2 receptors, even when activated with antigen/MHC, and that the addition of anti-IL-2 receptor antibodies to the cultures failed to prevent the growth inhibition caused by antigen activation (unpublished results). However, the possibility still exists that stimulation with antigen induced the expression of some other inhibitory lymphokine and its receptor.

Finally, for completeness, it should be mentioned that some hematopoietic neoplasms, such as erythroleukemia cells, can be induced to terminally differentiate and thereby cease their uncontrolled growth, by a wide variety of chemical (e.g., dimethylsulfoxide, butyrate) and physical (e.g., x and UV irradiation) agents (47). Some myeloid leukemic cells can be similarly induced to differentiate in the presence of colony stimulating factors (48). In view of the fact that normal effector T lymphocytes do not seem to undergo terminal differentiation (e.g., mature and functionally competent T cells seem to have a virtually unlimited capacity to renew themselves in vitro upon repetitive antigenic stimulation), and that antigenic stimulation of T cell hybridomas results in an apparently irreversible cell cycle block and death, as judged by the release of $^{51}$Cr (data not shown), it seems unlikely that such an event accounts for the inhibition of growth in T cell hybridomas. Furthermore, differentiation of hematopoietic cells is accompanied by a decrease in singular cell size (49); the increase in cell size accompanying the $G_1/S$ block reported in the present study more likely reflects unbalanced growth leading to cell death, such as that due to deoxyadenosine $G_1$ arrest of leukemic T cells (50).

The antigen-driven growth arrest of antigen-specific T cell hybridomas may have some useful applications. As demonstrated in Fig. 1, as long as the T cell hybrid population is fairly uniform in its ability to respond to antigen, the measurement of the inhibition of growth is as sensitive and quantitative as the measurement of IL-2 release. Moreover, the growth inhibition assay has a number of distinct advantages over lymphokine assays; it is more rapid (requiring ~24 h vs. 48 h), does not require sequential cell cultures, and most importantly, obviates the need to serially dilute supernatants to achieve quantitative results. It should be noted, however, that because IL-2 is not produced by unactivated T cell hybrids (i.e., the background activity is low), the detection of IL-2 in a nonhomogeneous population might be more sensitive than the assessment of growth inhibition. For example, the detection of a low level of IL-2 might convincingly indicate that at least some small portion of the cells in a mixed population had responded, whereas a small decrease in the incorporation of [$^3$H]thyidine might be considered insignificant because of the high background, resulting in a false negative.

Another potential use for the growth inhibition of antigen-stimulated T cell hybrids is the selection of mutants in the activation/growth inhibition pathway. As found with C10.9 and 2B4.11, small subpopulations that are relatively resistant to activation with antigen/MHC can continue to grow in the presence of cells that are prevented from proliferating, although the experience with 2B4.11 suggests that multiple rounds of selection may be necessary to allow substantial expansion of such clones. Therefore, it should be possible to treat a homogeneous in vitro line with mutagens and select for T cell hybridomas whose
growth is no longer inhibited by antigen/MHC. As demonstrated in this report, such mutants and/or phenotypic variants should include T cells that have lost molecules associated with the recognition of antigen (as with the antigen-specific T cell receptor on 2B4.11, and the L3T4 molecule on C10.9). They would also probably include cells in which the loss or modification of other molecule(s) involved in antigen recognition and/or signal transduction (perhaps such as elements of the T3 complex) had occurred, and might allow the separation of the IL-2 secretory response from the growth inhibitory response. The generation of such cells would be useful in the study of how antigen receptor occupancy leads to lymphokine release and growth inhibition. Finally, the finding that the activation of a variety of independently-derived T cell hybridomas can arrest their growth raises the intriguing possibility that a therapeutic role might be found for similar treatment of antigen-specific T cell tumors in vivo. Studies are currently underway in our laboratory to investigate the potential of such treatment in a model murine tumor system.

Summary

Stimulation of antigen-specific T cell hybridomas with the appropriate antigen/MHC combination, at concentrations that resulted in the secretion of the lymphokine interleukin 2, resulted in a dose-dependent decrease in both [³H]-thymidine incorporation and cell growth. Flow cytometric studies demonstrated that stimulation with antigen resulted in a cell cycle block that was most evident at the G₁/S border, and mixing studies revealed that bystander T cells of different antigen specificities were not affected. For at least the large majority of T cells, the G₁/S cell cycle block appeared to be irreversible after 24 h of exposure to antigen. This cell cycle block may be useful as a rapid and quantitative measure of T cell hybridoma activation, as a means of selecting T cell hybridomas that have functional alterations in the reception of stimulatory signals, and may serve as a model of the induction of tolerance in immature T cells.

We thank Dr. R. Schwartz for the gift of the A2B4.2-coupled Sepharose beads, Dr. B. Fox for providing purified A2B4.2, and Dr. P. Allen for providing the T cell hybridomas A4.A1 and A6.A2. We are also grateful to Drs. R. Schwartz and R. Germain for critical review of this manuscript.

Received for publication 10 September 1986.

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ASHWELL ET AL. 193

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