

# Production of functional human T-T hybridomas in selection medium lacking aminopterin and thymidine

(azaserine/hypoxanthine/lymphokines/interleukin 2)

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Communicated by Henry S. Kaplan, September 9, 1982

**ABSTRACT** The production of hybridomas between immunologically activated T cells and malignant T-cell lines offers a potentially unlimited source of soluble T-cell-derived products. Recently, human T-T hybrids have been described; however, their use has been hampered by slow growth and chromosomal instability due at least in part to the presence of thymidine in the traditional hypoxanthine/aminopterin/thymidine (HAT) selection medium. In this report, we describe the development of a rapidly growing hypoxanthine phosphoribosyltransferase-deficient human T-cell line designated J3R7, the use of azaserine/hypoxanthine (AH) medium as an alternative selection medium to HAT medium, and the production of functional T-T hybrids by using the J3R7 line and the AH selection technique. Hybrids selected in AH medium were 4-fold greater in number and 3-fold faster in growth rate than hybrids grown in HAT medium. No stable clones were obtained from HAT cultures whereas AH-derived hybrids could be readily cloned by the method of limiting dilution. Evidence for hybridization included (i) the presence of approximately twice the number of chromosomes in hybrids than in J3R7 cells; (ii) the presence on hybrid cells of the Leu-3a surface antigen, present on normal helper T cells but not on J3R7 cells; (iii) the expression of HLA antigens of both the normal T-cell partner and the J3R7 line; and (iv) the constitutive secretion of interleukin 2 from multiple hybrid clones but not from the J3R7 cell line. Thus far, these clones have maintained their rapid growth, chromosome number, surface phenotype, and constitutive secretion of interleukin 2 for 4 months.

T lymphocytes produce a variety of immunologically active mediators (lymphokines) on stimulation with antigens or mitogens (1, 2). These mediators can be divided into two broad categories: antigen nonspecific, such as growth factors for T cells (interleukin 2, IL-2) and B cells (3), and antigen-specific helper and suppressor factors (4). Due to the limited availability of these factors, knowledge of their structure and function lags far behind that of immunoglobulin, the major B-cell product. Typically, the T-cell products are derived from bulk cultures of normal lymphocytes stimulated with mitogens such as phytohemagglutinin (PHA) or concanavalin A (Con A). Although this approach has yielded biologically active factors, the presence of multiple lymphokines as well as the mitogens themselves have hindered large-scale preparation of homogeneous material. In mice, the propagation of T-cell clones *in vitro* has overcome some of these problems (5). In man, however, such clones have been difficult to maintain and have not yet proved to be reliable sources of lymphokines.

The use of T-T hybridomas produced by the fusion of an activated T cell with a malignant T-cell line offers the theoretical advantages of a single cell source of factor, rapid and continuous growth in the absence of exogenous growth promoters, and an

unlimited supply of product free of mitogen. This technique has been used successfully for the production of murine T-cell factors (5, 6). Although reports of human T-T hybrids have appeared (7-10), their utility has been limited by slow growth rates and chromosomal instability. It seems likely that a major factor in the problems encountered with human T-T hybrids is the presence of thymidine in the culture medium, which is known to inhibit the growth of T-cell lines (11). In this communication, we report a rapidly growing 6-thioguanine (SGua)-resistant T-cell line, a method for the selection of T-T hybrids that avoids the inhibitory effects of thymidine, and the use of this method for the production of IL-2-secreting human T-T hybridomas.

## MATERIALS AND METHODS

**Cell Lines.** A SGua-resistant human T-leukemia cell line, CEMT3 (a subclone derived from a line provided by Warner Greene, National Institutes of Health), was initially used as a fusing parent to produce T-T hybrids. A second permanent T-cell line, J3R7, was derived from the Jurkat human T-leukemia cell line (provided by R. Levy, Stanford University). The Jurkat cells were placed in RPMI 1640 medium (GIBCO)/25 mM HEPES/2 mM L-glutamine/10% fetal calf serum/1  $\mu$ M SGua (Sigma) and grown in humidified 95% air/5% CO<sub>2</sub> at 37°C. After 1 wk, the viable cells were separated by Ficoll/Hypaque gradient centrifugation (12) and placed in the same nutrient medium with a higher concentration of SGua. Over a 6-wk period, the level of SGua was increased to 0.2 mM. A rapidly growing clone, designated J3R7, was derived by the method of limiting dilution, and this clone was used in hybridization experiments.

**Cell Isolation and Activation.** Peripheral blood lymphocytes (PBL) were obtained from normal donor blood by Ficoll/Hypaque gradient centrifugation and  $1 \times 10^7$  cells/10 ml were stimulated with Con A (10  $\mu$ g/ml; Pharmacia) for 48 hr or with PHA (1  $\mu$ g/ml; Burroughs-Wellcome, Beckenham, England) for 24 hr in RPMI 1640/25 mM HEPES/2 mM glutamine/10% fetal calf serum.

Peripheral blood monocytes were enriched by adherence to Petri dishes at 37°C for 1 hr and harvested by pipetting with calcium-free medium containing 0.1% EDTA (GIBCO). These were used as feeder cells after irradiation with 5,000 rads (1 rad = 0.01 gray) from a cesium source (model 143 irradiator, J. L. Shepherd, Glendale, CA). Mouse macrophages were added as an alternative feeder at the same concentration.

**Fusion Procedure.** Stimulated PBL and CEMT3 or J3R7 cells were fused at a ratio of 1:1. Usually,  $4 \times 10^7$  PBL and 4

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Abbreviations: AH medium, azaserine/hypoxanthine medium; Con A, concanavalin A; HAT medium, hypoxanthine/aminopterin/thymidine medium; HPRT, hypoxanthine phosphoribosyltransferase; IL-2, interleukin-2; PBL, peripheral blood lymphocytes; PHA, phytohemagglutinin; SGua, 6-thioguanine.

Table 1. Yield of human T-T hybrids selected in either AH or HAT medium

	Medium	
	AH	HAT
Onset of visible hybrid growth, days	10	30
Wells with hybrids,* %	21	5
Wells positive for IL-2,* %	20	0
Cloning efficiency <sup>†</sup>	50	0
Clones positive for IL-2, <sup>‡</sup> %	25	0

Equal numbers of T-T hybrids derived from PHA-activated cells were distributed into wells containing either AH or HAT medium.

\* Determined after 30 days of culture.

<sup>†</sup> To analyze cloning efficiency, hybrids from each of four wells positive for IL-2 were seeded at 0.5 cell per well into 60 microwells. Cloning efficiency is defined as the percentage of seeded wells with cell growth 30 days after cloning.

<sup>‡</sup> Mean obtained at 30 days from wells in which cloning efficiency was determined.

$\times 10^7$  mutant tumor cells were mixed in 1 ml of 50% (vol/vol) polyethylene glycol ( $M_n$ , 1,500; BDH Chemicals, Poole, England) in RPMI 1640 over 1 min. The suspension was centrifuged for 3 min at  $500 \times g$ . After 8 min, the cell pellet was suspended in 1 ml of RPMI 1640 with slow mixing over 1 min, followed by addition of 6 ml of RPMI 1640/10% fetal calf serum. The cells were then pelleted at  $250 \times g$  for 10 min, and the pellet was washed twice in RPMI 1640/10% fetal calf serum. After this, the cells were suspended in either RPMI 1640/25 mM HEPES/2 mM glutamine/10% NCTC 109 (Microbiological Associates, Walkersville, MD)/15% fetal calf serum/100  $\mu$ M hypoxanthine/100 nM aminopterin/16  $\mu$ M thymidine/10  $\mu$ M deoxycytidine (Sigma) (HAT medium) or RPMI 1640/25 mM HEPES/2 mM glutamine/15% fetal calf serum/100  $\mu$ M hypoxanthine/azaserine (1  $\mu$ g/ml) (Sigma) (AH medium). The cells were cultured in 24-well trays at a concentration of  $10^6$  tumor cells per well in a 1-ml volume with a feeder layer of  $2 \times 10^5$  adherent cells per well. The cultures were maintained at 37°C in humidified 95% air/5% CO<sub>2</sub>. After 10 days, the medium was altered by removing aminopterin or azaserine, respectively. In general, the medium was changed every 3 days.

**Chromosome Analysis.** Chromosome preparations were produced according to standard techniques. Approximately  $10^6$

cells/ml were placed in basic growth medium containing vinblastine at 0.5  $\mu$ g/ml (Lilly). After 3 hr of incubation, the cells were harvested by centrifugation at  $250 \times g$  for 10 min. The cell pellet was suspended in 5 ml of hypotonic solution (growth medium/distilled water, 1:2) for 10 min. The cells were then pelleted, washed twice in fixative (methanol/glacial acetic acid, 3:1), suspended in a few drops of fixative, air dried on microscope slides, stained with Giemsa (Sigma), and examined microscopically.

**IL-2 Assay.** The presence of IL-2 was determined by the ability of culture supernatant to support the growth of IL-2-dependent T-cell lines. The assay is a modification of one described by Gillis *et al.* (13). Approximately  $1 \times 10^4$  HT-2 cells, a murine IL-2-dependent T-cell line (provided by G. Fathman, Stanford University), or an equivalent number of human T cells activated with PHA and maintained in IL-2 for at least 2 months were cultured for 24 hr in 96-well round-bottomed microtiter trays (Flow Laboratories, McLean, VA) containing RPMI 1640/penicillin/streptomycin/2 mM L-glutamine/10% fetal calf serum and serial dilutions of test supernatants. The microcultures were then pulsed with 1  $\mu$ Ci of [<sup>3</sup>H]thymidine (1 Ci =  $3.7 \times 10^{10}$  becquerels; New England Nuclear) for 4 hr, harvested on a Mash II harvester, and assayed in a liquid scintillation counter.

A standard IL-2 preparation used for comparison was derived from MLA 144, a gibbon leukemia line that constitutively produces IL-2 and has been shown to support murine and human IL-2-dependent T-cell clones (14).

**Cell Surface Marker Analysis.** Monoclonal antibodies anti-Leu-1, anti-Leu-2a, anti-Leu-3a, and anti-Leu-4 were obtained from Becton-Dickinson (Mountain View, CA). T-cell lines and hybridomas were analyzed for the expression of Leu 1-4 T-cell-specific antigens by using these monoclonal antibodies in combination with a cytofluorograph (FACS III; Becton-Dickinson FACS Systems) as described (15).

## RESULTS

In initial experiments, the CEMT3 line was fused with Con A-activated normal T cells. After 6 wk of continuous culture in HAT medium, cell growth was observed in approximately 5% of wells. Chromosomal analysis of these cells revealed 60-80

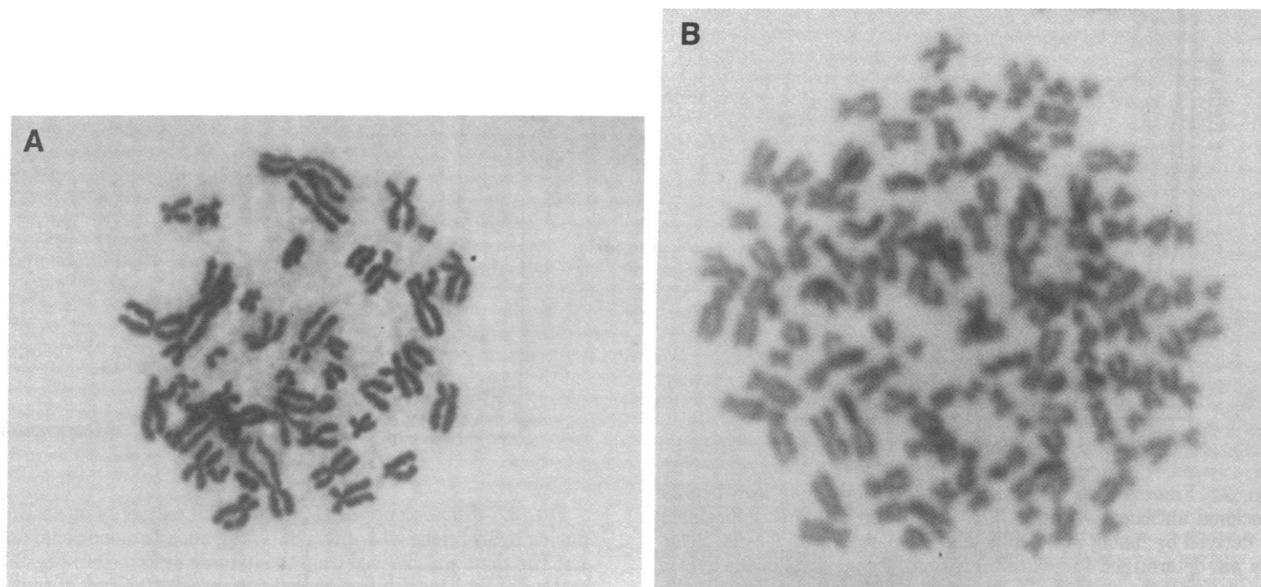


FIG. 1. Photomicrographs of chromosome preparations of J3R7 cells (A) and a typical T-T hybridoma (B). ( $\times 400$ .)

chromosomes compared with 40–46 in the CEMT3 line. Analysis of the cell surface by indirect immunofluorescence using monoclonal antibodies revealed the presence on 90% of hybrid cells of the Leu-4 T-cell marker, which was absent from the CEMT3 line. Seven of 24 supernatants from different lines secreted IL-2, based on the ability of supernatants from these lines to support the growth of IL-2-dependent murine and human T cells (data not shown). Although growth of these lines was maintained for an additional 6 months, the doubling time was slow (5 days), and five of seven lines stopped secreting IL-2 after 3 months. Analysis of these lines showed a significant reduction in the number of chromosomes—from a mean of 80 at 6 wk to a mean of 60 at 16 wk. Although multiple attempts to isolate a secreting hybrid clone by the limiting-dilution technique resulted in growing cells, no IL-2-secreting clones were obtained.

To address the problem of instability of lines, we developed a SGua-resistant T-cell line, J3R7, derived from the Jurkat human cell line. This line grew more rapidly than CEMT3; however, in the presence of thymidine, the growth rate of both lines slowed by 60–90% (data not shown). In preliminary experiments, T-T hybrids were produced with the J3R7 line and mitogen-activated T cells and, although the doubling time of these hybrids was less than those of CEMT3-derived hybrids (3 days compared with 5 days), the frequency of hybrids was not significantly greater (data not shown). On this basis, a selection agent was sought that avoided the inhibitory effects of thymidine.

Azaserine, a diazo analog of L-glutamine, is one such agent (17) and, in combination with hypoxanthine (AH medium) offers an alternative to HAT medium. To compare the HAT and AH selection media, T cells were cultured for 24 hr with PHA, fused in the usual manner with J3R7 cells, and distributed equally in HAT or AH medium. Hybrids appeared approximately 20 days earlier and were 4 times more frequent in AH medium than in

HAT medium (Table 1). Cells growing in AH medium had a doubling time of approximately 48 hr compared with 72–120 hr for cells growing in HAT medium. As shown in Fig. 1, analysis of growing hybrid cells revealed 70–90 chromosomes compared with 40–45 in J3R7 cells. Expression of the Leu-3a marker (Figs. 2 and 3), and the presence of HLA-A and -B antigens of both J3R7 and the normal T-cell partner confirmed hybridization. Thus, the Jurkat line has HLA-A3, -Aw32; -B8, -B13, -Bw4 and the normal donor has HLA-A1, -A3; -B8, -Bw45, -Bw6. The hybrid cells have HLA-A1, -A3, -Aw32; -B8, -B13, -Bw45, -Bw6. Unlike the Leu-3 and HLA-A, -B antigens, the Leu-1 and Leu-4 pan-T markers were expressed by the J3R7 line and all J3R7-derived hybrids and, hence, were not informative.

Although the J3R7 cells did not produce IL-2, 4 of 20 hybrids tested secreted IL-2 in the concentration range of the MLA 144 gibbon line. As shown in Fig. 4, the titer of IL-2 in one supernatant of a J3R7-derived hybrid is slightly less than that of the MLA-144 gibbon cell line. After 16 wk of continuous culture, there has been no detectable chromosomal loss or decrease in IL-2 activity in the supernatants of parent hybrids from J3R7 fusions; 12 wk after fusion, clones were obtained by the limiting-dilution method. In contrast to our experience with hybrids derived from CEMT3 cells, clones obtained from J3R7-derived hybrids continued to secrete IL-2 up to the time of submission of this report (Table 1, Fig. 5). As shown in Fig. 5, the IL-2 activity from some clones exceeded that obtained from parent cultures. The cloning efficiency was approximately 50% at a limiting-dilution level of one-half cell per well (Table 1).

## DISCUSSION

We have demonstrated the production of T-T hybridomas secreting IL-2 from the fusion of activated normal T cells with two mutant T-cell lines, CEMT3 and J3R7. In the initial fusions with CEMT3, a major problem was slow growth rate, probably due to the intrinsically slow growth rate of CEMT3 and the inhib-

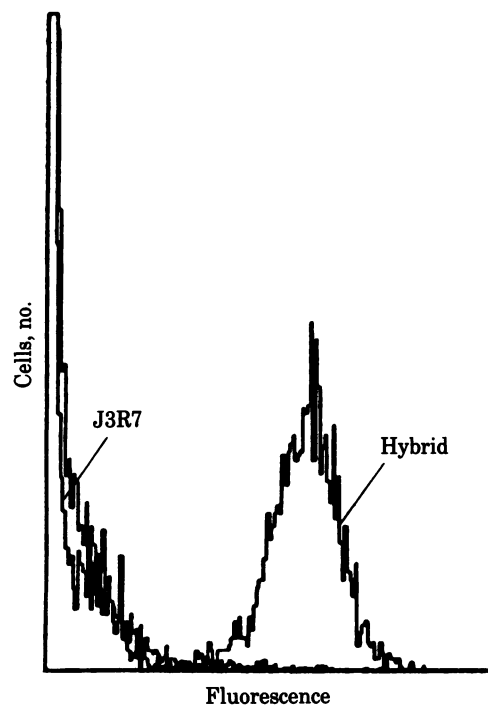


FIG. 2. Fluorescence histograms of the reactivity of anti-Leu-3a monoclonal antibody with the J3R7 T-cell line and with hybridoma cells derived by fusing J3R7 cells with PHA-activated T cells. Reactivity was determined 4 weeks after fusion. The majority of the hybrid cells are brightly stained. By contrast, J3R7 cells are minimally stained and fluorescence did not exceed that obtained with normal mouse serum (not shown).

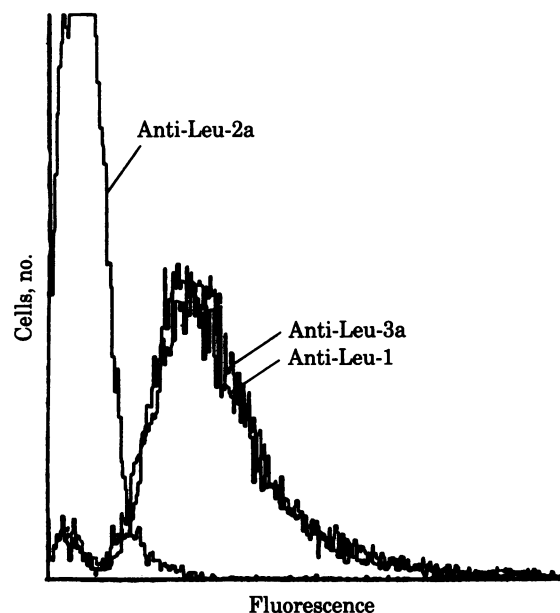


FIG. 3. Fluorescence histograms of the reactivity of an IL-2-producing hybrid clone with anti-Leu-1, -2a, and -3a monoclonal antibodies. The clone was derived from the hybrids described in Fig. 2 by the method of limiting dilution and was analyzed after 4 wk of continuous culture. At least 95% of the cells express the Leu-1 and Leu-3 antigens whereas no cells express detectable amounts of Leu-2.

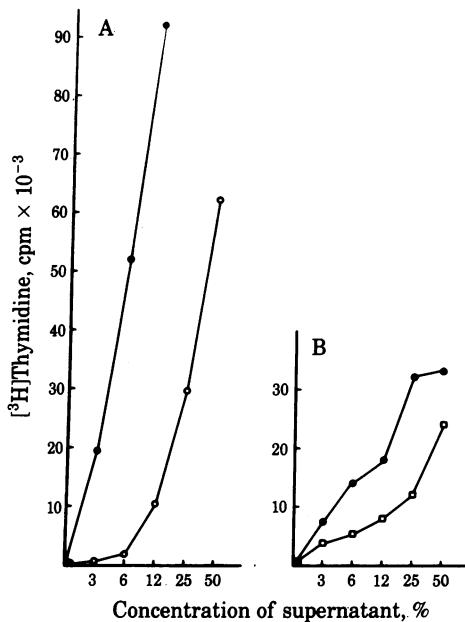


FIG. 4. Growth of IL-2-dependent T cells in supernatants from a J3R7-derived human T-T hybridoma. Approximately  $1 \times 10^4$  IL-2-dependent HT-2 murine (A) or human T (B) cells were suspended in supernatants from a human T-T hybrid (J3R-A11;  $\circ$ ) and a gibbon T-leukemia line (MLA 144 cm;  $\bullet$ ). The cells were cultured for 24 hr and pulsed for 4 hr with [<sup>3</sup>H]thymidine (1  $\mu$ Ci per well) before harvesting. Results represent means of quadruplicate cultures.

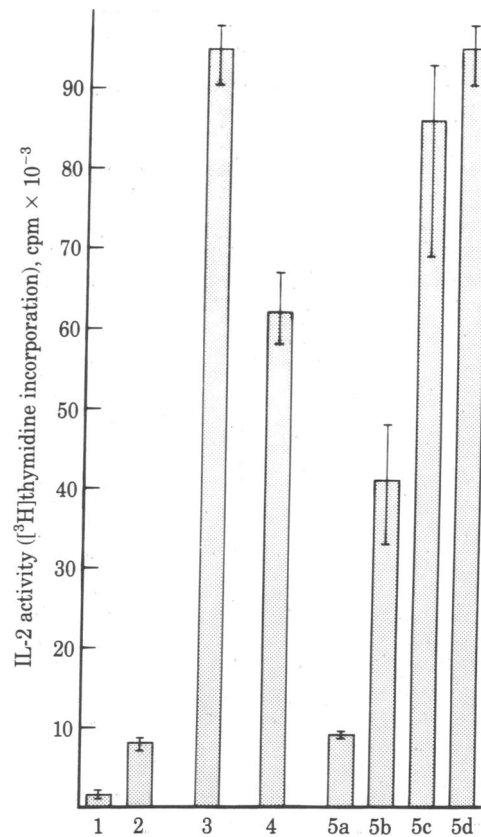


FIG. 5. Growth of IL-2-dependent T cells in supernatants from human T-T hybridomas. Supernatants from wells containing a 4-month-old parent culture of human T-T hybrids (bar 4), multiple monoclonal T-T hybrids (bars 5a–5d), a gibbon T-leukemia line (bar 3), PHA-activated normal T cells (bar 2), and the J3R7 line (bar 1) are compared. IL-2-dependent murine (HT-2) cells ( $1 \times 10^4$ ) were suspended in these supernatants, cultured for 24 hr, and pulsed for 4 hr with [<sup>3</sup>H]thymidine (1  $\mu$ Ci per well) before harvesting. Results represent means of quadruplicate cultures.

itory effect of thymidine. A second problem was progressive loss of functional activity. After 6 months of continuous culture and multiple attempts to isolate functional hybrid clones from CEMT3 fusions, a major portion of hybrids that secreted IL-2 were lost. This was attributed to chromosomal instability as reflected by a reduction in the number of chromosomes.

These problems were approached first with the development of a different SGua-resistant T cell line, J3R7, which has a doubling time of 24 hr. Second, a different selection medium was used in the later fusions with J3R7, which avoided the inhibitory effects of thymidine. Most mammalian cells have two pathways to synthesize DNA, the *de novo* pathway, which is dependent on folic acid as a coenzyme, and the salvage pathway, which is dependent on the enzyme hypoxanthine phosphoribosyltransferase (HPRT). Mutant tumor lines that are HPRT deficient depend on the *de novo* pathway for survival. In the conventional HAT selection medium, such HPRT-deficient tumor cells will die because the aminopterin blocks *de novo* nucleotide synthesis. Hybrid cells will survive if they have acquired the enzyme HPRT from the genes of the normal cell and are able to utilize the salvage pathway in HAT selection. Aminopterin blocks both purine and pyrimidine synthesis and, consequently, hypoxanthine and thymidine are necessary for hybrid cell survival.

Azaserine is an alkylating agent produced by the bacterium *Streptomyces fragilis* (16). It is a diazo analog of L-glutamine and, more importantly, its main action is irreversible binding to various L-glutamine amidotransferases that are necessary in *de novo* purine synthesis. Its effect on pyrimidine synthesis is minimal. Therefore, in a selection medium containing azaserine only, hypoxanthine is necessary for the salvage pathway whereas exogenous thymidine is not. Thus, the problem of thymidine inhibition is avoided with this selection method. In preliminary studies (data not shown), this system was tried with a murine B-B fusion and was successful in selecting murine hybrids. In

the human system, T-T hybrids have been produced with the mutant T-cell line J3R7. AH selection resulted in earlier appearance and greater number of hybrids (Table 1). The earlier appearance of hybrids in the J3R7 fusion in comparison with fusions with CEMT3 may also be influenced by the more rapid growth rate of the J3R7 mutant line.

After 4 months of continuous culture, there has been no detectable chromosomal loss or decrease in IL-2 activity in the supernatants from the J3R7-derived hybrids. Although the question of long-term stability of these hybrids requires continued surveillance, the use of AH selection appears to offer significant advantages over the traditional HAT medium in the selection of T-T hybrids. We believe that the approach described here should allow sufficient production of lymphokines for *in vitro* and *in vivo* studies of their roles in immunoregulation.

We thank Drs. A. Raubitschek and J. Larrick for helpful discussions. This work was supported in part by Grant CA24607 from the National Institutes of Health and a grant from the Cetus Corporation, Berkeley, CA.

1. Rocklin, R. E., Bendtzen, K. & Greinder, D. (1980) *Adv. Immunol.* 29, 55–136.
2. Bloom, B. R. (1971) *Adv. Immunol.* 13, 101–208.
3. Farrar, J. J., Benjamin, W. R., Hilfiker, M. L., Howard, M., Farrar, W. L. & Fuller-Farrar, J. (1982) *Immunol. Rev.* 63, 129–166.
4. Germain, R. N. & Benacerraf, B. (1981) *Scan. J. Immunol.* 13, 1–10.

5. Fathman, C. G. & Fitch, F. W., eds. (1982) *Isolation, Characterization, and Utilization of T Lymphocyte Clones* (Academic, New York).
6. Kapp, J. A., Araneo, B. A. & Clevinger, B. L. (1980) *J. Exp. Med.* **152**, 235–240.
7. Grillot-Courvalin, C., Brouet, J. C., Berger, R. & Berhnheim, A. (1981) *Nature (London)* **292**, 844.
8. Green, W. C., Fleisher, T. A. & Waldmann, T. A. (1981) *Clin. Res.* **29**, 368A.
9. Irrigoyen, O., Rizzolo, P., Thomas, Y., Rogozinski, L. & Chess, L. (1981) *J. Exp. Med.* **154**, 1827–1837.
10. Okada, M., Yoshimura, N., Kaieda, T., Yamamura, Y. & Kishimoto, T. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 7717–7721.
11. Fox, R., Tripp, E. & Tattersall, M. (1980) *Cancer Res.* **40**, 1718–1721.
12. Boyum, A. (1968) *Scand. J. Clin. Lab. Invest. Engl. Suppl.* **97**, 51–76.
13. Gillis, S., Ferm, M., Ou, W. & Smith, K. (1978) *J. Immunol.* **120**, 2027–2032.
14. Rabin, H., Hopkins, R., Ruscetti, F., Neubauer, R., Brown, R. & Kawakami, T. (1981) *J. Immunol.* **127**, 1852–1856.
15. Engleman, E. G., Benike, C. J., Grumet, F. C. & Evans, R. L. (1981) *J. Immunol.* **127**, 2124–2129.
16. Livingston, R., Venditti, J., Cooney, D. & Carter, S. (1970) *Adv. Pharm. Chemother.* **8**, 57–69.