T lymphocytes represent an extraordinarily heterogeneous population of cells with respect to antigen receptors, differentiation antigens, and distinct immunologic functions. Because of this heterogeneity, it is of obvious advantage in many studies to use restricted and homogeneous T cell populations. Recently, two approaches to isolate homogeneous clones of T lymphocytes have become feasible. One of them takes advantage of the observation that T lymphocytes can be maintained and propagated in vitro by culture in media containing the T cell growth factor interleukin 2 (1). Although this technique allows for the growth of clones of T cells with distinct helper, suppressor, or cytotoxic properties (2-4), the clones remain absolutely dependent on interleukin 2, and large numbers of cells are difficult to generate. The second approach uses techniques similar in principle to those defined by Kohler and Milstein (5) for the production of monoclonal antibodies. This technology has been developed in murine systems where a number of investigators have created T cell hybrids by fusing normal murine T cells with continuously growing, aminopterin-sensitive murine T lymphoma cell lines. Some of these hybrids express phenotypic properties of both parental cells and, in many instances, they have exhibited functional properties of the mature fusion partners (6-13).

In the experiments to be reported below, we have extended this latter technology to human T cells. First, we established 6-thioguanine (TG)-resistant, aminopterin-sensitive mutants from the continuously growing human T cell line CEM. One of these mutant T cell lines (CEM-T15) was fused with lectin-stimulated human T cells and, after selection in medium containing hypoxanthine, aminopterin, and thymidine (HAT), T cell hybrids were obtained. These human T cell hybrids grow independently from any added T cell growth factor(s). Some of these hybrids express the OKT3 antigen that is found on normal T cells but not on immature thymocytes or on the CEM-T15 mutant line (14). Functional analysis demonstrated that many of these human T cell hybrids exhibit helper function in vitro. Several of these helper T cell hybrids have been cloned repeatedly and maintain helper function.

Materials and Methods

**Generation of TG-resistant Mutants.** To increase the frequency of mutants, the human T lymphoblastoid line CEM was exposed to the mutagen ethyl methanesulfonate (EMS, Sigma

* Supported in part by grants AI 14969 and 11524 from the National Institutes of Health and by the Arthritis Foundation.

1 Abbreviations used in this paper: EMS, ethyl methanesulfonate; HAT, hypoxanthine-aminopterin-thymidine; HGPRT, hypoxanthine guanine phosphoribosyl transferase; IMDM, Iscove's modified Dulbecco's medium; PEG, polyethylene glycol; PFC, plaque-forming cells; PHA, phytohemagglutinin; PWM, pokeweed mitogen; TG, 6-thioguanine.
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Chemical Co., St. Louis, Mo.) (15). Thus, CEM cells growing in exponential phase at a concentration of $5 \times 10^6$ cells/ml were cultured with EMS at concentrations ranging from 5 to 160 $\mu$g/ml. The cultures were carried out for 16 h in a humidified 95% air, 5% CO$_2$ incubator at 37°C. Only 20% of the cells incubated with 5 and 10 $\mu$g/ml survived the treatment. At higher concentrations of EMS there were no surviving cells. The viable cells were washed, resuspended in final medium containing 7.5 $\times$ 10$^{-6}$ M TG, and plated in microwells. Final medium consisted of Iscove’s Modified Dulbecco’s Medium (IMDM) medium (Gibco Laboratories, Grand Island Biological Co., Grand Island, N. Y.) with 20% horse serum (Flow Laboratories, Rockville, Md.) and 50 $\mu$g/ml of chlortetracycline (Gibco Laboratories). Approximately 60% of the wells showed evidence of growth in this initial concentration of TG. 16 of those cultures were expanded to 25-cm$^2$ flasks and the concentration of TG was increased to 3 $\times$ 10$^{-5}$ M. Only 13 of the cultures survived. One of them, CEM-T15, was selected because of excellent growth characteristics. The rest were frozen and stored in a liquid nitrogen freezer. The CEM-T15 has a doubling time of ~22 h and has been steadily growing for >40 wk. At present, it is growing in medium containing 10$^{-4}$ M TG.

Lymphocyte Preparation and Fractionation. Fresh peripheral blood mononuclear cells were isolated from healthy human volunteers by Ficoll-diatrizoate density gradient centrifugation. Highly enriched populations of T and B lymphocytes were obtained by E rosetting and passage through a Sephadex G-200 rabbit anti-human F(ab')$_2$ column, as described (16). The B cells were treated with the monoclonal antibody OKT3 plus complement in order to eliminate residual T cells. Alternatively, B cells were obtained from tonsillar lymphocytes by E rosetting and subsequent treatment of the E$^+$ population with OKT3 antibody plus complement. The OKT4$^+$ cells were obtained by negative selection using complement-mediated lysis of the T cell population by the OKT8 monoclonal antibody as previously described (17).

Fusion Procedure. Human T lymphocytes at a concentration of $2 \times 10^6$ cells/ml were incubated with either 10 $\mu$g/ml of phytohemmaglutinin (PHA, Gibco Laboratories); 5 $\mu$g/ml of pokeweed mitogen (PWM, Gibco Laboratories); or 2 $\mu$g/ml of concanavalin A (Con A, Miles Laboratories Inc., Elkhart, Ind.) in a 5% CO$_2$ humidified atmosphere at 37°C for different lengths of time (see Results). These activated T cells were fused with the TG-resistant CEM-T15 mutant using either of two fusion techniques. I. Activated T cells and CEM-T15 mutants were mixed at ratios of 10:1 to 2:1. The mixture was centrifuged and the pellet resuspended over a period of 2 min in 1 ml of polyethylene glycol solution (PEG, J. T. Baker Chemical Co., Phillipsburg, N. J.). The suspension was then gradually diluted to 50 ml over the course of 10 min and centrifuged at 250 g for 10 min (6, 18). II. The pelleted cell mixture was resuspended in PEG and immediately spun down for 3 min at 580 g. Without removing the PEG, the cells were resuspended in 5 ml of medium and centrifuged at 250 g for 10 min (19). This resulted in a longer time of exposure to PEG than in technique I (8 vs. 2 min). Both procedures were performed at 37°C with prewarmed, serum-free IMDM. Solutions of PEG at different concentrations were adjusted to pH 8.2 immediately before use (20). After fusion by either technique, the cells were washed twice and resuspended in final medium containing HAT at various concentrations (see Results). The cells were cultured in microtiter plates at a concentration of $4 \times 10^5$ to $10^6$ mutant cells/well in 0.2 ml. The cultures were maintained at 37°C in a humidified 95% air, 5% CO$_2$ incubator and were fed every 5–6 d.

Surface Antigens Analysis. Phenotypic cell surface analysis was performed by indirect immunofluorescence using the monoclonal antibody OKT3 and a fluorescein-conjugated goat anti-mouse F(ab')$_2$ antibody (N. L. Cappel Laboratories Inc., Cochranville, Pa.). The cells were analyzed on a cytofluorograf (model 30-H, Ortho Instruments, Westwood, Mass.) as previously described (21). A cell population was considered positive if >20% of the cells expressed the OKT3 antigen. All experiments included a negative ascites control.

Chromosome Analysis. Chromosome preparations were performed according to standard techniques (22). Briefly, $4 \times 10^6$ exponentially growing cells were cultured with 0.01 pg/ml of Colcemid (Gibco Laboratories). After 2 or 3 h incubation, the cells were centrifuged and treated with 0.075 M potassium chloride for 12 min and then fixed with methanol/glacial acetic acid, 3:1. Drops of the fixed cell suspension were allowed to dry on slides and were stained with Giemsa for microscopic examination.

Polyclonal Induction of Antibody-secreting Cells. The culture conditions and the reverse hemolytic
plaque assay for the assessment of induction and regulation of plaque-forming cell (PFC) generation was previously described in detail (17). In brief, $10^8$ B cells were suspended in 1 ml of final medium containing 5 μg of PWM and were cultured in 16 × 150-mm tissue culture tubes. To these tubes were added $10^6$ of either normal T cells or the different cell hybrids to be tested. Control cultures consisted of B cells cultured alone or in the presence of PWM without added T cells. All cultures were incubated for 5-6 d in a humidified 5% CO₂ incubator at 37°C, and then assayed for antibody production using the reverse hemolytic plaque assay as described (17). The results are expressed as PFC per $10^9$ B cells at the beginning of culture.

Results

Differential Growth of CEM vs. CEM-T15 in Selective Media. The TG-resistant mutant CEM-T15 was compared with the parent line CEM for growth in medium containing $3 \times 10^{-8}$ M TG (Fig. 1). As expected, CEM-T15 maintained vigorous growth in TG medium, whereas CEM died within 24-48 h. These results demonstrate that, relative to the parental line CEM, CEM-T15 is unable to incorporate TG. These data suggest that CEM-T15 is deficient in hypoxanthine-guanine phosphoribosyl transferase (HGPRT). This was further tested by directly comparing the mutant and parental CEM lines for growth in HAT medium. Under conditions of aminopterin blockade of the de novo purine synthetic pathways, only those cells with HGPRT activity will be able to incorporate hypoxanthine and, therefore, survive. In these experiments, the cells were grown in graded concentrations of aminopterin while the concentrations of hypoxanthine ($10^{-4}$ M) and thymidine ($3 \times 10^{-5}$) were kept constant. As shown in Fig. 2, at an aminopterin concentration of $10^{-8}$ M both cell lines grow normally, whereas at a concentration of $10^{-7}$ M both cell lines rapidly die. Importantly, however, a differential growth was observed at aminopterin concentrations of $3.3 \times 10^{-8}$ and $6.6 \times 10^{-8}$ M. Under these conditions, CEM-T15 cells die rapidly (within 1-4 d), whereas CEM cells survive at least 7-10 d. These data, taken together with the differential growth in TG medium, confirm that CEM-T15 relative to CEM is deficient in HGPRT activity.

Hybridization of Activated Human T Cells with the Aminopterin-sensitive Human Lympho-
blastoid T Cell Line CEM-T15. The differential aminopterin sensitivity of CEM-T15 shown in the preceding experiments suggested that this mutant would be a suitable partner for fusion in hybridization procedures. Fusions of lectin-activated normal and leukemic T cells with CEM-T15 cells were performed under different experimental conditions. To date, 15 fusions successfully generated cultures containing rapidly dividing cells (Table I). In these fusion experiments the highest number of wells with growing cells was obtained when the fusions were performed according to technique II using PEG 1,000 mol wt at 50% concentration. A single fusion with PEG 6,000 mol wt at 40% concentration using technique I also yielded a high number of growing cultures. The chosen HAT concentrations and lengths of exposure of the fused cells to HAT were derived from experiments comparing CEM and CEM-T15 for survival under different concentrations and lengths of incubation in HAT medium (Fig. 2). We would emphasize that using technique II with 50% PEG 1,000 mol wt as the fusing agent, successful hybridization with a high number of growing wells was obtained in all experiments.

Since growth of cells in HAT medium following fusion does not necessarily imply successful hybridization, we randomly screened growing wells from all 15 fusions for the presence of cells expressing the OKT3 surface antigen. This screening strategy was selected because, in contrast to normal T cells or the leukemic T cells used, CEM-T15 cells lack the OKT3 antigen. As shown in Table I, a significant proportion of cultures growing 5–7 wk after fusion contain OKT3-positive cells. The data strongly suggest that the growing cells represent T cell hybrids since CEM-T15 does not express OKT3 and the majority of normal or leukemic T cells would not be rapidly dividing after 5–7 wk in culture.

**Table I**

| Fusion number | Activated parental T cell | PEG mol wt | Concentration | Fusion technique* | Viability after fusion§ | Wells seeded | Wells growing¶ | Wells with OKT3+ cells||
|---------------|---------------------------|------------|---------------|-------------------|------------------------|-------------|---------------|-----------------|
| 8             | Normal                    | PWM        | 8 d           | 1,000 35         | II >75 3.3 x 10^4     | 7 98 4 0     |               |                 |
| 22            | Normal                    | PHA        | 4 d           | 1,000 35         | I >75 3.3 x 10^4      | 5 180 16 20  |
| 23            | Normal                    | PHA        | 4 d           | 6,000 40         | I >75 3.3 x 10^4      | 5 240 12 40  |
| 24            | Normal                    | PHA        | 4 d           | 1,000 35         | I >75 3.3 x 10^4      | 5 240 2.5 0  |
| 25            | Normal                    | PHA        | 4 d           | 1,000 35         | II >75 3.3 x 10^4     | 5 240 >83 25 |
| 26            | Normal                    | PHA        | 4 d           | 1,000 35         | I >75 3.3 x 10^4      | 5 240 >83 55 |
| 27            | Normal                    | PHA        | 4 d           | 1,000 35         | II >75 3.3 x 10^4     | 5 240 >83 60 |
| 36            | Normal                    | Con A      | 2 d           | 1,000 40         | I >75 2.0 x 10^4      | 14 300 0 0  |
| 37            | Normal                    | PHA        | 4 d           | 1,000 40         | I >75 2.0 x 10^4      | 14 300 5 0  |
| 38            | Normal                    | PHA        | 4 d           | 1,000 40         | I >75 2.0 x 10^4      | 14 300 0 0  |
| 43            | Leukemic                  | PHA        | 12 h          | 1,000 30         | II >75 3.3 x 10^4     | 7 158 41 23 |
| 44            | Leukemic                  | PHA        | 12 h          | 1,000 30         | II >75 3.3 x 10^4     | 9 98 23 27  |
| 45            | Leukemic                  | PHA        | 12 h          | 1,000 30         | II >75 6.6 x 10^4     | 5 98 12 46  |
| 46            | Leukemic                  | PHA        | 6 h           | 1,000 30         | II >75 3.3 x 10^4     | 7 130 45 31 |
| 47            | Leukemic                  | PHA        | 6 h           | 1,000 30         | II >75 3.3 x 10^4     | 9 158 27 29 |
| 48            | Leukemic                  | PHA        | 6 h           | 1,000 30         | II >75 6.6 x 10^4     | 5 98 13 45  |

* See Methods.
§ Assessed by trypan blue exclusion.
¶ Assessed 5 wk after fusion.
† Analyzed by immunofluorescence in a cytofluorograf 5–7 wk after fusion.
To obtain further evidence for hybridization, samples from fusions 23-27 were also analyzed for chromosome number. When screened 10 wk after fusion, 90% of these cultures contained polyploid cells (range: 60-95 chromosomes), whereas the CEM and CEM-T15 lines contained diploid cells (range: 35-47 chromosomes). Fig. 3 shows a representative chromosomal spread from fusion 27. These results, taken together with the phenotypic analysis of OKT3, strongly suggest that the majority of the cultures from many of these fusions contain hybrid cells derived from the fusion of normal T cells with mutant CEM-T15 cells.

**Helper Function Analysis of Human T Cell Hybrids.** To determine whether these growing T cell hybrids maintained functions known to be present in the normal mature parent T cells, cultures from fusions 26 and 27 were analyzed for polyclonal induction of antibody production by B cells. In these experiments, growing hybrids were added to purified B cells in the presence of PWM, and PFC activity was measured 6 d later. Controls included normal helper T cells (OKT4+) as well as CEM and CEM-T15 mutant cells. As shown in Fig. 4, CEM and the mutant CEM-T15 cells did not generate helper activity. Furthermore, CEM-T15 inhibits the background level of PFC generated in cultures containing B cells and PWM. In marked contrast, several different hybrid cultures from both fusions expressed helper activity.

**Stability and Cloning of Human Helper T Cell Hybrids.** To assess the functional stability of the helper T cell hybrids, four hybrid cultures were periodically tested for helper activity over 20 wk of continuous culture (Table II). As can be seen, these uncloned hybrid cultures maintained helper activity for 13 wk. However, by 20 wk the majority of the cultures had lost function. This loss of activity could be secondary to either overgrowth by nonfunctional cells or, alternatively, by functional instability of a fraction of the cells. To address these possibilities the functional hybrids were cloned at limiting dilution early during culture (as soon as helper function could be

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**Fig. 3.** Representative chromosomal spreads from (A) CEM-T15 TG-resistant human T cell line; (B) 27.47 human T cell hybrid.
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Fig. 4. Human T cell hybrids with helper function in the polyclonal induction of antibody production by B cells. Each bar represents an individual T cell hybrid (10^5 cells) cultured with B cells and PWM. After 6 d, the cultures were harvested and assayed for PFC. The negative controls included B cells cultured with PWM in the absence of T cells or with PWM in the presence of CEM-T15 or CEM cell lines. The positive control consisted of B cells cultured with PWM in the presence of OKT4^+ T cells.

Fig. 6. Helper activity of the human T cell hybrid clone 436. Graded numbers of 436 cells were added to B cells and PWM. After 6 d, the cultures were harvested and assayed for PFC. The negative control consisted of B cells cultured with PWM in the absence of hybrid cells.

**Table II**

*Functional Assessment of Human T Cell Hybrids*

| Cells added to B cells and PWM | PFC per 10^6 B cells | Weeks after fusion |
|-------------------------------|----------------------|-------------------|
|                               |                      | 6  | 7  | 8  | 13 | 20 |
| None                          |                      | 75 | 380| 15 | 520| 150|
| CEM-T15                       |                      | 0  | 60 | 30 | 0  | 90 |
| 26.17                         |                      | 410| 930| 530| 2,640| 20 |
| 26.23                         |                      | 700| 460| ND*| 920| 130|
| 26.59                         |                      | 830| 750| 480| 1,240| 105|
| 27.02                         |                      | 440| 330| 700| 1,630| 440|

* Not done.

demonstrated) and functional clones were subsequently recloned (Table III). Every clone was given a number which followed the number of the culture of origin. For example, the hybrid 26.17 was cloned by limiting dilution in microtiter plates at a concentration of one cell every five wells. After 3 wk, all of the growing cells (23% of the total) were tested for helper activity. As seen in Table III, 9% had helper activity. One of these clones, designated 26.17.115, was recloned by limiting dilution as described above. After 4 wk, 13% of the wells contained growing cultures. In contrast with the relatively low percentage of helper cultures after the first cloning procedure, recloning of a positive culture resulted in 62% of clones with helper activity. The results of these cloning experiments suggest that the frequency of helper hybrids and therefore functional stability increases as cloning proceeds.

Interestingly, when the chromosome number was reassessed at 20 wk, many of these hybrids and their clones had a predominantly diploid karyotype, with some of them having only a small percentage of polyploid cells. This indicates that despite significant
loss of chromosomes during the first few months in culture, many of the diploid clones have retained genes encoding for helper activity. One example is a clone from 26.17.115, designated 436, which was chosen for further analysis. The clone 436 cells were evaluated for chromosome number, reactivity with OKT3 and helper function (Figs. 5 and 6). This clone has 46 chromosomes and is OKT3⁺. Furthermore, functional activity was maintained since graded numbers of 436 T cell hybrids added to B cells plus PWM induce a PFC response (Fig. 6). Taken together, these data indicate that functional stability of these human T cell hybrids can be preserved and consolidated by early and repeated cloning of the relevant hybrids.

Discussion

In this report we demonstrate that human T cell hybrids can be created by fusion of normal, lectin-activated human T cells with a TG-resistant, aminopterin-sensitive human T cell line. Our strategy was to (a) derive a variety of TG-resistant, aminopterin-sensitive mutants from the continuously growing T cell line CEM; (b) fuse those mutants with lectin-activated OKT3⁺ human T cells; and (c) select rapidly growing cultures that had survived HAT treatment for expression of the OKT3 differentiation antigen and for their capacity to polyclonally induce B cell differentiation.

It is of interest that, under our culture conditions, we did not observe the reported growth inhibition of CEM by thymidine (23, 24). More important, the comparison of the CEM line with one of its TG-resistant mutants, CEM-T15, for growth in aminopterin-containing medium showed a narrow, although clear difference in aminopterin susceptibility. By carefully selecting appropriate combinations of aminopterin concentration and length of exposure, we were able to minimize the possibility of outgrowth of CEM-T15 cells yet allow the recently created hybrids to grow in HAT medium. Because of our interest in obtaining functional T cell hybrids and also in discarding any surviving CEM-T15 cells, our initial screening strategy selected for cells expressing the OKT3 differentiation antigen. This surface antigen is not expressed on non-T cells nor on the CEM-T15 mutant line, but it is expressed on 85–95% of mature T cells and on virtually all normal helper T cells. This screening
was done 5 wk after fusion, at which time the possibility of survival of normal peripheral T cells is highly unlikely. Thus, most of the growing cells expressing the OKT3 antigen most likely represent hybrids between an OKT3-bearing T cell and a CEM-T15 mutant cell that has permanent growth potential but does not express OKT3. Evidence for hybridization was also obtained from chromosomal analysis after 10 wk of culture, which demonstrated that most of the cultures contained polyploid cells.

Additional evidence for successful hybridization resulted from functional analysis of several of these cultures. Our results clearly demonstrate that a proportion of the cultures contain helper cells. Thus, in the two separate fusions tested, human T cell hybrids were generated that were capable of inducing B cell differentiation into antibody-producing cells. The helper activity generated by these hybrids is even more striking in light of the fact that the parental mutant line CEM-T15 does not provide helper activity and, indeed, consistently inhibits B cell differentiation (Fig. 3 and O. Irigoyen et al., manuscript in preparation). We infer that following hybridization, genes coding for helper activity or, alternatively, for inactivation of inhibitory
mechanisms have been transferred from the normal T cell into the genome of the
CEM-T15 mutant.

The functional stability of these helper T cell hybrids was assessed both by periodic
testing for helper function and, more importantly, by repeated cloning of functioning
hybrids. An important point to emerge from these studies is that early and repeated
cloning is important for the preservation of functional stable hybrids. For example,
uncloned functional hybrids begin to lose activity after 13 wk of continuous culture.
In contrast, clones derived from these same hybrids early in culture give rise to
progenies with stable functional activity. Interestingly, functional stability is main-
tained despite significant loss of chromosomes after 10 wk of culture. For example, the
diploid clone 436 has been in culture for >21 wk and still expresses the OKT3 surface
antigen and maintains high levels of helper activity.

In summary, these experiments demonstrate that functional human T cell hybrids
can be created by fusion of normal PHA-activated T cells with CEM-T15 mutant
cells. These functional hybrids can be repeatedly cloned and maintain stable func-
tional activity. Experiments are now ongoing to determine whether antigen-activated
human T cells can be fused with CEM-T15 in an analogous manner to construct
antigen-specific human T cell hybrids with distinct functional activities. These cells
and their products will be of considerable interest with respect to further studies of
the immunobiology of human T cells.

Summary

Human T cell hybrids were generated by fusing lectin-activated normal and
leukemic human T cells with an aminopterin-sensitive human T cell line. This mutant
cell line, designated CEM-T15, was derived from the human T cell line CEM after
chemical mutagenesis with ethane methylsulfonate and subsequent culture in medium
containing 6-thioguanine. After polyethylene glycol-induced fusion, the cells were
cultured in hypoxanthine-aminopterin-thymidine selective medium. More than 5 wk
after fusion, evidence for successful hybridization was obtained by three independent
criteria: (a) The majority of the cultures contained cells expressing the OKT3 surface
antigen: this antigen is expressed on normal T cells but not on CEM-T15 cells. (b)
Most of the cultures contained polyploid cells. (c) Some of the cultures provided
helper activity in the generation of antibody-forming cells. This functional activity is
absent from the CEM-T15 parental cell line. Evidence for functional stability of the
hybrids >20 wk after fusion was provided by several clones that not only continue
growing exponentially but also maintain expression of OKT3 surface antigen and
high levels of helper function. These T cell hybrids and similar hybrids constructed
using antigen-specific human T cells should be of considerable importance in further
studies of the immunobiology of human T cells.

Received for publication 15 June 1981.

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