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Studies of Cultured Human T Lymphocytes (41558)
II. Initiation of Paired T- and B-Cell Lines from Healthy Donors

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Abstract. We report the sustained cultivation of both B- and T-lymphoblastoid cell lines from randomly selected healthy donors, and the results of studies defining the frequency with which these cell lines can be established. B-cell lines were initiated using the Epstein–Barr virus. Of 52 attempts, 40 B-cell lines (77% success) were obtained from 24 different donors. T-cell lines were started and propagated in long-term (>100 days) cultures using the T-cell growth factor interleukin-2 (IL-2). Of 55 attempts, 54 (98%) were successful in initiating IL-2-dependent T-cell lines, and these were derived from 28 healthy adults. Likewise, of 45 attempts, 32 (71%) were successful in producing paired lines in which both the B-cell line and T-cell line were cultivated from a single blood collection (N = 22 donors). Phenotypic profiles of these lines were defined using multiple marker assays, including rosette formation, surface immunoglobulins, cytochemistry, karyotype, as well as xenoantisera and monoclonal antibodies defining different membrane antigens. This work demonstrates the feasibility of propagating paired human B and T lymphoblastoid lines suitable for many comparative immunobiological studies.

During the last few years, several groups have reported the prolonged propagation of T cells from the blood and bone marrow of healthy human donors [reviewed in (1–4)]. The proliferation of these immunocompetent T cells, first described in 1976 (5) is dependent on a hormone-like T-cell growth factor present in the supernatant of phytohemagglutinin (PHA)- or antigen-activated short-term (24–72 hr) cultures of blood lymphocytes pooled from several healthy subjects. This factor, defined as interleukin-2 (IL-2) in the revised nomenclature of lymphokines (6), has been isolated and partially purified (7–11). IL-2 exhibits biochemical and biological properties distinct from the macrophage-derived monokine (Interleukin-1, IL-1) known formerly as lymphocyte activating factor (5, 12, 13). Results of these studies, as well as experiments of murine T cells *in vitro* and *in vivo*, suggest that IL-1 and IL-2 provide the first and second signals, respectively, initiating T-cell mediated immune responses (1–4, 7, 9–11).

The dependency of these mature T cells on IL-2 for sustained replication is but one of several features whereby they can be distinguished from other cells of long-term human lymphoblastoid cultures including leukemic T cells of lines derived from children in relapse of their acute lymphoblastic leukemia and B cells of lines initiated from healthy individuals or patients with different malignant and nonmalignant diseases.

Reported herein are the results of studies utilizing IL-2 and Epstein–Barr virus (EBV) for initiating T- and B-lymphoblastoid cell lines, respectively, from a given donor. This study not only defines the efficiency of initiating human lymphoblastoid cell lines, but also, and more importantly, illustrates the feasibility of utilizing paired B- and T-cell lines for a variety of comparative immunobiological studies.

Materials and Methods. *Leukocyte donors.* Leukocytes used for initiating B- and T-cell lines, and for generating IL-2, were obtained from the blood of adult (age: 17 to 65 years) men and women who appeared to be in good health and who were consenting volunteers of the Plasmapheresis Program at Roswell Park Memorial Institute (RPMI).

Initiation of B-cell lines. B-cell lines were initiated using blood leukocytes and the EBV-containing supernatant of the marmoset B-lymphoblastoid cell line B95.8 and procedures similar to those described previously (14). In brief, cells of the B95.8 line were maintained in log-phase growth using medium RPMI 1640 supplemented with L-glutamine (600 mg/liter), heat-inactivated (56°C,

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30 min) fetal calf serum (FCS; 10%), and PNS antibiotic mixture (1%; Grand Island Biological Co., Grand Island, N.Y., GIBCO). Supernatants used SN-B95.8) were freshly collected from static cultures (cell density, 1.0 to 1.3×10^6/ml; viability, >95%) that had been fasted for 4 days and were of low pH (~6.8 to 6.9). The cells were deposited by centrifugation (300*g*, 12 min); thereafter, the SN-B95.8 was filtered twice through micropore (0.45 μm) filters. Ficoll–Hypaque isolated blood leukocytes were then resuspended in RPMI 1640 containing 50% SN-B95.8 and 20% FCS to give a final mononuclear cell density of 0.5×10^6/ml. Twelve milliliters of this cell suspension was then added to 25-cm^2-style polystyrene flasks that were loosely capped and placed in an upright position in a 37°C incubator having a humidified atmosphere of 7% CO_2 and 93% air. Every 2–3 days thereafter, the spent medium was decanted and replaced with 1640 having 10% FCS. Pronounced aggregation of the cells, indicative of lymphocyte transformation, was noted within the first week, and log-phase growth, signaling the initiation of the cell line was recorded after 3 to 6 weeks. Mass propagation was achieved using larger vessels and, after 100 days of cultivation, samples of the phenotypically defined line were cryopreserved (*vide infra*).

Initiation of T-cell lines. T-cell lines were started from the same blood samples used for establishing the corresponding B-cell line. For this purpose, Ficoll-Hypaque separated leukocytes were suspended at a density of 0.5 to 1.0×10^6/ml of 1640 with 10% FCS and 1% PHA-M (GIBCO). Twelve milliliters of the mitogen-containing cell suspension lacking IL-2 was added to 25-cm^2 flasks; these were loosely capped and incubated in an upright position and with conditions similar to those for initiating B-cell lines. After 3 to 5 days, the spent medium was replaced with IL-2 and FCS; the optimal concentrations, determined empirically of these two supplements varied among different lots, but amounts ranging from 10 to 30% and 10 to 20%, respectively, were employed most frequently. Cell proliferation occurred during the first 7 to 10 days; thereafter, subcultures were initiated every 2 to 3 days using IL-2-supplemented medium and an optimal cell density of 1.0 to 2.0×10^5/ml. Mass propagation of these cells was performed using similar conditions, but in which 100 to 500 ml of cell suspension was cultivated in roller bottles or spinner vessels.

With reference IL-2-dependent T-cell lines of healthy or diseases donors, we have used the designation "long-term" to denote only those culures that have been propagated continuously for at least 100 days.

Production of IL-2. A detailed description of the IL-2 generation procedure has been presented elsewhere (15); accordingly, only a brief account will be presented herein. Leukocytes were isolated from the "buffy coats" from a unit (~500 ml) of blood from each of 8 to 14 healthy donors. The pooled leukocytes were then incubated at a density of 2.0 to 3.0×10^6/ml RPMI 1640 with 10% FCS in 2-liter spinner vessels designated as "Phase I" cultures. After 4 to 6 days, the leukocytes were collected and resuspended in a nutrient-enriched medium. These blood leukocytes were then mixed with an equal number of nonirradiated B cells pooled from 4 to 12 rapidly replicating long-term lymphoblastoid lines, and the B-cell lines used most frequently were derived from patients with Burkitt's lymphoma. The 1:1 mixture of blood leukocytes and cultured B cells were suspended to give a final density of 2.0 to 2.5 $\times 10^6$/ml medium with 1% FCS and 1% PHA-M. The SN of these "Phase II" spinner cultures was collected after 24 hr of cultivation. Following micropore filtration, this crude, unconcentrated supernatant, known to contain residual amounts of PHA, was used for all studies reported herein.

IL-2 assay. T-cell growth-promoting activity of different human supernatants was defined using procedures described previously (16) and IL-2-dependent T cells of long-term human (TPM-54) or murine lines (CTLL-2; kindly provided by Dr. S. Gillis). Ten milliliters of cell suspension was seeded in 25-cm^2-style flasks at $1\text{-}2 \times 10^4$/ml of RPMI 1640 containing FCS (5%), 2-mercaptoethanol (5×10^{-5} *M*), and different concentrations of human supernatant. Samples of the cultured cells were collected on assay Days 2 to 5 and growth was determined by the enumeration of the T cells using an automated counter

(Coulter Electronic, Inc., Hialeah, Fla.); reproducibility of the assay was good, SEM for replicate cultures was less than 3.0%. Conventional hemocytometer procedures and the trypan blue dye-exclusion test was used to insure the validity of the data obtained with the automated counter and to monitor cell viability.

Phenotyping. Identification and characterization of cells from EBV- or IL-2-initiated lines was defined using multiple marker procedures described elsewhere in detail (17, 18), and with conventional cytochemical and cytogeneic procedures.

Cryopreservation. After 100 days of culture, replicate samples of the B- and T-cell lines were cryopreserved using a programmable temperature controller to insure linear-rate freezing and methods described previously (19). The rate of recovery was good: B-cell lines, 90%; T-cell lines, 85%.

Results. Sustained cultivation of normal human T cells is dependent upon IL-2; accordingly, the selection of suitable supernatants is of paramount importance. Presented in Fig. 1 are the results of a representative experiment defining T-cell growth-promoting activity of 49 different human supernatants that had been generated using different experimental protocols prescribed either by ourselves or others. Growth of murine IL-2-dependent CTLL-2 T cells was defined using cultures initiated with different supernatant concentrations (20, 10, 5, 2.5, and 1.25%) in assays performed on days 2–5. Maximum density of viable cells was recorded on Day 4, and the results presented are those recorded for cultures containing 10% supernatant; of the better supernatants, similar levels of growth were achieved with concentrations as low as 1.25%. As illustrated, 22 of the 49 supernatants (45%) supported a 10-fold or greater increase in the number of T cells. Of these 49 supernatants, 14 had been prepared using the procedure described in Materials and Methods (*vide supra*) for sustaining the long-term normal human T-cell lines described in this report, and all 14 of these supernatants supported a high level of CTLL-2 T cell proliferation (range: 23- to 120-fold cell increase). The results of similar studies attempting to define the optimal culture conditions for producing maximal amounts of IL-2 in supernatants suitable for large-scale purification schemes will be presented elsewhere (Pauly *et al.*, manuscript submitted).

Presented in Fig. 2 are the results of efforts to establish lymphoblastoid cell lines from randomly selected healthy human donors.

In study 1A, attempts were made to establish both B- and T-cell lines from many, but not all of these 30 donors. In a subsequent experiment, Study 1B, conducted to define and monitor phenotypic changes, attempts were made to establish T-cell lines, but not B-cell lines, from this same group of subjects.

In these studies, a total of 40 B-cell lines were initiated, and these 40 lines were the successful products of 52 attempts; thus, the rate of success (40/52 attempts) was 77%. These 40 B-cell lines were derived from 24 of 29 donors from whom blood was obtained; using this data to calculate culture efficiency, the rate of success (24/29 donors) was 83%. All of these B-cell lines were from cultures in which the leukocytes were incubated initially in the presence of the EBV-containing SN-B95.8, and these lines had become established (i.e., exhibited log-phase growth kinetics) within 60 days (mean, ~37 days).

Similarly, 54 T-cell lines were established, and these were the successful products of 55 attempts (success rate, 98%). These 54 T-cell lines were derived from 28 different donors, and in no instance did we fail to propagate a T-cell line from a given subject. In all instances, these T-cell lines were initiated, and subsequently propagated, using IL-2-supplemented medium.

As illustrated in Fig. 2, multiple cell lines were initiated from a single subject, and this was achieved by establishing cell lines from blood collected on different occasions from a particular donor. The number of donors from whom one, two, three, or four B-cell lines were initiated were 15, 5, 4, and 1, respectively. Likewise, the number of individuals from whom one, two, three, four, five, and six T-cell lines were initiated were 14, 16, 6, 12, 0, and 6, respectively.

Paired B- and T-cell lines were ones in which both the B- and T-cell lines were derived from the same donor and from the same blood sample. A total of 32 paired lines were

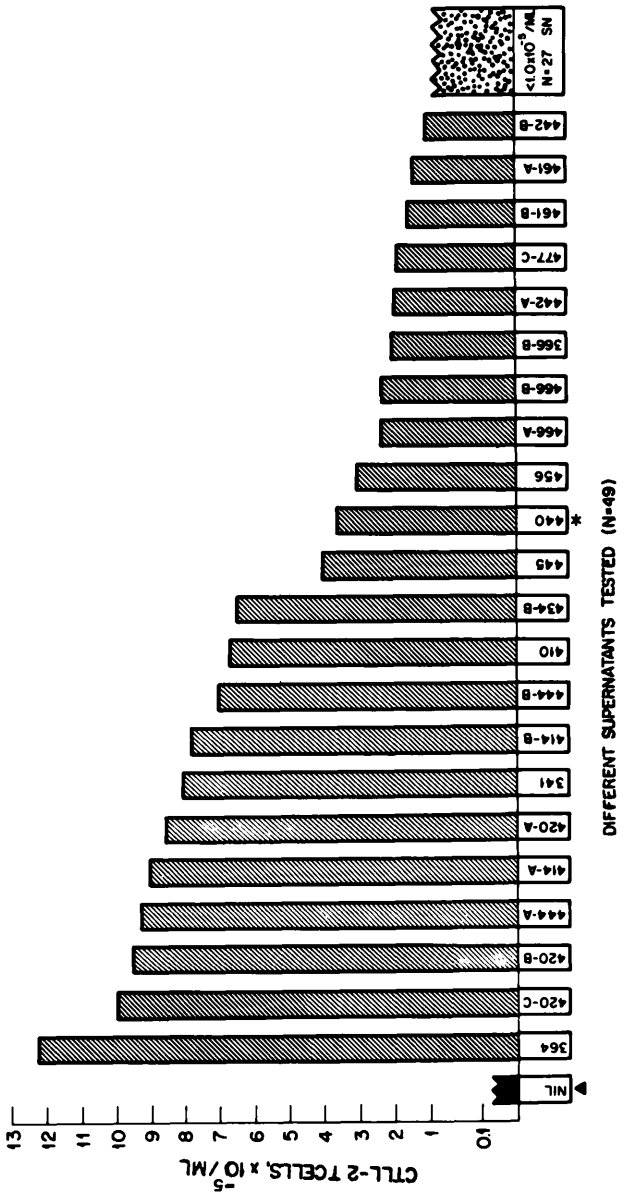

FIG. 1. Results of a representative experiment defining the T cell-growth promoting activity of 49 human supernatants produced under different experimental conditions as measured using IL-2-dependent murine T cells of the long-term line CTLL-2. Of the supernatants tested, 22 (45%) supported a 10-fold or greater increase in the number of T cells in a 4-day assay; positive control cultures received a laboratory IL-2-standard (SN-440; 10% v/v), and negative control cultures were prepared under similar conditions but without supernatants (NIL).

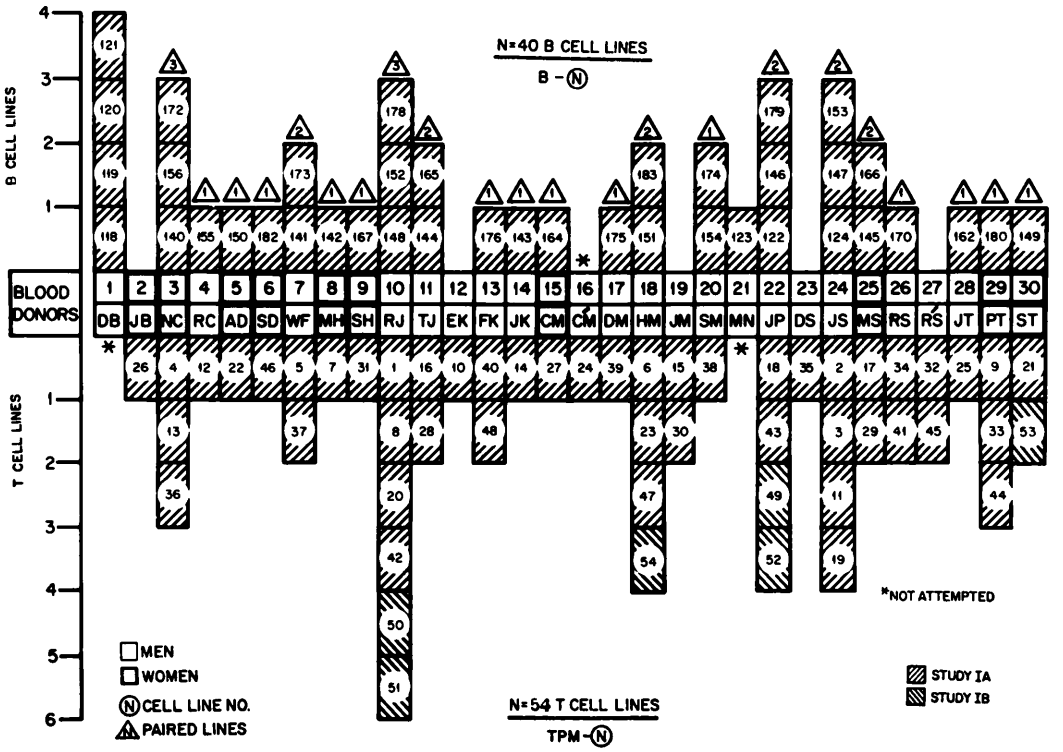

FIG. 2. Results of studies in which EBV- and IL-2-containing supernatants were successfully utilized for the long-term (>100 days) cultivation of 40 B-cell lines and 54 T-cell lines from randomly selected blood donors. Each man or woman is identified by an experiment aquisition number and their initials, and a cell-line number with the prefix B- or TPM- was used to designate each of the B- and T-cell lines, respectively. In 22 cases, both a B- and T-cell line were established from the same subject and the same blood sample, and the number of these paired lines initiated from a given individual is designated within the triangle.

obtained as a result of 48 attempts (success rate, 71%), and these lines were derived from all of the 13 men and 9 women who had been used in these experiments.

Initial screening of all cell lines was performed using fluorescein-conjugated goat anti-human immunoglobulin heavy-chain (α, δ, γ, and μ) or light-chain (κ and λ) specific reactions. Lines having positive staining cells (>5%) were tentatively designated as B-cell lines, and all of these lines ($N = 40$) had been derived from cultures initiated using the EBV-containing supernatant.

In contrast, none of the 54 cell lines established using IL-2-supplemented medium contained cells that reacted with these anti-immunoglobulin antibodies. Assays of rosette formation and T-cell antigen further substantiated their identity as T-cell lines. Moreover, selected lines were subjected to additional tests to further define their phenotypic profiles, and a detailed account of these studies will be presented elsewhere; a brief description is presented herein to further document the T-cell nature of these lines.

Presented in Table I are the results of a phenotypic analysis of a representative long-term T-cell line (TPM-54) initiated from healthy donor No. #18 (HM, Fig. 1) using IL-2.

About 80% of IL-2-dependent cells formed rosettes with sheep red blood cells (sE) or with sE that had been treated with 2-aminothyisothiouronium bromide (sE_{AET}). In contrast, less than 5% of the cells formed rosettes in assays identifying membrane receptors for bovine erythrocytes (bE) that had been complexed (bEA) to IgG or IgM of rabbit antibody to bE; this test identifies Fc receptors of T-cell subsets T_γ and T_μ, respectively. Moreover, IL-2-dependent T cells that had been cultivated for only 4 to 5 weeks demonstrated little or no reactivity in these Fc assays. T cells of long-term lines also failed to form bEAC

TABLE I. PHENOTYPIC ANALYSIS OF A REPRESENTATIVE LONG-TERM LINE (TPM-54) INITIATED FROM A HEALTHY HUMAN DONOR USING IL-2

ROSETTES:		ANTIGENS, HETEROANTISERA:	
sE	80%	Ia	80%
sEgr	60%	T-Ag	80%
sEaet	80%	MAg-1	NEG
bEA (IgM/IgG)	NEG	cALL	NEG
bEAC (IgM + C')	NEG	OKT-1	100%
IMMUNOGLOBULINS:		OKT-3	100%
sIg-A,-D,-G,-M	NEG	OKT-4	80%
cIg-A,-D,-G,-M	NEG	OKT-5	50%
CYTOCHEMISTRY:		OKT-8	65%
ALK. PHOSPHATASE	POS	OKT-9	100%
ACID PHOSPHATASE	POS	OKT-10	95%
CHLOR. ESTERASE	NEG	OKT-11	100%
NONSPECIFIC ESTER	NEG	OKI-1	80%
PAS	NEG	OKM-1	NEG
PEROXIDASE	NEG	ALLOANTIGENES:	
SUDAN BLACK	NEG	HLA-A1,A9;B7(40),B18	
TERM. TRANSFERASE	NEG	DR2, DR3	

KARYOTYPE:

DIPLOID MODE, N = 46; 50 FIGURES ENUMERATED: 3 ANEUPLOID; G-BANDING, UNREMARKABLE.

rosettes with bE that had been complexed with IgM of rabbit antibody and with mouse complement (C). Thus, the T cells had neither Fc nor C receptors.

About 80 to 90% of the T cells exhibited positive reactivity with appropriately absorbed and thoroughly characterized xenoantisera as well as with different monoclonal antibodies including those defining T-cell and Ia antigens. The profile obtained for a representative line (TPM-54) using monoclonal antibodies identifying T-cell subets (Ortho Diagnostic Systems, Inc., Raritan, N.J.; kindly provided by Dr. G. Goldstein) were as follows: (OKT-1, "pan" T cells; 100%), OKT-3 ("pan" human T cells; 100%), OKT-4 (inducer/helper T cells; 80%), OKT-5 (cytotoxic T cells; 50%), OKT-8 (cytotoxic T cells; 65%), OKT-9 (replicating T cells; 100%), OKT-10 (prothymocytes; 95%), OKT-11 ("pan" T cell; 100%), OKT-I (Ia antigen; 80%), OKM-1 (monocyte/macrophage cells; neg). These cultured T cells, however, did not react with antisera defining antigens associated with leukemic cells or cells of the myelocytic/monocytic series. HLA-antigens and DR specificities were defined easily with appropriate alloantisera. The profile obtained for both the TPM-54 T cell line and its corresponding paired B-cell line B-183 was: HLA-A1, -A9, -B7 (40), -B18, DR2, and DR3. Moreover, consistent HLA-typing results were obtained in repetitive assays of these and other cultures indicating that there was no cross contamination of the cells from the different lines.

Cytochemistry profiles (positive, +; negative, −) were as follows: alkaline phosphatase (−); acid phosphatase, with or without tartaric acid block (+); chloracetate esterase (−); nonspecific esterase (−); periodic acid-Schiff (−); peroxidase (−); Sudan black (−); and terminal transferase (−).

Karyotypic analyses have shown a normal diploid mode of 46 chromosomes, and less than 5% of the metaphase figures were aneuploid or contained atypical chromosomes as

defined using conventional banding techniques.

Discussion. The results of these studies demonstrate the establishment and long-term cultivation of both B- and T-cell lines from randomly selected healthy donors, and that the probability of success in initiating paired lines from a particular subject is about 71%.

The frequency of success in initiating B-cell lines was 77%. To be noted is that in undertaking these studies it became apparent that large numbers of cultures would require frequent feeding and routine maintenance. To reduce the number of flasks, these B-cell lines were initiated by seeding the leukocytes of a given donor into a single flask. A higher rate of success in initiating these lines were to have been anticipated had more flasks been prepared from a given donor. Moreover, most of our failures could be ascribed to technical errors, including defective plastic cultureware, laboratory accident, neglect, contamination, etc. Thus, higher rates of success would have been realized had we employed more carefully controlled laboratory conditions. Nevertheless, the success rate of 77% is substantially higher than values reported previously.

T-cell lines were initiated easily, and could be propagated in mass culture using IL-2. In subsequent studies, the results of which will be presented elsewhere, we have established IL-2-dependent T-cell lines from the thymus of children undergoing open heart surgery ($N = 10$), blood of youngsters with acute lymphoblastic leukemia ($N = 6$), and from adults with a cutaneous T-cell lymphoma (mycosis fungoides, $N = 2$; Sézary syndrome, $N = 1$).

We and others (4, 20–23) have noted a significant reduction and, in some instances a complete abrogation, in the proliferation of human and murine IL-2-dependent T cells after 4 to 6 weeks of culture. A varied degree of success has been obtained by different investigators in recovering cells from this "crisis" period (4, 20–23) for reinitiating sustained log-phase growth. The underlying cause of this event has not been elucidated, but several explanations have been proposed. Some have suggested that it is due to the depletion of short-lived population of macrophage-like accessory cell or other leukocyte subset whose function in promoting T-cell growth cannot be replaced by monokines (e.g., interleukin-1) or other lymphokines (20). In this respect it is noteworthy that the addition of irradiated allogeneic leukocytes as "feeder" cells (21, 22) has proven useful, particularly for cloning IL-2-dependent T cells of long-term lines. We have tentatively ascribed the crisis to mycoplasma which we have found to be associated with a marked reduction in the growth of human, but not murine, IL-2-dependent T-cell lines (15). Moreover, we speculate that this common culture contaminant probably arises from mycoplasma contaminated EBV-transformed B lymphoblastoid cells that are frequently used in generating this T-cell growth factor. Alternatively, others have argued that IL-2-dependent T cells are a normal diploid populations and, as with normal diploid fibroblasts (24, 25), have a finite life; accordingly, such cells can be propagated *in vitro* for a limited period (i.e., 25 to 35 replication cycles). This viewpoint, however, would prove difficult to sustain knowing that a number of investigators have sustained log-phase proliferation of T cells from healthy humans (1, 3, 15, 20) and mice (2, 4, 7, 9–11, 16) for many months or, in some instances, several years (e.g., CTLL-2, 16; HT-2, 23). Furthermore, it is thought that these are non-neoplastic T cells and have been defined as "normal" cells by different criteria, including; karyotype, phenotype, cytochemistry, functional assays, growth-factor dependency, and absence of heterotransplantibility.

Of particular relevance to the studies reported herein is the recent observation that cloned populations of nontransformed (i.e., devoid of the EBV or EBV nuclear antigen) human B lymphocytes can be propagated for several months using a crude culture supernatant from PHA-activated pooled human blood leukocytes and culture conditions similar to those used for generating IL-2 (26, 27). Also, IL-2-dependent T-cell lines as well as virus-transformed T- and B-cell lines from different primates have proven useful in recent studies of different serologically-defined membrane markers; most notable is that it has also been demonstrated that the Ia antigen expressed on T cells and B cells is identical (28).

In summary, studies reported herein describe the prolonged cultivation of both B- and T-cell lines from healthy human donors, and these experiments document the efficiency with which EBV- and IL-2-containing

supernatants can be employed for initiating paired lymphoblastoid lines suitable for comparative studies defining both phenotype and function.

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