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Transformation of human umbilical cord blood T cells by human T-cell leukemia/lymphoma virus

(type C retrovirus/adult T-cell leukemia/lymphoma/surface receptor for T-cell growth factor/T-cell growth factor/HLA-DR determinant)

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ABSTRACT **Several isolates of human T-cell leukemia/lymphoma virus (HTLV) were transmitted to normal human T cells obtained from the umbilical cord blood of newborns. T cells from seven specimens were immortalized by infection with different HTLV isolates and their properties were compared with those of activated uninfected normal T cells grown in the presence of T-cell growth factor (TCGF) and with those of HTLV-positive neoplastic T-cell lines derived from patients with T-cell malignancies. The HTLV-infected cells generally belonged to a class of mature T cells (OKT4$^+$ and Leu 3A$^+$) and differed from the normal uninfected cells in that they could be propagated in culture indefinitely; possessed altered morphology, including convoluted nuclei and some bi- and multinucleated giant cells; formed large clumps in culture; demonstrated a diminished requirement for TCGF; had an increased density of TCGF receptors; often became completely independent of exogenous TCGF; and expressed HLA-DR determinants. These properties of the HTLV-infected cord blood T cells contrasted to those of uncultured cord blood T cells and of cord blood cells stimulated with mitogen and grown with TCGF but resembled the characteristics of T-cell lines established previously from patients with HTLV-associated T-cell malignancies. This *in vitro* system offers a unique opportunity to study the basic mechanism involved in abnormal growth and neoplastic transformation of a specific class of human T cells.**

The discovery of T-cell growth factor (TCGF) in 1976 (1) enabled the development of the technology to grow normal and neoplastic mature T cells *in vitro* for considerable periods of time (2). Using purified TCGF (3), it was possible to grow in culture T cells that exhibited several characteristics of the primary tumor cells from patients with mature T-cell malignancies (2). A type C retrovirus designated human T-cell leukemia/lymphoma virus (HTLV) was isolated from some neoplastic T-cell lines (4–7). Detailed characterization of these isolates showed that HTLV is an exogenous human retrovirus closely linked to a subtype of mature T-cell malignancy and distinct from all known animal retroviruses (8–15). In our laboratory 15 HTLV isolates have been obtained from people of different parts of the world (4–7). In addition, human retrovirus isolates have been independently reported by workers in other laboratories (16–18) and comparative studies have shown that the new isolates are members of the HTLV group (19).

HTLV infection of normal T cells can induce long-term growth of the infected cells (16, 20). One source of HTLV, the MT-2 cell line, was derived from umbilical cord blood T cells designed to be a "feeder cell" for growing leukemia T cells in a cocultivation experiment, but the cord blood T cells incidentally became immortalized by an apparent infection with HTLV derived from the leukemia cells (16). Yamamoto *et al.* (21) recently confirmed this observation by using a MT-2 isolate for infection of human lymphocytes. We have transmitted numerous new HTLV isolates from virus-producing neoplastic T cells to T cells from blood of newborns (7). Here we describe the characteristics of cell lines established from cocultivation of the HTLV-positive cell lines with the cord blood lymphocytes. The recipient lymphocytes were infected with HTLV and the morphology, cell surface properties, and growth properties of these *in vitro*-infected cells were compared with those of their normal (uninfected) counterparts derived from parallel blood samples from newborns and with those of HTLV-positive neoplastic T-cell lines from patients with mature T-cell malignancies. The properties of the HTLV-infected cells are remarkably similar to the primary tumor cells. The cocultivation experiments appear to represent an *in vitro* model for HTLV-induced neoplastic transformation.

MATERIALS AND METHODS

Source of Virus and Cells. HTLV-positive T-cell lines from five different patients and one relative (T.K.) of a patient with ATL were used as a source of virus. The characteristics of the neoplastic T cells, including origin, establishment, HTLV expression, and T-cell characteristics, are described in detail elsewhere (7, 20). Briefly, HTLV is fully expressed in all the established cell lines used here as evidenced by reverse transcriptase activity in culture fluids and by the presence of type C particles by electron microscopic examination. All the cell lines from patients represent neoplastic mature T cells and with few exceptions (e.g., T.K.) exhibit mostly OKT4$^+$ and Leu 3A$^+$ phenotype.

HTLV Transmission. Cocultivation of HTLV-positive neoplastic T cells with cord blood T cells was carried out as described (7). After 4 to 5 wk, the cultures were processed for cell typing and HTLV detection. Both sex chromosomes and HLA antigens were used as markers to distinguish between the HTLV donor and recipient cells. HTLV expression in the infected cord blood T-cell lines has been described (7, 20). Briefly, the characteristics of uninfected (mitogen stimulated and cultured in the presence of 10% TCGF) and HTLV-infected cord blood T-cell lines used in this study were as follows. All of them were negative for B- (Epstein–Barr nuclear antigen and surface immunoglobulins) and positive for T-cell markers. Uninfected T cells were consistently negative for HTLV proteins. Their cell surface markers ranged from 90% to 98% for OKT3$^+$ and T$^+$101

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Abbreviations: HTLV, human T-cell leukemia/lymphoma virus; TCGF, T-cell growth factor; ATL, adult T-cell leukemia; PHA, phytohemagglutinin.

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(pan-T positive), 71% to 95% for OKT4$^+$ and Leu 3A$^+$, and 0% to 36% for OKT8$^+$ and Leu 2A$^+$. From 50% to >90% of the HTLV-infected cells produced HTLV proteins and were positive for pan-T-cell surface markers (OKT3$^+$ and T$^+$101), 70% to 92% were OKT4$^+$ and Leu 3A$^+$, and 0% to 10% were OKT8$^+$ and Leu 2A$^+$. The exception was the cord blood cells infected by HTLV-I_{MJ}. Most of these cells (called C5/MJ) were positive for both the OKT4 and Leu 3A antigen (59%) and the OKT8 and Leu 2A antigen (90%).

Fluorescence Analysis: TCGF Receptor and HLA-DR. Binding of a monoclonal antibody termed anti-TAC, which detects TCGF receptors (22), and a monomorphic anti-HLA-DR antibody 3.1 generously provided by T. Waldmann (National Cancer Institute) and J. Strominger (Harvard Biologic Laboratory), respectively, were determined by cytofluorometry using a fluorescence-activated cell sorter (23).

Morphology and Growth Characteristics of the Cultured Cells. Cell smears were prepared at regular intervals from cultures and stained with Wright–Giemsa. Thin-section electron micrographs were prepared of HTLV-infected and normal mitogen-stimulated T cells cultured in the presence of TCGF (24). Cumulative growth curves of HTLV-infected and uninfected T cells were determined in parallel experiments.

Cellular Requirement for Exogenous TCGF. The response of HTLV-transformed cord blood T cells to TCGF was compared with that of neoplastic T cells from patients with mature T-cell malignancies by a microassay (25, 26). The TCGF dilutions giving 50% maximal [^{3}H]thymidine incorporation of HTLV-infected and uninfected T cells were determined in parallel experiments with the same standard TCGF preparation. Values were determined by probit analysis (26). One unit of TCGF is defined as the amount necessary to give 50% maximal [^{3}H]thymidine incorporation into DNA of normal human T cells from cord or peripheral blood in the presence of the standard TCGF.

RESULTS

Morphologic Characteristics of Normal and HTLV-Infected Cord Blood T Cells. Cultures of normal (mitogen-stimulated) T cells after 2 wk grew predominantly as single-cell suspensions and in small clumps (Fig. 1*A*). Wright–Giemsa staining of cultured T cells from cord blood revealed a homogenous population of lymphoblastoid cells, as previously documented with *in vitro*-cultured normal T cells from adults (1) (Fig. 1*B*). The HTLV-infected T cells from cord blood briefly exhibited single-cell organization but soon displayed growth in large clumps (Fig. 1*C*). The growth in large clumps was a characteristic feature of those HTLV-infected T cells that became less dependent on or independent of exogenous TCGF. The cultures of transformed T cells were considerably less uniform in appearance than uninfected growing normal T cells. Wright–Giemsa staining of smears from these infected cultures showed that the cell size and number of nuclei per cell were variable (Fig. 1*D*). Mononucleated and binucleated cells were frequently found around multinucleated giant cells, the latter representing about 0.1–10% of the cell population. An example of growth in large clumps and of cell polymorphism of a neoplastic T-cell line established from neoplastic T cells from an HTLV-positive patient with cutaneous T-cell lymphoma is shown in Fig. 1 *E* and *F*.

The *in vitro*-transformed T cells and neoplastic T cells from patients with mature T-cell malignancies were compared by electron microscopy. Electron micrographs of HTLV-infected and mitogen-stimulated cord blood T cells are shown in Fig. 2. Unlike the mitogen-stimulated T cells, the HTLV-transformed T cells frequently exhibited convoluted nuclei with one or two nucleoli. Although the nuclear convolution was not as prominent as in the case of neoplastic T cells, it was a consistent feature of HTLV-infected cord blood lymphocytes.

Long-Term Growth of Mitogen-Stimulated and HTLV-Infected T Cells. Cumulative growth curves of mitogen-stimulated (uninfected) and HTLV-infected T cells from cord blood are shown in Fig. 3. The growth in the presence of TCGF of uninfected and HTLV-infected T cells was followed simultaneously in five separate experiments. After initial rapid growth, the uninfected T cells consistently entered a "crisis" period in which they failed to increase in cell number and showed degenerative cytologic changes, a decrease of viable cell number, and cessation of mitotic activity. The cultures thus resemble the so-called degenerative "phase III," analogous to the approaching end of the finite *in vitro* life span of human fibroblasts and glial cells (27). The onset of this crisis period varied from 3 to 8 wk, dependent on the donor. In the absence of HTLV infection, no permanent TCGF-dependent or -independent T-cell line from human cord blood was obtained in 20 attempts from 15 different blood samples regardless of the preparation and the concentration of TCGF used. No such crisis period was ob-

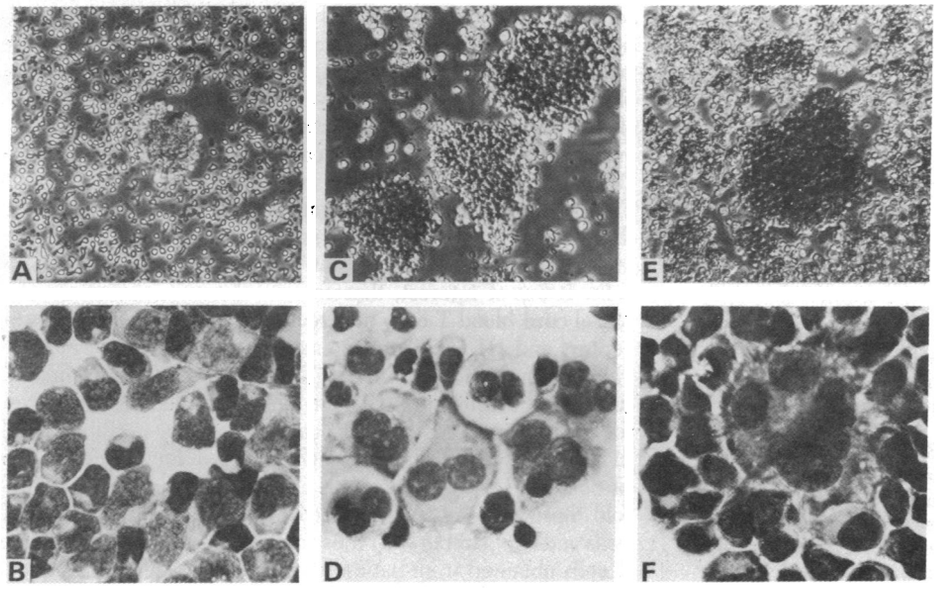

FIG. 1. Growth pattern (*A*, *C*, and *E*) and morphology (*B*, *D*, and *F*) of cultured normal (uninfected) and HTLV-transformed T cells from cord blood and from HTLV-positive neoplastic T cells from a patient (M.J.) with a cutaneous T-cell lymphoma (mycosis fungoides). (*A* and *B*) C5, normal (uninfected) cord blood T cells cultured with 10% TCGF for 18 days, showing growth as single cells, and the cells have uniform morphology. (*C* and *D*) Cord blood T cells shown in *A* and *B* but transformed by an M.J. isolate of HTLV. Growth is characterized by large clumps and many bi- and multinucleated cells. (*E* and *F*) M.J. cells in culture form large clumps and have some multinucleated cells. (*A*, *C*, and *E*, ×70; *B*, *D*, and *F*, Wright–Giemsa, ×325.)

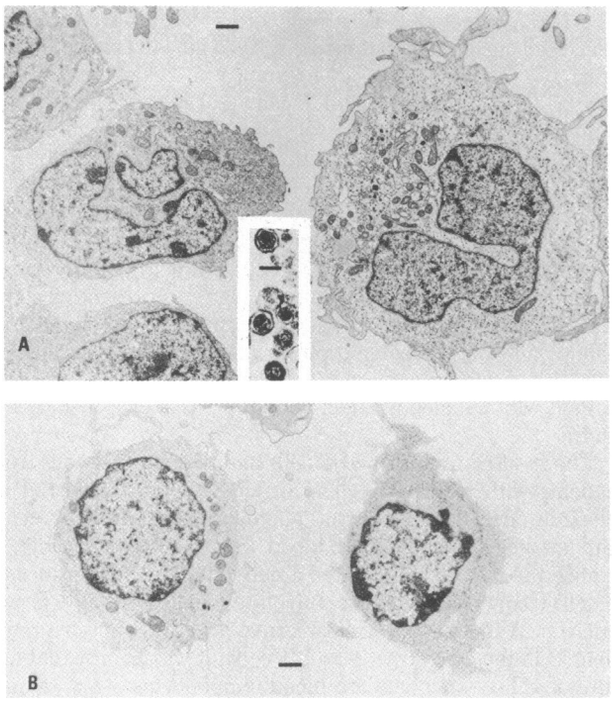

FIG. 2. Electron micrographs. (*A*) HTLV-transformed cord blood T cells (C5/MJ) consist of a proportion of cells with lobulated nuclei. (*Inset*) Extracellular C type particles. (*B*) Normal cord blood T cells (C5) were maintained in the presence of 10% TCGF for 2 wk before processing (24). Cells exhibit typical morphology of lymphoblasts with round nuclei. (*A* and *B*, bars = 1 μm; *Inset*, bar = 0.1 μm.)

served in any of the HTLV-infected T-cells, and all of them (30 infected T-cell lines) appeared to grow indefinitely. Unlike phytohemagglutinin (PHA)-stimulated cord blood T cells, the HTLV-infected T cells showed slower growth rates for the first 2 wk of *in vitro* cultivation, when apparently a small number of recipient cells were infected. After 4 wk of cultivation, the percentage of cells positive for HTLV p19 was 30–50, and the infected cells exhibited a rapid growth rate (doubling time, 25–30 hr).

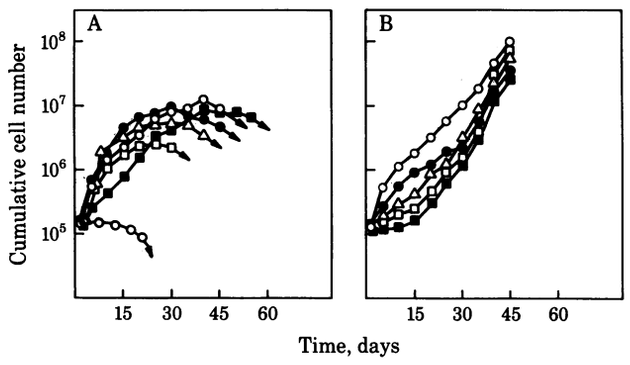

FIG. 3. Growth curves of mitogen-stimulated (*A*) and HTLV-infected (*B*) cord blood T cells in the presence of 10% TCGF in long-term cultures. (*A*) Normal cord blood T cells were either unstimulated (C7, ⊙), stimulated with PHA at 10 μg/ml (C5, ●; C7, ○; C8, △; C21, □), or stimulated with allogeneic T cells from peripheral blood of an adult treated with mitomycin C prior to cocultivation (C69, ■). (*B*) HTLV-infected cord blood T cells are from the same blood samples as the uninfected T cells (C5/MJ, ●; C7/TK, ○; C8/SK, △; C21/MI, □; C69/TK, ■). After 5 days in culture, the cells were seeded in culture medium with TCGF (2×10^5 cells per ml) and the cell number was regularly determined. The cumulative number was calculated as the potential number of cells per ml of culture medium obtainable from the second passage. Arrows indicate spontaneous death of cultures.

TCGF Independence of HTLV-Transformed T Cells. Activated normal T cells depend on exogenous TCGF for growth *in vitro*. T cells originating from clinical specimens obtained from patients with HTLV-positive mature T-cell malignancies differ from their normal counterparts in at least two respects. (*i*) They contain TCGF receptors and, therefore, they do not require lectin activation to respond to TCGF (2). (*ii*) They often have the capacity for indefinite growth (1, 2, 20, 21). Some become constitutive producers of TCGF, a possible reason for their growth independent of exogenous TCGF (25).

We determined the requirement for exogenous TCGF of the HTLV-infected cord blood T cells. The growth curves of uninfected (PHA-stimulated) cord blood T cells (C8), a "neoplastic" T-cell line (SK) from an adult T-cell leukemia (ATL) patient, and the C8 cord blood T cells infected by HTLV derived from the SK cell line (C8/SK) are shown in Fig. 4*A*. All cells were treated with a standard amount of TCGF (10%, vol/vol). The HTLV-infected cells, C8/SK, exhibited exponential growth by days 5 and 6 of culture, whereas both uninfected C8 and the HTLV-producing neoplastic T-cell line, SK, reached a plateau by day 4. The viability of the uninfected cord blood T cells was significantly diminished after the 4th day of culture. Thus, the growth curve of HTLV-infected T cells already suggested that these cells were less dependent on exogenous TCGF for growth. As shown in Fig. 4*B*, this was confirmed by TCGF dilution experiments. Estimates for the TCGF requirements for T cells from normal adult peripheral blood, cord blood, HTLV-positive neoplastic T cells, and *in vitro*-transformed cord blood T cells were compared (Fig. 4 *C* and *D*). The requirement for TCGF of both HTLV-positive neoplastic T cells from patients and the HTLV-transformed cord blood T cells is 1/10th to 1/2 of that of normal (uninfected) T cells from adult peripheral blood or from cord blood. In fact, four of these HTLV-transformed T-cell lines from cord blood (C1/MJ, C5/MJ, C91/PL, and C82/TK) are completely independent of exogenous TCGF for their growth and many more lines have subsequently been established.

Phenotypic Alterations of HTLV-Transformed Cord Blood T Cells: TCGF Receptors and HLA-DR. HTLV-positive neoplastic T cells from adults with T-cell malignancies can be induced to proliferate with TCGF without prior antigen or lectin activation *in vitro* (2). This suggested that the fresh neoplastic T cells express TCGF receptors, in contrast to the majority of normal T cells. This was recently shown by using a monoclonal antibody (anti-TAC) recognizing these receptors (T. Waldmann, personal communication). In the case of cultured "neoplastic" T cells of HTLV-positive established cell lines, >70% of cells reacted with anti-TAC monoclonal antibody (7). Moreover, >90% of the cultured T cells from adults possess HLA-DR determinants (28) whereas mitogen-stimulated cord blood T cells cultured *in vitro* are negative (29). Thus, it appears that the expression of both TCGF receptors and HLA-DR determinants may play an important role in proliferation of these cells. It was of interest, therefore, to determine whether umbilical cord blood T cells infected with HTLV have TCGF receptors and HLA-DR determinants. In parallel experiments, HTLV-transformed cord blood T cells and mitogen-stimulated uninfected T cells from the same blood samples were analyzed for the presence of these antigens. As shown in Table 1, all HTLV-infected T cells were highly positive for the expression of both TCGF receptors and HLA-DR, with values 10- to 100-fold those of mitogen-stimulated normal human cord blood T cells and comparable with those of "neoplastic" HTLV-positive T cells obtained from patients with T-cell malignancies.

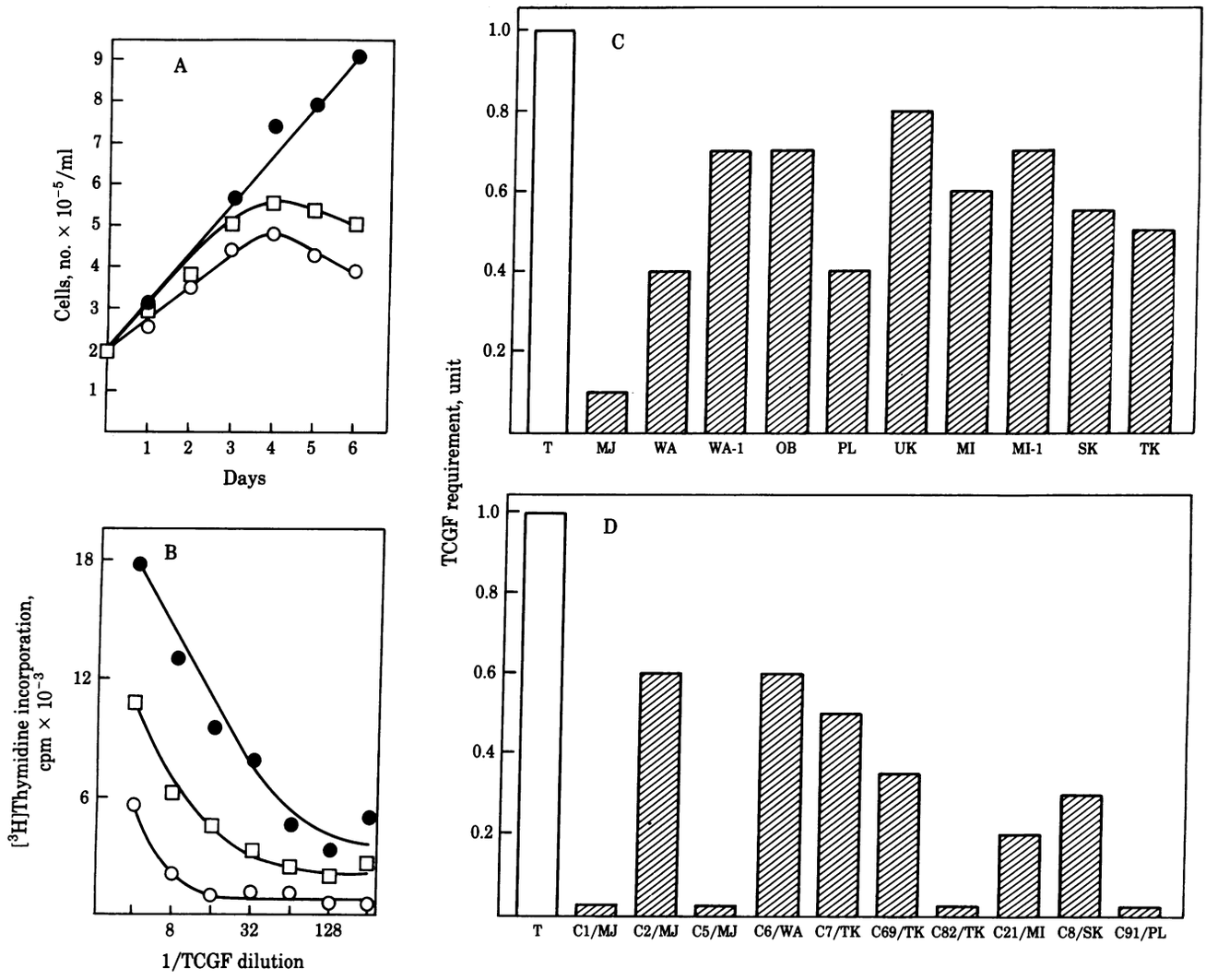

FIG. 4. Decreased TCGF requirement of HTLV-infected T cells. (*A*) Growth curves of normal (C8, ○) and HTLV-transformed cord blood T cells (C8/SK, ●) and of the neoplastic T cells (SK, □) used as a source of the virus infection. The cells were seeded at 2×10^5/ml in culture media containing 10% TCGF. The number of viable cells was determined daily. (*B*) Responses of normal cord blood T cells, HTLV-transformed cord blood cells, and neoplastic SK cells to different concentrations of TCGF were measured by [^{3}H]thymidine incorporation as described (15, 26). Symbols are as in *A*. (*C*) Comparison of TCGF requirement of HTLV-positive neoplastic T-cell lines (▨) with those of PHA-stimulated normal T cells (□) from adult blood. MI-1 and WA-1 are T-cell lines from patients M.I. and W.A., respectively. The characteristics of the established T-cell lines from patients have been described (7, 20). (*D*) Comparison of TCGF requirement of HTLV-transformed cord blood T cells (▨) with those of uninfected cord blood T cells (□).

DISCUSSION

The results reported here show that several new HTLV isolates can transform human T cells derived from the umbilical cord blood of newborns. The T-cell populations exhibit morphological alterations, infinite life span, diminished requirement for TCGF (or complete TCGF independence), and changes in surface phenotype. Comparison among HTLV-infected cord blood T-cell populations, their normal counterparts, and neoplastic T-cell lines showed that the properties associated with the neoplastic cells are consistently present in T-cell lines derived by infection of cord blood T cells with HTLV. Thus, they resemble the *in vitro* properties of the neoplastic cells derived from mature T-cell malignancies.

Normal (uninfected) T cells from adults cultured in the presence of TCGF express HLA-DR (28), and usually they grew only for a few months, although *in vitro* growth for considerable periods of time was observed in a few instances (1). Mitogen-stimulated cord blood T cells have absolute dependence on exogenous TCGF, morphological uniformity, limited life span, and lack of expression of HLA-DR during *in vitro* cultivation. The latter, as recently reported, may account for functional immaturity of cord blood T cells (29). With this "background" of cell characteristics, specific alterations induced by HTLV can be determined. However, the use of one parameter, as recently reported by Yamamoto *et al.* (21)—namely, *in vitro* growth of human lymphocytes for 3–7 wk in the absence of exogenous TCGF without quantitative estimation of cell proliferation and proper cell controls for comparisons—is not in our view a sufficient criterion for indicating that lymphocyte transformation was induced by the virus. There is also a possibility of *in vitro* selection of a biologically more active "laboratory" variant from the naturally occurring HTLV. This may have occurred in the case of the MT-2 cells used for HTLV infection by Yamamoto *et al.* (21) since these cells release a virus secondarily transmitted from an ATL patient and have been repeatedly passaged (16).

The frequency with which HTLV-transformed cord blood T-cell lines become independent of exogenous TCGF in our ex-

Table 1. Expression of HLA-DR determinants and TCGF receptors in mitogen-stimulated and HTLV-transformed human cord blood T cells

Cell line	HLA-DR		TCGF Receptors	
	% positive cells	Mean fluorescent units	% positive cells	Mean fluorescent units
Normal uninfected mitogen-stimulated human cord blood T cells				
C5	ND	ND	3	96
C6	2	2	2	28
C7	12	30	38	118
C8	10	200	12	212
C91	36	222	15	45
HTLV-transformed human cord blood T cells				
C1/MJ	86	3,284	56	1,504
C5/MJ	ND	ND	70	1,440
C6/WA	72	772	63	1,084
C7/TK	90	2,288	90	1,920
C8/SK	88	2,984	78	1,736
C82/TK	58	1,882	86	2,143
C91/PL	89	3,300	69	604

Both percentage of positive cells and mean fluorescence of HLA-DR determinants and TCGF receptors were detected by fluorescence-activated cell sorting using 3.1 and anti-TAC antibodies, respectively. Values of mean fluorescence represent relative density of the antigenic determinants on cell surface. P3 myeloma protein was used as a control. C91 cord blood cells were stimulated in the mixed coculture by using a mitomycin C-treated B-cell line positive for Epstein–Barr virus. ND, not determined.

perience is about 25%. All HTLV-infected T-cell lines are characterized by high percentage of TAC (TCGF receptor)-positive cells, high density of TAC antigen per cell, and decreased cellular requirement for exogenous TCGF. TCGF receptors and HLA-DR determinants may be induced by integration of the HTLV provirus, or HTLV may infect a cell type with these specific features already present and produce a clonal expansion of these cells. The capability of HTLV-infected T-cells to utilize exogenous TCGF more efficiently than normal T cells, irrespective of whether it is due to higher density of TCGF receptors or another mechanism coupled with the presence of constitutive TCGF producing cells within the HTLV-infected T-cell population, may represent the essential alteration leading to abnormal cell growth. Alternatively, HTLV infection may produce other genomic changes (30), and it is possible that these lead to abnormal cell growth in a manner that by-passes the TCGF–TCGF receptor system.

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