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Establishment and characterization of human T hybrid cells secreting immunoregulatory molecules

(human T hybridoma/azaguanine-resistant T-cell line/interleukin-2/killer helper factor)

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ABSTRACT **Hybridization of human T cells with an azaguanine-resistant human T cell line gave rise to T hybrid cell lines secreting several immunoregulatory molecules. Analyses of karyotypes, HLA phenotypes, and other surface phenotypes, such as T-cell-specific antigens or receptors for sheep erythrocytes and the patterns of mitogen responsiveness confirmed that the hypoxanthine/aminopterin/thymidine-resistant cell lines were human T–T hybridomas. One of the established hybrid clones (24-A) secreted human interleukin-2 (IL-2). The culture supernatants induced the proliferation of concanavalin A-stimulated murine T cells and supported the proliferation of an IL-2-dependent human cytotoxic T-cell line. In a clone (38-B) that did not show any IL-2 activity in culture supernatants, the addition of macrophages induced IL-2 production in the presence of phytohemagglutinin, suggesting that interleukin-1 induced IL-2 production in T hybrid cells. Hybrid cells secreting killer helper factor were also established. The culture supernatants from this clone, 55-A, helped the induction of cytotoxic T cells against UV-treated human B-blastoid cells but did not show any IL-2 activity.**

Application of cell-hybridization techniques to murine T lymphocytes has resulted in T hybrid cell lines that have several immunological activities—i.e., T hybridomas secreting IgE class-specific suppressor factor (1), antigen-specific suppressor factor (2), T-cell replacing factor (3), and the factor(s) responsible for the proliferation of T lymphocytes (interleukin-2; IL-2) (4). Homogeneous populations of such hybridomas capable of immune functions have obviously facilitated the isolation and chemical characterization of T-cell-derived immunoregulatory molecules.

Establishment of human T hybridomas should lead to production of purified lymphokines to allow more rapid progress in their chemical and biological characterization and exploration of their clinical potential. Thus, we attempted to establish human T hybrid cells by using an azaguanine-resistant mutant of a human leukemic T-cell line, CEM-AG^R, as a parental cell line. The results show that hybridization of human peripheral T cells with CEM-AG^R cells gives rise to human T–T hybridomas that secrete immunoregulatory molecules. One of several hybrid clones secretes human IL-2, which is effective in maintaining an IL-2 dependent human cytotoxic T-cell line, and the other clone produces helper factor(s) effective in the differentiation of cytotoxic T cells.

MATERIALS AND METHODS

Cell Lines. An azaguanine-resistant mutant T-cell line sensitive to 0.1 mM hypoxanthine/0.4 μM aminopterin/16 μM thymidine; (Sigma) was selected from a human T-leukemic cell line (CCRF-CEM) (5) provided by J. Minowada (RPMI, Buffalo, NY) by culturing the cells with 8-azaguanine (Sigma) at gradually increasing concentration (2–100 μM over 8 weeks). The Epstein–Barr virus-tranformed human B-blastoid cell line, CESS, was a gift from P. Ralph.

Cell Preparations. T lymphocytes from human peripheral blood or tonsils were separated by rosette formation with neuraminidase-treated sheep erythrocytes as described (6). Nonstimulated T cells, T cells stimulated with concanavalin A (Con A) (10 μg/ml) or T cells resensitized with mitomycin C-treated (80 μg/ml, 45 min, 37°C) CESS cells in secondary mixed lymphocyte cultures (MLC) were used for hybridization with CEM-AG^R cells. Human peripheral blood macrophages were separated from peripheral blood lymphocytes (PBL) by adherence to Petri dishes. Adherent cells were then detached by pipetting with Hanks' balanced salt solution/0.2% EDTA (Sigma) and used as macrophages.

Cell Fusion. Twenty million human T cells and 1×10^7 CEM-AG^R cells were pelleted by centrifugation and suspended in 0.3 ml of 45% polyethylene glycol 6000 (Koch-Light, Colnbrook Bucks, England) in minimal essential medium. After 6 min of incubation with gentle shaking at 37°C, prewarmed (37°C) minimal essential medium was added at a rate of 2 ml/min. Cells were pelleted again, suspended at 2×10^5 tumor cells per ml in RPMI 1640 medium/20% fetal calf serum (Centaurus, Santa Ana, CA), and distributed in wells of a Linbro multiwell plate. After 24 hr, the culture medium was replaced with HAT medium and incubation was continued for at least 4 weeks. Cloning of hybrid cells was performed by a limiting-dilution method.

Measurement of Mitogenic Activity of Culture Supernatants. Mitogenic activity of culture supernatants was assessed by the promotion of Con A-induced mitogenesis in murine thymocytes according to the method of Shaw *et al.* (7).

Assay for IL-2 Activity. Activity of human IL-2 in culture supernatants from T hybridomas was measured by an assay system similar to that described by Gillis *et al.* (8). IL-2 activity was tested with an IL-2-dependent human cytotoxic T-cell line, that had been induced by MLC reaction between normal human peripheral T cells and MMC-treated CESS cells and maintained in IL-2 for >16 weeks. Cytotoxic T cells (3×10^3) were cultured in the presence of supernatants from hybridomas for 24 hr and were pulsed with 0.5 μCi of [^{3}H]thymidine for 8 hr.

Assay for the Activity of Killer Helper Factor in the Differentiation of Cytotoxic T Cells. Helper activity of culture supernatants in the differentiation of cytotoxic T cells was mea-

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Abbreviations: HAT, hypoxanthine/aminopterin/thymidine; IL-2, interleukin 2 (formerly called T-cell growth factor); CEM-AG^R, azaguanine-resistant human T leukemic cell line CEM; CTL, cytotoxic T lymphocytes; MLC, mixed lymphocyte cultures; IL-1, interleukin 1; Con A, concanavalin A; PHA, phytohemagglutinin; PBL, peripheral blood lymphocytes.

sured in the primary MLC by using UV-treated stimulator cells as described (9). Briefly, MLC for the assay of the helper activity was set up with 1×10^6 human PBL T cells and 5×10^4 UV-treated CESS cells in the presence or absence of supernatants from T hybridomas in wells of a Linbro multiwell plate. After 5 days, cells were harvested and used as effector cells in a cytotoxic assay. The cytolytic activity was measured with a microcytotoxicity assay (9) using ^{51}Cr-labeled CESS targets.

Karyotype. Karyotype was analyzed as described (1).

HLA Typing. HLA typing of T cells and T hybridomas was performed as described (10).

Analysis of Cell Surface Phenotypes. Hybridomas were studied for their expression of cell surface antigens (i.e., Leu 1, Leu 2A, Leu 3A, DR antigens, and B-cell-specific antigens) by indirect immunofluorescence with monoclonal antibodies.

Partial Purification of IL-2. Crude IL-2 was obtained from culture supernatants harvested from 2-day cultures containing tonsillar T cells (1×10^6/ml) and 0.1% phytohemagglutinin. Crude IL-2 was partially purified by gel filtration on a Sephadex G-100 column and used as a positive control of IL-2 activity. The 1 unit/ml standard for IL-2 activity was defined as that of crude IL-2 obtained from 1×10^6 T cells.

RESULTS

Establishment of Human T Hybridomas. Hybridization experiments were repeated ≈60 times and representative results are summarized in Table 1. To confirm that cell lines growing in HAT medium after hybridization were hybridomas between CEM-AG^R cells and normal T cells, karyotypes, surface phenotypes, and mitogen responsiveness were studied after cloning of HAT-resistant cells. The mean number of chromosomes in four clones was 88–93, which definitively exceeded the number of chromosomes in CEM-AG^R cells, which were 69–87 or 78 on average. The analysis of HLA phenotypes of clone 24-A and its parental cells, CEM-AG^R and PBL from donor M.O. confirmed that the HAT-resistant clone, 24-A, was a hybridoma between two parental cells—i.e., the HLA phenotype of clone 24-A was A2, Aw24, A11, B5, B8, B37, Bw22 and the HLA phenotypes of the parental cell line, CEM-AG^R, and parental PBL were A2, A11, B8, B37, Bw22 and A2, Aw24, B5, B40, respectively. The result showed that all HLA phenotypes of both parental cells except B40 were expressed on clone 24-A and that this clone was a hybrid line between CEM-AG^r and normal T cells. Three out of four clones tested expressed Leu 1 and Leu 3A antigens but did not express Leu 2A antigen (Table 1). On the other hand, 35% of the cells of clone 55-A expressed Leu 2A antigen on their surface. Since the parental tumor cell line, CEM-AG^R, also expressed Leu 1 and Leu 3A antigens, the expression of Leu 1 and Leu 3A antigens on the surface of hybridomas did not necessarily mean that these hybrid clones were derived from T cells bearing Leu 1 and Leu 3A antigens. However, the expression of Leu 2A on the surface of 55-A cells indicated that parental T cells bore Leu 2A antigen and that 55-A cells were T–T hybridomas. Twenty percent of clone 55-A cells formed rosettes with sheep erythrocytes, further confirming that 55-A cells were T–T hybridomas. Although the analysis of T-cell-specific antigens with monoclonal antibodies did not confirm that hybrid clones except 55-A were T–T hybridomas, none of the clones expressed markers characteristic of B cells (i.e., immunoglobulin molecules, DR antigens, B-cell-specific antigens recognized by several human B-cell-specific monoclonal antibodies or complement receptors). Moreover, the patterns of mitogen responsiveness of these clones strongly suggested that they were T–T hybridomas. The addition of Con A (10–100 μg/ml) or 1–10% PHA partially inhibited the proliferation of the parental cell line CEM-AG^R but pokeweed mitogen and protein A did not affect the proliferation of these cells at any concentration. On the other hand, the proliferation of clone 24-A was almost completely inhibited with 1% PHA, and 0.5–1% pokeweed mitogen also showed the inhibitory effect on the proliferation of 24-A cells. The patterns of mitogen responsiveness of other clones were also apparently different from those of the (CM-AG^R parental cell line (data not shown).

Secretion of IL-2 by T Hybridomas. To study whether these hybrid clones could secrete any immunoregulatory molecules, the activity of IL-2 in their culture supernatants was assessed by using murine thymocytes or an IL-2-dependent human cytotoxic T-cell line. As shown in Table 2, the culture supernatant from clone 24-A induced significant proliferation of Con A-stimulated murine thymocytes and the mitogenic activity was more than that of conventional IL-2 obtained from 1×10^6 PHA-stimulated human T cells. On the other hand, supernatants of the parental cell line CEM-AG^R or of other hybrid clones, such as 36-B, did not show mitogenic activity. Mitogenic activity of supernatants from clone 24-A was greatly augmented by stimulation of the hybrid cells with Con A (Fig. 1). The parental cell line CEM-AG^R did not produce mitogenic factors even after stimulation with Con A.

As shown in Fig. 2, the M_r 13,000–20,000 fraction from the culture supernatant from 24-A cells could maintain the proliferation of an IL-2-dependent human cytotoxic T-cell line and the activity was dependent on the concentration of the factor. The fraction with the same molecular weight as the parental cell line CEM-AG^R did not show any IL-2 activity on a cytotoxic T-cell line at any concentration. The molecular weight of the factor with IL-2 activity from a hybrid clone was in close agreement with the M_r 15,000 of human T-cell-derived IL-2 reported by Gillis *et al.* (11).

Table 1. Characterization of human T hybridomas

Exp.	Parental T cells	Clone	Karyotype	T antigen,* %			Rosette, %		B marker, %		
				Leu 1	Leu 2A	Leu 3A	E	EAC	Ig	DR antigen	B antigen
24	PBL T (unstimulated)	24-A	70–97 (89)	++	–	++	–	–	–	–	–
36	Tonsil T (Con A stimulated)	36-B	79–100(89)	++	–	++	–	–	–	–	–
38	PBL T (Con A stimulated)	38-B	79–96 (88)	++	–	++	–	–	–	–	–
55	PBL T (alloantigen stimulated)	55-A	87–100(93)	42.6	35.1	87.5	21.3	–	–	–	–
	CEM-AG^R		69–87 (78)	++	–	++	–	–	–	–	–
	PBL T			93.1	53.6	58.5	++	1.8	6.5	7.4	5.4
	PBL B			6.2	4.4	6.6	2.4	87.0	75.0	65.7	59.2

E, erythrocyte; EAC, erythrocyte antibody complement; Ig, immunoglobulin; ++, >95% of cells were positive; –, <1% of cells were positive.

* Studied by indirect immunofluorescence with monoclonal antibodies from Becton Dickinson (Sunnyvale, CA).

† Studied by indirect immunofluorescence. Monoclonal anti-DR antibody (lot C0102) from Becton Dickinson. Monoclonal B-specific antibody used was reactive with human B cells and B-cell lines but not with T cells, T-cell lines, or macrophages.

Table 2. Mitogenic activity of the culture supernatant of 24-A cells on murine thymocytes

Supplement	Dilution	[^{3}H]Thd uptake, cpm Exp. 1	Exp. 2
None		673 ± 180	2005 ± 1398
24-A	× 2	7131 ± 1226	9120 ± 3168
	× 4	2736 ± 224	4435 ± 945
	× 8	1711 ± 908	2082 ± 488
	×16	890 ± 493	2185 ± 93
36-B	× 2	769 ± 198	
	× 4	684 ± 288	1236 ± 957
CEM-AG^R	× 2		1210 ± 640
IL-2 (1 unit)		3101 ± 492	6528 ± 994

24-A, 36-B, and CEM-AG^R cells were incubated at 1 × 10^5/ml in RPMI 1640/10% fetal calf serum for 24 hr and cell-free supernatants were recovered. Murine thymocytes (1 × 10^5) were cultured in 0.2-ml vol in flat-bottom microplates and stimulated with Con A at 2 μg/ml. Serial dilutions of culture supernatants were added and uptake of [^{3}H]Thd was measured on day 3.

Induction of IL-2 Activity in T Hybridomas by Stimulation with Adherent Cells. The three other clones tested did not show any IL-2 activity, even on stimulation with Con A. Several experiments have shown the requirement for macrophages or their products (interleukin 1; IL-1) in mitogen-induced production of murine IL-2. Thus, we studied whether human macrophages could induce IL-2 activity in those human T hybrid clones. As shown in Table 3, the culture supernatants from clone 38-B did not show any IL-2 activity, even when were stimulated with PHA. On the other hand, incubation of this clone with PHA in the presence of human adherent cells induced IL-2 activity in their culture supernatants. The addition of PHA and adherent cells to the CEM-AG^R culture did not induce IL-2 activity. These results suggest that macrophages or IL-1 stimulate hybrid clone 38-B to the production of human IL-2.

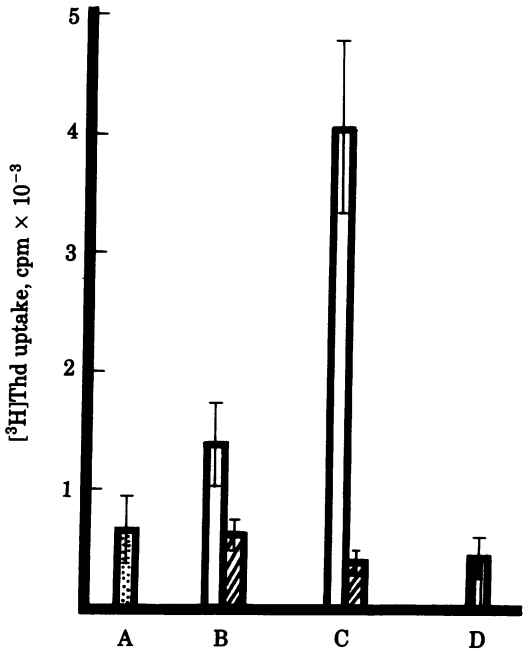

FIG. 1. Increase in IL-2 production in 24-A cells by stimulation with Con A. 24-A cells (□) or CEM-AG^R cells (▨) (1 × 10^5/ml) were incubated in the presence or absence of Con A (1 μg/ml) for 24 hr and cell-free supernatants were recovered. Murine thymocytes (1 × 10^5) were cultured in 0.2-ml vol in flat-bottom microplates and stimulated with Con A at 2 μg/ml. [^{3}H]Thymidine ([^{3}H]Thd) uptake was measured on day 3. Bars: A, without culture supernatant; B, with culture supernatant from nonstimulated cells; C, with culture supernatant from Con A-stimulated cells; D, with Con A, at 1 μg/ml. Results are mean ± SEM of triplicate cultures.

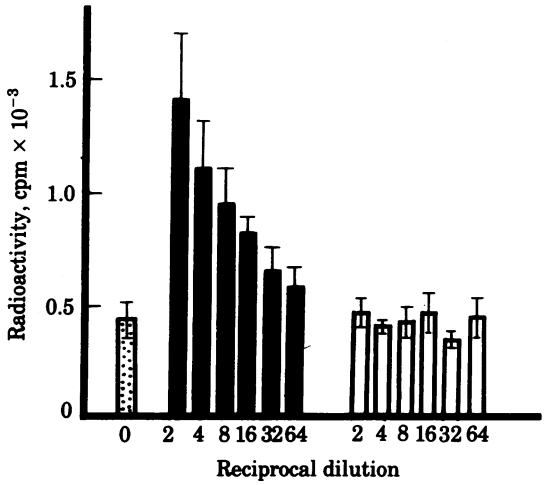

FIG. 2. IL-2 activity of the semipurified factor from 24-A cells was measured by using an IL-2-dependent human cytotoxic T-cell line. Supernatant (50 ml) from 24-A (■) or CEM-AG^R (□) cells cultured at a density of 1 × 10^5/ml was concentrated and applied to a Sephadex G-100 column and the M_r 13,000–20,000 fraction was concentrated to 5 ml on Amicon YM-5 membranes. IL-2 activity of the serially diluted semipurified factor was tested. ▦, Control (3 × 10^3 cells cultured in the absence of IL-2). All cultures were set up in triplicate.

Secretion of Killer Helper Factor(s) by T Hybridomas. Not only a hybrid clone secreting IL-2 but also a clone producing helper factor(s) for the differentiation of cytotoxic T cells has been established. As shown in Table 4, the coculture of normal T cells and UV-treated CESS cells did not induce any cytotoxic T cells against CESS cells. On the other hand, the addition of culture supernatants from hybrid clone 55-A permitted the induction of cytotoxic T cells in the coculture of normal T cells and UV-treated CESS cells. The activity of cytotoxic T cells induced by the addition of the culture supernatants from 55-A cells was comparable with that induced by the addition of culture supernatants from a MLC reaction. The culture supernatants from 55-A cells, which showed the helper effect on the induction of cytotoxic T cells, did not show any IL-2 activity when tested by using an IL-2-dependent cytotoxic T-cell line. These results suggested that clone 55-A produced a killer helper factor(s) that was distinct from IL-2.

Table 3. Effect of macrophages on IL-2 production by T hybrid clone 38-B

Supplement	[^{3}H]Thd uptake, cpm
None	1936 ± 838
38-B/Mϕ/PHA	5131 ± 1684
CEM-AG^R/Mϕ/PHA	2291 ± 865
38-B/PHA	2170 ± 628
CEM-AG^R/PHA	1844 ± 698
Mϕ/PHA	1995 ± 1098
PHA	711 ± 74
IL-2 (0.5 unit)	4214 ± 333

38-B and CEM-AG^R cells were incubated at a density of 1 × 10^5/ml in the presence or absence of macrophages (Mϕ; 0.5 × 10^5/ml) and stimulated with 0.1% PHA. After 48 hr, cell-free supernatants were recovered. IL-2 activity in culture supernatants was measured by using an IL-2-dependent human cytotoxic T-cell line.

Table 4. Effect of supernatants from T hybrid clone 55-A on the differentiation of cytotoxic T cells

Cells		Supernatant	Specific	IL-2 activity,†
Responder	Stimulator	added	cytolysis,* %	unit
T	$CESS_{MMC}$	—	48.0 ± 3.6	
T	$UV\text{-}CESS_{MMC}$	—	−0.6 ± 0.9	
T	$UV\text{-}CESS_{MMC}$	55-A	34.8 ± 0.6	<0.001
T	$UV\text{-}CESS_{MMC}$	MLC	34.4 ± 2.1	0.75

Human PBL T cells (1×10^6) were cultured for 5 days with 0.5×10^5 of mitomycin C (MMC)-treated CESS cells that had been (or had not been) exposed to UV irradiation. Supernatants were obtained from hybrid clone 55-A or a primary MLC. Hybrid clone 55-A cells were incubated at a density of 1×10^5/ml for 24 hr and supernatants were recovered. MLC was set up by using 1×10^6 human PBL T cells and 0.5×10^6 MMC-treated CESS cells for 48 hr. Cell-free supernatants harvested from these cultures were added at final concentrations of 30% and 50% (vol/vol), respectively.

* After 5 days MLC, cytotoxic activity of responder T cells against CESS cells was measured at an effector/target ratio of 10:1.

† IL-2 activity in supernatants was determined by using an IL-2-dependent human cytotoxic T-cell line and Con A-stimulated murine thymocytes. One unit of IL-2 activity represents IL-2 activity obtained from 1×10^6 PHA-stimulated normal T cells.

DISCUSSION

We have established a human T-T hybridoma continuously secreting IL-2 or killer helper factor by using a HAT-sensitive human T leukemic cell line (CCRF-CEM-AG^R) as parental cells. In murine T hybridomas, only two parental T-cell lines, BW5147 and EL-4, have been used for successful hybridization (1–4). Thus, we screened several 8-azaguanine-resistant human T-cell lines (HSB-2, JUR, HPB-ALL, MOLT-4, and CEM) and selected CEM-AG^R as a suitable parental cell line for hybridization with T cells. Establishment of human T hybrid cell lines was confirmed by the following evidence. (*i*) The mean number of chromosomes of hybrid clones exceeded the number of chromosomes of the parental cell line. (*ii*) Hybrid clones expressed HLA phenotypes of both parental cells. (*iii*) A clone, 55-A, expressed Leu 2A antigen, which was not detected on a parental cell line. (*iv*) Twenty percent of cells in clone 55-A formed rosettes with sheep erythrocytes. (*v*) The patterns of mitogen responsiveness of hybrid cells were different from those of the prental cell line. (*vi*) The culture supernatants of 24-A cells showed IL-2 activity, while the supernatants of the parental cell line did not, even when stimulated by Con A.

Not only Con A-induced production of IL-2 in the hybrid clone 24-A but also macrophage-induced production of IL-2 in another hybrid clone, 38-B, was shown. In a murine system, it has been reported that adherent cell-depleted spleen cells failed to produce IL-2 from splenic T cells by stimulation with Con A and that production of IL-2 was restored by the addition of macrophages or their products, IL-1 (12). Gillis and Mizel (13) recently obtained similar results with the murine monoclonal T lymphoma cell line LBRM-33-1A5. The present finding with a human T hybridoma is in agreement with their results that macrophages or IL-1 stimulate production of IL-2 from lectin-stimulated lymphocytes. The contamination of T cells in the adherent cell population could not be entirely excluded. However, it is unlikely that IL-2 activity was derived from the contaminating T cells because addition of the same adherent cell population to the parental cell line did not induce any IL-2 activity. Thus, the experimental model with T hybrid clone 38-B will prove useful for the analysis of IL-1-induced differentiation of helper T cells.

The culture supernatants from T hybrid clone 55-A helped the induction of cytotoxic T lymphocytes (CTL) in the primary MLC using UV-treated stimulator cells. Interestingly, however, IL-2 activity could not be detected in the supernatants when examined by using an IL-2-dependent human cytotoxic T-cell line or Con A-stimulated murine thymocytes. In the analysis of soluble factor(s) involved in the generation of CTL, it has been suggested that IL-2 is essential for the induction of CTL (14, 15). On the other hand, Farrar *et al.* (16) suggested that another soluble factor(s), immune-interferon, had helper activity in the generation of CTL and that IL-2 was required for the production of immune-interferon. The supernatants from 55-A cells showed helper activity in the induction of CTL but little IL-2 activity, suggesting that the hybrid cells produced factor(s) distinct from IL-2, which were effective mainly on differentiation of CTL. However, another possibility might be considered—i.e., the supernatants from 55-A cells might induce IL-2 production in T-cell populations involved in primary MLC. Actually, it has been suggested that supernatants from a macrophage tumor cell line (17, 18) can induce the primary CTL responses via IL-2 production.

Another approach for the large-scale purification of IL-2 has been reported by Gillis and Watson (19). They screened a large number of human leukemic T-cell lines for the production of IL-2 and found one, Jurkat-FHCRC, that was capable of secreting IL-2 after stimulation with Con A or PHA. No doubt this cell line will facilitate the chemical characterization of IL-2. However, it may be impossible to find T leukemic cell lines that produce several other lymphokines such as killer helper factor(s) or T-cell replacing factor. In such circumstances, the establishment of human T hybridomas should provide a tool for obtaining T-cell clones producing several different human immunoregulatory molecules. These may be applicable for the management and treatment of disorders in immune regulation, including immunodeficiency diseases, autoimmune diseases, immediate type hypersensitivity, and some types of cancer.

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