The EMBO Journal vol.6 no.9 pp.2705–2709, 1987

Retroviral expression of the human IL-2 gene in a murine T cell line results in cell growth autonomy and tumorigenicity

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Communicated by C.Weissmann

In mature T lymphocytes (T cells) the regulated expression of the genes for interleukin-2 (IL-2) and its receptor (IL-2R) constitutes an essential part in controlling the cell growth. Evidence has been provided which suggests the involvement of an aberrant function of the IL-2 system in developing T cell neoplasms, particularly the adult T cell leukemia/lymphoma (ATL). As an approach to examine the extent of the IL-2 system contribution to T cell neoplasms, we created the experimental conditions wherein both IL-2 and IL-2R are expressed constitutively in a murine T cell line. We made use of a retroviral vector to infect an IL-2-dependent CTLL-2 line and lead to the expression of human IL-2. Here, we show that the virus-infected cells not only proliferate *in vitro* in the absence of exogenously supplied IL-2 under certain conditions, but also develop tumors (lymphomas) in nude and syngeneic mice.

Key words: autocrine growth/IL-2/lymphoma/retrovirus/T lymphocyte

Introduction

Interleukin-2 (IL-2), the T cell growth factor, plays a key role in the antigen-specific clonal growth of T lymphocytes (T cells). Normally T cells reside in a resting stage and they transiently express IL-2 and its functional receptor (IL-2R) upon antigen stimulation, leading to the onset of cell multiplication. This transient expression of the IL-2 and IL-2R allows the T cells to go through only a limited number of rounds of proliferation and therefore it is believed to be an important feature in controlling T cell overgrowth (for review see Smith, 1980; Taniguchi *et al.*, 1986; Greene and Leonard, 1986). The regulatory DNA sequences responsible for the controlled expression of the human IL-2 and IL-2R (Tac antigen or p55) genes have been identified (Fujita *et al.*, 1986; Maruyama *et al.*, 1987; Durand *et al.*, 1987).

Many of the recent studies concerning the malignant transformation of cells focus on the potential oncogenic properties of growth factors and their receptors. In the concept of autocrine stimulation of cellular growth, a given cell can both produce and respond to a certain growth factor and it has been postulated that an aberrant operation of such an autocrine growth stimulation results in or contributes to the malignant transformation of the cells (Todaro *et al.*, 1977; Sporn and Todaro, 1980). Certain transformed cells express in perhaps a deregulated manner endogeneous genes encoding the growth factors to which they respond (De Larco and Todaro, 1978; Kaplan *et al.*, 1982; Graf *et al.*, 1986; see Sporn and Roberts, 1985 for review). In addition the oncogene of simian sarcoma virus encodes a factor that is very similar if not identical to platelet-derived growth factor (Doolittle *et al.*, 1983; Waterfield *et al.*, 1983). More recently, examples of the acquisition of tumorigenic properties following the introduction and constitutive expression of growth factor genes in non- (or less-) tumorigenic cells have also been reported for granulocyte-macrophage colony stimulating factor (GM-CSF) (Lang *et al.*, 1985), tumor growth factor-α (TGF-α) (Rosenthal *et al.*, 1986; Watanabe *et al.*, 1987) and epidermal growth factor (EGF) (Stern *et al.*, 1987).

In T cells, the involvement of the IL-2 system in T cell neoplasms has been studied particularly in relation to the development of adult T cell leukemia/lymphoma (ATL) (Hattori *et al.*, 1981; Gootenberg *et al.*, 1981; Depper *et al.*, 1984; Waldmann *et al.*, 1984; Greene *et al.*, 1986; Inoue *et al.*, 1986; Maruyama *et al.*, 1987; Shibuya *et al.*, 1987). Most if not all ATL cells are of $CD4^+$ helper T (i.e. IL-2 producer cell) origin and believed to be caused by a lymphotropic retrovirus, human T cell leukemia virus type I (HTLV-1) (for review see Gallo *et al.*, 1984). We recently provided experimental data which suggest that the aberrant activation of the IL-2 autocrine loop is caused in the virus-infected helper T clone(s) by the expression of the virus-coded *trans*-activator protein $p40^x$ (tat I) and subsequent extracellular stimulation by antigen(s) (Maruyama *et al.*, 1987; Shibuya *et al.*, 1987). We postulated that the aberrant operation of the IL-2 autocrine loop may induce the malignant state of certain virus-infected T cells. From this view point, an intriguing issue has been to what extent the IL-2 autocrine stimulation contributes to the leukemogenicity of T cells.

To examine if, under any circumstances, IL-2 autocrine stimulation contributes to the generation of tumorigenic T cells, we constructed a retrovirus which upon infection of cultured cells gives rise to the production of human IL-2. When an IL-2-dependent murine T cell line was infected with this virus, the cells gained growth autonomy *in vitro* under certain conditions and developed tumors (lymphomas) in nude and syngeneic mice.

Results

Construction and characterization of a retroviral vector for human IL-2 expression

We constructed a plasmid termed pZipSVIL2 by inserting a piece of the human IL-2 cDNA into a Moloney murine leukemia virus-derived vector, pZipSV(X)-1 (Cepko *et al.*, 1984). Essentially, the IL-2 cDNA from the *Mnl*I site at nucleotide 41 to the *Aat*I site at nucleotide 542 (see Taniguchi *et al.*, 1983) was inserted into the single *Bam*HI site of the vector through a series of cDNA manipulations (see Figure 1). The plasmid DNA was transfected into the virus-packaging line ψ_2 (Mann *et al.*, 1983) and four G418 resistant transformant clones were subsequently isolated. The infectious virus titer in the culture supernatants of each of

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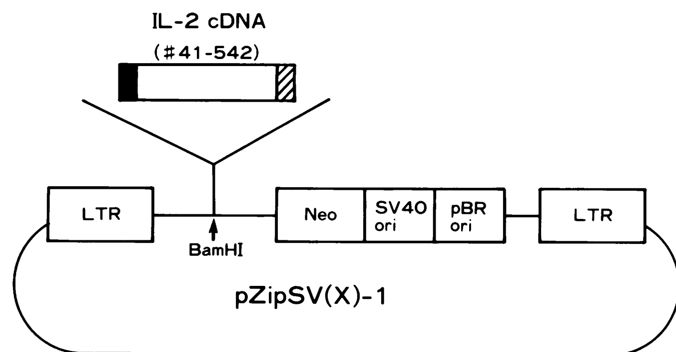

Fig. 1. Construction of pZipSVIL2. Human IL-2 cDNA was introduced into the *Bam*HI site of pZipSV(X)-1. The introduced cDNA fragment (nucleotide 41–542; Taniguchi *et al.*, 1983) contains additional sequences as illustrated by black and hatched boxes. The black box should consist of the following sequence; (5′) GATCCGAGCTTGGGCTGCAGGTC (3′). The hatched box should consist of the following sequence; (5′) CCTCGACG (3′).

Table I. Detection of IL-2 activity in the culture supernatant of the CTLL-2 transformant clones

CTLL-2 transformant clones		IL-2 activity (unit/ml) Culture supernatant collected on day 1	2	3
A3	Clones with IL-2 gene	0.8	4.2	12.2
B5		<0.1	0.1	0.8
B6		<0.1	<0.1	2.4
C2	No IL-2 gene	<0.1	<0.1	<0.1
C6		<0.1	<0.1	<0.1

Each of the transformant cells was seeded at 10^5 cells and, after 1–3 days, the culture supernatant was collected and IL-2 activity determined (see Materials and methods).

the four G418 resistant ψ_2 clones was monitored by the number of G418 resistant L929 cell colonies which appeared after virus infection, essentially as described by Cepko *et al.* (1984). The titer of the virus, designated HIL2V, ranged between 9×10^3 and 4×10^4 c.f.u./ml in those four ψ_2 transformant clones, and a clone which gave the highest titer was used for virus production for the subsequent studies. The level of the IL-2 secreted by the L929 cells infected with HIL2V was between 1.7×10^3 and 3.3×10^3 units/ml in 3 days conditioned medium. In contrast, culture media of the L929 cells infected with the virus recovered from pZipSV(X)-1-transfected ψ_2 cells (SVX virus; Cepko *et al.*, 1984) showed no IL-2 activity.

Characterization of IL-2-dependent murine T cell line infected with HIL2V

We next infected murine CTLL-2 cells with either HIL2V or SVX virus. CTLL-2 is a C57BL/6-derived cytotoxic T cell line that is dependent on exogeneously supplied IL-2 for its growth (Gillis *et al.*, 1978). One may then expect that infection of the cells with HIL2V results in the operation of IL-2 autocrine loop. One may also expect that the infected cells acquire growth autonomy, depending on the IL-2 and IL-2R expression levels. CTLL-2 cells were infected by exposing them to the culture supernatant of the ψ_2 transformant clones producing either HIL2V or the control SVX virus (see Materials and methods for the details). The CTLL-2 cells were initially selected for G418 resistance in the presence of conditioned medium of ConA-stimulated mouse spleen cells (ConA sup) and subsequently in

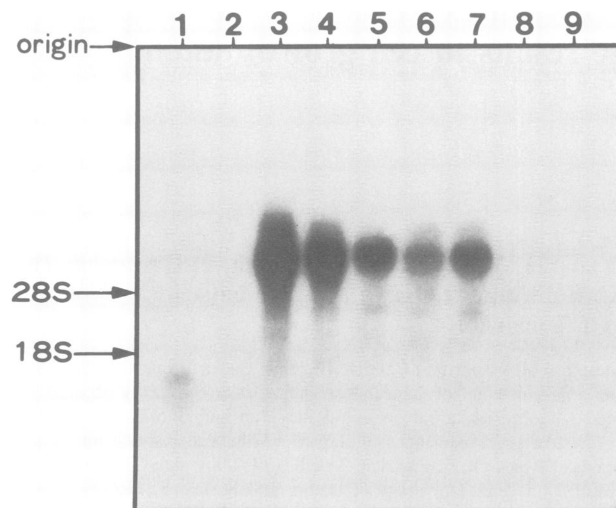

Fig. 2. Analysis of the IL-2 specific transcript in L929 and CTLL-2 transformant cells. The following RNA samples were subjected to the blotting analysis by using the nick-translated human IL-2 cDNA as the probe (see Materials and methods). **Lane 1**; 1 μg of poly(A) RNA from mitogen-activated human peripheral blood lymphocytes. Each lane from 2 to 9 contains 15 μg of total RNA from the following cells; **lane 2**, L929 transformants infected with SVX virus; **lane 3**, L929 transformants infected with HIL2V virus from ψ_2 clone 1; **lane 4**, L929 transformants infected with HIL2V virus from ψ_2 clone 4; **lane 5**, CTLL-2 clone B5; **lane 6**, CTLL-2 clone B6; **lane 7**, CTLL-2 clone A3; **lane 8**, CTLL-2 clone C2; **lane 9**, CTLL-2 clone C6.

the absence of the ConA sup in the case of HIL2V. The G418 resistant cells were further cloned by the limiting dilution method using a similar selection protocol (see Materials and methods and Discussion) and several clones were established. Neither those clones nor EL-4 thymoma cells formed colonies on a soft agar in our hands. All of the selected clones have been maintained in the absence of the ConA sup for more than 7 months. In the case of SVX virus, however, continuous supply of ConA sup was absolutely required to obtain any G418 resistant clones.

Southern blot analysis of the HIL2V-infected clones A3, B5 and B6, which were chosen arbitrarily for further studies, revealed the presence of a single copy of the proviral genome each integrated into a different site of the host chromosomes (result not shown). In contrast to HIL2V-infected L929 cells, all of the CTLL-2 transformant clones A3, B5 and B6 released little if any IL-2 in the culture media (Table I).

We then performed an RNA blotting analysis with each of the virus-infected L929 and CTLL-2 cells. As shown in Figure 2, all of the HIL2V-infected cells produced RNA containing the IL-2 gene sequence and the length of the major RNA was ~5000–5500 nucleotides. This RNA is diagnostic for the transcript of the entire provirus genome (5200 nucleotides long) which should direct the synthesis of IL-2. In this analysis, the level of the IL-2 specific RNA is only ~3 (clone A3, B5) to 6 (clone B6) fold lower in the CTLL-2 transformants compared to that of the L929 transformants. Hence, the difference in the IL-2 activity detectable in the culture supernatant between virally infected L929 and CTLL-2 cells may largely be due to the continuous consumption (i.e. internalization) of IL-2 in the infected CTLL-2 cells that is required for their autonomous growth (see below).

To gain more insight on the nature of the autonomous growth of the CTLL-2 transformants, we carried out an *in vitro* cell proliferation assay. As illustrated in Figure 3, the CTLL-2 transfor-

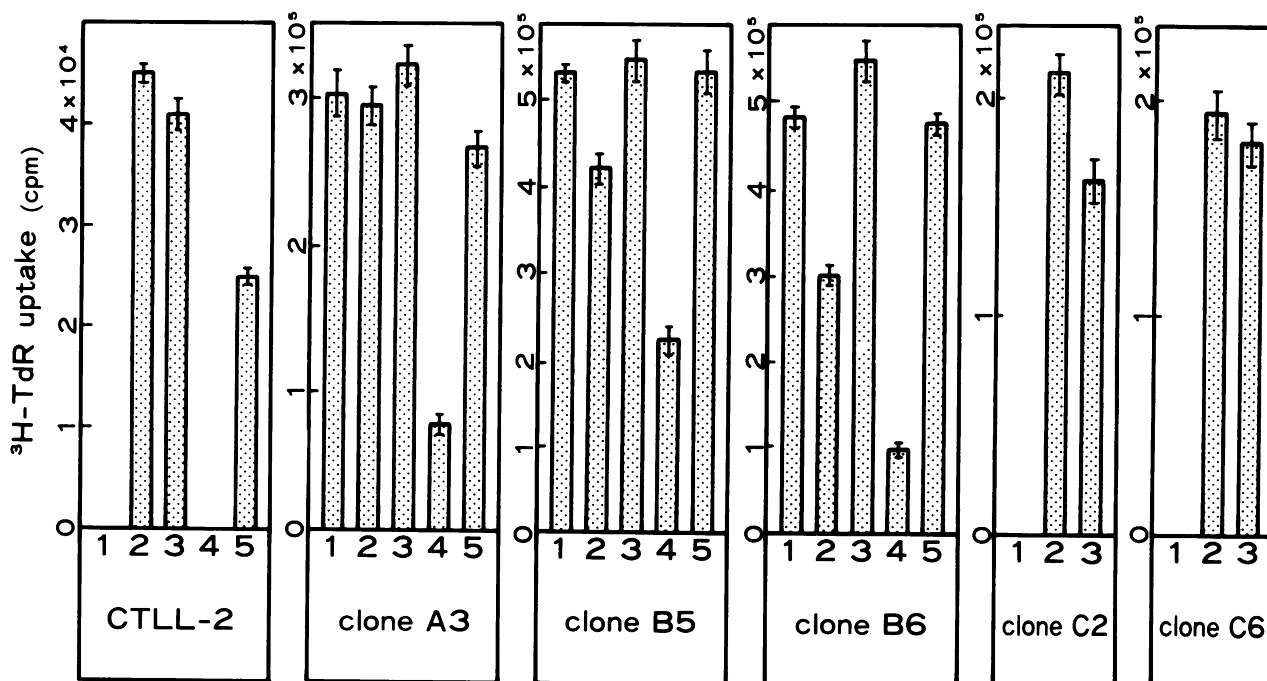

Fig. 3. *In vitro* cell proliferation assay of CTLL-2 cells and CTLL-2 transformant clones. Cell proliferation assay has been performed as described in Materials and methods. Cells were cultured in the following medium before the addition of [^{3}H]thymidine: **1**, RPMI1640 (10% FCS) alone; **2**, RPMI1640 (10% FCS) + human recombinant IL-2 (100 units/ml); **3**, RPMI1640 (10% FCS) + human recombinant IL-2 (20 units/ml); **4**, RPMI1640 (10% FCS) + antibody AMT-13 (at 1:200 dilution of ascites fluid); **5**, RPMI1640 (10% FCS) + antibody 23-7 (at 1:200 dilution of ascites fluid). In the case of parental CTLL-2 cells, the [^{3}H]TdR uptake is relatively lower compared to C2 and C5 clones. This difference is mostly due to the cell maintenance conditions, since we observed the comparable [^{3}H]TdR uptake in another set of assays.

Table II. Tumor formation in mice injected with CTLL-2 transformants

CTLL-2 derived clones injected	Mice with tumors/number injected			
	Exp. 1 BALB/c nu/nu i.p.	Exp. 2 BALB/c nu/nu s.c.	Exp. 3 BALB/c nu/nu s.c.	Exp. 4 300 rad-irradiated C57BL/6 i.p.
A3	5/5	5/5	N.D.	4/7
B5	5/5	N.D.	6/6	3/7
B6	4/4	5/5	N.D.	4/5
C2	0/5	0/4	0/4	0/5
C6	0/5	0/4	0/4	0/5

Five weeks after injection, mice with tumors >10 mm in diameter were scored positive. All the mice with the developed tumor died within 4–8 weeks. No salient effects were observed in mice injected with clone C2 or clone C6 within 12 weeks. N.D. = not done.

mant clones A3, B5 and B6 incorporated [^{3}H]thymidine in the absence of exogenously supplied IL-2 and the [^{3}H]thymidine uptake was not significantly augmented by the exogenously added IL-2. Rather, the [^{3}H]thymidine uptake was somewhat suppressed by IL-2 at higher concentration (100 units/ml) in those clones and this phenomenon was not seen in IL-2-dependent CTLL-2 cells (Figure 3). The significance of this observation is not clear at present. As expected, in parental CTLL-2 cells as well as in the G418 resistant CTLL-2 transformant clones (C2 and C6) which were established by infecting the SVX virus, cell growth was totally dependent on exogeneously added IL-2. The [^{3}H]-thymidine uptake of the clones A3, B5 and B6 was inhibited by an anti IL-2R monoclonal antibody, AMT13 (Osawa *et al.*, 1984) but not by a control antibody (H.Osawa, unpublished) (Figure 3), indicating that the cells secrete IL-2 and utilize it for their growth. Interestingly, we could not block completely the [^{3}H]-thymidine uptake of those clones at an antibody concentration sufficient to block >95% of the IL-2 dependent CTLL-2 growth at an IL-2 concentration of 20 units/ml (Figure 3). Moreover, no further growth inhibition was observed by 2-fold increase of the antibody (data not shown; see Discussion). As expected, flow microfluorimetric analysis of clones A3, B5, B6 as well as C2, C6 revealed that they all expressed IL-2R (p55) similar to the parental CTLL-2 cells (data not shown). Taken together, the IL-2 autocrine mechanism is most likely operative in these transformant clones and responsible for the cellular growth.

Tumorigenic phenotype of the HIL2V-infected T cell clones

Does the operation of the IL-2 autocrine mechanism in the cultured T cell line contribute to the development of malignant phenotype of the cells to any degree *in vivo*? We tested the IL-2-independent CTLL-2 clones for their ability to develop tumors (lymphomas) in mice, by injecting 10^6 to 5×10^6 cells of each clone either subcutaneously or intraperitoneally into BALB/c nu/nu and irradiated syngeneic C57BL/6 mice. Essentially all the nude mice injected with the IL-2-independent

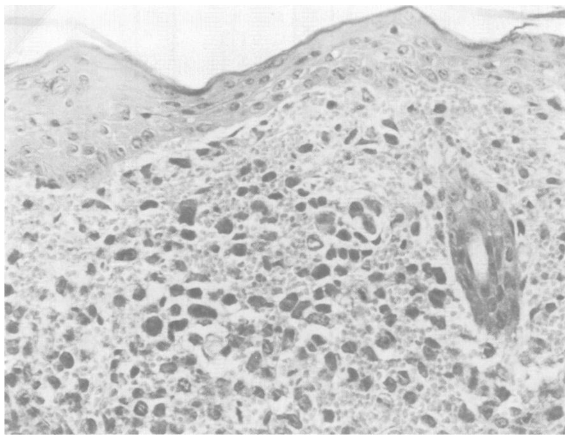

Fig. 4. Proliferation of clone B6 cells at the injection site. Clone B6 cells were injected subcutaneously with 10^6 cells into a BALB/c nu/nu mouse; the recipient was killed 4 weeks after the injection. H&E, ×280.

CTLL-2 clones developed progressively growing tumors and most of the animals died within 4–8 weeks following the transplantation whereas no salient effect was observed with the mice injected with the IL-2-dependent CTLL-2 clones (Table II). The transplanted tumor developed large diffusely infiltrating tumor mass at the site of the injection as shown in Figure 4. So far, we have not observed tumor invasion into organs such as spleen, liver and lung. The tumorigenic properties of the cells were also demonstrable following transplantation into irradiated syngeneic mice (Table II).

Discussion

We have introduced and expressed the gene encoding the human IL-2 in an IL-2-dependent murine T cell line by using a retrovirus vector. The T cells infected with the IL-2-producing retrovirus HIL2V proliferated in the absence of IL-2 *in vitro* (Figure 3). Preliminary results indicate that the initial presence of the ConA sup and feeder cells is required in the presently employed procedure for cloning. Such a requirement may depend on the culture conditions of the clones, and this possibility needs to be further investigated.

In all the virus-infected T cell clones, a cell proliferation assay revealed that the monoclonal antibody AMT-13 partially blocked the [^{3}H]thymidine uptake. Further increase of the antibody (2-fold) did not block further the [^{3}H]thymidine uptake. A somewhat similar observation has been reported with GM-CSF-dependent murine FDC-P1 cells infected with a GM-CSF producing retrovirus. An antiserum against GM-CSF was unable to inhibit the proliferation of the cells to a significant degree (Lang *et al.* 1985). Although the molecular basis for these phenomena is not clear at present, they may represent an advantageous feature of the autocrine growth of the cells.

Involvement of growth factors in the development and/or maintenance of the tumorigenic phenotype of the cells has been documented by numerous studies (see Sporn and Roberts for review, 1985). Acquisition of the tumorigenicity in hemopoietic and fibroblast cell lines by the introduction of the genes encoding the growth factors to which they respond has also been recently reported (Lang *et al.*, 1985; Rosenthal *et al.*, 1986; Watanabe *et al.*, 1987; Stern *et al.*, 1987). In the IL-2 system, several lines of evidence suggest the involvement of the IL-2 autocrine mechanism in developing T cell neoplasms. In the case of adult T cell leukemia/lymphoma (ATL), a number of studies suggest the involvement of the IL-2 system in the process of malignant cell transformation that is restricted to $CD4^+$ T cells *in vivo* (see Gallo *et al.*, 1984). We recently provided experimental data which suggest that the aberrant activation of the IL-2 autocrine loop is caused by the expression of HTLV-1-encoded $p40^x$ (tatI) and subsequent extracellular stimulation by an antigen(s) (Maruyama *et al.*, 1987; Shibuya *et al.*, 1987). In most cases of ATL, the leukemic T cells express IL-2R on their surface but respond poorly to IL-2 *in vitro*, suggesting the occurrence of an additional event(s) in the ATL establishment *in vivo* (Uchiyama *et al.*, 1985). On the other hand, some ATL cells manifest IL-2 dependency for their *in vitro* growth (Maeda *et al.*, 1985; T.Hattori, Kumamoto University, personal communication). In addition, IL-2 autocrine stimulation seems to account directly for the *in vitro* proliferation of another type of ATL cells (Arima *et al.*, 1986; K.Onoue, T.Hattori, Kumamoto University, personal communication). It remains to be shown how such *in vitro* properties of those ATL cells correlate with their leukemogenic property *in vivo*. A human T cell line, the growth of which is dependent on the IL-2 autocrine loop *in vitro*, has also been isolated from a patient with non-Hodgkin T cell lymphoma (Duprez *et al.*, 1985).

In the present study, we created the experimental conditions wherein a proviral genome was introduced into a cultured T cell line to produce IL-2 for the acquisition of growth autonomy. Our results demonstrate that the operation of the IL-2 autocrine loop indeed results in the generation of T cell clones each of which develops tumor (lymphoma) *in vivo*. While an intriguing possibility remains that further genetic changes have been induced in those T cell clones as a consequence of the continuous autocrine stimulation in the acquisition of their malignant phenotype *in vivo*, we think it more likely that the operation of the IL-2 autocrine loop is at least in part responsible for the tumor growth as both IL-2 and IL-2R mRNAs are still detectable in the RNA preparation of tumours (unpublished observation). The CTLL-2 is an *in vitro* established T cell line which constitutively expresses IL-2R and one may envisage that the cells might have already undergone multiple genetic alterations toward the acquisition of malignancy like other immortalized cell lines such as NIH3T3 and FDC-P1. In this regard, it may be of interest to examine whether or not introduction and constitutive expression of both IL-2 and IL-2R genes in normal T cells also result in the development of malignant T cells under certain circumstances. Whatever the mechanisms, our results provide further evidence that the IL-2 autocrine loop is of importance in the development and/or maintenance of lymphoma/leukemia cells under certain circumstances in naturally occurring T cell neoplasms.

Materials and methods

DNA manipulations

Construction of the retrovirus vector pZipSVIL2 was carried out according to the standard procedure (Maniatis *et al.*, 1982). Before cloning into the vector, the human IL-2 cDNA has gone through multi-step manipulations and, consequently, it is flanked by some additional DNA sequences derived from synthetic linkers (see legend to Figure 1). Essentially, a piece of cDNA expanding from nucleotide 41 to 542 (see Taniguchi *et al.*, 1983) was inserted into the *Bam*HI site of pZipSV(X)-1.

Cell cultures

Both L929 and ψ_2 cells (Mann *et al.*, 1983) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS). Parental CTLL-2 as well as virus-infected CTLL-2 cells were cultured in RPMI1640 medium supplemented with 10% FCS either in the presence or in the absence of mouse ConA sup (10%), as indicated in the text. Mouse ConA sup was prepared according to a published procedure (Gillis *et al.*, 1978).

DNA transfections

$5 \times 10^5 \psi_2$ cells were transfected with 10 μg of each vector DNA per 100 mm diameter Petri dish using the calcium phosphate co-precipitation methods as described by Wigler *et al.* (1979). Cells were then selected for G418 resistance as described by Cepko *et al.* (1984). Subsequently the resistant cells were cloned by the cloning cylinders.

Viral infections and IL-2 assays

Infectious retrovirus was collected in the supernatant of virus producing ψ_2 clones. To infect the virus to L929 cells, 5×10^5 cells were seeded in 100 mm diameter dishes and mixed with 1 ml of the ψ_2 culture supernatant plus polybrane (8 μg/ml) and cultured for 2 h at 37°C as described previously (Cepko *et al.*, 1984). Two days after infection, cells were selected for G418 resistance (700 μg/ml) and resistant colonies were detectable ~2 weeks later. The virus titers were expressed as G418-resistant c.f.u./ml of the ψ_2 supernatant. Similarly, 1×10^5 CTLL-2 cells were infected with the same amount of retrovirus in the presence of 10% mouse ConA sup. Two days after infection, the cells were cultured in the presence of G418 (700 μg/ml) and 10% mouse ConA sup. About 2 weeks later, the G418 resistant CTLL-2 cells were grown in the absence of the ConA sup. Those cells were cloned by the limiting dilution method in the presence of ConA sup and 3000 rad-irradiated C57BL/6 thymocytes as feeder cells (10^4 cells per well in a 96-well microtiter plate). After two weeks, cells were grown without ConA sup and the feeder cells. To measure the level of IL-2 released from L929 and CTLL-2 transformants, they were washed three times with RPMI1640 (10% FCS) and seeded at 10^5 cells per well in 24-well flat-bottomed culture plates. After 1–3 days of culture, the culture supernatant was collected and IL-2 activity was measured by the standard procedure (Gillis *et al.*, 1978).

RNA blotting analysis

Total cellular RNA was isolated from each cell by the standard procedure (Maniatis *et al.*, 1982) and subjected to the RNA blotting analysis (Thomas, 1980). Human PBL was isolated and cultured at 5×10^6 cells/ml in RPMI1640 (10% FCS) with PHA (5 μg/ml) and TPA (5 ng/ml) for 24 h. The IL-2 specific *Bam*HI fragment was derived from pZipSVIL2 and used as the probe.

Cell proliferation assay of parental and virus-infected CTLL-2 cells

Cells were washed three times with RPMI1640 (10% FCS) and plated at 1.5×10^4 per well in 96-well flat-bottomed microculture plates. Cells were cultured in RPMI1640 (10% FCS) in the presence or absence of human recombinant IL-2. In some cases, rat anti-mouse IL-2R monoclonal antibody AMT-13 (Osawa and Diamantstein, 1984) or control antibody 23-7 (the same subclass as AMT-13; i.e. IgG2a) was simultaneously added at 1:200 dilution of ascites fluid. After 24 h, 1 μCi of [^{3}H]thymidine was added to each culture well, incubation was continued for another 12 h and [^{3}H]thymidine incorporation was measured. The specific activity of the human recombinant IL-2 was 5×10^7 U/mg protein.

Transplantation of the virus-infected CTLL-2 clones

CTLL-2 transformant clones were washed three times with PBS and injected intraperitoneally with 5×10^6 cells or subcutaneously with 10^6 cells into BALB/c nu/nu mice (5 weeks old; purchased from Japan Clea Inc.) or 300 rad-irradiated C57BL/6 mice (5 weeks old; purchased from Shizuoka Agricultural Cooperative Association for Laboratory Animals, Hamamatsu, Japan).

Acknowledgements

We thank Drs H.Sugano, C.Weissmann, J.Hamuro, M.Hatakeyama, K.Yamasaki, Y.Fukushima and Y. Oikawa for their advice and help. We are also indebted to Dr E.Barsoumian for critical reading of the manuscript and Ms M.Nagatsuka for excellent assistance. The work is supported in part by a Research Grant of the Princess Takamatsu Cancer Research Fund and a grant-in-aid for Special Project Research, Cancer-Bioscience, from the Ministry of Education, Science and Culture of Japan.

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Received on April 16, 1987; revised on May 29, 1987