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MECHANISM OF T CELL PROLIFERATION *IN VIVO*: ANALYSIS OF IL-2 RECEPTOR EXPRESSION AND ACTIVATION OF c-*myc* and c-*myb* ONCOGENES DURING LYMPHATIC REGENERATION

M. Sihvola*[,1], L. Sistonen**, K. Alitalo** and M. Hurme*

*Department of Bacteriology and Immunology and the **Department of Virology, University of Helsinki, SF-00290, Helsinki, Finland

Received February 17, 1989

Summary The mechanism of T cell proliferation was studied using *in vivo* lymphatic regeneration as the model. Lymphatic regeneration was induced by injecting a sublethal dose (300 mg/kg) of cyclophosphamide (Cy) into mice. Majority of the regenerating splenic T cells were found to be in the cell cycle, nearly 30 % being found in S/G_2+M phases resembling the ratio obtained for mitogen activated T cells *in vitro*. Expression of interleukin-2 receptor (IL-2R) was defined by the monoclonal anti-IL-2R antibody, AMT-13. Only 1-3% of regenerating T cells were IL-2R positive (while about 30 % of the in vitro activated T cells were IL-2R positive). Accordingly, these cells did not respond to IL-2 *in vitro*. However, when the freshly isolated regenerating T cells were cultured in the presence of Con A or PMA + ionophore A 23187, IL-2R was readily induced. The regenerating T cells were further analyzed for the expression of the cellular oncogenes c-*myc* and c-*myb*. These cells expressed about three times more c-*myb* mRNA than Con A-stimulated T cells and the levels were comparable to those seen in thymocytes. By contrast, the amount of c-*myc* mRNA was similar in the regenerating T cells and in Con A-activated T cells, but weak or barely detectable in splenocytes and thymocytes. Taken together, our results imply that the vigorous T cell proliferation during cyclophosphamide-induced lymphatic regeneration is independent of the IL-2/IL-2R hormone system, like T-cell precursor proliferation in the thymus, and is characterized by both high c-*myb* expression typical for thymocytes and high c-*myc* expression typical for *in vitro* proliferation-activated T cells.

The peripheral T cell population is maintained constant throughout the adult life. New T cells are continuously produced in the thymus but vast majority of them die *in situ*, and only ca. 1% of thymocytes leave the thymus per day corresponding 2-3% of peripheral T cells (1). Although part of the peripheral T cells are long-lived cells (2-4), 30-50% of them belong to a short-lived cell population (3,5,6). Thus, thymic emigrants cannot be solely responsible for the renewal of peripheral T cell pool, but post-thymic expansion of T cells occurs (6-11).

Of the mechanisms which drive T cells to divide, interleukin-2/interleukin-2 receptor (IL-2/IL-2R) hormone system is well characterized (12-14). Thus, *in vitro* T cell proliferation has been shown to be strictly dependent on the induction of IL-2 production and IL-2R expression and the consequent IL-2/IL-2R interaction. There are, however, some antigen-specific T cell lines which have shown to proliferate indendently of IL-2 *in vitro* (15,16). The role of IL-2/IL-2R in regulating T

[1]Correspondence: M. Sihvola, Department of Bacteriology and Immunology, University of Helsinki, Haartmaninkatu 3, 00290 Helsinki, Finland.

Abbreviation used in this paper: Cy, cyclophosphamide; IL-2R, interleukin-2 receptor; NW, nylon wool; SI, stimulation index.

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cell division *in vivo* is not precisely known, and conflicting results exist (17-21). In the thymus, cell proliferation has been shown to occur independently of IL-2/IL-2R interaction (22-24).

We have studied the mechanism of peripheral T cell poliferation using a model of lymphatic regeneration after a sublethal dose of cyclophosphamide (300 mg/kg). The cell cycle distribution of regenerating T cells and the proportion of cells expressing the IL-2 receptor was evaluated. The cells were further analyzed for the expression of the cellular oncogenes c-*myc* and c-*myb* since these oncogenes have been implicated in T cell proliferation and differentiation (25-30). We show here that a large number of the regenerating T cells, 8 days after administration of Cy, is found to be in the cell cycle, still only a few of them express the IL-2 receptor. Furthermore, these cells do not respond to exogenous IL-2. When stimulated *in vitro*, however, IL-2R is induced. Thus these data indicate that the regenerating T cells proliferate independently of the IL-2/IL-2R system. The regenerating T cells are also characterized by the high expression of both c-*myc* and c-*myb* oncogenes.

MATERIALS AND METHODS

Mice. (CBAxC57BL/6)F_1 mice were obtained from the breeding unit of this department and were used at the age of 2-4 months.

Administration of cycloposphamide (Cy). Cy (Syklofosfamid, Lääke Oy, Turku, Finland) was dissolved in sterile water immediately before use, and a sublethal dose (300 mg/kg) was injected intraperitoenally.

Preparation of cells. Spleens were removed aseptically and single cell suspensions were prepared (in RPMI 1640 medium supplemented with 20mM HEPES and 5% fetal calf serum (FCS; Flow Laboratories, Irvine, Scotland)). T cells were prepared by passing spleen cells through a nylon wool column twice according to the method described by Julius et al (31).

Cell cultures. The *in vitro* cultures were carried out in RPMI 1640 medium supplemented with 10% FCS, L-Glutamine, $5x10^{-5}$ M 2-mercaptoethanol and antibiotics at 37 °C, in a 5% CO_2 atmosphere. As stimulants, we used 4 µg/ml Concavalin A (Con A; Pharmacia, Uppsala, Sweden), and 10 ng/ml phorbol myristate acetate (PMA; Sigma Chemical Co., St. Louis, Mo) together with 1 nM ionophore A 23187 (Calbiochem, San Diego, Ca), and recombinant IL-2 20 U/ml (Genzyme Corp., Boston, Ma). Cells ($1x10^5$) were cultured in flat-bottomed microtitre plates (Costar 3595, Cambridge, Ma) for 24-48 h, and they were pulsed with 1 µCi ^{3}H-Thymidine (24 Ci/mmol; Radiochemical Center, Amersham, UK) 6 h before harvesting on filters with an automatic cell harvester (Dynatech). The filters were air-dried and counted in a liquid scintillation counter. The data are expressed as mean of cpm of triplicate cultures. In some experiments, the stimulation index (SI) was calculated as cpm in stimulated cultures/cpm in unstimulated cultures.

Antibody treatments. Monoclonal anti-mouse interleukin-2 receptor antibody (clone AMT-13 (32,33), and monoclonal anti-Thy-1.2 (clone F7D5) were kindly provided by Dr Tibor Diamantstein (Freie Universität, Berlin, FRG) and Dr Elizabeth Simpson (Clinical Research Center, Harrow, UK), respectively. For staining, the cells (10^7 /ml) were first incubated for 30 min at 4 °C with the appropriate antibody, washed twice and incubated for an additional 30 min at 4 °C with fluorescein (FITC)-conjugated anti-rat (for AMT-13) or anti-mouse IgG (both products of Cappel Worthington, Malvern, Pa). After washings, cells were analyzed or isolated with the fluorescence-activated cell sorter FACS IV (Becton Dickinson, Sunnyvale, Ca).

Cell cycle analysis. For the determination of the DNA content of cells, they were stained with 50 µg/ml of the DNA binding dye propidium iodide (Sigma) in culture medium containing 0,1% NP 40 (34).

RNA preparation and analysis. The isolation of polyadenylated RNA from cell lysates was done by oligo(dt) chromatography as described (35). RNA was quantitated spectrophotometrically at 260 nm, samples were size-fractionated on 0,8% formaldehyde-agarose gels, transferred to a nylon membrane (Pall, Glen Cove, Ny), and baked under vacuum at 80 °C for 2 h. Filters were hybrizied overnight at 42 °C with ^{32}P-labelled probes c-*myc* insert of the plasmid pSVc-myc-1 (41029, American Tissue Type Collection, Rockville, MD) (36) and with PMB96, a full-lenght c-*myb* cDNA (37), a kind gift from Dr Thomas Gonda (Melbourne Tumour Biology

Branch, Ludwig Institute for Cancer Research, Royal Melbourne Hospital, Australia). After washings in 1xSSC (150mM NaCl, 15mM sodium citrate), 0,1% SDS at 60 °C, filters were exposed to Kodak AR X-Omat films at -70°C. Autoradiograms were analyzed by laser-densitometric scanning (LKB, Uppsala, Sweden).

T cells in the experiments were derived from spleens of 20-30 mice, and each experiment reported was repeated at least three times with concordant results. The values are given from representative experiments.

RESULTS

Cell-cycle analysis of regenerating T cells. Injection of mice with a sublethal dose of Cy (300 mg/kg) causes a rapid involution of the spleen within 1-2 days (decline from the normal 60-100x10^6 cells to 1-2x10^6 cells per spleen by day 2). This is followed by an extensive regeneration phase on days 6-9 after Cy administration (increase to 60-80x10^6 cells by day 8 after Cy) (38, 39).

The cell cycle distribution of regenerating splenic T cells was analyzed 8 days after Cy. As shown in Table 1, nearly 30 % of regenerating T cells were found to be in the S/G2+M phases resembling the cell cycle status of T cells activated with Con A for 24 h *in vitro*. Furthermore, only a small proportion of regenerating and Con A-activated T cells were found to be resting as judged by the acridine orange staining which separates resting cells from the cells entered the cell cycle (40; data not shown). As normal controls, we used 3-4 months old mice in which 7% of splenic T cells and 9% of thymocytes were in the S/G_2+M phases. In thymocytes derived from young, 2-3 weeks old animals the proportion of cells in the S/G_2+M phases was slightly higher (14%) than in the adults. To verify that cycling, NW-fractionated regenerating spleen cells were T cells, Thy-1^+ cells were isolated with FACS and analyzed by their cell cycle distribution; the proportion of cells in the resting

Table 1. The distribution of regenerating T cells over the cell cycle

Cell populations *	Cell cycle distribution (%)#		
	G_0/G_1	S	G2/M
T cells (adult)	92,5	7,3	0,2
thymocytes (adult)	90,6	8,2	1,1
thymocytes (2-3 weeks old)	85,4	12,8	1,8
Con A blasts	77,0	22,6	0,8
Cy day 8 T cells	72,6	24,2	3,2
Cy day 13 T cells	93,6	5,6	0,6

*) T cells derived from spleens of normal adult mice, or from mice injected with 300 mg/kg of Cy 8 or 13 days previously were fractionated in NW-columns. Thymocytes isolated from adult mice or young, 2-3 weeks old animals were analyzed immediately. Con A blasts were normal spleen cells activated with 4 µg/ml Con A for 24 h.
#) Percentage of cells in different phases was determined by propidium iodide staining followed by FACS analysis.

Table 2. IL-2 receptor expression on regenerating T cells

Cell population	IL-2 receptor expression (%)
thymocytes	1,2
normal T cells	1,5
Cy day 8 T cells	3,1
Cy day 13 T cells	2,0

$1x10^6$ freshly isolated thymocytes or NW-fractionated splenic T cells derived from normal mice or from mice treated with Cy 8 or 13 days previously were stained with the monoclonal AMT-13 anti-IL-2R antibody (plus FITC-coupled anti-rat IgG) and the percentage of IL-2R expressing cells was estimated with FACS.

state as well as the number of cells in the S/G_2+M phases was found to be of the same order as that obtained with NW-fractionated cells (data not shown).

IL-2 receptor expression on regenerating T cells. In order to find out whether T cell proliferation during regeneration would be dependent on the IL-2/IL-2R system, expression of the IL-2 receptor was tested by immunofluorescence and FACS using the monoclonal anti-IL-2 receptor antibody, clone AMT-13, which recognizes the 55 kD chain of the IL-2 receptor (32,33).

Table 3. Proliferation of regenerating spleen cells in vitro in the presence of IL-2

Cell culture	Stimulus	^{3}H-Thymidine incorporation (cpm) after culturing 24h	SI	48h	SI
normal spleen cells	-	444		1258	
	IL-2	1150	2,6	3438	2,7
Cy day 8 spleen cells	-	5036		3648	
	IL-2	8564	1,7	8412	2,3
Cy day 13 spleen cells	-	2138		1058	
	IL-2	4356	2,0	2900	2,7

10^5 spleen cells derived from normal mice or mice treated with 300 mg/kg Cy 8 or 13 days previously were cultured in the presence of 20 U/ml IL-2. Cell proliferation was measured by the ^{3}H-Thymidine incorporation.
SI= cpm of stimulated cultures / cpm of unstimulated cultures

Table 4. IL-2 receptor induction to regenerating T cells

Cell population	Stimulus	IL-2R expression (%)*
normal spleen cells	-	3,3
	Con A	39,9
	PMA+iono	28,1
Cy day 8 spleen cells	-	5,1
	Con A	23,5
	PMA+iono	22,7
Cy day 13 spleen cells	-	2,1
	Con A	19,7
	PMA+iono	26,2

*) $1x10^5$ cells were cultured in the presence of 4 µg/ml Con A or 10 ng/ml PMA + 1 nM ionophore A23187 for 24 h and IL-2R expression was analyzed with FACS after staining with the AMT-13 monoclonal anti-IL-2R antibody (plus FITC-coupled anti-rat IgG).

The proportion of IL-2R bearing cells among freshly isolated regenerating T cells was found to be similar to the proportion of IL-2R positive cells among normal splenic T cells ie. 1-3% (Table 2) (the variation between different experiments being 1.5-2.8 % in T cells derived from the normal mice versus 0,2-3,1 % in T cells derived from the regenerating spleens). If regenerating cells were cultured *in vitro* in the presence of exogenous IL-2 for 24-48 h, no further growth was noticed as judged by the SI (Table 3). The higher ^{3}H-Thymidine incorporation found in regenerating cells when compared with normal spleen cells was presumably due to the high cell division *in vivo* which still continued *in vitro* for some days (41). Addition of anti-IL-2 antibody to these cultures had no effect on the spontaneous proliferation of regenerating cells (data not shown).

The IL-2R was, however, readily induced if the regenerating T cells were cultured in the presence of Con A or PMA + ionophore (Table 4). Thus these data indicate that during lymphatic regeneration peripheral T cells were able to proliferate without the IL-2/IL-2R interaction.

The activity stage of regenerating T cells indicated by the expression of cellular oncogenes. Expression of cellular oncogenes c-*myc* and c-*myb* in regenerating T cells was studied by Northern blotting and hybridization analysis of poly(A)$^+$RNA (Fig. 1). The accumulation of c-*myc* and c-*myb* mRNA in regenerating T cells was compared with the corresponding mRNA levels in resting T cells, thymocytes, and splenic lymphocytes activated with Con A for 4 h or for 24 h. Regenerating T cells were found to express elevated levels of mRNA for both of these oncogenes. The level of c-*myc* mRNA was nearly the same in the regenerating T cells and in Con A-activated T cells, but weak or barely detectable in the splenocytes and thymocytes, respectively. By contrast, the expression of c-*myb* mRNA in the regenerating T cells was about 3-4 times greater

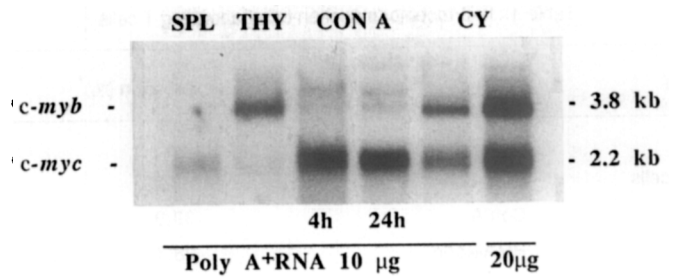

Figure 1. Northern blotting and hybridization analysis of c-*myc* and c-*myb* mRNAs from resting splenic T cells (SPL), thymocytes (THY), T cells activated with Con A for 4 h and for 24 h (CON A), and from regenerating T cells (CY). 10 µg or 20 µg of mRNA was applied to each lane and fractionated on 0,8% agarose-formaldehyde gel, transferred to nylon membrane, and then hybrizied to nick-translated probes.

than in Con A-stimulated cells and comparable to c-*myb* expression seen in thymocytes. Besides the major 3.8 kb c-*myb* species, other fainter c-*myb* RNA signals were observed in the 4.2-4.5 kb mobility region, especially in the Con A-stimulated T cells. These may represent c-*myb* RNA:s initiated in alternative upstream promoters which have been recognized in lymphocytes (42,43). By day 13 after Cy, the c-*myc* and c-*myb* mRNA levels had returned to the baseline level found in resting T cells (data not shown).

DISCUSSION

The data of the present study demonstrate that peripheral T cells have the capacity to proliferate vigorously without a known antigen challenge, and more importantly, that this proliferation is not dependent on the IL-2/IL-2R interaction.

In contrast to the B cell compartment which is continuously seeded from the bone marrow with 20-30x10^6 cells per day (44), the influx of T cell progenitors from the bone marrow to the thymus, and the consequent emigration from the thymus to the periphery involves only a small number of cells (1,45). It has been calculated that thymic emigrants cannot maintain the homeostasis of the peripheral T cell compartment (1,6-8,44-45). Post-thymic expansion defined as an antigen-independent expansion of thymus-educated T cells is thus probably contributing to the maintenance of the peripheral T cell pool. It is not, however, known whether all T cells have a renewal capacity (7). We have earlier shown that adult thymectomy, one week before administration of Cy had no effect on the regenerating capacity of T cells (39) arguing against the recent thymic origin of the regenerating cells.

The mechanism by which peripheral T cell expansion occurs is not yet understood. At present, the proliferation of mature T cells is thought to be strictly dependent on the IL-2/IL-2R interaction (12-14), IL-2 being the driving signal from the G_1 phase to the S phase (46). However, this model is based on *in vitro* experiments. The *in vivo* of IL-2 has been questioned (18,47,48). For example, Bandeira et al (18) noticed that *in vivo* mitogen-activated T cells did not respond to IL-2 *in vitro*.

When studying regenerating T cells after Cy injection, we noticed that they resemble in many aspects (eg. LDH isoenzyme pattern) more mature T cells than thymocytes (39). Now we have

demonstrated that these cells are actively dividing, their distribution over the different stages of the cell cycle resembling the cell cycle distribution of *in vitro*-activated T cells. However, the expression of the IL-2 receptor on regenerating T cells was similar to that of normal resting splenic T cells (Table 2). Like the few IL-2R positive thymocytes which are unable to respond to IL-2 *in vitro* (24), regenerating T cells did not proliferate in response to exogenously added IL-2 (Table 3). Thus the few IL-2R positive cells among regenerating cells could certainly not be responsible for the extensive T cell proliferation occuring during regeneration. IL-2R was, however, readily inducible after stimulation by lectin or PMA + calcium ionophore, speaking against the possibility that these cells would be unable to express IL-2R (Table 4). These data indicate that the IL-2/IL-2R interaction may not be the only mechanism which drives T cells to divide, and furthermore, the other mechanism(s) can induce proliferative responses as strong as the IL-2/IL-2R dependent mechanism. Participation of other growth factors eg. IL-4 or GM-CSF, however, has not been excluded.

Proto-oncogenes in T cell growth control have been implicated (49-50); the c-*myc* oncogene is inactive in resting (G_0) cells, rapidly induced upon cell cycle activation and relatively evenly expressed during the cell cycle (25-28,51). In regenerating T cells the enhanced expression of c-*myc* oncogene resembles that seen in *in vitro* activated T cells (Fig. 1). Expression of c-*myb* is also associated with the progression of the cell cycle in *in vitro* activated cells, but unlike c-*myc*, which is one of the early genes induced, c-*myb* is activated in the late G_1 phase (27,28,46). Thymocytes are known to contain expectionally high amounts of c-*myb* mRNA despite of the relatively low amount of thymocytes in the S/G_2 + M phases (29,30). We found that expression of c-*myb* mRNA was elevated also in regenerating T cells; their c-*myb* mRNA content was significantly greater than in Con A blasts although majority of both kind of cells was cycling and the proportion of cells in the S/G_2 +M phases were equal. It could be speculated that c-*myb* has some function in the maturation of regenerating T cells as suggested in the case of thymocytes (29,30). Data demonstrating that some IL-2 independent T cell lines accumulate high amounts of c-*myb* mRNA, and on the other hand IL-2 dependent cell lines depleted of IL-2 increase their c-*myb* expression (52) suggests that c-*myb* expression is somehow associated with IL-2 independent proliferation mechanisms.

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