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Growth factors can enhance lymphocyte survival without committing the cell to undergo cell division

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ABSTRACT **Growth factors have been defined by their ability to promote the proliferative expansion of receptor-bearing cells. For example, antigen-activated T cells expressing the $\alpha\beta\gamma$ form of the interleukin 2 (IL-2) receptor will proliferate in response to IL-2. In contrast, resting T cells, which express the IL-2 receptor β and γ chains, do not proliferate in response to IL-2. We demonstrate that the survival of resting T cells following γ irradiation is greatly enhanced by pretreatment with IL-2. The radioprotective effect of IL-2 is dose dependent, does not result from the induction of cell proliferation, and does not require expression of the IL-2 receptor α chain. Thus, the $\beta\gamma$ IL-2 receptor expressed on resting T cells can transduce signals that promote cell survival without committing the T cell to undergo cell division. IL-4 and IL-7, but not IL-1, IL-3, or IL-6, were also found to enhance the survival of quiescent T cells following γ irradiation. Thus, certain growth factor–receptor interactions can serve to maintain cell viability in a manner that is independent of their ability to initiate or maintain cell proliferation. These data may have important implications for the use of growth factors in patients being treated with radiation and/or chemotherapy.**

T cells in the peripheral immune system are quiescent, non-proliferating cells. However, each T cell bears a distinct antigen-specific receptor which allows the cell to initiate an immune response when the cell encounters a specific antigen. Antigen activation of a quiescent T cell renders the cell competent to proliferate in the presence of appropriate growth factors or lymphokines. Several lymphokines have been shown to promote the proliferative expansion of antigen-activated T cells. The best characterized of these growth factors is interleukin 2 (IL-2) (1). The ability of IL-2 to promote cell cycle progression of antigen-activated T cells involves the engagement of IL-2 receptor (IL-2R) complexes composed of the receptor α, β, and γ chains. In contrast to activated T cells, resting T cells do not proliferate in response to IL-2 (2). Nevertheless, resting T cells express IL-2Rs composed of β and γ chains (3-5). Despite the fact that the K_d of $\beta\gamma$ receptors for IL-2 is approximately 10^{-9} M (4), a function for the $\beta\gamma$ IL-2R on the surface of resting T cells has not been delineated.

An unexpected function for the $\beta\gamma$ IL-2R was uncovered while we were studying factors which regulate susceptibility of human peripheral blood T cells to undergo programmed cell death. Pretreatment of resting T cells with IL-2 was found to enhance the ability of resting T cells to survive following subsequent γ irradiation or cytotoxic chemotherapy. The ability of IL-2 to promote survival of resting T cells was not dependent on the ability of IL-2 to promote cell cycle progression or the expression of the IL-2R α chain (IL-2Rα). The ability of IL-2 to promote the survival of resting T cells was found to be shared by the lymphokines IL-4 and IL-7, but not IL-1, IL-3, or IL-6. The data suggest that certain growth factor receptors can function to directly promote cell survival without committing a cell to undergo proliferation. Under such conditions, these growth factors may more appropriately be considered survival factors that raise a cell's apoptotic threshold. Our studies suggest that such growth factors could be used to promote the selective survival of receptor-bearing cells.

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MATERIALS AND METHODS

Cell Culture. T lymphocytes were isolated from the buffy coats of healthy donors and cultured as described (6). T cells were activated with plate-immobilized anti-CD3 (G19.4) at 1 μg/ml in the presence of soluble antibody to CD28 (9.3) at 1 μg/ml. For the irradiation experiments, cells were stimulated for 12 hr, removed from the culture dish, and irradiated at 15 Gy with a cesium-source γ irradiator (J. L. Shepherd, Inc., San Fernando, CA). Prior to irradiation, cells were either left in the growth medium or washed three times and resuspended in fresh medium in the presence or absence of recombinant human IL-2 (Boehringer Mannheim), IL-1β (R & D Systems), or IL-3, IL-4, IL-6, IL-7, or transforming growth factor β (Boehringer Mannheim). After irradiation, cells were plated at 10^6 cells per ml and viability and total cell numbers were assessed by propidium iodide exclusion. For proliferation assays, [*methyl*-^{3}H]-thymidine [1 μCi (37 kBq)] was added for the final 8 hr of incubation. Cell surface receptor expression and cell cycle distribution were assessed as described (7, 8).

Western Blot Analysis. T cells were cultured for 24 hr in the presence or absence of IL-2 (200 units/ml, 6 nM). Cells (10^7) were isolated and cytoplasmic protein was prepared and subjected to Western blot analysis (8).

Propidium Iodide Exclusion Assays. At indicated time points, 2×10^5 cells were pelleted and suspended in 0.5 ml of phosphate buffered saline supplemented with 1% bovine serum albumin and 0.01% sodium azide. Two microliters of propidium iodide solution (0.5 mg/ml) was added and the samples were analyzed by flow cytofluorimetry with a FACSort and LYSIS II software (Becton Dickinson). Percent viability was determined by dividing the number of cells which excluded propidium iodide (viable) by the total number of cells.

RESULTS

Resting T Cells Are More Susceptible to Undergoing Programmed Cell Death Than Activated T Cells. It has been reported that resting T cells are more susceptible than phytohemagglutinin-activated T cells to induction of programmed cell death by agents such as radiation (9, 10). To further investigate the differential susceptibility of activated versus

Abbreviations: IL, interleukin; IL-2R, IL-2 receptor; IL-2Rα, IL-2R α chain.

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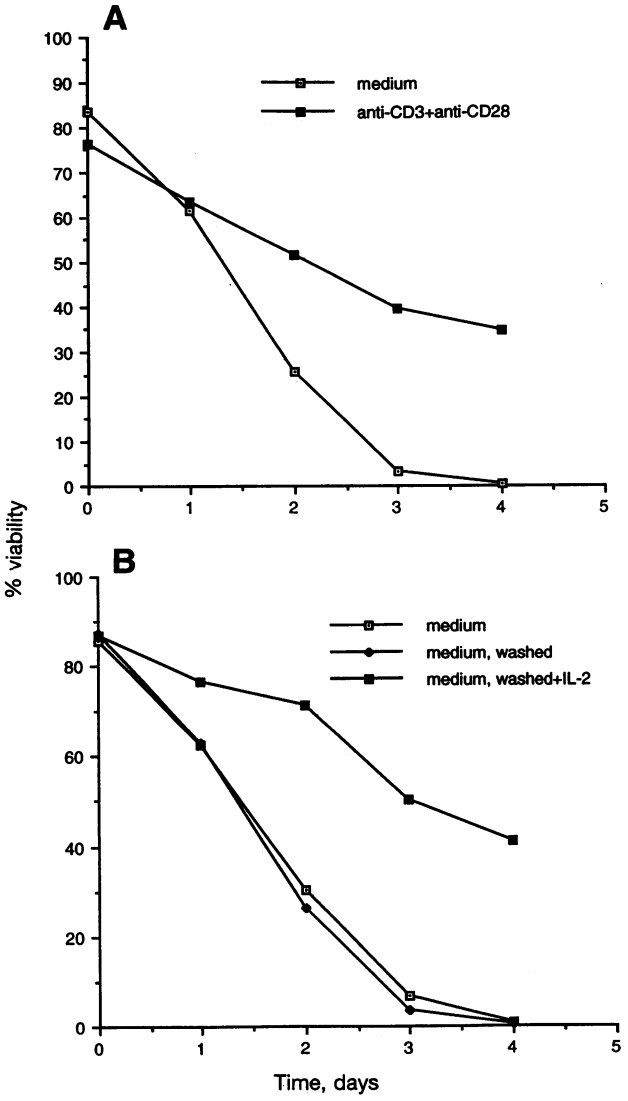

FIG. 1. Survival of resting and activated human T cells after γ irradiation. (*A*) T-cell activation enhances the survival of T cells after γ irradiation. Resting T cells were cultured in medium alone (⊡) or stimulated by a combination of antibodies against the T-cell receptor/CD3 complex and the costimulatory receptor CD28 (■) for 12 hr. Cells were then exposed to 15 Gy of γ radiation and cell viability was determined by propidium iodide exclusion. Each point represents the average of duplicate samples. The experiment shown is representative of three independent experiments using different donors. (*B*) Pretreatment with IL-2 enhances the survival of resting T cells after γ irradiation. Resting T cells were cultured overnight. Cells were then either kept in the original medium (⊡) or washed and placed in fresh medium in the presence (■) or absence (◆) of recombinant IL-2 at 200 units/ml (6 nM). All three populations of cells were then exposed to 15 Gy of γ radiation and cell viability was followed over time as described above. Data are representative of eight experiments using different donors.

resting T cells to cell death following γ irradiation, we examined whether the difference was dependent upon the presence of accessory cells. Human peripheral blood T cells were isolated and rigorously purified from non-T cells by negative selection. Both resting and activated T cells were exposed to 15 Gy of γ irradiation. This dose is sufficient for lethal DNA damage to occur in virtually all cells in the population. Thus the rate at which the cells die over the ensuing days can be used as a measure of their intrinsic ability to resist undergoing programmed cell death in response to DNA damage. We observed a dramatic difference in the rate of cell death between resting T cells and activated T cells (Fig. 1*A*). The activation-associated increase in the maintenance of cell viability was not the result of subsequent T-cell proliferation, as all the cells in the activated population were arrested within the cell cycle at either late G_1 or G_2. Irradiation did not appear to induce the resting T cells to enter the cell cycle. Cell death followed a classic apoptotic pattern, with cells first becoming crenated, followed by nuclear condensation and DNA fragmentation (data not shown).

IL-2 Treatment Enhances the Survival of Resting T Cells After γ Irradiation. One mechanism by which activated T cells differ from resting T cells is that activated T cells produce lymphokines. To test the significance of this potential difference, IL-2 (200 units/ml, 6 nM) was added to resting T cells immediately before the cells were γ irradiated. Cells cultured continuously in the same medium or resuspended in fresh medium prior to γ irradiation showed high susceptibility to irradiation-induced death. Addition of IL-2 dramatically enhanced the resistance of resting T cells to γ radiation (Fig. 1*B*). In contrast, the survival of cells stimulated with anti-CD3 plus anti-CD28 was not further enhanced by addition of IL-2 (data not shown). In fact, in the presence of IL-2 the survival of resting T cells was almost identical to that of activated cells (compare Fig. 1 *A* and *B*). In contrast, treatment of resting T cells with IL-1, IL-3, or IL-6 did not result in protection (Table 1 and data not shown). Both $CD4^+$ and $CD8^+$ T cells appeared to be protected equally by IL-2 (data not shown).

The Growth and Survival Functions of IL-2 Are Separable in Resting T Cells. IL-2 altered the susceptibility of resting T cells to γ-irradiation-induced death in a dose-dependent fashion (Fig. 2). Protective effects were reproducibly observed with as little IL-2 as 2 units/ml (0.06 nM) and reached a maximum with IL-2 at 200 units/ml (6 nM) (Fig. 2*A*). Cells pretreated with IL-2 did not enter the cell cycle or initiate proliferation as measured by progression through G_1 or incorporation of $[^3H]$thymidine (Fig. 2 *C* and *D*). Thus IL-2 does not promote resting T-cell viability by enhancing cell proliferation following irradiation. IL-2 had its greatest effects when given within 24 hr prior to γ irradiation to 6 hr following γ irradiation (Fig. 2*B*). A delay for 24 hr following γ irradiation significantly impaired the ability of IL-2 to enhance T-cell survival. Thus IL-2 appears to function by a mechanism distinct from that of the hematopoietic growth factors such as granulocyte-colony-stimulating factor, which are used to promote cell recovery following radiation treatments in human cancer patients (11). IL-2 is directly promoting the survival of cells in response to γ irradiation rather than supporting the recovery of a cell lineage by augmenting the proliferative expansion of cells surviving irradiation. In separate experiments, IL-2 was also found to protect resting T cells from death induced by the chemotherapeutic agent doxorubicin (data not shown).

IL-2-Induced Survival of Resting T Cells Does Not Require Expression of IL-2Rα. We considered the possibility that IL-2 was mediating its effects on resting T cells through the

Table 1. Effect of lymphokines on survival of resting T cells after γ irradiation

Cytokine (Conc.)	Radiation, Gy	Viability, %
None	0	87.8 ± 0.1
None	15	17.8 ± 0.6
IL-1 (500 units/ml, 0.6 nM)	15	17.3 ± 0.6
IL-2 (100 units/ml, 3 nM)	15	40.3 ± 0.5
IL-3 (100 units/ml, 3 nM)	15	13.7 ± 0.2
IL-4 (100 units/ml, 14 nM)	15	47.4 ± 0.3
IL-7 (100 units/ml, 0.1 nM)	15	50.6 ± 0.2

Viability was determined by propidium iodide exclusion assay performed in triplicate at 48 hr after the indicated radiation treatment. Results are expressed as mean ± SD.

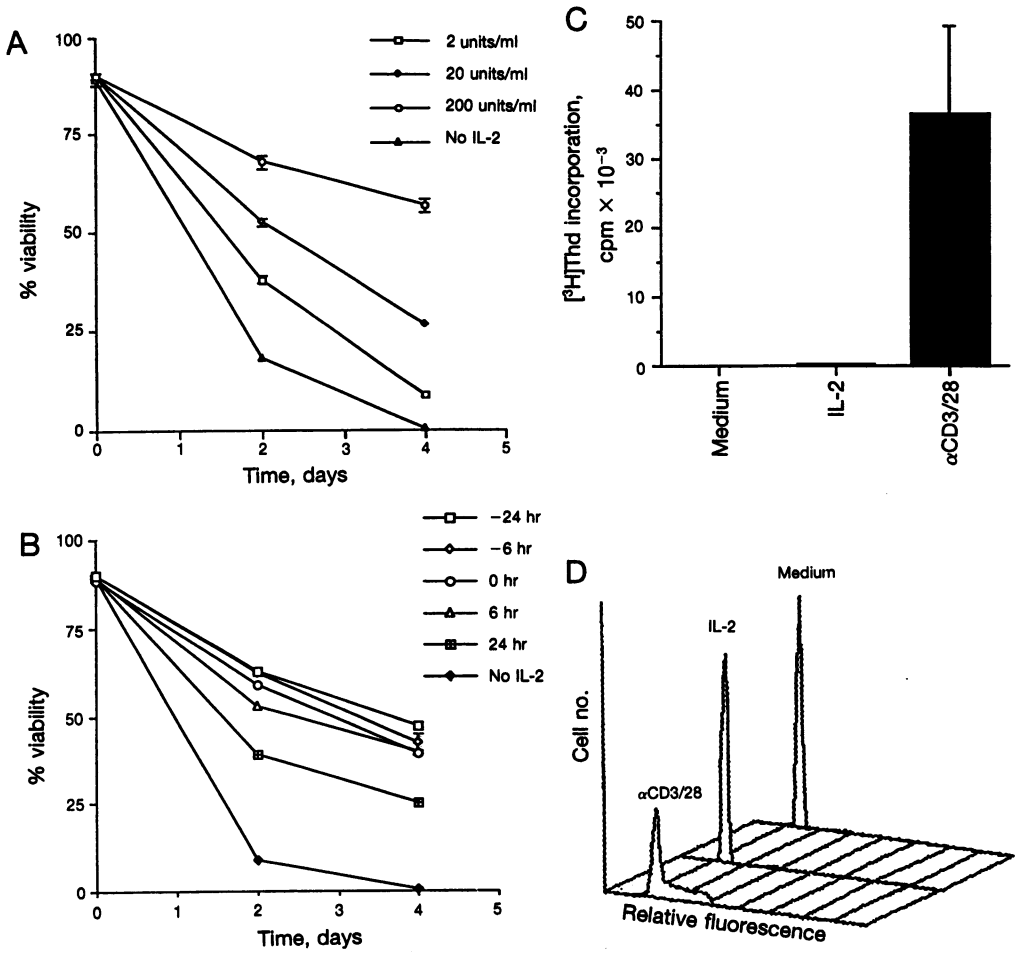

FIG. 2. IL-2 enhancement of resting-T-cell survival in response to γ irradiation depends on the dose and time of administration and does not induce a proliferative response. (*A*) Pretreatment of resting T cells with IL-2 prevents γ-irradiation-induced apoptosis. Various doses of IL-2 were added to cultures of purified resting peripheral blood T cells for 15 min and the cells were then exposed to 15 Gy of γ irradiation. Cell viabilities were determined in triplicate at the times indicated; mean and SD are presented. Data are representative of three independent experiments using different donors. (*B*) IL-2 enhances T-cell survival when given before or within 24 hr following γ irradiation. Resting peripheral T cells were placed in culture and IL-2 was added (200 units/ml) at various times from 24 hr before γ irradiation to 24 hr following γ irradiation. Cell viability was measured at the times indicated. Time zero is the time of irradiation. (*C*) IL-2 does not induce proliferation in resting T cells. Resting cells were cultured for 72 hr in the presence of medium alone or medium containing IL-2 (200 units/ml, 6 nM) or a combination of monoclonal antibodies directed against the T-cell receptor/CD3 complex and the costimulatory receptor CD28 (αCD3/28). [^{3}H]Thymidine (1 μCi) was added to cells for the final 8 hr of culture and [^{3}H]thymidine incorporation was measured. The experiment is representative of three independent determinations. (*D*) IL-2 treatment does not induce resting T cells to enter the cell cycle. T cells cultured for 72 hr in medium alone, medium containing IL-2 (200 units/ml, 6 nM), or a combination of anti-CD3 and anti-CD28 (αCD3/28). Cells were fixed in 75% ethanol and their cell cycle distribution was determined by flow cytometry following staining with propidium iodide (8). Greater than 95% of T cells cultured in medium alone or medium containing IL-2 had flow cytometry profiles characteristic of G_0 cells.

induction of a high-affinity IL-2R complex composed of the α, β, and γ subunits in response to irradiation. To test this possibility directly, we examined T cells for the expression of IL-2Rα by use of an α-chain-specific monoclonal antibody (Fig. 3). Less than 5% of the initial T cells used in these studies expressed detectable levels of IL-2Rα on their cell surface. In contrast, the majority of cells expressed detectable levels of IL-2Rβ (data not shown). Further, IL-2 stimulation of resting T cells did not induce IL-2Rα expression or proliferation of resting T cells (Fig. 3*B*). Most importantly, viable cells examined daily for up to 4 days after γ irradiation in the presence of IL-2 did not express detectable levels of IL-2Rα on their surface (Fig. 3*C* and data not shown).

IL-4 and IL-7 Also Enhance the Apoptotic Threshold of Resting T Cells. The IL-2R on resting T cells is composed of the IL-2-specific β chain and a γ chain which is also a component of the IL-4 and IL-7 receptors (12-14). The γ chain has been shown to play a role in receptor signal transduction from all three receptors (15-18). We therefore tested whether IL-4 and IL-7 were capable of enhancing the resistance of resting T cells to programmed cell death (Table 1). As measured by survival at 48 hr after γ irradiation, either IL-4 or IL-7 at 100 units/ml (14 nM and 0.1 nM, respectively) was at least as good as IL-2 at 100 units/ml (3 nM) at enhancing the survival of resting T cells. In contrast, neither IL-1 nor IL-3 at 100 units/ml (0.6 nM and 3 nM) enhanced resting-T-cell survival following γ irradiation. We were also unable to detect any protective effects from IL-6 (4 nM) or transforming growth factor β (0.4 nM) (data not shown).

Neither Bcl-x nor Bcl-2 Expression Is Affected by IL-2 Treatment of Resting T Cells. IL-2 does not appear to be promoting survival of resting T cells through the induction of genes known to be involved in regulating the intrinsic resistance of cells to programmed cell death. Two genes that can enhance resistance to cell death have been shown to be expressed in T cells, *bcl-2* and *bcl-x*$_L$ (19, 20). IL-2 did not enhance the baseline level of Bcl-2 expressed nor did it induce Bcl-x in resting T cells (Fig. 4). We also found that IL-2

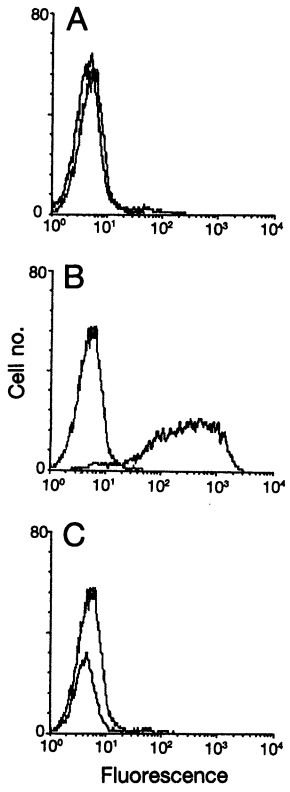

FIG. 3. IL-2Rα chain is not induced in resting T cells by IL-2 treatment. IL-2Rα expression was examined on various cell populations by staining with a fluorescein-conjugated IL-2Rα antibody (IL2R, Coulter). (*A*) IL-2Rα expression on resting T cells cultured with (thick line) or without (thin line) IL-2 for 4 days. (*B*) IL-2Rα expression on resting cells cultured in medium alone (thin line) or cells stimulated for 4 days with anti-CD3 + anti-CD28 (thick line). (*C*) IL-2Rα expression of viable cells 4 days after 15-Gy γ irradiation in the presence of IL-2. The thin line represents cells cultured in the absence of irradiation or IL-2, while the thick line represents viable cells remaining 4 days after γ irradiation in the presence of IL-2.

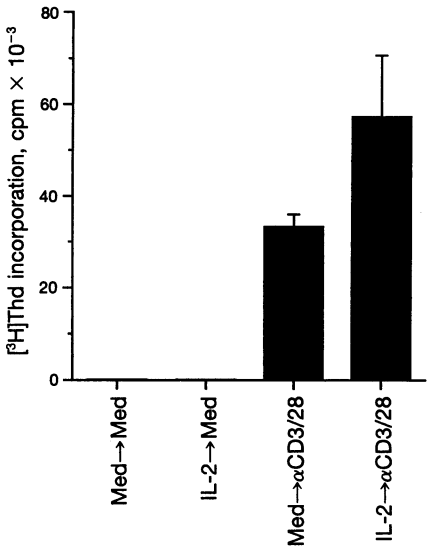

FIG. 5. Pretreatment of cells with IL-2 does not affect the subsequent proliferative capacity of resting T cells. Resting T cells were cultured for 24 hr in the presence or absence of IL-2 (200 units/ml, 6 nM). Cells were then washed three times in fresh medium, rested overnight, and cultured for 72 hr in medium (Med) or medium containing anti-CD3 plus anti-CD28 (αCD3/28). [3H]Thymidine incorporation (represented by bars) was determined at 72 hr. The first stimulation condition (medium or IL-2) is followed by an arrow, then the second stimulation condition (medium or anti-CD3 plus anti-CD28) is indicated. The experiment is representative of three independent experiments.

pretreatment did not downregulate the expression of the death effector Fas (data not shown).

IL-2 Pretreatment of Resting T Cells Does Not Predispose Cells to Die upon Subsequent Activation. Previous work suggested that IL-2 treatment of activated, IL-2Rα-positive T cells increased their susceptibility to cell death upon subsequent T-cell receptor crosslinking (21). This did not occur in IL-2-treated resting T cells (Fig. 5). When such cells were removed from IL-2 and stimulated with anti-CD3 and anti-CD28, the cells readily underwent blast transformation and proliferation. Under a variety of culture conditions, IL-2-pretreated cells consistently maintained higher viability and proliferative capacity than cells pretreated with medium alone.

DISCUSSION

The demonstration that IL-2 can promote the survival of resting T cells may have important physiological implications. Previous evidence suggests that T cells can be recruited into inflammatory lesions in the absence of antigen-receptor engagement (22, 23). Within inflammatory lesions, T cells which recognize antigens presented on antigen-presenting cells are induced to enter the cell cycle. Helper T cells begin the production of lymphokines such as IL-2 while cytotoxic T cells acquire cytolytic activity. Our data suggest that IL-2 produced by helper cells within inflammatory lesions may also promote the survival of T cells that are recruited to the lesions but do not encounter antigen. Since this effect of IL-2 does not promote T-cell activation and proliferation, the ability of IL-2 to promote survival of cells that have not encountered antigen does not contribute to nonspecific induction of the immune response by inappropriately inducing cell proliferation. Therefore, the response of resting T cells to IL-2 may maintain the organism's T-cell repertoire in the face of the need to recruit multiple T cells with distinct specificities to inflammatory lesions.

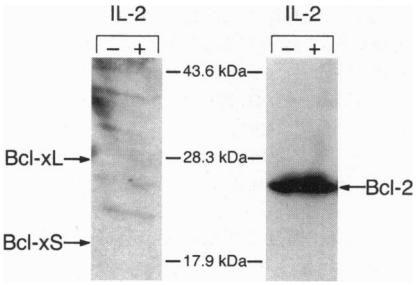

FIG. 4. IL-2 does not induce Bcl-x or Bcl-2 in resting T cells. Resting T cells were cultured in the presence or absence of IL-2 (200 units/ml, 6 nM) for 24 hr. Cytoplasmic extracts were subjected to Western blot analysis with antibodies specific for Bcl-x or Bcl-2, as previously described (8). The positions of Bcl-2, Bcl-x_L, and Bcl-x_S, as indicated by the arrows, were determined from positive controls ran on the same gel.

IL-2 appears to maintain resting T-cell survival by interacting with the βγ IL-2R. Flow cytometric analysis revealed that a high percentage of the cells were IL-2β-positive, whereas few cells expressed IL-2Rα. IL-2Rγ has been shown to be constitutively expressed in lymphoid cells (1). Further, the dose range in which IL-2 radioprotection was observed, 0.06–6 nM, correlates well with the established K_d for the βγ receptor, 1 nM. Since IL-2 can also provide a cell survival advantage to proliferating T cells (24), our data suggest that the basal function of the IL-2R βγ complex is to provide a signal transduction pathway that specifically enhances a T cell's ability to resist the induction of programmed cell death. IL-2 does not appear to be mediating its effects through altering the expression of the survival-gene products Bcl-2 and Bcl-x_L or the death effector Fas. The antigen-receptor engagement which induces IL-2Rα expression appears to be required for T cells to acquire the ability also to proliferate in response to IL-2. This suggests that the formation of the multimeric IL-2R complexes on the surface of activated T cells leads not only to higher affinity for the ligand, but also to the acquisition of additional receptor signaling properties (25, 26). Whether

IL-2Rα is sufficient to account for the change in the effector function of the receptor complex has not been determined. The ability of complex receptors to acquire additional signaling properties upon acquisition of additional subunits may explain how multisubunit receptor complexes have been selected during evolution.

The sharing of common receptor subunits may also confer similar functional properties on the receptors of distinct growth factors. The IL-2R shares a common γ chain with the IL-4 and IL-7 receptors (12-14). Loss of the γ chain results in X chromosome-linked severe combined immunodeficiency (27). Receptor subunit reconstitution studies and *in vitro* mutagenesis experiments suggest that the γ chain plays a critical role in regulating some of the common features of IL-2, IL-4, and IL-7 signaling (15-18). Like the $\beta\gamma$ IL-2R, IL-4 and IL-7 receptors are expressed on resting T cells (28, 29). We have found that IL-4 and IL-7 can enhance the survival of resting T cells following γ irradiation at least as well as IL-2. Thus, one of the shared functions of IL-2, IL-4, and IL-7 appears to be the ability to promote the survival of resting T cells.

Our observation that IL-2, IL-4, and IL-7 can protect resting T cells from the induction of programmed cell death following radiation exposure may also have important practical implications. One of the significant side effects of aggressive radiation and chemotherapeutic protocols has been that such treatments can lead to profound immunosuppression through the induction of lymphocyte cell death. Numerous clinical trials are underway which use growth factors following irradiation and/or chemotherapy to promote hematopoietic or immune cell recovery. Our data suggest that growth factors may also be used during irradiation and chemotherapy to enhance the resistance of a given cell population to cell death. Since cell survival factors will be selective for cell types possessing receptors for the given factor, one might get selective sparing of important cell populations such as hematopoietic, lymphoid, or endothelial cells through growth factor treatments administered prior to irradiation or chemotherapy.

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