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IL-1 INDUCES HIGH AFFINITY IL-2 RECEPTOR EXPRESSION OF CD4⁻8⁻ THYMOCYTES

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We investigated the role of cytokines for the growth of CD4⁻8⁻ thymocytes (double negative thymocytes) (DNT) in vitro and found that IL-1-induced IL-2-dependent proliferation of only the IL-2R-positive DNT subpopulation. The presence of IL-1 during the first 18 h of culture was sufficient for an optimal response and suggested that IL-1 induced DNT differentiation. We could indeed show by RNA dot blot analysis that IL-1 stimulated de novo expression of the p55 chain of the IL-2R thus initiating high affinity IL-2 binding and a proliferative response. Because macrophages and epithelial cells in the thymus produce IL-1 we propose that IL-1 is involved in early events during maturation of immature thymocytes.

CD4- and CD8-negative thymocytes (DNT)[2] represent the most immature T cells to immigrate into the thymic cortex. Although about 50% of DNT express IL-2R, they do not respond to IL-2 (1). Growth of DNT has been induced with nonphysiologic stimuli like mitogens and ionophores with or without the addition of exogenous IL-2 (2, 3). Under physiologic conditions, however, differentiation and growth of DNT might be induced by contact with cortical Ia-negative macrophages (4) or epithelial cells. Because these cells are capable of secreting IL-1 (5) (our unpublished data), we examined whether IL-1 could provide a physiologic growth and differentiation signal for murine DNT. We found that rIL-1 induced optimal IL-2-dependent proliferation of only the IL-2R-positive DNT subpopulation within 18 h of culture. Cytoplasmic RNA dot-blot and Northern analyses showed that IL-1 functions by initiating de novo expression of high affinity IL-2R. Thus, these in vitro data suggest that IL-1 may also serve as the first stimulus for DNT in vivo inducing their differentiation and clonal expansion.

MATERIALS AND METHODS

Preparation of DNT and antibodies used. DNT were prepared from thymuses of 4-wk-old C3H mice by antibody and C treatment (see also Reference 2). Briefly, thymuses were first treated with anti-Lyt-2 mAb (3.168.8.1) (6) in the presence of DNAse (20 U/ml) and in a second cycle of treatment by anti-Lyt-2, anti-L3T4 (RL172.4) (7), anti-IA^k (118-49-20, generously supplied by N. Koch) and anti-MAC-1 (TIB 128; American Type Culture Collection, Rockville, MD) antibodies. The cells were incubated with the antibodies for 30 min at 4°C, and C (Low-Tox C, Cedar Lane, Hornby, Ontario, Canada) lysis was done for 45 min at 37°C. At the end of the incubation, viable cells were purified on a Ficoll (Pharmacia, Freiburg, FRG) cushion. The typical yield was about 1% of total thymic cells. The purity of the cells was assessed by analysis with the fluorescence activated cell sorter. The cells were 80 to 90% Thy-1 positive and 45 to 50% IL-2R positive. L3T4, Lyt-2, and MAC-1 positive cells were not detected.

7D4 antibodies (anti-IL-2R) (8) were purified on an affinity column. Culture supernatant was applied to a Diasorb 1000 (Diagen, Düsseldorf, FRG) column coupled with mouse-anti-rat-K-chain mAb. 7D4 antibodies were applied at pH 7 and eluted at pH 4. Purity was controlled by SDS gel electrophoresis under reducing conditions.

Proliferation assays. DNT were cultured in flat bottom 96-well plates in RPMI 1640 supplemented with 10% FCS, 3×10^{-5} M mercaptoethanol and 10 μg/ml gentamicin. After 3 days of culture in 5% CO_2 in air and 90% relative humidity at 37°C the cells were pulsed for 4 to 6 h with 1 μCi/well of [^{3}H]TdR (Amersham, Frankfurt, FRG), specific activity 5 Ci/mMol) and incorporated radioactivity was measured in a liquid scintillation counter after harvesting of the DNA on glass fiber filters. [^{3}H]TdR incorporation is shown as cpm. Purified rIL-1a with a specific activity of 17×10^6 U/mg was a generous gift of Hoffmann-La Roche, Nutley, NJ. Purified human rIL-2 from *Escherichia coli* was kindly supplied by Cetus Corporation, Emeryville, CA.

Preparation of IL-2R-negative DNT. During the second cycle of DNT preparation aliquots of cells were treated with 7D4 antibodies in addition. At the end of treatment 7D4-treated and nontreated cell aliquots were suspended in the same volume and assayed. 7D4 treatment decreased the cells number by 50%. A total of 5×10^5 cells of both populations was incubated with 7D4 antibody at 10 μg/ml in 100 μl for 30 min at 0°C in the presence of 0.1% sodium azide. The cells were washed and incubated under the same conditions with FITC-conjugated rabbit-anti-rat Ig. The cells were again washed and the final pellet was suspended in 1% paraformaldehyde in saline. A total of 300 cells of each sample was counted by using a fluorescence microscope. A total of 5×10^4 cells of each population was seeded in triplicate into wells of microtiter plates as above. PMA (1 ng/ml, Sigma Chemical Co., St. Louis, MO), ionomycin (250 ng/ml, Calbiochem), and IL-2 (100 U/ml) were added to the cultures. Proliferation was assessed after 3 days of culture as above.

^{125}I-IL-2-binding assay. Cultured cells were washed twice and incubated for 2 h at 37°C in fresh medium. They were again washed twice and then used for binding of radiolabeled IL-2. The concentration of ^{125}I-IL-2 (NEX 229; NEN, Dreieich, FRG) used was 100 U/ml based on 10^7 U/mg. This concentration resulted in saturation binding when tested with an IL-2-dependent cell line under our assay conditions. A total of 6×10^5 cultured DNT was incubated in a total volume of 100 μl of medium containing the labeled IL-2 and 0.1% sodium azide on ice for 1 h. A 100-fold excess of nonlabeled IL-2 was used to measure nonspecific binding. The binding reaction was terminated by centrifuging the cells through an oil mixture. The bottoms of the tubes were cut off and bound radioactivity was counted. Specific binding is represented as the difference in cpm bound by cells with and without nonlabeled IL-2. Nonspecific binding was between 18 and 20% in this experiment.

For Scatchard analysis conditions were slightly different. ^{125}I-IL-2 from Amersham (IM 197, specific activity 779 Ci/mmol) was used. Incubation at 37°C before assay was 3 to 4 h. Binding was done after purification of viable cells on a Ficoll cushion for 45 min at 37°C in culture medium/PBS 1:1 containing 0.1% azide. Binding was determined between 12.5 and 200 pM concentrations by using 5 to 8×10^5 cells in a final volume of 0.1 ml. Nonspecific binding was determined in the presence of a 1000-fold excess of nonlabeled IL-2 and was less than 5%. Results represent the mean of duplicates.

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[2] Abbreviation used in this paper: DNT, double negative thymocytes.

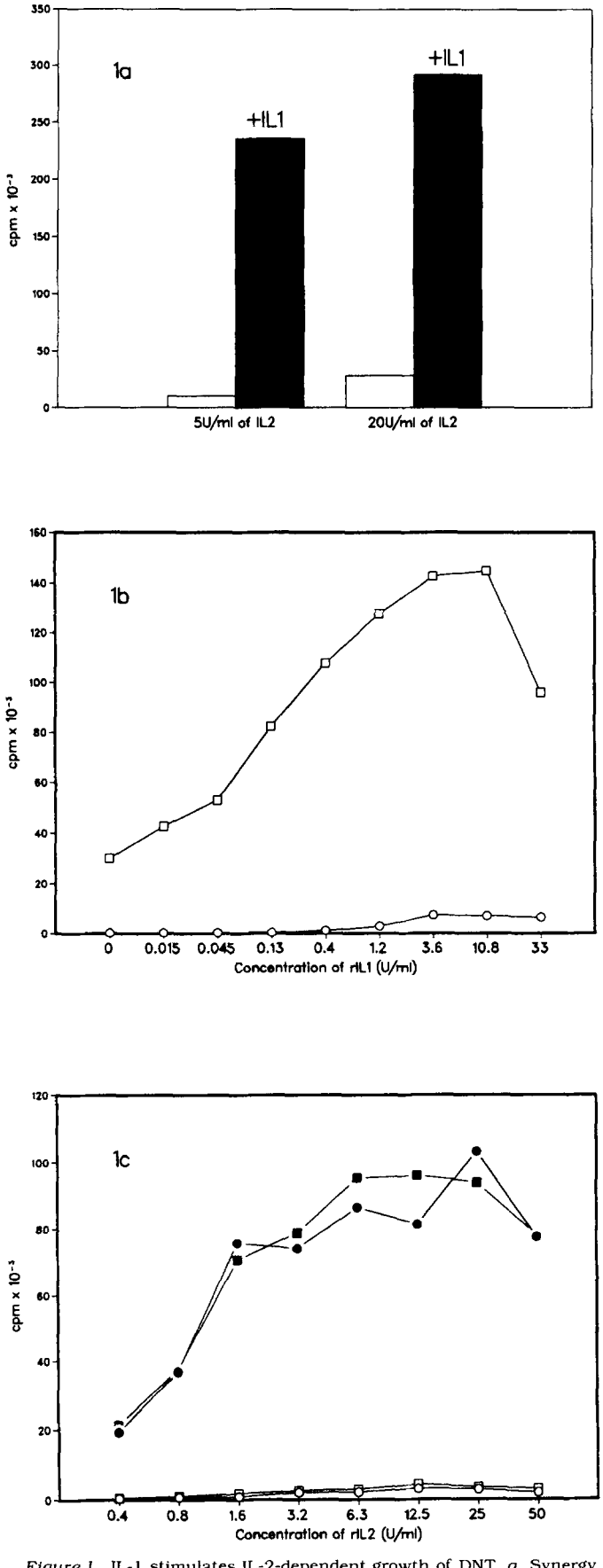

Figure 1. IL-1 stimulates IL-2-dependent growth of DNT. *a*, Synergy between IL-1 and IL-2. DNT (10^5 per well in 200 μl medium) were cultured for 3 days in presence of recombinant cytokines alone or in a combination of these cytokines. Concentration of rIL-2 was as indicated; concentration of rIL-1 was 10 U/ml. *Open bars* show the response to IL-2 alone; *closed bars* show the synergistic response to a combination of IL-1 and IL-2. Cells in medium or IL-1 alone did not proliferate. *b*, The synergism of IL-1 and IL-2 in growth induction of DNT is dependent on the concentration of IL-1. DNT (5×10^4/well) were cultured for 3 days with (□) and without (○) rIL-2 (50 U/ml) and different concentrations of rIL-1a. *c*, An 18-h pulse is sufficient for optimal activation. Four groups of DNT were pulsed for 18 h with rIL-2 (5 U/ml) with (●) and without (○) rIL-1a (10 U/ml) or rIL-2 (50 U/ml) with (■) and without (□) rIL-1a, respectively. After the pulse, the cells were washed three times, counted, and incubated for 2 days more in different concentrations of rIL-2. As a control, cells from all four groups were kept in rIL-2 (50 U/ml) and rIL-1 (10 U/ml) during the entire culture period. The cpm for the latter groups were: (●) 106,100; (○) 17,010; (■) 100,690; (□) 20,240, respectively.

Dot-blot and Northern analysis. Cytoplasmic RNA for dot-blots was prepared from 10^6 cells by the method of Cheley and Anderson (9). A $\log_2$ dilution of RNA in 10 × SSC was blotted and the first well represents the amount of RNA equivalent 5×10^5 cells. RNA for Northern analysis was prepared by the guanidine-iso-thio-cyanate/cesium-chloride method. RNA was denatured with glyoxal and electrophoresed in a 1% agarose gel. The blotting was done on nylon membranes (Compas, Genofit, Heidelberg, FRG). RNA was fixed with UV light. Hybridization was done at 42°C in the presence of dextran-sulfate. The filters were washed twice under high stringency conditions (65°C, 30 min, 2 × SSC containing 1% SDS). Probes were labeled with ^{32}P-GTP and ^{32}P-CTP (Amersham; specific activity 3000 Ci/mmol) by the random primer method with the use of a hexamer (Pharmacia). The IL-2R probe was generously provided by Dr. Thomas Malek, Miami, FL (10).

RESULTS

We have previously shown that in the absence of mitogens IL-1 and IL-2 synergistically induce expression of IL-2R and hence proliferation of thymocytes (11). We have also observed that isolated Ia-negative macrophages from the thymus of C3H mice spontaneously produce IL-1 in vitro (data not shown). This observation and the fact that DNT carry IL-1R and react to IL-1 in the presence of mitogens (12) prompted us to investigate whether DNT could be stimulated by IL-1 without mitogens.

DNT were prepared from thymuses of adult mice and it was tested whether a synergy in growth induction could be observed with a combination of IL-1 and IL-2. Figure 1*a* shows indeed a strong synergism between IL-1 and IL-2, and Figure 1*b* shows that this proliferation in the presence of IL-2 is strictly dependent on the concentration of IL-1. Neither IL-1 nor IL-2 alone had any effect. These data suggest that IL-1 is not sufficient to induce IL-2 secretion of DNT but is required for IL-2-dependent proliferation. In addition, the response of DNT to IL-1 showed a linear dependence on the number of DNT in culture (from 750 cells/well to 9.6×10^4 cells/well) suggesting a direct effect of IL-1 on DNT.

We hypothesized that such an effect might represent an IL-1-mediated differentiation signal necessary only during the initial culture period. Figure 1*c* shows that this assumption was correct. An 18-h pulse with IL-1 in the presence of IL-2 induced maximal DNT proliferation measured on day 3. This response was as high as when IL-1 was present during the entire culture period (see legend to the figure).

In order to show that the proliferation initiated by IL-1 was dependent on IL-2 it was tested whether the synergistic effect of IL-1 plus IL-2 was inhibited by antibody against the IL-2R, 7D4. As shown in Figure 2 this was possible. At suboptimal concentrations of IL-1 inhibition was better than 90% at a given concentration of 7D4. More importantly, this inhibition was reversed by higher concentrations of IL-1, suggesting an involvement of IL-1 in IL-2R expression.

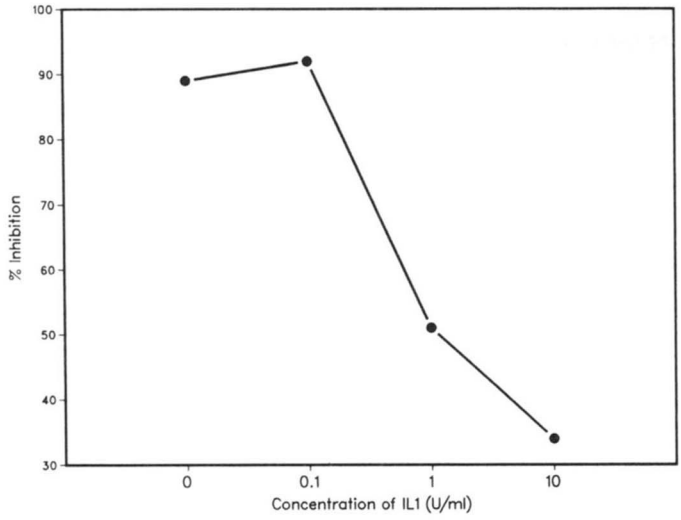

Figure 2. IL-1 induced proliferation of DNT is dependent on IL-2. DNT were cultured at a concentration of 5×10^4 cells per culture well in a total volume of 200 μl culture medium. All wells contained rIL-2 (10 U/ml) and affinity-purified monoclonal anti-mouse-IL-2R antibodies (7D4) (70 μg/ml) (8). The cells were cultured for 3 days and then pulsed for 4 h with 1 μCi/well of [^{3}H]TdR. Inhibition of [^{3}H]TdR incorporation in cultures with anti-IL-2R antibodies in comparision with cultures without antibodies is given in percentage. Controls: cultures with IL-2 (10 U/ml) alone 1930 cpm; IL-2 plus IL-1 (0.1 U/ml) 3180 cpm; IL-2 plus IL-1 (1 U/ml) 14210 cpm; IL-2 plus IL-1 (10 U/ml) 21450 cpm.

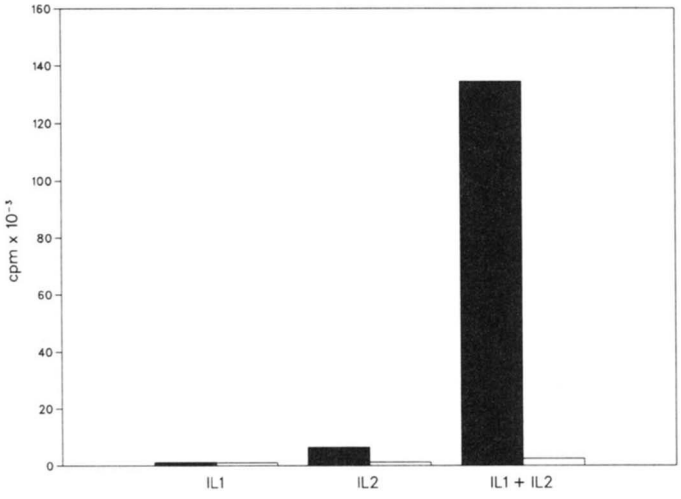

Figure 3. Only the IL-2R positive subpopulation of DNT is induced to proliferate in response to IL-1 plus IL-2. A mixture of IL-2R-positive and IL-2R-negative DNT (*closed bars*, 5×10^4/well) and IL-2R-negative DNT (*open bars*) were tested for a response to rIL-1a (10 U/ml) alone, rIL-2 (20 U/ml) alone, and a combination of the two cytokines at the same concentrations.

To address the question whether the IL-2R-positive and/or IL-2R-negative subpopulation comprised the responding cells, we eliminated the IL-2R-positive DNT subpopulation by anti-IL-2R antibodies plus C. More than 99% of IL-2R$^+$ cells were removed by this method as monitored by staining of the cells with 7D4 and FITC-rabbit-anti-rat antibodies. To test the viability of the remaining cells they were stimulated with PMA and ionomycin in the presence of exogenous IL-2. The magnitude of response of both populations was comparable (not shown). Figure 3 shows that elimination of the IL-2R-positive DNT dramatically reduced the IL-1-induced proliferative response of the remaining DNT.

For a more specific analysis of the involvement of IL-1 in IL-2R expression, IL-2R mRNA levels were measured by dot-blot analysis. DNT were cultured in the presence of rIL-1 (10 U/ml) and rIL-2 (50 U/ml) for 1 to 3 days (Fig. 4). Cytoplasmic RNA from such cells was blotted in serial twofold dilutions and probed with an IL-2R L chain probe. In addition, on day 0 of culture, DNT RNA was prepared for Northern analysis with the same probe. In a separate experiment, DNT were cultured in the same way but high affinity IL-2 binding was measured by using ^{125}iodine-labeled IL-2. The results of the hybridization experiments are shown in Figure 4. Figure 4, *left panel*, shows relatively little IL-2R-specific mRNA on day 0 of culture (*lane 3*). On day 1, however, IL-2R mRNA was undetectable (*lane 4*). In contrast, a considerable amount of IL-2R mRNA was expressed on day 2 (*lane 5*) and increased on day 3 (*lane 6*). ^{125}I IL-2 high affinity binding to IL-2R on DNT followed this pattern exactly (Table I).

Controls for hybridization specificity were included in the dot-blot and the Northern analyses. *Lane 7* (dot-blot) and *lane 4* (Northern blot) show RNA from activated cells of the macrophage cell line PU5.18. No hybridization was detected even after longer exposure times. The known pattern of mRNA (10) of the mouse IL-2R was detected in DNT RNA on day 0 (*lane 3*). Exactly the same pattern was found when cells were cultured for 4 days in IL-1 and IL-2 (not shown). This shows that the defect in response to IL-2 of day 0 DNT is not caused by the nature of the transcripts. It was not possible to obtain mRNA of cells that were cultured in either lymphokine alone be-

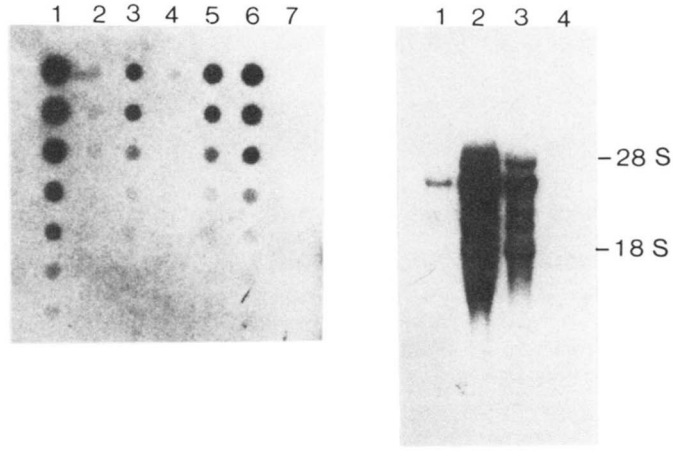

Figure 4. The synergistic action of IL-1 and IL-2 induces de novo expression of high affinity IL-2R. *Left panel*, RNA dot-blot hybridized with a probe for the mouse IL-2R. The lanes show total RNA from *1*) IL-2-dependent cell line W2, *2*) stimulated EL4 thymoma cells, *3*) DNT on day 0 of culture, *4*) DNT on day 1 after stimulation with IL-1 and IL-2, *5*) DNT on day 2 of culture, *6*) DNT on day 3 of culture, and *7*) cells of the activated PU5.18 macrophage cell line. *Right panel*, Northern blot probed with mouse IL-2R probe. The lanes show total RNA from *1*) stimulated EL4, *2*) IL-2-dependent cell line W2, *3*) DNT on day 0, and *4*) activated PU5.18 cells. Estimated from methylene blue staining of the filter, the amount of RNA in *lanes 1*, *2*, and *4* was comparable; about one-third of RNA was applied in *lane 3*.

TABLE I

Binding of ^{125}I-IL-2 to cultured DNT[a]

Day of Culture	^{125}I-IL-2 Bound[b] (cpm)
0	1501
1	132
2	2712
3	3482

[a] DNT were cultured in IL-1 and IL-2 and prepared for binding as given in *Materials and Methods*.

[b] The numbers represent specific binding by 6×10^5 cells.

cause those cells die during the 1st day of culture.

To substantiate the interpretation that the functional IL-2R is of high affinity, dose-dependent IL-2 binding was measured on day 1 and day 3 of DNT cultured in the presence of IL-1 and IL-2. Binding of ^{125}I-IL-2 to an IL-2-dependent cell line was used as an internal experimental control (yielding a K_d of 76 pM and 33,000 binding sites/cell). Binding was measured in concentrations between 12.5 and 200 pM. Under these conditions, no significant binding was measured on day 1 of culture (not shown). This was consistent with data from Table I and Figure 4. On day 3, however, dose-dependent high affinity binding was observed as shown in the Scatchard plot (Fig. 5). In several experiments the K_d derived from these plots were between 23 and 38 pM with numbers of binding sites between 690 and 3100. The costimulatory effect of IL-1 and IL-2 in these experiments was also controlled and was consistent with data in Figure 1, showing no response to either lymphokine alone but a strong synergistic effect on proliferation measured on day 3. No viable cells were recovered from cultures stimulated with lymphokine alone and, therefore, it was not possible to obtain binding data from such cells. Taken together, our data lead to the conclusion that in the presence of IL-1 and IL-2 the IL-2R$^+$ DNT subpopulation expresses de novo high affinity IL-2R as shown by the ability to proliferate IL-2 dependently and to bind IL-2 with high affinity.

DISCUSSION

Thymic epithelial cells can produce IL-1 (5). In addition, we observed that thymic macrophages produce IL-1 in vitro without stimulation. We therefore investigated whether IL-1 was able to initiate DNT growth in the absence of mitogens. The data in Figure 1 illustrate clearly that the combination of IL-1 and IL-2 induces growth of DNT whereas either lymphokine alone had no effect. It is not surprising that others (12) did not observe this synergism. Compared to mitogenic stimulation at the respective cell numbers used the effect of IL-1 and IL-2 on [^{3}H]TdR incorporation is low and may thus have escaped detection.

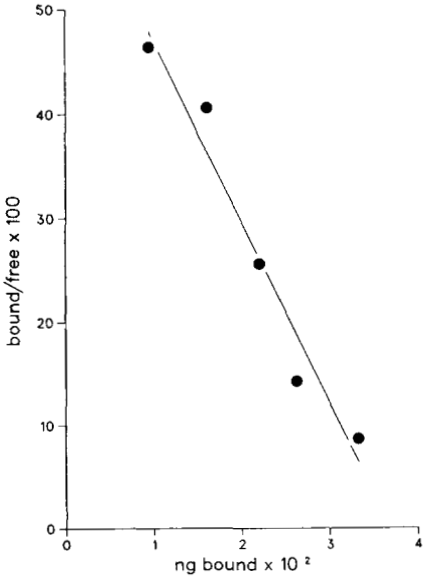

Figure 5. Scatchard plot of DNT-IL-2 binding after 3 days of culture. After culture in IL-1 (5 U/ml) and IL-2 (25 U/ml) cells were cultured for additional 3 to 4 h in IL-2-free medium. Viable cells were purified on Ficoll and incubated at 4.8×10^6 cells/0.1 ml with $\log_2$ dilutions of ^{125}I-IL-2 in concentrations between 12.5 and 200 pM. Nonspecific binding was measured in the presence of a 1000-fold excess of nonlabeled IL-2. Bound and free radioactivity was counted after separation of the cells through an oil mixture. Data represent the mean of duplicates.

An 18-h pulse with IL-1 at the onset of culture is sufficient to initiate this effect (Fig. 1*c*). This suggests that IL-1 gives a differentiation signal to DNT and does not act as a growth factor. Because DNT proliferation was strongly inhibited by anti-IL-2R mAb and because this inhibition was reversed by higher concentrations of IL-1 (Fig. 2) we concluded that IL-1 modulates the expression or affinity of IL-2R on DNT. Thus, IL-1 may either induce new IL-2R on IL-2R negative DNT (50% of DNT) or change IL-2R function on the IL-2R-positive DNT subpopulation. The first possibility was ruled out by the fact that the responder population was eliminated together with the IL-2R-positive cells. The latter would be explained by induction of de novo high affinity IL-2R. This is also supported by the finding of Lowenthal et al. (13) who reported that the high affinity IL-2R on freshly isolated DNT is not functional. We tested this possibility by monitoring IL-2R expression on the mRNA level. The data from Figure 4 show that stimulation by IL-1 leads to a strong de novo expression of the p55 chain of the IL-2R paralleled by strongly increased binding of IL-2 and appearance of high affinity IL-2R on day 3 as shown in Figure 5. Whether or not the p75 chain of the IL-2R is also regulated by IL-1 cannot be answered by these experiments. It can only be said that after IL-1 stimulation the IL-2R on DNT has the properties of a high affinity IL-2R with respect to IL-2 binding and proliferation of the cells. There is evidence, however, derived from experiments using p55 transgenic mice, that the p75 chain is constitutively expressed on thymocytes (14). The question whether IL-1 also regulates the p75 chain can easily be answered as soon as antibodies or cDNA probes for this chain become available.

Another important negative finding was the fact that under our mitogen-free culture conditions synthesis of IL-2 was never detected. This was supported by two observations: 1) no significant proliferation was induced by IL-1 alone (Fig. 1), and 2) no IL-2 mRNA could be detected in DNT (blots from Fig. 4 hybridized with an IL-2 probe, not shown). These findings also show that stimulation of DNT by IL-1 differs from stimulation of DNT with mitogens, because in the latter case endogenous IL-2 secretion by DNT was detected (15, 16).

Taken together, our in vitro data show that the role of IL-1 is the induction of high affinity IL-2R expression on DNT. DNT then become responsive to growth signals provided by other cells in the thymic environment. Analogous events may take place under in vivo conditions in the thymus. Indeed, De Luca and Mizel (17) previously reported evidence for a role of IL-1 in fetal thymus organ cultures. Nonlymphocytic thymic cells that produce membrane and/or secreted IL-1 may induce IL-2R expression on DNT and may play a key role for the growth and differentiation of these cells.

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