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Immunology

Unfractionated human thymocytes have a lower proliferative capacity than $CD3^-4^-8^-$ ones but have a similar capacity for expression of interleukin 2 receptors and production of interleukin 2

(phorbol 12-myristate 13-acetate/calcium ionophore)

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ABSTRACT **$CD3^-4^-8^-$ and unfractionated thymocytes were compared for their capacity to proliferate, to express interleukin 2 (IL-2) receptor, and to secrete IL-2. Phorbol ester and Ca^{2+} ionophore were used as mitogens. $CD3^-4^-8^-$ thymocytes responded vigorously when stimulated with phorbol ester in the presence of IL-2 or in combination with Ca^{2+} ionophore. In contrast, unfractionated thymocytes responded weakly when stimulated with either of these mitogens. Surprisingly, however, the stimulation of these populations with either phorbol ester plus IL-2 or phorbol ester plus ionophore induced a high and similar level of IL-2 receptor expression in both thymocyte populations. A similar level of IL-2 secretion in both populations was also obtained when they were stimulated with a combination of phorbol ester plus ionophore. These results suggest that during the maturation process, the majority of thymocytes lose their capacity to be activated by some mitogens, although they maintain their capacity to secrete IL-2 and to express the IL-2 receptor.**

The analysis of the cell surface antigen phenotype has shown that the thymus is composed of a complex and highly heterogeneous population of cells (1, 2). The relationship among the different subpopulations and their functional capabilities are still uncertain. There are several discrepancies between the various proposed models of thymocyte maturation (1, 3–5), although all of them describe the $CD3^-4^-8^-$ population (or its equivalent in mice, $L3T4^-$ $Ly\text{-}2^-$) as the most immature one. The evidence in favor of considering this population as the most immature is (*i*) that it is the first to appear in the embryonic thymus (6); (*ii*) that some of the cells of this population can give rise to other thymocyte subpopulations *in vitro* (7); and (*iii*) the order of appearance of the gene rearrangements and the transcripts of the α and β chains of the antigen-specific T-cell receptor (8).

Recently, it was reported that double-negative ($L3T4^-$ $Ly\text{-}2^-$) murine thymocytes have a strong proliferative capacity (9) and are able to secrete interleukin 2 (IL-2) (10). In contrast, it is well known that in both murine and human systems, thymocytes respond poorly to mitogens such as lectins (11). One way to explain these apparently contradictory data consists in assuming that the maturation process is accompanied by an important loss in the proliferative capability of the cells.

To assess this point and to find out whether thymocytes become unresponsive to mitogenic signals at a certain stage in their maturation, we compared the proliferative capability of $CD3^-4^-8^-$ human thymocytes in relation to unfractionated ones. For this purpose we chose mitogens that do not need specific membrane proteins to be effective. The mitogens used were phorbol 12-myristate 13-acetate (PMA) and the Ca^{2+} ionophore ionomycin. The expression of IL-2 receptor and the secretion of IL-2 were also analyzed in these populations.

MATERIALS AND METHODS

Thymocyte Populations. Thymus samples were obtained from children undergoing reparative cardiac surgery. Thymocytes were isolated by teasing the thymus fragments in complete medium [RPMI 1640 supplemented with 10% fetal bovine serum (Flow Laboratories), 2 mM glutamine, and 10 μg of gentamicin per ml]. $CD3^-4^-8^-$ thymocytes were isolated from thymocyte suspensions by treatment with ascites fluids (1:100 dilution) containing mouse monoclonal antibodies directed against CD3 (Cris-7; ref. 12), CD4 (EDU-2; ref. 12), and CD8 (109-2D4; ref. 13) plus complement. Cells ($5–9 \times 10^7$ per ml) in RPMI 1640 containing 10% fetal bovine serum were incubated with antibodies at 4°C for 1 hr, pelleted by centrifugation, and resuspended in selected rabbit complement (1:10 dilution). After incubation for 1 hr at 37°C, the surviving cells (about 10% of the starting number) were centrifuged on Ficoll-Hypaque. The thymocyte subpopulations thus obtained were checked for purity by cytofluorimetry.

Immunofluorescence. To assess the purity of the cell preparations, treated cells were suspended (10^6 cells in 50 μl) in phosphate-buffered saline (PBS, without Ca^{2+} and Mg^{2+}) containing 2% fetal bovine serum and 0.1% NaN_3 and were incubated for 30 min at 4°C with monoclonal antibodies against CD3 (Cris-7), CD4 (EDU-2), and CD8 (109-2D4), washed three times, and resuspended in 50 μl of the same medium containing absorbed fluorescein-conjugated $F(ab')_2$ goat anti-mouse immunoglobulin (Cappel Laboratories, Cochranville, PA). After incubation for 30 min at 4°C, the cells were washed three times, resuspended in 0.5 ml of the phosphate-buffered saline/serum/NaN_3 solution, and analyzed by flow cytofluorimetry (FACS, Becton Dickinson). The percentage of $CD3^+$, $CD4^+$, or $CD8^+$ cells in the treated population was <5%. The expression of IL-2 receptor (CD25) was also measured by immunofluorescence, using two monoclonal antibodies, anti-CD25 (Immunocytometry Systems, Becton Dickinson) and Mar-108 (14).

Cell Cultures. Aliquots of cell suspensions were placed in 96-well plates (Costar, Cambridge, MA) at 5×10^4 cells per well. Cells were stimulated with various concentrations of PMA (Sigma) and ionomycin (Calbiochem). The Ca^{2+} iono-

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Abbreviations: IL-2, interleukin 2; rIL-2, recombinant interleukin 2; PMA, phorbol 12-myristate 13-acetate.

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phore was stored as a 1-mg/ml stock in dimethyl sulfoxide at −20°C. The phorbol ester was stored as a 10-μg/ml stock in 95% ethanol. Recombinant IL-2 (rIL-2; Boehringer Mannheim) was added to the appropriate cultures at a final concentration of 20 units/ml. Cultures in triplicate were incubated at 37°C under 5% CO_2 for 3–5 days (kinetic studies indicated that the proliferative responses peaked on these days of culture). Sixteen hours before the cultures were harvested, [*methyl*-^{3}H]thymidine (2.5 μCi per well; 1 μCi = 37 kBq) was added. The incorporation of [^{3}H]thymidine was measured with a liquid scintillation counter. To measure the expression of IL-2 receptor, cells suspended in complete medium (10^6 per ml) were plated in 24-well plates with the appropriate stimuli. After the incubation period, cells were washed twice with PBS. The presence of CD25 antigen was measured by immunofluorescence.

Production and Assay of IL-2. Thymocytes were cultured with or without activation stimuli in 24-well plates (5×10^6 cells per ml) for 18 hr. Cells were harvested, washed, and suspended again with complete medium (5×10^6 per ml) and incubated at 37°C for 24 hr. Supernatants were harvested and assayed for the presence of IL-2 activity. Supernatants of washings were used as controls to rule out the presence of residual activators (PMA, ionomycin) that could stimulate target cells. IL-2 activity was assayed by determining the ability to sustain the growth of an IL-2 dependent T-cell line, as described by Gillis *et al.* (15). CTLL-2 cells (16) suspended in complete medium (3×10^3 in 50 μl) were plated in round-bottom microwells and 50 μl of supernatant was added to each well. Samples were assayed in triplicate. After 48 hr at 37°C, cells were incubated with 2.5 μCi of [^{3}H]thymidine for 5 hr and harvested, and incorporation of [^{3}H]thymidine was measured as above. For each experiment a standard curve was constructed by using serial dilutions of a solution containing rIL-2 at 200 units/ml.

RESULTS

CD3$^-$4$^-$8$^-$ Thymocytes But Not Unfractionated Thymocytes Present a Marked Response to Phorbol Ester in the Presence of IL-2. Neither CD3$^-$4$^-$8$^-$ immature thymocytes nor unfractionated ones showed a significant proliferative response when they were incubated with PMA at 0.01 ng/ml to 10 μg/ml (Fig. 1*A*). The addition of rIL-2 induced a vigorous response in the CD3$^-$4$^-$8$^-$ thymocytes (Fig. 1*B*), suggesting that PMA induced the expression of IL-2 receptor, as was later confirmed (see Table 1).

In contrast to the CD3$^-$4$^-$8$^-$ cells, the unfractionated population hardly responded to any of the concentrations of PMA tested in the presence of rIL-2 (Fig. 1*B*), even with rIL-2 concentrations as high as 100 units/ml (data not shown). The poor response of the unfractionated thymocytes was neither due to the fact that these cells require different concentrations of PMA (Fig. 1) nor to the possibility of having a peak response on a different day. The CD3$^-$4$^-$8$^-$ thymocytes presented their peak response on the fifth day, but the unfractionated population did not respond strongly at any time in the period analyzed (Fig. 2).

The data suggest that the proliferation induced by PMA in human CD3$^-$4$^-$8$^-$ thymocytes is IL-2-dependent. Unfractionated thymocytes appear to be unable to mount a clear proliferative response through the IL-2 pathway in the system used.

Proliferation of CD3$^-$4$^-$8$^-$ Thymocytes in Response to Phorbol Ester plus Ca^{2+} Ionophore Is Greater Than That of Unfractionated Thymocytes. PMA and ionomycin were used in combination at various concentrations to stimulate thymocytes. At concentrations of PMA ranging from 1 ng/ml to 1 μg/ml, the addition of ionomycin at 0.1 or 1 μg/ml induced a strong proliferative response in the CD3$^-$4$^-$8$^-$ thymocytes (Fig. 3*B*). Concentrations of ionophore <0.1 μg/ml or >1 μg/ml induced only a very weak response. The unfractionated thymocytes did not present a strong proliferative response at any combination tested, and only a modest response was observed with the combinations of PMA plus ionomycin that produced the greatest response in CD3$^-$4$^-$8$^-$ thymocytes.

The addition of rIL-2 to CD3$^-$4$^-$8$^-$ thymocyte cultures containing PMA plus ionophore induced a proliferative response of similar magnitude for all tested combinations of PMA (1 ng/ml–1 μg/ml) ionophore (≤1 μg/ml) (Fig. 3*A*). In contrast, unfractionated thymocytes showed a proliferative pattern similar to the one observed when no IL-2 was added.

These data indicate that, at high ionophore concentration, a synergistic effect of PMA and ionophore could be observed even when the concentration of PMA was low. At low concentration of ionophore, an induction of IL-2 receptor expression could be indirectly detected by the vigorous proliferative response obtained when rIL-2 was added to the cultures. That was not the case when high concentrations of

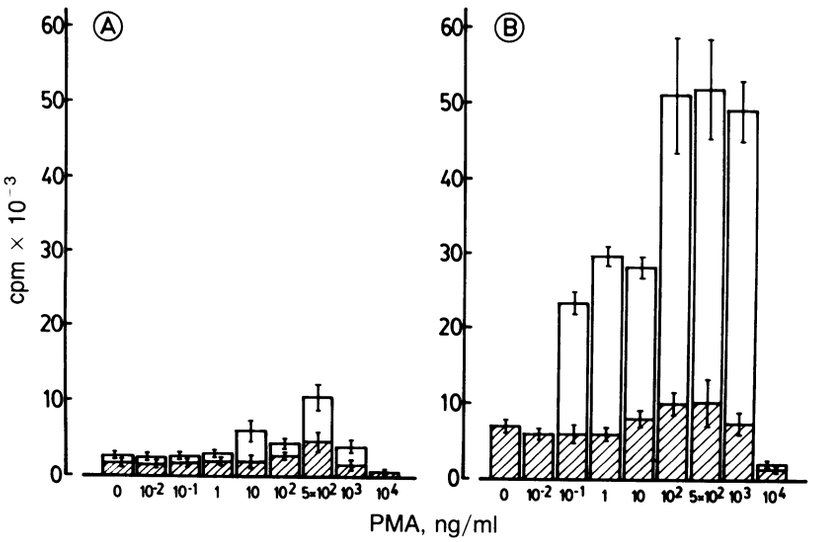

FIG. 1. Responses of CD3$^-$4$^-$8$^-$ (bars) and unfractionated (hatched portions of bars) thymocytes to various concentrations of PMA in the absence (*A*) or presence (*B*) of rIL-2 (20 units/ml). Each value is the mean ± SD of triplicate cultures. Cultures were harvested on day 5.

Table 1. Percentage of IL-2 receptor (CD25) expression in thymocyte populations

Stimulation	% CD25⁺ cells	
	$CD3^-4^-8^-$	Unfractionated
None	4.5 ± 1.0	0
PMA	6 ± 3	3 ± 1
PMA + IL-2	44 ± 9	40 ± 7
PMA + ionophore	46 ± 10.7	42 ± 10.3

Thymocytes were stimulated at 10^6 viable cells per ml. Final concentrations for the activating agents: PMA, 500 ng/ml; Ca^{2+} ionophore (ionomycin), 500 ng/ml; IL-2, 20 units/ml. Cultures were harvested at day 3. Results represent mean ± SD of five experiments.

ionophore were used. The addition of IL-2 did not further increase the high response obtained in these conditions (Fig. 3).

To rule out the possibility that these patterns of response were due to differences in either the sex or the age of the individual thymus donors, six thymus samples from patients of both sexes and of ages ranging from 2 months to 7 years were tested (Fig. 4). Although some variations between patients were observed in the amount of [^{3}H]thymidine incorporated by the cells, the same pattern of response was observed in all the cases. When PMA plus ionophore was used at high concentrations, the unfractionated thymocytes presented a response that, although weak in relation to the one presented by $CD3^-4^-8^-$ thymocytes, was higher than the one observed with PMA plus IL-2.

The data on proliferation kinetics presented in Fig. 2 show that the difference between the $CD3^-4^-8^-$ and unfractionated thymocyte populations is not due to different kinetics. In addition, neither treatment with complement (Fig. 4) nor treatment with anti-CD3 (data not shown) induced a proliferative response in $CD3^-4^-8^-$ thymocytes.

Unfractionated Thymocytes Are Able to Express the IL-2 Receptor (CD25) at a Level Similar to the Immature Thymocytes. Similar percentages of both $CD3^-4^-8^-$ and unfractionated populations expressed the IL-2 receptor antigen (CD25) when they were stimulated either with PMA plus ionophore or with PMA plus IL-2. As the fact that the unfractionated thymocytes expressed the IL-2 receptor at the

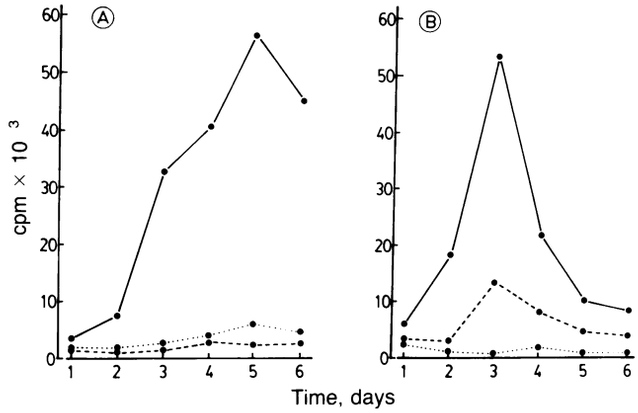

FIG. 2. Kinetic analysis of the proliferative responses of $CD3^-4^-8^-$ and unfractionated thymocytes to PMA (500 ng/ml) plus rIL-2 (20 units/ml) (*A*) or plus ionomycin (500 ng/ml) (*B*). Solid line, $CD3^-4^-8^-$ thymocytes; dotted line, unfractionated thymocytes; dashed line, unfractionated thymocytes treated with complement.

same frequency as the $CD3^-4^-8^-$ thymocytes was very surprising, kinetic analysis was performed to exclude the possibility that the results were due to an overgrowth of the $CD3^-4^-8^-$ thymocytes present in the bulk population.

The kinetics of the appearance of CD25 was similar in the two populations (Fig. 5). These data, together with the fact that the cell yield and viability in both populations remained very high during the cell culture period, show that the CD25 expression in the total thymus population was not merely due to expansion of IL-2 receptor-positive cells of the $CD3^-4^-8^-$ thymocytes included in the unfractionated population.

$CD3^-4^-8^-$ and Unfractionated Thymocytes Secrete Similar Levels of IL-2. $CD3^-4^-8^-$ and unfractionated thymocytes secreted IL-2 when stimulated with PMA plus ionophore at high concentrations (Table 2). Ionophore alone did not induce a detectable production of IL-2. PMA alone induced detectable production of IL-2 only if it was used at a concentration of 500 ng/ml. This result does not agree with published data indicating that PMA alone does not induce the secretion of IL-2 (17). This discrepancy could be due to the higher concentrations of PMA used in the present study. An indirect

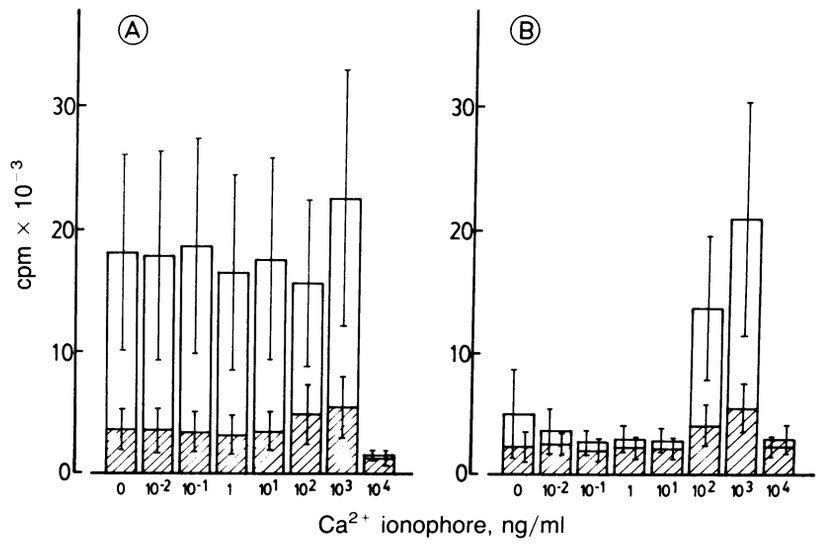

FIG. 3. Incorporation of [^{3}H]thymidine by $CD3^-4^-8^-$ (bars) and unfractionated (hatched portions of bars) thymocytes after stimulation by several combinations of PMA plus ionomycin in the presence (*A*) or absence (*B*) of rIL-2 (20 units/ml). PMA was used at concentrations of 1, 10, 100, and 1000 ng/ml. All of these concentrations of PMA were tested in combination with concentrations of ionomycin ranging from 10^{-2} to 10^4 ng/ml. As the different concentrations of PMA gave similar results to each concentration of ionomycin, each column represents the mean value ± SD of the results obtained for all PMA concentrations with each concentration of ionomycin. Cultures were harvested on day 3.

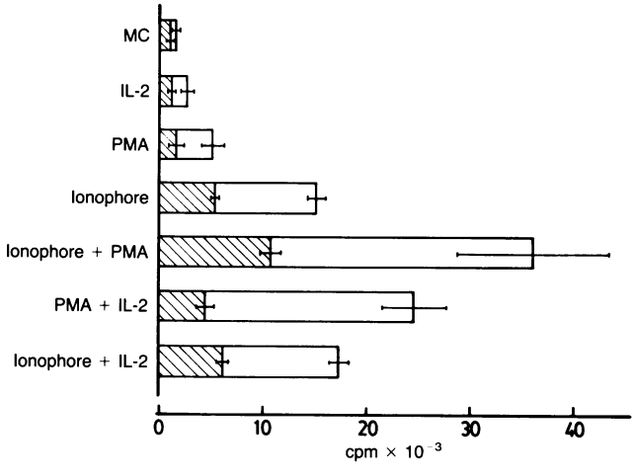

FIG. 4. Proliferative responses of $CD3^-4^-8^-$ (bars) and unfractionated (hatched portions of bars) thymocytes after stimulation with various mitogens. Thymus samples were obtained from six children (three boys and three girls) aged 2 months, 14 months, 18 months, 2 years, 4 years, and 7 years. Each value is the mean ± SD for the six thymus samples. Cultures were harvested on day 3. MC, control (culture medium).

confirmation of these data is shown in Fig. 1A, where it can be observed that $CD3^-4^-8^-$ thymocytes showed some proliferation when they were stimulated with 500 ng of PMA per ml in the absence of exogenous IL-2.

These data on the production of IL-2 show that human $CD3^-4^-8^-$ thymocytes, like the murine $L3T4^-$ $Ly\text{-}2^-$ ones, are able to secrete IL-2. Our data also indicate that unfractionated thymocytes are able to produce IL-2 at levels that are only slightly lower than the ones obtained with $CD3^-4^-8^-$ thymocytes. Both populations produced IL-2 in a lower quantity than did peripheral T cells.

DISCUSSION

Our results show that human $CD3^-4^-8^-$ thymocytes exhibit a marked proliferative response to stimulation with PMA plus IL-2 or PMA plus Ca^{2+} ionophore. In contrast, unfractionated thymocytes, although they express the IL-2 receptor,

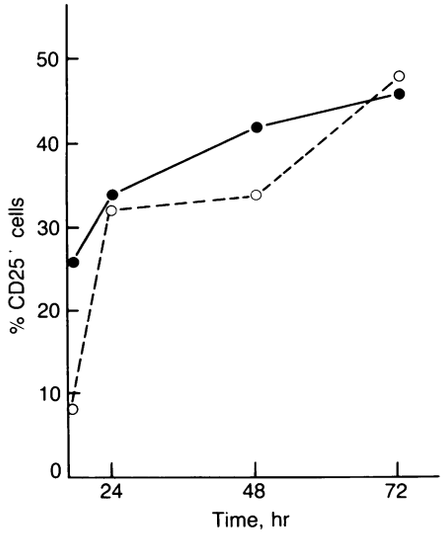

FIG. 5. Kinetics of IL-2 receptor expression (CD25) in $CD3^-4^-8^-$ (●) and unfractionated (○) thymocytes stimulated with PMA (500 ng/ml) plus ionomycin (500 ng/ml).

Table 2. IL-2 production by thymocyte populations and peripheral blood mononuclear cells (PBMCs)

Stimulation	IL-2 activity, units/ml		
	Thymocytes		
	$CD3^-4^-8^-$	Unfractionated	PBMCs
Ionophore	<0.05	<0.05	<0.05
PMA	0.43 ± 0.32	0.73 ± 0.21	0.84 ± 0.23
PMA + ionophore	1.85 ± 1.63	1.74 ± 1.32	5.16 ± 2.12

PBMCs were obtained by Ficoll-Hypaque separation of heparinized venous blood drawn from healthy human volunteers. Cells were stimulated at 5×10^6 per ml. Final concentrations for the activating agents: PMA, 500 ng/ml; Ca^{2+} ionophore, 500 ng/ml. Results represent mean ± SD of four experiments.

hardly proliferate. The two thymocyte populations secrete fairly similar quantities of IL-2.

Unfractionated thymocytes comprise all the subpopulations present in a cell suspension obtained from the thymus (1, 18). Although there is some disagreement on the exact number and type of subpopulations present in the thymus, a consensus exists on the main populations. Most reports agree that the double-positive ($CD4^+8^+$) population comprises about 70–80% of the total population (1, 18) and that the mature, immunocompetent subpopulations ($CD3^+4^+8^-$ and $CD3^+4^-8^+$) comprise about 7% and 6%, respectively (18). A recent report (19) concerning these mature populations in mice showed that the $Ly\text{-}2^+$ population (equivalent to the human $CD3^+4^-8^+$) is the only one of the two mature thymocyte populations that shows a large proliferative response to PMA plus IL-2. Since the $CD3^-4^-8^-$ subpopulation comprises only about 10–15% of the total thymocyte population in humans, it can be assumed that these two populations ($CD3^-4^-8^-$ and $CD3^+4^-8^+$), which could be considered as high responders, comprise about 20% of the total population. Thus, if we take into account the levels of response obtained with the unfractionated population, we could consider that these two subpopulations are the main set of cells that respond when the unfractionated thymus is stimulated with PMA plus IL-2, suggesting that the rest of the subpopulations do not contribute in a significant way to this response. This assumption is confirmed by the results obtained with the cells containing the antigen (OX-44), which has recently been described in the rat (20). This antigen is present in 12% of the thymus population, and the cells positive for this antigen respond intensively to several mitogens. In contrast, thymocyte suspensions depleted of OX-44 cells do not respond to these mitogens. The $OX\text{-}44^+$ cells are mainly the $CD4^-8^-$, $CD4^+8^-$, and $CD4^-8^+$ subpopulations.

The mechanism of activation that uses the interaction between IL-2 and its receptor seems to be the most common one (21). It has been shown with several experimental models that thymocytes also use this pathway to proliferate (22, 23). In this work we have confirmed that human $CD3^-4^-8^-$ thymocytes can also use this activation mechanism.

Under appropriate stimuli, human $CD3^-4^-8^-$ thymocytes are able to express the IL-2 receptor and proliferate in the presence of IL-2. Murine double-negative ($L3T4^-$ $Ly\text{-}2^-$) thymocytes also express IL-2 receptor and proliferate when they are stimulated with PMA and ionophore (9). Nevertheless, the optimal conditions for proliferation described in this paper for human $CD3^-4^-8^-$ thymocytes differ from the ones described for murine double-negative thymocytes. These differences in stimulation conditions are not surprising, because murine and human peripheral T cells also have different requirements for stimulation with PMA or Ca^{2+} ionophore (reviewed in ref. 24). What is surprising is the fact that although unfractionated thymocytes can be induced to

express IL-2 receptor, they respond weakly in the presence of saturating amounts of IL-2. It can be argued that unfractionated thymocytes are able to express only low-affinity IL-2 receptors. This seems very unlikely, as both $CD3^-4^-8^-$ and unfractionated thymocytes incubated with PMA alone either do not express IL-2 receptor or express it at low levels (ref. 25 and Table 1). In contrast, when IL-2 is added together with PMA, both thymocyte populations express the IL-2 receptor at a high frequency (ref. 25 and Table 1). It is probable that the PMA induces the expression of a small number of IL-2 receptors of high affinity and that the interaction IL-2 with its receptor induces an increase in this expression. An up-regulation of the expression of IL-2 receptors, such as the one originally described for peripheral T lymphocytes (26), probably occurs. If unfractionated thymocytes were unable to express high-affinity receptors, no initial interaction between IL-2 and its receptor would have taken place. An alternative explanation of the weak response of unfractionated thymocytes could be based on a lack of internalization of the IL-2/IL-2 receptor complex.

The production of IL-2 by murine double-negative thymocytes has been described by several authors, although there is some controversy over the reported quantities of IL-2 secreted by these cells (10, 27). Table 2 shows that both $CD3^-4^-8^-$ and unfractionated thymocytes produce IL-2 when stimulated with PMA plus ionophore. Our results show that the quantity of IL-2 produced by $CD3^-4^-8^-$ thymocytes is similar to the quantity produced by unfractionated thymocytes.

These data show that the human $CD3^-4^-8^-$ thymocytes, like the murine $L3T4^-$ $Ly\text{-}2^-$ ones, have a high proliferative capacity and are able to produce IL-2 and to express the IL-2 receptor. On the other hand, the unfractionated thymocytes, although able to express the IL-2 receptor and to produce IL-2, show a very poor proliferative capacity, at least with the mitogenic stimuli used in this work. These data lead us to postulate that during the maturation process the thymocytes lose their proliferative capability but preserve their capacity to express the IL-2 receptor and to secrete IL-2. It seems as if the thymocytes follow a terminal maturation similar to the one described in other hematopoietic systems such as the myeloid and erythroid. In these systems the terminal cells (granulocytes, erythroblasts) lose their proliferative capabilities but keep some of their functional capacities. In their terminal maturation stages these cells live only for a few days. A similar situation seems to occur with the thymocytes, as a high percentage of these cells lose their proliferative capability. Most probably these cells also die inside the thymus. This assumption is based on the well-known fact that the majority of thymocytes die inside the thymus (28). Only a minority of thymocytes would become competent and would leave the thymus and move to the periphery. In the latter case a blockade would be produced on the way to terminal differentiation and the cells would be switched to follow another maturation pathway, becoming immunologically competent. Since we used mitogens that act nonspecifically, it seems very unlikely that the poor response of the unfractionated thymocytes could be due to suppressor mechanisms.

What still remains to be proven is the way in which the cells would be selected to become immunologically competent instead of following a terminal differentiation. Some published results (29) and preliminary experiments in our laboratory show that if $CD3^-4^-8^-$ thymocytes are stimulated in a nonspecific way, they can acquire a mature phenotype and keep proliferating *in vitro* for several weeks. This leads us to postulate, as has already been proposed for B cells (30), that a proliferative stimulus would be sufficient to switch the differentiation pathway from the one leading to terminal differentiation to the one that ends in the formation of mature, competent T cells. One important question to be answered is whether the recognition of the self major histocompatibility complex product is the main proliferative stimulus (31, 32) or whether other activation signals are also operative.

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