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Expression of cytokines and their receptors by human thymocytes and thymic stromal cells

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SUMMARY

The repertoire of cytokine and cytokine receptor mRNA expressed by unstimulated human thymocytes and thymic stromal cells was explored by a quantitative polymerase chain reaction (PCR) using sequence specific internal standards. Of the 18 cytokines tested we found a considerable overlap in the expression of cytokines by human thymocytes and by thymic stromal cells; both cell types express the mRNA for interleukin-1β(IL-1, IL-6, IL-7 and tumour necrosis factor-α (TNF-α). However, there are substantial differences in the levels of cytokine mRNA expressed in these two types of cells as revealed by the quantitative PCR assay. Stromal cells express considerably higher levels of IL-1β and IL-6 than thymocytes (14- and 27-fold respectively). In addition, a number of cytokines such as lymphotoxin and interferon-γ (IFN-γ), are expressed exclusively in thymocytes whereas others such as stem cell factor (SCF), IL-1 receptor antagonist-2 (IRAP-2) and granulocyte–macrophage colony-stimulating factor (GM-CSF) are produced only in stromal cells. There is a complete overlap in the expression of a group of cytokine receptors tested in thymocytes and thymic stromal cells; these include IL-1R, IL-2R, IL-6R, IL-7R, TNFR and stem cell growth factor receptor (c-KIT). The expression of specific cytokines by thymic stromal cells and the parallel expression of their receptors on thymocytes under physiological conditions, support the hypothesis that these cytokines participate in paracrine interactions between these two cell populations during thymocyte differentiation.

INTRODUCTION

The T-cell precursors originate from hematopoietic stem cells in the bone marrow and colonize the thymus during development. Here the cells undergo a complex maturation process involving genotypic and phenotypic changes in the expression of different surface markers.[1-4] The differentiation of T lymphocytes in the thymus is affected by the thymic microenvironment,[3,5] which consists of non-lymphoid epithelial cells and fibroblasts as well as bone marrow-derived dendritic cells and macrophages.[1] Immature T cells migrate from the thymic cortex toward the medulla where they bind to epithelial cells, macrophages, or dendritic cells which directs their maturation process into differentiated $CD4^+$ or $CD8^+$ cells.[1-4] Despite the abundant documentation for the role of the thymic microenvironment in T-cell differentiation, very little is known about the mechanisms by which stromal cells influence T-cell differentiation. Since the growth and differentiation of intrathymic T lymphocytes is largely affected by a variety of cytokines,[6-10] the identification of the cellular source for the production of these cytokines may shed light on a possible mechanism by which stromal cells affect thymocyte development.

Studies involving the addition of cytokine-specific antibodies and recombinant cytokines to cultured thymocytes have demonstrated the requirement of several cytokines like interleukin-2 (IL-2) and IL-7 for precursor cell proliferation and differentiation to mature T cells.[11-13] A number of other cytokines, including IL-2, IL-4, IL-7 and tumour necrosis factor-α (TNF-α), promote the growth and differentiation of immature thymocytes *in vitro*.[7-10,14] However despite the evidence that these cytokines are responsible of intrathymic T-cell development, their cellular source(s) and the interactions within the thymus which control their production under physiological conditions are not well documented.

It has been shown that immature thymocytes have the potential to produce a number of cytokines, such as IL-2, IL-3, IL-4, interferon-γ (IFN-γ) and TNF-α, in response to stimulation by lectins or phorbol esters.[15-17] However, these non-physiological stimuli may mask or override the normal control

Abbreviations: c-KIT, stem cell growth factor receptor; ENC, encephalin; GM-CSF, granulocyte–macrophage colony-stimulating factor; IFN-γ, interferon-γ; IL-1R, interleukin-1 receptor; IRAP, IL-1 receptor antagonist; IRAP-2, IL-1 receptor antagonist-2; LIF, leukaemia inhibitory factor; LT, lymphotoxin; SCF, stem cell factor; TGF-α, transforming growth factor-α; TGF-β, transforming growth factor-β.

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of cytokine production. Unstimulated murine thymocytes were found to produce IL-2, IL-3 and IL-4 during foetal development,[18-20] and there is little information available about the production of other cytokines or the corresponding receptors either in the murine system or in unstimulated human thymocytes especially during the foetal maturation process. Thymic epithelial cells, on the other hand, were found to produce a number of cytokines including IL-1, granulocyte colony-stimulating factor (G-CSF), macrophage (M)-CSF, IL-6 and IL-7.[7-10,12] The expression by thymic stromal cells of a number of other cytokines and cytokine receptors known to affect T-cell development was not tested.

To address this question, we examined the expression of various cytokines and their receptors in unstimulated human thymocytes and in cultured stromal cell. We used a quantitative polymerase chain reaction (PCR) for mRNA determinations using sequence-specific synthetic internal standards.[21,22] The results demonstrate that unstimulated thymic stromal cells are a major source for a number of cytokines including IL-1, IL-6, IL-7, GM-CSF, TNF-α, IL-1 receptor antagonist-2 and stem cell factor (SCF). Unstimulated fresh thymocytes express some of these cytokines but at a much reduced level. Both thymocyte and stromal cell populations express the receptors for these cytokines as well as for IL-2 and SCF.

MATERIALS AND METHODS

Cell preparations

Thymocytes were obtained from patients (2 weeks–3 years old) undergoing corrective cardiac surgery. Viable thymocytes were isolated by Ficoll–Hypaque (Pharmacia, Uppsala, Sweden) density centrifugation. Human stromal cells were isolated and anchored as explants in culture flasks. Stromal cell cultures were washed daily with fresh RPMI–1640 medium supplemented with 10% foetal calf serum (FCS) (Hyclone, Logan, UT) so that only adherent cells were cultured. In addition, deoxyguanosine (0·2 mM) was added to the medium during the first days of culture to remove thymocytes, macrophages and dendritic cells as described by others.[11] After 2 weeks the stromal cells were trypsinized (0·25% Trypsin; Sigma, St Louis, MO), total cell RNA was extracted, the mRNA was transcribed to cDNA and the cDNA was amplified by the PCR. Cells were cultured in RPMI–1640 medium supplemented with penicillin (100 μg/ml), streptomycin (100 μg/ml), and L-glutamine (2 mM) and 10% FCS.

RNA preparations

Total cellular RNA was extracted according to the previously described method from Chomczynski and Sacchi.[23] The RNA concentration was measured with a Beckman spectrophotometer (Beckman Instruments DU-40, Fullerton, CA) and the integrity of the RNA was confirmed by gel electrophoresis using denaturing conditions on a 0·9% agarose gel.[22]

Reverse transcriptions of mRNA

After denaturation of the fresh prepared RNA at 65° for 10 min a single-stranded cDNA was produced by reverse transcription at 37° for 60 min in an end volume of 40 μl containing 5 μg of total cellular RNA, 0·5 μg oligo dT (12-18 mer; Pharmacia), 1 mM dithiothreitol, 125 μM dNTP, 80 U of RNA guard (Pharmacia) and 100 U of the recombinant MMLV reverse transcriptase (Gibco BRL, Burlington, Ontario, Canada). After heating at 65° for 10 min the cDNA was used for amplification.[24]

Amplification of the cDNA by PCR

cDNA was amplified as previously described using synthetic DNA as an internal standard as described in detail by Wang *et al.*[21] The thymus-derived cDNA and the internal standard DNA were co-amplified in the same tube. The target cDNA and the internal standard DNA utilize identical primer sequences but yield PCR products of different sizes that are easily separated by gel electrophoresis.[22] Quantitation is achieved through reference to a standard curve, generated by varying amounts of internal standard DNA.[21] The sizes of the internal standards and of the corresponding primers for the cytokines and their receptors are listed in Table 1 and most of the sequences are described previously.[22] The nucleotide sequences for additional upstream and downstream oligonucleotide primers are as follows:- IL-9, 5′ATCCTGGACATCAACTTCTCTATC3′ and 5′CTTGCCTCTCATCCCTCTCATC3′;[25] IL-11, 5′GCACTGGGAGCTCTACAGCTC3′ and 5′CAGCCGAGTCTTCAGCAGCAG3′;[26] SCF, 5′CCCAGGCTCTTTACTCCTGAAG3′ and 5′CTGCCCTTGTAAGACTTGGCTG3′;[27] stem cell growth

Table 1. The size of the PCR products received by using cytokine and cytokine receptor specific primers

mRNA species	Size of PCR products in bp	
	mRNA	Standard
IL-1β	350	238
IL-2	368	234
IL-3	342	256
IL-4	312	255
IL-5	312	257
IL-6	462	236
IL-7	361	255
IL-9	355	294
IL-11	348	291
TNF-α	352	252
IFN-γ	349	234
GM-CSF	345	217
SCF	349	292
IL-1R	369	237
IL-2R	356	238
IL-4R	350	253
IL-6R	350	235
IL-7R	370	248
TNF-αR	354	245
IFN-γR	368	234
GM-CSFR	400	291
c-KIT	351	291
IRAP	337	251
IRAP-2	346	289
ENK	355	293
LT	347	240
TGF-β	352	217

Sequences of the corresponding primers were chosen as described in Materials and Methods and the PCR products for the various cytokine cDNA of 312 bp to 462 bp, and an internal standard product of 217 bp to 293 bp are listed above.

factor receptor (c-KIT), 5'TGGGAGCTGTTCTCTTTAGGAAG3' and 5'GACATCGTCGTGCACAAGCAG3';[28] GM-CSFR, 5'CCAGCAATGTCACCGTACGTT3' and 5'TGTGGAACTGGCGGGAACAG3';[29] IRAP-2, 5'CTAGTTGCTGGATACTTGCAAGG3' and 5'GGTGACCATGACGCCTCCGTC3'.[30]

All the used primers were designed to give a melting temperature greater than 58°. The sizes of the PCR products for the various cDNA were between 312 base pair (bp) and 462 bp, and for the internal standard DNA between 217 bp and 293 bp as shown in Table 1.

Different dilution series of the internal standard DNA (0·03 pg, 0·05 pg or 1 pg, respectively) and a constant concentration of the patient-derived cDNA (200 ng) were amplified in the presence of 1 U of *Thermus aquaticus* DNA polymerase (Taq polymerase; Cetus, Emeryville, CA), the appropriate primers (500 ng), the [γ^{32}P]end labelled primer (10^6 c.p.m.) where indicated, 200 μM dNTP, baker yeast tRNA (1 μg) (Boehringer Mannheim, Mannheim, Germany) and the PCR buffer containing 15 mM $MgCl_2$, 500 mM KCl, 100 mM Tris-HCl (pH 8·3) and 0·01% gelatin in a total volume of 100 μl.[22]

The DNA was amplified for 29 cycles by denaturing at 95° for 30 seconds, primer annealing at 53° for 30 seconds, and extending at 72° for 1 min in a BIOSYCLER OVEN (BiosCorp., New Haven, CT). Oligonucleotides were end labelled with [γ^{32}P]ATP and separated from unincorporated nucleotides on Sephadex G-50 column as described previously.[22] Twenty microlitres of the PCR product were electrophoresed using 5% polyacrylamide gels or precast 4–20% polyacrylamide mini-gels (Novex, San Diego, CA). When radiolabelled oligonucleotides were incorporated into the amplied product, the gels were dried and autoradiographed. For quantitation, the amounts of radioactivity recovered from excised gel bands were plotted against the corresponding concentrations of the internal standard and the amount of cDNA present in each sample was calculated from this standard curve. The range of concentrations for both templates and the number of amplification cycles are chosen in the way that the PCR reaction for the target sequence remain within the exponential phase of the PCR.[21] Also negative controls without using target DNA were necessary for monitoring false positive and to give the amount of background counts.

RESULTS

The repertoire of cytokines expressed by human thymocytes and stromal cells

We first analysed the repertoire of cytokine mRNA expressed by human thymocytes and by cultured thymic stromal cells using the PCR with specific primers for 18 different cytokines. These primers were used to amplify the respective cytokine cDNA yielding a product in each case of approximately 350 bp (ref. 22 and Table 1). In addition, each tube contained a known amount of synthetic DNA standard which contained specific sequences of all the cytokine tested and gave in each case a PCR product of approximately 250 bp (ref. 22 and Table 1). These internal standards provide both, positive controls for the amplification process and means of quantitation the levels of each cytokine mRNA by using different dilutions of the internal standard.

The analyses of cytokine mRNA expression in thymocytes and thymic stromal cells is presented in Fig. 1. Thymocytes as well as stromal cells express the mRNA for IL-1β and TNF-α, although the ethidium bromide-stained gels suggest that the level of expression of these cytokines varies between these two cell types (Fig. 1). In addition, the two cell types exhibit qualitative differences in the expression of mRNA for other cytokines, thus IFN-γ and lymphotoxin (LT) mRNA are detected in thymocytes but not in stromal cells, while IL-6, SCF, GM-CSF, encephalin (ENC) and IRAP-2 mRNA are present in stromal cells only (Fig. 1). No mRNA for IL-2, IL-3, IL-4, IL-5, IL-9, IL-11 and transforming growth factor-β TGF-β is detectable in unstimulated thymocytes or in stromal cells.

On the other hand, there is a complete overlap in the expression of cytokine receptors mRNA between thymocytes and thymic stromal cells. The mRNA for the receptors of IL-1β, IL-2, IL-6, IL-7, TNF-α, IFN-γ and SCF are expressed in both thymocytes and in stromal cells (Fig. 1c, d, g, h). The mRNA for IL-4 and GM-CSF receptors were not expressed in either cell population (Fig. 1c, d, g, h).

Expression of cytokine and cytokine receptor genes measured by quantitative PCR

In order to compare the relative mRNA levels for individual cytokines expressed in thymocytes and in stromal cells we have employed a quantitative PCR assay in which we compared the incorporation of radioactive oligonucleotides into thymocytes and stromal cell-derived cDNA with their incorporation into internal synthetic internal standard DNA.[22] The assay detects as little as 0·01 pg mRNA of each cytokine, a sensitivity 10-fold higher than achieved with non-quantitative assay (Fig. 1). Using this assay we were able to detect the mRNA for the cytokines IL-1β, IL-6, IL-7, TNF-α and GM-CSF as well as the mRNA for the cytokine receptors IL-1R, IL-2R, IL-6R, IL-7R, TNF-αR (Fig. 2). However, the levels of individual cytokine mRNA expressed vary greatly between thymic stromal cells and thymocytes. The expression of IL-1β and IL-6 mRNA was markedly higher in stromal cells as compared to thymocytes (14 times and 27-fold respectively) while the levels of IL-7 mRNA were twice as high in stromal cells as in thymocytes (Fig. 2). In contrast, IL-7R mRNA was expressed at eight-fold higher levels in thymocytes than in stromal cells. The levels of mRNA for other cytokines were similar between thymocytes and stromal cells.

The levels of mRNA for several cytokines (IL-2, IL-3, IL-4 and IL-5) were undetectable in either unstimulated thymocytes or in stromal cells ($< 0{\cdot}01$pg $mRNA_{cytokine}/\mu g$ RNA total; Fig. 2 and data not shown), while GM-CSF mRNA was expressed in stromal cells but not in thymocytes (Fig. 2).

DISCUSSION

The involvement of cytokines in intrathymic T-cell development is well documented; a number of cytokines can be used as growth factors by thymocytes[12,18,20,31,32] and a variety of cytokines are produced by stimulated thymocytes.[33–37] In addition, the thymus microenvironment is required for precursor cell differentiation *in vitro*.[5,6,38–40] However, the identification of cytokines expressed by the human thymus under physiological conditions, and the cellular sources of their production are still unclear.[18] One hypothesis proposes that the thymic stromal cells

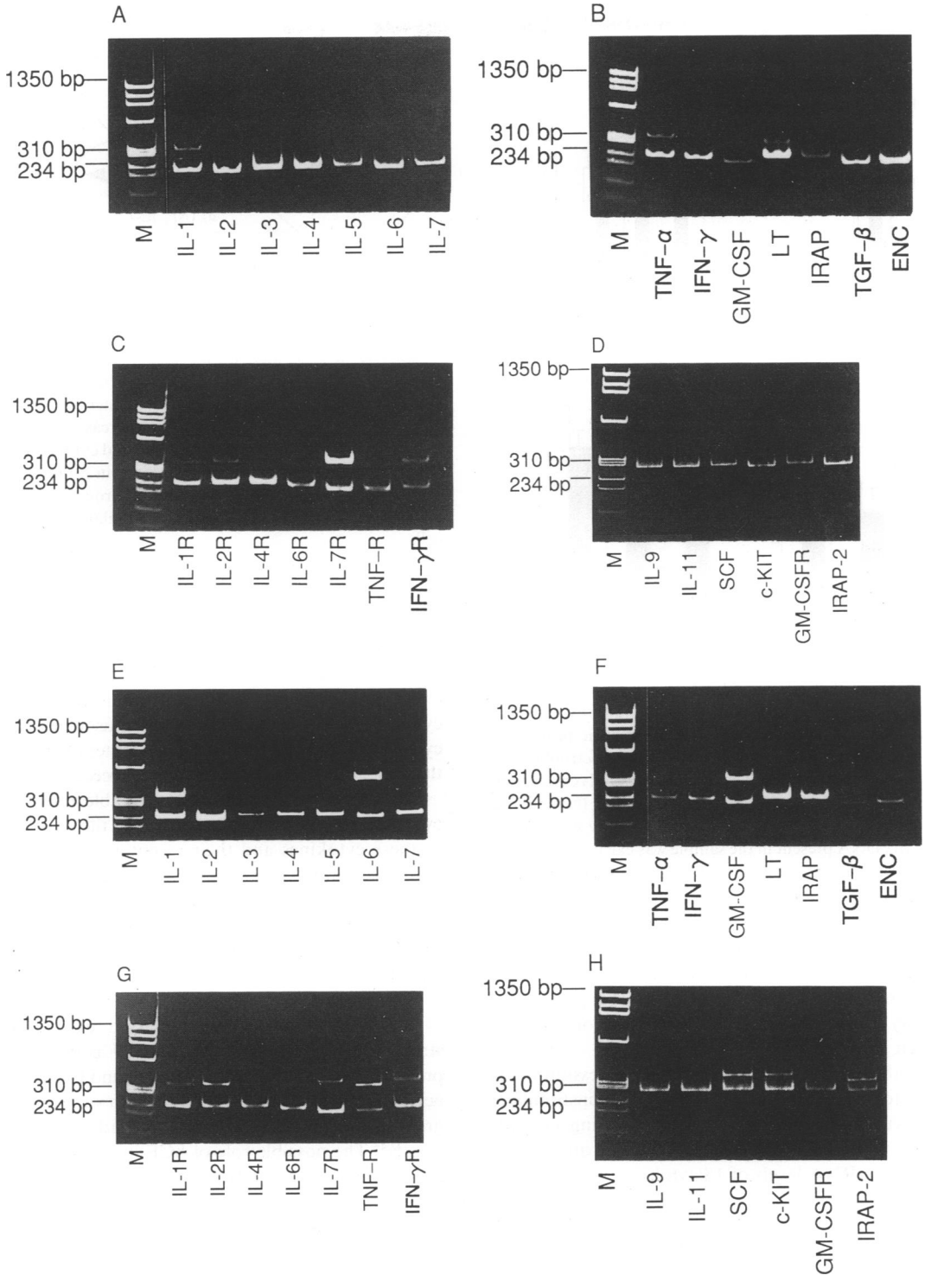

Figure 1. Expression of cytokine and cytokine receptor mRNA in human thymocytes and in thymic stromal cells. RNA prepared from thymocytes (A, B, C, D) and stromal cells (E, F, G, H) was reversed transcribed using oligo dT, as described under Materials and Methods. The reverse transcription products (cDNA, 0·1 μg/reaction) of cytokines and cytokine receptor mRNA were amplified by 29 cycles of PCR using specific primers as described in Materials and Methods. Amplification of each of the cytokines or cytokine receptors was performed in the presence of an internal standard DNA containing the primer sequences of the cytokines and cytokines receptors. The internal standard DNA gives rise in each case to a predicted PCR product of ~250 bp (lower band), whereas the same primers amplify PCR products of ~350 bp (upper band) for the individual cytokine or cytokine receptor cDNA. The nucleic acid standard ΘX174 RN DNA/HAE III fragments was used as the marker (M) for determining the molecular size of the corresponding fragments. PCR products were electrophoresed and stained with ethidium bromide as described in Materials and Methods. The data are from a single thymus representative of three newborn thymi analysed.

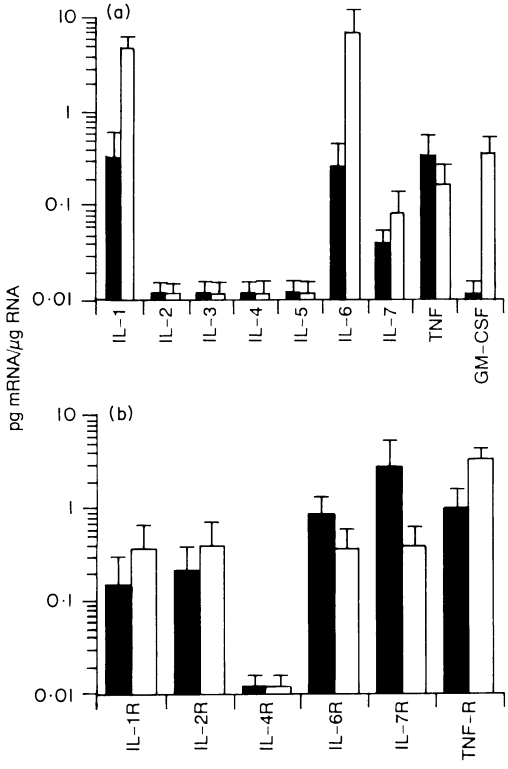

Figure 2. Quantitation of cytokine and cytokine receptors of human thymocytes (■) and stromal cells (□). After quantitative PCR as described under Materials and Methods with the specific primers for cytokines and cytokine receptors, the products were electrophoresed, the gel dried and autoradiographed. The radioactive bands corresponding to the dilution series of the internal standard, and the amplified cDNA were excised from the dried gel, the radioactivity was counted and the amount of cDNA present in the sample was calculated from the co-amplified internal standard as described in Materials and Methods. The results represent the mean of three determinations of cytokine mRNA (a) and cytokine receptor mRNA (b).

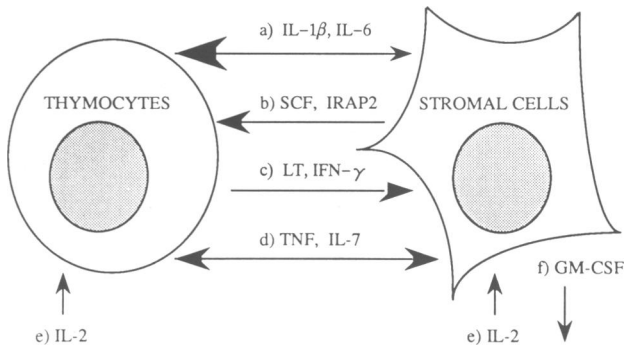

Figure 3. Schematic model for cytokine interactions between human thymocytes and thymic stromal cells. Cytokines are divided into six groups (a, b, c, d, e, f) according to their pattern of expression by thymocyte or thymic stromal cells. Cytokines belonging to group a are primarily produced by stromal cells, whereas cytokines from group b and f are produced by stromal cells only and cytokines from group c are produced by thymocytes only. Cytokines in group d are produced by both cell populations at similar levels. Group e represents cytokines which were not produced by either unstimulated thymocytes or stromal cells.

may support thymocyte differentiation by the production of specific cytokines.[5] In addition, it has been suggested that thymocytes may engage in autocrine production of cytokines supporting their own growth.[6,31]

To address this question, we studied the expression of the mRNA for cytokines and their receptors by unstimulated human thymocytes and by thymic stromal cells using a quantitative PCR assay. This assay allowed us to achieve quantitative determinations of mRNA levels of 18 cytokines and several of their receptors in fresh thymocytes and cultured stromal cells. Although these measurements do not demonstrate the secretion of these cytokines they nevertheless represent the repertoire of expressed cytokines and their receptors in separated thymocyte and stromal cell populations under physiological conditions.

We found a considerable overlap in the cytokines expressed in human thymocytes and stromal cells, thus both types of cells express the mRNA for IL-1β, IL-6, IL-7 and TNF-α (Figs 1, 2). However, there are substantial differences in the levels of cytokine mRNA expressed in these two types of cells revealed by the quantitative PCR assay. Stromal cells express much higher levels of IL-1β and IL-6 than thymocytes (14- and 27-fold respectively; Fig. 2), and a number of cytokines were expressed exclusively either in thymocytes (LT, IFN-γ) or in stromal cells (SCF, IRAP-2, GM-CSF). In contrast, there was a complete overlap in the expression of cytokine receptors in thymocytes and thymic stromal cells and both cell types express most of the receptors tested including IL-1R, IL-2R, IL-6R, IL-7R, TNFR and c-KIT (Figs 1, 2). Thus it seems that the specificity of cytokine interactions within the thymus depends mainly on the cytokines produced by either thymocytes or stromal cells rather than through selective expression of receptors.

Based on these results we were able to divide the thymic cytokines into six groups according to the pattern of expression of these cytokines and their receptors in stromal cells and in thymocytes (Fig. 3). Cytokines in group a, which includes IL-1β and IL-6, are expressed as higher levels in stromal cells than in thymocytes. These cytokines are likely involved in paracrine control of thymocyte growth or differentiation by stromal cells. Indeed both IL-1β and IL-6 were reported to induce thymocyte proliferation *in vitro*.[7,8,41]

SCF and IRAP-2 are expressed exclusively by thymic stromal cells (group b in Fig. 3). SCF is the growth factor for primitive hematopoietic progenitors and for mast cells where it acts synergistically in conjunction with various CSF to promote growth and differentiation of myeloid and erythroid precursors.[42,43] The possible role of SCF in lymphoid cell differentiation has not yet been tested. However, our observations that the mRNA for SCF is expressed by stromal cells, and that the mRNA for its receptor (c-KIT, SCFR) is expressed by both stromal cells and thymocytes (Fig. 1), suggest a role for SCF in T-cell proliferation.[44] IRAP-2, a specific product of epithelial cells, competes with IL-1 binding to its receptor and thus controls its biological activity.[30] The production of IRAP-2 by epithelial cells may regulate the effects of IL-1.[45]

LT and IFN-γ (group c in Fig. 3) are products of activated peripheral T cells.[46,47] Their expression by unstimulated thymocytes may indicate a role for these cytokines in interaction between thymocytes and stromal cells or an autocrine action on thymocytes (both populations express the receptors for these cytokines; Fig. 1). It was shown that both cytokines affect thymocyte proliferation *in vitro*.[46,49] TNF-α and IL-7 are

reported to support the proliferation of immature thymocytes *in vitro*;[56-60] our results indicate that these cytokines (group c in Fig. 3) are expressed at similar levels by thymocytes and stromal cells, again, suggesting the involvement of both paracrine and autocrine interactions driving intrathymic T-cell proliferation.

Thymocytes and epithelial cells express similar amounts of the mRNA for the α-subunit of IL-2R (group e in Fig. 3). The significance of the expression of the IL-2R by thymocytes and stromal cells is unclear since there was no detectable expression of IL-2 mRNA by either type of cell (Figs 1, 2). Moreover, using the PCR assays, we were unable to detect any levels of the mRNA for IL-2, IL-3 or IL-4 in either thymocyte or thymic stromal cell populations (Fig. 1). The radioactive PCR assay allows the detection of cytokine mRNA in the order of one molecule cell; nevertheless mRNA for IL-2, IL-3 and IL-4 (Fig. 2) is not detectable by this assay. The failure to detect mRNA for these cytokines is not due to unsuccessful amplification since these specific primers were able to detect IL-2, IL-3 and IL-4 mRNA in phorbol ester-activated T cells (data not shown). In addition, as a positive control synthetic DNA was amplified using identical primers in the same tube. Although we cannot exclude the possibility that a minor population of thymocytes or stromal cells may express low levels of the mRNA for these cytokines, it is unlikely that these cytokines play a major role in T-cell differentiation in the thymus of newborn. These results are in agreement with observations made in the mouse where it was shown that IL-2, IL-3 and IL-4 mRNA are expressed in the thymus only during embryonic development and not in the thymi of newborn mice.[18-20] These results suggest that T-cell differentiation can proceed without the participation of IL-2, IL-3 or IL-4 in the newborn human thymus.

The mRNA for GM-CSF was found exclusively in the stromal cell populations (group f in Fig. 3). The significance of GM-CSF expression by thymic stromal cells is unclear; no mRNA for GM-CSFR was found in thymocytes or stromal cells (Fig. 1). Again, it is still possible that a mRNA species expressed in a minor subpopulation of cells at a very low level will not be detected in this assay. In any case GM–CSF production is unlikely to play a role in the large majority of thymocyte undergoing differentiation in the thymus.

Previous studies documented the expression of a number of cytokines including IL-1, IL-6, IL-7, TNF-α, TGF-α, LIF, GM-CSF, G-CSF and M-CSF by thymic epithelial cells.[7-10,12,17,38,53] In the present study we extended the known cytokine repertoire expressed by stromal cells to include SCF and IRAP-2, as well as a number of cytokine receptors such as IL-1R, IL-2R, IL-6R, IL-7R, TNFR and c-KIT. These observations support the hypothesis that a network of cytokines direct intrathymic T-cell development. The expression of specific cytokines by thymic stromal cells and the parallel expression of their receptors on thymocytes under physiological conditions support the hypothesis that these cytokines participate in paracrine interactions between these two cell populations during thymocyte differentiation.

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