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The influence of IL-7 on V(D)J recombination

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SUMMARY

The influence of interleukin-7 (IL-7) on V(D)J recombination was investigated directly in the V(D)J recombination competent pre-B-cell line 38B9. The addition of IL-7 to the medium reduced the V(D)J recombination rate by 52–64%. This reduction was insensitive to the addition of cyclosporin A, indicating that the repression by IL-7 is not mediated by phosphatase 2B. The repression mechanism of IL-7 did not synergize with those of the protein kinase C activator 12-*O*-tetradecanoylphorbol-13-acetate (TPA) and the intracellular Ca^{2+} mobilizer thapsigargin. The action of IL-7 was blocked by the addition of the protein kinase A stimulator caffeine and the synthetic glucocorticoid dexamethasone. IL-7 did not change the m-RNA levels of the V(D)J recombination activating genes *RAG-1* and *RAG-2*, therefore IL-7 must exert its influence on V(D)J recombination either by post-transcriptional regulation of the *RAG* genes or by the regulation of other genes that are involved in V(D)J recombination. Although IL-7 may be necessary for the induction of and maintenance of V(D)J recombination during some stages of lymphocyte precursor development, it reduces the V(D)J recombination activity in pre-B cells.

INTRODUCTION

Interleukin-7 (IL-7) is a cytokine that is essential for lymphocyte precursors during several stages of their development. This IL-7 dependence occurs during the same stages in which the V(D)J recombination of the B- and T-cell receptors takes place.[1–3] The recombination activating genes *RAG-1* and *RAG-2* which are essential for V(D)J recombination[4–7] are also expressed during these stages. The simultaneity of IL-7 dependence and V(D)J recombination led to the assumption that V(D)J recombination and *RAG* gene expression are initiated and stimulated by IL-7. Experiments with thymocytes from fetal mouse thymuses showed that IL-7 promotes the rearrangement of the T-cell receptor (TCR) β chain gene and sustains the expression of recombination activating genes *RAG-1* and *RAG-2*.[8] Similar results were obtained with T cells from fetal liver in which an increased expression of the TCR γ V genes and the *RAG* genes was observed after the addition of IL-7 to these cells *in vitro*.[9]

From these data one may suppose that IL-7 generally increases the V(D)J recombination rate. However, these experiments only showed the parallel increase of *RAG* gene expression and V(D)J recombination in the presence of IL-7 and the direct effect of IL-7 on V(D)J recombination was not tested.

To test directly the effect of IL-7 on V(D)J recombination, the V(D)J recombination activity in 38B9 pre-B cells was measured in the presence and absence of IL-7.

V(D)J recombination is regulated differently by several signalling pathways. It is stimulated by protein kinase A (PKA) signalling pathway and down-regulated by the PKC and Ca^{2+} pathways.[10,11] To see whether IL-7 synergizes or antagonizes with these signal transduction pathways, the V(D)J recombination activity in 38B9 cells was also tested in the presence of IL-7 and activators of the several signal transduction pathways mentioned above. The effect of IL-7 on the *RAG* gene transcription was also monitored.

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Abbreviations: CsA, cyclosporin A; DEAE, diethylaminoethyl; EDTA, ethylenediamine tetra-acetic acid; IL-7, interleukin-7; IPTG, isopropyl β-D-thiogalactopyranoside; LB, Luria–Bertani broth; PKA, protein kinase A; PKC, protein kinase C; PP-2B, phosphatase 2B; *RAG*, recombination activating gene; TBS, Tris-buffered saline; TCR, T-cell receptor; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside.

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MATERIAL AND METHODS

Cell culture and transient DNA transfection

38B9 cells were grown in HEPES-buffered RPMI-1640 medium with 2 mM glutamax, supplemented with 10% fetal calf serum (FCS), 2·5 mg/ml amphotericin B, 5 mg/ml gentamicin and 25 mM β-mercaptoethanol (all from Life Technologies A.G., Basel, Switzerland) and transfected transiently as described by Hesse *et al.*[12] The cells were incubated in the DNA/diethylaminoethyl (DEAE) mixture in 0·5 ml medium for 25 min, washed twice with medium and incubated for a further 48 hr.

Extrachromosomal V(D)J recombination substrate and determination of the recombination rate

38B9 cells were transiently transfected with the artificial V(D)J recombination substrate pBlueRec[13] and treated with corresponding agents (protein kinase activators, etc., Sigma Chemie, Buchs, Switzerland) after the removal of the remaining DNA. Cells were

harvested 48 hr after transfection, and plasmid DNA was extracted as described below. Plasmid solution (2–10 μl) was digested with 5 units of the restriction enzyme *Dpn*I (New England Biolabs, Beverly, MA) for 15 min at 37° to remove non-replicated plasmid. The digestion mix was added to 100–200 μl freshly thawed *E. coli* DH5 α cells which had been made competent following the method of Inoue *et al.*,[14] and kept on ice for 1 hr. The cells were then heat shocked for 20 s in a 42° warm water bath and put back on ice for 1 min. Half a millilitre of 37° warm SOC medium (2% bacto tryptone, 0·5% yeast extract, 10 mM NaCl, 2·5 mM KCl, 10 mM $MgSO_4$, 10 mM $MgCl_2$, 20 mM glucose) was added and the bacteria were incubated for 1 hr at 37°. The bacteria were then plated on Luria–Bertani broth plates, containing 80 μg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) and 150 μg isopropyl β-D-thiogalactopyranoside (IPTG; both from Boehringer Mannheim). Plates were incubated overnight at 37° and the recombination rate was determined by dividing the number of blue colonies by the sum of blue and white colonies.

Isolation of plasmid-DNA from eucaryotic cells

Plasmid DNA from eucaryotic cells was recovered by the method of Birnboim and Doly,[15] modified by Göran Magnusson (unpublished). Cells were washed twice with Tris-buffered saline (TBS) and lysed in a 1 : 2 mixture of solution I (25 mM Tris pH 8, 10 mM ethylene diamine tetra-acetic acid (EDTA) pH 8 and solution II (0·2 M NaOH, 1% sodium dodecyl sulphate (SDS)). For a 90 mm petri dish 400 μl of this mixture were used. The dishes were gently shaken until the mix became viscous and transferred into an Eppendorf vial. Two hundred microlitres of solution III (1 vol. 3 M potassium acetate + 1 vol. 2 M acetic acid pH 4·8) were added to neutralize the solution. Precipitated cell debris was removed by centrifugation in an Eppendorf centrifuge for 10 min, and the supernatant was purified by phenol/chloroform (1 : 1) and chloroform extraction. The DNA was precipitated by 1 vol. of isopropanol for 1 hr at −20°, centrifuged, washed with 80% ethanol, dried and resolved in 200 ml TE (10 mM Tris, 1 mM EDTA pH 8).

Statistical evaluation of the experiments

For each experiment three different transfections were performed. The cell extract of each transfection was tested by two transformations of *E. coli* DH 5 α cells. From the resulting six measurements for each experiment the average recombination activity and the standard deviation was determined. Depending on the recombination rate 11 000–18 000 bacterial colonies for each experiment were evaluated.

RNAse protection assay

Antisense RNA for the RNA protection of the *RAG-1* gene was obtained by *in vitro* transcription from the T3 promoter of the pBS gar-1T3 plasmid. This plasmid is a derivative of pBlueScript into which 482 bp of the 5′ end of a *RAG-1* cDNA[6] was inserted. *RAG-2* antisense RNA was transcribed from the T7 promoter of the PSCT-gar-2 plasmid containing 270 bp of the 5′ end of a *RAG-2* cDNA.[7] RNA isolation, hybridization, signal detection and quantification were performed as described earlier.[16]

RESULTS

IL-7 decreases the V(D)J recombination rate

To test the influence of IL-7 on the recombination ration rate different concentrations of IL-7 were added to the medium. Five nanograms of IL-7 per millilitre of medium caused a reduction of the V(D)J recombination rate by 52% and 10 ng IL-7 per millilitre of medium reduced the recombination rate by 64% (Fig. 1, bars 1–3 from the top). Higher IL-7 concentrations led to the formation of large cell clusters. As an increased tendency towards larger cell clusters was already noticed at 10 ng IL-7 per millilitre of medium, only 5 ng/ml IL-7 were used for further experiments to avoid the effects caused by the formation of cell clusters which may interfere with the effects of IL-7.

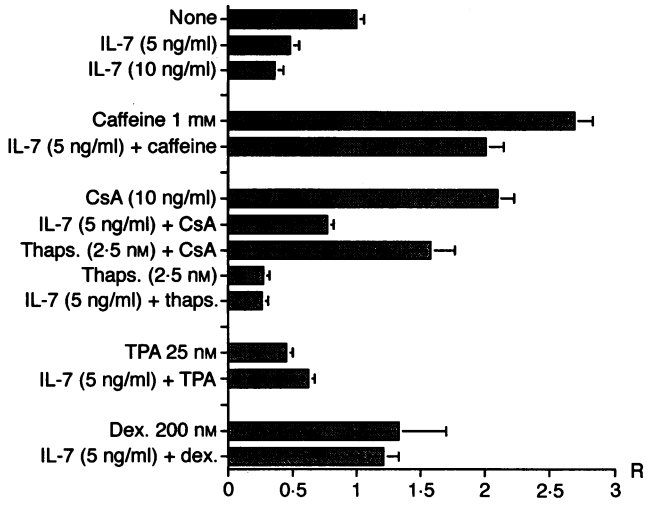

Figure 1. The effect of IL-7 on V(d)J recombination, alone and in the presence of other substances influencing the V(d)J recombination rate in 38B9 cells. The relative recombination rate is given by the abscissa and the values correspond to those given in Table 1 in the last column. For better comparison the recombination rate of untreated 38B9 cells was set as 1·0.

The interplay between IL-7 and activators or inhibitors of V(D)J recombination

V(D)J recombination activity can be influenced by several agents. Caffeine a stimulator of the protein kinase A (PKA) pathway increases the V(D)J recombination rate by enhancing the transcription of the *RAG* genes (Fig. 2).[10,11] In the presence of 1 mM caffeine IL-7 has only a moderate effect (25% reduction) indicating that activation of the PKA pathway can at least partially overcome the effect of IL-7 (Fig. 1, bars 4 and 5 from the top).

Cyclosporin (CsA) is a blocker of the phosphatase-2B (PP-2B, calcineurin, see 17 for review) which is able to abolish the suppression of V(D)J recombination by intracellular Ca^{2+} mobilized by thapsigargin.[11] The increase of the V(D)J recombination rate by the addition of CsA to 38B9 cells may be therefore due to the inhibition of a constitutive PP-2B activity. In contrast to the results obtained with the Ca^{2+} mobilizer thapsigargin, CsA is not able to reduce the down regulating effect of IL-7 completely (see the bars 6–10 from the top in Fig. 1). The relative inhibition factor of IL-7 in the presence of CsA is even greater than that observed in untreated cells (2·7-fold versus 2·1-fold). These results suggest that the inhibitory effect of IL-7 is not mediated by raising intracellular Ca^{2+} levels.

It was also tested whether IL-7 can synergize with other agents

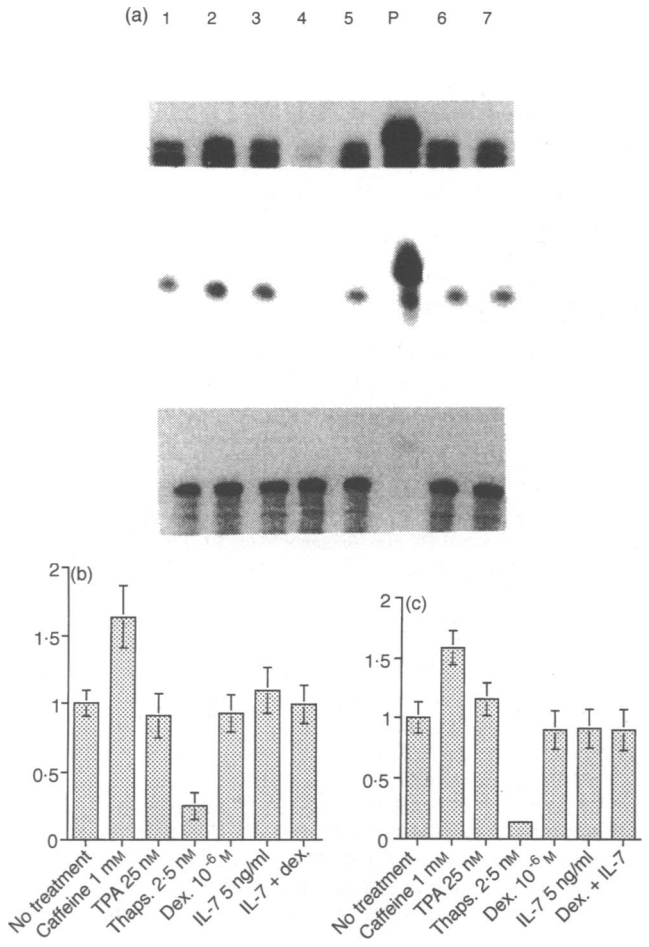

Figure 2. The influence of IL-7 and other regulators of V(d)J recombination on the mRNA levels of the *RAG-1* and *RAG-2* genes. (a) RNA protection assays with antisense probes of *RAG-1* (top), *RAG-2* (middle) and mouse β actin (bottom). Lane 1: no treatment, lane 2: 1 mM caffeine, lane 3: 25 nM TPA, lane 4: 2·5 nM thapsigargin, lane 5: 200 nM dexamethasone, lane P: probe, lane 6: 5 ng/ml IL-7, lane 7: 5 ng/ml Il-7 and 200 nM dexamethasone. (b) and (c) Evaluation of the signals for *RAG-1* (b) and *RAG-2* (c) mRNAs in (a) by phosphoimager. The signal of untreated 38B9 cells was set as 1·0.

that lower the V(D)J recombination rate. The simultaneous addition of IL-7 and the phorbol ester TPA or IL-7 and the intracellular Ca^{2+} liberator thapsigargin had no additional effect (see bars 3 and 4 and 6 and 7 from the bottom of Fig. 1), the addition of IL-7 to 12-*O*-tetradecanoylphorbol-13-acetate (TPA) seems to have even a weak enhancing effect.

The effect of the anti-inflammatory synthetic glucocorticoid hormone dexamethasone on IL-7 mediated V(D)J recombination repression was also tested. Figure 1 (bars 1 and 2 from the bottom) shows that dexamethasone itself has no influence on V(D)J recombination, but was able to block the effect of IL-7 totally, indicating that signalling through the IL-7 receptor is sensitive to glucocorticoids.

IL-7 has no effect on the RNA levels of the *RAG* genes

To test whether IL-7 influences the RNA levels of the *RAG* genes which are essential RNA protection assays were performed using *RAG-1* and *RAG-2* antisense probes. Figure 2 shows that IL-7 has no influence on the RNA levels of the two *RAG* genes. Dexamethasone, which antagonizes the effect of IL-7 on V(D)J recombination, also did not show any influence on the *RAG* RNA levels. Both agents therefore must work in another way, probably by post-transcriptional mechanisms, as has been shown by Lin and Desiderio (reviewed in 18).

DISCUSSION

The result that IL-7 does not stimulate, but reduces V(D)J recombination may seem at first glance somewhat surprising, as some experiments with T-cell precursors have suggested that IL-7 is necessary for the induction of the *RAG* genes[4,5] which are essential for V(D)J recombination. However, this view has recently been compromised by the finding that IL-7 can be replaced by IL-3 as a growth factor for B-cell precursors.[19] IL-7 may be therefore of different importance in T- and B-cell precursors.

The down-regulation of V(D)J recombination by IL-7 can be explained by the fact that IL-7 is a growth factor and as a such it will accelerate the cell cycle by shortening the time of the G phases. This reduction of the lengths of the G phases would lead to a reduction of V(D)J recombination, as V(D)J recombination is restricted to the G phases, since V(D)J recombination during the S or M phases would lead to double strand breaks and consequently lead either to apoptosis or transformation of the cell. Indeed at low cell densities the doubling time of 38B9 cells (22 hr) was shortened by 1–2 hr by the addition of 5 ng/ml IL-7. It has been shown that the activity of the RAG proteins is tightly controlled during the cell cycle and that the RAG proteins are inactivated during the S and M phases (reviewed in 18).

As the RAG RNA levels are not reduced by IL-7 it may play a double role as inducer of the RAG RNAs and as a growth factor. It ensures high levels of RAG RNAs during the cell cycle so that during the G phases these RNAs can be translated immediately into functional RAG proteins. As a growth factor IL-7 shortens the G phases and attenuates the V(D)J recombination rate as the RAG proteins are inactivated by phosphorylation during the S and M phase.[18]

In summary the results of this study show that IL-7 represses V(D)J recombination by a glucocorticoid sensitive pathway that does not influence the expression of the *RAG* genes. Experiments with CsA showed that the effect of IL-7 on V(D)J recombination is not mediated by increasing the levels of intracellular Ca^{2+} High intracellular Ca^{2+} levels induce the phosphatase PP-2B which in turn inhibits protein kinase A[20] which is a stimulator of V(D)J recombination.[10,11] The PP-2B inhibitor CsA, which totally blocks the effect of high intracellular Ca^{2+} levels induced by thapsigargin, did not block the down-regulating effect of IL-7. Instead IL-7 totally abrogated the V(D)J recombination enhancing effect of CsA in 38B9 cells. Therefore Ca^{2+} cannot be the mediator of the IL-7 effects. The RNAse protection experiments also argue against Ca^{2+} and PP-2B as the mediators of the IL-7 effect on V(D)J recombination, because IL-7 did not decrease the levels of the *RAG* RNAs like high levels of intracellular Ca^{2+} do (see Fig. 2a). The lack of synergism with TPA also argues against Ca^{2+} as the mediator of the IL-7 effect, as such a synergism has been observed in nearly all tested signalling systems.

The way of action of IL-7 has some similarities with the PKC pathway. Like IL-7 TPA does not lower the *RAG* genes' RNA levels and in contrast to thapsigargin TPA is, like IL-7, able to reduce the enhancing effect of CsA.[21] The effect of IL-7 is like that

of TPA, sensitive to dexamethasone.[22–24] Whether these similarities are proves for same way of action or just are coincidental has still to be proven. The lack of synergism between IL-7 and TPA can be explained by the fact that long-term stimulation of the PKC pathway leads to the destruction of this enzyme.[25,26]

How dexamethasone interferes with IL-7 remains to be elucidated. Glucocorticoids can interfere with signal transduction on the level of phospholipases (reviewed in 27) and the transcription factors AP-1[22–24] and NFkB.[28,29] AP-1 is a more likely candidate, as it has been shown to down regulate V(D)J recombination[11] and NFkB is also induced by the PKA pathway which enhances V(D)J recombination.

The stimulation of the PKA pathway by caffeine could largely reduce the down-regulating effect of IL-7 indicating that the IL-7 and PKA pathway act antagonistically. For the reasons mentioned above only the lower dose of IL-7 was tested. Therefore one may suppose that higher doses of IL-7 could further reduce the effect of caffeine.

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