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CONSTITUTIVE ACTIVATION OF NF-KB IN HUMAN THYMOCYTES

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NF-kB is a eukaryotic transcription regulatory factor. In T cells and T cell lines, NF-kB is bound to a cytoplasmic proteic inhibitor, the IkB. Treatment of T cells with mitogens (phorbol esters) or cytokines (TNFα) induces NF-kB nuclear translocation and the subsequent expression of NF-kB dependent T cell genes. Here we examined the activation of NF-kB in human T cell thymic progenitors. We report differences in $(Ca^{2+})i$ requirement for NF-kB activation in thymocytes as compared to mature T cells. Furthermore, our results indicated that thymocytes have a constitutively active form of NF-kB, suggesting that they are activated *in vivo*.

NF-kB is a eukaryotic transcription factor which was shown to interact with a specific DNA consensus sequence called kB (1). The kB sites are widely distributed among enhancer domains of both cellular (Ig k light chain, cytokines, receptors) and viral (SV 40, HIV, cytomegalovirus) genes (for review see 2). The transcriptionnally active form of NF-kB is composed of at least two polypeptides, p50 and p65 (3). Except in Ig k light chain producing-B cells and macrophages, NF-kB was found to be complexed to a cytosolic inhibitor, IkB (4). NF-kB activation in non-B cells results from dissociation of the IkB/NF-kB complex followed by nuclear translocation of the p50-p65 complex. This activation can be induced *in situ* by treatment of cell cultures with various activators (mitogens, cytokines, viral gene products). In T cells, among other functions, NF-kB could play an important role in polyclonal T cell proliferation by enhancing the transcription of interleukin 2 (IL2) and its

Abbreviations used: Ig, immunoglobulin; TNFα, Tumor necrosis factor α or cachetin; PMA, phorbol 12-myristate 13-acetate; PHA, phytohemagglutinin; HIV, human immunodeficiency virus-1; HIV-LTR, HIV-long terminal repeat; PRE, positive regulatory element from the HIV-LTR; EMSA, electrophoretic mobility shift assay.

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receptor α (5,6). Furthermore, NF-kB was reported to transactivate HIV transcription in HIV infected T cell cultures (7). Indeed, theHIV-LTR enhancer is composed of a tandem of the kB site (8).

In order to acquire immunocompetence, T cell progenitors undergo genetic and molecular transformations in the thymus. This series of events results in the mature T cell phenotypic repertoire. In the present work, we examined the potential role of NF-kB in these thymic events by studying NF-kB activation in thymocytes. Our results indicate that in human thymocytes, NF-kB was activated by TNFα as well as by phorbol ester. Interestingly enough, we found differences in NF-kB activation in thymocytes, as compared to mature T cells. In particular, we found that NF-kB was present in nuclear extracts of untreated thymocytes. We interpret this last result as a *in vivo* constitutive activation of human thymocytes.

MATERIALS AND METHODS

Cells:

Jurkat cells, a human CD4+ tumor T cell line were cultured in RPMI 1640 supplemented with 10% fetal calf serum and standard concentrations of L-glutamine and antibiotics. Human thymuses were obtained from patients undergoing cardiovascular surgery. Thymocytes were separated by Dounce homogeneisation and purified by Ficoll centrifugation, 0.16 M ammonium chloride treatment and monocytes adsorption on plastic. B cells and monocytes contamination was determined by FaxScan analysis using B4 (CD19 specific) and My4 (CD14 specific) antibodies (Coulter) respectively.Both B cells and monocytes represented less than 0.5 % of the total cell population. Purified thymocytes were suspended in RPMI 1640 with 10% fetal calf serum (10^7 cells/ ml) and were used immediately for NF-kB activation analysis.

Cell activation experiments were carried out using TNFα (100 Units/ml), phorbol 12-myristate 13-acetate (PMA, Sigma) (50 ng/ml), PMA plus phytohemagglutinin (PHA, Welcome) (50 ng/ml and 1 μg/ml respectively). Cells were treated for 1 hour at 37°C, 5% CO_2.

Nuclear protein preparation:

Protein from the Jurkat cells and thymocytes nuclei were prepared as described previously (9). Protein concentration was determined according to Bradford (10).

Oligonucleotides and Electrophoretic DNA Mobility Shift Assay (EMSA):

All oligonucleotides were a kind gift of Dr. Leo Lee (NCI, Frederick, Md. USA). Rabbit serum with polyclonal antibodies (Pab 350), which are specific for NF-kB recombinant p50 subunit, was a generous gift of Dr. A. Israel (Pasteur Institute, Paris, France) . NF-kB specific binding oligonucleotide (PRE) was a tandem of the kB site from the HIV-LTR: 5'-

ACAAGGGACTTTCCGCTGGGGACTTTCCAGG-3. Sequences of oligonucleotides used as competitors were as follows: 5'-AGGGGCTTTGACGTCAGCC-3' (CRE) which is not related to kB sequence, 5'-CTGGAGGGGACTTTATGACA-3' (TH), 5'-GCAGGGAATCTCCCT-3' (TAC), and
5'-CTCACTTTCCGCTGCTCACTTTCCGCTGCTCACTTTCCAAA-3' (MkB). TH and TAC oligonucleotides present 80% identity with the kB sequence of PRE. MkB is a trimer of a mutated form of kB, where GGG was replaced by CTC (underlined).

The gel shift assays (EMSA) were performed as described previously (9).

RESULTS AND DISCUSSION

To investigate activation of NF-kB, we extracted nuclear proteins from freshly prepared thymocytes and analysed their NF-kB content by EMSA of ^{32}P-PRE. Jurkat cells were used as a model for mature T cells. Moreover, activation of NF-kB by phorbol ester and TNFα in Jurkat cells was described previously (9); we thus used the Jurkat cell nuclear extracts as control for NF-kB migration in EMSA experiments. The results, shown in figure 1, demonstrate that thymocyte nuclei contained a kB binding protein with identical migration in EMSA to NF-kB extracted from activated Jurkat cells. The thymocyte NF-kB was activated by TNFα alone and PMA alone, whereas in Jurkat cells PHA or ionomycine co-treatment was necessary for efficient activation of NF-kB by PMA (figure 1 and ref.9). We have shown previously that PHA or ionomycine alone potentiate PMA-induced

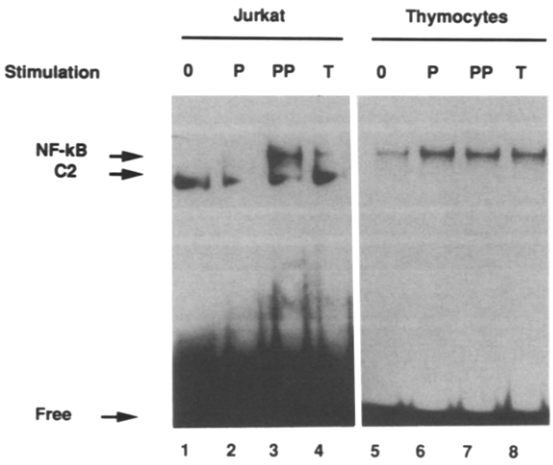

Figure 1. *EMSA of ^{32}P-PRE with Jurkat cells and thymocyte nuclear extracts.* Cells were treated with PMA (P) , PMA and PHA (PP), TNFα (T) or untreated (0) for one hour prior to protein extraction (see Materials and Methods). For both Jurkat cells (lanes 1-4) and thymocytes (lanes 5-8), amounts of protein extracts used in the assay were 13 to 14 μg per lane. Unbound oligonucleotide is indicated by the arrow (Free).

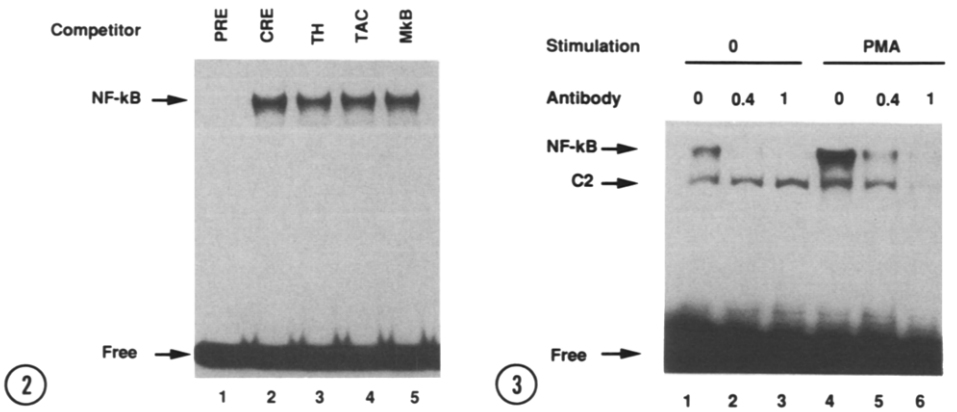

Figure 2. *Competition assay with unlabeled oligonucleotides.* Nuclear extracts from thymocytes were incubated with 100 ng of either PRE oligonucleotide itself or CRE, TH, TAC or MkB oligonucleotides (see Material and Methods for sequences) prior to incubation with 32P-labelled PRE oligonucleotide. Each lane contained the same amount of protein extract (14 μg) from PMA treated thymocytes. Unbound oligonucleotide is indicated by the arrow (Free).

Figure 3. *Inhibition of NF-kB binding by p50-specific antibodies (Pab 350):* Nuclear extracts from unstimulated (lanes 1-3) or PMA-stimulated (lanes 4-6) thymocytes were preincubated 30 minutes with 0.4 μl(lanes 2 and 5) or 1 μl (lanes 3 and 6) of Pab 350 antiserum prior to adding ^{32}P-labelled PRE oligonucleotide. Unbound oligonucleotide is indicated by the arrow (Free).

activation by increasing $[Ca^{2+}]i$, although alone they do not activate NF-kB. Here, our results indicate that in thymocytes Ca^{2+} influx is not required for PMA activation of NF-kB. Furthermore, in Jurkat cells, TNFα activation of NF-kB is generally less potent than activation by PMA plus PHA, whereas in thymocytes TNFα activated NF-kB with similar potency to PMA alone and PMA plus PHA (figure 1). These results indicate that the mechanism of NF-kB activation is different in thymocytes and mature T cells. The cause of these differences is unknown but it might reside within the structure of NF-kB/IkB complex. Alternatively, the enzymatic systems which contribute to NF-kB activation might be different in T cells and their thymic progenitors. For example, the PKC isoforms which mediate PMA activation of NF-kB might be different in thymocytes and in mature T cells.

To further characterize thymocyte NF-kB, we performed competition experiments with a 100-fold molar excess of unlabelled oligonucleotides (figure 2) and Pab 350 antibodies specific for the p50 sub-unit of NF-kB (figure 3). Pab 350 inhibits NF-kB binding to kB oligonucleotides (A. Israel,

personnal communication). The results show that only the PRE oligonucleotide was able to compete for NF-kB binding (figure 2). Mutated (MkB) and irrelevant (CRE) oligonucleotides did not inhibit ^{32}P-PRE/NF-kB interaction. Interestingly, oligonucleotides with only two mismatches (TH and TAC) did not inhibit, even partially, NF-kB binding to ^{32}P-PRE. This result indicates that NF-kB from thymocytes possesses a higher affinity for the HIV-kB sequence than for the IL2 receptor α (TAC) sequence. This finding is in agreement with previously described affinities of Jurkat cells NF-kB (11) as well as placental NF-kB for kB sequences (12).

Pab 350 antiserum was able to inhibit thymocyte-NF-kB binding to PRE in a dose dependent manner (figure 3). When using 0.4 μl of the antiserum, the inhibition was specific for NF-kB, since the faster migrating C2 complex was not affected by the presence of the antiserum. This result identifies thymocyte kB binding protein as NF-kB. When the amount of antiserum was increased to 1 μl, partial inhibition of the faster-migrating C2 complex was seen in the PMA-treated cells, but not in the untreated cells. We have previously shown that, in Jurkat cells, the C2 complex was composed of kB-specific as well as non-specific proteins (11). The differential effect of Pab 350 antibodies on the thymocyte C2 complex in PMA stimulated and unstimulated cells is not understoood yet but was due, perhaps, to different amounts of the specific protein. Altogether, these results suggest that, even though NF-kB activation involves different mechanisms, the binding characteristics are similar in thymocytes and mature T cells.

In the absence of cell treatment, thymocytes contained NF-kB in their nuclei, even if in much lower amounts than after PMA or TNFα treatment (figure 1). In contrast, using comparable amount of Jurkat cell nuclear extracts, NF-kB was not detected by EMSA without cell stimulation (figure 1). In order to determine whether detection of NF-kB in unstimulated cells was due to the amount of protein in the assay or not, we performed a "dose-response" experiment using increasing concentrations of proteins extracted from Jurkat and thymocyte cells nuclei. The results, represented in figure 4, clearly show that increasing amounts of protein from both unstimulated and TNFα-stimulated thymocytes retarded ^{32}P-PRE migration in a dose dependent manner. In contrast, increasing concentration of proteins did

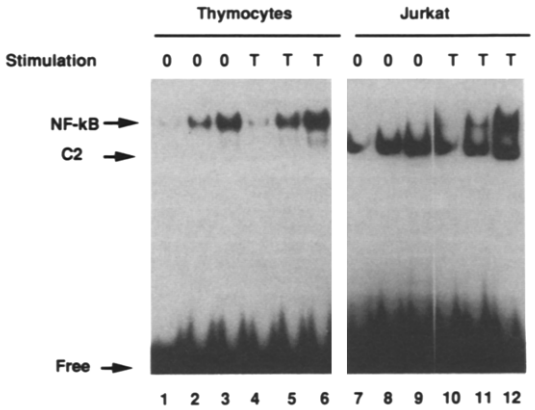

Figure 4. *EMSA of ^{32}P-PRE using increasing amounts of protein extract.* Untreated (0) or TNFα treated (T) thymocytes (lanes 1-6) and Jurkat cells (lanes 7-12) were assayed for NF-kB by EMSA. The following amounts of nuclear extract per incubation were used: 13.7, 27.4, 41 μg in lanes 1-3; 6.9, 13.9, 20,9 μg in lanes 4-6; 9.1, 18.3, 27.5 μg in lanes 7-9; 12, 23.6, 35,4 μg in lanes 10-12. Unbound oligonucleotide is indicated by the arrow (Free).

not reveal active NF-kB in untreated Jurkat cells, although the unidentified C2 complex was proportionally increased. In order to verify that the lack of active NF-kB in untreated Jurkat cells is not due to the tumoral nature of these cells, we performed identical experiment with freshly prepared peripheral blood T lymphocytes, and we did not find constitutively activated NF-kB (results not shown). Furthermore, the fact that Pab 350 antibodies inhibited the binding of NF-kB from both, unstimulated and PMA-treated thymocytes in a comparable manner (figure 3) argues in favor of a molecular identity between NF-kB extracted from *in vitro* untreated and treated cells. Altogether our results indicate that there is a difference between mature T cells and their thymic progenitors at the level of the basal activation of NF-kB. The presence of active NF-kB in unstimulated thymocytes might be due to a sub-population of thymocytes which was activated *in situ* in the thymus. Alternatively, thymocytes at every step of thymic differentiation could present detectable amounts of NF-kB in their nuclei. This latter situation would reflect a state of activation specific for T cells in the thymus. In any case, and even though the target genes of NF-kB in thymocytes are unknown, our results suggest that NF-kB could play an active role during T cell intra-thymic processing. We are currently investigating NF-kB distribution in thymocyte sub-populations

as well as the genes which are transcriptionally regulated by NF-kB activation in the thymus.

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