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Cellular redox status influences both cytotoxic and NF-κB activation in natural killer cells

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SUMMARY

The role of cellular redox status in both cytotoxic activity and NF-κ B activation in natural killer (NK) cells was investigated. The results indicate that stimulation of NK cells, either freshly isolated from peripheral blood lymphocytes (PBL) or long-term cultured NK clones, with specific cell targets results in an increased binding activity of NF-κB and AP-1 transcription factors measured by gel retardation. Pretreatment of NK cells with the antioxidant pyrrolidine dithiocarbarmate (PDTC) leads to the inhibition of NF-κB activation but the AP-1 binding to DNA was superinduced. The inhibition of NF-κB by PDTC paralleled with an inhibition of spontaneous cytotoxicity mediated by NK cells. Moreover, the inhibitors of serine proteases, *N*-α-tosyl-L-lysine chloromethyl ketone and *N*-α-tosyl-L-phenylalanine chloromethyl ketone, also blocked the cytolytic activity of NK cells against the sensitive target K562. In contrast, NK activity was not affected by pretreatment of the effector cells with the proteasome inhibitor *N*-acetyl-leu-leu-norleucinal which selectively inhibits NF-κB activation. Altogether, these results support the hypothesis that the activation of NK cells involved transcriptional and post-transcriptional events, and that reactive intermediates may play an important role in the molecular processes related with the generation of a cytotoxic response by NK cells.

INTRODUCTION

Natural killer (NK) cells constitute a small population of cytotoxic lymphocytes found in peripheral blood and lymphoid tissues, which are capable of killing target cells such as tumour cells, virus- and bacteria-infected cells in a non-major histocompatibility complex (MHC)-restricted manner without previous sensitization.[1,2] The molecular mechanisms for NK cell recognition and activation by target cells are not entirely clarified. NK cells can be activated to lyse target cells through the Fc receptor (CD16) and the CD2 molecule.[3,4] However, other NK receptors are involved in natural killing because NK cells lacking expression of CD16 and CD2 can still perform spontaneous cytotoxicity.[1,3]

At least two pathways of NK-mediated cytolysis have been identified. The first, called the granule exocytosis is mediated by releasing mediators stored in the cytoplasmic granules such as the pore-forming perforin, granzymes and fragmentins.[5–7] The second pathway is mediated by a Fas ligand protein that binds Fas molecules present at the surface of some target cells, thereby triggering their apoptotic death.[8] Moreover, cytokines produced by activated NK cells may contribute to its cytolytic function.[9] Thus, NK-mediated cytotoxicity appears to be mediated by separated signalling transmission pathways.

Although membrane receptors by which NK cells mediated natural cytotoxicity have to be entirely defined, intracellular changes occurring upon ligation with NK-sensitive target cells have been described. These changes include protein tyrosine phosphorylation, phosphoinositide hydrolysis, activation of protein kinase C, intracellular calcium mobilization and cytoplasm acidification.[10–13] These proximal intracellular events induced by cell contact have to be followed by distal events that in turn may alter the activity of transcription factors, which induce a certain number of activation-associated genes that in the case of NK cells may participate in the regulation of both cytolytic response and cytokine production.

NF-κB, formerly described as a κ enhancer-binding transcription factor constitutively present in the nuclei of mature B cells,[14] is a pleiotropic family of transcription factors involved in the regulation of many genes whose products participate in the immune or inflammatory response.[15,16] It is made of hetero- or homodimeric combinations of several proteins belonging to the same family. The most common species are heterodimers consisting of a 50 000 MW subunit (p50) and either a 65 000 MW protein (p65 or RelA) or the

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Abbreviations: N-ALLN, *N*-acetyl-leu-leu-norleucinal; PDTC, Pyrrolidine dithiocarbamate; ROI, reactive oxygen intermediates; TLCK, *N*-α-tosyl-L-lysine chloromethyl ketone; TPCK, *N*-α-tosyl-L-phenylalanine chloromethyl ketone.

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product of the *c-rel* oncogene. In most cell types, NF-κB is found in an inactive cytosolic form, retained by association with its inhibitor, IκB.[15,16] Upon cell activation by phorbol esters, mitogens, cytokines, ultraviolet and viral proteins, IκB undergoes specific modifications and is subsequently degraded, then releasing the active NF-κB complex to the nucleus.[17,18] Evidence suggests that the formation of reactive oxygen intermediates (ROI) may be a common mediator of the diverse NF-κB-activating signals. NF-κB can be activated by exogenous hydrogen peroxide in some type of cells[19] and also the activation of this factor by a different stimulus can be inhibited by antioxidant thiols or iron chelators by preventing IκB phosphorylation,[20] a process that it is absolutely required for the IκBα ubiquitination and degradation by the 26S proteasome.[21]

We show here the activation of NF-κB and AP-1 in NK cells activated by sensitive target cells. NF-κB binding activity was increased in both peripheral blood NK cells stimulated by sensitive K562 cells, and NK clones stimulated by C1R cells. This binding was abolished by the presence of the antioxidant pyrrolidine dithiocarbamate (PDTC). This antioxidant, as well as the serine protease inhibitors *N*-α-tosyl-L-phenylalanine chloromethyl ketone (TPCK) and *N*-α-L-lysine-chloromethyl ketone (TLCK) were able to inhibit the cytotoxic activity of NK cells against K562. In contrast, incubation of NK cells with the proteasome inhibitor *N*-acetyl-leu-leu-norleucinal (N-ALLN) did not affect its cytotoxic activity. These results suggest that activation of NK cells by a specific target may result in the generation of ROI that mediate both granule exocytosis and activation of some transcription factors that may regulate the immune response of NK cells at the transcriptional level.

MATERIALS AND METHODS

Reagents and monoclonal antibodies

Complete culture medium was RPMI-1640 (Bio-Whittaker, Belgium) supplemented with 2 mM L-glutamine, 1 mM HEPES, antibiotics (Gibco Ltd., Paisley, UK), and 10% fetal calf serum (FCS; Bio-Whittaker). The following monoclonal antibodies (mAb) were used: anti-leu4 (anti-CD3), was purchased from Becton-Dickinson (Mountain View, CA). The mAb NKH-1 (anti-CD56) was purchased from Coulter (Hialeah, FL). The mAb 3G8 (anti-CD16), RMO52 (anti-CD14), and J4.119 (anti-CD19) were purchased from Immunotech (Marseille, France). Antisera used to detect p50 (#350), RelA (#1226) and c-rel were previously described.[22] The (γ-^{32}P) ATP (6000 Ci/mmol) was obtained from ICN (Costa Mesa, CA). All other reagents were from Sigma Chemical Co (St Louis, MO).

Target cell lines

C1R and K562 were used as target cells. C1R is a HLA-A and HLA-B deficient Epstein–Barr virus-transformed B-cell line and K562 is an erythroleukaemic cell line. The cell lines were cultured in complete medium at 37° in 5% CO_2.

NK-cell purification

Polyclonal NK cells from healthy donors were used as effector cells. These NK cells were obtained from venous blood from healthy donors by Ficoll–Hypaque (Pharmacia, Uppsala, Sweden) density gradient centrifugation. Macrophages were depleted by plastic adherence for 1 hr at 37° and B cells were removed through a nylon wool column. Purified non-adherent cells were incubated with anti-CD3 mAb (anti-leu4) for 1 hr at 4°, washed three times in cold phosphate-buffered saline (PBS) and then incubated with goat anti-mouse coated magnetic beads (Dynal, Oslo, Norway). After immunomagnetic separation, the negative selected cells consisted of 80–90% $CD56^+$, $CD16^+$ cells as determined by cytometry and were considered the peripheral blood NK fraction. $CD3^+$, $CD19^+$, or $CD14^+$ cells were not detectable (<5%).

Generation of NK-cell clones

Peripheral blood lymphocytes (PBL) derived from a typed blood donor were isolated on Ficoll–Hypaque gradients and NK cells were purified as described above. Viable cells $CD3^-$ $CD4^-$ $CD8^-$, were isolated and cloned by limiting dilution at concentration of one to five cells/well in 96-well U-bottom plates in the presence of phytohaemagglutinin (PHA; 0·1% v/v; Gibco), and recombinant interleukin-2 (rIL-2) (1000 U/ml, Hoffmann-La Roche, Nutley, NJ), 10% leucocyte-conditioned medium[23] and feeder cells. These consisted of 10^5 irradiated (6000 rads) peripheral blood mononuclear cells (PBMC) well. After 10 days, proliferating cultures were transferred to 96-well flat-bottomed plates and further expanded in complete medium. NK-cell clones were restimulated with feeder cells every 3–4 weeks. Two clones (104 and 123) used in this study showed cytotoxic activity against the C1R target cells and display a $CD3^-/CD56^+/CD16^+$ phenotype.

Cytotoxicity assays

K562 target cells were combined with purified polyclonal NK cells at the indicated effector: target ratios using 5×10^3 targets in a final volume of 200 μl per V-bottom microtitre plate in triplicate. The plates were centrifuged at 500 ***g*** for 2 min, then incubated for 4 hr at 37°. Plates were then centrifuged at 1000 r.p.m. for 5 min, 50 μl aliquots were removed and tested for lactate dehydrogenase (LDH) activity using the CytoTox 96 kit (Promega, Madison, WI). Percentages of specific cytotoxicity were calculated following the manufacturer's instructions.

Preparation of protein extracts

Polyclonal NK cells or NK clones ($2{\cdot}5 \times 10^5$ cells) were incubated with 5×10^3 of the indicated target cells in 200 μl of medium in V-microtitre plates. As positive control effector cells were stimulated with phorbol 12-myristate 13-acetate (PMA; 50 ng/ml) in the absence of target cells. Plates were centrifuged and cells were incubated at 37° for the indicated times. After that, the cells were recovered and centrifuged in an Eppendorf tube, washed with cold PBS and centrifuged again. Sedimented cells were gently resuspended and disrupted for 20 min at 4° in 50 μl of lysis buffer containing 20 mM HEPES pH 7·9, 0·35 M NaCl, 20% glycerol, 1 mM $MgCl_2$, 0·5 mM dithiothreitol (DTT), 0·5 mM ethylene diaminetetraacetic acid (EDTA), 0·1 mM ethylene glycol-bis(β-aminoethyl)ether (EGTA), protease inhibitor (1 μg/ml each of apronitin, leupeptin and pepstatine), 1 mM phenylmethylsulphonyl fluoride (PMSF), and 0·2% nonidet P-40 (NP-40). After lysis, samples were centrifugated for 5 min at 10 000 ***g***, supernatants were carefully recovered and placed in new Eppendorf tubes.

Electrophoretic mobility shift assay (EMSA)

Double-stranded oligonucleotides containing the κB binding site located in the enhancer region of the H-2^b promoter (KBF) or the AP-1 binding site located in the metallothionine promoter were used. The binding reaction contained 10 μg of proteins, 1 μg poly(dI-dC), 20 mM HEPES pH 7, 50 mM NaCl, 2 mM DTT, 0·01% NP-40, 100 μg/ml BSA, 4% Ficoll, and 100 000 c.p.m. of end-labelled DNA fragments in a total volume of 20 μl. When indicated, 0·5 μl of rabbit anti-p50, anti-RelA, anti c-rel, or preimmune serum was added to the standard reaction prior to the addition of the radiolabelled probe. For cold competition a 100-fold excess of the double-stranded oligonucleotide competitor was added to the binding reaction. After 30 min incubation at room temperature, the mixture was electrophoresed through a 6% polyacrylamide gel under non-denaturing conditions and was subsequently dried and exposed at −80° to XAR film.

RESULTS

Induction of NF-κB-binding activity in NK cells stimulated by its interaction with target cells

Treatment of purified peripheral blood NK cells with K562 cells rapidly activates the transcription factor NF-κB, as shown by electrophoretic mobility shift assay (EMSA) using a ^{32}P-labelled KBF oligonucleotide. The binding was already detected after 15 min of K562 activation and was maintained up to 90 min after stimulation (Fig. 1, left panel). This binding complex migrated to an extent similar to that of the complex found in PMA-stimulated NK cells. The specificity of such binding was demonstrated by cold competition experiments using a 100-fold excess of the related IgκB, or unrelated AP-1 and SP-1 oligonucleotides. Figure 1 (right panel) shows that IgκB but not AP-1 or SP-1 oligonucleotides competed for the NF-κB-binding activity with the end-labelled KBF probe. In addition, supershift experiments demonstrated that this binding protein was by p50/RelA heterodimer of the NF-κB family of transcription factors (data not shown).

To ascertain that NF-κB was activated in NK-cell clones, 104 and 123 clones were treated with PMA or incubated with the sensitive C1R target cells, and both NF-κB and AP-1 binding detected by EMSA. Figure 2 shows that NF-κB as well as AP-1 were activated in both NK cell clones. Proteins extracted from 5×10^3 C1R cells did not show detectable NF-κB or AP-1-binding activities, ruling out that the binding activities detected were from target cells. The lower band that appears in proteins extracted from C1R cells (lane 14) was not specific for AP-1 since an excess of cold AP-1 oligonucleotide did not outcompete it (data not shown). To identify the members of the NF-κB/Rel family involved in the NF-κB-binding activity observed in activated NK-cell clones, we performed supershift retardation assays with a specific antisera. NF-κB-binding activities detected in both cell clones were composed of p50/p65 (p50/RelA) and p50/c-rel heterodimers (Fig. 3). Preimmune antisera did not affect the binding of proteins (data not shown).

Effect of PDTC on NF-κB and AP-1 binding activities in NK-stimulated cells

The differential regulation of NF-κB and AP-1 transcription factors in response to an oxidative stress has been previously

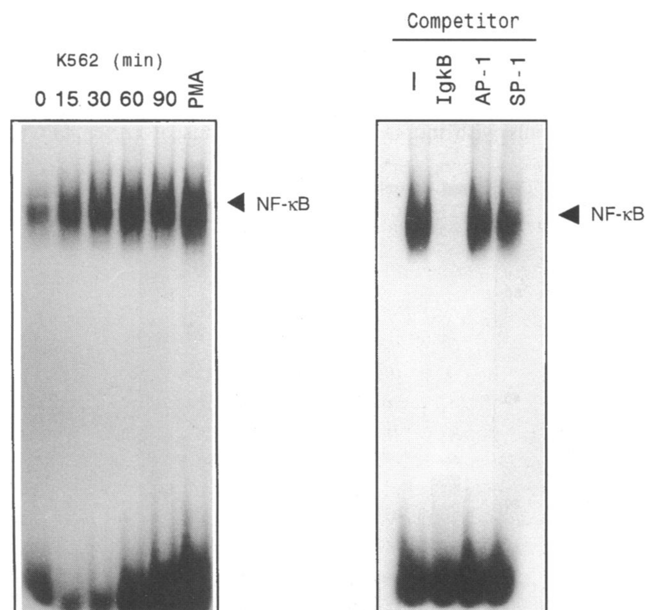

Figure 1. Activation of NF-κB binding activity in NK cells. Purified peripheral blood NK cells ($2{\cdot}5 \times 10^5$) were incubated with k562 cells (5×10^3) for the indicated times or activated with PMA (50 ng/ml) for 30 min. Total proteins were extracted and subjected (10 μg in each lane) to EMSA using an end-labelled KBF probe (left panel). Proteins from the 60 min stimulation were subjected to cold competition by using a 100-fold excess of unlabelled IgκB, AP-1 or SP-1 double-stranded oligonucleotides (right panel).

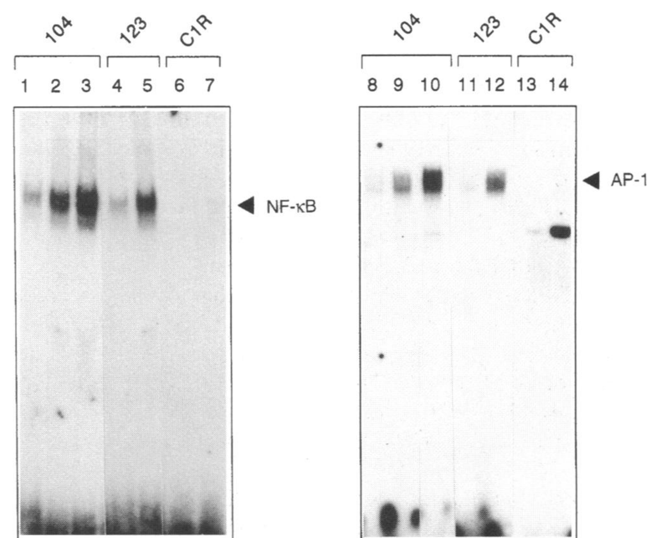

Figure 2. Activation of NF-κB and AP-1 DNA binding in stimulated NK clones. NK clones 104 and 123 (2×10^5 cells) were either incubated with 5×10^3 C1R target cells for 60 min or stimulated with PMA (50 ng/ml) for 30 min, and binding activity for NF-κB (lanes 1 to 7) and AP-1 (lanes 8 to 14) sites were detected by EMSA. Lanes 1, 4, 8 and 11 (unstimulated clones); lanes 2 and 9 (PMA-stimulated clones); lanes 3, 5, 10 and 12 (C1R-stimulated clones); lanes 6 and 13, and 7 and 14 represent the binding activity of proteins obtained from 5×10^3 unstimulated or PMA (50 ng/ml) stimulated for 30 min C1R cells respectively.

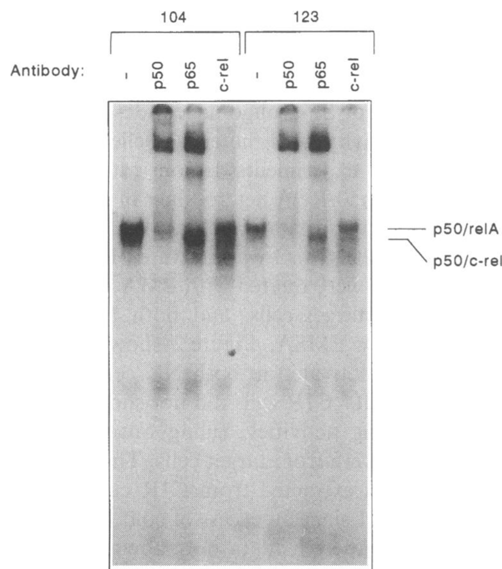

Figure 3. Identification of NF-κB complexes in NK-cell clones; 104 and 123 NK-cell clones were incubated with C1R target cells as indicated in Fig. 2. Proteins (10 μg) were preincubated with the specific NF-κB antisera as described in the Materials and Methods and the NF-κB complexes identified by EMSA. Antisera anti-p50 and anti-p65 induce a supershift of the binding complexes, and c-*rel* antiserum prevent the DNA binding of c-*rel* complexes.

described in other cells.[19,24] Thus, to study whether or not the NF-κB and AP-1 activities in stimulated NK cells were affected by the intracellular redox system, we preincubated the NK cells with the radical scavenger PDTC for 1 hr prior to the stimulation with PMA or with K562 cells. We show in Fig. 4 that 100 μM of PDTC blocked the binding of the nuclear NF-κB induced in NK cells by both PMA and K562 cells. In contrast, the AP-1-binding activity was superinduced by PDTC in K562-stimulated NK cells but was inhibited in PMA-stimulated cells, suggesting that PMA and K562 mediate different signalling pathways leading to the AP-1 activation.

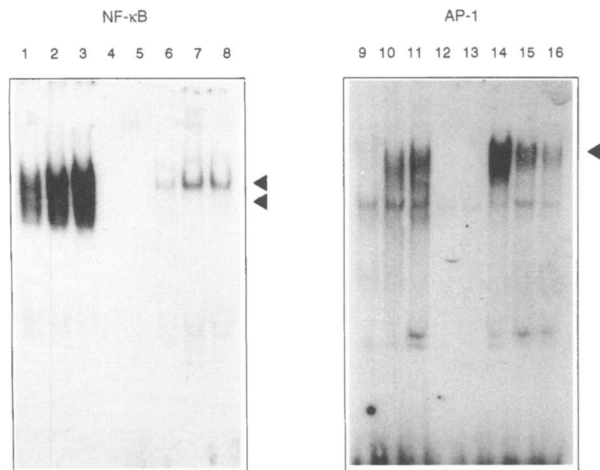

Figure 4. The effect of PDTC on NF-κB and AP-1 binding in activated NK cells. NF-κB (lanes 1 to 8) and AP-1 (lanes 9 to 16) binding activities were detected in protein extracts of purified peripheral NK cells stimulated as follow: lanes 1 and 9, unstimulated cells; lanes 2 and 10, stimulated with PMA (50 ng/ml) for 30 min; lanes 3 and 11, stimulated with K562 cells for 60 min at the same ratio as in Fig. 1; lanes 4 and 12, unstimulated NK cells in the presence of PDTC (100 μM); lanes 5 and 13, same as in the presence of PDTC; lanes 6 and 14, same as in the presence of PDTC. Lanes 7 and 15 and 8 and 16 correspond to K562 cells (5×10^3) treated or no with PDTC.

Effect of PDTC on NK-cell cytotoxic activity

In order to analyse the effect of the antioxidant PDTC in the cytotoxic activity of NK cells, we treated purified peripheral blood NK cells with PDTC for 1 hr and then incubated with the target cells at different effector : target ratios. The cytotoxic activity was measured by the release of LDH from target cells as described above. PDTC was a potent inhibitor of the spontaneous cytotoxicity mediated by polyclonal NK cells (Fig. 5). PDTC alone was not toxic for the target cells because K562 cells treated with this antioxidant showed normal spontaneous LDH release comparable to that of untreated control cells. The inhibition of NK activity by PDTC was concentration dependent, and while 100 μM PDTC resulted in 100% inhibition, 50 and 25 μM mediated 72% and 40% of inhibition respectively. Moreover, pretreatment of purified NK cells with *N*-acetyl-L-cysteine, a cysteine derivative that can raise intracellular glutathione (GSH) levels and thereby protect cells from the effects of ROI, resulted in a complete inhibition of NK activity (data not shown).

Effect of serine proteases and proteasoma inhibitors on NK-cell cytotoxic activity

It has been demonstrated that in addition to antioxidants, serine protease inhibitors such as chloromethyl ketones and the proteasome inhibitor, N-ALLN, are potent inhibitors of NF-κB activation by blocking the degradation of the cytoplasmic κB inhibitor.[25,26] To study whether these compounds were able to inhibit NK cell-mediated cytotoxicity, we treated effector cells with increasing concentrations of either TPCK,

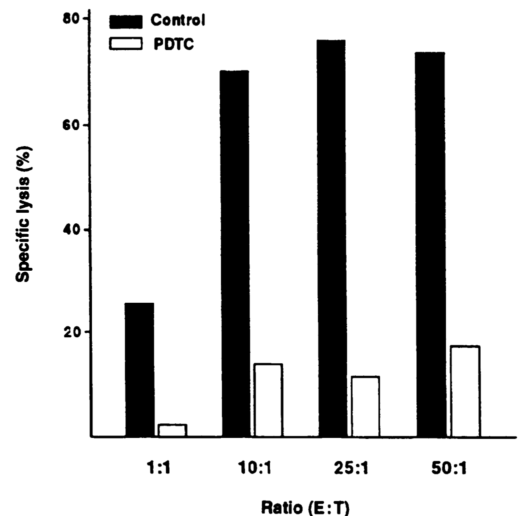

Figure 5. Inhibition of NK cytolytic activity by PDTC. Purified NK cells were incubated with or without PDTC (100 μM) for 60 min, and then tested for cytolytic activity against K562 cells using a LDH detection system. One representative out of the three independent experiments is shown.

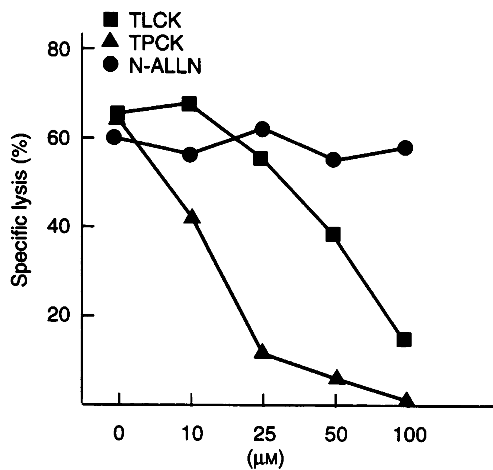

Figure 6. Effects of chloromethyl ketones and N-ALLN on NK activity. The cytolytic activity of purified NK cells against K562 cells was studied in the presence or absence of TPCK, TLCK or N-ALLN at the indicated concentrations. One representative out of three independent experiments is shown.

TLCK, or N-ALLN for 30 min prior to incubation with the K562 target cells at the ratio 25:1 (E:T). In Fig. 6 it is shown that both serine protease inhibitors, TPCK and TLCK, were able to inhibit NK cytolytic activity. By contrast, N-ALLN at concentrations ranging from 25 to 100 μM, did not affect the lysis of K562 cells mediated by NK effector cells.

DISCUSSION

Eukaryotic cells continuously produce ROI as side products of the redox reaction. The generation of ROI is mostly governed by mitochondria and the ROI comprise hydrogen peroxide, hydroxyl radicals and superoxide anions. All of them are essential compounds of the oxidative metabolism.[27] Under physiological conditions the intracellular levels of ROI are finely tuned by cellular enzymes with radical scavenging activities.[27]

We have shown in this report that the cytotoxic activity of NK cells is completely abrogated in the presence of the thiol scavenger PDTC and by serine protease inhibitors. Such inhibition cannot be explained by the ability of these compounds to inhibit NF-κB activation since the proteasome inhibitor, N-ALL-N, which did not affect the cytotoxic activity of NK cells was able to inhibit the NF-κB binding to DNA in NK cells stimulated by its specific target (M. V. Blazquez and E. Muñoz, unpublished results). Based on these results we propose that activation of NK cells by cell contact with its specific targets, would result in the generation of ROI, which in turn may regulate two sequential processes. First, ROI can activate a cascade of kinases and/or proteases leading to the releasing of pore-performing granules by post-transcriptional mechanisms. It is also possible that chloromethyl ketones may inhibit the activity of granzymes rather than their release from cytoplasmic granules. Secondly, this cascade of signalling may result also in the ubiquitination and phosphorylation of IκBα,[21] a previous step required for degradation of this protein and nuclear translocation of NF-κB. In addition, ROI generated by cell contact may influence the binding of AP-1. It has been proposed that activation of AP-1 is governed by a complex pathway that involves transcriptional and post-transcriptional mechanisms. Thus, it is possible that NK-cell contact with sensitive target cells results in c-Jun phosphorylation and this conformational change of pre-existing AP-1 complexes, allows the binding to DNA. As previously described, the action of antioxidants on AP-1 binding may be mediated by transcriptional mechanisms that induce c-*fos* and c-*jun* gene transcription.[24]

It is likely that the lytic pathway is distinct from the proliferative pathway in NK cells, but the generation of ROI may be a very early step common to those pathways. This concept is supported by a previous report indicating that thiol compounds, such as 2-mercaptoethanol (2-ME) or reduced glutathione, potentiates the ability of IL-2 to induce both the cytotoxic activity and the proliferation murine T cells and human NK cells.[28] Moreover, the inhibition of NK cytotoxicity by dithiocarbamates may be overruled by sulphydryl compounds which abolish the capacity of this compound as a free radical scavenger.[29]

Recent evidence has demonstrated that cytotoxicity by NK cells is controlled by activating and inhibitory receptors.[30,31] Among those that activate, the best characterized is the rat NKR-P1, a type II integral protein that after ligation with specific mAb, activates phosphoinositide turnover and increases the levels of intracellular calcium in NK cells.[32] The signalling pathways activates by NKR-P1 receptor and other NK antigens depend on the presence of CD45,[33] a tyrosine phosphatase that participate in the cytolytic response of NK cells.[34] The role of tyrosine phosphatases in the regulation of NF-κB activation has been previously suggested.[35,36] Much less is known about the signalling pathways mediated by inhibitory receptors, although they apparently block the activation pathways generated by the activating receptors.[37] As a working hypothesis it is possible that such blocking occurs at the very early stages of activation and we are currently investigating whether this inhibition affects the intracellular redox state of activated NK-cell clones.

Independently of the immediate lytic activity mediated by NK cells after interaction with specific target, the activation of NF-κB and AP-1 in these cells may be of special relevance in the transcriptional regulation of cytokines such as tumour necrosis factor-α and -β, whose genes are regulated at the transcriptional level by these transcription factors.[15] Besides soluble mediators, other cell surface markers with important functions in NK-cell activation such as IL-2R,[15] CD69,[38] intracellular adhesion molecule-1,[15] and other integrins involved in cell adhesion[15] may be also regulated by NF-κB. Thus, it is likely that this transcription factor is essential for cytotoxic cells to acquire a fully activated phenotype.

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