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ORIGINAL ARTICLE

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Protein-bound polysaccharide K and interleukin-2 regulate different nuclear transcription factors in the NKL human natural killer cell line

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Abstract The activation of natural killer cells and induction of cytotoxicity are complex processes whose molecular mechanisms have not been clearly elucidated. Stimulation of the NKL human NK cell line with interleukin-2 (IL-2) or protein-bound polysaccharide K (PSK) leads to sustained growth and cytolytic activity in comparison to unstimulated NKL cells. However, it is not known whether both agents give rise to the same or different intracellular signals. To determine the molecular basis for the action of IL-2 and PSK, the binding activity of AP-1, CRE, NF-κB, PU.1, SP-1, NFAT, STAT1, STAT5/6, GAS/ISRE and IRF-1 transcription factors was compared in IL-2- and PSK-stimulated NKL cells. Here we report that PSK enhanced AP-1 and CRE binding activities, whereas IL-2 increased AP-1 and SP-1 and modified GAS/ISRE, IRF-1 and STAT5. Our results indicate that IL-2 and PSK regulate different nuclear transcription factors in NKL cells, and that the signal transduction pathway used by these inducers is different.

Key words Natural killer cells · Interleukin 2 · PSK Cytotoxic activity · Transcription factors

Introduction

Protein-bound polysaccharide K (PSK) is a biological response modifier that has been widely used for cancer immunotherapy in Japan [19, 22, 30]. This polysaccharide, obtained from the basidiomycete fungus *Coriolus versicolor*, restores host immunity impaired by tumor or antitumoral chemotherapeutic agents [21, 35]. However, the exact mechanism of its immunomodulatory effect is unknown. We previously showed that PSK is able to inhibit metastatic colonization in a mouse fibrosarcoma model, and that this inhibition is due to the activation of NK cells in vivo [2, 3].

To further analyze the mechanisms responsible for this NK activation we studied the effect of PSK in the human cell line NKL, derived from a patient with a large granular lymphocyte (LGL) leukemia that showed natural cytotoxic activity [26]. We have previously shown that PSK maintains the viability of NKL cells in culture and also induces NK cytotoxicity [23]. Both this polysaccharide and interleukin-2 (IL-2) induce similar levels of NK cytotoxic activity against different tumor cell lines (K562, Daudi and U937). The activated NKL phenotypes observed after PSK and IL-2 treatment were very similar ($CD2^+$, $CD16^+$, $CD18^+$, $CD38^+$, $CD44^+$, $CD98^+$, $CD25^+$, $CD94^+$, ILT-2^+, MHC class I^+ and class II^+); however, the mechanism(s) used by these two modulators to produce these effects might not be the same. It was previously reported that PSK can activate human NK cells independently of IFN and the IL-2/IL-2R system [16].

Several transcriptional factors have been studied in IL-2-treated NK and T cells [20, 37], in which treatment with IL-2 increased AP-1-specific binding activity [5]. In the NK3.3 NK cell line IL-2 also increased NF-κB-specific DNA binding activity [34]. IRF-1 plays a crucial role in the induction of NK cell-mediated cytotoxicity in IRF-1-deficient mice [10]. ETS transcriptional factors are involved in the development of NK cells in mice [7], and in the development of NK and NK1.1 + T (NKT) cell lineages [32]. In contrast, T-lineage commitment is characterized by the repression of PU.1, a binding activity found in non-differentiated, but not in differentiated, T cells [4]. In NKL cells, activation of the natural cytotoxicity program increased DNA binding activity of AP-1 transcriptional factors, with the appearance of a new complex formed by jun-fos heterodimers [8]. This new activity was impaired by the

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engagement of NK cell inhibitory receptors for MHC class I molecules.

To dissect the molecular mechanisms involved in proliferation and NK cytotoxicity induced by PSK in NKL cells, and to compare these mechanisms with those used by IL-2, we monitored the levels of specific DNA binding activity of different nuclear transcription factors known to be involved in the regulation of transcription. The DNA binding activities analyzed correspond to the nuclear transcriptional factors AP-1, cyclic AMP response element (CRE), NF-κB, PU1, SP-1, GAS/ISRE, STAT1, STAT5/6, NFAT and IRF-1. In this study we show that PSK and IL-2 led to different variations in the binding activities of some of these transcriptional factors in NKL cells. Only AP-1 binding activity was increased by both agents. Treatment with PSK, but not with IL-2, also increased CRE binding activity. IL-2 treatment increased SP-1 binding activity and modified GAS/ISRE, IRF-1 and STAT5 binding activities.

Materials and methods

Protein-bound polysaccharide K

PSK was kindly provided by Kureka Chemical Ind. (Tokyo, Japan). It is prepared by extracting cultured mycelia of *Coriolus versicolor* with hot water. The precipitate is separated from the clear supernatant with saturated ammonium sulfate, then desalted and dried [30]. PSK was dissolved in RPMI medium and heated at 50 °C for 20–30 min until a clear solution appeared. The PSK preparation was filter-sterilized and diluted in culture medium to the desired concentration. PSK was titrated for NKL cells previously [23] and the working dilution we used was 100 μg/ml.

Culture of NK cells, cytokines and reagents

Cell line NKL [26], which provided the effector cells for this study, was established from peripheral blood lymphocytes of a patient with LGL leukemia by Dr. J. Ritz, and was kindly provided by Dr. M. Lopet-Botet (Hospital de la Princesa, Madrid, Spain). NKL cells were maintained in culture with RPMI–1640 and 10% heat-inactivated human AB serum (Sigma Chemical, St. Louis, Mo., USA). Recombinant human IL-2 was purchased by Hoffmann–La Roche (Nutley, N.J.) (purity >97%; specific activity, 2×10^6 U/mg). Unit definition: one unit is the amount required to induce half-maximal incorporation of ^{3}H-thymidine into IL-2-dependent cytolytic T cell lymphocytes. For flow cytometric analysis and NK assays, cells were cultured in medium alone (unstimulated cells) or supplemented for 96 h with either human recombinant IL-2 (1000 U/ml) or PSK (100 μg/ml).

In vitro NK cytotoxicity assays

Cytotoxicity tests in treated and untreated NKL cells were done with a standard 2–3 h ^{51}Cr-release assay against Daudi tumor cells. Spontaneous release of radioactivity, determined by incubating the target cells in 100 μl of medium, was always less than 20% of maximum release; the variation between replicates was always less than 15%. The results were expressed as a percentage of specific lysis.

Electrophoretic mobility shift assay (EMSA)

Cell lines were grown to log phase before harvest for nuclear protein extraction. Nuclear proteins were extracted from unstimulated NKL cells or cells incubated for 96 h with IL-2 or PSK, and were prepared as previously described [27] with modifications [1]. Briefly, 10×10^6 cells were collected, washed twice in phosphate-buffered saline, and resuspended in 400 μl cold buffer A (10 mM HEPES pH 7.6, 10 mM KCl, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM spermidine, 0.15 mM spermine) and allowed to swell for 15 min on ice. The nuclei were collected by centrifugation at 400*g* for 5 min, washed once in buffer A, and resuspended in 50 μl of buffer C (20 mM HEPES pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride). All buffers contained aprotinin (2 μg/ml), leupeptin (2 μg/ml) and pepstatin (1 μg/ml) as additional protease inhibitors. The tubes were vigorously rocked on a shaking platform at 4 °C for 30 min and centrifuged at 12,000*g* for 5 min at 4 °C; the supernatant was then aliquoted and rapidly frozen on liquid N_2 and stored at −80 °C. The protein concentration in the nuclear extracts was determined by using the Bradford protein assay (Bio-Rad, Madrid, Spain).

Binding activity was assessed using DNA oligomers corresponding to the binding sequences of the transcriptional factors shown in Table 1. Complementary oligodeoxynucleotides containing the required binding sequence were synthesized in solid phase according to the phosphoramidate method on a Beckmann Oligo 1000 automated DNA synthesizer (Beckmann Instruments, Palo Alto, Calif., USA). Probes were annealed with the complementary strand by heating to 95 °C for 3 min, then slowly cooling for 150 min to 25 °C.

Fifty nanogram amounts of double-stranded oligonucleotide were labeled by filling-in with Klenow fragment DNA polymerase I and [α-^{32}P] dCTP or dATP (3000 Ci/mmol) (Amersham, Little Chalfont, UK). A quantity amounting to 0.5 ng (4×10^4 cpm) of labeled oligomers was then incubated at 4 °C for 20 min with 2–6 μg of nuclear extract in 20 μl of binding buffer containing 5 mM HEPES (pH 7.9), 6 mM $MgCl_2$, 0.2 mM EDTA, 15 mM KCl, 15% glycerol, 1 mM DTT, and 2 μg poly(dI-dC). The specificity of binding was examined by competition with 100-fold excess unlabeled oligomer and incubation at 4 °C with the cellular extract for 15 min before the radioactive probe was added. An oligonucleotide containing the Oct-1 binding site was used to control for the quality and quantity of nuclear extracts (Table 1). Reactions were electrophoresed through 4–6% low-ionic-strength acrylamide gels (29/1), 0.5 × TBE (1 × TBE is 89 mM Tris-base, 89 mM boric acid and 2 mM EDTA), fixed in 10% acetic acid, dried and exposed on XAR film (Kodak) at −20 °C.

Results

Cytotoxic activity of NKL cells stimulated with IL-2 or PSK

We previously reported that NKL cells stimulated by PSK continued to proliferate and showed cytotoxic activity [23]. Before preparation of the nuclear extracts, we measured viability and cytotoxic activity of unstimulated NKL cells and NKL cells treated with IL-2 or PSK. Viability of NKL cells was determined by trypan blue staining. We found no differences in viability between NKL cells treated with IL-2 or PSK (data not shown). Cytotoxic activity was measured in a standard 3 h ^{51}Cr-release assay against a Daudi target tumor cell line. There were no significant differences in proliferation or cytotoxic activity between NKL cells treated with IL-2 or PSK (Fig. 1).

Table 1 Oligonucleotides used in this study

Oligonucleotide	Sequence
AP-1	5′GTGACTCATGACTCATGACTCATGACTC 3′ 3′ACTGAGTACTGAGTACTGAGTACTGAGT 5′
NF-κB	5′GCATTAGGGGGCTTCCACGGCCTGA 3′ 3′GTAATCCCCCGAAGGTGCCGGACTT 5′
SP-1	5′GGTCAGGACCCGCCCCTTCTGGTCCG 3′ 3′CAGTCCTGGGCGGGGAAGACCAGGCT 5′
CRE	5′GGTGATTGCCTGACGTCAGAGAGCAT 3′ 3′CACTAACGGACTGCAGTCTCTCGTAT5′
Oct-1	5′GCAGGGCAGGGATTTGCATCCATCTA 3′ 3′GTCCCGTCCCTAAACGTAGGTAGATT 5′
GAS/ISRE	5′TCGTACTTTCAGTTTCATATTACTCT 3′ 3′GCATGAAAGTCAAAGTATAATGAGAG 5′
STAT1	5′GATCATATGGATATTCCTGTAAGTGT 3′ 3′TAGTATACCTATAAGGACATTCACAT 5′
STAT5/6	5′GTCTTTCCCAGAAAAGGATC 3′ 3′AGAAAGGGTCTTTTCCTAGT 5′
IRF-1	5′GCTAGCGAAAATGAAATTGCA 3′ 3′GATCGCTTTTTACTTTAACGTT 5′
NFAT	5′TGCTTGGAAAATTTGTTTCATAGTTCA 3′ 3′CGAACCTTTTAAACAAAGTATCAAGTG 5′

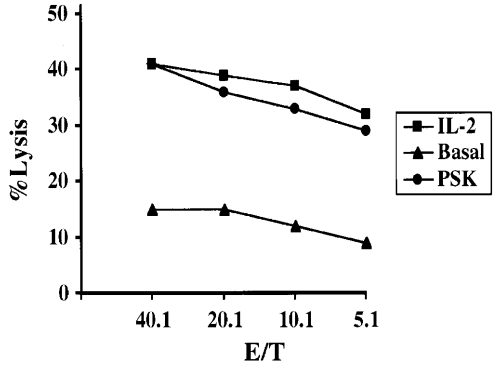

Fig. 1 Cytotoxic activity of unstimulated NKL cells and in cells stimulated with IL-2 or PSK. In vitro 3 h ^{51}Cr-release cytotoxic assays against Daudi tumor cells were done with unstimulated NKL effector cells (*Basal*), NKL cells treated with 100 μg/ml PSK (*PSK*) or cells treated with 1000 U/ml IL-2. The results represent the average of three different experiments (*E*/*T* effector-target ratio)

Increased AP-1 DNA binding activity in NKL cells treated with IL-2 or PSK

The involvement of AP-1 transcription factors in growth and natural cytotoxicity of NKL cells was investigated by comparing the DNA binding activity of AP-1 present in protein extracts prepared from NKL cells alone (not treated during 4 or 7 days) and from NKL cells treated with IL-2 or PSK for 4 days. DNA binding activity was measured by EMSA, and binding of AP-1 to its cognate DNA enhancer element was evaluated with a radiolabeled double-strand oligonucleotide containing the consensus sequence (Table 1). Protein extracts from untreated NKL cells showed a very low binding affinity for AP-1 oligonucleotide, and EMSA revealed a faint retarded band that represented the basal AP-1 level in unstimulated NKL cells (Fig. 2A). In contrast, nuclear extracts prepared from NKL cells treated with PSK showed greater binding to the AP-1 oligonucleotide, and new AP-1 complexes were seen with EMSA as a wide retarded band that comprised several bands corresponding to different complexes (Fig. 2A). Binding activity in the nuclear extracts from PSK-stimulated cells was completely inhibited in the presence of a 100-fold excess of cold AP-1 double-strand oligonucleotide (Fig. 2A). The formation of complexes was abrogated in the presence of wild type, but not mutant, oligonucleotides (data not shown).

In nuclear protein extracts prepared from NKL cells treated with IL-2, AP-1 binding activity was very similar to that of the nuclear extracts from PSK-stimulated cells, and EMSA revealed a wide retarded band of the same intensity as the band obtained for the protein extracts prepared from PSK-treated cells (Fig. 2A). These results show that IL-2 and PSK induce AP-1 DNA-binding activity in NKL cells, and that this activity is very similar in both cases.

Each nuclear extract was also incubated with radiolabeled oligonucleotide Oct-1 (Table 1) to verify that the amount of nuclear extract used in each reaction was the same (Fig. 2B). The Oct-1 DNA binding activity in nuclear extracts from NKL cells alone (unstimulated for 4 or 7 days) showed that these cells maintained good viability and metabolic activity.

CRE binding activity induced by PSK but not by IL-2

To determine whether the functional activity of CRE was increased in stimulated NKL cells, we monitored the

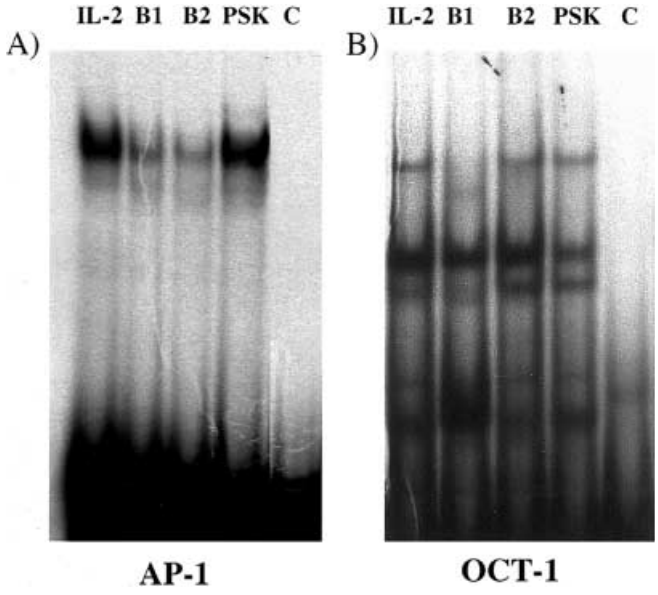

Fig. 2A Characterization of AP-1 DNA binding activity in NKL cells. Radiolabeled AP-1 oligonucleotide (Table 1) was incubated with nuclear extracts (4 μg) prepared from NKL cells grown for 4 days (*lane B1*) or 7 days (*lane B2*) without stimulation, NKL cells stimulated for 4 days with IL-2 (*lane IL-2*), and NKL cells stimulated for 4 days with PSK (*lane PSK*). Nuclear extracts of PSK-stimulated cells were preincubated with a 100-fold excess of the unlabeled AP-1 oligonucleotide before incubation with radiolabeled AP-1 oligonucleotide (*lane C*). **B** Oct-1 binding activity in NKL cells. Radiolabeled Oct-1 oligonucleotide (Table 1) was incubated with nuclear extracts (4 μg) prepared from NKL cells grown without stimulation for 4 days (*lane B1*) or 7 days (*lane B2*), IL-2-stimulated NKL cells (*lane IL-2*) or PSK-stimulated cells (*lane PSK*). Nuclear extracts from PSK-stimulated cells were preincubated with a 100-fold excess of the unlabeled Oct-1 oligonucleotide (*lane C*). The experiments shown are representative of four independent assays

Fig. 3 Effects of IL-2 and PSK on CRE binding activity in NKL cells. Nuclear extracts prepared from NKL cells grown without stimulation for 4 days (*lane B*), IL-2-stimulated NKL cells (*lane IL-2*) or PSK-stimulated NKL cells were incubated with a radiolabeled CRE oligonucleotide (Table 1). Nuclear extracts of PSK-stimulated cells were also preincubated with unlabeled CRE oligonucleotide to verify the specificity of the band (*lane C*). The results shown are from one of three experiments

levels of specific CRE DNA binding activity in nuclear extracts from unstimulated and stimulated cells. In nuclear extracts from unstimulated NKL cells, CRE DNA binding activity was very low (Fig. 3). Stimulation with PSK increased CRE binding activity (Fig. 3). When the specificity of the band was analyzed with a 100-fold excess of unlabeled CRE double-strand oligonucleotide, the formation of complexes was completely abrogated (Fig. 3). In contrast, when NKL cells were treated with IL-2 for 4 days, the nuclear extracts showed very low CRE binding activity weaker than that found in unstimulated cells (Fig. 3). The quality and amount of nuclear extracts used in each reaction were checked by incubation with radiolabeled oligonucleotide Oct-1 (Table 1) (Fig. 2B).

These results clearly indicated that stimulation with PSK of NKL cells increased CRE DNA binding activity, whereas the treatment with IL-2 decreased this binding activity.

NF-κB and SP-1 binding activities are regulated differently by IL-2 and PSK

We analyzed binding to NF-κB and SP-1 probes (Table 1) by EMSA in unstimulated and stimulated NKL cells. Unstimulated NKL cells showed NF-κB and SP-1 binding activity, and these activities were not modified by treatment with PSK (Fig. 4). In contrast, IL-2 affected the regulation of NF-κB and SP-1 transcriptional activities in NKL cells in opposite ways: NF-κB binding activity decreased slightly, whereas SP-1 binding was slightly increased by treatment with this cytokine (Fig. 4). Nuclear extracts of PSK-stimulated cells were also preincubated with unlabeled NF-B and SP-1 oligonucleotides to verify the specificity of the bands (Fig. 4).

Changes in GAS/ISRE, IRF-1 and STAT5 binding in IL-2-stimulated NKL cells

Binding to the GAS/ISRE, IRF and STAT5 oligonucleotide probes (Table 1) was analyzed by EMSA. In unstimulated NKL cells we detected binding activity to the GAS/ISRE, IRF-1 and STAT5 probes (Fig. 5). These activities were not modified by treatment with PSK (Fig. 5). However, in IL-2-treated NKL cells a new more retarded band was seen instead of the band found in unstimulated or PSK-stimulated cells (Fig. 5). The appearance of this *upper* band, of greater molecular weight and different electrophoretic mobility, corre-

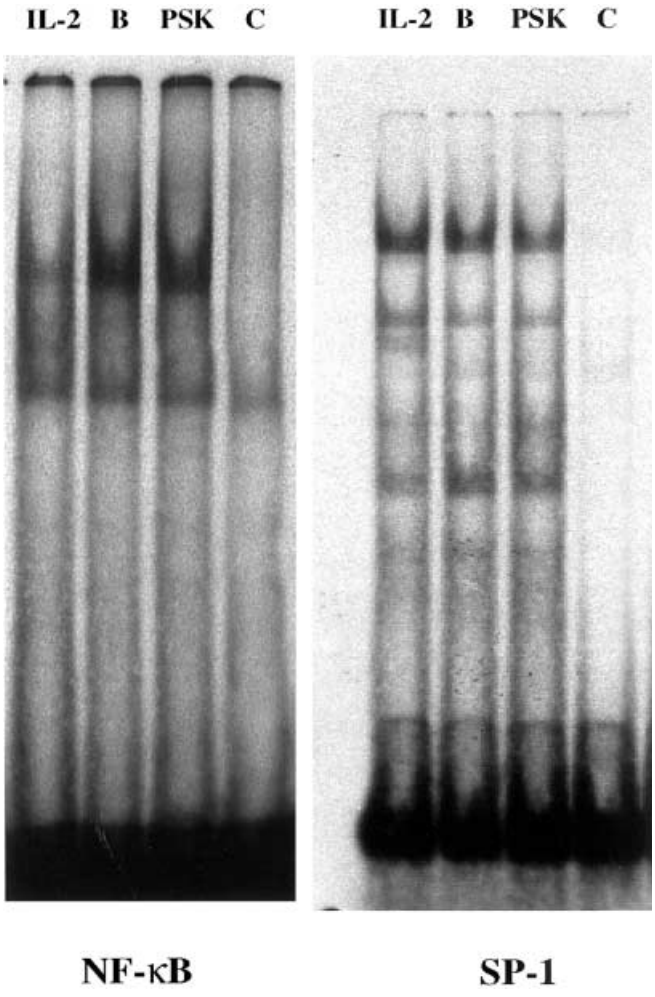

Fig. 4 Alterations in NF-κB and SP-1 binding activities in IL-2- and PSK-stimulated NKL cells. Radiolabeled NF-κB and SP-1 oligonucleotides were incubated with nuclear extracts (4–6 μg) prepared from unstimulated NKL cells (*lanes B*), IL-2-treated NKL cells (*lanes IL-2*), and PSK-treated NKL cells (*lanes PSK*). Treatment with PSK did not modify the binding activities in unstimulated cells, whereas treatment with IL-2 enhanced SP-1 activity and decreased NF-κB activity. Nuclear extracts of PSK-stimulated cells were also preincubated with unlabeled NF-κB and SP-1 oligonucleotides to verify the specificity of the bands (*lanes C*). The gel shows a representative assay from three independent experiments

sponds to posttranslational modifications of complexes present in unstimulated cells, because the band present in unstimulated cells did not appear in IL-2-treated cells. Nuclear extracts of IL-2-stimulated cells were also preincubated with a 100-fold excess of the unlabeled oligonucleotides before incubation with radiolabeled oligonucleotides (Fig. 5).

NKL cells treated with IL-2 or PSK showed no variation in NFAT, STAT1 or PU.1 binding activity

In this study weak signals for NFAT and STAT1 complexes were found in nuclear extracts from unstimulated

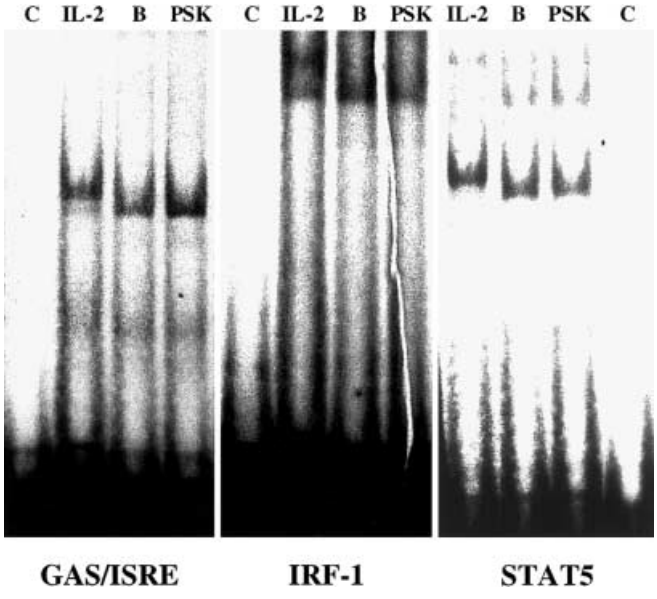

Fig. 5 IL-2, but not PSK, activated GAS/ISRE, IRF-1 and STAT5 binding activities in NKL cells. EMSA analysis was done with nuclear extracts isolated from unstimulated NKL cells (*lanes B*), PSK-stimulated cells (*lanes PSK*) or IL-2-stimulated cells (*lanes IL-2*). IL-2 induced complexes with the greatest molecular weight with all three probes. Nuclear extracts of IL-2-stimulated cells were preincubated with a 100-fold excess of the unlabeled oligonucleotides before incubation with radiolabeled oligonucleotides (*lanes C*). Three independent experiments were done

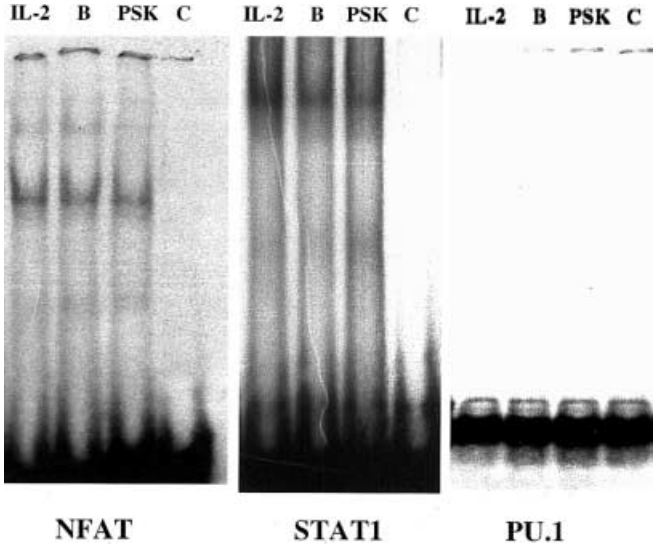

Fig. 6 Weak NFAT and STAT1 binding activities were detected in NKL cells, but no PU.1 binding activity was detected. STAT1, NFAT and PU.1 radiolabeled double-strand oligonucleotides (Table 1) were incubated with nuclear extracts prepared from unstimulated cells (*lane B*), IL-2-stimulated cells (*lane IL-2*) or PSK-stimulated cells (*lane PSK*). No changes in binding activities were detected

NKL cells, but no PU.1 complexes were detected (Fig. 6). When NKL cells were treated with IL-2 or PSK, neither NFAT nor STAT1 binding activity was

modified; also, PU.1 activity was not detected. When induction experiments were repeated with different incubation periods (1–72 h) and other amounts of IL-2 and PSK, these DNA binding activities were not modified (data not shown).

Discussion

The intracellular pathways that give rise to NK cell activation and natural cytotoxicity have not been clearly elucidated. Interleukin-2 stimulates T cell and NK cell proliferation and also augments cytotoxic function in NK cells [25, 15, 29], leading to the activation of different transcriptional activities [20, 37]. Protein-bound polysaccharide K has a favorable effect on the immune system, and it has also been reported to have chemotherapeutic effects in cancer patients [21, 35, 17]. In mice, PSK produces NK activation in vivo, and abrogates tumor growth and metastasis formation [2, 3]. In a previous study we showed that PSK can imitate IL-2 in stimulating proliferation and natural cytotoxic activity in the NKL cell line [23]. However, other cytokines, such as IL-1, IL-4, IL-6, IL-7, IL-12, TNF-α and IFN-γ, are not able to activate NKL cells [26]. These findings suggested the possibility that IL-2 and PSK may induce the same intracellular signaling events. In view of this hypothesis, we aimed to distinguish between the roles of different nuclear transcription factors in the stimulation of NKL cell proliferation and induction of natural cytotoxicity by PSK and IL-2. We report here that AP-1 binding activity was increased by treatment with IL-2 or PSK. The polysaccharide also increased CRE activity, but did not modify other binding activities. Interleukin-2 augmented SP-1 binding, and activated GAS/ISRE, IRF-1 and STAT5 activities by phosphorylation, but decreased CRE and NF-κB binding activities.

Interleukin-2 is an activator of AP-1 binding activity in T and NK cells [5], and an increase in AP-1 DNA binding activity has been observed in human NK clones during natural cytotoxicity [31]. In NKL cells new AP-1 complexes appear during the early stage of NK cell cytolytic programs, and engagement of NK cell inhibitory receptors for MHC class I molecules impairs the very early activation of AP-1 [8]. These results indicate that activation of AP-1 occurs during cytolytic activity of NK cells and is necessary for the development of the cytolytic program. However, before the development of cytolytic activity, we detected increased AP-1 binding activity in IL-2- and PSK-stimulated NKL cells in comparison with unstimulated cells (Fig. 2A). This IL-2- or PSK-induced AP-1 activity seems likely to be necessary for natural cytotoxicity in NKL cells, as suggested by the fact that unstimulated NKL cells showed neither cytotoxic activity nor AP-1 binding activity (Figs. 1 and 2A).

These results might be taken to suggest that PSK and IL-2 use the same mechanism – activation of AP-1 binding activity – to maintain proliferation and induce natural cytotoxicity in NKL cells. However, we detected differences in the induction of other binding activities by these two agents. PSK and IL-2 affected CRE binding activity in opposite ways: PSK induced CRE activity in NKL cells, whereas IL-2 decreased this activity (Fig. 3). Several studies have demonstrated that the CRE transcription factor is involved in the development and function of T cells. Stimulation with IL-2 of T lymphocytes induces CRE activity [11]. Moreover, CRE activity has been implicated in IL-2 gene expression, and the failure to produce IL-2 is an important determinant of anergy induction [9]. In transgenic mice that express a dominant negative form of CREB, T cell development is normal, but thymocytes and T cells display a profound proliferative defect characterized by markedly decreased IL-2 production, G1 cell cycle arrest, and reduced activation of AP-1 activity [6]. Our data indicate that the action of IL-2 in NKL cells is not mediated by CRE binding activity, and that the increase in AP-1 is not dependent on this activity. In contrast, CRE activity does appear to be involved in the mechanism used by PSK. This conclusion is significant, as it implies that IL-2 and PSK do not produce the same signal cascades in NKL cells.

In addition to the difference in CRE binding activity, we found other differences between the intracellular signals produced by PSK and IL-2. In cells stimulated with PSK, the intensity of GAS/ISRE, IRF-1 and STAT5 binding activities was similar to that seen in unstimulated cells (Fig. 5). However, treatment with IL-2 produced a similar variation in all three of these binding activities, leading to the appearance of a higher band with lower electrophoretic mobility than that which appeared in PSK-stimulated or unstimulated NKL cells (Fig. 5). The appearance of this higher band of greater molecular weight, and the absence of the band present in unstimulated cells, indicates that these complexes were formed as a result of posttranslational modifications, principally phosphorylation of the complexes present in unstimulated NKL cells. That the same variation was seen in all three transcriptional activities may be explained by the fact that the consensus sequence for GAS/ISRE can bind STAT5 and IRF-1 transcriptional factors. Thus, activation of the GAS/ISRE, IRF-1 and STAT5 transcription factors may be involved in the proliferation and natural cytotoxicity induced by IL-2, but not by PSK. This idea is supported by previous studies that have shown that IL-2 produces activation and interaction between STAT5 and ETS transcription factors in human T cells, binding these complexes to the GAS sequence [24]. Interleukin-2 phosphorylates STAT proteins [28, 33], a transcriptional factor that is a mediator of IL-2 signaling in T cells. Moreover, STAT5b binding activity plays an essential, non-redundant role in NK cell-mediated proliferation and cytolytic activity [18, 12]. With regard to IRF-1 binding activity, studies in IRF-1-deficient mice have shown that this transcriptional factor is essential for the induction of NK cell-mediated cytotoxicity and for the

Table 2 Role of nuclear transcriptional factors in the NK and T cell activities. Variation in IL-2 and PSK-activated NKL cells

Transcriptional factor	Role	NKL basal	IL-2	PSK
AP-1	Induction by IL-2 NK cytolytic activity	+	+ + + +	+ + + +
CRE	T cell development and functions IL-2 gene expression AP-1 activation G1 cell cycle arrest	+	–	+ + + +
GAS/ISRE-IRF-1-STAT5	NK cell proliferation and cytolytic activity	+ +	New complexes	+ +
NF-κB	Induction by IL-2 Not implicated in NK activity	+ +	+	+ +
SP-1	IL-2 gene expression Fas-ligand mediated cytotoxicity	+ +	+ + +	+ +

in vivo effector functions mediated by this activity [10]. These results, together with our data, are evidence of an important role for GAS/ISRE, IRF-1 and STAT5 in NK cell activation by IL-2.

Other binding activities that were affected differently by PSK and IL-2 treatment are SP-1 and NF-κB. NF-κB and SP-1 binding activities were detected in unstimulated NKL cells. Treatment with IL-2 increased SP-1 and decreased NF-κB; and treatment with PSK did not change these activities (Fig. 4). These results show that the effect of IL-2 on NKL cells is not mediated by NF-κB activation, but may be mediated by SP-1 activity. Earlier studies showed that SP-1 transcriptional factor is involved in the expression of the IL-2 gene, and it appears to play an important role in FAS ligand-mediated cytotoxicity of activated peripheral T cells treated with IL-2 [36]. Moreover, NF-κB binding activity is increased by stimulation with IL-2 of NK3.3 cells [34], and is also is increased in NK human cell clones stimulated with specific cell targets. However, pre-treatment of the cells with the proteasome inhibitor N-acetyl-leu-leu-norleucina, which selectively inhibits NF-κB activation, did not affect NK activity [31]. In addition, NF-κB activation is not required for IL-2-mediated survival or cell cycle progression in activated primary human T cells [13]. These results and our data indicate that NF-κB activity is probably not necessary for NK proliferation and induction of natural cytotoxicity.

No changes in NFAT, PU.1 or STAT1 binding activities were detected in stimulated NKL cells with respect to unstimulated cells (Fig. 6). Therefore, these transcriptional factors appear not to be involved in the activation of NKL cells by IL-2 or PSK.

In conclusion, nuclear extracts of unstimulated NKL cells showed AP-1, CRE, NF-κB, SP-1, GAS/ISRE, IRF-1 and STAT5 binding activities, weak STAT-1 and NFAT binding, and no binding of PU.1. IL-2-stimulated NKL cells showed increased AP-1 and SP-1 binding activities, modified GAS/ISRE, IRF-1 and STAT5 binding activities, and decreased NF-κB and CRE binding. In contrast, PSK increased AP-1 and CRE binding, but did not modify other binding activities present in unstimulated cells. Table 2 shows the results obtained and the role previously described in the NK and T cells for these transcriptional factors. The signal transduction pathway induced by IL-2 in NKL cells may involve AP-1, SP-1, GAS/ISRE, IRF-1 and STAT 5 binding activities. In contrast, the PSK pathway might involve principally AP-1 and CRE.

Our results show that the signal transduction pathway used by IL-2 and PSK in NKL cells is different; and suggest that, despite the differences in the mechanism of intracellular signaling, these inducers may ultimately have the same effect on proliferative and cytotoxic behavior of NKL cells. Moreover, like previous reports indicating that PSK does not influence IL-2 in T or NK cells [14, 16], our data provide additional evidence that the action of PSK does not occur through the induction of IL-2 in NKL cells.

The molecular mechanisms implicated in the antitumor effect of PSK have not been previously elucidated. Our data provide a molecular basis to explain that the immunomodulatory effect of PSK may be due to selective activation of natural killer cells mediated by the AP-1 and CRE transcriptional factors. Thus, understanding these signaling components may shed light in cancer immunotherapy and could potentially lead to the application of new antitumoral drugs. Moreover, these results provide indications that the NKL cytotoxicity system is a good model to further analyze the molecular mechanisms responsible for PSK activity.

Further experiments with decoy strategies are in progress to specifically determine which of the IL-2- or PSK-induced binding activities are necessary for proliferation and cytotoxicity in the NKL cell line.

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