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NK-lysin, a novel effector peptide of cytotoxic T and NK cells. Structure and cDNA cloning of the porcine form, induction by interleukin 2, antibacterial and antitumour activity

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A 78 residue antimicrobial, basic peptide, NK-lysin, with three intrachain disulfide bonds was purified from pig small intestine and characterized. A corresponding clone was isolated from a porcine bone marrow cDNA library. The 780 bp DNA sequence had a reading frame of 129 amino acids which corresponded to NK-lysin. The clone was used to show that stimulation with human interleukin-2 induced synthesis of NK-lysin-specific mRNA in a lymphocyte fraction enriched for T and NK cells. Lower levels of mRNA were detected in tissues known to contain T and NK cells, such as small intestine, spleen and colon. Interleukin-2 also induced both proliferation of the lymphocyte fraction and cytolytic function in these cells. Immunostaining showed that NK-lysin was present in cells positive for CD8, CD2 and CD4. NK-lysin showed high antibacterial activity against *Escherichia coli* and *Bacillus megaterium* and moderate activity against *Acinetobacter calcoaceticus* and *Streptococcus pyogenes*. The peptide showed a marked lytic activity against an NK-sensitive mouse tumour cell line, YAC-1, but it did not lyse red blood cells. The amino acid sequence of NK-lysin exhibits 33% identity with a putative human pre-proprotein, NKG5, of unknown function but derived from a cDNA clone of activated NK cells. We suggest that NK-lysin is a new effector molecule of cytotoxic T and NK cells.

Key words: antibacterial peptide/cytotoxic peptide/DNA sequencing/protein purification/small intestine

Introduction

Antibacterial peptides are considered to be an important part of the innate immunity of mammals (Zasloff, 1992; Boman *et al.*, 1994; Boman, 1995) and have been isolated from the small intestine of pig (Lee *et al.*, 1989; Agerberth *et al.*, 1991, 1993) and mouse (Eisenhauer *et al.*, 1992; Selsted *et al.*, 1992; Ouellette *et al.*, 1992, 1994), while genes for such peptides have also been found in mouse (Huttner *et al.*, 1994) and in human Paneth cells (Jones and Bevins, 1992, 1993). The intestine is not only an organ with a rich supply of blood vessels, but its epithelium is also a major site for T lymphopoiesis and contains the largest collection of T cells in the body (Poussier and Julius, 1994). The cytotoxic T and NK cells, together with neutrophils and macrophages, are the primary effector cells of innate immunity and some (or all) of these cell types contain granules with antibacterial peptides.

There is a wealth of information available concerning the recognition mechanisms of cytotoxic T and NK cells (Paul, 1994), while the effector mechanisms are less clearly defined. Two mutually non-exclusive theories about the effector mechanism have received much attention: one claims that the target cells are killed by induction of apoptosis (Berke, 1991), the other that they are killed by perforin, a protein that forms lytic pores in the target cell membrane (Krähenbuhl and Tschopp, 1991). We now describe the structure and properties of a novel 78 residue peptide, NK-lysin (NKL), which is most probably an additional component of the cytotoxic machinery of T and NK cells. This peptide was isolated from porcine intestinal tissue on the basis of its antibacterial activity, but it also shows lytic activity against an NK-sensitive tumour cell line of murine origin. Since another antibacterial peptide, PR-39, previously isolated from the same intestinal material (Agerberth *et al.*, 1991), is transcribed in bone marrow rather than in the intestine (Storici and Zanetti, 1993), there was an early need for both an NK-lysin-specific clone and an antiserum in order to identify the tissue(s) and cells that produce NK-lysin. Moreover, studies on the mechanism of action of the peptide would require peptide material, obtained from either the cells normally producing NK-lysin or cultures of cell lines with a vector designed for the synthesis of NK-lysin. We have therefore studied the conditions for gene activation, to allow future *in vitro* production of the peptide. The fractions from the purification of intestinal hormones (Mutt, 1986) that were originally used for the isolation of NK-lysin gave in a whole year just sufficient amounts of the peptide for the structural and functional studies here reported and for raising the antiserum used.

Results

Isolation of NK-lysin

Figure 1A shows gel filtration of a crude peptide fraction obtained from a concentrate of thermostable intestinal polypeptides (CTIP) by further purification steps, as described in Materials and methods. Fractions with anti-*E.coli* activity (indicated by the bar in Figure 1A) were

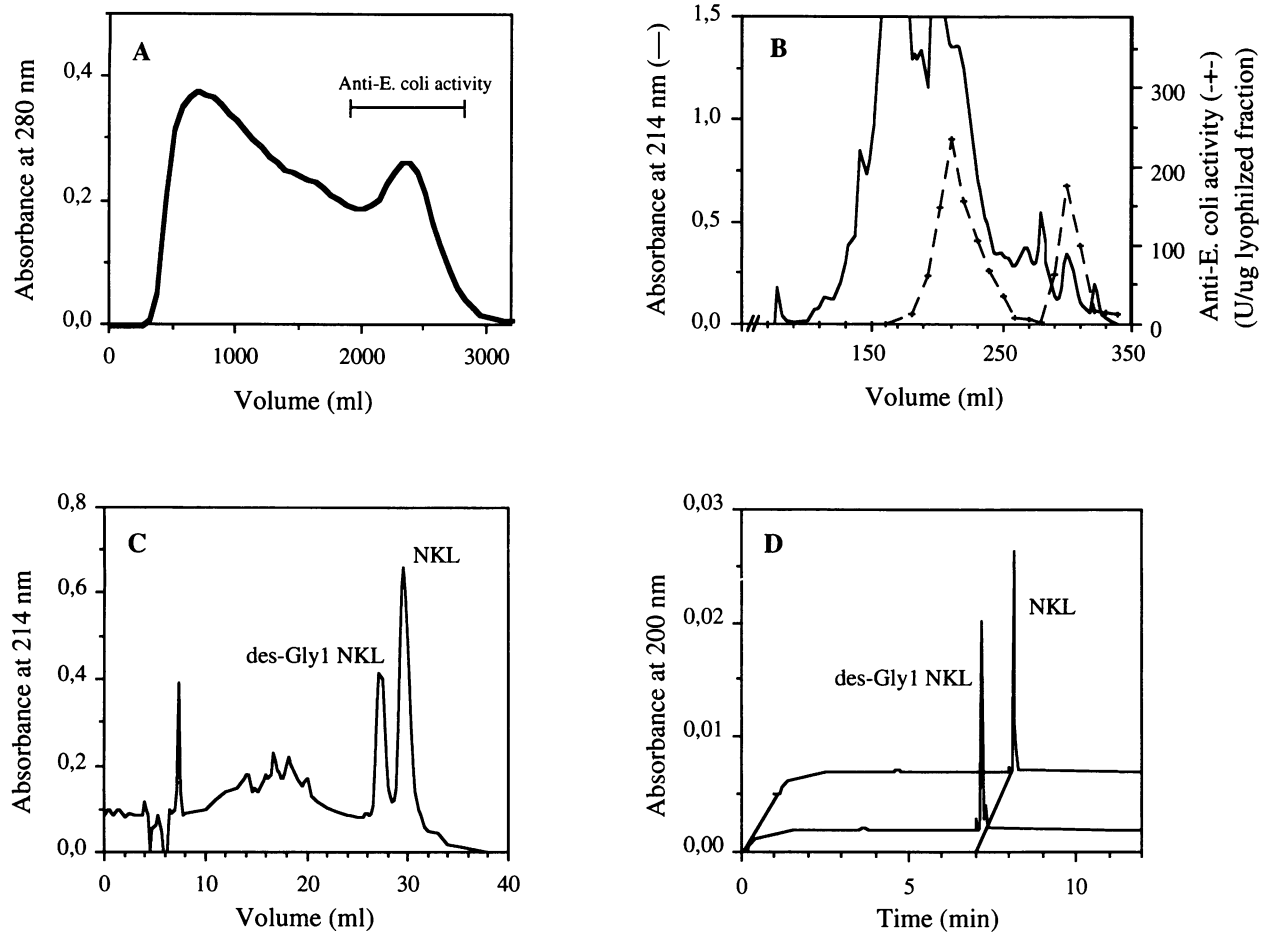

Fig. 1. Purification of NK-lysin. (**A**) Gel filtration on Sephadex G-25 fine. Anti-*E.coli* activity is indicated by the bar. (**B**) Reverse-phase HPLC on a semi-preparative Vydac column with trifluoroacetic acid as counter-ion. The most active fractions from the second peak of anti-*E.coli* activity were pooled and used in the next purification step. Units of anti-*Ecoli* activity were determined for all fractions. (**C**) Final purification on an analytical reverse-phase HPLC Vydac column with heptafluorobutyric acid as counter-ion. Two peaks with antibacterial activity were collected and identified after analysis as des-Gly1 NK-lysin and NK-lysin. (**D**) Capillary zone electrophoresis used for analysis of purity of the material in the two main peaks from the last chromatographic step.

pooled and gave ~600 mg crude freeze-dried material with a specific activity of 6–7 U/μg. For the next purification step, 80 mg batches were dissolved in water and the peptides fractionated on a reverse-phase HPLC column using a linear gradient of 20–50% acetonitrile in 0.1% trifluoroacetic acid. Anti-*E.coli* activity of the dried material from each fraction was determined and two peaks of activity were obtained (Figure 1B). Material corresponding to the second peak, eluting with 50% acetonitrile, was pooled from the three fractions with the highest activity. Hydrolysis and amino acid analysis showed the material corresponding to the first antibacterial peak (Figure 1B) to contain a high proportion of arginine and proline, most likely originating from PR-39 (Agerberth *et al.*, 1991). However, the material in the second region of activity was further purified (in batches of 1.0–1.5 mg) by analytical reverse-phase HPLC with a gradient of acetonitrile in 0.1% heptafluorobutyric acid (Figure 1C). The change in counter-ion resolved the antibacterial activity into two main components eluting at 27–31 ml. Analyses identified the peptides as des-Gly1 NK-lysin and NK-lysin. Starting from 600 mg of the active material in Figure 1A, the yields of the two peptides were 1.2 and 3.9 mg respectively. Both peptides eluted as single peaks upon capillary zone electrophoresis (Figure 1D) and had identical specific antibacterial activities of 230 U/μg, assuming that the slope of the standard curve for these peptides is the same as for cecropin A.

Structure analysis of NK-lysin

Sequence data from intact NK-lysin and fragments generated enzymatically (by Lys-C and Asp-N) or chemically (by CNBr) gave the entire 78 residue amino acid sequence (Figure 2). The molecular mass (8923.2 ± 1 kDa) determined by electrospray mass spectrometry is in good agreement with the value of 8924.8 calculated from sequence data (assuming that the half-cystines form three intramolecular S−S bonds). Similarly, total composition after hydrolysis of NK-lysin and des-Gly1 NK-lysin in the native state and after oxidation by performic acid gave results in agreement with the sequence data. Mass spectrometric analysis of des-Gly1 NK-lysin gave a molecular mass of 8866.3 ± 1 kDa, or 56.9 mass units less than for NK-lysin, which is close to 57.2, the mass of the missing glycine residue. Edman degradation of the material in the first peak showed amino acid residues 1–18 to be identical to residues 2–19 of NK-lysin. Thus we conclude that the second component is des-Gly1 NK-lysin.

Fig. 2. Structural analysis of NK-lysin. The fragmentation was made chemically with CNBr (CN1−CN3) or enzymatically with Asp-N (D1−D6) or Lys-C (K1). The top arrow (N1) shows the N-terminal sequence obtained with intact peptide. Other arrows show the sequences obtained for the respective fragments. K1 was generated after fragmentation of CN3. Dotted lines indicate sequences in agreement with total compositions. The disulfide bridges are shown below the sequence.

Peptide preparations normally produced 75% of the full-length NK-lysin and 25% of des-Gly1 NK-lysin. The latter peptide could originate from a difference in N-terminal processing or, more likely, from exopeptidase degradation of NK-lysin during isolation.

For determination of the disulfide bridges, NK-lysin was digested with trypsin and the fragments were separated by reverse-phase HPLC. One of two fragments containing a half-cystine was then digested with endoproteinase Glu-C, giving two further half-cystine-containing fragments. All three fragments containing half-cystines were analysed by Edman degradation. In each case the results showed two sequences, indicating that segment GYFCE (residues 1–5) was associated with ICKE (residues 75–78), suggesting a Cys4−Cys76 bond, that segment SCRK (residues 6–9) was associated with KPQAICVDIK (residues 65–74), suggesting an S−S bond between Cys7 and Cys70, and that the segment starting with LEDM (residues 14–37) was associated with GLCK (residues 43–46), suggesting a Cys35−Cys45 bond. These results are consistent with the disulfide bridge pattern shown in Figure 2.

Searching the SwissProt database revealed that the sequence of NK-lysin showed similarities to the C-terminal portions of two almost identical proteins, 519 and NKG5, both predicted but never isolated and with unknown functions. They were deduced from cDNA clones isolated from activated human T and NK cells respectively (Jongstra *et al.*, 1987; Yabe *et al.*, 1990). The C-terminal region of 519 and NKG5 both showed ~33% identity with NK-lysin (using the program BESTFIT; UWGCG).

cDNA cloning and gene expression stimulated by interleukin-2 (IL-2)

Since we suspected that NK-lysin originated from lymphocytes, total RNA from porcine bone marrow was used for 3′-RACE-PCR (Frohman *et al.*, 1988). The PCR fragment obtained was sequenced and found to code for a peptide very similar to a segment of NK-lysin (for details see Materials and methods). The PCR product was used as a probe for screening a cDNA library from porcine bone marrow. Two identical clones were isolated and sequenced, both coding for NK-lysin (Figure 3). The reading frame of the mature peptide in our NK-lysin clones and the peptide isolated from domesticated pig differ at seven internal positions, six compatible with single base replacements. Only the replacement of tryptophan with lysine at position 58 requires a two base shift.

Total RNA was prepared from a pig lymphocyte fraction containing T and NK cells (see Materials and methods) and from a number of different porcine tissues. Of these RNA samples, 16 were used in Northern blot analysis with the NK-lysin clone as a probe. Figure 4 shows that a mixed population of T and NK cells incubated with IL-2 gave a marked increase in NK-lysin-specific mRNA. There were also clear signals detected in RNA from spleen, bone marrow and colon and, after prolonged exposure, weak signals were obtained with RNA prepared from blood and small intestine. These tissues are all known to contain significant numbers of lymphocytes. We used material from both wild and domesticated pigs, because in-breeding could have resulted in the loss of some relevant genes, but no difference was seen in the size of the corresponding NK-lysin transcripts.

Characterization of the pig lymphocyte fraction used

A fraction enriched in porcine T and NK cells was isolated from whole blood of domesticated pigs. This cell population was divided and incubated in culture medium with or without human IL-2. The cells were harvested after 6 days and tested for cytotoxic activity against ^{51}Cr-labelled YAC-1 cells (an NK-sensitive mouse tumour cell line). Figure 5 shows that an IL-2-activated population of porcine T and NK cells was clearly more cytolytic for YAC-1 than unstimulated control cells. As the lymphocyte concentration increased, target cell lysis rose from 15 to 35%, while unstimulated cells gave <9% lysis. Thus the lymphocytes were clearly functional and, as is often the case, a higher effector cell concentration was inhibitory. The human cell line K562 was less sensitive to porcine lymphocytes than YAC-1; the highest concentration of effector cells gave 25% lysis of K562.

The same lymphocyte fraction was also analysed by flow cytometry and immunocytochemistry. Figure 6 shows that incubation with IL-2 gave an enrichment of the R1 gated cell population. When a polyclonal antibody against NK-lysin was used in FACscan analyses the strongest staining was found in the R1 population (not shown). Monoclonal antibodies showed that the R1 gated population of T and NK cells after IL-2 stimulation contained 90% $CD2^+$, 6% $CD4^+$, 75% $CD8^+$ and 22% $N1^{c+}$ cells.

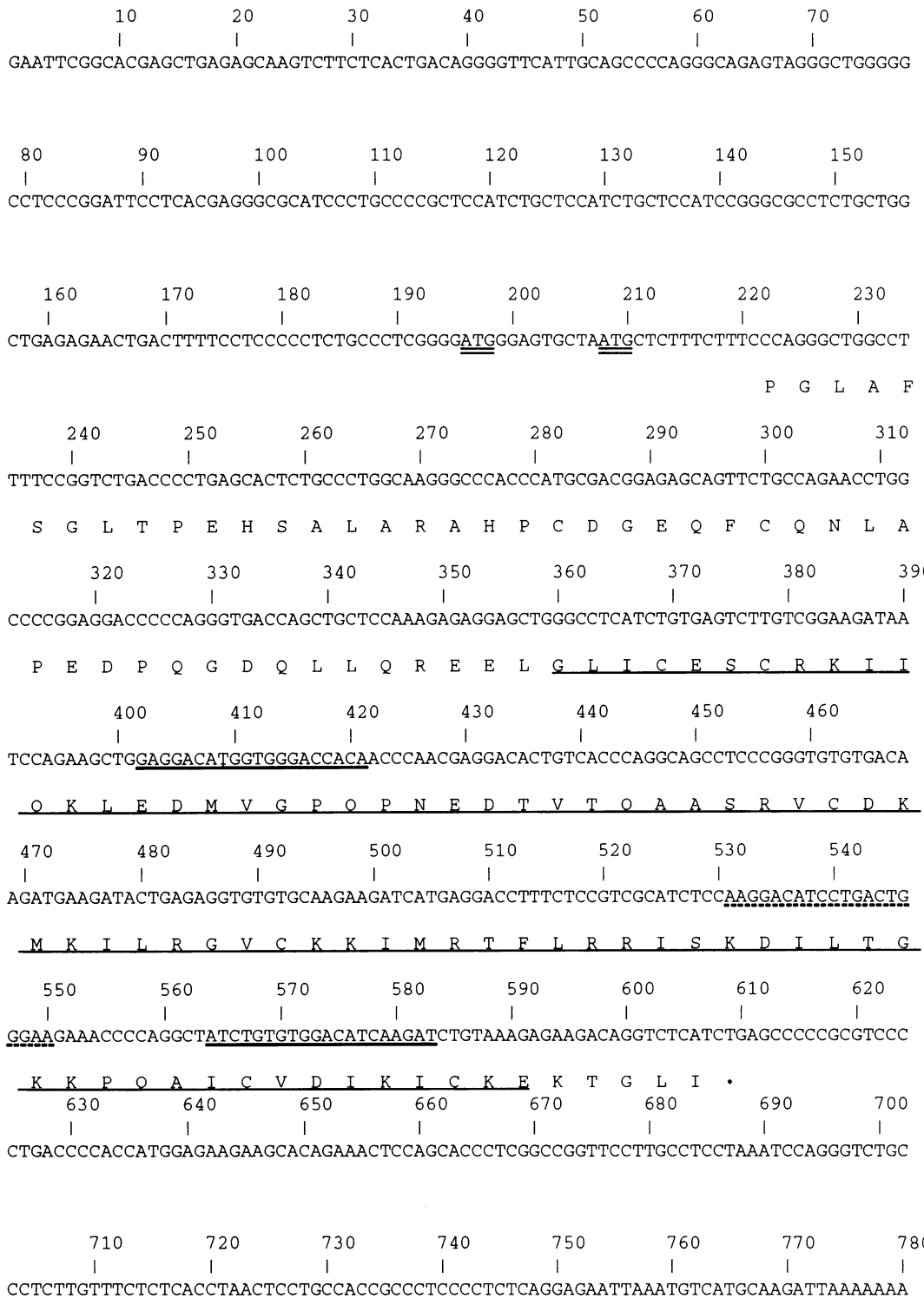

Fig. 3. Nucleotide sequence of the clones corresponding to NK-lysin. The part of the reading frame representing the mature NK-lysin is underlined. The sites corresponding to the two primers used in the second step of the PCR synthesis are underlined with bold lines, the preliminary probe used during the amplification steps is underlined with a dotted line. The first of these oligonucleotides (bp 402–421) corresponds to the sequence given in the figure, the two others are complementary sequences (corresponding to bp 564–583 and 531–550 respectively). The suggested start codons are double underlined. For further details see Materials and methods. EMBL accession number: X85431.

As the next step, the stimulated T and NK cell fraction was characterized by immunostaining using polyclonal rabbit anti-NK-lysin IgG antibodies (see Materials and methods) and the monoclonal antibodies used in the FACscan analyses. Figure 7 shows that the lymphocyte fraction contained three cell types with NK-lysin. The first (Figure 7a and b) was $CD8^+$ and ~50% of these cells were also NKL^+ and ~20–25% were either NKL^+ and $CD8^-$ or $CD8^+$ and NKL^-. The second (Figure 7c and d) was $CD2^+$ and almost all of these cells were NKL^+. A few cells were identified as $CD2^+$ and NKL^-, but no cells were found to be NKL^+ and $CD2^-$. For the third

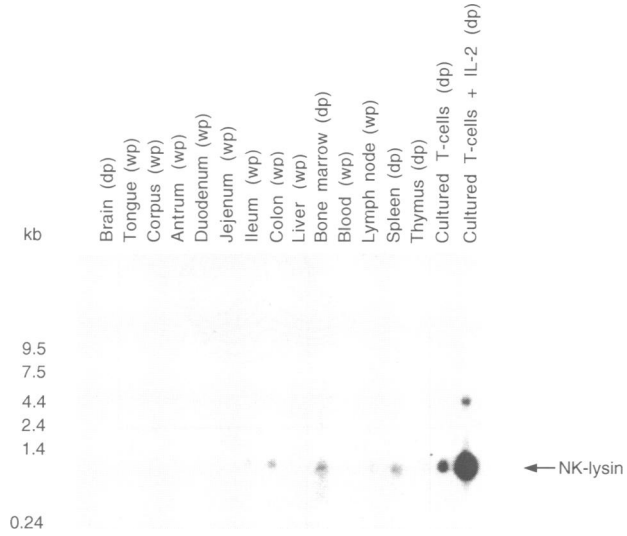

Fig. 4. Northern blot analysis of total RNA prepared from porcine lymphocytes incubated for 6 days with or without interleukin 2 (IL-2). Also included are total RNA samples from 14 tissues of either domesticated (dp) or wild pig (wp).

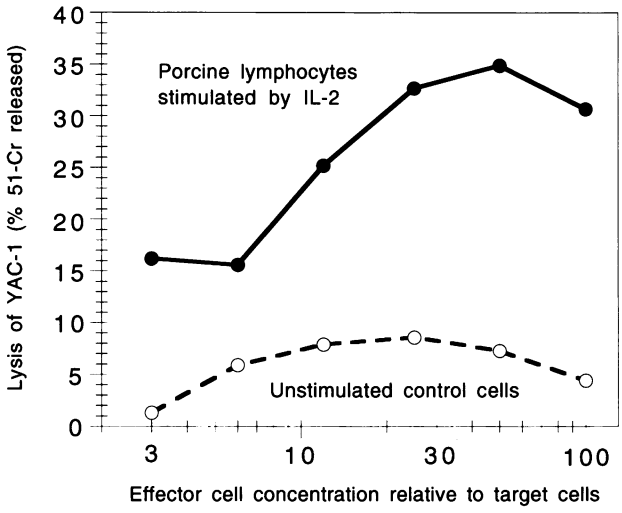

Fig. 5. Lysis of ^{51}Cr-labelled mouse YAC-1 cells after 2 h incubation at 37°C with increasing concentrations of a population of porcine T and NK cells. These cells were isolated using a Ficoll gradient followed by passage through nylon wool and then pre-incubated for 6 days with human IL-2 (50 U/ml). Control cells were incubated for the same time in the same medium, but without any addition.

cell type (Figure 7e and f) ~25% of the cells were NKL$^+$ and CD4$^+$, ~50% were CD4$^+$ and NKL$^-$ and a few were NKL$^+$ and CD4$^-$. When the antibodies against NK-lysin were pre-absorbed with NK-lysin, no specific staining was detected.

Antimicrobial and cytotoxic activity

The LC values (lowest concentration lethal for the bacteria) for NK-lysin and des-Gly1 NK-lysin were found to be almost identical, as were the antibacterial spectra (Table I). High activity was found against *Escherichia coli* and *Bacillus megaterium*, with moderate activity against a pig pathogenic strain of *E.coli*, *Acinetobacter calcoaceticus* and *Streptococcus pyogeneis*. For both of the peptides, medium E gave a pronouced increase in activity. NK-lysin was also found to have some antifungal activity and the LC value for *Candida albicans* (30 μM) was the same as found by us for defensin NP-2 (Lehrer *et al.*, 1993) using the same assay. There was no activity against *Pseudomonas aeruginosa*, *Staphylococcus aureus* or *Salmonella* LT-2 (LC > 170 μM), the latter being an organism known to be susceptible to NK cells (Garcia-Penarrubia, 1992). Neither peptide gave a lytic zone in an assay with sheep red blood cells at a concentration of 170 μM.

NK cells are known to lyse certain tumour cell lines (NK-sensitive cells) and our lymphocyte fraction did show such activity (Figure 5). However, purified NK-lysin is more potent than the lymphocyte fraction. Figure 8 shows that NK-lysin at 50 μg/ml was able to give 90% lysis of ^{51}Cr-labelled YAC-1 cells in a medium with 2% fetal calf serum (FCS) and ~75% lysis in phosphate-buffered saline (PBS), while there was only ~15% lysis if the cells were suspended in medium E, designed for bacterial growth. FACscan experiments with our anti-NK-lysin IgG fraction showed that NK-lysin at 1 and 10 μg/ml was 100% bound to YAC-1 cells (data not shown).

Discussion

NK-lysin belongs to a novel class of physiologically active peptides

During the last decade antibacterial peptides have been isolated from both vertebrates and invertebrates (Zasloff, 1992; Boman *et al.*, 1994; Boman, 1995). Using procedures similar to those applied for the purification from intestine of cecropin P1 (Lee *et al.*, 1989) and PR-39 (Agerberth *et al.*, 1991), we have now isolated a 78 residue antimicrobial peptide, NK-lysin. Cecropin P1 and PR-39 are free of cysteine, while in NK-lysin there are three disulfide bridges. Myeloid defensins also have three disulfide bonds and they constitute a group of peptide antibiotics well studied with respect to structure and function (Lehrer *et al.*, 1993). X-ray crystallography of the human defensin HNP-3 (Hill *et al.*, 1991) showed a dimeric three-dimensional structure with three antiparallel strands. Other mammalian peptides with six half-cystines in intrachain bonds are TAP from bovine trachea (Diamond *et al.*, 1991) and the β-defensins from bovine phagocytes (Selsted *et al.*, 1993). Antibacterial peptides with only four half-cystines are smaller in size and they constitute a separate group with the tachyplesins (Miyata *et al.*, 1989) from horse shoe crab and protegrins from pig phagocytes (Kokryakov *et al.*, 1993). The larger antibacterial peptides (6–7 kDa) found in horse leukocytes contain eight cysteines (Couto *et al.*, 1992).

There is no obvious relationship between NK-lysin and the previously isolated antibacterial peptides with three disulfide bridges. NK-lysin, with its molecular weight close to 9 kDa, is larger than defensins (3–4 kDa), the disulfide bridges are not similarly located and there are no clear sequence similarities. Functionally, NK-lysin is more effective against *E.coli* than defensin NP-2, but the activities against *C.albicans* are similar and both molecules have cytotoxic properties. On the other hand, the defensins are inactive at high salt concentrations (Lehrer *et al.*,

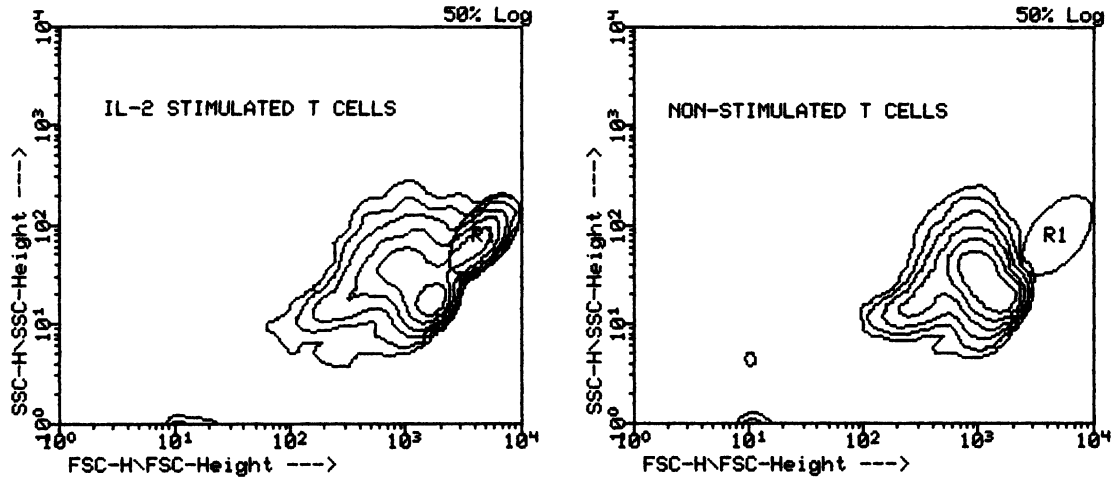

Fig. 6. Flow cytometry illustrating the effects of IL-2 on the T and NK cell populations isolated using a Ficoll gradient followed by passage through nylon wool. These cells were also used for the results shown in Figures 4 and 7. The instrument was a Becton and Dickinson FACscan and the R1 population was characterized by monoclonal antibodies against CD2, CD4, CD8 and N1[c].

Table I. Antimicrobial activity of NKL and des-Gly 1 NKL

Bacterial and fungal species	Strain	Lethal conc. (μM) of Peptides			
		NKL		des-Gly 1 NKL	
		+Med. E	−Med. E	+Med. E	−Med. E
Escherichia coli	D21	0.5	8	0.8	10
Escherichia coli	Bd2221/75	10	39	11	40
Pseudomonas aeruginosa	OT97	>190	ND	>160	ND
Acinetobacter calcoaceticus	Ac11	7	ND	12	ND
Bacillus megaterium	Bm11	1.6	0.8	1.4	0.9
Staphylococcus aureus	Cowan I	ND	>190	ND	>160
Streptococcus pyogenes	w.t.	ND	34	ND	30
Candida albicans	w.t.	ND	31	ND	ND

Thin plates (1 mm thick) were poured with LB broth, with (+Med. E) and without (−Med. E) medium E, 1% agarose and ~6×10^4 cells/ml of the test bacteria. Lethal concentrations (LC values, i.e. the lowest concentrations that will kill the bacteria) were calculated from inhibition zones of dilution series (Hultmark *et al.*, 1983). *E.coli* Bd2221/75 is a strain pathogenic for piglets, originating from Olof Söderlind (SVA, Uppsala, Sweden).

1993), while NK-lysin is ~10-fold more active against *E.coli* in medium E (Table I), a basal salt medium developed for *E.coli* (Vogel and Bonner, 1956). On a molar basis, NK-lysin in medium E is almost as active against *E.coli* D21 as cecropin P1 and PR-39 are without this medium (LC values of 0.5 compared with 0.3 μM). des-Gly1 NK-lysin shows the same activity as NK-lysin towards all bacteria and fungal strains tested, which suggests that the antimicrobial determinants of NK-lysin are not located at the very N-terminus.

Alignment of NK-lysin and positions 64–142 of 519 (Jongstra *et al.*, 1987) and NKG5 (Yabe *et al.*, 1990) shows that five of the six half-cystine residues in NK-lysin are conserved in the two postulated proteins 519 and NKG5 (Figure 9). The sixth half-cystine residue in NK-lysin is aligned with a tyrosine residue in 519 and NKG5 (position 67), a replacement that only requires a single base substitution. This loss of a cysteine in 519 and NKG5 does not rule out structural similarities with NK-lysin. The disulfide bridge pattern in NK-lysin (Figure 2) suggests that the peptide chain folds back on itself and NKG5 may be stabilized in a similar manner, but with two bridges. Chou−Fasman predictions indicate that the N-terminal and central parts of NK-lysin could form amphipathic α-helices, while other parts are likely to form β-sheets. Circular dichroism analyses are in line with these predictions (not shown). The predicted helical regions may explain the effects of medium E (Table I), because this medium was recently found to induce helix formation and increase activity of FALL-39, another antibacterial peptide (Agerberth *et al.*, 1995). Of the known structures for half-cystine-containing peptides, only insect defensins are in part α-helical and in part β-sheet structure (Hoffmann and Hetru, 1992).

The clone obtained (Figure 3) does not have an open reading frame starting with methionine. However, the clone contains one reading frame with homology to 519 (Jongstra *et al.*, 1987) and NKG5 (Yabe *et al.*, 1990) at positions 17–147 (see Figure 9). The 5′ regions of our two clones were sequenced by three different methods and in one case both strands were sequenced. These results showed no indication of a compression that could explain a missing base pair. Moreover, the size of the clone is approximately the same as estimated for the mRNA in the Northern blot (Figure 4). Thus we believe that one of the methionine codons, at position 196 or 208 in Figure 3

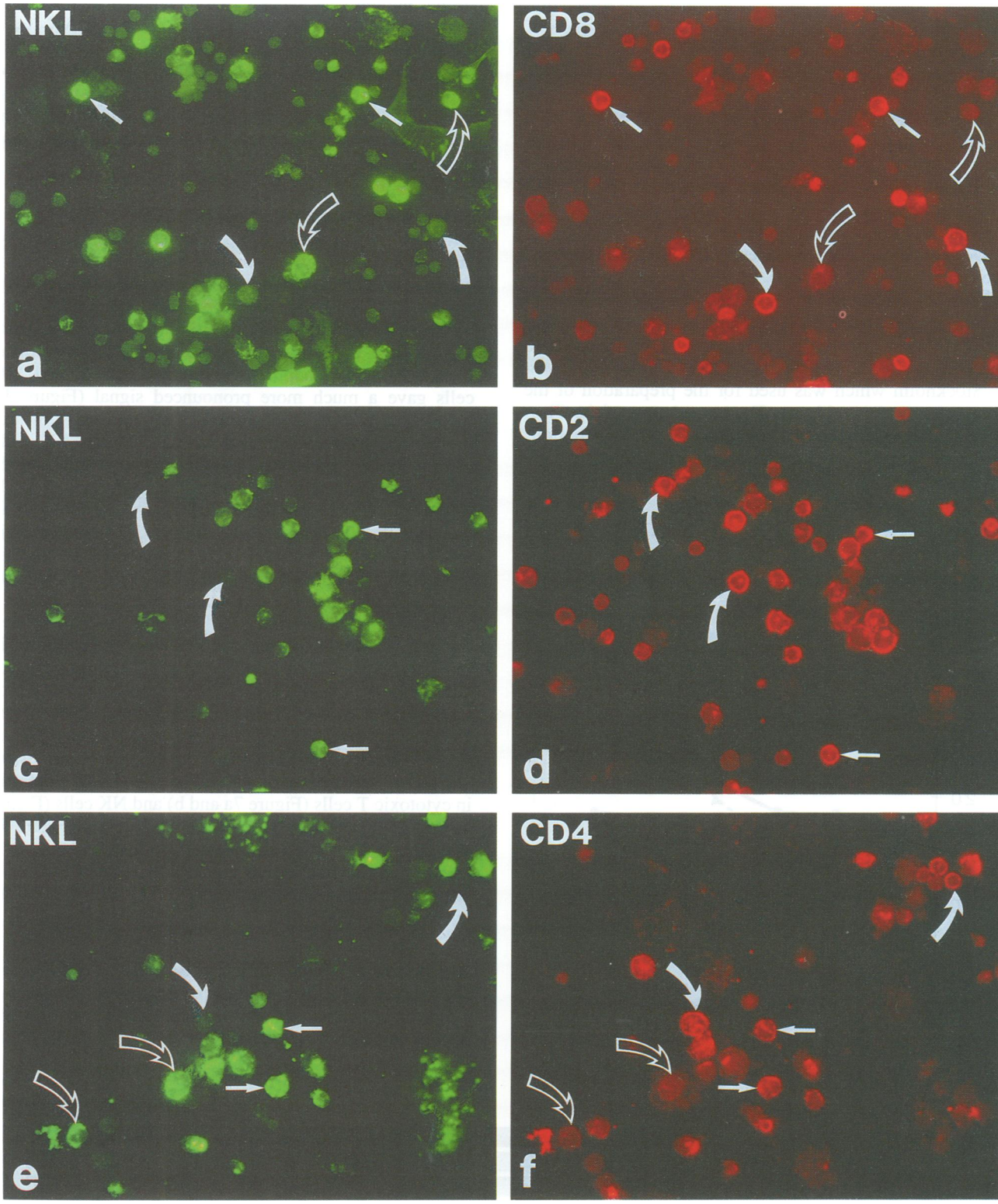

Fig. 7. Immunofluorescence micrographs of a T and NK cell fraction stimulated with IL-2 and incubated with antibodies against NK-lysin, CD2, CD4 or CD8 respectively. The cells are the same as those used for RNA preparation (Figure 4) and in flow cytometry (Figure 6). NK-lysin-positive cells are shown in green (left side), cells positive for CD2, CD4 or CD8 in red (right side). (**a** and **b**) Cells positive for both NK-lysin and CD8 (straight filled arrows), NKL-positive and CD8-negative cells (open curved arrows), cells negative for NK-lysin and positive for CD8 (filled curved arrows). (**c** and **d**) Cells positive for both NK-lysin and CD2 (straight filled arrows), cells negative for NK-lysin and positive for CD2 (filled curved arrows). No cells were NK-lysin-positive and CD2-negative. (**e** and **f**) Cells positive for both NK-lysin and CD4 (straight filled arrows), cells positive for NK-lysin and negative for CD4 (open curved arrows), cells negative for NK-lysin and positive for CD4 (filled curved arrows). The monoclonal antibodies were the same as given in the legend to Figure 6. $N1^c$ did not work in these experiments.

(double underlining), is the start codon for the translation of pre-proNK-lysin. One explanation for the absence of an open reading frame starting with ATG in the clones is that a frame-shift could have occurred during our cDNA synthesis.

Figure 9 gives the amino acid sequence of NK-lysin together with the translation of pre-proNKL derived from the clones now isolated (Figure 3). Also included are the published putative amino acid sequences for NKG5 and 519. Furthermore, NKG5 is also identical to LAG-2, a patented but unpublished sequence.

Does the clone isolated correspond to the NKL peptide? We think this to be the case, because the internal residue differences between the clone and the peptide seen in Figure 9 are most likely allelic variations between slaughterhouse pigs (from a restricted area in southern Sweden) and one pig originating from a farm southwest of Stockholm which was used for the preparation of the bone marrow cDNA library. The second exon of the Class II antigen in the pig shows more than 20 allelic variations in 80 amino acid residues (L.Andersson, personal communication). Since both NKL and Class II genes may have been subject to similar selection pressures (the defense against bacteria) they can be expected to show similar numbers of alleles in the population. There is also a C-terminal difference between the clone and NK-lysin; the last five residues of the reading frame of the clone are absent in the isolated peptide. This difference can be explained by two hypothetical processing steps, the first involving a trypsin-like enzyme, the second the action of a putative carboxypeptidase B removing the remaining lysine residue.

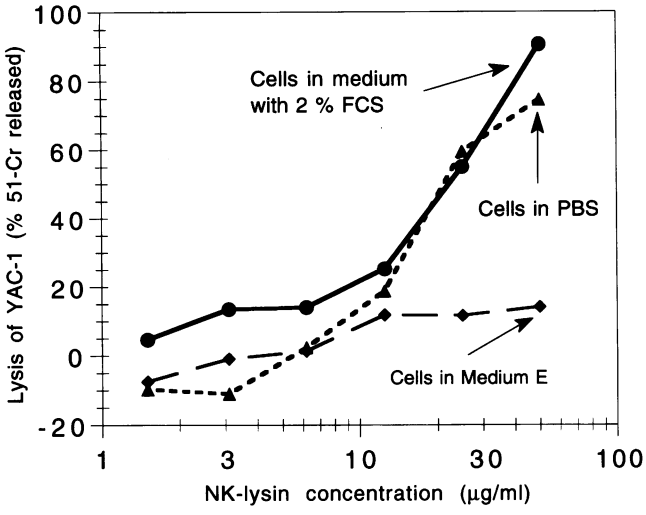

Fig. 8. Dose–response curves for NK-lysin-mediated lysis of mouse YAC-1 cells suspended in different media. Media with 2% FCS and PBS are physiological for animal cells, while medium E is formulated for bacteria. The media are described in Materials and methods.

Place of synthesis of NK-lysin

NK-lysin was isolated from porcine small intestine by large scale peptide fractionation, which is optimized for peptide hormones (Mutt, 1986). At the mRNA level, signals for NK-lysin transcript were detected in the different parts of the small intestine (duodenum, jejenum and ileum), but only after prolonged exposure times. However, a cell population enriched for T and NK cells gave a much more pronounced signal (Figure 4). Incubation of these cells with IL-2, a treatment known to preferentially proliferate and activate T and NK cells (Smith, 1993) gave an increase in cell number (Figure 6) and a clear increase in the cytolytic activity of these cells (Figure 5). Stimulation with IL-2 also gave an ~30-fold increase in NK-lysin-specific mRNA (Figure 4). This mRNA was also present in tissues known to contain significant amounts of lymphocytes, like bone marrow, colon and spleen.

Finally, immunostaining of our IL-2-treated T cell fraction (Figure 7) shows that NK-lysin was present in three cell types (often localized to granules), positive for CD8 (Figure 7a and b), for CD2 (Figure 7c and d) or for CD4 (Figure 7e and f). In the first group, ~50% of the $CD8^+$ cells were also NKL^+. In the second group, almost all cells positive for CD2 were also NKL^+. These results are in line with the interpretation that NK-lysin is produced in cytotoxic T cells (Figure 7a and b) and NK cells (Figure 7c and d) activated by IL-2. In the third group (Figure 7e and f), ~25% of the cells were NKL^+ and $CD4^+$. It is known that sub-fractions of mouse and human $CD4^+$ cells can be cytotoxic (Greenberg, 1991), but no NK cells are known to be $CD4^+$.

Antibacterial and antitumour activity of NK-lysin

On a molar basis, the antibacterial activity of NK-lysin is almost comparable with those of cecropin P1 and PR-39, peptide antibiotics isolated earlier from pig intestine (Lee

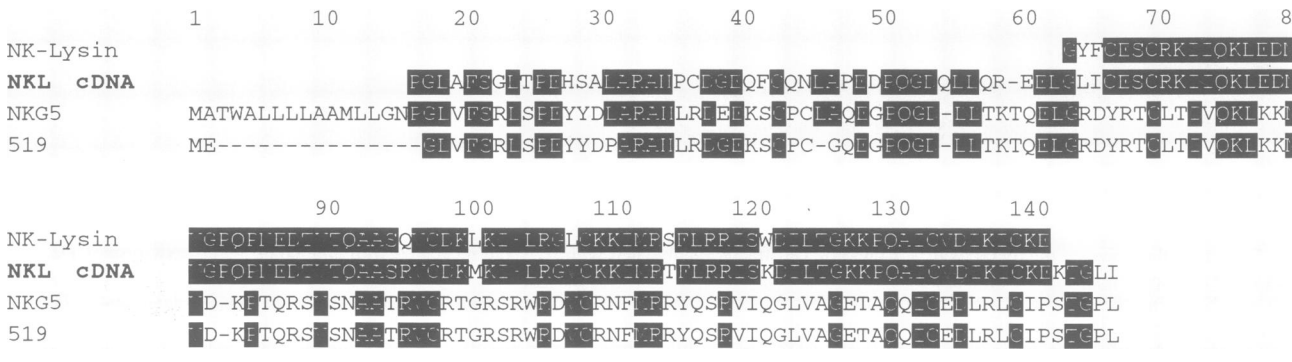

Fig. 9. Comparison of the amino acid sequence of pig NK-lysin with pre-proNK-lysin as obtained from the clone in Figure 3. Also included are the open reading frames for NKG5 and 519 respectively. The latter two clones are expressed in activated human NK and T cells. Their mature products have not been isolated. Residues identical to NKL cDNA are marked in Courier Black.

et al., 1989; Agerberth *et al.*, 1991), and as potent as tetracycline (Boman *et al.*, 1993). On the other hand, the size of the novel peptide is twice that of PR-39. The data available indicate that the spectrum is slightly narrower than those for the two other peptides.

The pig lymphocyte fraction isolated by Ficoll gradient and nylon filtering shows a clear cytolytic activity (maximum 35%) towards the mouse cell line YAC-1 (Figure 5). However, compared with porcine lymphocytes, purified NK-lysin is a much more potent lytic agent for YAC-1. A concentration of 50 μg NK-lysin/ml gave 90% lysis of YAC-1 cells in medium with 2% FCS (Figure 8). Almost the same lysis was obtained with YAC-1 in PBS, while cells in medium E (Vogel and Bonner, 1956) only showed ~14% lysis. Medium E was designed for bacteria and was found to increase the antibacterial activities of both NK-lysin (Table I) and FALL-39. Since medium E increased both activity and helix formation in FALL-39 (Agerberth *et al.*, 1995), our results in Table I and Figure 8 could imply that different conformations of NK-lysin are optimal for certain antibacterial and antitumor activities.

The mouse line YAC-1 was more sensitive to NK-lysin than the human K562 line (data not shown), but with a homologous peptide the activity might be more potent. The structural similarity between NK-lysin and the two putative human gene products, 519 (Jongstra *et al.*, 1987) and NKG5 (Yabe *et al.*, 1990), suggests that the unknown function(s) of these two pre-propeptides are related to those of NK-lysin. It has been suggested that 519 and NKG5 are alternative splicing products from the same human gene (Houchins *et al.*, 1993). It remains an open question whether NK-lysin is a pig homologue of the gene products of the NKG5 gene or if they are different members of a gene family present in both species.

Two main mechanisms have earlier been suggested for the cytotoxic action of T and NK cells on target cells: activation of apoptosis or killing by perforin-mediated lysis (Berke, 1994). Recent investigations of perforin-deficient mice demonstrated the importance of perforin for the activity of cytotoxic T lymphocytes and NK cells, but the data also point to the existence of alternative cytolytic mechanisms (Kägi *et al.*, 1994a,b; Lowin *et al.*, 1994). However, the fact that NK-lysin alone can lyse NK-sensitive tumour lines (Figure 8), together with the data for the place of synthesis (Figures 4 and 7), strongly suggest that this peptide is an additional component of the cytolytic machinery of T and NK cells. It is of importance that NK-lysin shows antibacterial activity, because NK cells have been shown to kill bacteria (Garcia-Penarrubia, 1992), a property that can be explained neither by perforin nor by apoptosis.

However, the effector molecules presently known do not fully explain all the effector functions of T and NK cells. NK-lysin does not kill *Salmonella* LT-2, while NK cells are known to kill *Salmonella* without direct cell–bacterial contact (Garcia-Penarrubia, 1992). Moreover, perforin *in vitro* is known to lyse erythrocytes (Henkart, 1985), while NK-lysin does not give any lysis of sheep red blood cells. Thus, compared with perforin, NK-lysin better meets the requirements for an effector molecule of T and NK cells. However, the total effector machinery—'one of the holy grails of immunology' (Clark, 1994)—could possibly include both perforin, NK-lysin and/or the peptide(s) made from the NKG5 gene and perhaps other effector molecules not yet identified. This can ultimately be settled only by isolation of all the respective effector molecules in two homologous systems—of mice and men.

Materials and methods

Materials

Carboxymethyl cellulose CM 23 (CM cellulose) was from Whatman (Kent, UK) and Sephadex G-25 fine from Pharmacia (Uppsala, Sweden). Trypsin, endoproteinase Lys-C, endoproteinase Asp-N and endoproteinase Glu-C (staphylococcal V8), all sequence grade, were from Boehringer Mannheim (Mannheim, Germany). Rabbit defensin NP-2 was kindly provided by R.I.Lehrer (UCLA, Los Angeles, CA).

Peptide purification

An aqueous solution of CTIP was fractionated with ethanol as described (Lee *et al.*, 1989). The precipitate obtained at −20°C was suspended in 22.5 mM sodium phosphate, pH 6.4, containing 0.5% thiodiglycol and dissolved peptides were adsorbed onto CM cellulose. After washing with the buffer (first without and then with 0.2 M NaCl), a peptide fraction was eluted with 0.2 M HCl, the eluate was adjusted with sodium acetate to pH 4 and peptides were precipitated by saturation with NaCl. The basic fraction from CM cellulose (12 g) was dissolved in 0.2 M acetic acid and fractionated on Sephadex G-25 (10×110 cm). Anti-*E.coli* activity was found in the later fractions, indicated by a bar in Figure 1A. These fractions were lyophilized and applied to a semi-preparative Vydac 218TP152022 column (22×250 mm; The Separation Group, Hesperia, CA). The sample was then eluted at a flow rate of 10 ml/min in a linear gradient of 20–50% acetonitrile with 0.1% trifluoroacetic acid over 30 min and then 50–70% over 5 min. Two peaks with antibacterial activity were separated. Fractions from the second peak were lyophilized and applied to a Vydac 218TP54 column (4.6×250 mm) eluted at 1 ml/min with a linear gradient of 32–48% acetonitrile with 0.1% heptafluorobutyric acid over 5 min and then 48–64% over 30 min. Purity of the isolated peptides was ascertained by capillary zone electrophoresis with a Beckman P/ACE system 2000, using capillaries of fused silica (inner diameter 75 μm, total length 57 cm) and 50 mM phosphate, pH 2.5.

Structural analysis

Total compositions were determined with a Pharmacia-LKB Alpha Plus 4151 amino acid analyser, after hydrolysis for 22 h at 110°C in evacuated tubes with 6 M HCl–0.5% phenol (w/v). Peptides were reduced, carboxymethylated (Agerberth *et al.*, 1989) and desalted on Sep-Pak C18 (Waters, Milford, MA). Separate sets of carboxymethylated peptides were cleaved with endoproteinases Asp-N (in 10% acetonitrile, 1% NH_4HCO_3 for 24 h at 37°C) and Lys-C (in 1% NH_4HCO_3 for 8 h at 37°C) at enzyme:peptide ratios of 1:20 (w/w) or chemically with CNBr in 70% formic acid for 20 h. For determination of the disulfide bridge pattern, 18 nmol intact NK-lysin was digested with trypsin [in 0.1% NH_4HCO_3 for 6 h at 37°C, enzyme:peptide ratio 1:15 (w/w)]. After purification by reverse-phase HPLC, one fragment was incubated with endoproteinase Glu-C [in 0.5% NH_4HCO_3 for 16 h at 37°C, enzyme:peptide ratio 1:2 (w/w)]. All fragments were separated by reverse-phase HPLC on a Vydac 218TP54 column (4.6×250mm). N-Terminal amino acid sequences were analysed with an Applied Biosystems 477A instrument coupled to a 120A analyser or with an Applied Biosystems 470 instrument using a separate HPLC for phenylthiohydantoin detection.

Assay of antibacterial activity

Thin plates (1 mm thick) were poured with LB broth, 1% agarose and ~6×10^4 cells/ml test bacteria, with and without medium E (Vogel and Bonner, 1956). Small wells (diameter 3 mm) were punched in the plates and 3 μl test samples were applied in the wells. After overnight incubation at 30°C, the diameters of inhibition zones were recorded with a magnification lens with an internal millimetre scale. During purification, the activity was monitored as inhibition zones on *E.coli* D21 and units were read from a standard plot of zone diameter against the log of the amount of cecropin A. One unit was equal to 1 ng cecropin A. For purified peptides, lethal concentrations (LC values) were calculated from the zones of a dilution series of the peptide (Hultmark *et al.*, 1983).

Assay of antitumour activity

An NK-sensitive mouse tumour cell line, YAC-1, was used in most experiments. The cells were maintained suspended in RPMI 1640 culture medium supplemented with 5% heat-inactivated fetal calf serum, 200 IU/ml penicillin and 100 μg/ml streptomycin (GIBCO, UK). They were subcultured twice weekly and routinely checked for lack of mycoplasma infection. The cells (in total 2×10^6) were labelled with [^{51}Cr]Na_2CrO_4 (Amersham International, Amersham, Bucks, UK) at a 1.0 mCi/ml concentration in 0.1 ml for 45 min, washed, counted and adjusted to 2×10^5 cells/ml. Fifty microlitres containing 10^4 target cells, suspended as indicated, were incubated for 2 h at 37°C with purified NK-lysin in a 96-well V-bottom microtitre plate. Total label was determined by resuspension of the cell lines in 1% Nonidet P-40. Triplicate determinations were made and the per cent lysis was calculated. Standard error of the means from triplicate c.p.m. measurements did not exceed 5%.

RNA preparation and analysis

Samples of different tissues from wild and domesticated pigs were collected within 1 h of death of the animals and frozen in liquid nitrogen. Later the frozen tissues were ground to a fine powder and total RNA isolated using an RNA separator kit (Clontech). RNA was separated by electrophoresis in denaturating formaldehyde gel and hybridizations were carried out under high stringency conditions (Sambrook *et al.*, 1989).

PCR and cDNA cloning

Total RNA from bone marrow of a domesticated pig was used for 3′-RACE-PCR (Frohman *et al.*, 1988). The primer used for the reverse transcription in the 3′-RACE was 5′-TCGAATTCCTCGAGAAGC$(T)_{18}$. The primers used for amplification were 5′-TCGAATTCCTCGAGAAGC and 5′-GA(GA)GA(TC)ATGGTNGGNCCNCA-3′ (the latter corresponding to bp 402–421). These primers (at 0.4 μM) and template DNA (6 ng/μl) were used in a PCR experiment with the following thermal cycle profile: 95°C 3 min, 40 cycles of 95°C 1 min, 55°C 1 min, 72°C 1 min and an extension step of 72°C 7 min. In the following step a nested primer [5′-AT(CT)TT(TAG)AT(AG)TCNAC(AG)CA(ATG)AT-3′] directed to the C-terminal part of NK-lysin (corresponding to bp 564–583) was used in a second round of amplification, together with the primer corresponding to bp 402–421. This round of PCR cycles gave a single band when screened with the probe corresponding to bp 531–550 [5′-TTNCCNGTNA(AG)(GAT)AT(AG)TCCCA-3′]. Sequencing of this product, corresponding to bp 402–583 in Figure 3, confirmed that it was derived from an NK-lysin transcript. This PCR product was then used as a probe for screening a cDNA library from porcine bone marrow made in λZAP II (Stratagene). Positive clones were purified and plasmids rescued by *in vivo* excision (Stratagene). The subsequent sequencing of the clones showed the probe to contain 16 base differences, 10 of these in the primer regions (cf. sequences given above and in Figure 3). cDNA inserts of the clones were sequenced on an ALF (Pharmacia, Sweden), an Applied Biosystems sequenator and by the dideoxy chain termination method with a Sequenase kit (US Biochemicals). The screening hybridization was done in 6× SSC, 5× Denhardt's, 1% SDS and 100 μg/ml denatured herring sperm DNA at 55°C overnight. Final washing was in 2× SSC and 0.1% SDS at 55°C.

Preparation of antibodies against NK-lysin

Antiserum against NK-lysin was obtained by a standard immunization scheme. Doses of 100 μg peptide, mixed with Freunds' complete adjuvant, were injected intramuscularly into a rabbit. The first injection was at time zero, the second after 3 weeks and the third after an additional 10 days. The antiserum collected was assayed by ELISA, then used for preparation of an IgG fraction by purification on a protein G column (Pharmacia). A similarly purified IgG fraction from the preserum of the rabbit was used as a negative control in all experiments. The anti-NK-lysin IgG fraction did not cross-react with anything in human lymphocytes and it did not bind to the human T cell line Molt-16.

Characterization of lymphocytes

Whole blood from a pig, collected in heparin-containing bottles, was centrifuged and the lymphocyte population was further enriched for T and NK cells using a Ficoll gradient followed by passage through nylon wool. The collected cells were divided into two cultures, one kept as a control, the other stimulated with human IL-2 (50 U/ml) for 6 days. The cells in the two cultures were characterized by flow cytometry using a Becton and Dickinson FACscan. The R1 population (see Figure 6) was characterized with monoclonal antibodies (mAbs) against CD2, CD4, CD8 and N1^c (VMDR Inc., Pullman, WA). Similar cells were used for the results given in Figures 4–7.

For immunostaining, a fraction enriched in T and NK cells and stimulated with IL-2 was collected onto glass microscopic slides pretreated with chrome alum gelatin using a cytospin centrifuge. The cells were immediately immersed for 90 min at 4°C in a solution consisting of 4% paraformaldehyde in 0.16 M phosphate buffer (pH 6.9) containing 0.4% picric acid and rinsed for 2 h in 10% sucrose containing 0.02% sodium azide (Sigma, St Louis, MO) and 0.01% bacitracin (Bayer AG). After a brief rinse in 0.01 M PBS, pH 7.2, sections were incubated at 4°C for 18–24 h with IgG from a polyclonal antiserum raised against NK-lysin and mAbs against the surface markers CD2, CD4 and CD8 (VMDR Inc.). Cells were rinsed in PBS for 20 min and incubated at 37°C for 30 min with either fluorescein isothiocyanate-conjugated secondary goat anti-rabbit antibodies (1:80; Boehringer, Mannheim, Germany) or donkey anti-mouse indocarbocyanine (CY3)-conjugated antibodies (1:400; Jackson Immunoresearch Laboratories Inc., West Grove, PA). Afterwards the slides were rinsed in PBS, mounted in PBS–glycerol (3:1) containing 0.1% *p*-phenylenediamine and analysed in a Nikon Microphot-FX microscope equipped with filter combinations for epifluorescence. As a control, the NKL antiserum was pre-treated with an excess of the peptide (1 μM). No immunofluorescence was then observed.

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