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The Antimicrobial Peptide NK-2, the Core Region of Mammalian NK-Lysin, Kills Intraerythrocytic *Plasmodium falciparum*▽

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In a time of dramatically increasing resistance of microbes to all kinds of antibiotics, natural antimicrobial peptides and synthetic analogs thereof have emerged as compounds with potentially significant therapeutical applications against human pathogens. Only very few of these peptide antibiotics have been tested against protozoan pathogens that are a major cause of morbidity and mortality in large parts of the world. Here, we studied the effect of NK-2, a peptide representing the cationic core region of the lymphocytic effector protein NK-lysin, on the malaria parasite *Plasmodium falciparum*. Whereas noninfected red blood cells were hardly affected, human erythrocytes infected with the parasite were rapidly permeabilized by NK-2 in the micromolar range. Loss of plasma membrane asymmetry and concomitant exposure of phosphatidylserine upon infection appears to be the molecular basis for the observed target preference of NK-2, as can be demonstrated by annexin V binding. The peptide also affects the viability of the intracellular parasite, as evidenced by the drop in DNA content of cultured parasites. Accumulated evidence derived from permeabilization assays using parasites and liposomes as targets and from fluorescence microscopy of infected erythrocytes treated with fluorescently labeled NK-2 indicates that the positively charged peptide electrostatically interacts with the altered and negatively charged plasma membrane of the infected host cell and traverses this membrane as well as the parasitophorous vacuole membrane to reach its final target, the intracellular parasite. The apparent affinity for foreign membranes that resulted in the death of a eukaryotic parasite residing in human host cells makes NK-2 a promising template for novel anti-infectives.

The vast majority of antimicrobial agents have been derived from natural products. In a time of emerging resistance against classical antibiotics, peptides from natural sources have attracted increasing attention. These natural peptides are widespread in nature and are an essential part of the internal defenses of animals and plants against potential pathogens (20, 44). Albeit their existence on earth dates back millions of years, these weapons are still effective, pointing to the fact that their mode of action, i.e., the permeabilization of the microbial membrane (44), makes the emergence of complete microbial resistance highly unlikely. Currently more than 700 antimicrobial peptides have been identified from natural sources (http://aps.unmc.edu/AP/main.php), and a plethora of synthetic analogs with improved activity and selectivity have been constructed (1, 4, 7, 9, 13, 18, 31, 32, 39, 45). Moreover, cell-penetrating peptides have become interesting, as they are capable of delivering a broad range of compounds into a target cell (16, 23). The recently reported idea that antimicrobial peptides, fusogenic peptides, and cell-penetrating peptides may better be unified as "membrane active peptides" makes the enormous variety of presumably interesting templates for potentially biomedically valuable peptides even clearer (21). However, only a very limited number of antimicrobial peptides are known to be effective against eukaryotic pathogens (5, 19, 42, 44).

Protozoan parasites and the devastating diseases they cause are a major burden of the tropics, and in particular, *Plasmodium falciparum*, the causative agent of falciparum malaria, creates an ever-burgeoning public health problem in many areas of the world. Successful vaccines against malaria are not applicable to patients, and the available drugs are becoming more and more inefficient. Among the very few antimicrobial peptides tested against parasites (see references 19 and 42 and references therein) are the dermaseptins from tropical frogs that were previously used as templates to develop promising antiplasmodial agents (10, 17, 25).

In the present study, we have investigated the antiplasmodial activity of a synthetic shortened analog of the mammalian defense protein NK-lysin, a 78-residue effector molecule originally identified in porcine cytotoxic lymphocytes (2). The peptide termed NK-2 represents the positively charged core region (K39–K65) of NK-lysin and was previously found to display activity against gram-positive and gram-negative bacteria and against the fungal pathogen *Candida albicans*, but was found to be virtually nontoxic to human cells (3, 4). Moreover, we demonstrated that NK-2 can kill the intracellular parasite *Trypanosoma cruzi*, the causative agent of Chagas' disease, leaving the host cell unharmed (24). Here, we demonstrate that NK-2 is

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potently active against intraerythrocytic plasmodia and preferentially permeabilizes infected erythrocytes at micromolar concentrations. When we employed annexin V as a phosphatidylserine-specific agent and lipid vesicles of defined composition, it became apparent that the changes of the phospholipid composition of erythrocytes induced by the parasite are the molecular basis for the target cell selectivity of NK-2. Furthermore, we used a lissamine derivative of NK-2 to demonstrate its cellular distribution in infected red blood cells (iRBC).

MATERIALS AND METHODS

Peptides. The 27-residue peptide NK-2 was synthesized on commission (Biosyntan GmbH, Berlin, Germany) by solid-phase techniques utilizing an F-moc-based protecting group strategy. The sequence was KILRGVCKKIMRTFLRR ISKDILTGKK, comprising an amidated C terminus. The peptide was obtained in a purity grade of more than 95%, and aliquots of 1 mg were dissolved in 0.01% trifluoroacetic acid. Additionally, NK-2 was obtained as a fluorescent derivative (called LNK-2) by N-terminal labeling with the fluorophore lissamine (Biosyntan GmbH, Berlin, Germany). Melittin, of a high-performance liquid chromatography grade, was purchased as a synthetic peptide (Sigma-Aldrich).

Culture of parasites. *P. falciparum* 3D7 cells were maintained in continuous culture according to the method of Trager and Jensen (40). Parasites were cultured in human RBC (blood group A^{Rh+}) in RPMI 1640 medium supplemented with 25 mM HEPES (Life Technologies, Inc.) and with 20 mM sodium bicarbonate, 5% heat-inactivated human plasma (blood group A), and 0.25% AlbuMAX I (Life Technologies, Inc.) at 2.5% (vol/vol) hematocrit. Cultures were maintained at 37°C with a gaseous phase of 90% N_2, 5% O_2, and 5% CO_2.

Assay for antiplasmodial activity. Unsynchronized parasites were incubated under normal culture conditions at 5% hematocrit and 1% parasitemia in the presence of increasing concentrations of NK-2 (V_{EXP}) or in culture medium alone (V_T). Cells were harvested 45 h later, and the content of parasite DNA as a measure of viability was determined by using the fluorescent dye Hoechst 33258 (Molecular Probes) (36). Fluorescence was monitored in a fluorospectrometer (LS50B; PerkinElmer) at emission and excitation wavelengths of 365 and 460 nm, respectively. Samples from parasites harvested before the incubation period were used (V_0) to determine the initial DNA content. Percent viability was calculated according to the following formula: $(V_{EXP} - V_0)/(V_T - V_0) \times 100$. The viability of parasites was determined in triplicate (median and range). The peptide concentration at which the viability is diminished to 50% was calculated by nonlinear regression using Prism software (GraphPad Software, Inc., San Diego, CA).

Assay for hemolytic activity. RBC infected with mature parasites were enriched by a cushion of 80% Percoll (Amersham Bioscience) in phosphate-buffered saline (PBS) containing 3% (wt/vol) L-alanine (Fluka) (12). The resulting parasitemia was about 95%, as determined by Giemsa-stained smears. Noninfected RBC and iRBC were washed separately in PBS, resuspended in 100 μl PBS in 96-well microtiter plates, and exposed to increasing concentrations of NK-2 at 2.5% hematocrit and at 37°C for 2 h. After the incubation period, cells were pelleted by centrifugation at 600 × *g* and 20°C for 1 min. Supernatants were removed and diluted 1:10 in PBS, and the release of hemoglobin from RBC was measured photometrically at 405 nm (experimental lysis [L_{EXP}]). As controls, the same numbers of cells were incubated in buffer alone (spontaneous lysis [L_0]) or lysed in distilled water to obtain complete hemolysis (L_T). Percent hemolysis was calculated by the following formula: $(L_{EXP} - L_0)/(L_T - L_0) \times 100$. Hemolytic activity was determined in triplicate (median and range). In parallel, NK-2-treated and untreated parasites were visualized by Giemsa staining of iRBC smears and viewed by light microscopy.

Time dependence of NK-2 activity. For monitoring the kinetics of hemolytic activity of NK-2 toward parasitized RBC, Percoll-enriched parasites (~90% parasitemia, 5% hematocrit) were incubated in the presence of 10 μM NK-2 in PBS at 37°C (900 μl final volume). Samples (50 μl) were taken at different time intervals and diluted in 800 μl PBS, cells were sedimented, and hemolysis was monitored as described above. To estimate spontaneous lysis during the time of incubation, samples of parasitized RBC incubated in the absence of peptide were taken at the same time intervals. Total lysis (100%) was reached by adding distilled water to the cells instead of PBS, and the corresponding maximum absorbance was measured after the end of the experimental period.

In an additional experiment, the time-dependent permeabilization of the plasmodial plasma membrane was monitored using Sytox Green (Invitrogen, Molecular Probes). We adapted a procedure described previously for other target cells (22, 29, 38) to in vitro-cultured *P. falciparum*. Briefly, a plasmodial culture (~5% parasitemia, mixed stages) was washed in PBS supplemented with 10 mM glucose (PBS-G). The hematocrit was adjusted to 5% in PBS-G. Sytox Green was added at a final concentration of 2 μM, and the cells were incubated in the dark for 30 min at 37°C. After that period, 50 μl of the cell suspension was added to the same volume of either 10 μM NK-2, 20 μM NK-2, or 0.1% Triton X-100 each in PBS-G or PBS-G alone using flat-bottomed black microtest plates (Costar; Corning, NY). Fluorescence was measured at different time points after the addition of cells with a TECAN SpectraFluor fluorescence spectrometer at excitation wavelength of 485 ± 10 nm (bandwidth) and an emission wavelength of 530 ± 20 nm. Fluorescence values were corrected by the negative control (PBS-G), and the percentage of permeabilized plasmodia was calculated using the positive control (0.05% Triton X-100 in PBS-G) as the 100% value.

Annexin V binding to *P. falciparum*-iRBC. iRBC from unsynchronized cultures were subjected to either 65% or 75% Percoll for the enrichment of RBC that primarily contained schizonts or trophozoites, respectively. After centrifugation, preparations were controlled by Giemsa-stained smears routinely yielding a purity of the respective stage of more than 70% parasitemia. After purification, iRBC were washed several times with Ringer solution and 5×10^5 cells in 100 μl of the same solution were incubated with 1 μg fluorescein isothiocyanate (FITC)-labeled annexin V (Roche Diagnostics, Mannheim, Germany) on ice for 15 min. Cells were subsequently washed with ice-cold PBS and fixed with 1% paraformaldehyde in PBS on ice for 20 min. Fixed cells were washed with PBS containing 1% bovine serum albumin and analyzed by flow cytometry on a FACScan cytometer using the CellQuest software (Becton-Dickinson, Heidelberg, Germany).

Hemolytic activity of NK-2 in the presence of annexin V. iRBC enriched to a parasitemia of 97% were washed in Tris-buffered saline supplemented with 10 mM glucose and 2.5 mM $CaCl_2$ (TBS-GC). The parasites were incubated under culture conditions in the presence or absence of 10 μg/ml annexin V in the same buffer for 1.5 h. After this period, the cells were added at 1% hematocrit to serial dilutions of NK-2 in 100 μl of TBS-GC in the presence or absence of recombinant chicken annexin V (10 μg/ml) (annexin V was a kind gift of Martin Herrmann, Erlangen, Germany) (8). The suspensions were incubated under culture conditions for 1 h and centrifuged, and the supernatants were diluted 1:5 with TBS-GC before the release of hemoglobin was measured as described above.

In a similar experiment, 100 μg/ml annexin V was used for all concentrations of NK-2 and for controls either in TBS-GC or in TBS supplemented with 10 mM glucose and 2.5 mM Na_2-EDTA (TBS-GE). Cells were incubated in 100 μg/ml annexin V in TBS-GC or in TBS-GE for the measurement of spontaneous hemolysis or lysed in distilled water to reach maximum lysis. Each experiment was performed in triplicate (median and range). Results were statistically analyzed by SigmaPlot 10.0 (Systat Software, Inc., San Jose, CA).

Liposome permeabilization assay. Pure synthetic phospholipids, i.e., 1-palmitoyl-2-oleoyl-/sn/-glycero-3-phosphocholine and 1,2-dipalmitoyl-/sn/-glycero-3-[phospho-L-serine] (sodium salt) (both from Sigma) or mixtures thereof (total, 2.5 mg) were dissolved in chloroform-methanol (2:1, by volume). The solvent was removed by a constant stream of nitrogen, and the resulting lipid film was dried under vacuum overnight. Subsequently, the lipid film was hydrated in buffer (10 mM HEPES, 1 mM EDTA, pH 7.4) containing 30 mM calcein (Molecular Probes, Leiden, The Netherlands). The suspension was applied to four freeze-and-thaw cycles using liquid nitrogen and a water bath of 37°C and passed 17 times through a 100-nm-pore-size membrane by using a LiposoFast extruder (Avestin, Inc., Ottawa, Canada). After extrusion, the liposomes were passed through a PD-10 column (Amersham Bioscience, Uppsala, Sweden) to remove untrapped calcein. Elution was performed with 10 mM HEPES, 150 mM NaCl, 1 mM EDTA, pH 7.4. Lipid concentration was determined by using a phosphorous assay (6). Liposomes (2.5 μM of phospholipids) were treated with various amounts of peptide at 20°C, and peptide-induced lysis was monitored fluorometrically by the detection of the release of fluorescent dye at excitation and emission wavelengths of 490 and 520 nm, respectively. After signals had been recorded for 10 min (F_{EXP}), complete dye release was achieved by the treatment of liposomes with 0.1% Triton X-100 (F_T). Percent lysis was calculated with the following formula: $(F_{EXP} - F_0)/(F_T - F_0) \times 100$. F_0 is the fluorescence level before the addition of peptide. The spontaneous dye release from liposomes was always less than 2% throughout the experiment.

Fluorescence microscopical imaging. Fluorescence microscopy and confocal laser scanning microscopy were performed using a Zeiss Axio Imager Z1 apparatus and a Zeiss Axiovert 100 M, respectively. In brief, a plasmodial culture was incubated in the presence of 5 μM LNK-2 and 5 μg/ml Hoechst 33258 in complete medium at 25°C for 2 h. After the incubation period, the cells were washed twice in PBS to reduce background fluorescence. The cells were smeared

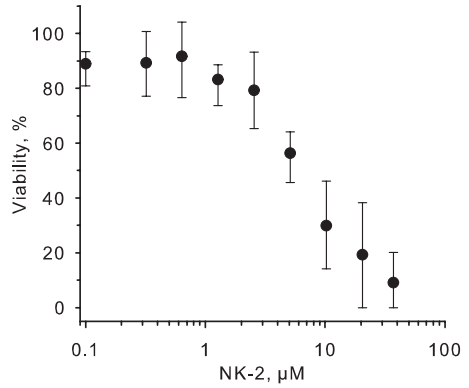

FIG. 1. Dose-dependent effect of NK-2 on the viability of *P. falciparum*. Parasites were incubated for 45 h in the presence of the indicated concentrations of NK-2 peptide, and samples were assayed for DNA content. Bars mark the range around the median (circles) for the experiment performed in triplicate.

on a slide, and fluorescence was visualized by using the filter settings for rhodamine and DAPI (4′,6′-diamidino-2-phenylindole).

RESULTS

The antiplasmodial activity of the synthetic peptide NK-2 was determined by using the increase of parasite DNA after one developmental cycle as a measure of viability. The peptide was found to be active against asexual stages of *P. falciparum* and exerted a dose-dependent effect on the growth of the parasite. At 6.2 μM of NK-2, viability was found to be decreased by 50% (Fig. 1). As normal culture conditions were used during the incubation period, the antiplasmodial activity of NK-2 is not inhibited by heat-inactivated human serum.

To compare the effect of NK-2 peptide on normal RBC and plasmodium-iRBC, NK-2 was incubated in increasing concentrations in two different sets, with either highly enriched infected (95% parasitemia) or noninfected erythrocytes (Fig. 2). Hemolysis was determined spectrometrically by measuring the absorbance maximum specific for hemoglobin in the supernatants. A very strong hemolytic activity was found toward host cells that are infected with *P. falciparum* at trophozoite and schizont stages. In contrast, noninfected RBC were found to resist the cytolytic activity of NK-2 in the micromolar range. The data suggest that NK-2 is selective against parasitized RBC inducing lysis of infected cells in concentrations that have no substantial effect on noninfected erythrocytes after 2 h of incubation. The data are in good agreement with the results obtained by measuring the viability of parasites. Notably, lissamine-conjugated NK-2, which was also used in this study as a fluorescently labeled derivative (LNK-2), was found to possess hemolytic activity to the extent of NK-2.

To directly examine the effect of the peptide on the intracellular parasite, iRBC were incubated with 10 μM NK-2 for 1 h, stained with Giemsa, and analyzed by light microscopy (Fig. 2). Relative to the case for parasite-iRBCs that were incubated under the same conditions as those described above but without NK-2, it became apparent that the plasmodia inside the host cell had been damaged by the peptide, as only hemozoin crystals were visible as residuals of the parasites. Apparently, none of the two membranes surrounding the parasite, i.e., the host cell plasma membrane and the parasitophorous vacuolar membrane, constitutes a barrier for the peptide, assuming that the plasmodial plasma membrane is the final target.

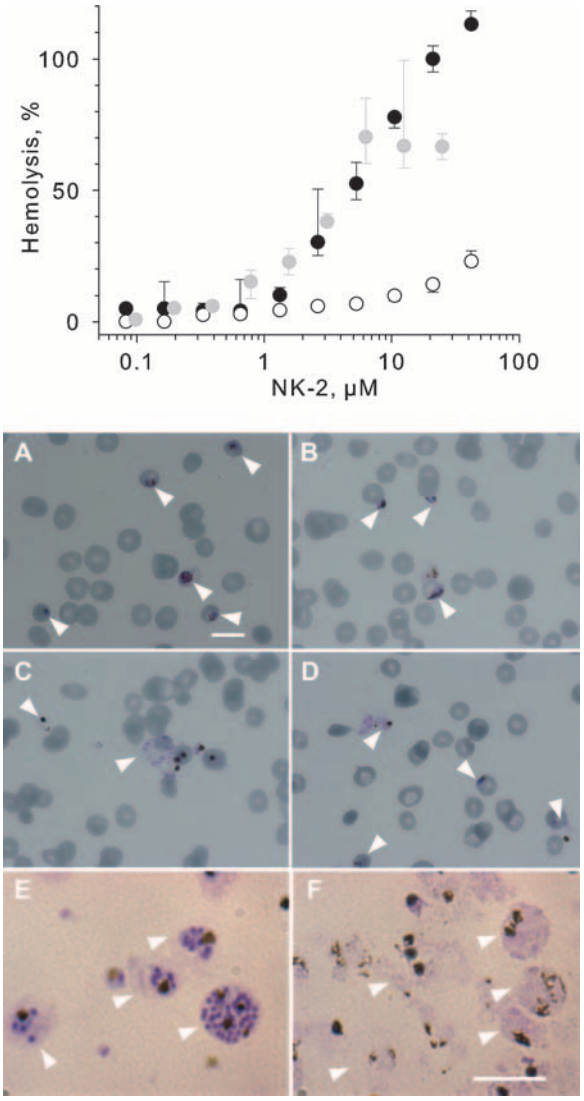

FIG. 2. Lytic activity of NK-2 on infected erythrocytes. In the diagram, the hemolytic activities of NK-2 and LNK-2 are shown. Enriched parasites were incubated with increasing concentrations of NK-2 (filled circles) or LNK-2 (gray circles) at 37°C for 2 h. For a control, the experiment was performed using NK-2 and noninfected erythrocytes (empty circles). The absorbance of the supernatant was measured spectrometrically at 405 nm to monitor the release of hemoglobin. Complete lysis of the cells was obtained by adding distilled water instead of the NK-2 solution. Error bars mark the range around the median for the experiment, which was performed in triplicate. In the lower microscopic images, the effect of NK-2 on intraerythrocytic parasites is demonstrated. Infected erythrocytes were incubated without NK-2 (A) or in the presence of 5 μM (B), 10 μM (C), or 20 μM (D) NK-2 for 1 h, smeared on slides, Giemsa stained, and monitored by light microscopy. (E and F) The experiment was repeated using Percoll-enriched, late-stage parasites in the presence of 10 μM NK-2. Arrowheads indicate the positions of infected erythrocytes or NK-2-compromised plasmodia. Bars, 10 μM.

To assay the time dependence of hemolytic activity of NK-2,

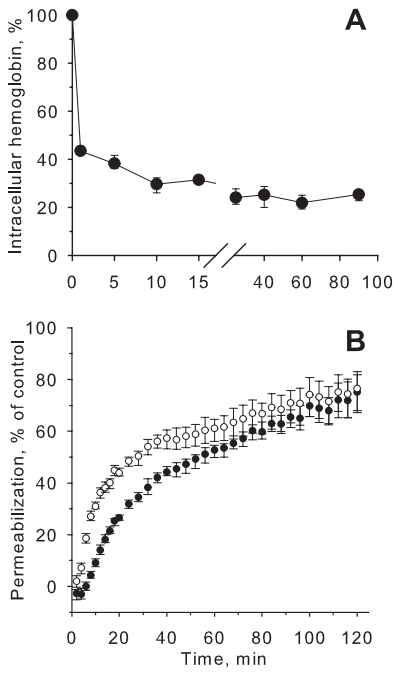

FIG. 3. Kinetics of antiplasmodial activity of NK-2. (A) Time dependence of NK-2 action on infected erythrocytes. Membrane permeabilization activity is expressed as a percentage of intracellular hemoglobin of iRBC treated with NK-2 relative to plasmodia unexposed to the peptide over time. At the same time, an untreated control was used to correct for spontaneous lysis. Error bars show the maximum and minimum of the experimental triplicates. (B) Time dependence of Sytox Green dye uptake by plasmodia as a measure of membrane permeabilization in the presence of 5 μM NK-2 (filled circles) and 10 μM NK-2 (white circles). The percentages of eight parallel measurements were plotted over time and represented as medians and ranges.

enriched parasites were incubated in the presence of 10 μM NK-2 in PBS for various time intervals (Fig. 3). At defined time points, cells were sedimented and the release of hemoglobin was measured. It became apparent that NK-2 exerted activity already after a few minutes of incubation, suggesting that hemolysis occurs very rapidly toward infected erythrocytes. Complete lysis was not reached after a 90-min incubation period, presumably because of residual noninfected erythrocytes in the cell preparation. For control, a different set of iRBC was incubated without NK-2 to monitor spontaneous lysis at the same time points. No hemolysis was detected throughout the experimental period. As a consequence, the time course of the transfer of NK-2 to and effect on intracellular parasites was monitored. We introduced the cell membrane-impermeant Sytox Green fluorescent dye in the experiment to investigate the parasite's membrane integrity over time. As a result, we found that NK-2 does compromise plasmodial membranes within minutes after contact. As shown in Fig. 3B, 10 μM NK-2 accelerated the kinetics compared to the result with 5 μM NK-2. Note that at higher concentrations of NK-2 (>20 μM), the fluorescence signals were found to be quenched, presumably due to the binding of NK-2 to DNA and the competition between NK-2 and the dye.

To evaluate the differences in the plasma membrane composition of infected and noninfected erythrocytes and, in particular, the differences in phosphatidylserine (PS) exposure, FITC-labeled annexin V binding to externalized PS on RBC was performed. Annexin V is known to bind specifically to the polar head group of PS in the presence of micromolar concentrations of calcium (37). By using flow cytometry, we found that FITC-labeled annexin V-binding to erythrocytes correlates with the parasitemia of cultures used (Fig. 4). Erythrocytes that were not infected by *P. falciparum* show very low annexin V-binding in the histogram (0.2% of cells) (Fig. 4A). A parasitemia of less than 10% in culture increased the FITC-annexin signal substantially, and 13% of cells became positive (Fig. 4B). In samples using trophozoite-infected erythrocytes (more than 70% parasitemia), the percentage of FITC-annexin-positive cells was raised to 45%. Using schizont-infected erythrocytes at the same parasitemia (more than 70%), a high FITC-annexin V signal intensity was found, representing 71% of cells in the histogram and indicating a signal shift in flow cytometry, depending on the parasitemia and maturity of the parasites (Fig. 4C and D, respectively). These results suggest that elevated exposure of PS on the outer surface of the RBC membrane occurs in infected erythrocytes and that the rate of PS exposure increases during the maturation of parasites.

In a corresponding experiment, whether annexin V is capa-

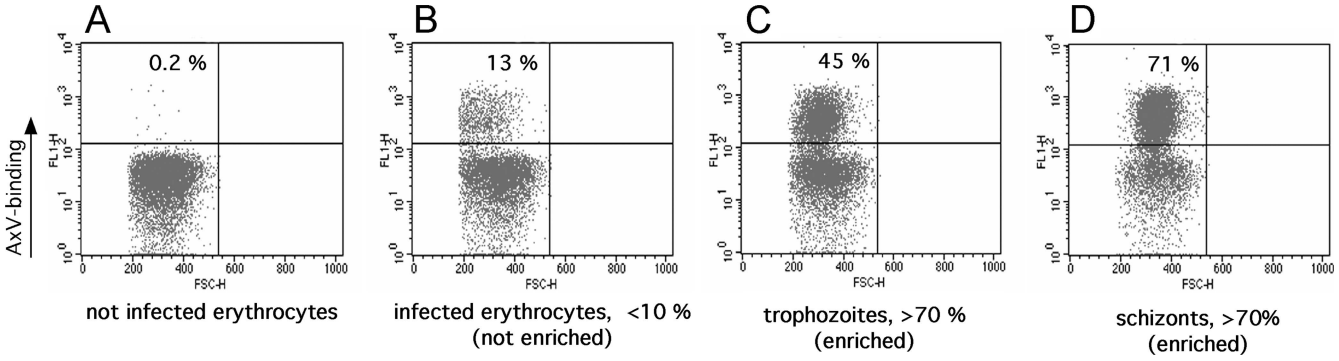

FIG. 4. FITC-labeled annexin V binding of *P. falciparum*-infected erythrocytes. Noninfected erythrocytes (A), erythrocytes infected at low parasitemia (B), and erythrocytes infected at high parasitemia (C and D) were incubated with FITC-labeled annexin V (AxV) and subjected to flow cytometric analysis. (B) Signals of a culture with less than 10% parasitemia. (C and D) iRBC were enriched by Percoll to a parasitemia of greater than 70%, either infected with trophozoites (C) or infected with schizonts (D). For clarity, the percentages of signals for AxV-binding found in each upper left panel are indicated. The forward scatter (FSC-H) is an indication of the size of the cells.

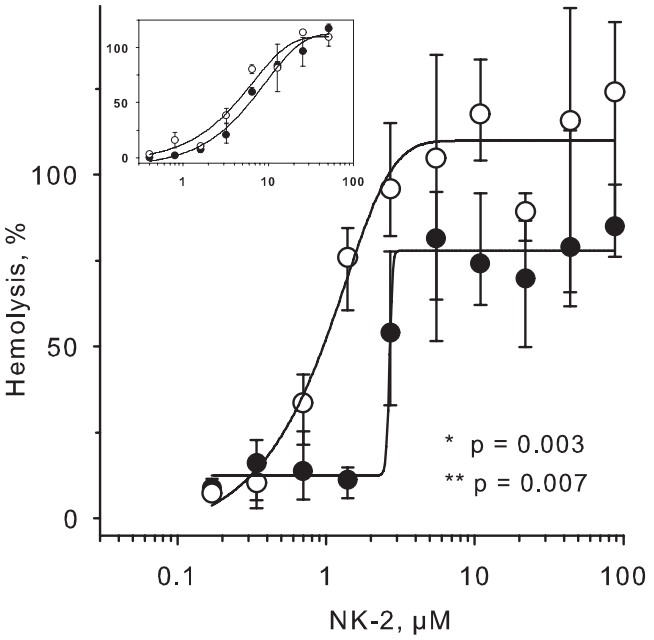

FIG. 5. Hemolysis of infected erythrocytes by NK-2 in dependence of annexin V and calcium. The effect of annexin V on the hemolytic activity of NK-2 against iRBC was tested in two different experiments: 100 μg/ml annexin V was used in the presence (filled circles) or absence (empty circles) of calcium at various concentrations of NK-2. (Inset) A total of 10 μg/ml annexin V was applied (filled circles) or omitted (empty circles) in the presence of various concentrations of NK-2 and in the presence of calcium. The experiments were performed in triplicate, and the medians and ranges are indicated. The asterisks indicate the *P* values of the *t* test of both of the experiments performed with 100 μg/ml annexin V (*) and 10 μg/ml annexin V (**).

ble of diminishing the NK-2-induced lysis of iRBC was investigated. Either 10 μg/ml annexin V (Fig. 5, inset) or 100 μg/ml annexin V was applied to enriched iRBC prior to the addition of NK-2. It was found that annexin V substantially reduces the dose-dependent cell lysis induced by NK-2, particularly at an elevated annexin V concentration of 100 μg/ml in the presence of calcium. A complete blockage of hemolysis was not observed; however, in the presence of high concentrations of annexin, the effect was much more pronounced (Fig. 5).

To prove the effect of NK-2 in dependence of PS, a liposome permeabilization assay was performed. Calcein-entrapped liposomes of various lipid compositions were used (Fig. 6). For a control, melittin was used to gain peptide-induced lysis without a phospholipid preference (11). No lytic activity of NK-2 was found by using liposomes that were prepared with phosphatidylcholine (PC) only, even at high concentrations of peptide (Fig. 6A). By contrast, NK-2 exerts lytic activity depending on the amount of PS in the liposomal membrane (Fig. 6B and C). At a molar ratio of PC and PS of 90:10, NK-2 displays a liposome lysis activity that equals the activity measured for melittin (Fig. 6C).

Notably, the fluorescently labeled lissamine derivative of NK-2, named LNK-2, was found to label the parasite within its host cell (Fig. 7) but did not decorate noninfected erythrocytes. This observation supports our finding that NK-2 specifically permeates infected erythrocytes and attacks the parasite within its host cell.

DISCUSSION

In the present study, an antiplasmodial activity of NK-2, a peptide representing the cationic core structure of porcine NK-lysin, was found and the molecular basis of the effect was investigated. It has previously been shown (3) that NK-2 has a cytotoxic activity against gram-positive and gram-negative bacteria and the yeast *C. albicans*, but was found to have less of an effect on the human keratinocyte cell line HaCaT. Interestingly, NK-2 showed low hemolytic activity toward human RBC.

An important property of antimicrobial peptides is the specificity for bacterial targets. Such a selectivity usually would exclude the killing of most eukaryotic cells and, in particular, the killing of intracellular parasites.

The properties of NK-2 reported previously prompted us to investigate the effect of NK-2 on human RBC infected with *P. falciparum*. The hemolytic activity of NK-2 toward normal RBC and RBC that were infected at high parasitemia was compared. The small hemolytic effect on noninfected erythrocytes, even at high concentrations of NK-2, was in contrast to the substantial activity against parasitized host cells. Consistently, NK-2 treatment of iRBC decreased the viability of the parasites in the same order of magnitude as that found for the hemolytic activity toward iRBC. Investigations of the time dependence of activities of NK-2 were performed by monitoring the hemoglobin release of the host cell as well as the Sytox Green uptake of the parasite. The experiments showed that both the host cell membrane and the plasmodial plasma membrane were compromised by NK-2 within minutes (Fig. 3).

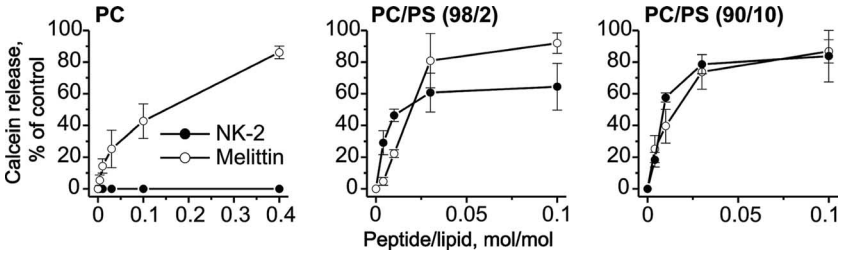

FIG. 6. Lysis of liposomes by NK-2. Peptide-induced lysis of calcein-containing liposomes of defined composition with various phospholipid compositions was monitored fluorometrically by the detection of dye release. The molar ratio of PC and PS within the liposome membranes is indicated. For a comparison, melittin was used in the same concentrations as those of NK-2. Error bars indicate the standard deviations of the mean of at least three measurements.

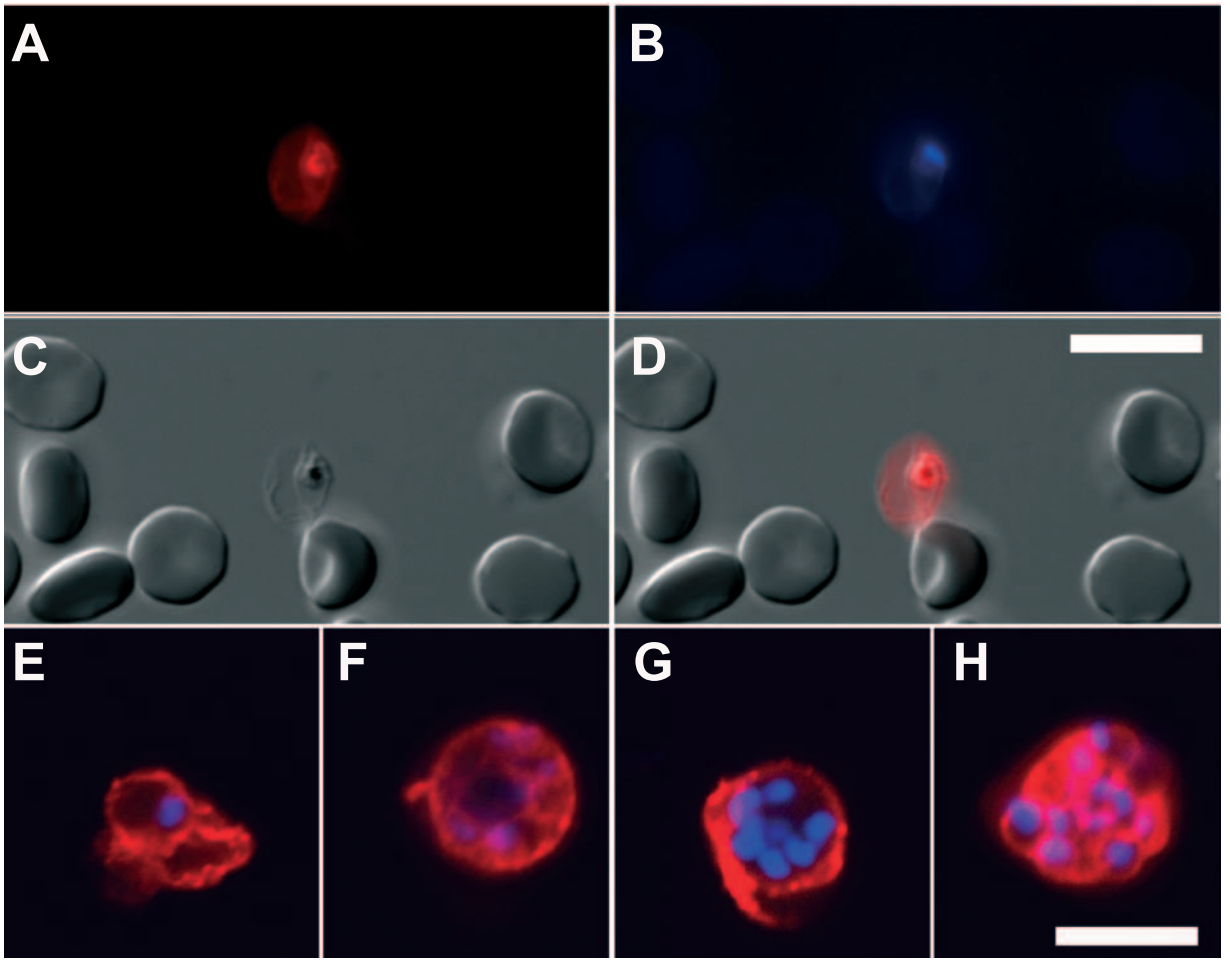

FIG. 7. Microscopic images of LNK-2 distribution in an infected erythrocyte. Fluorescence of LNK-2 (red) (A) and of nuclei stained by Hoechst 33258 (blue) (B) in the same parasite (trophozoite). (C) Transmission image of the same section. (D) Overlay image of panels A and C. The noninfected RBCs do not fluoresce. Panels E, F, G, and H show enlarged images of LNK-2-labeled and Hoechst 33258-stained parasites of trophozoite, young schizont, mid-schizont, and late-schizont stages, respectively. Bars, 10 μm.

However, the binding kinetics of the dye to the DNA of affected parasites leading to an increased fluorescence signal appears more slowly than it does in the lysis of iRBC. Notably, to allow binding of the fluorescent dye to the plasmodial nuclei, NK-2 has to pass through both the parasitophorous vacuolar membrane and the plasma membrane. The Sytox Green assay evidences that NK-2 readily destroys the integrity of the parasite plasma membrane. Consequently, the viability of the parasites is affected not only by host cell hemolysis but by transfer to and permeabilization of intracellular plasmodia. These findings are confirmed by Giemsa staining of iRBC incubated with NK-2, indicating not only that the host cell plasma membrane was affected by NK-2 but also that the morphology of the parasite was altered (Fig. 2). However, the lysis of iRBC was supposed to be caused by a physical disruption of the host cell membrane. Therefore, the hemolysis of the host cell by NK-2 may be a result of an increased binding affinity and insertion efficiency of the peptide to membranes of infected host cells compared to the binding affinity and insertion efficiency of normal RBC. *P. falciparum* is known to alter permeability, fluidity, and protein and lipid composition of the erythrocyte membrane (35, 41), resulting in a reorganization of the host cell plasma membrane and altered cytoadherence (14). Noninfected erythrocytes have an asymmetrical distribution of phospholipids throughout the life span of the cell, locating PS exclusively in the inner leaflet (26). Pathological or experimental changes in distribution of PS, i.e., the exposure of PS to the outer surface of the cell, lead to an increased adhesion of erythrocytes to the vascular endothelium in vivo (30). In the present study, it was shown by annexin V binding to RBC that the exposure of PS is correlated with infection of the cell. Accordingly, PS exposure in iRBC may be an alternative strategy of the parasite for sequestration in vivo. The annexin V-binding experiments are consistent with the studies of Maguire et al. (28), who showed the presence of PS in the outer layer of the plasma membrane of *P. falciparum*-iRBC by a PS-sensitive prothrombinase assay. The increased affinity of NK-2 to infected, PS-exposing RBC was found to be decreased by annexin V cloaking the surface of the iRBC. Annexin V forms two-dimensional arrays on membrane surfaces containing PS in the outer layer. Consequently, we tested whether annexin V cloaking on infected erythrocytes can inhibit or

decrease the lytic activity of NK-2 toward iRBC. In the presence of annexin V, activity of NK-2 toward iRBC is decreased significantly (*t* test, with a *P* value of 0.003 for 100 μg/ml annexin V). It is not clear whether annexin V spatially blocks the binding of NK-2 to insertion into the RBC plasma membrane or whether annexin V decreases activity of NK-2 by masking the anionic head groups of PS on the outer layer of the membrane. The reason why NK-2 still affects iRBC in the presence of annexin V may be reversible binding on the annexin V to RBC. Even high concentrations of annexin V (10 μg/ml and 100 μg/ml or 0.3 μM and 3 μM, respectively, were used in this assay) could not inhibit the effect of NK-2 completely. Using concentration of NK-2 higher than 12 μM, we found the effect of annexin V to be diminished. This result may be due to the high molar ratio of NK-2 to annexin V, in addition to annexin V's reversible binding to the RBC surface mentioned before or to a higher affinity to the RBC surface of NK-2 compared to that of annexin V. Moreover, it might be possible that PS is not the only component of the outer layer of the erythrocyte plasma membrane to which NK-2 binds. Other candidates may be phosphatidylethanolamine (PE) (33, 43) and phosphatidylgycerol (4, 43). It is known that like the case with PS, the amount of PE is also increased in the outer leaflet of the iRBC membrane, whereas the amount of PC is lower compared to that in normal RBC (28).

To prove the direct relationship between the membrane-permeabilizing activity of NK-2 and the presence of PS on the outer surface of membranes, the release of a fluorescent dye from a minimalistic system comprising defined phospholipids only was monitored. The lysis of liposomes by NK-2 was clearly dependent on PS content, confirming that PS in the outer surface of the target membranes is an important target for the membranolytic action of the peptide. As NK-2 is a linear amphipathic helical peptide with a net charge of +10 when the amidated C terminus is considered (3), it has previously been suggested that NK-2 preferentially binds to the negatively charged head group of PS (34) in the outer leaflet of the iRBC membrane and is membranolytically active after reaching a threshold concentration. The stoichiometry of this process is unclear, but it seems likely that NK-2 forms transient oligomers at the membrane.

The consensus view is that antimicrobial peptides play a pivotal role in the innate defensive systems of animals. However, their in vitro activities are often substantially diminished under the physiological conditions of human body fluids, particularly in the presence of 100 to 150 mM NaCl (27). It is therefore interesting to note that NK-2 is markedly active against blood-stage plasmodia at physiological salt concentrations. Moreover, it appears that the presence of serum components does not abrogate the activity of NK-2, as the peptide was active under normal culture conditions.

At the cellular level, the fluorescently labeled lissamine derivative LNK-2 was found to label the intracellular parasite very intensively. Preferentially, the parasite surface was labeled, suggesting that the plasmodial plasma membrane is the final target of the peptide rather than cytoplasmatic structures of the parasite. However, it was reported that trophozoite membranes contain low levels of PS, despite of the fact that it is the most abundantly biosynthesized phospholipid in iRBC (15). As PS is a source for PE synthesis in *Plasmodium*-infected erythrocytes by a decarboxylation event (15), PE in the parasite plasma membrane could be the molecular target of NK-2, leading to the enrichment of the peptide on the parasite surface. Conclusively, NK-2 is a host cell-permeating antiplasmodial peptide with a preference for negatively charged phospholipids and, as such, may represent a valuable template for novel anti-infectives that also kill intracellular parasites.

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