Inhibition of Macrophage iNOS by Selective Targeting of Antisense PNA†

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ABSTRACT: Peptide nucleic acids (PNAs) are synthetic polynucleobases that bind to DNA and RNA with high affinity and specificity and with poor membrane permeability. Although PNAs have an enormous potential as antisense agents, the success of antisense PNA applications will require efficient cellular uptake. In this study, a unique antisense 14-mer anti-inducible nitric oxide synthase (iNOS) was encapsulated into erythrocytes (RBC) by hypotonic dialysis. RBC loaded with PNA (10.5 ± 3.5 μmol/mL RBC) were targeted specifically to murine macrophages, taking advantage of an in vitro opsonization induced by $ZnCl_2$ and bis-sulfosuccynimidil-suberate (BS^3). This in vitro opsonization enhanced the phagocytosis of loaded RBC and the delivery of PNA into macrophages (0.72 pmol/10^6 macrophages). The efficacy of this delivery system is demonstrated by decreases in NO production and iNOS protein expression inside the macrophage. Therefore, we can suggest this novel approach for biomedical application.

During inflammation, macrophages synthesize various growth factors, cytokines, and other autacoids involved in cell signaling (*1*). These messengers modulate blood and vascular cell function and are involved in both local inflammation and systemic inflammatory response of septic shock (*2*). In vitro, exposure of macrophages to the proinflammatory bacterial lipopolysaccharide (LPS) induces the synthesis of cytokines and secondary mediators such as nitric oxide (NO) and prostaglandins (PGs) (*3*, *4*).

NO, a gaseous, short-living free radical, is an important signaling molecule with pleiotropic activities including vasodilatation, neurotransmission, microbial and tumor cell killing, and target-tissue damage in organ-specific autoimmune disorders (*5*). The production of NO from L-arginine results in the formation of L-citrulline and is catalyzed by the enzyme nitric oxide synthase (NOS). To date, three different isoforms of NOS have been characterized: two of them are termed neuronal constitutive (ncNOS or type I) and endothelial constitutive (ecNOS or type III) (*6*). These isoforms are Ca^{2+}- and calmodulin-dependent and produce relatively small amounts of NO.

The third NOS isoform, the inducible NOS (iNOS or type II), which produces larger amounts of NO, is present only after stimulating the host or isolated cells with bacteria or bacterial products such as LPS and/or inflammatory cytokines (TNF-α, IL-1β, IFN-γ) (*7*).

In these circumstances, NO mediates antimicrobial and antitumor activities of the immune system, but, under certain conditions, high levels of NO cause tissue damage and autoimmune diseases (*8*). Therefore, it is crucial to regulate NO production by iNOS when considering the inflammatory reaction (*9*, *10*).

Most of the currently used pharmacological inhibitors of iNOS may also affect the other two isoforms and to some degree interfere with unrelated metabolic cellular pathways (*1*, *2*, *6*−*8*). Therefore, an antisense strategy might result in a more selective pharmacological control of iNOS (*11*).

The oligodeoxynucleotides (ODNs) have great potential as antisense and antigenic agents due to their ability to bind to RNA and/or double-stranded DNA. The fast degradation of natural oligonucleotides in biological fluids suggests the synthesis of modified oligonucleotides (*12*). Phosphorothioate ODNs have now reached phase I and II in clinical trials for the treatment of cancer and viral infections (*13*). However, many obstacles still exist in the development of this technology (*14*).

A new type of DNA analogue, a peptidic nucleic acid (PNA), was designed by Nielsen et al. (*15*). In PNAs, the natural nucleic acid backbone is replaced by an uncharged mimic consisting of repeating 2-aminoethyl-glycine units. The resulting decrease in electrostatic repulsion allows the formation of a PNA−DNA hydrogen-bonded double helix, which possesses many properties that DNA oligos do not possess (*16*). These properties include high thermal stability, greater specificity of interaction, and resistance to nucleases and proteases. Applications for PNAs can be antisense, genotyping, mutation detection, analysis of single nucleotide polymorphisms, etc. (*17*, *18*).

Most studies on the use of PNAs in gene therapy or as antisense have been conduced in cell-free systems (*19*, *20*); in fact, PNAs suffer from poor membrane permeability (*21*, *22*). For this reason, studies regarding cell targeting and the delivery of PNA to tissue need to be improved.

In the past few years, we have developed a method based on the use of autologous erythrocytes, for the selective

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administration of drugs, peptides, and plasmids to the monocyte/macrophage compartment (*23*−*26*).

Now, we propose the use of loaded opsonized autologous erythrocytes to selectively deliver PNAs to monocytes/macrophages. The loading procedure includes hypotonic dialysis and isotonic resealing that permits the encapsulation of PNAs into red blood cells. PNA-loaded erythrocytes are then properly modified to induce their opsonization and consequently to enhance their phagocytosis by macrophages (*27*).

Given their polyamide backbone and low molecular weights, PNAs seem to possess chemical properties that allow them to be encapsulated into erythrocytes. On the basis of these considerations and on previous experience from our laboratory (*26*), we have developed a targeting system for the selective delivery of PNA to murine macrophages, which play a central role in the inflammatory processes.

Macrophages are known to have a lot of functions (*28*) and can be expected to influence every chronic inflammation. The chemical mediators of inflammation can be arachidonic acid metabolites, cytokines, and nitric oxide. Approaches to inhibit iNOS preserving NOS activity may be beneficial.

In this paper, we report the results obtained by the evaluation of the possibility of utilizing a homopyrimidine PNA designed to bind specifically to murine iNOS mRNA and consequently to inhibit its translation in murine macrophages. In particular, we have studied the inhibition of the expression and the activity of the target enzyme iNOS after stimulation with LPS following the selective targeting of loaded erythrocytes to macrophages and the delivery of PNA from erythrocytes to macrophages.

MATERIALS AND METHODS

Peptide Nucleic Acid Synthesis and Purification. The 14-mer antisense PNA (ELA24) was composed exclusively of pyrimidinic monomers complementary to the homopurinic region 238−251 of murine iNOS cDNA (GenBank accession number M84373). The same basis composition, but disposed arbitrarily, was maintained also for the synthesis of a scrambled control PNA with no specificity. Nucleotidic sequences of the synthesized antisense and scrambled PNA molecules were 3′ Gly−C−T−T−T−C−T−C−C−T−T−T−T−C−C−Lys 5′ (PNA ELA24) and 3′ Gly−C−T−T−T−T−T−C−C−C−T−C−T−T−C−Lys 5′ (scrambled PNA).

The molecules were manually synthesized using the standard method of solid-phase peptide synthesis, which follows the *tert*-butoxycarbonyl (Boc) strategy (*29*, *30*), with minor modifications (*31*).

The synthesized compounds were purified by reverse phase high performance liquid chromatography (RP-HPLC), and their molecular weights were confirmed by electrospray mass spectrometry (ESMS) (*31*). After purification, the synthesis had a final yield of 53−55% and the molecules had a purity of 95% after preparative HPLC purification of products.

The purified PNA, analyzed with ESMS, showed a mass of 4003.6 consistent with the molecular weight of the expected molecules.

Preparation of Macrophages. Swiss mice were injected with 1.0 mL of 10% thioglicollate (Sigma, USA) intraperitoneally. On day 4, the peritoneal exudate cells were obtained by peritoneal lavage with 10 mL of ice-cold Hank's balanced salt solution supplemented with 10 U/mL of heparin. The cells were washed twice, resuspended in DMEM (International PBI, Italy) culture medium supplemented with 10% heat-inactivated fetal calf serum (FCS), 1% antibiotics, 2 mM glutamine, and (4×10^6/plate) overlaid on plastic dishes (Φ 35 mm, Sarstedt, Italy). The plates were incubated in a humidified 5% CO_2 atmosphere at 37 °C overnight to allow macrophage adherence. Plates were washed with gentle agitation with warmed DMEM medium for dislodging nonadherent cells, and a macrophage monolayer was obtained. About 95% of the adherent cells were macrophages as determined by immunostaining and surface marker analysis.

Encapsulation of PNA in Erythrocytes. PNA was encapsulated into erythrocytes following a procedure of hypotonic dialysis, isotonic resealing, and reannealing (*32*). Briefly, blood was centrifuged at 750*g* to collect plasma; cells so obtained were then washed twice in 10 mM Hepes, 154 mM NaCl, 5 mM glucose, pH 7.4 (buffer A) to remove leukocytes and platelets. RBC were resuspended at 70% hematocrit in the buffer A and dialyzed for 1 h and 45 min using a tube with a cutoff of 12−14 kDa against 50 vol of 10 mM $NaHCO_3$, 10 mM NaH_2PO_4, 20 mM glucose, 4 mM $MgCl_2$, pH 7.4 containing 3 mM reduced glutathione, 2 mM ATP, and PNA ELA24 and scrambled PNA in the range of 50−230 μM. The osmolarity of the dialyzing buffer was 65−70 mOsM. All these procedures were performed in sterile conditions and at 4 °C. Resealing and reannealing of erythrocytes were obtained as previously described (*32*).

Resealed erythrocytes were then centrifuged two times at 450*g* in the buffer A and processed to increase their recognition by macrophages (*32*). The final concentration of PNA in erythrocytes was evaluated in perchloric acid ($HClO_4$) extracts by HPLC analysis.

Cell Activation. Murine macrophages, prepared as above, were incubated for 4 h in the presence of erythrocytes at a ratio of four RBC per macrophage. Nonphagocytosed RBC were carefully removed by repeated washes, and macrophages were maintained in fresh medium for 4 h prior to stimulation to complete the uptake of the remaining adhering RBC.

After this time, macrophages were further incubated for 18 h with or without 50 ng/mL of LPS from *Escherichia coli* (serotype 0111:B4, Sigma USA) to elicit the expression of iNOS and the production of NO. For in vivo studies, Swiss mice were injected intraperitoneally with 200 μL of PNA-loaded erythrocytes at 5% hematocrit. The following day, mice were sacrificed and cells obtained by peritoneal lavages were washed and overlaid as described above. The plates were incubated for 2−3 h to allow macrophage adherence. After this time, cells were stimulated with 50 ng/mL of LPS, washed, and lysed as described above.

Chromatographic Conditions. $HClO_4$ extracts were prepared, neutralized, and processed as previously described (*33*). Fifty microliter of extracts were analyzed by HPLC (JASCO, Japan). A 5-μm C18 column (150 × 460 mm, Varian, U.S.A.) protected by a guard column (Pelliguard LC-18, 20 × 4.60 mm, pellicular packing material 40-μm particles) was used for these studies. The mobile phase used for the separation of PNA consisted of two eluents: 0.1% trifluoroacetic solution (buffer A) and acetonitrile 0.1%

trifluoracetic acid solution (buffer B). All buffer solutions, as well as standards and sample solutions, were filtered through a 0.22-μm membrane filter (Millipore, Italy).

The elution conditions used were 2 min 100% buffer A, 10 min up to 35% buffer B, 15 min up to 65% buffer B, 5 min up to 100% buffer B, 3 min up to 100% buffer A. The flow rate was 1 mL/min, and the detection wavelength was 260 nm. The analyses were performed at room temperature. Quantitative measurements were carried out by injection of standard solutions of known concentration. The retention time for PNA was 6.5 min.

PNA Stability in Erythrocytes. The stability of PNA ELA24 and scrambled PNA in loaded erythrocytes was evaluated at 37 °C by incubation of PNA-loaded RBC and scrambled-loaded RBC at 7% hematocrit in 0.9% (w/v) NaCl containing 5 mM glucose and 10 mM Hepes (pH 7.4). At time intervals of 0, 3, 18 h, 250 μL of erythrocyte suspension were centrifuged at 450*g* to separate packed erythrocytes by supernatant. Both were extracted with 10% (v/v) $HClO_4$ and then analyzed by HPLC.

Nitrite Determination. NO production was estimated by measurement of nitrite in the culture supernatant using Griess reagent as described by Stuehr and Nathan (*34*). Briefly, after macrophages were treated with PNA-loaded RBC or unloaded-RBC in the absence or presence of LPS, the supernatants were immediately centrifuged at 750*g* in an Eppendorf microcentrifuge to remove cells in suspension. Two hundred microliter aliquots of culture supernatants were mixed with 200 μL of Griess reagent (1% sulfanilamide, 0.1% *N*-(1-naphthylethyl-enediamine dihydrochloride in 2.5% H_3PO_4) and incubated at room temperature for 10 min. The absorbance at 540 nm was measured with a spectrophotometer. Nitrite levels were determined using $NaNO_2$ as a standard.

Preparation of Cellular Extracts and Immunoblotting. After stimulation with LPS, cells were harvested by scraping with a solution containing 50 mM Tris-HCl, pH 7.6, 0.25 M sucrose, 1% (w/v) SDS, 2 μg/mL leupeptin, 1 μg/mL pepstatin, 2 mM phenylmethylsulfonylfluoride (PMSF), 5 mM EDTA, and 5 mM *N*-ethylenmaleimide. Cellular extracts were immediately boiled for 5 min, sonicated to shear the DNA and centrifuged at 10000*g* in an Eppendorf microcentrifuge to remove insoluble debris. Protein content was assayed by the method of Lowry (*35*). Equal amounts of protein extracts (4 μg) were resolved on 8% SDS−polyacrylamide gels, transferred to nitrocellulose membranes (Amersham Pharmacia Biotech, UK), and then detected with an antibody specific against iNOS (M-19, Santa Cruz Biotechnology Inc., USA). The secondary antibody was horseradish-peroxidase-conjugated goat anti-rabbit, and immune complexes were visualized with the ECL detection kit (Amersham Life Science, UK) according to the manufacturer's instructions. Membranes were stripped and reprobed with an antibody against p65 (Rel A) (C-20, Santa Cruz Biothecnology, USA). Immunoreactive bands were quantitated by laser densitometry, and iNOS levels were normalized to p65.

Detection of iNOS mRNA by RT-PCR Analysis. Total RNA was isolated from macrophages using the Trizol LS reagent (GIBCO-BRL, Life Technologies, Italy) according to the manufacturer's instructions. Total RNA was analyzed by RT-PCR for the expression of iNOS and β-actin mRNA. Each RNA sample was diluted to a concentration of 5 ng/μL before use in the one-step protocol for RT-PCR performed with the "Ready-To-Go RT-PCR Beads" (Amersham Pharmacia Biotech, UK), following the manufacturer's instructions. Twenty-five nanograms of total RNA was reverse-transcribed into first-strand cDNA with 10 pmol of a gene-specific primer in a final volume of 25 μL. The RT reaction was performed at 42 °C for 25 min, followed by a denaturation step of 5 min at 95 °C to ensure complete denaturation of the template DNA and inactivation of the M-MuLV reverse transcriptase. For iNOS cDNA amplification, the PCR was performed using iNOS-upper (5′-ACAGGGAAGTCTGAAGCACTAG-3′) and iNOS-lower (5′-CATGCAAGGAAGGGAACTCTTC-3′) (*36*) at the following conditions: 15 s at 95 °C, 10 s at 62 °C, and 40 s at 72 °C for 30 cycles (selected to perform the PCR in a linear range) followed by a final extension at 72 °C for 7 min. The selected pair of primers was expected to give a 1033 bp PCR product corresponding to nt 1805 to nt 2837 in the iNOS mRNA (GenBank accession number M84373).

To verify that an equal amount of total RNA was used, the content of β-actin mRNA, a housekeeping gene, was also determined as an internal invariant control. RT-PCR of β-actin mRNA was carried out on 25 ng of total RNA under the following one-step PCR conditions: 15 s at 95 °C, 50 s at 72 °C for 25 cycles with a final extension at 72 °C for 7 min. The sequences of the primers used were β-actin-upper 5′-ATCTGGCACCACACCTTCTACAATGAGCTGCG-3′ and β-actin-lower 5′-CGTCATACTCCTGCTTGCTGATC-CACATCTGC-3′ with an expected PCR product of 838 bp in length (nt 349−1186, GenBank accession number AF195094). iNOS amplification products were resolved by electrophoresis on 1.8% agarose gel and quantified by ethidium bromide staining using the Gel Doc 2000 apparatus (Bio-Rad, USA) with the Quantity One software (Bio-Rad) and normalized to the corresponding β-actin PCR products.

RESULTS

PNA Encapsulation and Stability in Erythrocytes. PNA ELA24 was encapsulated in erythrocytes by a procedure of hypotonic dialysis, isotonic resealing, and reannealling as described under Materials and Methods at a final concentration of 10.5 ± 3.5 μmol/mL RBC and with a percentage of encapsulation of 24.3 ± 2.5% (mean ± SD, $n = 3$). Stability of PNA ELA24 loaded into erythrocytes was tested at 37 °C. After 18 h of incubation, 82% of the PNA encapsulated was still inside the RBC.

A scrambled PNA was encapsulated at a final concentration of 6.3 ± 0.6 μmol/mL RBC and with a percentage of encapsulation of 14.1 ± 1.2% (mean ± SD, $n = 3$). After 18 h of incubation, 98% of the scrambled PNA encapsulated was still inside the RBC (Figure 1).

In all experiments, unloaded RBC, which are submitted to the same procedure as loaded RBC but without adding PNA, were used as a control.

Targeting of PNA-loaded RBC to murine macrophages was obtained by promoting the clusterization of the erythrocyte transmembrane band 3 protein (*27*). This clusterization was induced by the addition of $ZnCl_2$ and BS^3 (Zn^{2+} is the clustering agent, and BS^3 is a cross-linker that makes the cluster irreversible upon Zn^{2+} removal). Once these clusters are formed, erythrocytes are opsonized by autologous IgG

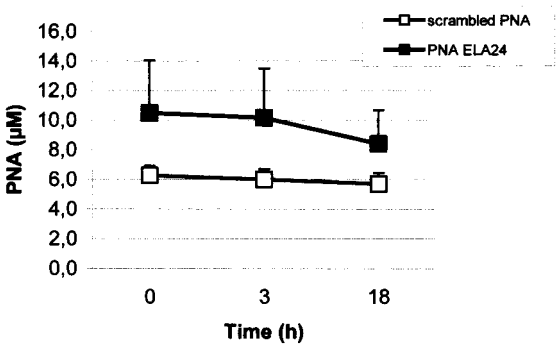

FIGURE 1: Stability of PNA ELA24 and scrambled PNA into erythrocytes. PNA ELA24 was encapsulated in eythrocytes at a final concentration of 10.5 ± 3.5 μM, and scrambled PNA was encapsulated at a final concentration of 6.3 ± 0.6 μM. PNA-loaded RBC were resuspended at an hematocrit of 0.5% in DMEM medium containing 10% heat-inactivated (30 min at 56 °C) autologous serum and stored at 37 °C. At time intervals as indicated, the content of PNA in the cells was determined. The results are mean values of three experiments ± SD.

Table 1: Phagocytosis of PNA-Loaded RBC by Murine Macrophages[a]

	PNA ELA24
conc of PNA/RBC (pmol)	0.78×10^{-6}
conc of PNA/macrophage (pmol)	0.72×10^{-6}
molecules of PNA/RBC	469,000
molecules of PNA/macrophage	436,000

[a] PNA was encapsulated into RBC by a procedure of hypotonic dialysis and isotonic resealing as described under Materials and Methods at a final concentration of 10.2 μM. Macrophages were prepared and phagocytosis was evaluated as described under Materials and Methods.

and C3b and are recognized by macrophages through their Fc and C3b receptors. Erythrocytes treated as described above were actively phagocytosed by macrophages, allowing the delivery of PNA into the phagocytic cells.

Using HPLC chromatography, it was possible to establish the quantity of PNA targeted to murine macrophages. This determination permitted us to conclude that in agreement with previous data (*26*), each macrophage phagocytosed one RBC (Table 1).

Effects of PNA on iNOS Expression. iNOS is an inducible protein synthesized in consequence of external stimuli such as cytokines or various pathogens.

To examine the effect of PNA ELA24 on iNOS protein expression, murine macrophages were treated with unloaded or PNA-loaded RBC and subsequently stimulated with LPS for 18 h. The cellular extracts were prepared and tested for iNOS expression by Western blot analysis as described under Materials and Methods.

The evaluation of iNOS levels by laser densitometry of the immunoreactive band, normalized to p65 (Rel A), is reported (Figure 2).

Immunoblot analysis of unstimulated macrophage cellular extracts revealed that no iNOS expression was detectable upon phagocytosis of unloaded or PNA-loaded RBC in control macrophages (Figure 2A, lanes 1, 3, and 5). Moreover, after macrophage stimulation with LPS there was no difference in iNOS levels of murine macrophages that had received or had not received unloaded erythrocytes (Figure 2A, lanes 2 and 4).

Finally, LPS activated macrophages treated with PNA-loaded RBC in which PNA was encapsulated at the concentration of 5.0 ± 2.0 μM showed an iNOS level inhibition of 40.4 ± 5.9% with respect to macrophages receiving unloaded RBC (Figure 2A, lanes 4 and 6). When we used scramble PNA-loaded RBC we obtained results comparable with those obtained using unloaded erythrocytes (not shown).

Similar results were obtained from in vivo studies. In fact, the iNOS level of macrophages obtained from mice treated intraperitoneally with PNA-loaded RBC was inhibited by 46.3 ± 5.9% with respect to macrophages receiving unloaded RBC (Figure 2B, lanes 4 and 6).

Effects of PNA on NO_2^- Production. NO_2^- production was measured in the culture medium of murine macrophages treated with PNA-loaded RBC (PNA concentration 5.0 ± 2.0 μM) or unloaded RBC in the absence or presence of LPS as described under Materials and Methods.

The results obtained show similar values of NO_2^- production in unstimulated macrophages treated or untreated with unloaded or PNA-loaded RBC. Stimulated macrophages treated with PNA-loaded RBC showed a reduced nitrite production of 30% (Figure 3).

Similar nitrite reduction was obtained by the experiments performed in vivo (not shown).

Effects of PNA on iNOS mRNA Level. The iNOS mRNA levels determined by RT-PCR of total RNA are represented in Figure 4.

iNOS mRNA levels in LPS stimulated macrophages were about 3 times higher than those found in unstimulated macrophages. The values of iNOS mRNA were similar in stimulated macrophages treated or untreated with unloaded or PNA-loaded RBC.

DISCUSSION

Peptide nucleic acid (PNAs) (*15*) are biomolecules of great interest in biomedicine, for their well-known in vitro antisense or antigene activity (*17*, *37*).

In PNAs, the pseudopeptide backbone is composed of *N*-(2-aminoethyl)glycine units (*15*) to which normal DNA bases are linked. This structure gives to PNAs resistance to DNAses and proteases (*38*) and the capacity to efficiently hybridize with complementary RNA and DNA sequences (*39*).

PNAs are mostly studied for their potential antisense and antigene activities (*35*, *37*, *39*, *40*), even if their natural low permeability across the cellular membrane (*22*) limits their application. It has been previously reported that modifying PNAs, for example by means of a covalent linkage to a hydrophobic peptide, results in a more efficient cellular internalization (*11*). This goal can be achieved in several other ways but in our opinion the selective targeting of PNAs to a specific compartment might be the most advantageous. For this reason, we developed a new system for the delivery of PNAs to macrophages. This delivery system is based on the peculiar capacity of macrophages to recognize and phagocytose autologous senescent RBC that can be easily transformed into carrier cells (*25*). Encapsulation in carrier erythrocytes has been already used to extend the pharmacological activity of drugs or enzymes (*41*), to reduce the toxicity of antiviral (*42*) or antitumoral drugs (*43*).

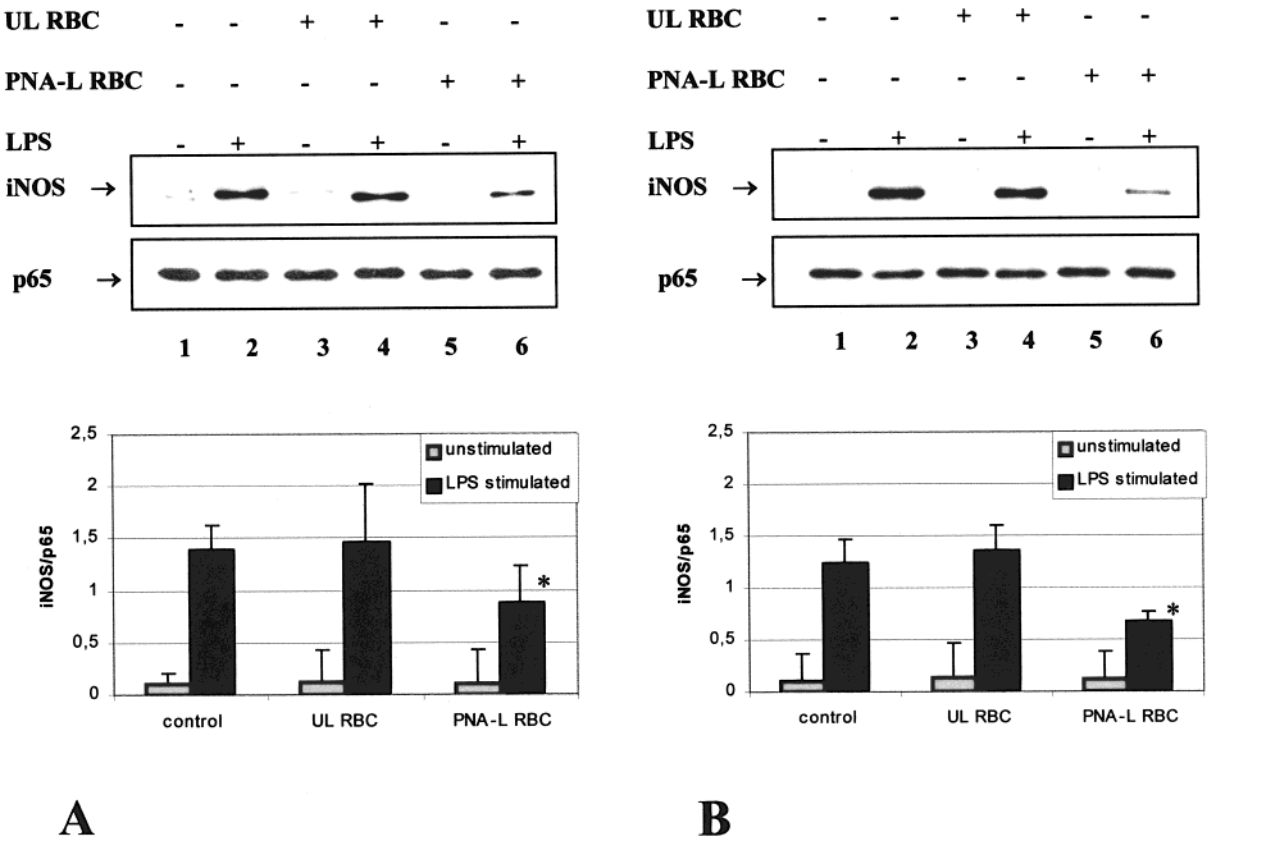

FIGURE 2: Inhibition of iNOS by PNA ELA24 in vitro experiments (A) and in vivo experiments (B). Above: immunoblot analysis of iNOS levels in murine macrophages not receiving RBC (lanes 1 and 2), in comparison with macrophages targeted with unloaded RBC (UL RBC) (lanes 3 and 4) or PNA-loaded RBC (PNA-L RBC) (lanes 5 and 6). Lanes 1, 3, and 5 show unstimulated cells, whereas in lanes 2, 4, and 6 cells were stimulated for 18 h with 50 ng/mL LPS. Blots were probed with anti-iNOS, anti-p65 (Rel A) antibodies. Below: quantification by laser densitometry. iNOS levels were normalized to p65 (Rel A) and expressed as iNOS/p65 ratio ± SD from three different experiments. * $p \leq 0.02$ (vs UL RBC and control).

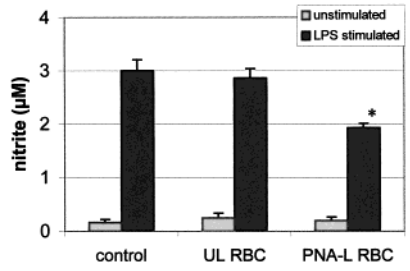

FIGURE 3: Effects of PNA ELA24 on NO^{2-} production in murine macrophages. Mouse macrophages (3×10^6 cells) were incubated for 4 h with PNA-loaded RBC (PNA-L RBC) or unloaded RBC (UL RBC) in DMEM (without phenol red) culture medium and further incubated for 18 h with or without LPS (50 ng/mL). NO^{2-} levels in the supernatants of culture medium were measured using Griess reagent as described in Materials and Methods. The values are the mean ± SD of three different experiments. * $p = 10^{-3}$ (vs UL RBC and control).

The selective targeting of carrier erythrocytes to macrophages can be achieved by opsonization. PNA-loaded RBC are treated with $ZnCl_2$ and BS^3, to induce irreversible band-3 clustering, opsonization by IgG and C3b binding (*27*). Thus, the immune system recognizes these "clusters" as nonself, promoting phagocytosis by macrophages (*44*).

The aim of this study was the evaluation of the capacity of PNA-loaded RBC to control the production of a mediator of inflammation (*8*). To do this, we have chosen a specific homopyrimidinic antisense PNA able to inhibit the translation of murine iNOS.

In particular, we have studied the inhibition of the expression and activity of iNOS, after stimulation with LPS following the selective targeting of loaded RBC to murine macrophages.

The results obtained show that erythrocytes are useful carriers to deliver PNA to murine macrophages, where an inhibition of the inflammatory response mediated by NO release can be obtained. This was demonstrated by evaluating iNOS expression and measuring NO_2^- production.

Experiments with PNA-loaded RBC in which PNA was encapsulated at different concentrations (range 2−20 μM) were performed and the highest inhibition percentage of iNOS expression was 40.4 ± 5.8%.

Similar results were obtained in NO_2^- production in culture medium, and they are particularly encouraging if we consider that similar inhibition has been obtained with higher concentration of antisense oligonucleotide given free in culture medium in a similar cellular model (*36*).

The results of RT-PCR of total RNA isolated from the macrophages revealed no differences in the iNOS mRNA levels between cells treated with PNA-loaded RBC and those untreated or treated with unloaded RBC. So we can suggest that the inhibition of the iNOS expression occurs at a translation and not at a transcription step. Furthermore, it is likely that ribonuclease H is not activated by PNA when hybridized to mRNA (*19*).

This study was extended to an in vivo murine model, and the results obtained show an inhibition of iNOS expression

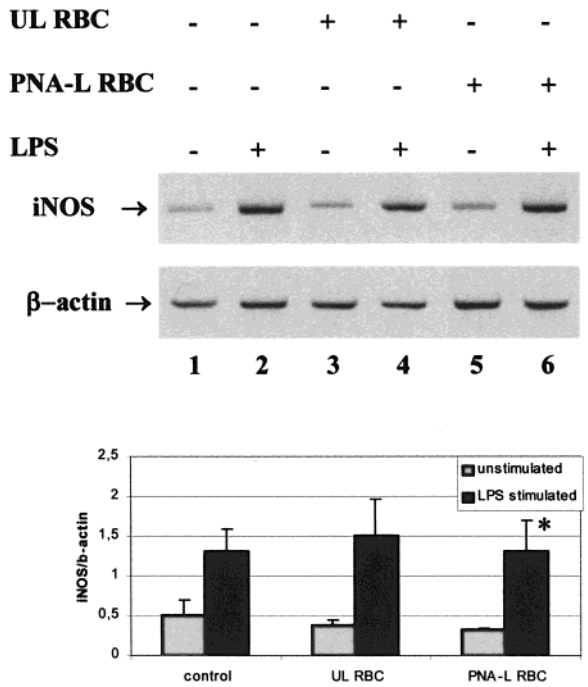

FIGURE 4: iNOS mRNA expression in macrophages treated with PNA ELA24. RT-PCR analysis of total RNA obtained from macrophages targeted with PNA-loaded RBC (PNA-L RBC) (lanes 5−6) in comparison with macrophages targeted with unloaded RBC (UL RBC) (lanes 3−4) and macrophages not receiving RBC (lanes 1−2). Lanes 1, 3, and 5 show unstimulated cells, whereas in lanes 2, 4, and 6 cells were stimulated for 18 h with 50 ng/mL LPS. RT-PCR products of iNOS mRNA (1033 bp) and β-actin mRNA (838 bp) were resolved by electrophoresis on 1.8% agarose gel. iNOS mRNA RT-PCR products were quantified by ethidium bromide staining with Quantity One software using the Gel Doc 2000 apparatus and normalized to β-actin. Data represent iNOS/β-actin ratio $\pm$ SD from two different experiments. * Not significantly different (vs UL RBC).

of 46.3 $\pm$ 5.9% using PNA concentrations of 4.0 $\pm$ 1.0 μM into loaded erythrocytes confirming the results discussed above.

We can conclude that it is possible to encapsulate PNAs into erythrocytes where they are particularly stable and that carrier RBC are able to deliver PNA to macrophages, where they can efficiently inhibit iNOS expression and NO_2^- production. A more refined analysis of iNOS messenger would probably permit us to identify the "best" antisense sequence.

This system permits us to overcome the obstacle of crossing intact macrophage membranes by PNAs. Moreover, it represents a good alternative to the drugs already disposable that are often toxic and lacking of specificity, suggesting its possible useful therapeutic application.

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