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# PRODUCTION OF POLYCLONAL AND MONOCLONAL ANTIBODIES FOR SPECIFIC DETECTION OF NITROSATION-PROFICIENT DENITRIFYING BACTERIA IN BIOLOGICAL FLUIDS

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Polyclonal and monoclonal antibodies were raised against the nitrosating enzyme previously isolated and purified from a denitrifying bacteria Pseudomonas aeruginosa. Using the polyclonal antibodies, a preliminary ELISA test was set up which allowed the detection of the nitrosating enzyme in 2 ml urine samples with a minimal total bacteria count of $\geq 10^5$ cells/ml. The use of such a rapid immunological screening test in clinical settings should ascertain whether individual subjects at higher risk for cancer of the stomach or bladder harbour more nitrosation-proficient microorganisms in their microflora. The availability of monoclonal antibodies provides a tool for studying the mechanisms of bacteria-mediated nitrosamine formation from precursor amines. 

Endogenous formation of carcinogenic N-nitroso compounds in the presence of bacteria has been postulated as an important etiological factor in human carcinogenesis at sites such as the infected urinary bladder (1) and the colonized stomach (2,3). Previous results clearly demonstrated that nitrosation at neutral pH is catalysed by various bacterial species (4-7); subsequently, an enzyme catalysing the nitrosation reaction at pH 7.25 has been isolated and purified from two denitrifying microorganisms, P. aeruginosa and Neisseria mucosae (8). This article describes the production of polyclonal and monoclonal antibodies against the nitrosating enzyme and the setting up of an immunological assay for the rapid detection of this protein in human urine from subjects infected with nitrosation-proficient denitrifying bacteria.

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**Abbreviations**
PBS, 0.01 M phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay; BSA, bovin serum albumin; Ig, immunoglobulin; DMSO, dimethylsulfoxide; SDS, sodium dodecyl sulfate.

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## Materials and Methods

Chemicals: 3,3',5,5'-Tetramethylbenzidine, 3,3'-diaminobenzidine, peroxidase coupled anti-rabbit IgG, peroxidase coupled anti-mouse IgG, dimethylsulfoxide (DMSO), bovine serum albumin (BSA), $H_2O_2$ were supplied by Sigma (St Louis, MO, USA); acrylamide, bis-acrylamide, N,N,N',N'-tetramethyl-ethylenediamine, nitrocellulose membrane, ammonium persulfate by Bio-Rad (Richmond, CA,USA); sodium nitrite, sodium nitrate, morpholine hydrochloride and N-methyl-pentylamine by Merck (Darmstadt, Germany). All other chemicals were of analytical grade and obtained from either Merck or Sigma.

Polyclonal antibody production: Two HY/CR rabbits were injected subcutaneously with 200 µg and 400 µg, respectively, of the nitrosating enzyme isolated previously (8), in Freund's complete adjuvant. Fourteen days later, they were boosted with the same amount of antigen. They were bled ten days after the boost injection and titers were determined by ELISA (see below). The highest immune response was obtained with the serum taken from a rabbit immunized with 200 µg antigen.

Polyclonal antibodies were purified by chromatography on a 3 ml Avid AL column (BioProbe International Inc., Tustin, CA, USA). IgG were eluted with 0.1 M sodium acetate pH 3.0, and dialysed against phosphate buffered saline (PBS). Protein concentration was measured according to Lowry et al. (9).

Titration of polyclonal antibodies by solid-phase ELISA: Falcon 96-well microtest plates were coated with 25 ng/well of nitrosating protein in PBS and incubated at 4°C overnight. The remaining protein-binding sites were blocked by incubation with 100 µl/well of 0.2% BSA in PBS for 30 min at 37°C. The plates were then washed twice with the same solution. Following addition of 100 µl of 1:1000 dilution serum per well, the plates were incubated for 60 min at 37°C. The plates were again washed, and 50 µl of peroxidase coupled anti-rabbit IgG (1:5000 dilution in PBS) was added and incubated for 60 min at 37°C. After the plates were washed, 50 µl of a substrate solution containing 1 mg 3,3'5,5'-tetramethyl-benzidine in 0.1 ml DMSO and 2 µl $H_2O_2$ (30 wt %) in 10 ml citrate buffer, pH 5.0 was added and incubated for 15 min at room temperature. The reaction was stopped with 50 µl 1 M HCl and the absorbance was read at 450 nm with a Bio-Tek EL 312 plate reader.

Immunoblotting: SDS-polyacrylamide gel electrophoresis was carried out on 12.5% acrylamide gel (10). Gels were stained with Coomassie blue or subjected to Western transfer (11). Following Western transfer, the nitrocellulose membrane was blocked in 3% BSA in washing buffer (150 mM NaCl, 50 mM Tris HCl, pH 7.2 plus 0.05% Tween 20), for 1 h at room temperature, washed twice in washing buffer, incubated with antibody (1:5000 diluted rabbit serum) for 1 h at room temperature. The immunoblots were washed four times in washing buffer and developed with a peroxidase-conjugated anti-rabbit IgG reacted with 0.03% $H_2O_2$ in buffer containing 0.2% 3,3'-diaminobenzidine.

Sample preparation for non-competitive ELISA: Three types of samples were treated: urine, urine spiked with P. aeruginosa ($10^6$ bacteria/ml), P. aeruginosa suspension in PBS ($10^6$ bacteria/ml). Ten ml of each sample was filtered successively through a 5.0 µm cut-off filter (Millipore, Millex-SC) and a 0.45 µm cut-off filter (Millipore, Ultrafree CL). The 0.45 µm cut-off filters were resuspended in 1 ml PBS and incubated at 37°C for 15 min. The suspension was then tested by non-competitive ELISA.

Non-competitive ELISA on samples: Falcon 96-well microtest plates were coated with 50 µl of suspension obtained as above, and incubated at 4°C overnight. After blocking with 100 µl/well of 0.2% BSA in PBS for 30 min at 37°C, the plates were washed and incubated with a 1:1000 dilution of rabbit serum for 1 h at 37°C. The procedure used was as described above.

Monoclonal antibody production: Two female BALB/c mice were injected intraperitoneally with 25 µg of the nitrosating enzyme isolated from P.

aeruginosa (8) in Freund's complete adjuvant. This was followed by two booster shots of similar doses of the protein in Freund's incomplete adjuvant and in PBS respectively, at 12-day intervals. Three days before the fusion, each mouse was given 25 µg of protein in PBS.

Cell lines producing antibodies that recognize the nitrosating protein were obtained from the fusion of spleen cells of immunized mouse with Sp2 myeloma cells (12), and tested by ELISA (see below). Hybridoma cells secreting the antibody of interest were grown to stationary phase. Cells were removed by centrifugation at 4000 g for 10 min. Immunoglobulins were purified by chromatography on Protein A-Sepharose (13).

Screening of clones by solid-phase ELISA: This was carried out under conditions similar to those used for the titration of polyclonal antibodies; the plates were incubated with 100 µl of culture supernatant (instead of a 1:1000 diluted serum); the second antibody solution was a 1:5000 diluted peroxidase-coupled anti-mouse IgG.

Culture conditions for the microorganisms and isolation of nitrosating enzymes from P. aeruginosa (8) and the procedure for nitrosation assay and analysis of nitrosamines (5) have been previously reported.

## Results and Discussion

In previous experiments, we have isolated and purified the protein (nitrosating enzyme) catalysing nitrosamine formation in P. aeruginosa (8). Subsequently, two rabbits were immunized with the nitrosating enzyme from P. aeruginosa in Freund's complete adjuvant and boosted once with enzyme in PBS. One of the two rabbits was found to produce significant anti-enzyme titers as measured by ELISA.

An aliquot of the serum was purified by affinity chromatography on an Avid AL column, and the eluted Ig were tested by non-competitive ELISA (Fig 1). The linear relationship between the molar ratio Ig/enzyme and the absorbance at 450 nm showed that the Ig produced by the infected rabbit were specific towards the bacterial enzyme.

Immunoblot analysis of the rabbit serum revealed reactivity with different purified fractions, as assayed by both SDS-polyacrylamide gel and on nitrocellulose membrane (Fig 2). The presence of a single 66 kD band (lane 8) on the membrane suggested that the crude serum could be used to obtain a specific immunological response. The presence of this single band on lanes 2, 3, 4, 6 and 7 demonstrated that the nitrosating protein present in a crude bacterial extract (lane 2) or in different fractions with nitrosating activity (lanes 3, 4, 6 and 7) was specifically bound by the serum Ig. Similar results were obtained with a non-competitive ELISA test (data not shown).

The specificity of these Ig was used to ascertain the absence of the nitrosating enzyme in a suspension of P. aeruginosa in which this enzyme was not

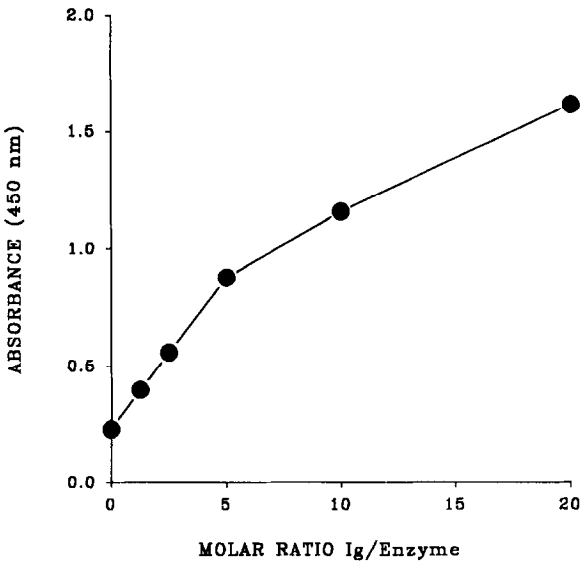



Fig. 1. Binding curve as determined by direct antibody binding ELISA using a rabbit serum purified on Avid AL column (Ig: immunoglobulin).

induced by pro-incubation with nitrate or nitrite (Fig 3, lanes 2 and 3). The absence of any band on lane 3 indicated that the bacterial suspension that was unable to nitrosate morpholine did not contain the 66 kD protein; thus the nitrosating enzyme is inducible in culture by nitrate or nitrite.

The serum showed high specificity and did not bind any protein from nitrosating non-denitrifying bacterial species (Escherichia coli, Klebsiella pneumoniae) that

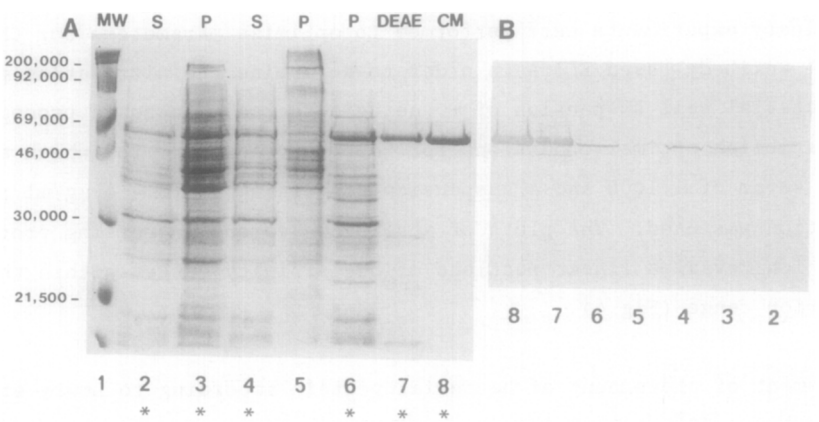
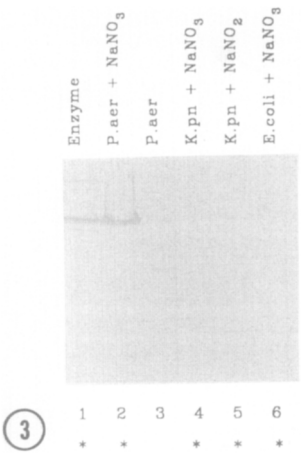



Fig. 2. SDS-polyacrylamide gel (A) and immunoblot (B) analysis of purified fractions containing the nitrosating enzyme. Lanes 2 and 3: sonicated P. aeruginosa suspension; lanes 4 and 5: 40% $(NH_4)_2SO_4$ precipitation; lane 6: 75% $(NH_4)_2SO_4$ precipitation; lane 7: purification after DEAE-cellulose; lane 8: purification after CM-cellulose; P:pellet; S:supernatant; MW, molecular weight markers; (*): fractions containing nitrosating enzyme.



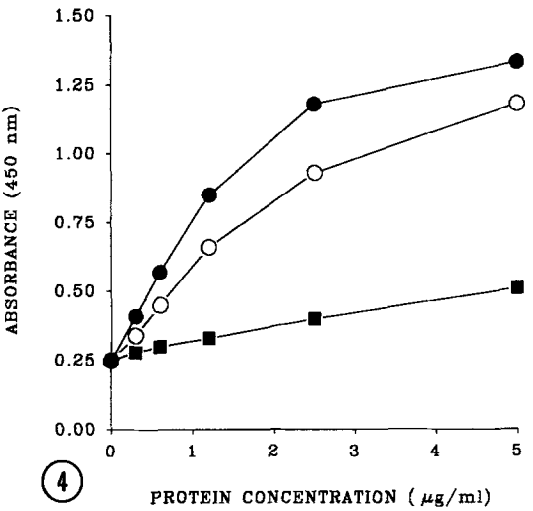




Fig. 3. Immunoblot analysis of nitrosating enzyme (lane 1) in different bacterial strains. P.aer, Pseudomonas aeruginosa; K.pn, Klebsiella pneumoniae; E.coli, Escherichia coli; (*): fractions containing nitrosating activity.

Fig. 4. Analysis of P. aeruginosa added to PBS (●-●) or urine (o-o) and analysis of unspiked urine (■-■).

were cultured in the presence of nitrate or nitrite (Fig 3, lanes 4, 5 and 6). These results confirm that the nitrosating enzyme in these strains differs from that found in denitrifying strains, as proposed earlier (14, 15).

The sensitivity of both immunoblot and ELISA procedures enabled us to develop an immunoassay for application to biological samples.

Preliminary experiments were performed to optimize parameters for the polyclonal antibody-based ELISA in order to determine the nitrosating protein in intact bacterial cell suspension of P. aeruginosa. A checkboard procedure was used to establish optimal conditions for color development, for which a dilution of rabbit serum of 1:1000 and a suspension containing a 0.3 - 5 µg/ml protein concentration was used. The plots of absorbance versus increasing protein concentration revealed linear portions of the affinity curves within this protein concentration range (Fig 4).

Measurement of the amount of bacterial protein according to Lowry et al. (9) indicated that a $10^8$ bacteria/ml suspension contained a 50 µg/ml protein.

The assay was validated using (i) PBS spiked with known quantities of bacteria, (ii) human urine spiked with the same quantities of bacteria and (iii) unspiked human urine samples. PBS and urine were spiked with $10^6$ P. aeruginosa

cells/ml. Samples were prepared and analysed by non-competitive ELISA, as described in Materials and Methods. The results are shown in figure 4. The urine spiked with bacteria exhibited a curve similar to that obtained with the bacterial suspension in PBS. A control assay was performed with a 1:1000 dilution of serum taken from a non-injected rabbit. The response was negative for all three samples.

Different storage conditions were also checked. Results from ELISA showed that the urine samples cannot be frozen, but can be stored for 24 h at 4°C (data not shown), to allow a reproducible assay performance.

The present immunological assay, therefore, allows the specific detection of the nitrosating enzyme in denitrifying bacteria, when urine samples are infected with at least $10^5$ bacteria/ml. This test, requiring a quite small sample volume (10 ml) and a short filtration time, would be a simple assay to detect nitrosation-proficient bacteria in biological fluids such as infected urines.

In addition, monoclonal antibodies were produced from two BALB/c mice immunized with the nitrosating enzyme from P. aeruginosa (Materials and Methods). Six stable positive clones were grown to stationary phase; the cells were removed and the supernatant purified by chromatography on Protein A-Sepharose.

The use of monoclonal antibodies against the enzyme catalysing nitrosation by denitrifying bacteria provides a tool to elucidate more clearly the mechanism(s) of bacterial nitrosation, that so far has only been partially characterized (14).

As non-denitrifying Enterobacteria are more prevalent in the human microflora from infected urines, gastric juice from subjects with achlorhydric stomach, colon and nasopharynx (7, 4, 16), it remains important to develop similar immunoassays for the nitrosating enzyme in E. coli.

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