Cancer Immunol Immunother (2002) 51: 121–129
DOI 10.1007/s00262-001-0262-2

ORIGINAL ARTICLE

NaMi Cho · Pin-Ju Chueh · Chinpal Kim
Sara Caldwell · Dorothy M. Morré · D. James Morré

Monoclonal antibody to a cancer-specific and drug-responsive hydroquinone (NADH) oxidase from the sera of cancer patients

Received: 1 August 2001 / Accepted: 29 November 2001 / Published online: 27 February 2002

Abstract Monoclonal antibodies were generated in mice to a 34-kDa circulating form of a drug-responsive hydroquinone (NADH) oxidase with a protein disulfide–thiol interchange activity specific to the surface of cancer cells and the sera of cancer patients. Screening used Western blots with purified 34-kDa tNOX from HeLa cells and the sera of cancer patients. Epitopes were sought that inhibited the drug-responsive oxidation of NADH with the sera of cancer patients, but which had no effect on NADH oxidation with the sera of healthy volunteers. Two such antisera were generated. One, designated monoclonal antibody (mAb) 12.1, was characterized extensively. The NADH oxidase activity inhibited by mAb 12.1 also was inhibited by the quinone site inhibitor capsaicin (8-methyl-*N*-vanillyl-6-noneamide). The inhibition was competitive for the drug-responsive protein disulfide–thiol interchange activity assayed either by restoration of activity to scrambled RNase or by cleavage of a dithiodipyridine substrate, and was uncompetitive for NADH oxidation. Both the mAb 12.1 and the postimmune antisera immunoprecipitated drug-responsive NOX activity and identified the same 34-kDa tNOX protein in the sera of cancer patients that was absent from sera of healthy volunteers, and was utilized as immunogen. Preimmune sera from the same mouse as the postimmune antisera was without effect. Both mouse ascites containing mAb 12.1 and postimmune sera (but not preimmune sera) slowed the growth of human cancer cell lines in culture, but did not affect the growth of non-cancerous cell lines. Immunocytochemical and histochemical findings showed that mAb 12.1 reacted with the surface membranes of human carcinoma cells and tissues.

Keywords Monoclonal antibody · NADH (hydroquinone) oxidase · Pancancer (all forms of cancer) · tNOX

N. Cho · P.-J. Chueh · C. Kim · S. Caldwell · D.J. Morré (✉)
Department of Medicinal Chemistry
and Molecular Pharmacology,
Hansen Life Sciences Research Building,
Purdue University, West Lafayette, IN 47907, USA
Tel.: +765-494-1388
Fax: +765-494-4007

P.-J. Chueh · D.M. Morré
Department of Foods and Nutrition,
Purdue University, West Lafayette, IN 47907, USA

Introduction

Our laboratory has described a drug-responsive enzymatic activity of the cancer cell surface, referred to as tNOX, that catalyzes both NADH oxidation (NOX) [8] and protein disulfide–thiol interchange [19]. While NOX activity has been measured most often in terms of oxidation of NADH [9], the physiological substrates appear to be hydroquinones of the plasma membrane, such as reduced coenzyme Q [5].

Human carcinoma cells (HeLa and BT-20) exhibit two forms of NADH oxidase activity. One is inhibited by quinone site inhibitors such as the vanilloid capsaicin (8-methyl-*N*-vanillyl-6-noneamide) [13, 17] or the antitumor sulfonylurea *N*-(4-methylphenylsulfonyl)-*N*′-(4-chlorophenyl) urea (LY181984) [14]. This drug-inhibited and tumor-associated tNOX protein in the sera of cancer patients was shown to reside with a 34-kDa sulfonylurea- and capsaicin-binding protein [15] found on the external cell surface [7]. It is shed into the culture media [18, 24] and into the sera [12, 20]. The sera of cancer patients display an activity (tNOX) corresponding to the capsaicin- [20] or sulfonylurea- [12] inhibited NADH oxidase of the cancer cell surface.

The serum form of tNOX exhibits the same degree of drug responsiveness as the cell surface membrane-associated form [12, 20]. Pooled sera from patients with a variety of cancers including leukemias, lymphomas and solid carcinomas have a drug-responsive NADH oxidase activity [12, 20] corresponding to a 34-kDa tNOX protein that is absent from the sera of healthy volunteers or patients not diagnosed as having cancer. A constitutive

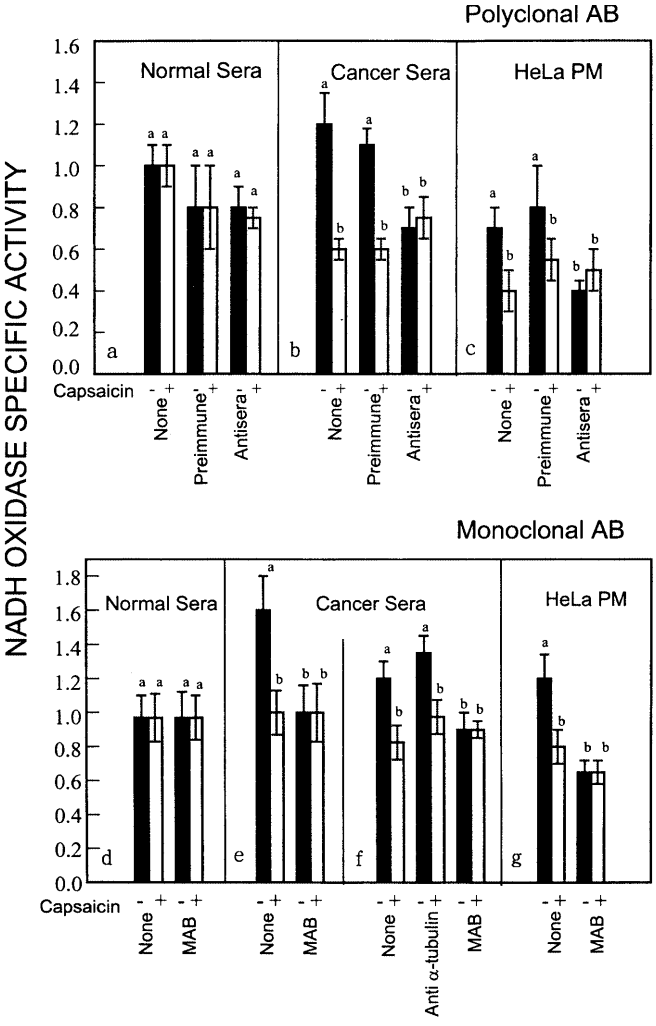

Fig. 1 NADH oxidase specific activity expressed in nmol NADH oxidized min^{-1} 100 μl^{-1} in the pooled sera of healthy volunteers (**a**) and that of of cancer patients (**b**, **e**, **f**) or expressed as NADH oxidized min^{-1} mg $protein^{-1}$ for plasma membranes from HeLa cells (**c**, **g**). Unfractionated crude postimmunization sera (2.5 µl) were added to the 2.5-ml enzymatic reaction in **a**--**c**. In **d**–**g**, 5 µg mAb IgG was added to the 2.5-ml enzymatic reaction (dilution of 1:1,000). Values are from three independent determinations ± SD. Values not followed by the same letter are significantly different ($P < 0.003$). The open bars denote the presence of 1 µM capsaicin (+). The solid bars indicate its absence (–)

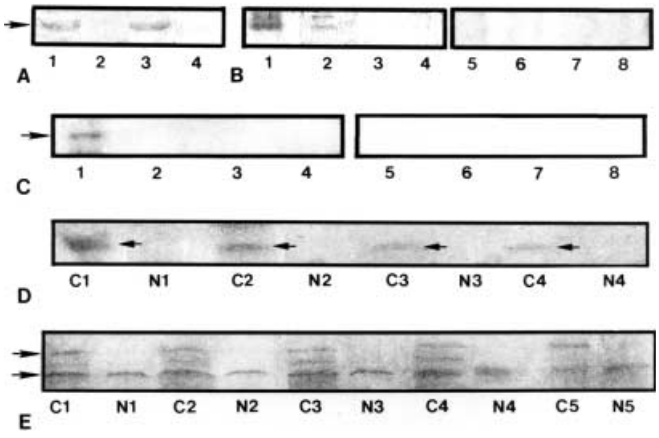

Fig. 2A–E Western blot analyses of 34-kDa tNOX (*arrows*). **A**, **B** Unfractionated postimmunization antisera (180 µg; 1:1,000). **A** *Lanes 1 and 2*: HeLa cells (20 µl of 0.5 µg/µl). *Lanes 3 and 4*: tNOX-enriched fraction from HeLa cells used for mAb screening. *Lanes 1 and 3*: postimmunization sera. *Lanes 2 and 4*: preimmunization sera. **B** Immunoprecipitates comparing pooled sera from cancer patients (*lanes 1, 2, 5 and 6*) or sera from healthy volunteers (*lanes 3, 4, 7 and 8*). *Lanes 1–4*: post-immunization sera. *Lanes 5–8*: preimmunization sera. **C–E** 5 µg mAb 12.1 IgG (1:1,000). **C** Immunoprecipitation with mAb 12.1 (1:250) (*lanes 1–4*) or ascites generated only by implantation of unstimulated myeloma cells (*lanes 5–8*). *Lanes 1, 3, 5 and 7*: pooled sera from cancer patients. *Lanes 2, 4, 6 and 8*: sera from healthy volunteers. **D** Sera from individual cancer patients (C) or healthy volunteers (N) (3 µl of 1:10 diluted 5 µg/µl sera). *Arrows* indicate the 34-kDa component. Cancers represented were: breast (C1), prostate (C2), ovarian (C3), and leukemia (C4). N1 to N4: sera from healthy volunteers. **E** Sera were first delipidated with Cleanascite (CPG, Lincoln Park, N.J.) and treated with proteinase K (200 µg/ml for 2 h at 37°C followed by heating to 70°C for 20 min to inactivate proteinase K). Cancers represented were: prostate (C1), lymphoma (C2), ovarian (C3), leukemia (C4) and breast (C5). N1 to N5: sera from healthy volunteers. The common band at ca. 29 kDa (*lower arrow*) was proteinase K that cross-reacts unspecifically with mAb 12.1 at the large amounts of proteinase K added to provide an index of uniformity of loading

NADH oxidase (CNOX) with pooled sera from healthy volunteers is not inhibited either by capsaicin or by LY181984. The property of inhibition by capsaicin has been utilized to monitor the isolation and purification of the capsaicin-inhibited activity from the sera of cancer patients [2]. Serum activity was characterized by the following: molecular weight (34 kDa); isoelectric point (pH 5.6); cofactor requirements (none); sensitivity to thiol reagents (inhibited); resistance to proteases [4]; and conditions for assay [2, 3, 7, 10, 13, 14, 15, 17, 24]. Plasma membranes isolated from non-transformed cells [13, 16, 17] or from sera from healthy individuals [12, 20] lacked biochemically detectable levels of drug-inhibited activity, but expressed the constitutive CNOX form of the activity that was drug- [13] and thiol reagent-[10] resistant. In the present report, we describe the generation of a mAb to the 34-kDa peptide associated with the shed form of tNOX activity obtained from the sera of cancer patients [2].

Materials and methods

Sera

Sera were selected at random from patient populations with active metastatic disease treated at the Michiana Hematology-Oncology Clinic (South Bend, Ind.) or from patients at the St. Elizabeth Medical Center (Lafayette, Ind.) and frozen as previously described [12, 20].

Enzyme assays

NADH oxidation was measured spectrophotometrically from the disappearance of NADH measured at 340 nm [13]. Protein disulfide–thiol interchange was determined either by restoration of

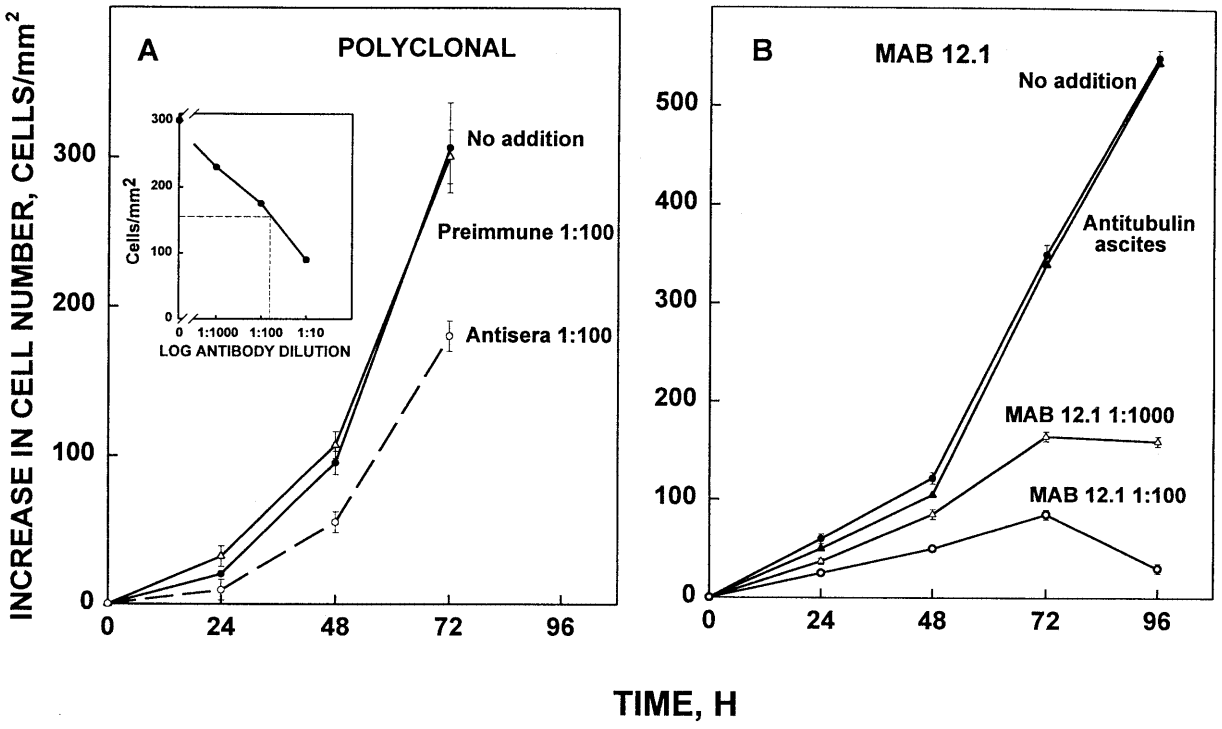

Fig. 3A, B HeLa cell growth. **A** Unfractionated postimmunization antisera added once at the beginning of the experiment (*open circles*) but not preimmune sera (*open triangles*) had an inhibitory effect compared to no addition (*solid circles*) after 72 h at a dilution of 1:100 (72 μg total protein/μl). A dilution of about 1:200 elicited 50% inhibition (*inset*). **B** mAb 12.1 at a concentration of 2 μg/ml diluted 1:100 (*open circles*) or 1:1,000 (*open triangles*) resulted in dead cells at both dilutions by 168 h. Results are the averages of two independent experiments ± SD. Growth of HeLa cells was unaffected by ascites containing mAb to α-tubulins generated in parallel (1:1,000 dilution) (*solid triangles*) compared to no addition (*solid circles*)

activity to scrambled and inactive RNase prepared from native RNase A as described with cCMP as the RNase substrate [6], or spectrophotometrically from the cleavage of the dithiodipyridine substrate, dipyridyldithionicotinic acid (DTNA) [21].

Proteins were estimated by the bicinchoninic acid (BCA) procedure [23] with bovine serum albumin (BSA) as standard. Plasma membrane vesicles were isolated by aqueous two-phase partitioning [7, 13, 14].

Isolation of antigen

tNOX antigen was purified from the pooled sera of cancer patients with active metastatic disease as previously described [2]. The purified tNOX proteins were further separated by preparative sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Diced gel bands containing the activity were macerated in 0.5 μl Freund's complete adjuvant (FCA) and 0.5 ml phosphate-buffered saline (PBS), homogenized by forcing through a 22-gauge and then an 18-gauge needle and used as antigen.

Production of hybridomas

Two female BALB/C mice, aged 10 weeks, were injected subcutaneously with 10–20 μg of the purified tNOX emulsified in FCA in a total volume of 0.4 ml according to the following schedule: first injection, 20 μg of antigen, boost 1 (1 week, 7.5 μg); boost 2 (2 weeks, 1.5 μg); boost 3 (7 weeks, 1.5 μg). Boosts 4 to 8 with 1 μg antigen each were spaced at intervals of 3 to 5 weeks. The final boost was with 1.5 μg antigen in 0.2 ml of PBS in the absence of adjuvant injected intravenously and intraperitoneally. Hybridomas (the fusions were with SP 2 myeloma cells) and ascites were generated in the Monoclonal Antibody Facility of the Purdue Cancer Center following standard protocols [22].

Hybridoma fusion screening

Cell-free conditioned medium supernatants of fusions were screened for tNOX antibody-producing hybridomas by Western blot analyses with partially purified tNOX protein from HeLa cells [3] resolved by SDS–PAGE and transferred onto nitrocellulose membranes as antigen. Additionally, positive clones from primary screening reactive on Western blots were screened further on the basis of the inhibition of the drug-responsive NADH oxidase activity with the sera of cancer patients, and no inhibition with the sera from healthy volunteers. The clones selected recognized both cell surface and serum forms of tNOX. The initial titers were 20 mg IgG/ml. Later titers were much less, i.e., 2 mg/ml for the last major ascites production (see Discussion). The antibodies were purified and antibody amounts were based on amounts of IgG corresponding to purified monoclonal antibodies (mAb). Immunoblotting and immunoprecipitation were as previously described [24].

Growth measurements and determination of apoptosis

Attached cells were grown and treated in 35×10-mm plastic dishes in 2.5 ml culture medium and analyzed for apoptosis as described [13].

Results

Pooled sera from cancer patients (Fig. 1b, e, f) or plasma membranes from HeLa cells (Fig. 1c, g) displayed NADH oxidase activity, a component of which (up to

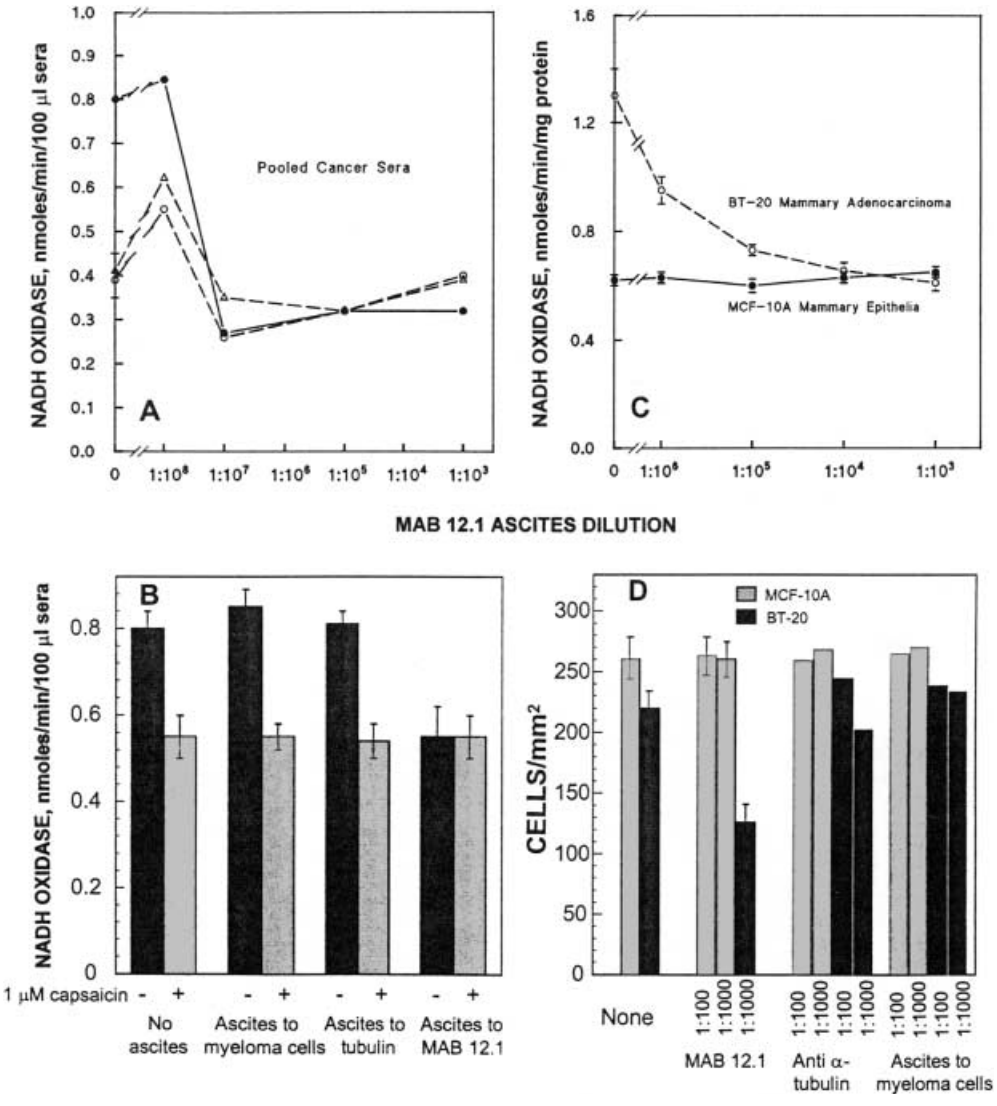

Fig. 4 Inhibition of NADH oxidase activity (**A**–**C**) and cell proliferation (**D**) by mAb 12.1 (20 µg antibody/µl). **A** Pooled sera from cancer patients. *Solid symbols*: no capsaicin; *open circles*: 1 µM capsaicin; *open triangles*: 100 µM capsaicin. **B** Supernatants from immunoprecipitates of pooled sera from cancer patients as in Fig. 2. Three experiments ± SD. **C** Plasma membrane vesicles from BT-20 human mammary adenocarcinoma and MCF-10 A human mammary epithelia (non-cancer). Ascites containing mAb to α-tubulins were without effect (not shown). Two experiments ± mean average deviation. **D** As described for Fig. 3. Inhibition was significantly different ($p > 0.003$) from no treatment or ascites containing α-tubulin mAb (2.5 µl of 2.4 µg IgG/µl). Ascites generated only as a result of implantation of SP 2 myeloma cells at a comparable volume and protein amount as for mAb 12.1 were without effect. No response was observed with MCF-10 A cells which lack tNOX

50% of the total activity) was inhibited by capsaicin at a final concentration of 1 µM. NADH oxidation with pooled sera from healthy volunteers (Fig. 1a, d) was not inhibited by capsaicin.

Characterization of mouse postimmunization antisera

To permit a comparison with preimmunization sera, results with crude postimmunization antisera were included along with those of the mAb. These comparisons are important to establish the cancer specificity of the antibodies generated, especially for enzyme inhibition and immunoprecipitation experiments where lack of effect of preimmunization sera provides a compelling argument for antibody specificity that is not possible with mAb 12.1 alone. Anti-tNOX antisera (Fig. 1a–c) generated by both immunized mice, inhibited the drug-responsive component of NADH oxidase in the sera of cancer patients to the same extent as did 1 µM capsaicin (Fig. 1b). Postimmunization antisera and preimmunization sera were similar in their effects on NOX activity in the sera from healthy volunteers (Fig. 1a). The postimmunization antisera but not the preimmunization sera blocked NADH oxidase activity of plasma membrane vesicles from HeLa cells (Fig. 1c).

Reacting specifically with the postimmunization antisera on Western blots but not with the preimmunization sera (Fig. 2A) was a 34-kDa component of the plasma membranes of HeLa cells as well as a 34-kDa component of a NADH oxidase-enriched fraction from the HeLa cell surface corresponding to the antigen used for immunization. A corresponding 34-kDa protein was immunoprecipitated with the postimmunization antisera from the sera of cancer patients (Fig. 2B; lane 1) but not from the sera of healthy volunteers (Fig. 2B; lane 3). With preimmunization sera, no 34-kDa band was immunoprecipitated (Fig. 2B; lanes 5–8).

When added to the culture media, the postimmunization antisera (Fig. 3A) inhibited the proliferation of HeLa cells, as did the mAb (Fig. 3B). At a dilution of

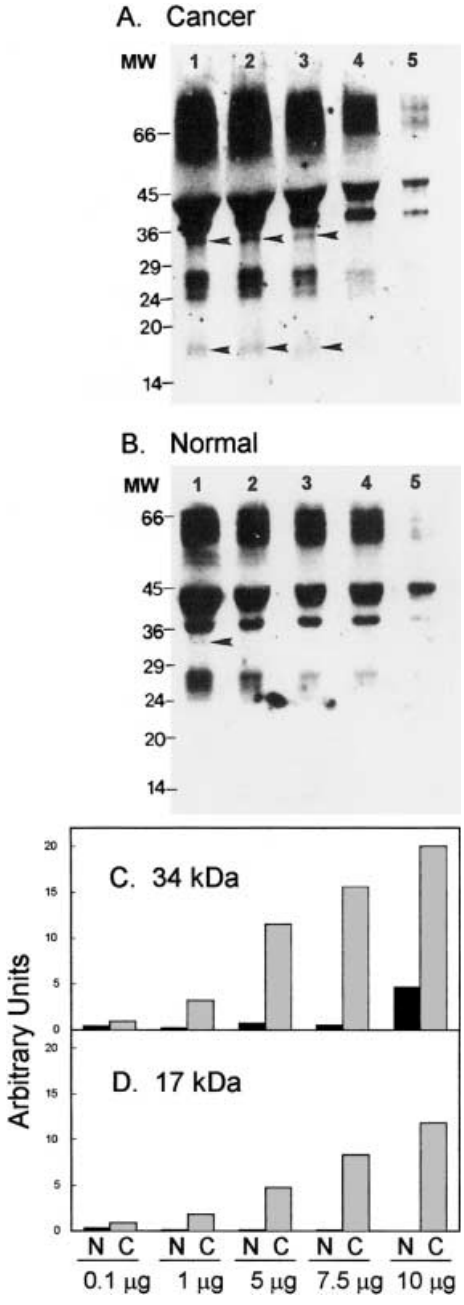

Fig. 5A, B Western blot analysis (mAb 12.1, 2 µg/µl dilution of 1:250) of proteinase K (1.4 µg/ml; overnight at room temperature)- and heat -treated (50°C, 1 h) pooled sera from cancer patients (**A**) or sera from healthy volunteers (**B**) using a chemiluminescent method necessary to allow for quantitation over the wide range of serum dilutions reported. Only the bands at 34 and 17 kDa reacted specifically with mAb 12.1. Other components present reacted with the chemiluminescence reagents to the same extent in either the presence or the absence of antibody. Total serum protein: *lane 1*: 10 µg; *lane 2*: 7.5 µg; *lane 3*: 5 µg; *lane 4*: 1 µg; *lane 5*: 0.1 µg. **C,D** Quantitation of 34 kDa (**C**) or 17 kDa (**D**) bands

1:100, the postimmunization antisera reduced the cell numbers by 40% after 72 h of treatment (Fig. 3A). Preimmunization sera or PBS (no addition) were without effect on HeLa cell proliferation. The proliferation response of HeLa cells was log-linear with respect to antisera dilution (Fig. 3A; insert), with an EC_{50} of about 1:200.

Monoclonal antibody screening

mAb 12.1 and 12.5 were selected from a total of 35 primary clones after screening with Western blots based on NADH oxidase inhibition of the activity of sera from cancer patients and the lack of effect on NADH oxidase activity of sera from healthy volunteers (Fig. 1d–f). The two clones, designated 12.1 and 12.5, both stereotyped as IgG, were equivalent. All antibody amounts were based on IgG content.

mAb 12.1 ascites inhibited NOX activity of sera from cancer patients to the same extent as capsaicin (Fig. 1e, f) and was without effect on NADH oxidase activity of sera from healthy volunteers (Fig. 1d). Ascites containing mAb to α-tubulins generated with the same strain of mouse by a procedure comparable to that used to generate mAb 12.1 were without effect (Fig. 1f). The initial mouse ascites containing mAb 12.1 blocked the capsaicin-inhibited component of the NOX activity of pooled sera from cancer patients at a dilution of $1{:}10^7$ (Fig. 4A).

mAb 12.1 quantitatively immunoprecipitated the capsaicin-inhibited activity of pooled sera from cancer patients (Fig. 4B). Ascites generated only in the presence of unstimulated myeloma cells or containing mAb to α-tubulins raised as for mAb 12.1 were ineffective in immunoprecipitating tNOX (Fig. 4B).

mAb reacts with a 34-kDa component of sera from cancer patients on western blots

Sera from individual cancer patients or healthy volunteers were evaluated for the presence of the 34-kDa processed tNOX protein by Western blot analysis using mAb 12.1-containing ascites. The four samples of patient sera (from patients with ovarian cancer, prostate cancer, leukemia and lymphoma) revealed discernible 34-kDa bands, whereas corresponding immunoreactive bands at 34 kDa were not found in the sera from four healthy volunteers (Fig. 2D). The results were clearer when the sera were first delipidated, and total protein loading was reduced by pretreatment with heat and proteinase K (Fig. 2E).

mAb 12.1 inhibits plasma membrane tNOX and the growth of transformed cells but neither CNOX nor the growth of non-transformed cells

mAb 12.1 (at a dilution of 1:1,000 with 5 µg antibody in 2.5 ml of culture medium) slowed the growth and eventually killed HeLa cells after 72 h by apoptosis (Fig. 3B). Control ascites with α-tubulin as antigen or control ascites taken from the same strain of mice inoculated with unstimulated myeloma cells were not cytotoxic.

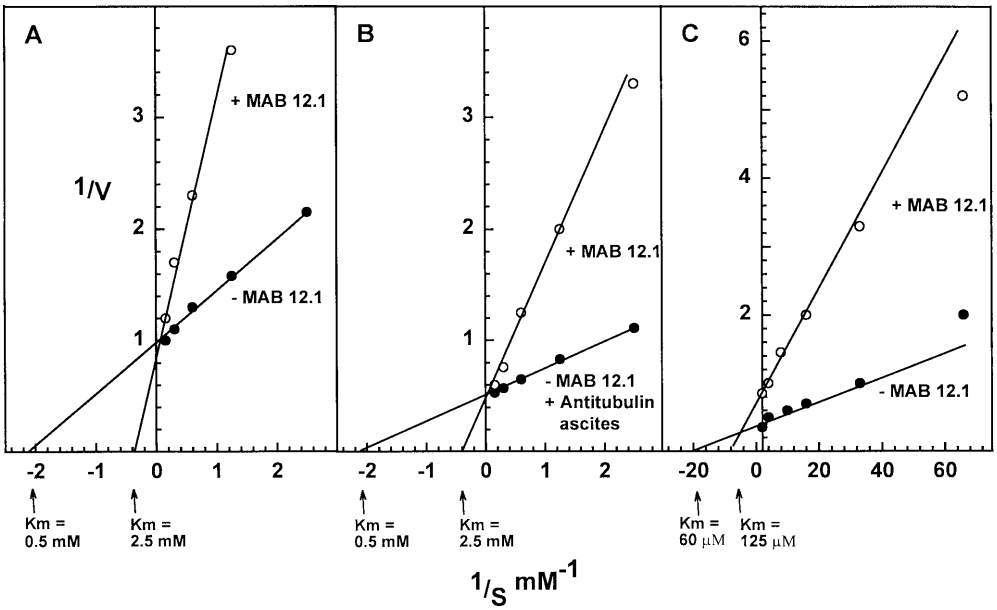

Fig. 6A–C Double reciprocal kinetic analysis of activity of HeLa plasma membranes in the presence and absence of mAb 12.1 at a final dilution of 1:1,000 (2.5 μl of 2 μg/μl antibody concentration in 2.5 ml total reaction mixture). **A** Protein disulfide–thiol interchange with dithiodipyridine substrate [21]. The apparent K_m at a higher substrate concentrations compared to no antibody and no effect on maximum velocity (same ordinate intercept) indicated competitive inhibition. **B** As in **A** except the mAb 12.1 assays were carried out in the presence of ascites containing mAbs to α-tublins. **C** NADH oxidation. Both the apparent K_m and the maximum velocity were changed relative to those for no antisera indicative of uncompetitive inhibition. Units of specific activity are nmol DTNA cleaved min^{-1} mg $protein^{-1}$ for **A** and **B** and nmol NADH oxidized min^{-1} mg $protein^{-1}$ for **C**

mAb 12.1 inhibited NOX activity (Fig. 4C) and growth (Fig. 4D) of human mammary adenocarcinoma (BT-20) cells, but did not inhibit NOX activity or the growth of non-malignant MCF-10 A (mammary epithelial), 3T3 (mouse fibroblast) or WI-38 (human diploid fibroblast) cell lines.

Even the hybridoma cells producing ascites generated by fusion of the mouse splenocytes with SP 2 myeloma cells grew slowly, and their growth was prevented if antibody titers reached critical levels. This response of hybridoma cells to the mAb 12.1 of ascites represented an obstacle for the large-scale production of ascites. The highest mAb titers were achieved in culture medium concentrates conditioned by the growth of hybridoma cells in a Cellmax system where secreted antibodies were removed continuously by the circulating culture medium.

Quantitation of immunoreactive tNOX in sera

After proteinase K digestion, the relative amounts of the band at 34 kDa and an additional band at 17 kDa, representing a product of extended proteinase K cleavage that retains enzymatic activity [3, 4], showed a proportional increase as the amount of protein loaded was varied between 0.1 and 10 μg per lane (Fig. 5). The amount of the 34-kDa component in the sera of cancer patients (Fig. 5A, C) was at least 10-fold higher than that in the sera of healthy volunteers (Fig. 5B). With the 17-kDa component, the absorbance in the 17 kDa region in the sera of healthy volunteers was at background levels (Fig. 5D).

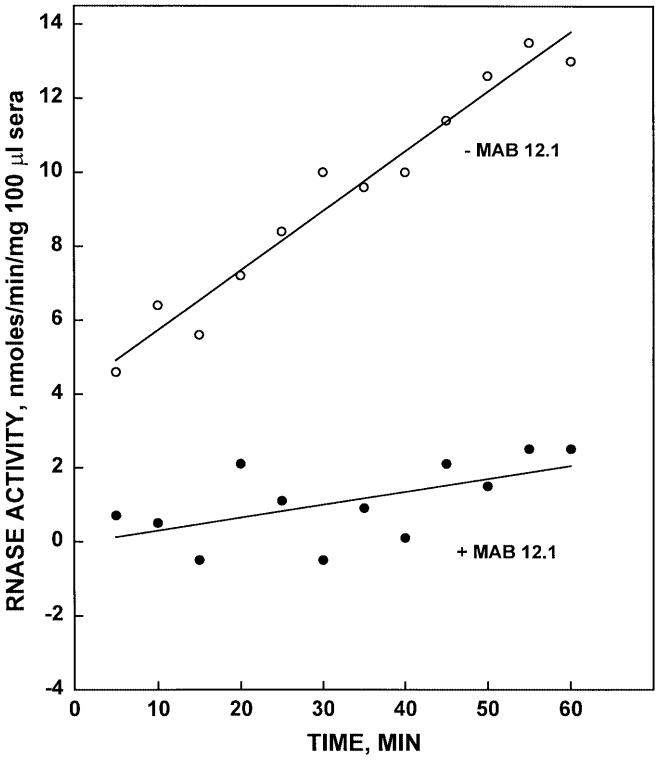

Fig. 7 Time course of protein disulfide–thiol interchange activity of pooled sera from cancer patients estimated from the activation of scrambled RNase in the presence of cCMP as substrate [6]. The mAb 12.1 was diluted 1:1,000 (2.5 μl of 2 μg/μl IgG in a total reaction volume of 2.5 ml)

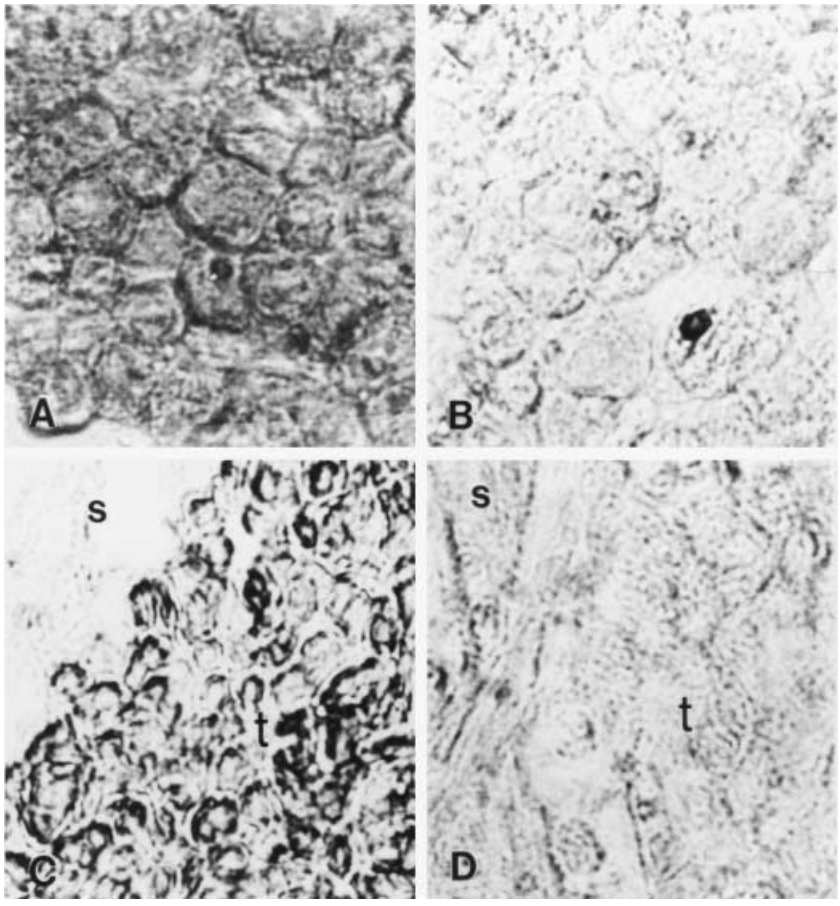

Fig. 8 Reaction of mAb 12.1 with cell borders of formalin-fixed (10% neutral formalin) and paraffin-embedded HeLa cells (**A**, **B**) or human renal carcinoma (**C**, **D**) reacted with affinity purified IgG from culture media of mAb 12.1 hybridoma grown in a Cellmax system. Deparaffinized slides were hydrated, treated for 5 min with hydrogen peroxide and carried through an antigen retrieval protocol (0.01 M sodium citrate, pH 6.0; microwave 700 W for 5 min). Tissue sections were covered with normal goat serum followed by mAb diluted to achieve a final antibody concentration of ~0.2 μg/μl (**A, C**) or Tris buffer (**B**, **D**). Slides were incubated 1 h at room temperature. After incubation, Super Sensitive MultiLink was added (5 min) followed by Super Sensitive Label (5 min). In **C** and **D**, the surrounding tissues (s) were not immunoreactive whereas cell surfaces were stained both with the HeLa cells and the tumor tissue (*t*). Visualization was carried out with alkaline phosphatase-conjugated streptavidin and New Fuscin substrate. Magnification ×1,000

mAb inhibits tNOX protein disulfide–thiol interchange activity

mAb 12.1 was a competitive inhibitor of protein disulfide–thiol interchange both in the plasma membranes from HeLa cells (Fig. 6A, B) and in the sera of patients with breast cancer. Ascites containing mAb to α-tubulins did not compete (Fig. 6B). With NADH, the competition was uncompetitive (Fig. 6C). The inhibition by mAb 12.1 of the tNOX-catalyzed restoration of activity to scrambled and inactive ribonuclease [19] was nearly complete (Fig. 7).

Immunohistochemistry

mAb 12.1, when presented to HeLa S cells, was localized to the cell surface (Fig. 8A). In the absence of mAb, there was no significant signal (Fig. 8B). Also stained were the cell borders of renal (Fig. 8C, D), colon and ovarian carcinomas in paraffin-embedded pathological specimens. The borders of the surrounding stromal cells were unstained. For cytological reaction, it was necessary first to restore cell surface NADH oxidase activity (1 μM GSH for 10 min followed by 0.03% hydrogen peroxide or 100 μM oxidized glutathione for 10 min, both in the presence of 150 μM NADH).

Discussion

The mAb-producing clones selected, 12.1 and 12.5, produced mAb specific for the 34-kDa protein at the surface of cancer cells and the 34-kDa protein shed into the sera of cancer patients initially at a working dilution of ~$1{:}10^7$ (Fig. 4A). The specificity of the antisera was confirmed by the inhibition of activity (Fig. 1), immunoprecipitation and Western blot analyses (Fig. 2). The antibodies acted as classical competitive inhibitors of the capsaicin-inhibited activity, as revealed by kinetic analyses using a dithiodipyridine substrate for the protein disulfide–thiol interchange activity of

the tNOX protein (Fig. 6A,B). With NADH as substrate this activity also was inhibited, but the inhibition was uncompetitive (Fig. 6C). Thus, the mAb appears to react at or near the cancer-specific drug site of the protein, and apparently only when the protein is in an enzymatically active and non-denatured conformation. The screening procedure was designed to select for such an epitope.

Findings with drugs targeted to NADH oxidase first suggested the role of the NOX proteins in growth [8]. The inhibited cells failed to enlarge following cytokinesis [11], and as a result [1] did not reenter mitosis. After several days of growth arrest, the cells underwent apoptotic cell death. The response to mAb 12.1 was similar to that observed with capsaicin [13] or with LY181984 [14]. The NOX proteins are externally located [7] so that the mAb did not need to enter cells for inhibition to occur.

The specificity of cell killing was demonstrated from comparisons of the response of BT-20 human mammary adenocarcinoma cells and the lack of response of MCF-10 A human mammary epithelia. The BT-20 cells contained a constitutively activated component of NADH oxidase activity, inhibited by capsaicin, that was absent from the surface of MCF-10 A cells [13]. Specificity (absence from normal cells and tissues) was further indicated by an apparent absence of tNOX in the sera of healthy volunteers and patients with diseases other than cancer. The presence of a tNOX band in the pooled sera from patients diagnosed as not having cancer at a level one-tenth of that in the pooled sera from cancer patients was consistent with previous studies where drug-responsive NOX activity was found in approximately 6% of sera from such patients [12] and could be considered as representing undetected neoplasms.

Acknowledgements We thank J. Wilder and D. Asai (Purdue University Cancer Center Monoclonal Antibody Facility) for assistance in hybridoma production, S. Wang (Department of Foods and Nutrition) for the preparation of antigens for hybridoma screening, C. Chalko and the Michiana Hematology-Oncology Clinic of South Bend, Indiana, for the provision of sera, L.-Y. Wu for cell culture and carrying out the growth experiments, D. Sedlak for assistance with the Western blots, W. Jacobson, MD (St. Elizabeth Hospital) for samples of sera and preliminary histological evaluations, D. Werderitsh for photography and P. Runck for assistance in preparing the manuscript.

References

1. Baserga R (1985) The biology of cell reproduction. Harvard University Press, Cambridge
2. Chueh P-J, Morré DJ, Wilkinson FE, Gibson J, Morré DM (1997) A 33.5-kDa heat- and protease-resistant NADH oxidase inhibited by capsaicin from sera of cancer patients. Arch Biochem Biophys 342:38
3. Del Castillo-Olivares A, Yantiri F, Chueh P-J, Wang S, Sweeting M, Sedlak D, Morré DM, Burgess J, Morré DJ (1998) A drug-responsive and protease-resistant peripheral NADH oxidase from the surface of HeLa S cells. Arch Biochem Biophys 358:125
4. Kelker M, Kim C, Chueh P-J, Guimond R, Morré DM, Morré DJ (2001) Cancer isoform of a tumor-associated cell surface NADH oxidase (tNOX) has properties of a prion. Biochemistry 40:7351
5. Kishi T, Morré DM, Morré DJ (1999) The plasma membrane NADH oxidase of HeLa cells has hydroquinone oxidase activity. Biochim Biophys Acta 1412:66
6. Lyles MM, Gilbert HF (1991) Catalysis of the oxidative unfolding of ribonuclease A by protein disulfide isomerase: dependence of the rate on the composition of the redox buffer. Biochemistry 30:613
7. Morré DJ (1995) NADH oxidase activity of HeLa plasma membranes inhibited by the antitumor sulfonylurea *N*-(4-methylphenylsulfonylurea)-*N*′-(chlorophenyl) urea (LY181984) at an external site. Biochim Biophys Acta 1240:201
8. Morré DJ (1998) NADH oxidase: a multifunctional ectoprotein of the eukarotic cell surface. In: Asard H, Bérczi A, Caubergs R (eds) Plasma membrane redox systems and their role in biological stress and disease. Kluwer Academic Publishers, Dordrecht, pp 121–156
9. Morré DJ, Brightman AO (1991) NADH oxidase of plasma membranes. J Bioenerg Biomemb 23:469
10. Morré DJ, Morré DM (1995) Differential response of the NADH oxidase of plasma membranes of rat liver and hepatoma and HeLa cells to thiol reagents. J Bioenerg Biomemb 27:137
11. Morré DM, Morré DJ (1995) Mechanism of killing of HeLa cells by the antitumor sulfonylurea *N*-(4-methylphenylsulfonylurea)-*N*′-(chlorophenyl)urea (LY181984). Protoplasma 184:188
12. Morré DJ, Reust T (1997) A circulating form of NADH oxidase activity responsive to the antitumor sulfonylurea *N*-(4-methylphenylsulfonylurea)-*N*′-(chlorophenyl) urea (LY181984) specific to sera from cancer patients. J Biomemb Bioenerg 29:281
13. Morré DJ, Chueh P-J, Morré DM (1995) Capsaicin inhibits preferentially the NADH oxidase and growth of transformed cells in culture. Proc Natl Acad Sci USA 92:1831
14. Morré DJ, Wu LY, Morré DM (1995) The antitumor sulfonylurea *N*-(4-methylphenylsulfonylurea)-*N*′-(chlorophenyl) urea (LY181984) inhibits NADH oxidase activity of HeLa plasma membrane. Biochim Biophys Acta 1240:11
15. Morré DJ, Wilkinson FE, Lawrence J, Cho N, Paulik M (1995) Identification of antitumor sulfonylurea binding proteins of HeLa plasma membranes. Biochim Biophys Acta 1236:237
16. Morré DJ, Merriman R, Tanzer R, Wu L-Y, Morré DM, MacKellar WC (1995) Inhibition of the NADH oxidase activity of plasma membranes isolated from xenografts and cell lines by the antitumor sulfonylurea *N*-(4-methylphenylsulfonylurea)-*N*′-(chlorophenyl) urea (LY181984) correlates with drug susceptibility of growth. Protoplasma 188:151
17. Morré DJ, Sun E, Geilin C, Wu L-Y, de Cabo R, Krasagakis K, Orfanos CE, Morré DM (1996) Capsaicin inhibits plasma membrane NADH oxidase and growth of human and mouse melanoma lines. Eur J Cancer 32:1995
18. Morré DJ, Wilkinson FE, Kim C, Cho N, Lawrence J, Morré DM, McClure D (1996) Antitumor sulfonylurea-inhibited NADH oxidase of cultured HeLa cells shed into media. Biochim Biophys Acta 1280:197
19. Morré DJ, Jacobs E, Sweeting M, de Cabo R, Morré DM (1997) A protein disulfide–thiol interchange activity of HeLa plasma membranes inhibited by the antitumor sulfonylurea *N*-(4-methylphenylsulfonylurea)-*N*′-(chlorophenyl) urea (LY181984). Biochim Biophys Acta 1325:117
20. Morré DJ, Caldwell S, Mayorga A, Wu L-Y, Morré DM (1997) NADH oxidase from sera of cancer patients is inhibited by capsaicin. Arch Biochem Biophys 342:224
21. Morré DJ, Gomez-Rey ML, Schramke C, Em O, Lawler J, Hobeck J, Morré DM (1999) Use of dipyridyl-dithio substrates to measure directly the protein disulfide–thiol interchange activity of the auxin stimulated NADH: protein disulfide reductase (NADH oxidase) of soybean plasma membranes. Mol Cell Biochem 207:7

22. Schook LB (1987) Monoclonal antibody production techniques and applications. Immunology series, vol. 3. Marcel Dekker, NewYork, pp 99–101
23. Smith PK, Krohn RI, Hermanson GT, Mailia AK, Gartner FH, Provenzano MD, Fujimoto EK, Goeke NM, Olson BJ, Klenk DC (1985) Measurement of protein using bicinchoninic acid. Anal Biochem 150:70
24. Wilkinson F, Kim C, Cho N, Chueh P-J, Leslie S, Moya-Camarena S, Wu L-Y, Morré DJ, Morré DM (1996) Isolation and identification of a protein with capsaicin-inhibited NADH oxidase activity from culture media conditioned by growth of HeLa cells. Arch Biochem Biophys 336:275