Polyclonal Antimetallothionein Antibodies: Isolation, Specificity, and Immunospecific Staining on Histological Specimens

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Highly specific polyclonal rabbit antimetallothionein antibodies are isolated and characterized. The possibility of using these antibodies in immunohistochenical assay was verified on normal and tumor mammary tissues, the common metallothionein-positive models.

Key Words: *metallothioneins; polyclonal antibodies; specificity; immunohistochemistry*

Metallothioneins (MT) is a family of inducible cytoplasmic proteins (6.5 kD) consisting of 61 amino acid residues, of them 20 strictly arranged cystein residues are responsible for unique metal-binding properties [7]. MT-positive cells were immunochemically detected in various organs and tumors [1,5, 13]. It was shown that MT participate in the storage and metabolism of essential metals (Zn, Cu, Mn, etc.) and elimination of toxic metals (Cd, Pb, Hg, etc.) [2]. MT possess antioxidant activity and protect cells and organisms from ionizing and UV radiation, chemical agents, and other inductors of free radical processes [2,11].

In the present study we describe isolation of rabbit polyclonal anti-MT antibodies and analysis of their specificity by enzyme-linked immunosorbent assay (ELISA) and demonstrate the possibility of using these antibodies for immunolocalization of MT in histological sections.

MATERIALS AND METHODS

Random-bred male rats weighing 180±20 g were subcutaneously injected with $CdCl_2$ (0.2 ml, Chemapol) in doses of 1 mg/kg on day 1 and 3 mg/kg during the subsequent 3 days. One day after the last injection, the liver was removed and homogenized, and MT fraction was precipitated with alcohol and lyophilized [4]. The MT fraction was purified on a Sephadex G-75 preparative G-75 column (Pharmacia), the MT-peak fractions were pooled, lyophilized, dissolved and desalted on a Sephadex G-10 column (Pharmacia). MT was measured spectrophotometrically (Philips) (ε_{220}=48,200 $M^{-1}cm^{-1}$ at pH 1) [14].

MT aggregates prepared by incubation with an excess of glutaraldehyde (Serva) [6] were used for immunization. The immunogen consisting of insoluble MT aggregates was resuspended by passing through a syringe and dialyzed against 20-fold volume of 0.01 M boron-borate buffer, pH 8.5 at 4°C for 24 h and stored at -40°C. Four Chinchilla rabbits weighing 2-2.5 kg were subcutaneously injected with 2 ml immunogen in Freund's complete adjuvant (1:1, Calbiochem, multipoint injection regimen). The doses of MT for the 1st, 2nd (after 30 days), and 3rd (after 90 days) were 1.5, 2, and 3.1 mg per rabbit, respectively. Blood was sampled on days 44, 60, and 100 after the first immunization.

The antigen was adsorbed on polystyrene plates (Nunc). To this end, 0.1 ml/well (0.2 μg/ml) MT in 0.1 M carbonate buffer (pH 9.6) was incubated in the plates at 4°C for 18 h. At all stages of ELISA, free components were removed by 3-fold washout with a buffer containing 0.01 M phosphate buffer saline (pH 7.2), 0.85% NaCl, 0.05% Triton X-100

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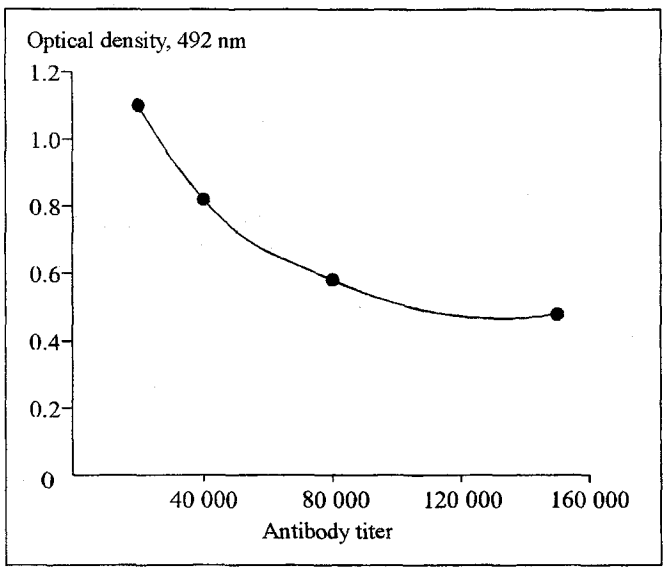

Fig. 1. Binding of antimetallolathionein antibodies to immobilized metallothionein.

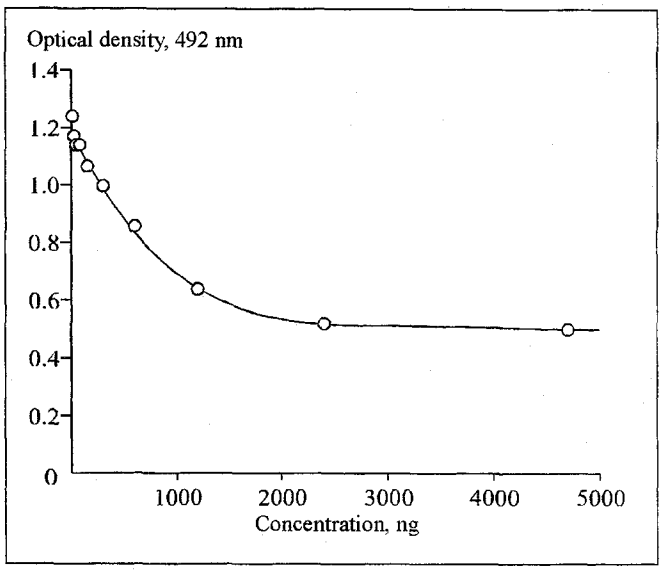

Fig. 2. Standard curve of enzyme-linked immonosorbent assay for metallothionein.

(Sigma), 0.01% sodium azide, and 0.01% bovine serum albumin (Serva). Optimal dilution of anti-MT for ELISA was determined by test binding of various anti-MT concentration to immobilized MT. Calibration curve was constructed using competitive ELISA. To this end, 0.1 ml mixture of MT standards and diluted 1:20,000 anti-MT (1:1) were incubated for 18 h at room temperature and transferred to wells with immobilized MT. The plates were incubated for 30 min at 37°C, washed, and incubated with 0.1 ml peroxidase-conjugated anti-rabbit IgG (1:100) at 37°C for 30 min. The reaction was assessed photometrically (492 nm) using o-phenylene diamine as a chromogen substrate.

Immunohistochemistry with anti-MT antibodies was performed on mammary gland tumor samples obtained during surgery. The samples from the boundary between the tumor and normal tissue were fixed in Bouin's fluid for 24 h and embedded in paraffin. Histological sections (7 μ) were mounted onto poly-L-lysine-treated slides (Sigma) and deparaffinized. Endogenous peroxidase was blocked by 0.3% H_2O_2 in Coons physiological saline (CPS) containing 0.01 M phosphate buffer (pH 7.4) followed by washout with CPS containing 0.2% Triton X-100. The sections were incubated with anti-MT (from 1:500 to 1:8000) in a humidified chamber at 4°C for 18 h. Further procedures (3-fold washout with CPS, incubation with biotinylated anti-rabbit and streptavidin-peroxidase complex, and visualization of the marker enzyme with diaminobenzidine and hydrogen peroxide) were performed according to Super Sensitive Anti-rabbit Immunostaining Kit manual (BioCenex). Finally, the sections were washed with CPS and distilled water, dehydrated in alcohols, treated with xylene and embedded in Entellan (Merck). To control the specificity of immunohistochemical reaction, anti-MT was substituted with normal rabbit serum in the corresponding dilutions.

RESULTS

Production of antibodies against monomeric MT (titer from 1:1 to 1:4) was detected in Ouchterlony immunodiffusion test as soon as after the 2nd injection of immunogen (on day 44 after first immunization). On day 100 of immunization (after the 3rd injection) the antibody titer attained 1:8. Figures 1 and 2 present binding curve and standard curve of competitive ELISA for MT, respectively. The sensitivity of ELISA for NT was no less than 40 ng. The major serum proteins albumin and hemoglobin in concentrations of 250 μg/ml and 16 mg/ml had no effect on the binding of anti-MT to immobilized MT, which confirms the specificity of anti-MT.

TABLE 1. Intensity of Immunostaining in Myoepithelial Cells and Background Staining at Different Anti-MT Dilutions

Dilution of anti-MT	Intensity of reaction	
	immunostaining	background
1:500	Intense	Quite intense
1:1000	Intense	Moderate
1:2000	Intense	Practically absent
1:4000	Quite intense	Absent
1:8000	Weak	Absent

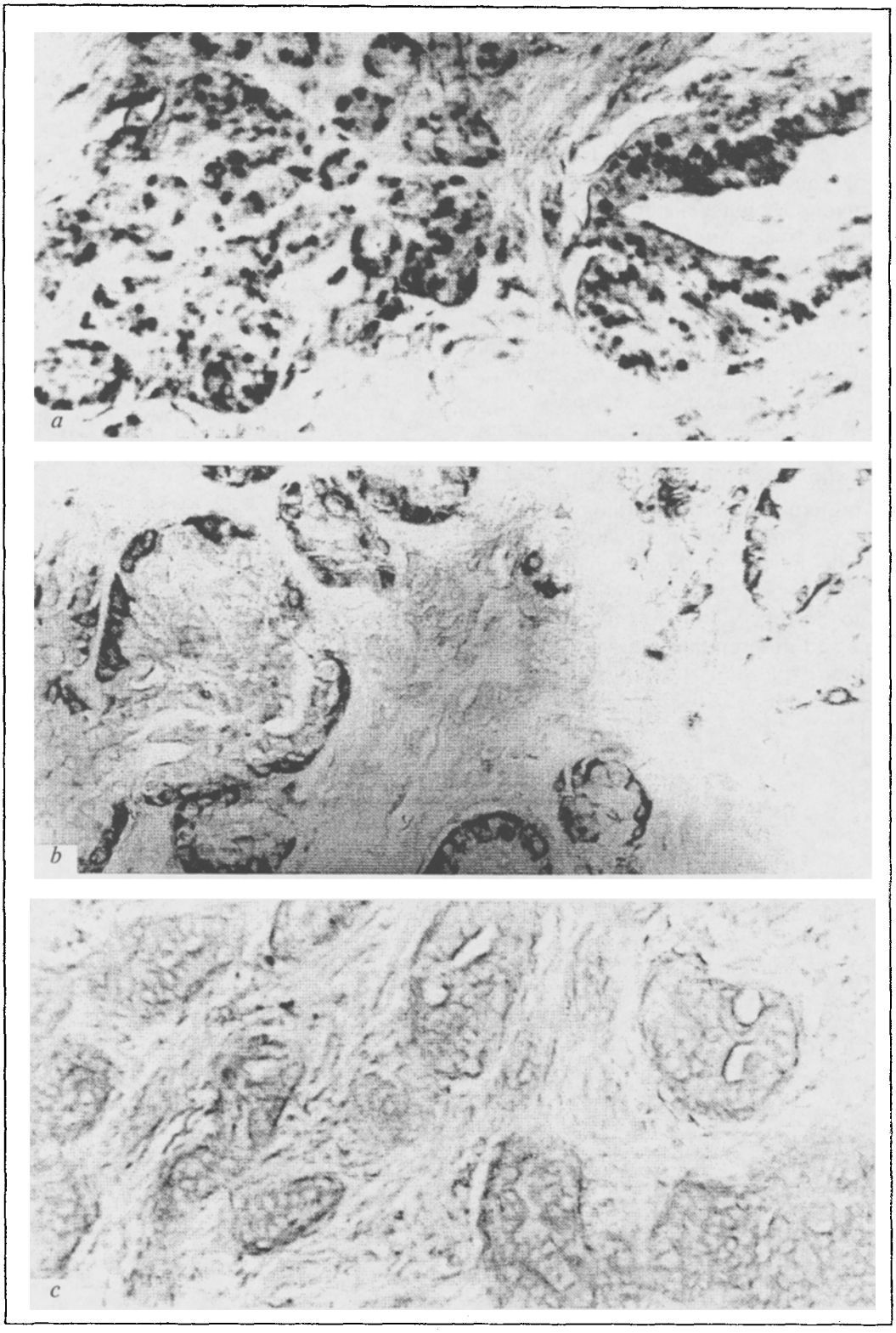

Fig. 3. Infiltrating mammary gland carcinoma. Immunostaining for metallothionein (MT) in normal and tumor tissues, antibody titer 1:2000. Streptavidin-biotin-peroxidase-complex method, diaminobenzibine, ×320. *a*) normal mammary gland tissue. MT in myoepithelial cells of a distal lobule and interlobular duct. *b*) peripheral tumor zone, enhanced MT expression in the zone of invasive tumor growth. *c*) central tumor zone. Negative reaction to MT.

In normal mammary gland tissue positive immunochemical reaction to MT was found in myoepithelial cells of distal lobules and ducts. This reaction was positive at all tested anti-MT concentrations, specific to background intensities was optimal at anti-MT titer of 1:2000 (Fig. 3, *a*, Table 1). In peripheral tumor zones, enhanced positive staining was noted in myoepithelial cells forming rings or semirings around actively proliferating tumor cells (Fig. 3, *b*). In the central tumor zones no MT-positive staining were usually noted (Fig. 3, *c*).

Thus, we prepared rabbit polyclonal antibodies against rat MT and demonstrated their specificity and the possibility of using these antibodies for immunolocalization of MT in histological sections. Since mammalian MT are not species specific [8], these antibodies can be used for MT identification in tissues of other mammals including men. MT distribution pattern in human normal and tumor mammary gland observed by us are consistent with published data obtained with the use of monoclonal anti-MT antibodies [10,12,13].

According to current views, MT participate in cell proliferation and differentiation and are markers of aggressive tumor expansion and can play a role in chemotherapy of tumors [3,10,12,15]. MT prevent carcinogenesis induced by some chemical toxicants and antitumor drugs [9].

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