American Journal of Pathology, Vol. 137, No. 1, July 1990

Immunohistochemical Localization of Antioxidant Enzymes in Adult Syrian Hamster Tissues and During Kidney Development

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Tissues from adult Syrian hamsters were studied with immunoperoxidase techniques using polyclonal antibodies to three antioxidant enzymes (copper, zinc and manganese forms of superoxide dismutase, and catalase). Tissues from labile organs, in which cell renewal is prominent (uterus, intestine, and transitional epithelium of the urinary tract), showed strong antioxidant enzyme immunostaining in differentiated cells but not in stem cells. In stable organs, in which cell renewal occurs at a high rate only in response to injury (kidney and adrenal), each cell type showed a specific pattern of antioxidant enzyme immunostaining. In permanent organs (brain and heart), antioxidant enzymes were regionally specific markers. Axons of the cerebellum showed more intense antioxidant enzyme staining than those of the cerebral cortex; in the heart, atria stained more intensely than ventricles. Germ cells of the testis resembled cell renewal systems in their antioxidant enzyme-immunostaining pattern: spermatogonia were negative, whereas spermatozoa were strongly positive. The tubules of the kidney showed no antioxidant enzyme immunostaining until after birth. Our results suggest that there is a prominent role for antioxidant enzymes in cell differentiation during development and cell renewal. (Am J Pathol 1990, 137:199–214)

Reactive oxygen species are constantly generated intracellularly in aerobic organisms[1–13] and are released extracellularly during the respiratory burst of phagocytes.[2] These reactive oxygen species, which include superoxide anion ($O_2^{\cdot-}$), hydroxyl radical ($^{\cdot}OH$), hydrogen peroxide (H_2O_2), organic peroxide radicals ($ROO^{\cdot}$), and singlet molecular oxygen ($^1O_2$), are thought to be the major mediators of oxygen cytotoxicity.[4–6] The discovery of an intracellular system that detoxifies reactive oxygen species in aerobic cells suggested that an oxidant–antioxidant equilibrium exists in these cells.[7,8] Major constituents of the antioxidant system include 1) certain enzyme systems (superoxide dismutase-catalase-peroxidase and glutathione peroxidase-glutathione reductase-glucose-6-phosphate dehydrogenase), 2) nonenzymatic cellular components (vitamins E, C, and A), and 3) low molecular weight-reducing agents (glutathione and other thiols).

Reactive oxygen radicals have been implicated in many pathologic processes, including mutagenesis, carcinogenesis, xenobiotic toxicity, postischemic tissue injury, diabetes, and inflammatory disease.[12] Most biochemical studies linking oxygen radicals to pathologic processes have used whole organs, and this has been a problem with these studies; in mammalian systems, each organ contains multiple cell types, each of which may be affected differently by oxidative stress. Oxygen radicals also have been implicated in three major physiologic processes: aging,[13] mitosis,[14,15] and cell differentiation.[16] Because of the possible importance of oxygen radicals in both pathologic and physiologic processes, we thought it was important to localize antioxidant enzymes (AE) in various adult mammalian tissues. The present study demonstrates that each cell type in the adult hamster has a unique AE profile.

Materials and Methods

Animals

Noninbred Syrian golden hamsters (*Mesocricetus auratus*) of both sexes, weighing 90 to 100 g, were purchased

Supported by grant CA-41267 from the National Cancer Institute, NIH, DHHS (Larry W. Oberley), and by the Department of Veterans Affairs Medical Research Service (Terry D. Oberley).

Accepted for publication February 26, 1990.

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from Harlan Sprague-Dawley (Indianapolis, IN). They were housed according to the guidelines of the United States Department of Health and Human Resources (NIH 1985). They were maintained on a 12-hour light-dark cycle and fed rodent Certified Chow (5002 Purina) and tap water, *ad libitum*. To obtain tissue for analysis, animals were killed with methoxyflurane anesthesia. Three 6-month-old hamsters were used for Western blot analysis of AEs. Six 6-month-old hamsters (3 male and 3 female) were used for immunohistochemical analysis. Hamsters were routinely screened for infection in our animal care unit, and only infection-free animals were used for our study. In addition, routine hematoxylin and eosin staining of sections from major organs of the study animals showed no evidence of infection or inflammation.

Fetal tissues and kidneys from newborn hamsters were also examined. Pregnant female hamsters were maintained as described above. Determination of estrus period and breeding techniques followed standard procedures.[17] The gestation period of Syrian hamsters is 16 days, counting the day after mating as day 1. Fetuses were obtained on the 13th and 15th days of gestation by caesarean section while the mother was under methoxyflurane anesthesia. The uterus was opened and the fetuses were killed by decapitation. Some pregnant hamsters were allowed to deliver, and the pups were killed 1 and 4 days after birth to obtain their kidneys. Two pups of each age were examined by immunohistochemistry. Two additional animals were killed at 4 weeks of age and their kidneys were removed for immunohistochemical examination.

Antibody Preparation and Specificity Testing

Preparation and specificity testing of antibodies to manganese superoxide dismutase (MnSOD), copper, zinc superoxide dismutase (CuZnSOD), and catalase (CAT) have been described.[18,19] Bovine liver catalase (Sigma Chemical Co., St. Louis, MO), bovine liver CuZnSOD (Diagnostic Data, Inc., Mountainview, CA), and human kidney MnSOD (isolated by Dr. M. L. McCormick) were used as antigens. The catalase preparation was protected from inactivation by 4.5 μmol/l (micromolar) NADPH.[20] Antigen purity was determined by native (7.5%) and denaturing (12.5%) polyacrylamide gel electrophoresis; gels were stained with Coomassie blue or silver reagents. New Zealand rabbits were immunized by subcutaneous injection of 500 μg of antigen in complete Freund's adjuvant. After 4 weeks a boost of 750 μg of antigen was given in incomplete Freund's adjuvant. For catalase, the rabbit was exsanguinated 2 weeks later. For CuZnSOD, a 1-mg boost of antigen was given intravenously 1 month later, followed after 1 week by an intravenous 0.3-mg boost, and the rabbit was exsanguinated 3 days after the final boost. For MnSOD, two boosts of 0.3 mg were given subcutaneously at 3-week intervals and the rabbit was exsanguinated 1 week after the final injection.

For Western blot analysis of antibody specificity, protein was transferred to nitrocellulose from 12.5% (catalase, MnSOD) or 15% (CuZnSOD) denaturing polyacrylamide gels by the method of Towbin et al.[21] The nitrocellulose sheets were blocked overnight at 4°C in a mixture of 10% bovine serum albumin, 10% fetal calf serum, and TRIS-buffered saline (TBS: 10 mmol/l [millimolar] TRIS HCl, 140 mmol/l NaCl, pH 8.0). The sheets were then rinsed with TBS containing 0.05% Tween 20 and incubated in a 1:250 (catalase), 1:300 (CuZnSOD), or 1:500 (MnSOD) dilution of whole rabbit antiserum for 4 hours, after which they were rinsed and incubated in a 1:10,000 dilution of goat anti-rabbit IgG antibody conjugated with alkaline phosphatase (Sigma) for 2 hours. The blots were rinsed twice with TBS-0.05% Tween 20 and once in alkaline phosphatase-staining buffer containing 100 mmol/l TRIS, 100 mmol/l NaCl, and 5 mmol/l $MgCl_2$ at pH 9.5. Staining was carried out in alkaline phosphatase-staining buffer by adding 0.45 mmol/l 5-bromo-4-chloro-3-indolyl phosphate and 0.244 mmol/l nitroblue tetrazolium.

To test the specificity of the antibodies, Western blot analysis was performed on homogenized hamster kidney cortex, heart, and skeletal muscle, and results were compared with those from the purified antigen. For MnSOD and CAT, the purified antigen comigrated with the immunostained hamster protein, and only one band was present on the Western blot. For CuZnSOD, the major band migrated with the purified antigen; however, a few minor bands were present. The same minor bands were recognized by a commercial antibody (The Binding Site, Inc., San Diego, CA), suggesting that they are variants of CuZnSOD or proteins with strong homology to CuZnSOD (unpublished observations). By Western analysis, there was no evidence of cross-reactions of specific antibodies with other AEs.

Immunohistochemistry

Tissues were fixed for 1 hour in 4% formalin in phosphate-buffered saline (PBS: 10 mmol/l sodium phosphate, 133 mmol/l sodium chloride, pH 7.4) and embedded in paraffin. Four micrometer-thick sections were mounted on glass slides coated with poly-L-lysine (Sigma) to prevent detachment and deparaffinized in three changes of xylene, 2 minutes each, and 100% ethanol. Results from these sections were the same as results from unfixed frozen sections (unpublished results).

Endogenous peroxidase activity was blocked by a 5-minute incubation in 3% H_2O_2 methanol. Slides were rehydrated in an ethanol series (1 minute each in 100%, 95%, 70%, and 50% ethanol) and 1 minute each in distilled wa-

ter and 0.05 mol/l (molar) TBS (pH 7.4, 32°C). All sections were treated with a blocking agent, 10% normal goat serum, for 20 minutes to reduce nonspecific staining of the bridging antibody. Sections were incubated with the primary antibody (1:200) for 1 to 2 hours and rinsed with TBS for 2 minutes. They were treated with bridging antibody (biotinylated goat anti-rabbit 1:100) for 30 minutes. Slides were rinsed twice for 2 minutes with TBS and incubated with the avidin-peroxidase complex (1:100) for 30 minutes. They were then rinsed three times in TBS for 6 minutes, after which the chromogen diaminobenzidene (0.025% in TBS) was added together with a 3% aqueous solution of H_2O_2. Color development was monitored and stopped after 3 to 10 minutes, depending on the control. Sections were rinsed in TBS, counterstained in 1% Harris hematoxylin for 5 to 10 seconds, decolorized in Li_2CO_3 solution, rinsed, and dehydrated. Coverslips were mounted with Permount. Duplicate slides were prepared for each stain. Normal rabbit serum or preimmune serum controls were run for each organ and antibody tested, and antibody plus 100 μg of antigen were preincubated and used for staining; these controls were uniformly negative.

Results

Immunostaining of Antioxidant Enzymes in Epithelial Cell-renewal Systems

Immunostaining for AEs was studied in the 25 tissues listed in Table 1, which summarizes the results by organ system. When these organs are classified as labile (cell renewal continuously occurs at a high rate), stable (cell renewal occurs at a very low rate), or permanent (mitosis does not occur in the adult), generalizations can be made about staining patterns of AEs. As an example of a cell renewal system, we examined the uterine epithelium (endometrium) (Figure 1). The deep glands of the uterus did not stain with antibodies to AEs, whereas the more differentiated cells of the epithelium lining the uterine cavity showed moderate to intense immunostaining for all AEs. The same was true for other cell renewal systems (hair follicle, small and large intestine (Figure 2), and transitional epithelium of the urinary tract) (Table 1). That is stem cells did not stain, but differentiated cells stained intensely. In general the most intense immunostaining in differentiated epithelium of the cell renewal systems was for MnSOD, which always stained in a granular fashion. The epidermis was the only cell renewal system we studied in which the differentiated cells did not stain more intensely than the progenitor (basal) cells (see Discussion below).

Immunostaining of Antioxidant Enzymes in Stable Organs

Stable organs such as kidney (Figure 3) and adrenal (Figure 4) have several differentiated cell types. Each of these cell types showed unique antioxidant enzyme staining. In the kidney, intense immunostaining with anti-MnSOD was found in segments of the nephron that contain many mitochondria, including the S_1 and S_2 but not the S_3 segments of the proximal tubule. In contrast, the glomeruli and collecting ducts of the renal papilla showed no immunostaining with anti-MnSOD. The thin loop of Henle showed light immunostaining with anti-MnSOD; the distal tubule showed light immunostaining in the convoluted portion and heavy immunostaining in the straight portion (thick loop of Henle). In all segments of the nephron where immunostaining with anti-MnSOD was observed, staining was granular and in the cytoplasm. In contrast, immunostaining with anti-CuZnSOD was uniformly diffuse in the cytoplasm. Glomeruli did not show immunostaining with anti-CuZnSOD, whereas tubules stained moderately throughout the nephron. Cytoplasm of the proximal tubules showed granular immunostaining with anticatalase but glomeruli did not stain. The remaining tubules showed moderate, uniform, diffuse cytoplasmic immunostaining. However, the collecting ducts in the renal papilla showed intense, uniform, diffuse cytoplasmic immunostaining with the anticatalase antibody.

The adrenal also showed cell type-specific patterns of antioxidant enzyme immunostaining. The zona fasciculata of the adrenal cortex showed intense granular cytoplasmic immunostaining with anti-MnSOD, while the zona glomerulosa, zona reticularis, and adrenal medulla were negative. The zonae glomerulosa and reticularis of the adrenal cortex showed moderate, diffuse cytoplasmic immunostaining with anti-CuZnSOD, while the zona fasciculata and adrenal medulla did not stain. Antibody to catalase immunostained the cytoplasm of cells of the zonae fasciculata and reticularis in a granular fashion but did not immunostain the zona glomerulosa or the adrenal medulla.

Immunostaining of Antioxidant Enzymes in Permanent Organs

The cerebellum (Figure 5) and heart (Table 1) were chosen as examples of permanent organs in which mitosis does not occur in adult life. The axons of the cerebellum showed intense diffuse cytoplasmic immunostaining with antibodies to catalase, whereas anti-CuZnSOD and anti-MnSOD showed minimal staining. Cell bodies did not show immunostaining with any of these antibodies. The axons of the cerebral cortex also showed immunostaining

Table 1. *Immunoperoxidase Staining of Selected Syrian Golden Hamster Tissues*

Organ or system	Immunoperoxidase staining*†		
	CuZnSOD	MnSOD	Catalase
Cardiovascular System			
Heart			
Atrium	2+ DC (diffuse)	3+ GC (diffuse)	2+ GC (diffuse)
Ventricle	2+ DC (focal)	4+ GC (focal)	4+ GC (focal)
Respiratory System			
Trachea			
Epithelium	2+ DC	2+ GC	2+ DC
Cilia of epithelium	4+ D	0	0
Cartilage	2+ DC	2+ GC	2+ DC
Lung			
Bronchial epithelium	3+ DC	2+ GC	1+ DC
Alveolus	0	0	2+ DC‡
Urinary System			
Kidney (see Figure 3)			
Glomerulus	0	0	0
Proximal tubule	2+ DC	4+ GC	3+ GC
Basal cell, transitional epithelium of renal pelvis	0	0	0
Superficial cell, transitional epithelium of renal pelvis	2+ DC	1+ GC	3+ GC
Female Reproductive System			
Ovary			
Follicle	0	0	0
Stroma	2+ DC	0	0
Oviduct (see Figure 8)			
Epithelium (cytoplasm)	3+ DC	2+ GC	1+ DC
Cilia of epithelium	4+ D	0	0
Uterus (see Figure 1)			
Deep glands	0	0	0
Superficial glands	2+ DC	4+ GC	3+ DC
Liver			
Hepatocyte	1+ DC	1+ GC (focal 4+)	3+ GC
Bile duct	tr DC	1+ GC	3+ DC
Endocrine Organs			
Pancreas			
Islet	2–3+ DC	tr GC	2+ DC (one cell type 4+)
Duct	tr DC	0	2+ DC
Acinus	0	0	0
Thyroid			
Epithelium	1+ DC	0	1+ DC
Parathyroid			
Chief cell	3+ DC	0	0
Adrenal (see Figure 4)			
Zona glomerulosa	3+ DC	tr GC	tr GC
Zona fasciculata	tr DC	3+ GC	3+ GC
Zona reticularis	2+ DC	tr GC	3+ GC
Medulla	0	0	0
Skin			
Epidermis	2+ DC	1+ GC	1+ DC
Sebaceous gland	2+ DC	0	1+ DC
Sweat gland	1+ DC	1+ GC	tr DC
Hair follicle epithelium (root)	0	0	0
Hair follicle epithelium (shaft)	2+ DC	4+ GC	3+ DC
Digestive System			
Stomach			
Surface mucous cell	2+ DC	2+ GC	3+ DC
Gland (neck)	0	0	0
Gland (base)	2+ DC	0 (3+, parietal cells)	0
Small intestine			
Villus	tr DC	1+ GC	3+ DC
Crypt	0	0	0
Large intestine (see Figure 2)			
Superficial epithelium	1+ DC	4+ GC	4+ DC
Goblet cell	0	0	0
Hematopoietic Organs			
Spleen	0	0	0
Bone marrow			
Megakaryocyte	0	2+ GC	0
Lymphoid cell	0	0	0
Myeloid cell	0	0	2+ DC
Erythrocyte precursor	0	0	0

Table 1. (*continued*)

Organ or system	Immunoperoxidase staining*†		
	CuZnSOD	MnSOD	Catalase
Male Reproductive System			
Seminal vesicle epithelium	0	0	0
Prostate epithelium	1–2+ DC, N	0	tr DC
Testis (see Figure 6)			
Mature sperm	1+ DC	3+ DC	1–2+ DC
Spermatogonium	0	0	0
Leydig cell	4+ DC	4+ GC	4+ DC
Sertoli cell	0	0	0
Central Nervous System			
Brain			
Choroid plexus	1+ DC	3+ GC	3+ DC
Neuron	0	Occasional 1+ GC	0
Axon	0	0	2+ DC
Cerebellum (see Figure 5)			
White matter	2+ DC	tr DC	3+ DC
Purkinje cell	1+ DC	0	0
Mammary Gland			
Large duct	4+ DC	0 (basal cells) 4+ (lumenal cells) GC	4+ DC
Small duct	2+ DC	1+ GC	3+ DC
Adipose tissue	0	0	0

* Staining scored on a scale of 0 (negative) to 4+ (strongly positive); tr, trace.
† G, granular staining; D, diffuse staining; C, cytoplasm; N, nucleus.
‡ One cell type, probably alveolar macrophage.

with anticatalase, but this was much less than in the cerebellum.

Both atria and ventricles showed antioxidant enzyme immunostaining of cardiac muscle fibers. However, immunostaining for AEs was much more intense in atria than in ventricles.

Immunostaining of Antioxidant Enzymes in Testis

To study antioxidant enzyme immunostaining in germ line cells, we immunostained hamster testes (Figure 6). Mature sperm showed cytoplasmic immunostaining for all AEs, whereas spermatogonia were uniformly negative. The strongest immunostaining was with anti-MnSOD.

Antioxidant Enzymes During Kidney Development

The kidney was chosen as a stable organ in which to study antioxidant enzyme immunostaining during development (Figure 7). Kidneys of 13- and 15-day-old fetuses showed no antioxidant enzyme immunostaining. One day after birth, proximal tubules in the inner cortex showed light granular immunostaining for all three enzymes, but the outer cortex did not stain. By 4 days after birth, the number and intensity of proximal tubules immunostained had increased significantly; however, the adult pattern of intense immunostaining of proximal tubules in both inner and outer cortex was not observed until 4 weeks (Figure 3).

CuZnSOD in Ciliated Epithelium

Both trachea and oviduct (Figure 8) showed intense immunostaining of the ciliated epithelium with anti-CuZnSOD. This was not observed in other epithelia.

Discussion

The present study presents a detailed immunoperoxidase study of the distribution of AEs in adult hamster tissues and during kidney development. We have confidence in our results because 1) for controls we used normal serum, preimmune serum, and preincubation of antigen with antibody, 2) Western blot analysis indicated that the hamster proteins identified were indeed the expected AEs because immunostained hamster protein(s) comigrated with purified antigens and only one major band was present, and 3) our previous studies of enzyme activity in the kidney have yielded compatible results—specifically, fetal kidney has much less SOD and catalase than does adult tissue,[22] and glomeruli have very low levels of antioxidant enzyme activity.[23] It should be emphasized that a negative immunoperoxidase result does not mean that the organ has no antioxidant enzyme activity. Rather, it means that the amount of enzyme is less than the level of immunohistochemical detection. For example, we found

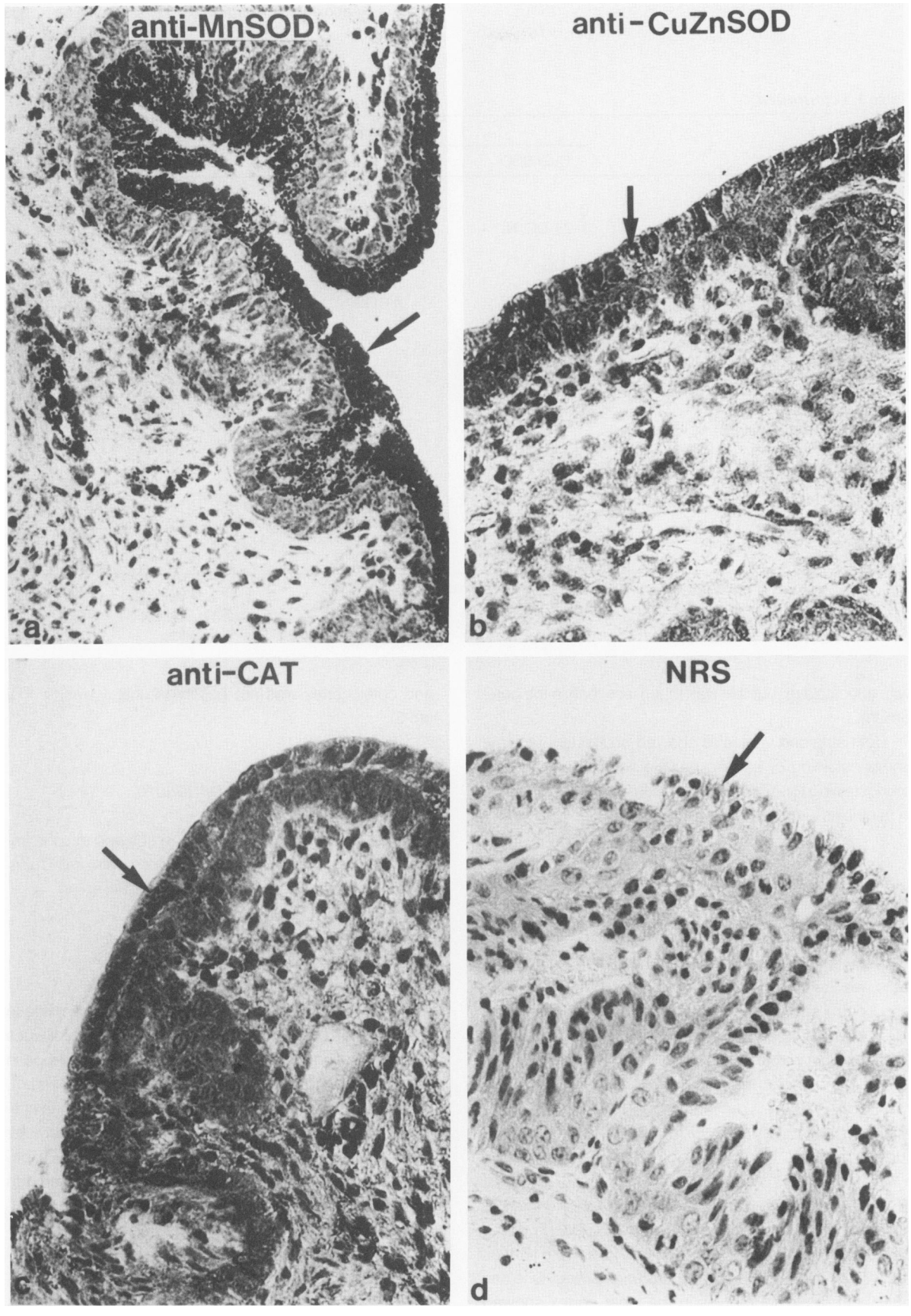

Figure 1. *Immunoperoxidase staining of hamster uterus. The epithelium lining the endometrial cavity shows impressive staining for all three antioxidant enzymes (arrows), and anti-MnSOD (a) and anti-CAT (c) are especially pronounced. Not shown are the deep endometrial glands embedded in the uterine stroma, which were uniformly negative for AE immunostaining. A control treated with normal rabbit serum (NRS) did not show staining (d) (a–d: ×833).*

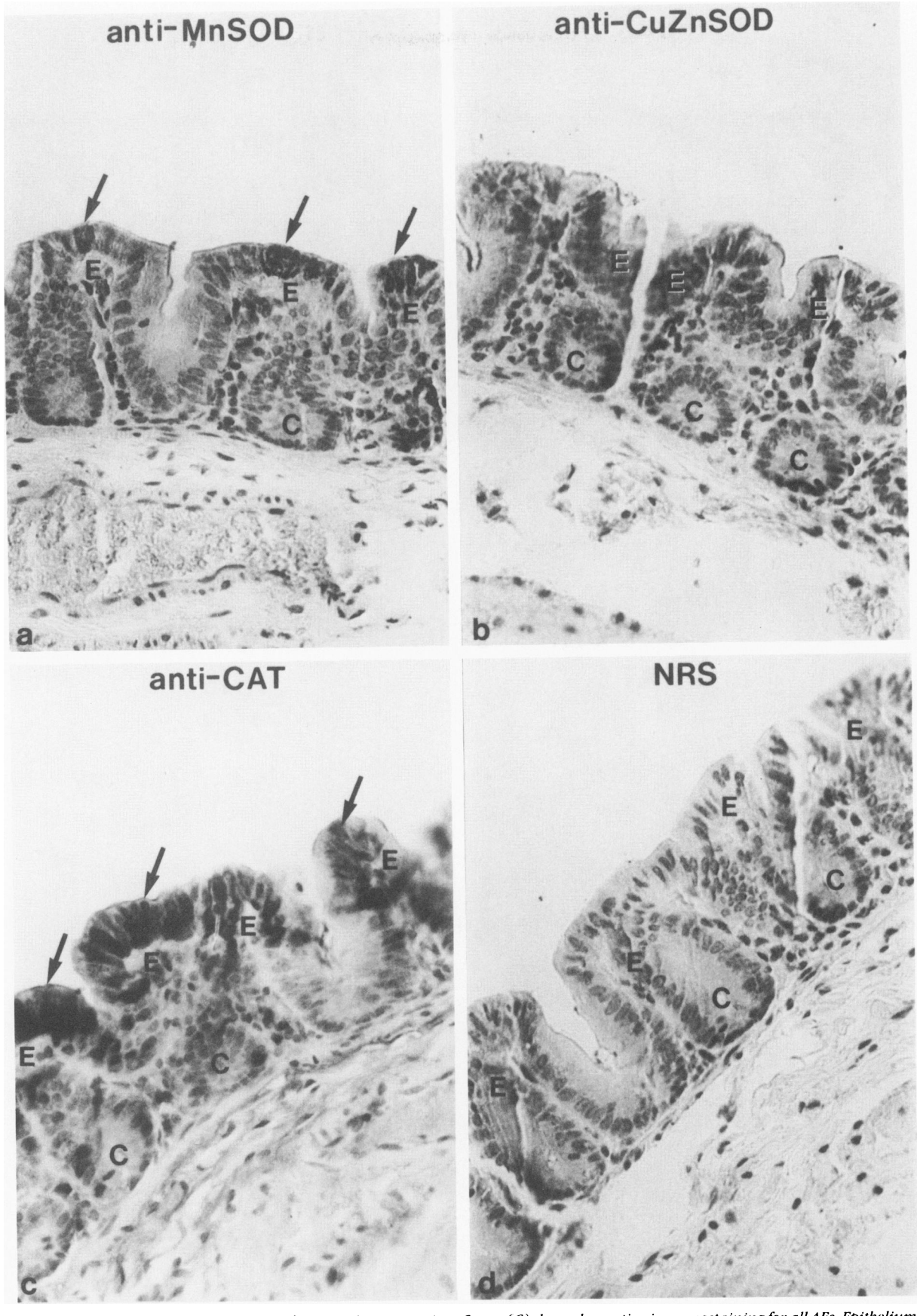

Figure 2. *Immunoperoxidase staining of hamster large intestine. Crypts (C) showed negative immunostaining for all AEs. Epithelium (E) showed strong staining for MnSOD* (a) *and CAT* (c) *(arrows), whereas CuZnSOD showed minimal staining* (b). *A control treated with normal rabbit serum (NRS) did not show staining* (d) (a–d ×833).

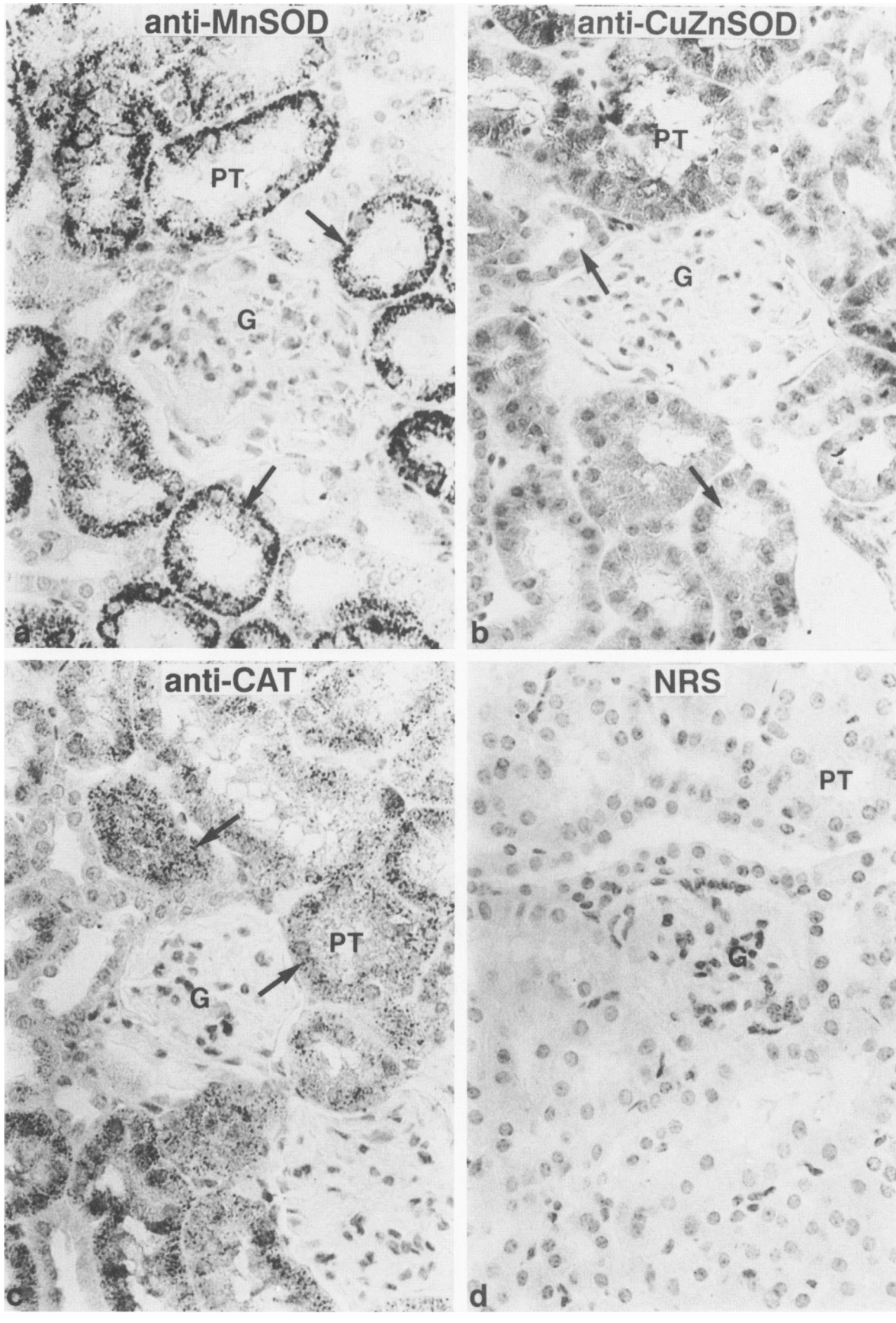

Figure 3. *Immunoperoxidase staining of hamster kidney. Glomeruli (G) were negative for all antibodies studied. Proximal tubular cytoplasm (PT) showed strong granular immunostaining (arrows) with anti-MnSOD* (a) *and anti-CAT* (c) *and moderate diffuse immunostaining (arrows) with anti-CuZnSOD* (b). *A control treated with normal rabbit serum (NRS) did not show staining* (d) (a–e ×*833*).

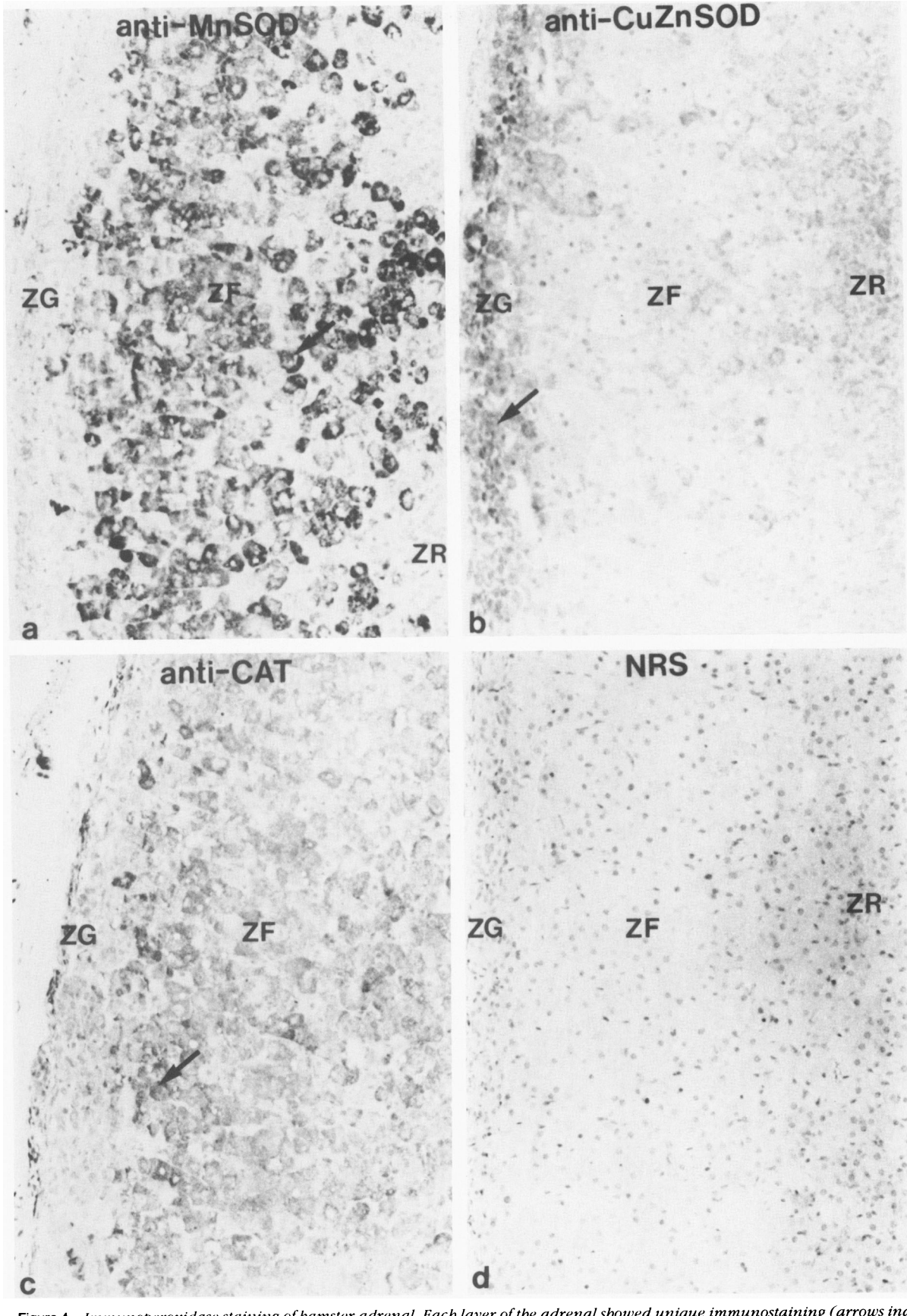

Figure 4. *Immunoperoxidase staining of hamster adrenal. Each layer of the adrenal showed unique immunostaining (arrows indicate positive staining). ZG = zona glomerulosa; ZF = zona fasciculata; ZR = zona reticularis. Not shown is adrenal medulla, which showed negative immunostaining for all three AEs. A control treated with normal rabbit serum (NRS) did not show staining* (**d**) (**a**–**e** ×*833*).

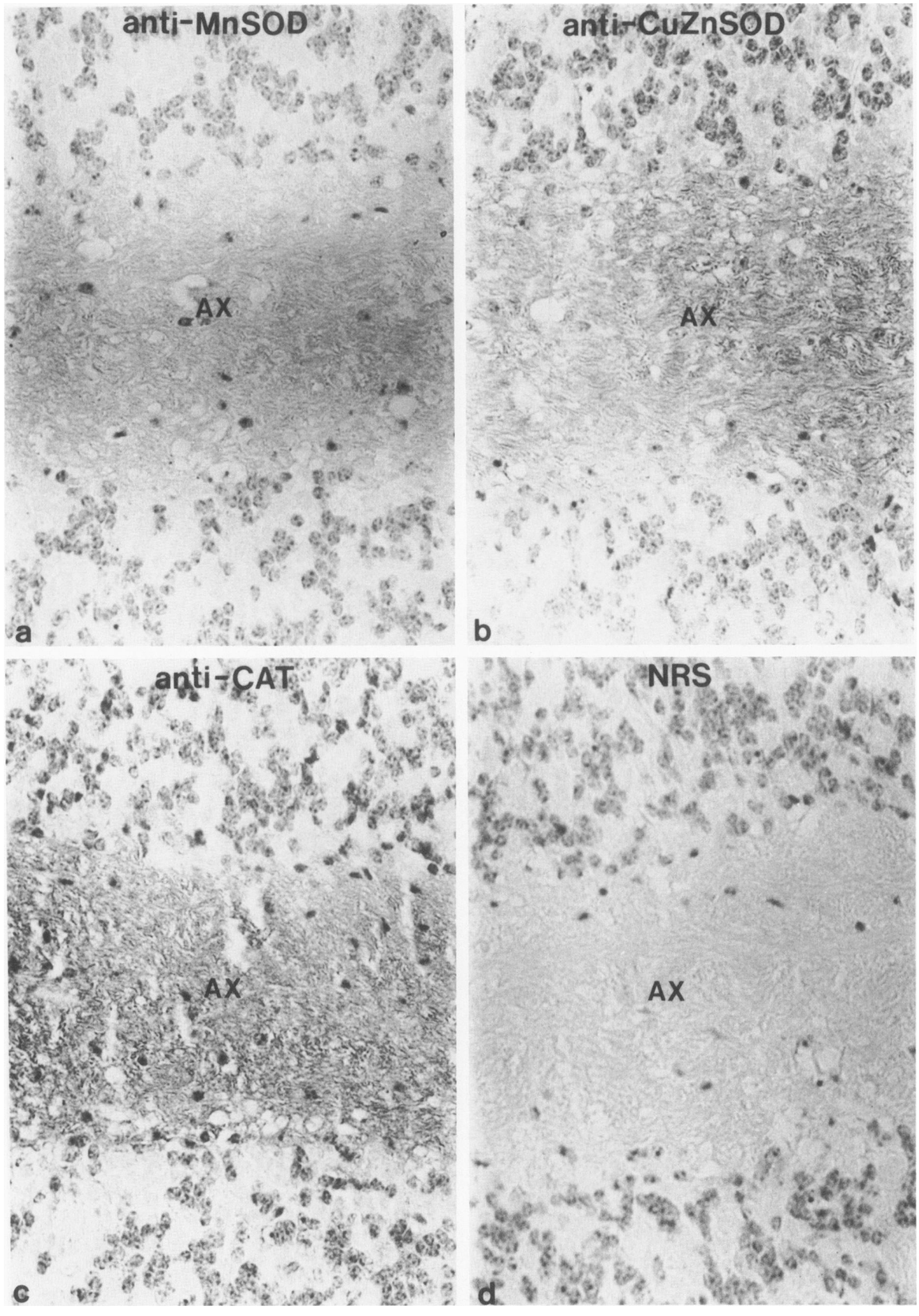

Figure 5. *Immunoperoxidase staining of hamster cerebellum. Axons (Ax) showed strong immunostaining with anticatalase* (c). *A control treated with normal rabbit serum (NRS) did not show staining* (d) (a–e ×833).

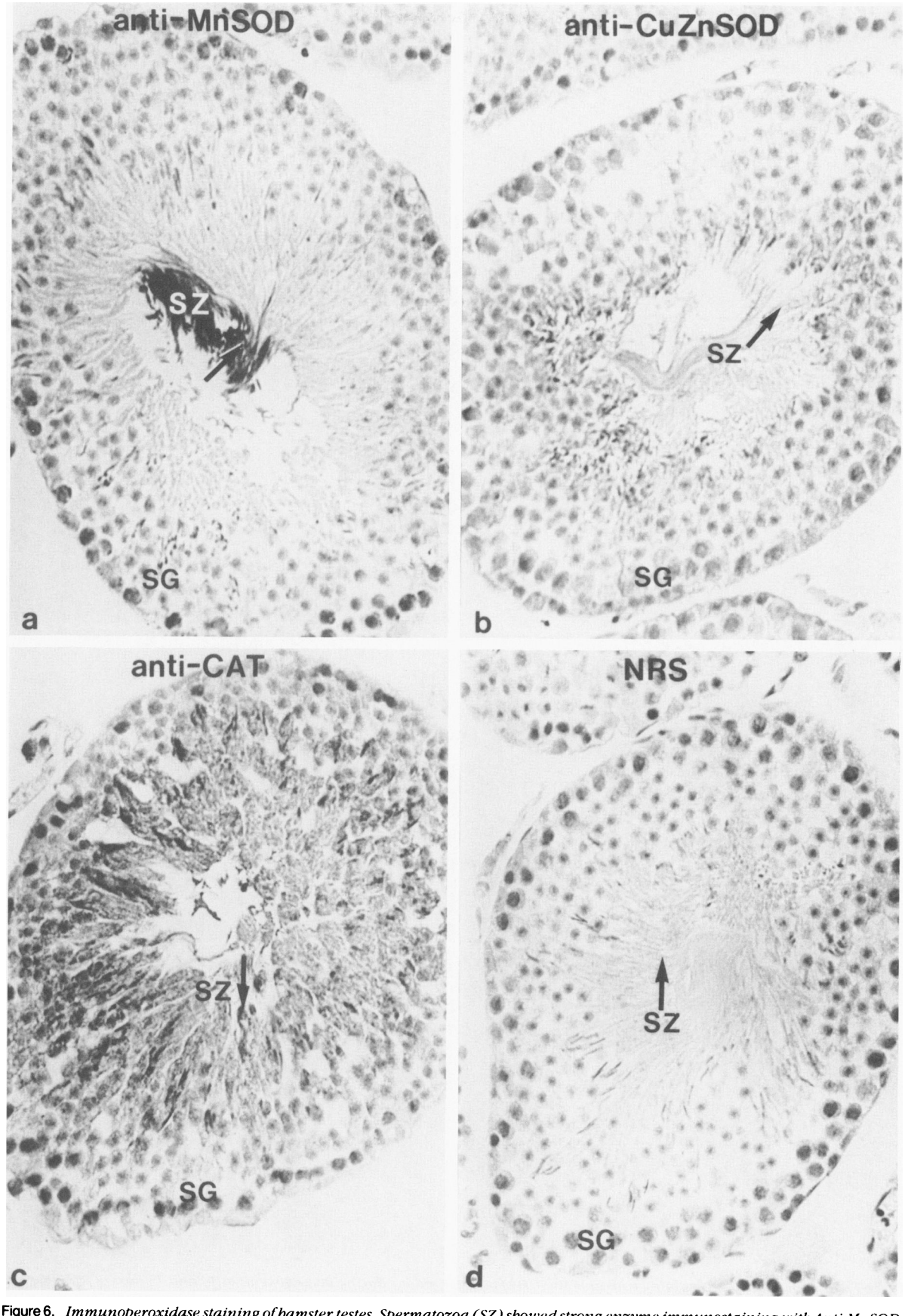

Figure 6. *Immunoperoxidase staining of hamster testes. Spermatozoa (SZ) showed strong enzyme immunostaining with Anti-MnSOD and anti-CAT (arrows), but spermatogonia (SG) did not. Anti-CuZnSOD stained spermatozoa lightly (b). A control treated with normal rabbit serum (NRS) did not show staining (d)* (a–e ×833).

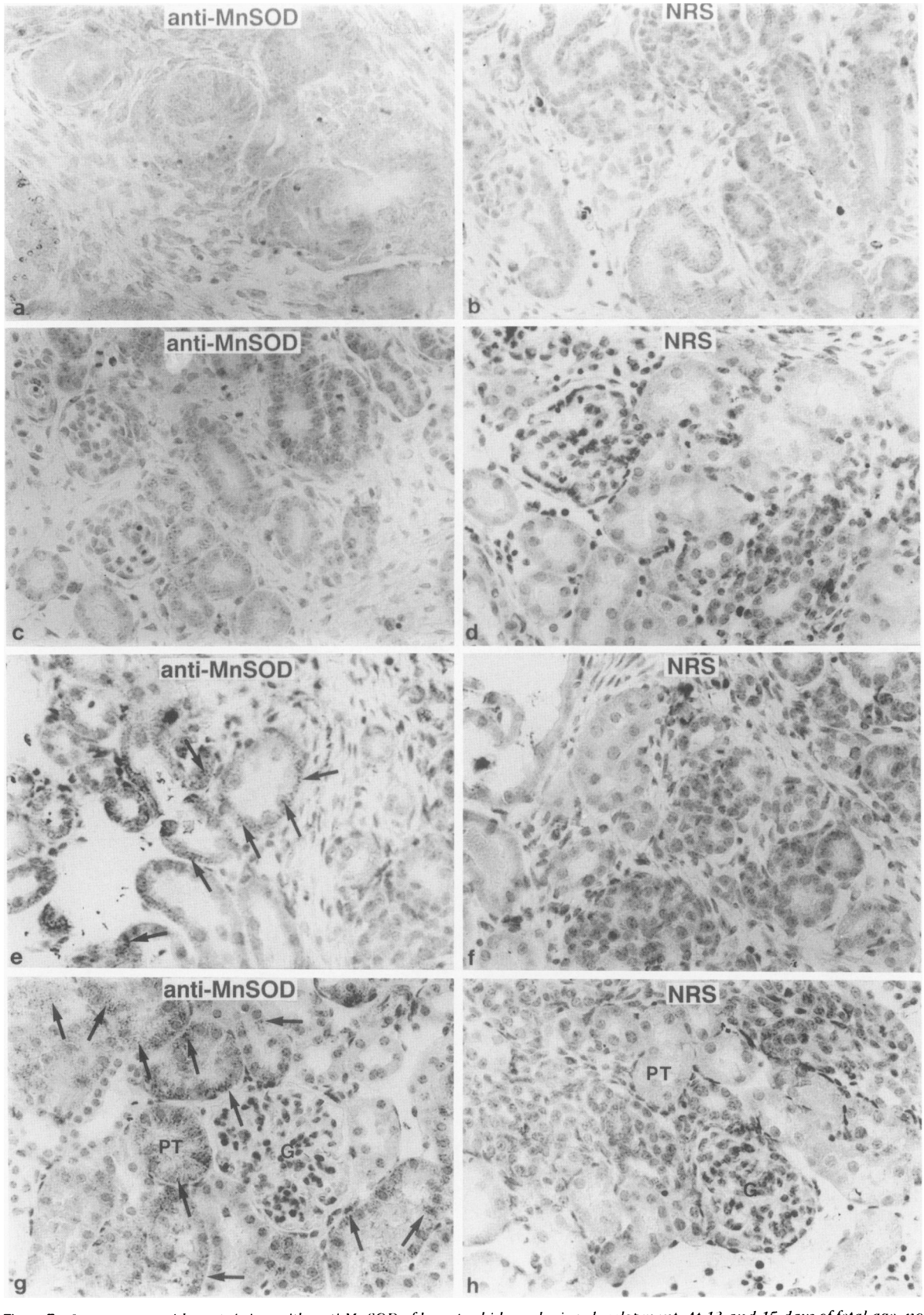

Figure 7. *Immunoperoxidase staining with anti-MnSOD of hamster kidney during development. At 13 and 15 days of fetal age, no immunostaining was seen with anti-MnSOD. At 1 and 4 days after birth, both the intensity of immunostaining and the number of proximal tubules (PT) stained increased. At 1 day* (**e**)*, only proximal tubules in the deep cortex showed granular staining (arrows). After 4 days* (**g**) *the number of positive tubules increased (arrows). Pt, proximal tubules; G, glomerulus.* **a**: *Thirteen-day-old fetus.* **b**: *Normal rabbit serum (NRS).* **c**: *Fifteen-day-old fetus.* **d**: *Normal rabbit serum.* **e**: *One-day-old pup.* **f**: *Normal rabbit serum.* **g**: *Four-day-old pup.* **h**: *Normal rabbit serum* (**a–h** ×333).

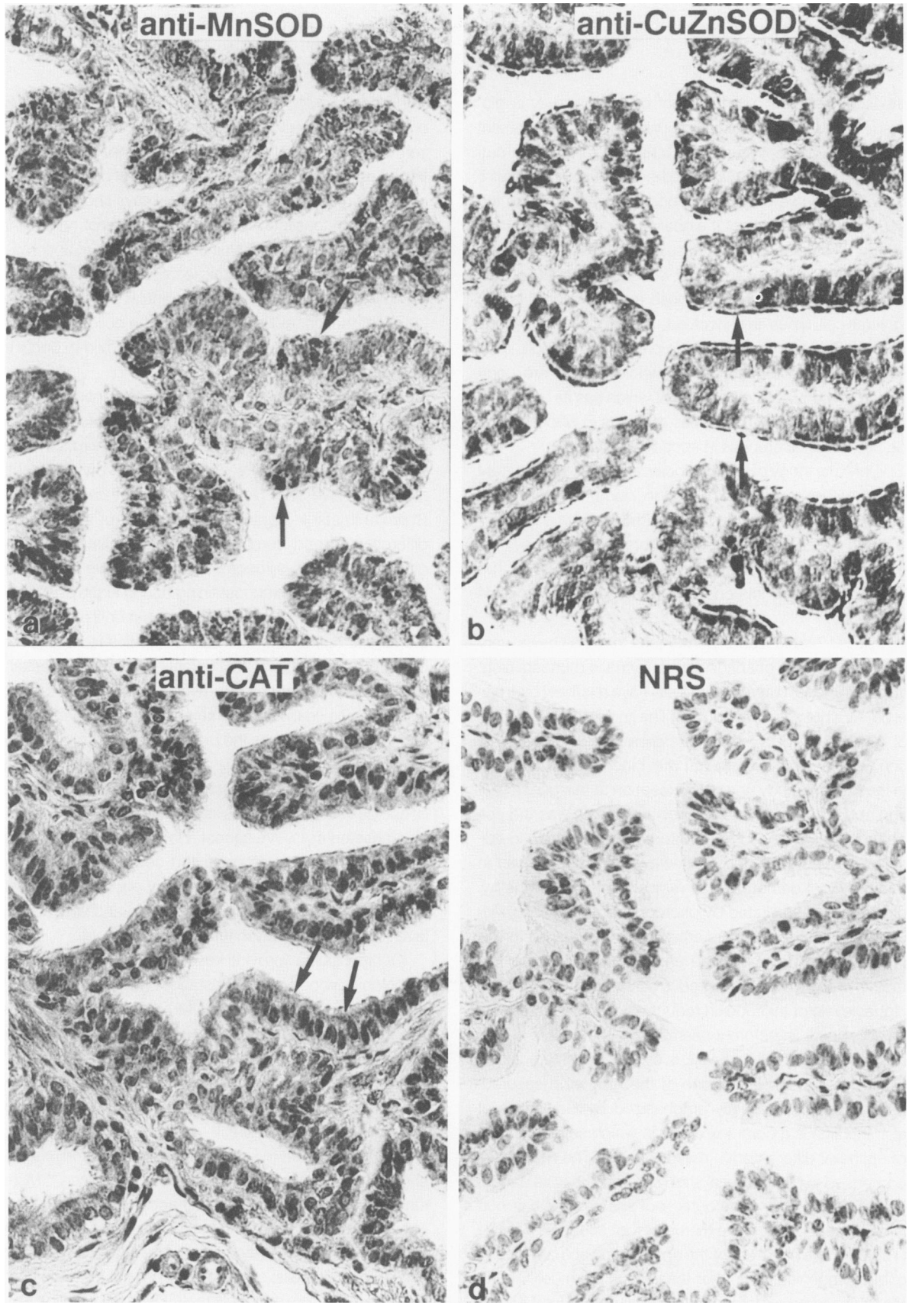

Figure 8. *Immunoperoxidase staining of hamster oviduct. Cytoplasm of epithelial cells showed granular staining with anti-MnSOD (a) and anti-CAT (c) (arrows). Brush border of oviduct epithelium showed intense immunostaining with anti-CuZnSOD (arrows). A control treated with normal rabbit serum (NRS) did not show staining (d) (a–e ×833).*

measurable levels of antioxidant enzyme activity in glomeruli, yet no immunostaining was seen in the present study. The present study was intended to evaluate patterns of protein levels. Thus methods that increase sensitivity (eg, double bridging antibodies) would change the semiquantitation but not the qualitative patterns reported.

Many studies have measured antioxidant enzyme activities in various organs of several mammalian species.[24–26] The problem with these measurements is that several cell types are involved, making generalizations about pathologic or physiologic processes difficult. As illustrated in the present study, whole organs are composed of many cell types, each of which has its own characteristic AE levels. Thus measurements in whole organs do not reveal differences in specific cell types.

Only one study of immunolocalization of an antioxidant enzyme (using anti-CuZnSOD, in dogs) has been reported.[27] Our results correlate with those of Thaete et al,[27] with the exception that these authors reported heavy immunostaining of the thick ascending limb of Henle. The differing results in this one case may be due to species specificity.

Labile, stable, and permanent organs have very different patterns of antioxidant enzyme immunostaining. In labile organs, differentiated cells have relatively high levels of AEs but stem cells do not. The finding of lower levels of AEs in stem cells than in differentiated cells of the intestine was seen earlier in the rat with biochemical studies.[28] In the present study, the only exception to this generalization about AE levels in cell renewal systems was the epidermis. It was previously reported that SOD is low in epidermis.[29] The epidermis is, however, a unique organ in that the most damaging free radical generated is the hydroxyl radical produced by photochemical reactions with ultraviolet light.[30] A unique antioxidant enzyme system is present, which includes the enzyme thioredoxin reductase.[31] Epidermal cells allowed to differentiate *in vitro* have higher levels of thioredoxin reductase than do undifferentiated cells.[31] Therefore it seems likely that in this cell renewal system at least one AE is elevated in differentiated cells, but it is simply not one of the AEs we measured. Furthermore, as recently emphasized by Coulombe et al,[32] the skin is a complex organ in which several stages of epithelial differentiation may be found. The hair bud is composed of pluripotential stem cells, whereas its daughter cells are found in the outer root sheath. The hair bud is negative for AEs, while the outer root sheath is positive (Table 1). The basal cell of the epidermis is actually a committed progenitor cell rather than a true stem cell. Therefore it would be expected to be positive for AEs if it were true that all stem cells have low AEs. In fact, we find that the epidermis stains relatively uniformly for AEs, reflecting its relatively late stage of skin epithelial cell development. In stable organs, each cell type has a unique antioxidant enzyme-staining pattern. We have confirmed this finding in the kidney using biochemical assays.[22,23] In permanent organs, there is regional specificity of antioxidant enzyme immunostaining patterns. This finding has been confirmed using biochemical assays in the rat brain.[33]

It is of interest that anti-MnSOD always showed granular staining, consistent with its mitochondrial origin.[34] In contrast, anti-CuZnSOD stained the cytoplasm uniformly, consistent with its cytoplasmic localization.[35] It is noteworthy that catalase showed both granular (in the proximal tubule of the kidney) and diffuse (in the collecting tubule of the kidney) staining. This enzyme is known to occur in peroxisomes[36] and also in cytoplasm.

It has been suggested that increased antioxidant enzyme levels and cell differentiation are directly correlated.[16,37] The present study apparently confirms this hypothesis in cell-renewal systems, in which only differentiated cells have AEs. However, the hypothesis is difficult to prove in stable organs because most of the cells are differentiated and comparison of differentiated and undifferentiated cells is impossible. Therefore we studied antioxidant enzyme immunostaining during kidney development and found that AEs do not appear until after birth in the tubular epithelium. The earliest tubules formed (deep cortex) showed the first antioxidant enzyme immunostaining. Four weeks after birth, almost all proximal tubules showed antioxidant enzyme immunostaining. Even in permanent organs such as the brain, antioxidant enzyme immunostaining was not fully developed until the animals were 4 weeks old (unpublished observations). Thus we conclude that lack of AEs is a general property of undifferentiated cells. We cannot rule out the possibility that undifferentiated and differentiated cells contain different forms of the AEs (not recognized by our antibodies), although if they do, it is difficult to understand why activity levels are uniformly low in fetal tissue.[22]

Our results may provide keys to understanding several pathologic processes. For instance, we found only small amounts of immunostaining for MnSOD in the islets of the pancreas. This could explain sensitivity to oxygen radical attack after administration of radical-generating drugs.[38] Similarly, the absence of MnSOD in the renal papilla may be a clue to the pathogenesis of papillary necrosis secondary to analgesic abuse.[39]

The present findings may provide clues to the relationship between antioxidant enzymes and cancer.[40] Many studies have found altered SOD activity in tumor cells.[41] These cells usually have lowered activity of both CuZnSOD and MnSOD. There is evidence that many tumors arise from stem cells, even in stable organs.[42–44] The present study therefore suggests that SOD is lowered in tumor cells because of their stem cell origin. In future studies we will test this hypothesis.

The present study also may provide important clues to the physiologic function of cell organelles. Thus it is intriguing that the ciliated epithelium of only those two or-

gans involved in the movement of particles (trachea and oviduct) have extremely elevated levels of CuZnSOD. Future studies are needed to determine why CuZnSOD is necessary in these locations.

The present study documents cell-type specificity in the distribution of AEs. These regional differences may provide important clues to mechanisms of many pathologic processes.

References

1. Freeman BA: Biological sites and mechanisms of free radical production. *In* D Armstrong, RS Sohal, RG Cutler, TF Slater, eds. Molecular Biology, Aging, and Disease. New York, Raven Press, 1984, pp. 43–52
2. Fridovich I: The biology of oxygen radicals. Science 1978, 201:875–880
3. Mason RP, Chignell CF: Free radicals in pharmacology and toxicology. Pharmacol Rev 1983, 33:189–211
4. Beauchamp C, Fridovich I: A mechanism for the production of ethylene from methional: The generation of hydroxyl radicals by xanthine oxidase. J Biol Chem 1970, 245:4641–4646
5. Frank L: Oxygen toxicity in eukaryotes. *In* LW Oberley, ed. Superoxide Dismutase. Vol 3. Boca Raton, FL, CRC Press, 1985, pp. 1–44
6. Sies H, Cadenas E: Oxidative stress: Damage to intact cells and organs. Philos Trans R Soc Lond 1985, 311:617–631
7. Brawn K, Fridovich I: Superoxide radical and superoxide dismutase: Threat and defense. Acta Physiol Scand Suppl 1980, 492:9–18
8. Fridovich I: Superoxide dismutase. *In* Hayaishi O, ed. Molecular Mechanism of Oxygen Activation. New York, Academic Press, 1974, pp. 453–477
9. Freeman BA, Crapo JD: Biology of disease: Free radicals and tissue injury. Lab Invest 1982, 47:412–426
10. Meister A: Selective modification of glutathione metabolism. Science 1983, 220:472–476
11. Witting LA: Vitamin E and lipid peroxidation in free radical-initiated reactions. *In* Pryor WA, ed. Free Radicals in Biology, Vol. 4. New York, Academic Press, 1980, pp. 295–305
12. Oberley LW, ed. Superoxide Dismutase. Boca Raton, FL, CRC Press, 1985
13. Phillips JP, Campbell SD, Michand P, Charbonneau M, Hilliker AJ: Null mutation of copper/zinc superoxide dismutase in *Drosophila* confers hypersensitivity to paraquat and reduced longevity. Proc Natl Acad Sci USA 1989, 86:2761–2765
14. Armato U, Andreis PG, Romano F: Exogenous Cu, Zn-superoxide dismutase suppresses the stimulation of neonatal rat hepatocytes' growth by tumor promoters. Carcinogenesis 1984, 5:1547–1555
15. Shibanuma M, Kuroki T, Nose K: Induction of DNA replication and expression of proto-oncogene *c-myc* and *c-fos* in quiescent Balb/3T3 cells by xanthine/xanthine oxidase. Oncogene 1988, 3:17–21
16. Allen RG, Balin AK: Oxidative influence on development and differentiation: An overview of a free radical theory of development. Free Radicals Biol Med 1989, 6:631–661
17. Magalhaes H: Hamsters. *In* Hafez ESE, ed. Reproduction and Breeding Techniques for Laboratory Animals. Philadelphia, Lea and Febiger, 1970, pp 258–272
18. Oberley LW, Kasemset St Clair D, Autor AP, Oberley TD: Increase in manganese superoxide dismutase activity in the mouse heart after x-irradiation. Arch Biochem Biophys 1987, 254:69–80
19. Spitz DR, Elwell JH, Sun Y, Oberley LW, Oberley TD, Sullivan SJ, Roberts RJ: Oxygen toxicity in control and H_2O_2-resistant Chinese hamster fibroblast cell lines. Arch Biochem Biophys (In press)
20. Kirkman H, Galiano S, Gaetani G: The function of catalase-bound NADPH. J Biol Chem 1987; 262:660–666
21. Towbin H, Staehelin T, Gordon J: Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets. Proc Natl Acad Sci USA 1979; 76:4350–4354
22. McCormick M, Oberley LW, Oberley TD, Li JJ: Decreased superoxide dismutase and catalase levels in fetal hamster tissues and hamster kidney tumors. (Submitted for publication)
23. Yang AH, Oberley TD, Oberley LW, Ramanathan R: Effect of cell substrate on antioxidant enzyme activities in cultured renal glomerular epithelium. Am J Pathol 1988, 130:616–628
24. Marklund SL, Westman NG, Lundgren E, Roos G: Copper- and zinc-containing superoxide dismutase, manganese-containing superoxide dismutase, catalase, and glutathione peroxidase in normal and neoplastic human cell lines and normal human tissues. Cancer Res 1982, 42:1955–1961
25. Marklund SL: Extracellular superoxide dismutase and other superoxide dismutase isoenzymes in tissues from nine mammalian species. Biochem J 1984, 222:649–655
26. Marklund SL: Extracellular superoxide dismutase and other superoxide dismutase isoenzymes in human tissues and human cell lines. J Clin Invest 1984, 74:1398–1403
27. Thaete LG, Crouch RK, Schultz BA, Spicer SS: The immunolocalization of copper-zinc superoxide dismutase in canine tissues. J Histochem Cytochem 1983, 31:1399–1406
28. Loven DP, Oberley LW, Rousseau FM, Stevens RH: Superoxide dismutase activity in 1,2-dimethylhydrazine-induced rat colon adenocarcinoma. J Natl Cancer Inst 1980, 65:377–381
29. Carraro C, Pathak MA: Characterization of superoxide dismutase from mammalian skin epidermis. J Invest Dermatol 1988, 90:31–36
30. Scott G: Antioxidants in vitro and in vivo. Chemistry in Britain 1985, 21:648–653
31. Schallreuter KU, Wood JM: Free radical reduction in the human epidermis. Free Radical Biol Med 1989, 6:519–522
32. Coulombe PA, Kopan R, Fuchs E: Expression of keratin K_{14} on the epidermis and hair follicle: Insights into complex programs of differentiation. J Cell Biol 1989, 109:2295–2312
33. Benzi G, Marzatico F, Pastoris O, Villa RF: Relationship between aging, drug treatment and the cerebral enzymatic antioxidant system. Exp Gerontol 1989, 24:137–148
34. Weisiger RA, Fridovich I: Mitochondrial superoxide dismutase. Site of synthesis and intramitochondrial localization. J Biol Chem 1973, 248:4793–4796
35. Chang LY, Slot JW, Geuze HM, Crapo JD: Molecular immunocytochemistry of the CuZn superoxide dismutase in rat hepatocytes. J Cell Biol 1988, 107:2169–2179

36. Novikoff AB, Goldfischer S: Visualization of microbodies for light and electron microscopy. J Histochem Cytochem 1968, 16:507(Abstr)
37. Oberley LW, Ridnour LA, Sierra-Rivera E, Oberley TD, Guernsey DL: Superoxide dismutase activities of differentiating clones from an immortal cell line. J Cell Physiol 1989, 138:50–60
38. Grankvist K, Marklund SL, Taljedal IB: CuZn-superoxide dismutase, Mn-superoxide dismutase, catalase and glutathione peroxidase in pancreatic islets and other tissues in the mouse. Biochem J 1981, 199:393–396
39. Kincaid-Smith P: Analgesic nephropathy. Aust N Z J Med 1988, 18:251–255
40. Oberley LW, Buettner GR: Role of superoxide dismutase in cancer: A review. Cancer Res 1979, 39:1141–1149
41. Oberley LW, Oberley TD: Free radicals, cancer, and aging. *In* Johnson JE, Walford R, Harmon D, Miguel J, eds. Free Radicals, Aging, and Degenerative Diseases. New York, Alan R. Liss, pp. 325–371
42. Pierce GR, Shikes R, Fink LM: Cancer: A Problem of Developmental Biology. Englewood Cliffs, NJ, Prentice-Hall, 1978
43. Sell S, Dunsford HA: Evidence for the stem cell origin of hepatocellular carcinoma and cholangiocarcinoma. Am J Pathol 1989, 134:1347–1363
44. Rao MS, Duivedi RS, Yeldani AV, Subbarao V, Tan X, Usman MI, Thangada S, Nemali MR, Kumar S, Scarpelli DG, Reddy JK: Role of periductal and ductual epithelial cells of the adult rat pancreas in pancreatic hepatocyte lineage: A change in the differentiation commitment. Am J Pathol 1989, 134: 1069–1086