

High Concentrations of Antibodies to Xanthine Oxidase in Human and Animal Sera

Molecular Characterization

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Abstract. The widespread occurrence of antibodies (IgG) specific to xanthine oxidase in both normal (nonimmune) human and animal sera, and in antisera raised against a diversity of unrelated antigens is described. A study of sera from 81 humans revealed that xanthine oxidase-specific IgG represents a high proportion (1–8%) of total IgG. No obvious correlation to pathological events or symptoms of disease could be found. These xanthine oxidase-specific antibodies could be isolated by immunoaffinity chromatography on purified human or bovine xanthine oxidase and showed specific binding to the enzyme polypeptide of M_r 155,000 in immunoblotting experiments. By immunofluorescence microscopy they displayed the same cell type-specific reaction as experimentally induced antibodies, i.e., the staining of lactating mammary gland epithelium and capillary endothelium.

The naturally occurring xanthine oxidase-specific antibodies consisted of polyclonal IgG of various subclasses. $F(ab')_2$ preparations gave immune-reactions identical to those of IgG. The human xanthine oxidase-specific IgG cross-reacted with the bovine enzyme and both human and animal antibodies partially inhibited its activity. The xanthine oxidase activity of human milk lipid globules and supernatant fractions from various human tissues was extremely low when compared with that of the bovine antigen. The enzyme protein, however, was effectively precipitated from these sources by both the human and bovine antibodies.

We suggest that the exceptionally high concentrations

of antibodies against one protein, xanthine oxidase, are due to self-immunization to the xanthine oxidase antigen present in endothelial cells of capillaries. We do not exclude, however, nutritional contributions of bovine milk antigen to the appearance of xanthine oxidase antibodies in human sera. The possible biological functions of this immunological reaction are discussed.

Introduction

Recently, evidence has accumulated for the occurrence of spontaneous antibodies, i.e., antibodies formed without intentional immunization in healthy individuals, which are commonly interpreted as autoantibodies (e.g., references 1–3). In some cases the antigenic targets appear to be constituents of intracellular structures such as actin, tubulin, spectrin, myoglobin, vimentin, and keratin. It has therefore been proposed that the presence of such putative autoantibodies in sera is the result of cell lesions in the body, which lead to an exposure of such antigens. In the case of tissue or cell type-specific antigens, such autoantibodies might provide useful indicators of cell-specific damage.

In immunolocalization studies, we previously demonstrated the occurrence of appreciable amounts of xanthine oxidase, an iron-sulfur-molybdenum-containing flavoprotein, in the cytoplasm of mammary-gland epithelium, and in capillary endothelial cells of a variety of bovine tissues (4). An immunologically related protein containing a polypeptide of the same molecular weight (M_r 155,000) has been identified in human milk and tissues (5; and these authors, unpublished observations). Therefore, we have speculated that the appearance of human xanthine oxidase in the blood stream might be an indication of capillary endothelial cell damage (4). However, attempts to identify xanthine oxidase in human sera have been unsuccessful, even in patients with severe and extended capillary lesions. Reports of the occurrence of antibodies in human sera assumed to react with bovine xanthine oxidase (6, 7; for review see reference 8) prompted us to identify such antibodies and to characterize the antigen at the molecular level. Sera from many humans, from cows and

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goats, and from various laboratory animals (mice, guinea pigs, and rabbits) that had been immunized against different antigens all contained surprisingly high concentrations (1–8% of total Ig) of antibodies binding to bovine xanthine oxidase.

Methods

Tissues, cells, and fractionation procedures. Bovine tissues were taken at a local slaughterhouse from 3–4-yr-old Holstein cows within 5 min after death. For immunofluorescence microscopy, samples from liver, heart muscle, pancreas, parotid gland, as well as lactating and nonlactating mammary gland, were cut into pieces of 2–4 mm diam and frozen in isopentane at -150°C . For biochemical studies, tissue slices of <1 cm diam were directly incubated in ice-cold phosphate buffered saline (PBS; 0.15 M NaCl, 10 mM sodium phosphate, pH 7.4) supplemented with 0.4 mM phenylmethylsulfonyl fluoride. Supernate fractions (10^5 g) from tissues and from milk lipid globules were prepared as described previously (4). Samples of human tissue were obtained from biopsy material at the University Hospitals of Heidelberg, Mannheim (Federal Republic of Germany), and Vienna (Austria). These tissues included liver, lung, heart, kidney, epidermis, and placenta from a woman after delivery. The time between surgical removal of the specimens and further processing for immunolocalization and biochemical studies was as described for bovine material and usually took less than 1 h.

The following established cell lines were used for comparison: (Madin-Darby bovine kidney cells (ATCC No. CCL 22), hormone-dependent bovine mammary gland epithelial cells (see reference 9), HeLa (human cervical adenocarcinoma cells; for conditions of growth see reference 10), and MCF-7 (human breast adenocarcinoma; see reference 11).

Test sera and antibodies. Serum was prepared from blood samples of BALB/c mice, New Zealand white rabbits, and Holstein cows. Human blood samples were obtained and serum was prepared from 10 healthy volunteers of both sexes ranging from 22 to 56 yr, and from 71 people at the University Hospitals. These included two babies at days 2 and 4 after birth, respectively, one patient who had undergone immunosuppression by treatment with cyclosporin A after kidney transplantation, patients suffering from melanoma or lupus erythematosus discoides, and age- and sex-matched controls without indications of disease. Outdated blood samples from the University of Heidelberg Blood Bank were also used. In all cases, Ig fractions were prepared from the individual sera as described below.

The following antibodies were used: lyophilized human IgG (Miles Laboratories, Inc., Frankfurt); γ -chain-specific anti-human IgG (developed in goat; Sigma Chemical Co. München); anti-mouse IgG (from rabbit), human F(ab')₂ fragment, and anti-human IgG F(ab')₂-fragment (from rabbit) from Dianova (Hamburg); anti-mouse IgG (from goat) from Cappel Laboratories, Inc., (Cochranville, PA); and rabbit serum against human factor VIII antigen (Behringwerke, Marburg, Federal Republic of Germany). Other commercially available immunoreagents will be mentioned later.

Affinity-purified guinea-pig antibodies (Ig) directed against purified bovine milk xanthine oxidase (M, 155,000) have been described (5). A monoclonal murine antibody (IgG₁) against a nuclear protein from *Xenopus* oocytes was a gift from B. Hügle, and specific guinea-pig antibodies (Ig) to murine vimentin (12) and cytokeratin component D (13) were kindly provided by W. W. Franke (both from the Institute of Cell and Tumor Biology, German Cancer Research Center).

Purification of specific IgG. Total Ig was precipitated from serum with 40% saturated $(\text{NH}_4)_2\text{SO}_4$ solution (pH 7.4), dialyzed against 20 mM sodium phosphate buffer (pH 8.0), chromatographed on DEAE-Affi-Gel Blue (Bio-Rad Laboratories, München), and then applied to a Sephadex G-200 column (Pharmacia Fine Chemicals, Freiburg, Federal Republic of Germany) in the presence of 0.1 M sodium phosphate buffer (pH 8.0). The major fraction containing IgG was further purified by binding to protein A-Sepharose 4B. Bound IgG was eluted with 3 M KSCN and extensively dialyzed against PBS. This preparation was rotated end-over-end in a sealed column with purified human or bovine xanthine oxidase (for enzyme purification see reference 4), covalently linked to CNBr-activated Sepharose 4B, for either 2 h at room temperature or overnight at 4°C . Specifically bound IgG was eluted from the column with 3 M KSCN.

Determination of IgG subclasses. Antibodies against human IgG subclasses (developed in sheep; Miles Laboratories, Inc.) were immobilized on nitrocellulose paper ("dot-blot"), incubated first for 2 h with 0.5% bovine serum albumin (BSA) in PBS and then for another 2 h at room temperature with radioiodinated (see below) anti-xanthine oxidase IgG. Autoradiography was performed as described (14).

Preparation of F(ab')₂ fragments. Ig chromatographed on DEAE-Affi-Gel Blue was incubated with pepsin (Boehringer Mannheim Biochemicals, Mannheim, Federal Republic of Germany), according to the method of Johnstone and Thorpe (15). Undigested Ig and F_c fragments were removed by chromatography on a protein A-Sepharose 4B column. Unbound material was separated on a Sephadex G-200 column. Fractions containing the F(ab')₂ fragments were pooled and subjected to immunoaffinity chromatography with immobilized xanthine oxidase as described above for IgG.

Gel electrophoresis. One-dimensional gel electrophoresis was as described by Laemmli (16). Two-dimensional gel electrophoresis in the range of pH 2–11 was according to the method of O'Farrell et al. (17). Staining of proteins was either with Coomassie Blue or with silver nitrate, according to the method of Ansorge (18).

Immunoblotting. $(\text{NH}_4)_2\text{SO}_4$ precipitate fractions from sera and purified xanthine oxidase-specific antibodies were tested for specificity by modifications (19) of the immunoblot technique of Towbin et al. (20).

Immunoprecipitation and enzyme assays. Xanthine oxidase-specific antibodies (IgG) were rotated end-over-end overnight in tightly sealed test tubes with the 10^5 g supernate preparations of various tissues. The mixture was then placed on ice for 30 min and then centrifuged for 15 min at 4,000 g. Soluble complexes and unbound IgG were precipitated by the addition of an anti-species antibody (anti-human IgG from rabbit; anti-human IgG from goat; or anti-bovine IgG from rabbit [Sigma Chemical Co.]) that had been pre-absorbed on immobilized human and/or bovine xanthine oxidase. All immunoprecipitates and the corresponding supernates were assayed for xanthine oxidase and xanthine dehydrogenase activities as described (21).

*Enzyme-linked immunosorbent assay (ELISA).*¹ The enzyme immunoassay in general followed the procedure of Hautanen and Linder (22). Each well of preactivated microtiter plates (Titertek; Flow Laboratories Inc., McLean, VA) was coated with 10 $\mu\text{g}/50$ μl PBS of soluble xanthine oxidase purified from the 10^5 g supernate of human or bovine milk lipid globules (for purification see reference 4). Remaining active sites were blocked with 200 μl of 0.1% BSA in PBS. Then the test sera, as well as the $(\text{NH}_4)_2\text{SO}_4$ precipitates from test sera and their

1. *Abbreviations used in this paper:* FITC, fluorescein isothiocyanate; ELISA, enzyme-linked immunosorbent assay.

1:10 and 1:100 dilutions in 0.1% BSA (in PBS), were added and incubated for 2 h at room temperature. Bound IgG was detected by the addition of protein A conjugated with alkaline phosphatase and by the subsequent reaction with *p*-nitrophenylphosphate (both, Sigma Chemical Co.) as a substrate, diluted in 1 M diethanolamine buffer (pH 9.8). The enzymic reaction was monitored after 1, 3, and 5 h by a reading of the absorbance at 405 nm in a photometer (Titertek Multiscan; Flow Laboratories Inc.).

Radiolabeling of antibodies. Xanthine oxidase-specific IgG was ¹²⁵I-labeled by the Bolton and Hunter reagent (Amersham Buchler, Braunschweig, Federal Republic of Germany) using the procedure described by Dingwall et al. (23).

Immunofluorescence microscopy. Indirect immunofluorescence microscopy was performed on cryostat sections of frozen tissue and on cells grown on coverslips as described by Krohne et al. (24). Fluorescein isothiocyanate (FITC)-conjugated antisera to IgG from mouse and guinea pig (developed in rabbit) and from rabbit and human (developed in goat) were from Miles-Yeda (Rehovot, Israel). Rhodamin-B-conjugated antiserum to guinea pig IgG (developed in rabbit) was from Medac (Hamburg).

Results

Xanthine oxidase-specific Ig is present in diverse species. When we tested various monoclonal murine antibodies with anti-murine Ig developed in goats, rabbits, or guinea pigs (distributed by Miles-Yeda, Sigma Chemical Co., or Behringwerke), we noted that all of these antibody preparations revealed a specific immunoblot reaction with an *M_r* 155,000 polypeptide present in the 10⁵ g supernate fractions from many tissues.

On sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis the reacting antigen co-migrated with xanthine oxidase protein purified from milk lipid globules, which gave an identical immunoreaction. When typical anti-mouse Ig preparations (e.g., from goat or guinea pig) were incubated with purified xanthine oxidase immobilized on Sepharose 4B, ~2% of the applied Ig was immunoabsorbed by xanthine oxidase (Table I). The nonabsorbed Ig-fraction and the specifically bound Ig-fraction, which was obtained by elution with 3 M KSCN, were used to detect xanthine oxidase with the immunoblotting technique. With the 10⁵ g supernate fractions from bovine liver, with bovine and human milk lipid globule membranes, and with xanthine oxidase purified from milk lipid globules, only the xanthine-oxidase-specific Ig-fraction reacted with the *M_r* 155,000 xanthine oxidase polypeptide after transfer to nitrocellulose paper.

The Ig preparations from the sera of several other species, including goat, cow, guinea pig, rabbit, and mouse, also contained remarkably high concentrations of xanthine oxidase-specific Ig, as determined by the immunoaffinity absorption method (Table I). Antibodies contained in these preparations reacted with both human and bovine xanthine oxidase. Only bovine anti-xanthine oxidase samples did not cross-react with the corresponding human antigen.

Xanthine oxidase-specific IgG is present in human serum. In order to test human serum for the presence of xanthine

Table I. Concentration of Xanthine Oxidase-specific Ig in Ig Fractions from Different Species

Antibody source	Xanthine oxidase-specific Ig*
	% total Ig
Goat IgG (anti-mouse)	2.0
Goat IgG (anti-human)	1.4
Bovine IgG	4.5
Guinea-pig Ig (anti-mouse)	2.1
Guinea-pig Ig (anti-butyrophilin)	positive‡
BALB/c mouse Ig	4.0
Rabbit IgG (anti-human)	5.8
Rabbit Ig (anti-galactosyltransferase)	positive‡
Human Ig	1.8§

* The xanthine oxidase-specific Ig was purified and the concentration was determined by exhaustive immunoaffinity chromatography.

‡ In these cases specificity of Ig was demonstrated by immunoblotting test.

§ Mean value from 81 people.

oxidase-specific antibodies, one of us (Dr. Heid) donated several blood samples. Total Ig was prepared and fractions, after filtration on a Sephadex G-200 column, were tested for specific binding to immobilized xanthine oxidase (see Methods). Only the IgG fraction (characterized by electrophoresis on SDS-polyacrylamide gels, see below) revealed specific binding. Specifically bound IgG recognized the *M_r* 155,000 antigen in both human and bovine samples as shown by the immunoblot test (Fig. 1). IgG that was not retained by immobilized xanthine oxidase did not bind to any protein in the electrophoresed fractions (Fig. 1 *b*). IgG preparations of blood samples from the same individual which were donated at different times (approximately weekly intervals) varied in their xanthine oxidase-specific IgG concentration from 1 to 6% of total IgG. Seven colleagues² and the other two authors also donated samples. All proved to be positive for xanthine oxidase-specific IgG by the immunoblot technique with ¹²⁵I-protein A. No obvious correlation with dietary preferences, especially with respect to dairy products, was found. The sera from five patients of the Department of Dermatology at the University Hospital in Mannheim were also monitored for xanthine oxidase-specific IgG, and 0.2–1.8% of their total IgG was found to bind to immobilized xanthine oxidase.

In order to screen a large number of individuals ELISA was used. Purified xanthine oxidase from human milk lipid globules was coated on pre-activated microtiter plates, test samples were allowed to bind to the immobilized xanthine oxidase, and specific binding was detected with enzyme-linked

2. We thank B. Czernobilsky, W. W. Franke, B. Hügle, H.-P. Kapprell, W. Keller, A. Krämer, I. Moll, all from the Institute of Cell and Tumor Biology, German Cancer Research Center.

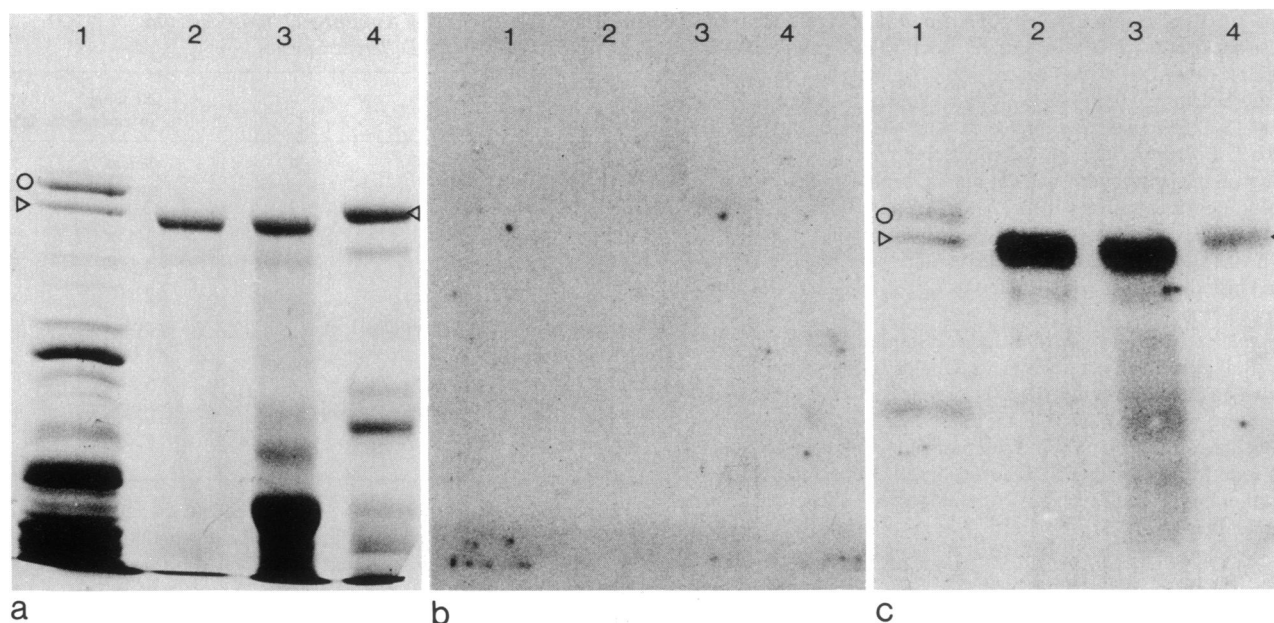


Figure 1. Immunoblot reaction of xanthine oxidase-specific IgG from human serum. Proteins were separated on SDS-polyacrylamide (6%) gels. The specific electrophoresed polypeptides were from 10^5 g supernate of bovine liver (lane 1), chromatographically purified xanthine oxidase from bovine milk lipid globules (lane 2), bovine milk lipid globule membranes (lane 3), and human milk lipid globule membranes (lane 4). Shown are blots of (a) Coomassie Blue-stained control gel, run in parallel with two identical gels, used for electrophoretic transfer of polypeptides to nitrocellulose. The nitrocellulose paper was incubated with human IgG (from Dr. Heid) that had been

applied to immobilized xanthine oxidase from bovine milk lipid globules. Also shown are blots of (b) nonabsorbed IgG and (c) specifically absorbed IgG, which was then incubated with ^{125}I -labeled protein A. Exposure times were identical for both autoradiographs (b and c). Note the reaction of the affinity-purified IgG with the xanthine oxidase polypeptide of M_r 155,000 (arrowheads) but the absence of reaction with nonabsorbed IgG. In lane 1, a polypeptide of M_r 160,000 (circles) is also detected by the antibodies. This polypeptide is typical for liver tissue and is biochemically very similar to xanthine oxidase of M_r 155,000 (reference 5).

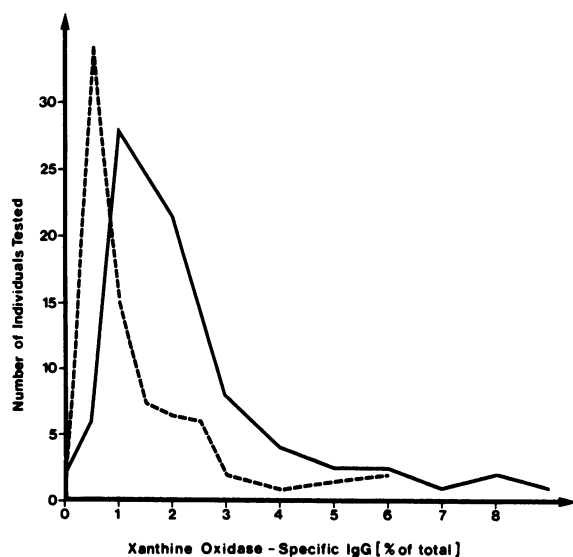


Figure 2. Concentration of xanthine oxidase-specific antibodies in the sera of 74 human individuals. Values were obtained by ELISA (for details see Methods). The graph represents the mean of two different

protein A (for details see Methods). We used protein A for two reasons: one, all tested animal sera contained xanthine oxidase-specific antibodies (e.g., anti-human IgG from goat or rabbit; see Table I); and two, in all human sera analyzed in detail specific binding to xanthine oxidase were confined to the IgG fraction. The xanthine oxidase-specific IgG (the same as that used in Fig. 1 c) from one individual (Dr. Heid) was used in serial dilution to calibrate the assay for specific binding. The unbound IgG from immunoaffinity chromatography (of the same individual) that showed no specific binding with xanthine oxidase in the more sensitive immunoblot reaction (same as in Fig. 1 b) was used as negative control. In parallel, the binding of human IgG to bovine milk xanthine oxidase was also measured. In the majority of the sera, 0.5–3% of the total IgG (based on the calibration above) were xanthine oxidase-specific antibodies (Fig. 2). Two individuals were neg-

determinations. The solid line represents the binding of IgG to human milk xanthine oxidase coated to the microtiter plates, and the dashed line shows binding to bovine milk xanthine oxidase coated in the same way. In most individuals 0.5–3% of total IgG bound to human xanthine oxidase. Titers to the bovine enzyme were on average lower, equivalent to 0.2–1.5% of total human IgG.

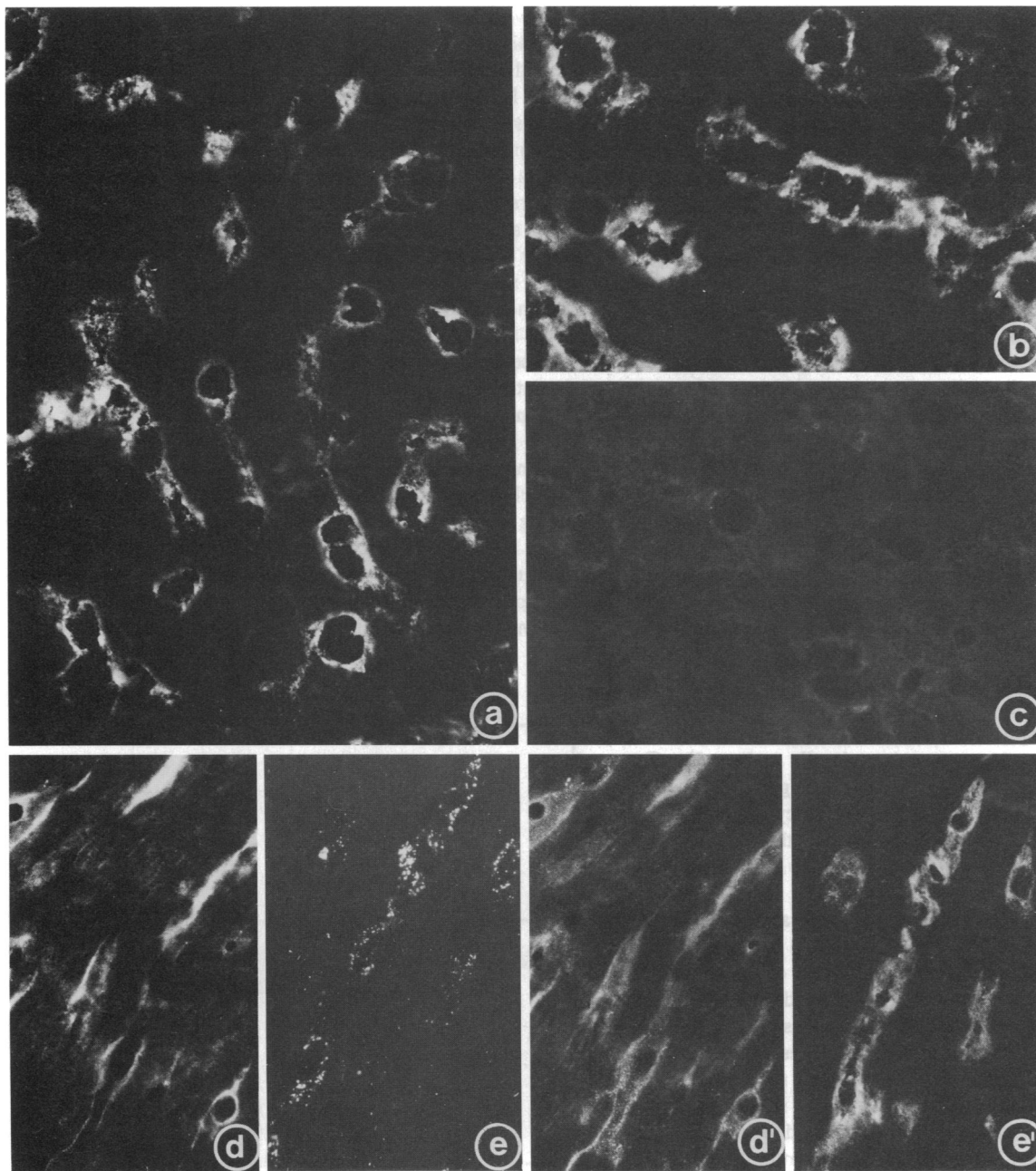


Figure 3. Immunofluorescence microscopy on cryostat sections by use of human xanthine oxidase-specific antibodies (*a*, *b*, and *d*). Sections through bovine (*a*) and human (*b* and *c*) liver as well as bovine heart (*d* and *e*) are shown. The sinusoidal lining of liver (*a* and *b*) and the capillaries of heart muscle (*d*) are brightly stained by xanthine oxidase-specific IgG by the use of FITC-coupled anti-human IgG as second antibodies but not by nonspecific IgG from the same human serum (*c*). Staining of capillaries by the xanthine oxidase-specific IgG was confirmed by colocalization on the same sec-

tions (*d* and *d'*; *e* and *e'*) by the use of affinity-purified guinea-pig Ig to authentic bovine xanthine oxidase and Rhodamin B-coupled anti-guinea pig IgG (*d'* and *e'*) or rabbit antiserum to Factor VIII antigen and FITC-coupled anti-rabbit Ig (*e*). Staining for xanthine oxidase appears diffuse throughout the cytoplasm of endothelial cells whereas antibodies to Factor VIII show a distinct punctate, i.e., granular, pattern. Magnification: 640 (*a*); 600 (*b* and *c*); 400 (*d* and *d'*); and 450 (*e* and *e'*).

ative in the ELISA assay. The serum sample of one of them had been taken 10 d after a kidney transplant and treatment with the immunosuppressive drug cyclosporin A, but with the immunoblot technique, xanthine oxidase-specific IgG was detected in the serum, albeit at a low level (data not shown). On the average, the binding to human xanthine oxidase was higher than binding to bovine xanthine oxidase by a factor of two. However, four individual sera showed a lower binding affinity to the human xanthine oxidase when compared with the bovine antigen. Seven individuals contained antibodies to human xanthine oxidase in very high concentrations, i.e., between 6 and 9%. The sera of two newborn children (2 and 4 d old) had values of 1 and 8%, respectively.

Localization of antigen in capillary endothelium. Using human IgG that specifically bound to xanthine oxidase, we examined the location of antigen in several human and bovine tissues by immunofluorescence microscopy. Fig. 3 shows the staining of xanthine oxidase-specific IgG on frozen sections of human and bovine liver and of bovine heart, in comparison with the staining of affinity-purified guinea-pig Ig to bovine milk xanthine oxidase and antiserum to human factor VIII antigen, a glycoprotein known to be secreted by endothelial cells (e.g., reference 25). Decoration by human IgG specific for xanthine oxidase was restricted to the sinusoidal lining of both bovine (Fig. 3 a) and human (Fig. 3 b) liver, whereas the liver parenchymal and bile duct epithelial cells were negative. The staining pattern was distinct from that observed with antibodies to vimentin, which reacted strongly with endothelial cells but also with other mesenchymal cells such as Kupffer cells (4). It was also different from the staining with antibodies to cytokeratins which are specific for hepatocytes and the bile ducts (13). The IgG fraction of human sera that did not bind to immobilized xanthine oxidase showed no specific staining (Fig. 3 c).

Human xanthine oxidase-specific IgG also stained the capillary endothelial cells of bovine (Fig. 3 d) and human (not shown) heart muscle. No specific decoration of the capillaries was observed with the human IgG that did not bind to immobilized xanthine oxidase. For comparison, the staining of heart capillaries with guinea pig antibodies to bovine milk xanthine oxidase (Fig. 3 d' and e') and with rabbit antiserum to human Factor VIII antigen (Fig. 3 e) is shown. Whereas the xanthine oxidase appeared to be located throughout the endothelial cells, as judged from the immunofluorescence studies with all xanthine oxidase-specific antibodies, the staining for Factor VIII antigen revealed a distinct punctate pattern. This difference in location could be explained by observations that xanthine oxidase is a cytoplasmic protein of the endothelial cells (4, 21), whereas the Factor VIII antigen is located in endoplasmic reticular cisternae and cytoplasmic granules (e.g., 26).

Immunofluorescence studies were also performed with a variety of other tissues and cells in cultures. Human xanthine oxidase-specific IgG reacted with capillaries and other small

blood vessels of human placenta and kidney. The antibodies also stained capillaries of bovine pancreas and parotid as well as capillaries and secretory epithelial cells of lactating mammary gland. By contrast, nonlactating mammary gland epithelial cells displayed only a very weak reaction, and no reaction was seen on various human and bovine cells growing in culture such as HeLa, MCF-7, Madin-Darby bovine kidney cells and the hormone-dependent bovine mammary gland cell line (for details see Methods). In contrast to human xanthine oxidase-specific IgG, which cross-reacted with the bovine antigen, the bovine xanthine oxidase-specific IgG did not react with human xanthine oxidase. With all the xanthine oxidase antibodies tested, immunofluorescence staining was abolished when the tissue sections had been fixed with formaldehyde, even at low concentrations (0.5%).

Characterization of human xanthine oxidase-specific antibodies. Ig fractions from a number of human sera, purified by ammonium sulfate precipitation, chromatography on DEAE-Affi-Gel Blue, and Sephadex G-200 filtration were tested for binding to xanthine oxidase by immunoaffinity chromatography and by immunoblotting. Fractions that reacted positively with xanthine oxidase were analyzed by SDS-polyacrylamide gel electrophoresis (data not shown). Analyses of Sephadex G-200 fractions by immunoaffinity chromatography revealed the ex-

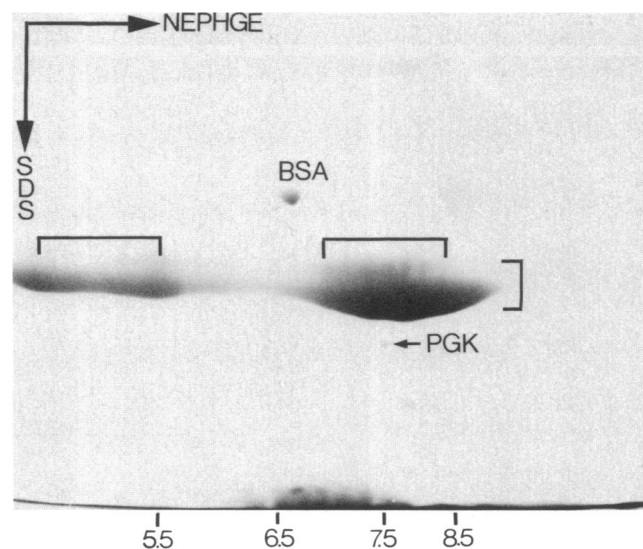


Figure 4. Two-dimensional gel electrophoresis of xanthine oxidase-specific human IgG. About 250 μ g specific IgG (identical preparation to that used in Fig. 1 c) was used for isoelectric focusing. BSA and phosphoglycerokinase (PGK) were coelectrophoresed as markers. Staining was with Coomassie Blue. Direction of nonequilibrium pH gel electrophoresis (NEPHGE) was from left to right, and gel electrophoresis (9% acrylamide) in the presence of SDS was from top to bottom. Numbers at the bottom indicate pH values. Brackets demarcate three subgroups of isoelectric variants of IgG heavy chain.

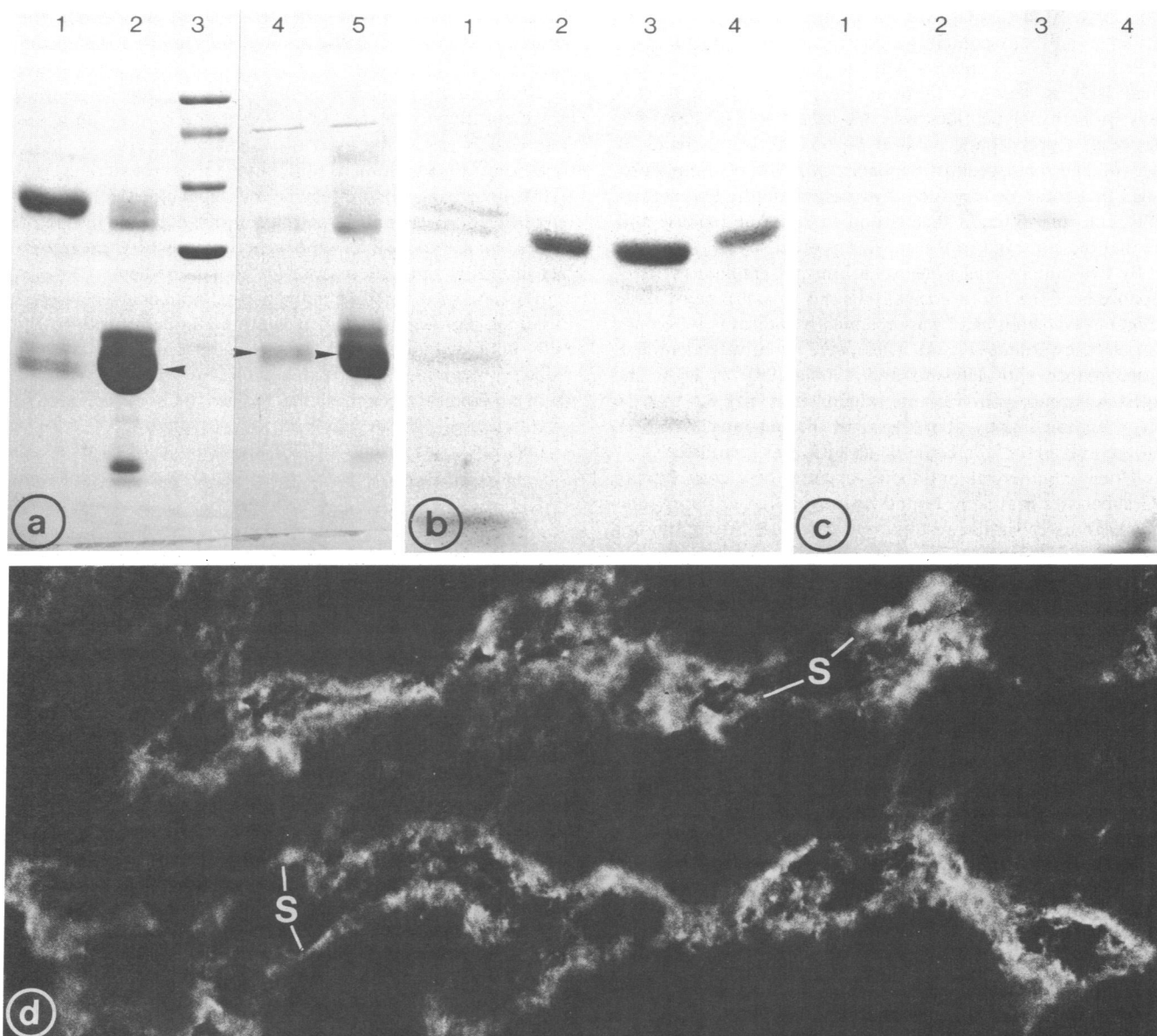


Figure 5. Gel electrophoresis and immune reactions of $F(ab')_2$ fragments. (a) One-dimensional gel electrophoresis (12% acrylamide, staining with Coomassie Blue) of untreated human IgG (lane 1), a commercial human $F(ab')_2$ preparation (lane 2), molecular weight markers (lane 3, from top to bottom: phosphorylase a, transferrin, catalase, and actin), human $F(ab')_2$ fragments specifically binding to immobilized human and bovine xanthine oxidase (lane 4), and human $F(ab')_2$ fragments that did not bind to immobilized xanthine oxidase (lane 5). Arrowheads denote $F(ab')_2$ fragments. (b) Autoradiograph of the immunoblot reaction with human xanthine oxidase-specific $F(ab')_2$ fragments. Lanes 1-4 contain the same protein preparations as those described in Fig. 1. Note specific reaction with the M_r 155,000 xanthine oxidase polypeptide and the M_r 160,000 polypeptide of liver (lane 1), similar as described for intact IgG in Fig. 1

c. (c) Autoradiograph of the immunoblot reaction with the human $F(ab')_2$ fragments that did not bind to immobilized human and bovine xanthine oxidase (same exposure time). Note only weak reaction of some residual amount of specific $F(ab')_2$ fragments (lane 3).

In these experiments, $F(ab')_2$ was detected by the addition of rabbit IgG against human $F(ab')_2$ fragments that had been preabsorbed against human and bovine xanthine oxidase, followed by the addition of ^{125}I -labeled protein A. (d) Immunofluorescence microscopy on cryostat section of human liver by use of human xanthine oxidase-specific $F(ab')_2$ fragments. Rabbit IgG against human $F(ab')_2$ was preabsorbed on immobilized bovine xanthine oxidase and used as second antibodies, which in turn were reacted with FITC-conjugated goat anti-rabbit IgG. Only cells of the sinusoidal lining (S) are stained (compare Fig. 3). Magnification: 960.

istence of xanthine oxidase-specific IgG in two major fractions: (a) Larger antibody-containing complexes were eluted from the Sephadex G-200 column with the void volume and contained IgG, as was evident from the presence of light and heavy γ -chain polypeptides with M_r values $<55,000$ and the absence of a polypeptide band at M_r 65,000, i.e., μ -chains of IgM. (b) The main peak of Sephadex filtration also consisted of IgG that could be separated, by immunoaffinity chromatography, into one fraction that bound to xanthine oxidase and one that did not bind to the enzyme protein.

By two-dimensional nonequilibrium pH gradient gel electrophoresis of the IgG specifically bound to xanthine oxidase, the IgG heavy-chain band was separated into distinct subgroups of isoelectric variants (Fig. 4). These were tentatively identified, in accordance with Howard and Virella (27), as IgG₁ (the largest subgroup, with variants ranging from pH 8.5 to 6.5), IgG₂ (banding slightly above IgG₁ in the pH range 7.7–6.7), and IgG₄ (at pH <5.7). Little or no IgG₃ was identified.

When commercial antibodies against these four human IgG subclasses that were bound on nitrocellulose paper (dot-blot) were used, similar results were obtained. Strong binding of radiolabeled xanthine oxidase-specific human IgG was observed with antibodies to IgG₁, IgG₂, and IgG₄, and only a weak reaction with anti-IgG₃ was seen. Similar results, with a marked preference for IgG₁, were obtained in other xanthine oxidase-specific human IgG examined. It was obvious from these experiments that xanthine oxidase-specific antibodies of human sera were IgG of polyclonal origin and were not confined to one distinct IgG subclass.

In order to examine whether the xanthine oxidase binding site of the xanthine oxidase-specific IgG was in the Fab' or in the F_c-region, human IgG fractions were digested with pepsin. F(ab')₂ fragments were purified by affinity chromatography on protein A-Sepharose 4B and Sephadex G-200 filtration and analyzed by gel electrophoresis (data not shown). After chromatography on immobilized xanthine oxidase, the specifically absorbed and the nonabsorbed F(ab')₂ fragments were analyzed by gel electrophoresis. As expected, the polypeptide composition of both fractions was the same (Fig. 5 a, lanes 4 and 5). Their protein concentration varied widely: The xanthine oxidase-specific F(ab')₂-fraction amounted to only $\sim 3\%$ of the total F(ab')₂. Binding to the M_r 155,000 xanthine oxidase polypeptide separated on gels was determined by immunoblotting on nitrocellulose paper by use of anti-F(ab')₂ IgG and ¹²⁵I-labeled protein A. The specifically absorbed F(ab')₂ showed a strong reaction (Fig. 5 b) in contrast to the very weak residual binding activity recovered in the large ($\sim 97\%$) fraction of nonabsorbed F(ab')₂ (Fig. 5 c). Similarly, a commercially available preparation of human F(ab')₂ fragments (Fig. 5 a, lane 2) also contained $\sim 5\%$ xanthine oxidase-specific fragments.

When the F(ab')₂-fraction that was absorbed to immobilized xanthine oxidase was used for immunofluorescence studies, the staining pattern observed was the same as that of the xanthine oxidase-specific IgG-fraction (compare Fig. 3). Cap-

illaries and sinusoids were intensely stained (Fig. 5 d). The nonabsorbed F(ab')₂ fraction showed only weak, insignificant reaction with capillaries.

Characterization of human and bovine antigen. In samples of various human tissues tested, xanthine oxidase activities were usually below the limit of detection, i.e., at least 1,000 times lower than in human milk lipid globule membranes and 60,000 times lower than in bovine milk lipid globules (compare reference 28). Because of these low enzyme activities in human tissues, we had to look for other criteria to identify the antigen recognized by xanthine oxidase-specific antibodies.

We have used experimentally induced antibodies (developed in guinea pig) against purified xanthine oxidase from bovine milk lipid globules, which cross-reacted with the xanthine oxidase in human milk lipid globules. As shown in Fig. 3, these antibodies recognized the antigen in both bovine and

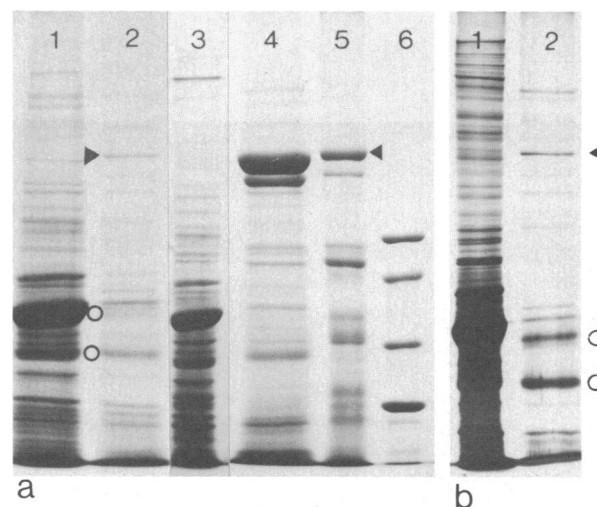


Figure 6. Immunoblotting of xanthine oxidase from human tissues. 10^5 g supernatant fractions from homogenized tissues were incubated with immobilized purified guinea-pig Ig against bovine xanthine oxidase. Specifically retained material was eluted with 3 M KSCN and analyzed by gel electrophoresis (7% acrylamide). (a) Comparison of polypeptides of 10^5 g supernatant fraction of human lung (lane 1) and the immunoabsorbed fraction thereof (lane 2); 10^5 g supernatant fraction from human kidney (lane 3) and the immunoabsorbed fraction thereof (lane 4). Polypeptides of human milk lipid globule membranes are shown for reference (lane 5), together with M_r marker proteins (lane 6; same as in Fig. 5 a). Staining was with Coomassie Blue. (b) 10^5 g supernatant fraction from human epidermis (lane 1) and immunoabsorbed fraction thereof (lane 2). Staining was with silver nitrate. Arrowheads denote position of xanthine oxidase, and circles denote positions of proteolytic fragments of xanthine oxidase (identified by immunoblotting procedures with experimentally induced guinea-pig Ig against xanthine oxidase, not shown). Note the relatively large proportion of xanthine oxidase in lung and kidney comigrating with milk xanthine oxidase polypeptide of M_r 155,000 (lane 5, arrowhead).

human tissues, in the same location, i.e., in capillary endothelial cells. When these xanthine oxidase-specific antibodies from guinea pig were used in immunoaffinity chromatography with supernate fractions from human tissue homogenates, a protein was specifically enriched that had the same molecular weight as xanthine oxidase in human milk lipid globules (Fig. 6). From these data we conclude that both antibody preparations—that developed in guinea pig against xanthine oxidase and that purified from human sera by immunoabsorption—recognized the same antigen, i.e., xanthine oxidase.

The xanthine oxidase-specific IgG from human sera precipitated the M_r 155,000 polypeptide from human and bovine milk lipid globules (Fig. 7). IgG that did not bind to immobilized xanthine oxidase (the same preparation as that used in Fig. 1 b) did not precipitate. Xanthine oxidase activity of human

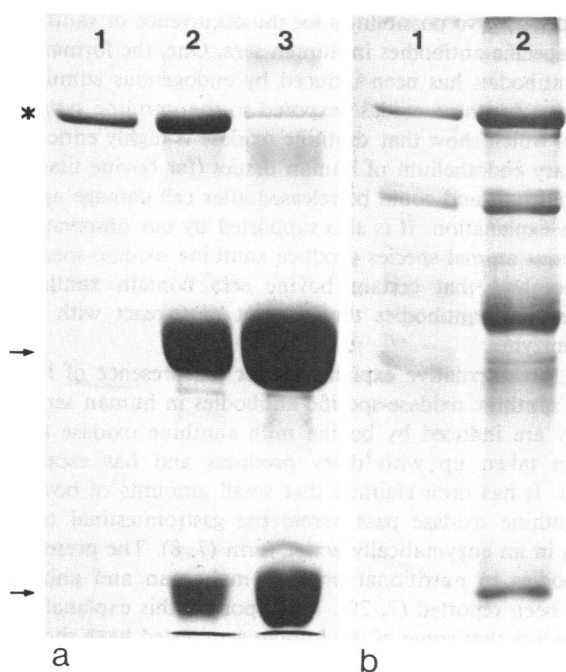


Figure 7. Gel electrophoresis of milk xanthine oxidase material immunoprecipitated with xanthine oxidase-specific IgG. (a) Coomassie Blue-stained immunoprecipitates analyzed on 10% polyacrylamide gels after incubation of protein from 10^5 g supernate fractions from bovine milk lipid globules (lane 1) with xanthine oxidase-specific bovine IgG that had been purified from bovine sera by immunoabsorption on immobilized bovine xanthine oxidase: the immunoprecipitate is shown in lane 2. IgG not bound on immobilized xanthine oxidase was also incubated with the 10^5 g supernate from bovine milk lipid globules: the precipitate is shown in lane 3. (b) 10^5 g supernate fraction from human milk lipid globules (lane 1) was incubated with xanthine oxidase-specific IgG purified from human serum, and the immunoprecipitate obtained is shown in lane 2. Arrows denote positions of precipitated heavy and light chains of immunoglobulins; asterisk denotes position of xanthine oxidase.

milk lipid globule fractions was too low to be measured accurately in the immunoprecipitates obtained with xanthine oxidase-specific antibodies. We therefore used the bovine system, where xanthine oxidase activity is high (compare references 4 and 21). From bovine serum, we succeeded in isolating xanthine oxidase-specific IgG by immunoabsorption. This IgG precipitated the enzyme protein (Fig. 7) and the enzyme activity (Table II). The bovine xanthine oxidase-specific IgG inhibited the homologous enzyme activity by $\sim 35\%$, whereas the nonabsorbed IgG fraction produced no inhibition. About 25% of the activity measured in the presence of xanthine oxidase-specific IgG was recovered in the immunoprecipitate. In contrast, no enzyme activity and very little enzyme protein was found in the immunoprecipitates obtained after incubation with nonabsorbed IgG (Table II and Fig. 7).

By immunoblotting, the bovine xanthine oxidase-specific IgG did not cross-react with human xanthine oxidase. On the contrary, the IgG from several human individuals not only cross-reacted with bovine xanthine oxidase but also showed an even stronger reaction with the bovine compared with the

Table II. Effect of Xanthine Oxidase-Specific IgG from Bovine Serum on Xanthine Oxidase Activity in Bovine Milk Lipid Globules

Fraction and treatment	Enzyme activity	
	nmol uric acid/min	
10^5 g supernatant of bovine milk lipid globules (0.3 mg protein)	945	100
In the presence of 0.3 mg xanthine oxidase-specific IgG from bovine serum	610	65
After immunoprecipitation with 0.3 mg xanthine oxidase-specific IgG: immunoprecipitate*	142	15
After immunoprecipitation with 0.3 mg xanthine oxidase-specific IgG: remaining supernatant	362	38
In the presence of 0.48 mg nonabsorbed IgG from bovine serum	945	100
After immunoprecipitation with 0.48 mg nonabsorbed IgG from bovine serum: immunoprecipitate*	4	0.4
After immunoprecipitation with 0.48 mg nonabsorbed IgG from bovine serum: remaining supernatant	926	98

* The gel electrophoretic analysis of immunoprecipitates is shown in Fig. 7 a.

human antigen (see also data obtained by ELISA and Fig. 1 c). In addition, some human sera (an example is documented in Fig. 8) contained IgG not only to xanthine oxidase but also IgG that bound to certain glycoproteins of bovine milk lipid globule membranes (Fig. 8, lane 3).

Discussion

Our results show that surprisingly high concentrations of IgG binding to xanthine oxidase are found in the sera of various animals as well as in human sera. Specificity for xanthine oxidase has been demonstrated by immunoaffinity chromatography, ELISA, immunoblotting, by immunolocalization in lactating mammary gland and capillary endothelium, and by

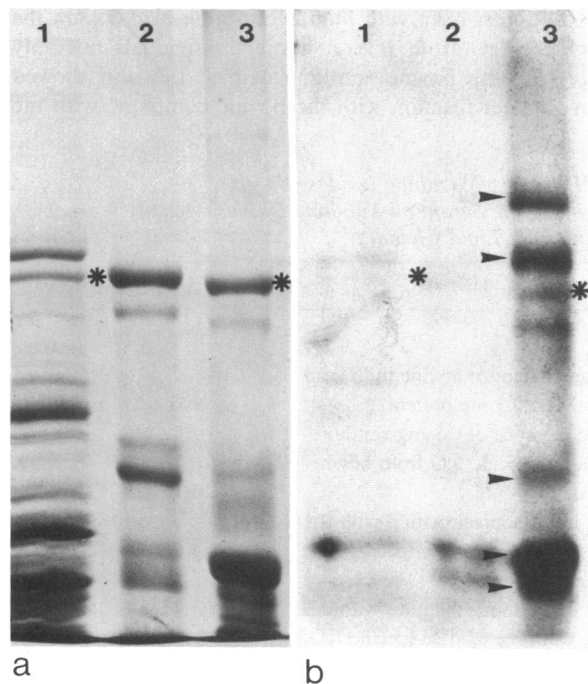


Figure 8. Immunoblotting reactions of a special human serum showing a preferential reaction with xanthine oxidase and other proteins of the bovine milk lipid globule membrane. Gel electrophoresis ([a] 6% acrylamide; Coomassie Blue staining) shows major polypeptides of $10^5 g$ supernate fraction from homogenates of bovine liver (lane 1), human milk lipid globule membranes (lane 2), and bovine milk lipid globule membranes (lane 3). The corresponding autoradiograph (b) shows the IgG binding to these proteins as detected by ^{125}I -labeled protein A. This particular serum was taken from a 44-yr-old volunteer who had suffered from extensive hemorrhagic gastritis and was on a diet rich in dairy products for 1 y. It contains, in addition to xanthine oxidase-specific IgG (asterisks), IgG binding to a M, 200,000 polypeptide (uppermost arrowhead, lane 3) and several major glycoproteins of bovine milk lipid globule membranes (arrowheads), including butyrophilin (lane 3, second arrowhead from bottom).

immunoprecipitation. $F(ab')_2$ fragments show the same xanthine oxidase-specificity as intact immunoglobulin molecules. In most cases the antibodies have shown cross-species reactivity with the human and bovine M, 155,000 xanthine oxidase polypeptide, although differences of intensities in binding to human and bovine xanthine oxidase have been noted.

The xanthine oxidase-specific antibodies consist mostly of polyclonal IgG of different subclasses, suggesting that they originate from different antigenic stimulation events. Pronounced differences in xanthine oxidase-specific IgG concentrations can be seen between different individuals and also in the same person at different times.

Xanthine oxidase-specific IgG from human and bovine sera can precipitate xanthine oxidase enzyme activity from bovine tissues. The immunoabsorbed protein from human tissues is similar to that absorbed by experimentally induced antibodies to authentic bovine xanthine oxidase.

We discuss two possibilities for the occurrence of xanthine oxidase-specific antibodies in human sera. One, the formation of the antibodies has been induced by endogenous stimulus, i.e., human xanthine oxidase exposed to the immune system. Our data which show that xanthine oxidase is highly enriched in capillary endothelium of human tissues (for bovine tissues, see reference 4) and could be released after cell damage agree with this explanation. It is also supported by our observation that various animal species produce xanthine oxidase-specific antibodies and that certain bovine sera contain xanthine oxidase-specific antibodies that do not cross-react with the human enzyme.

Two, an alternative explanation for the presence of high titers of xanthine oxidase-specific antibodies in human sera is that they are induced by bovine milk xanthine oxidase that has been taken up with dairy products and has escaped digestion. It has been claimed that small amounts of bovine milk xanthine oxidase pass across the gastrointestinal tract epithelia in an enzymatically active form (7, 8). The presence of antibodies to nutritional proteins in human and animal sera has been reported (7, 29). In support of this explanation is our finding that some of the human sera tested have shown stronger binding to bovine than to human xanthine oxidase and that they also contained antibodies that bound to other bovine milk proteins that are not noted in human milk and tissue samples (e.g., Fig. 8).

However, we cannot rule out the possibility that the xanthine oxidase-specific IgG in human and animal sera are directed against an unknown antigen that shares many epitopes with xanthine oxidase.

Antibodies to endogenous proteins are well known in pathological situations such as autoimmune diseases (for review see reference 30). In the last 10 yr antibodies against self-components have been described, although at relatively low concentrations, in sera of apparently healthy animals and humans (3, 31–33). Such antibodies can be directed against a variety of antigens: examples include intermediate filament

proteins of the keratin (34, 35) and the vimentin (36) types, actin, tubulin, and spectrin (e.g., references 1, 2, and 37) and a variety of soluble cytoplasmic and secretory proteins such as thyroglobulin, myoglobin, albumin, and transferrin (33). Most of these natural autoantibodies examined have been found to belong to the IgM class (1, 3, 36, 38).

From our arguments given above we think that most of the xanthine oxidase antibody formation is due to immunization against the endogenous protein. However, the antibodies against the cytoplasmic protein xanthine oxidase in human and animal sera differ from autoantibodies described in other reports in the heterogeneity of the antibody population and the extraordinarily high titers (20 to 100 times higher than values reported for natural autoantibodies) (2, 33). This suggests to us that self-immunization with xanthine oxidase is a multiple event, perhaps induced by accidental harmless lesions of capillary endothelium. However, we cannot explain why the immune system has not acquired immunological tolerance to this protein.

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