Immunological relationship between peroxidases in eosinophils, uterus and other tissues of the rat

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A rabbit antibody against purified pig intestinal peroxidase was shown by means of Western blotting and immunodetection to bind to peroxidases in various rat tissues, including eosinophils, uterus, uterine fluid and mammary tumours, and also to bind to bovine lactoperoxidase. The peroxidase in all rat tissues had an M_r of 53 000, except for uterine fluid, in which the cross-reacting band had an M_r of 80 000. The results indicate that while some of the peroxidase present in uterine tissue could be derived from eosinophils, the enzyme secreted into the lumen of the uterus is likely to have a different origin. They also suggest that mammary tumour peroxidase could originate from infiltration by eosinophils.

There has been considerable work to determine whether most animal peroxidases (EC 1.11.1.7) are derived from eosinophil granulocytes, rich in peroxidase (Rytömaa & Teir, 1961; King *et al.*, 1981) or whether this haem enzyme is produced by the tissues themselves. This is important because oestrogen-sensitive organs, such as the uterus, show large increases in peroxidase activity after hormonal treatment, making this enzyme a useful marker with which to follow oestrogen action (DeSombre & Lyttle, 1979; Jellinck *et al.*, 1979). To this end, peroxidases from various tissues of different species have been purified, characterized and their properties shown to be very similar to each other (Archer *et al.*, 1965; Stelmazyńska & Zgliczyński, 1971; Hosoya *et al.*, 1981; Jörg *et al.*, 1982; Kimura & Jellinck, 1982; Olsen *et al.*, 1982). Few immunological studies have been reported (Morrison *et al.*, 1965) but the identity of rat spleen and rat uterine peroxidase has now been demonstrated with a monoclonal antibody (Keeping & Lyttle, 1982). Peroxidases from rat uterus and rat eosinophils were shown to be enzymologically very similar, and were both inhibited by a rabbit antiserum raised against the uterine enzyme (Olsen & Little, 1982).

Recently, it has been suggested (Hosoya & Saito, 1981; Hosoya *et al.*, 1981) that peroxidase in rat uterine fluid, and lactoperoxidase, which are both

soluble enzymes, differ from membrane-associated peroxidases inside uterine cells or eosinophils. It was therefore considered of value to determine the immunological relationship of peroxidases in various tissues of the rat.

In the present paper, we compare the peroxidase from eosinophils with the peroxidases from other tissues of the rat, including the uterus and uterine fluid, by using an immunological technique.

Materials and methods

Sample preparation

Female Sprague–Dawley rats (140–170 g) from Charles River (La Prairie, Quebec, Canada) were used to obtain tissue samples for gel electrophoresis. The rats were given oestradiol (10 µg) in 0.2 ml of sesame oil subcutaneously, 20 h before killing by cervical dislocation, and the lack of effect of oestradiol on peroxidase activity in tissues other than uterus was confirmed. The small intestine, uterus, spleen, and lung were removed and uterine fluid collected as described by Hosoya *et al.* (1981). Cellular particles in the uterine fluid were removed by centrifugation at 600 g for 15 min (uterine fluid pellet). Eosinophils were obtained by peritoneal lavage with 0.15 M-NaCl and treated with hypo-osmotic saline to remove any contaminating erythrocytes (Hosoya *et al.*, 1981). Mammary tumours were induced in 45–50-day-old female Sprague–Dawley rats by the gastric instillation of a single dose (15 mg) of dimethylbenz[*a*]anthracene in sesame oil (1.0 ml). Tumours

Abbreviation used: IgG, immunoglobulin G.

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began to appear 6–7 weeks after treatment and only those with high peroxidase activity were used. Immature female rats (20–22 days old) were given daily injections of oestradiol (1 μ g in 0.2 ml of oil) for 3 days before excision of the uteri and extraction of peroxidase. Pig small intestine was washed thoroughly with water and 0.15 M-NaCl and the mucosa obtained by scraping. The entire washed intestine was used with the rat. All tissues were homogenized in 5 vol. of 10 mM-EDTA/10 mM-Tris/HCl, pH 7.2, using a Potter–Elvehjem homogenizer with a Teflon pestle, and the homogenate was centrifuged at 40000 g for 30 min at 4°C. After washing with the same buffer, the precipitate was rehomogenized in 10 mM-EDTA/10 mM-Tris/HCl (pH 7.2)/1.2 M-NH₄Cl, and centrifuged again at 40000 g for 30 min to obtain a supernatant fraction. The 1.2 M-NH₄Cl extracts were used for gel electrophoresis without dialysis and some were freeze-dried to increase the concentration of protein. Pig small intestine peroxidase was purified by the method of Stelmazyńska & Zgliczyński (1971) with slight modifications. Bovine lactoperoxidase, cytochrome *c* from rat, pig, bovine and horse heart, myoglobin from horse heart and sperm whale muscle, and horseradish peroxidase were obtained from Sigma.

Antibody preparation

Antisera were raised in three female New Zealand white rabbits by intramuscular injections of purified pig small intestinal peroxidase, initially and at 4 weeks with doses of 400 μ g of protein in Freund's complete adjuvant, and at 10 and 20 weeks with 200 μ g of protein in Freund's incomplete adjuvant. Rabbits were bled 10 days after injection, and sera showing a strong antibody response by double immunodiffusion were pooled and stored at –20°C with 0.1% NaN₃.

Purification of IgG

IgG specific for the peroxidases was purified from crude antiserum by immunoaffinity chromatography using bovine lactoperoxidase as ligand. (All procedures were carried out at room temperature.) Lactoperoxidase (10 mg) was coupled to 1 g of CH-Sepharose-4B by the carbodi-imide method (Schleuning & Fritz, 1976). The immunoabsorbant was incubated with 15 ml of phosphate-buffered saline containing 2.5% (w/v) bovine serum albumin for 1 h, washed with phosphate-buffered saline, and then incubated with 1.4 ml of rabbit anti-(pig intestinal peroxidase) serum overnight. The gel was poured into a column and washed with phosphate-buffered saline and with 0.6 M-NaCl/10 mM-sodium phosphate, pH 7.4, and then the specifically-bound IgG was eluted with 0.2 M-glycine/0.6 M-NaCl, pH 2.4. The fractions of low pH were

immediately adjusted to pH 8 with 0.5 M-Tris and 2 M-NaOH and tested by immunodiffusion against pig intestinal peroxidase and against goat anti-(rabbit IgG) serum. Approx. 300 μ g of specific IgG was recovered.

Assay of peroxidase

Peroxidase activity was determined at 25°C using guaiacol as substrate (Himmelhoch *et al.*, 1969). The reaction mixture contained 13 mM-guaiacol and 0.33 mM-H₂O₂ in a total volume of 3.0 ml of 10 mM-Tris/HCl, pH 7.2, and 3–50 μ l of the sample, depending on its activity. The linear increase in *A*₄₇₀ resulting from the oxidation of guaiacol was measured with a Beckman model 25 recording spectrophotometer. One unit of enzyme activity is defined as the amount of enzyme required to produce an increase of 1 *A* unit/min. Protein was determined by the method of Bradford (1976) with γ -globulin as standard.

Western blotting

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis was performed as described (Laemmli, 1970). The transfer procedure was according to Towbin *et al.* (1979) and Burnette (1981) using a Trans-Blot cell (Bio-Rad) at constant voltage of 35 V. After blotting overnight, the nitrocellulose sheet was incubated for 2 h in phosphate-buffered saline containing 2.5% (w/v) bovine serum albumin and 5% (v/v) normal goat serum (Woodlyn Laboratories, Guelph, Ontario, Canada) and washed with 10 mM-Tris/HCl (pH 7.6)/0.15 M-NaCl/0.05% Tween 20 (Tris/NaCl/Tween). The sheet was then incubated in a heat-sealed plastic bag for 4 h in 50 mM-Tris/HCl (pH 7.4)/0.15 M-NaCl/5 mM-EDTA/0.25% bovine serum albumin/0.05% Nonidet P40 (antibody buffer), together with 2.5% (v/v) purified IgG and 5% (v/v) normal goat serum. It was then washed in Tris/NaCl/Tween, and incubated for 2 h in antibody buffer containing 0.1% alkaline phosphatase-conjugated goat anti-(rabbit IgG) (Boehringer Mannheim) and 5% (v/v) normal goat serum. The sheet was again washed in Tris/NaCl/Tween, then in phosphate-buffered saline, and finally in 0.5 mM-MgCl₂/0.1 M-Tris/HCl, pH 10.0 (reaction buffer). The reaction with alkaline phosphatase on the nitrocellulose sheet was carried out by addition of 0.1% Naphthol ASBI phosphate and 0.1% Fast Red TR salt in reaction buffer at pH 9.3 (Mason & Sammons, 1978). After pink bands had appeared, the sheet was washed with water and dried. All incubations were performed at room temperature. Control blots were incubated as above, except that non-specific rabbit serum was used in place of the specific antiserum. For molecular weight determination (Bio-Rad molecular weight standards), the nitrocellulose sheet was

stained with Amido Black [0.1% in methanol/acetic acid/water (45:7:48, by vol.)] and destained with methanol/acetic acid/water (40:7:53, by vol.).

Results

Purified pig intestinal peroxidase gave two precipitin lines on immunodiffusion against the crude antiserum, but only one line against the affinity-purified IgG fraction recovered from a lactoperoxidase–Sephadex column. Lactoperoxidase itself gave no precipitin line against either crude or purified antibodies and the crude antiserum inhibited the activity of pig intestinal peroxidase but not that of lactoperoxidase. Antibodies not retained by the lactoperoxidase–Sephadex column were still able to bind pig peroxidase on a Western blot, but their reaction with lactoperoxidase and rat peroxidase was barely detectable. As shown in Fig. 1, however, purified pig intestinal peroxidase (lane *b*), a fresh crude extract of pig intestine (lane *a*), and a fresh crude extract of rat intestine (lane *d*) all reacted on a Western blot with the affinity-purified IgG, displaying only one major band of M_r of about 53 000. Lane (*c*) contained lactoperoxidase and the immunoreactive band had an M_r value of about 78 000, which is consistent with the reported molecular weight of this enzyme (Carlström, 1969). Blots made from duplicate gels and incubated with control rabbit sera gave no visible bands.

The affinity-purified IgG recovered from the lactoperoxidase–Sephadex column was then used to detect peroxidases in other rat tissues (Fig. 2) known

to have relatively high peroxidase activity (Neufeld *et al.*, 1958; DeSombre *et al.*, 1975; Hosoya & Saito, 1981).

Fig. 1. Immunodetection of peroxidases from different sources

The samples were run on a sodium dodecyl sulphate/polyacrylamide gel, transferred electrophoretically to nitrocellulose, and screened immunologically as described in the Materials and methods section. (*a*), Fresh crude extract of pig small intestine (0.1 unit of peroxidase activity, 143 μ g of protein); (*b*), purified pig intestinal peroxidase (0.6 unit, 0.6 μ g); (*c*), bovine lactoperoxidase (0.6 unit, 1.4 μ g); (*d*), fresh crude extract of rat small intestine (0.1 unit, 36 μ g). M_r values derived from standards are shown on the right.

Fig. 2. Immunodetection of peroxidases in rat tissue extracts

The experimental method was as described in Fig. 1. (*a*), Bovine lactoperoxidase (0.6 unit of peroxidase activity, 1.4 μ g of protein). The other lanes contained fresh crude extracts of rat tissues as follows: (*b*), small intestine (0.1 unit, 36 μ g); (*c*), uterus (0.1 unit, 37 μ g); (*d*), uterine fluid (0.1 unit, 153 μ g); (*e*), eosinophils (0.1 unit, 9 μ g); (*f*), spleen (0.1 unit, 96 μ g); (*g*), lung (0.1 unit, 267 μ g); (*h*), mammary tumour (0.1 unit, 191 μ g).

Fig. 3. Immunodetection of peroxidase in extracts of uteri from control and oestradiol-treated rats

The experimental method was as described in Fig. 1. The lanes contained extracts from the following tissues: (a), mature rat uterus (0.1 unit of peroxidase activity, 37 μ g of protein); (b), uteri from four immature rats treated with oestradiol (0.1 unit, 287 μ g); (c), uteri from six immature rats (no peroxidase activity, 287 μ g).

The extracts of rat uterus (lane c), eosinophils obtained by peritoneal lavage (lane e), spleen (lane f), lung (lane g) and mammary tumour (lane h) all showed a major band at M_r 53 000. The most significant finding, however, was that uterine fluid (lane d) showed a band with an M_r value of about 80 000, and no trace at M_r 53 000. In spleen, lung, and mammary tumour, an additional minor immunoreactive band appeared of slightly higher molecular weight (M_r 58 000) with some variation from one preparation to another. This band was never observed in extracts of the intestine and uterus but the extract of the sediment obtained, on one occasion, from uterine fluid contained only this higher molecular weight material.

The uterine extract of oestrogen-treated immature rats showed the expected band at M_r 53 000 (Fig. 3);

using the same amount of protein from untreated immature uterus, this band was not detectable.

No immunological cross-reaction was observed using crude antiserum on a Western blot of horseradish peroxidase, horse and sperm whale myoglobin, and cytochrome c from various species.

Discussion

The finding that most animal peroxidases, except myeloperoxidase, have similar properties (Archer *et al.*, 1965; Stelmazyńska & Zgliczyński, 1971; Hosaya *et al.*, 1981; Jörg *et al.*, 1982; Kimura & Jellinck, 1982; Olsen *et al.*, 1982) led to the suggestion that the peroxidase found in most tissues arises from infiltration of the tissues by eosinophils, which are rich in peroxidase (Rytöma & Teir, 1961; King *et al.*, 1981). The identity between rat spleen and rat uterine peroxidases (Keeping & Lyttle, 1982) and between rat eosinophil and rat uterine peroxidases (Olsen & Little, 1982) has further been shown recently by immunological procedures. The work reported here extends the range of immunological cross-reaction, and has shown that peroxidases identical in M_r value to the eosinophil peroxidase are present in every tissue tested, with the exception of uterine fluid and of the cellular material sedimented from uterine fluid. It was also shown that this peroxidase band appeared in extracts of immature uterus only after oestrogen treatment, consistent with the known appearance of peroxidase activity in the oestrogen-stimulated uterus.

The Western blot procedure has the advantages that many tissues may be tested at once, that crude extracts may be applied to the gels without purification, and that non-precipitating antibodies (as found here for lactoperoxidase) are readily detected.

The antibodies used on the Western blots are considered to be monospecific for peroxidase for several reasons. They were obtained by affinity chromatography on a bovine lactoperoxidase column, and then gave a single immunoprecipitin line on immunodiffusion against pig peroxidase; they revealed only one immunoreactive band in crude tissue extracts containing very many different proteins; and the M_r of 53 000 of the band in most rat tissue extracts was similar to that of purified pig intestinal peroxidase on the same gel, and close to the M_r values reported for the purified peroxidase from rat tissues (McNabb & Jellinck, 1975; Lyttle & DeSombre, 1977; Olsen & Little, 1981; Kimura & Jellinck, 1982).

It was also shown that the soluble peroxidases, bovine lactoperoxidase and rat uterine fluid peroxidase, are very similar to each other in molecular weight, and share at least one antigenic determinant with bovine and rat peroxidases of the eosinophil

type. The similarity of lactoperoxidase and rat uterine fluid peroxidase had been shown recently by Hosoya *et al.* (1981). The reported M_r values of 90000 for uterine fluid peroxidase (by gel filtration) and 78000 for lactoperoxidase (by sedimentation equilibrium; Carlström, 1969) are consistent with those observed on the Western blot in the present work.

The nature of the cross-reacting band of M_r 58000 seen, in variable amounts, in rat spleen and lung, but not at all in uterus, and exclusively present in the sediment recovered from uterine fluid, remains to be investigated. The existence of yet other types of rat peroxidase which do not react with this antibody preparation cannot be excluded, but no other major bands were detected on the Western blots even when the crude anti-(pig peroxidase) serum was used in place of the affinity-purified antibodies.

The peroxidase in dimethylbenzanthracene-induced mammary tumours was also similar to eosinophil peroxidase, and could therefore have originated from eosinophil leukocytes. This must be considered in studies on the correlations between peroxidase activity, oestrogen receptor concentration, and hormonal responsiveness of tumours (DeSombre *et al.*, 1975; Jellinck *et al.*, 1979).

Two major conclusions may be drawn. First, bovine lactoperoxidase, rat uterine fluid peroxidase, and rat tissue peroxidases, all share antigenic determinants with pig intestinal peroxidase. Second, a peroxidase similar to eosinophil peroxidase is present in many rat tissues, and is probably due to infiltration by eosinophils. This does not exclude the possibility that this particular peroxidase gene is expressed both in the different tissues and in eosinophils. In contrast, the soluble peroxidase of rat uterine fluid, while sharing antigenic determinants with the eosinophil enzyme, has a higher molecular weight, and is unlikely to be derived from eosinophils.

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