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IDENTIFICATION AND TISSUE LOCALIZATION OF AN EOSINOPHIL 17 kDa PROTEIN ACCUMULATING IN RAT UTERUS UPON ESTRADIOL TREATMENT

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Summary—In a previous paper (*J. Steroid Biochem.* **29** (1988) 475–480), the isolation of a 17 kDa protein that was dramatically induced in the uterus of estrogen-treated spayed rats was presented. We now describe a new purification procedure that is compatible with micro-sequencing of the 17 kDa protein. The protein partial *N*-terminal amino acid sequence analysis gave 28 residues that revealed a strong homology to the human major basic protein (MBP) of eosinophils described by Wasmoen *et al.* (*J. Biol. Chem.* **263** (1988) 12559–12563). Polyclonal rabbit antibodies were raised against this protein and used for tissue or blood cell analysis after electrophoresis and Western blotting. The 17 kDa protein was found to be constitutively present in the stomach and small intestine of the rat and guinea-pig. Estrogen treatment had a clearcut effect in guinea-pig uterus, but not as drastic as that observed in rat uterus. The protein was abundant in purified rat eosinophils. The antibodies cross-reacted with human MBP and an equivalent molecular weight human polymorphonuclear leukocyte protein. Immunohistochemical staining of rat uterus sections showed that the protein was first only associated with eosinophils that emigrate upon estrogen treatment; it then spread throughout the stroma and the deep glandular epithelium. It was not found in the myometrium. In conclusion, the appearance of a 17 kDa protein that is presumably the rat MBP is clearly regulated in the rat uterus.

INTRODUCTION

The uterus is a target for steroid hormones, and estrogen action in rat uterus has frequently been used as a model for the study of steroid hormone action. In this particular model, estrogens are able to regulate biological events in two distinctive ways.

The first one involves a cellular regulation of specific gene expression. This regulation is mediated through an interaction of the hormone receptor with specific responsive DNA elements, located in the vicinity of the regulated gene, which enhances or represses its transcriptional level [1–3].

Secondly, the prominent presence of eosinophils in the endometrium of rodents undergoing estrus has been known for many years. This is particularly true for the rat whose eosinophil leukocytes migrate from the blood stream to the uterus under estrogen stimulation, as described in detail by Tchernitchin [4, 5]. Eosinophil leukocytes are not the only cells that infiltrate the rat uterus; a study using monoclonal antibodies against epitopes of selected cells has shown that macrophages and T-helper cells also infiltrate the immature rat uterus upon estrogen treatment [6]. This infiltration could be explained by the presence of an estradiol-regulated chemotactic factor in the immature rat uterus, first demonstrated by Lee *et al.* [7]. Such an eosinophil infiltration is not restricted to the uterus, glucocorticoids induced a similar massive migration of eosinophil leukocytes from the blood stream to the thymus, spleen and lymph nodes [8]. In the rat uterus, eosinophils accumulate at the stromal–myometrial junction, undergo lysis just before estrus and are phagocytized by macrophages after estrus [9], suggesting that they are normal components of physiological stromal remodelling during the cycle. The exact role of this organ infiltration of eosinophil leukocytes under the influence of hormones is still to be elucidated.

Many studies have focused on uterine peroxidase levels that are drastically increased by

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estrogen treatment, and it has been demonstrated that much of this peroxidase is brought by the infiltration of eosinophils [10]. However, peroxidase is only one of the many eosinophil protein constituents: the most important ones include the major basic protein (MBP), the eosinophil cationic protein (ECP), and two proteins termed EDN/EPX that are probably identical (for a review see [11]). Other proteins like the lysophospholipase of the Charcot–Leyden crystals [12], phosphatases, collagenases, arylsulphatases, should also be mentioned. Some of these proteins might also accumulate in the rat uterus, concomitantly with eosinophil infiltration, as peroxidase does. To our knowledge, there are no studies that have dealt with localization of proteins brought by eosinophils other than peroxidase in the rat uterus.

Our earlier interest in the effect of steroids on uterine contractility [13] led us to study pattern variation of calmodulin-binding proteins by an *in vitro* overlay procedure [14]. We thus detected a hydrophobic 17 kDa protein induced by estrogenic treatment of spayed rats. In normal rats, the level of this 17 kDa protein was low and its appearance, presumably inhibited by the presence of ovarian progesterone, was stimulated by the antihormone RU38486.

In the present study, we describe a purification procedure that is compatible with the microsequencing of the 17 kDa protein. The partial *N*-terminal amino acid sequence analysis of the protein revealed a strong homology to the human MBP. Rabbit polyclonal antibodies raised against the purified protein were used to analyze various rat, guinea-pig and human tissues after tissue protein electrophoresis and Western blotting, and to localize the 17 kDa protein in rat uterus by immunohistochemistry.

EXPERIMENTAL

Materials

Acrylamide was from Serva (Heidelberg, Germany). Urea was purchased from Schwarz Mann Research (Spring Valley, N.Y., U.S.A.). Bis-acrylamide, Coomassie brilliant blue R, ammonium persulfate, *N,N,N′,N′*-tetramethylethylene diamine (TEMED) and Nonidet P-40 were from Bio-Rad (Watford, U.K.). Brij 35 was from Merck AG (Darmstadt, Germany). 17β-estradiol 3-benzoate, dithiothreitol, 5-bromo-4-chloro-3-indolyl phosphate (BCIP), nitro blue tetrazolium (NBT), diaminobenzidine tetrahydrochloride (DAB), polymyxin B sulfate and common chemicals were from Sigma Chem. Co. (St Louis, Mo., U.S.A.), as well as complete or incomplete Freund's adjuvant. Cyto paraffin 56–58C and solvents were from Prolabo (Paris, France). Mayer's Hemalum and a smear staining kit were from RAL (Villers Saint Paul, France). Goat anti-rabbit IgG (H + L) chain antiserum (GAR), and peroxidase-anti-peroxidase complex of rabbit (R/PAP) were from Nordic Immunology (Tilburg, Holland). Goat polyclonal antibody anti-rabbit IgG (H + L) chain F(ab′)2 linked to alkaline phosphatase was from Immunotech (Marseille, France). Alkaline phosphatase conjugated AffiniPure F(ab′)2 fragment Goat Anti-Rabbit IgG (H + L), was from Immunotech (Marseille, France). Heparin was from Roche (Neuilly, France). Permount mounting glue was from Fisher (Fair Lawn, N.J., U.S.A. Immobilon-P transfer membranes were from Millipore (Bedford, Mass. U.S.A.). Semi-dry transfer apparatus was from Biometra (Göttingen, Germany), and Mini-slab gel apparatus was from Hoefer Sci. Instr. (San Francisco, Calif., U.S.A.). The Biotrap electroelution apparatus was from Schleicher and Schuell (Dassel, Germany). Human native MBP was a gift from Drs G. J. Gleich and J. Checkel (Mayo Clinic, Rochester, Minn., U.S.A.).

Animals and animal treatments

Animals were acquired and used in compliance with the regulations of the French Ministry of Agriculture.

Wistar rats from Iffa-Credo (Les Oncins, France), weighing 200–220 g, were housed under controlled lighting (14 h light/10 h dark) and temperature (21–22°C) conditions. The various stages of the estrous cycle of normal rats, referred to as cycling rats, were observed by vaginal smears.

In order to induce the appearance of the 17 kDa protein maximally, rats were treated as in [14]. Briefly, they were lightly anaesthetized with diethyl ether, ovariectomized, and implanted concomitantly with silastic tubes (5 mm length, 1.9 mm i.d.) filled with estradiol benzoate. The hormone treatment lasted for 10 days. Animals used for control were ovariectomized 2 weeks prior to sacrifice. In the kinetic study of the appearance of the 17 kDa protein, rats were ovariectomized 2 weeks before the beginning of the experiment and they were implanted on day 0, and sacrificed on days 1, 2, 3, 4, 5 and 6. In the study on the disappearance of the protein,

rats were treated so as to induce the protein maximally. The silastic tubes were then removed on day 0 and animals were sacrificed on days 1, 2, 3, 4, 5, 6, 11 and 15.

In order to induce eosinophil migration, two different polymyxin B treatments were performed on rats:

—4 daily intradermal injections of 250 μg in 0.2 ml 0.9% NaCl,
—or 7 weekly intraperitoneal injections of the same polymyxin B dose.

Ovariectomized Hartley guinea-pigs from Charles River France (Cléon, France), weighing 300–350 g were implanted or not with estradiol silastic tubes twice as big as those in rats.

Each rat and guinea-pig group consisted of three animals.

Four New Zealand white rabbits, from the Institut National de la Recherche Agronomique (Montpellier), were used in the immunization procedure.

Human materials

Blood from allergic patients was collected using heparin (20 IU/ml).

The stomach fundus sample (0.25 mm^2) was obtained from a 72-yr old patient during a digestive tract operation.

Healthy endometrium samples were obtained from the uteri of 5 women (45–51 yr old) undergoing hysterectomy for abnormal uterine bleeding or prolapse.

All human samples were collected at the Centre Hospitalier Régional of Montpellier.

Analysis of tissues and leukocytes

Leukocyte preparations. Rat leukocytes from heparinized (25 IU/ml) blood were obtained by centrifugation at 1500 *g* for 5 min, collection of the buffy coat, and red cell hypotonic lysis.

Rat peritoneal eosinophils were obtained by the method of Jong *et al.* [15] from animals given i.p. injections of polymyxin B.

Polymorphonuclear and mononuclear human leukocyte enriched fractions were obtained on a Percoll discontinuous gradient according to Roberts and Gallin [16].

Cells plated on a slide using a Cytospin centrifuge were fixed and stained with the RAL kit (eosin and methylene blue staining), to allow the detection of eosinophils.

Powder preparations from tissues and leukocytes. We looked for the 17 kDa protein in several rat, guinea-pig and human tissues. Tissues were processed as previously described [14]. In short, tissues were cut into small pieces and ground to a thin powder in liquid nitrogen. The powder proteins were precipitated with 10% trichloracetic acid, washed twice in diethyl ether/ethanol 95:5 (v/v), and then twice in diethyl ether alone. Leukocyte proteins were directly precipitated by adding 10% trichloracetic acid to the cell pellet. The resulting tissue or leukocyte powder was then dried under vacuum and kept frozen at −20°C before being electrophoresed.

Electrophoresis and Western blot analyses. SDS–polyacrylamide gel electrophoreses (SDS–PAGE) were conducted according to the system of Laemmli [17]. 2 mg of the tissue or leukocyte powders were solubilized in 100 μl of lysis buffer: 0.05 M Tris, 5% SDS, 5 M urea, 0.5 M dithiothreitol, pH 6.8. The mixture was centrifuged for 5 min in an Eppendorf centrifuge. The resulting supernatant was fractionated by electrophoresis using standard gels (15 × 15 × 0.2 cm) for preparative studies, or mini-gels (6 × 8 × 0.075 cm) for analytical studies.

After electrophoresis, the gel was either stained with Coomassie blue for protein detection or Western blotted. Proteins were electrotransferred onto Immobilon membranes for 1 h from mini-gels, and for 2 h from standard gels, with a 200 mA constant current, in the following buffer: 50 mM Tris, 0.7 M glycine, pH 9.5. This buffer has been described as improving the transfer efficiency of hydrophobic proteins [18].

Immuno-staining of the Immobilon membrane. After transfer, the Immobilon membrane was blocked by immersion for 1 h at 25°C with gentle shaking, in buffer A: 0.3 M NaCl, 36 mM citrate, 20% horse serum pH 7. It was then incubated for 1 h in the appropriate dilution in buffer A of rabbit preimmune of anti-17 kDa protein serum obtained as described below. Serum test dilutions ranged from 1:500 to 1:2000. The membrane was then rinsed twice in solution B: 0.5 M NaCl, 0.09% Brij 35, and then incubated for 2 h in a 1:5000 dilution in buffer A of an alkaline phosphatase conjugated anti-rabbit antibody. This was followed by two washings in solution B in and two washings in buffer C: 50 mM Tris, 0.1 M NaCl, 0.02 M $MgCl_2$, pH 9.5. Finally the staining was performed in 0.4 mM BCIP and 0.4 mM NBT in buffer C. The membrane was then washed in deionized water, and blotted dry.

The 17 kDa protein purification procedure

Because the purification steps described in [14] were not suitable for the sequencing procedures, they were modified as follows:

Uteri from five maximally induced rats were cut into small pieces and homogenized in buffer D: 25 mM Tris, 0.1 mM $CaCl_2$, 0.1 mM $MgCl_2$, pH 7, in a glass-glass Potter–Elvejehm tissue grinder. The homogenate was centrifuged at 100,000 *g* for 1 h and the cytosol discarded. The pellet was resuspended in buffer D supplemented with 0.5% SDS and left at laboratory temperature for 15 min in order to dissolve the most soluble proteins, in particular the low molecular weight proteins. The mixture was then centrifuged at 100,000 *g* for 1 h, and the resulting pellet was ground to a thin powder in liquid nitrogen; the powder proteins were precipitated with trichloracetic acid as described above.

The resulting powder was subjected to preparative 13.5% acrylamide SDS–PAGE. The electrophoresis was run at 30°C to obtain the highest 17 kDa protein yield (the yield decreased when the gel was cooled). The right and left borders of the gel were stained with Coomassie blue, so that the 17 kDa protein could be located in a narrow band and it could be excised from the rest of the gel without staining or fixing the proteins. Electroelution of the proteins from this gel slice was carried out in the BioTrap apparatus with the electrophoresis buffer: 20 mM Tris, 0.2 M glycine, 0.1% SDS. The electroelution efficiency was estimated by comparing the Coomassie blue staining of the 17 kDa protein and known amounts of a standard protein, run on the same gel. In order to check the extracted protein purity, an aliquot of the protein was subjected to a 20% acrylamide gel electrophoresis, transferred onto an Immobilon membrane, and immunostained with the anti-17 kDa protein serum obtained as described below.

The purified protein, stored at −20°C, was used for the immunization procedures and amino acid sequence analyses.

Immunization against the 17 kDa protein

The purified 17 kDa protein solution (about 50 μg in 200 μl electrophoresis buffer) was emulsified with an equal volume of complete Freund's adjuvant and injected subcutaneously into the rabbits. Booster injections of 50 μg protein emulsified with incomplete adjuvant were performed 3 and 6 weeks after the first injection. Blood was collected from the ear vein 1 week after each booster injection, and left to settle for a few hours at room temperature to allow serum coagulation. Collected sera were stored at −20°C without any further purification step. They were tested against uterus protein powders from maximally induced rats that were fractionated by a 13.5% or 20% acrylamide SDS–PAGE transferred onto Immobilon membranes and immunostained as described above.

Amino acid sequence

Automated sequence analysis of the 17 kDa protein was performed with a gas-phase micro Sequenator (model 470, Applied Biosystem, Foster City, Calif., U.S.A.), connected to a model 120 A PTH analyzer. For this purpose, 1–5 nmol of the purified protein was spotted on a piece of Immobilon membrane. It was then rinsed extensively in deionized water to remove the excess glycine before being sequenced.

Computer search

Sequence homology of the 17 kDa protein to other proteins was screened through the Protein Sequence Data Base (Atlas of Protein Sequence and Structure, National Biochemical Research Foundation, Georgetown University, Washington, D.C., U.S.A.).

Immunostaining of rat uterus sections

Tissue preparation. Uteri from rats treated as indicated above were cut into 3 mm long cylindrical segments. The segments were fixed by immersion overnight in ethanol/chloroform/acetic acid 6:3:1 (v/v), 24 h in ethanol, and 10 h in isoamylacetate. They were then embedded in paraffin at 60°C.

Immunohistochemistry. Tissue sections (7 μm-thick) were placed on egg-white coated slides, heated at 50°C for 1 min and kept overnight at 37°C. The sections were deparaffinized in toluene and rehydrated through ethanol to water, washed in 0.05 M Tris–HCl buffered saline pH 7.5 (TBS), incubated for 30 min in TBS containing 5% BSA (TBS–BSA) to reduce background staining, and then incubated at 4°C overnight in TBS–BSA containing a 1:1000 dilution (or 1:750) of rabbit anti-17 kDa protein serum. The sections were then washed in TBS (2 × 20 min), incubated in TBS–BSA (20 min) and then in a 1:50 GAR dilution in TBS–BSA for 30 min. They were washed in TBS (2 × 20 min), incubated in TBS–BSA (20 min) and finally in TBS–BSA containing a 1:100 R/PAP dilution (30 min), washed in TBS (3 × 10 min) and

stained with a solution containing 1.4 mM DAB and 0.03% H_2O_2.

Sections were rinsed in TBS, counterstained in Mayer's Hemalum (10 s) and rinsed in tap-water. They were dehydrated through ethanol to toluene and mounted with a drop of Permount under a coverslip. They were examined with an Olympus BH-2 microscope and photographed using a 200 Asa Ektacolor film.

Controls. Two types of controls were performed:

—preimmunization rabbit serum was substituted for rabbit anti-17 kDa protein serum,
—for endogenous peroxidase detection, all of the serum incubations were omitted.

RESULTS

Purification

In this purification procedure, a protein electroelution was substituted for the extraction in ammonium bicarbonate buffer as previously described [14]. The purification was performed by 13.5% acrylamide SDS–PAGE of uterus powdered proteins (Fig. 1). In powdered proteins form estrogen-treated spayed rat uterus, a 17 kDa molecular weight protein band appeared on the gel while no protein was seen in that place on a spayed rat uterus gel. Higher concentration gels could not be used because they become clogged by the high molecular weight proteins of the tissue powder. The estimated electroelution yield was approximately 70% giving 50 μg protein from 30 mg tissue powder. As with the previously described method [14], the electro-eluted protein co-migrated with its initial counterpart when subjected to a 13.5% acrylamide SDS–PAGE. When the electroeluted protein was run on more concentrated gels (up to 20%), only one spot was still observed after

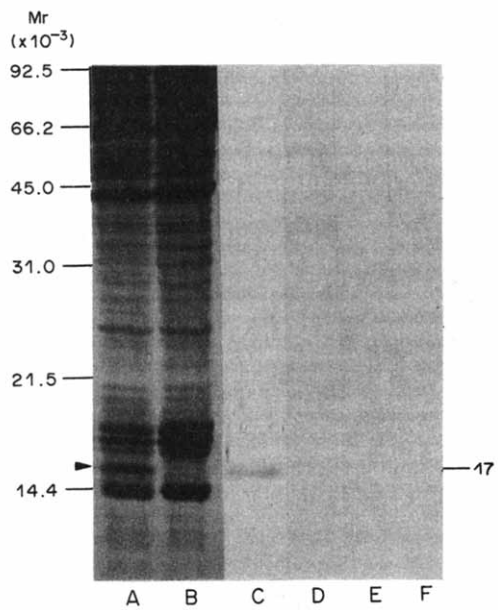

Fig. 1. Analysis of the specificity of rabbit anti-17 kDa protein serum. Uterine tissue powders from estradiol-treated (lane A) or untreated (lane B) spayed rats were run on 13.5% acrylamide gels, and then transferred onto Immobilon membranes. The membranes were incubated with a 1:2000 dilution of anti-17 kDa protein serum (lanes C and D, for estradiol-treated or untreated spayed rats, respectively) or of the preimmune serum (lanes E and F, for estradiol-treated spayed rats or untreated, respectively). For further details see Experimental.

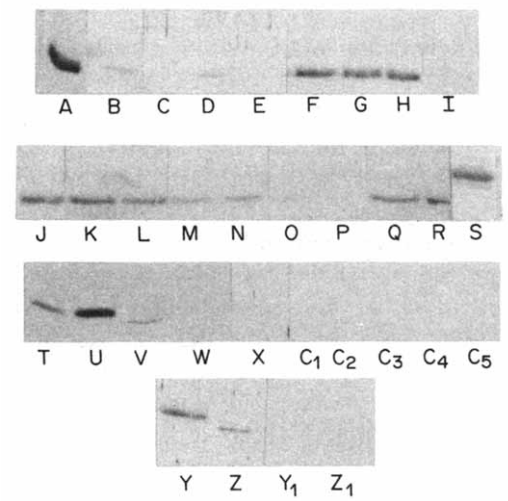

Fig. 2. Presence of the 17 kDa protein in different tissues from rat and other species. Tissue powders (lanes A–R), and white cell powders (lanes U–X) were run on 13.5% acrylamide mini-gels and transferred onto Immobilon membranes. The same powder weight was used for all lanes. Purified 17 kDa protein was run on a 20% acrylamide mini-gel (lane S). The 17 kDa protein was revealed with the rabbit anti-17 kDa protein serum (1:500). Each test was done in triplicate, and the immunostained transfer with the intermediate staining intensity was chosen for the figure. "Rat" here stands for "female rat". *Uterus* from: estrogen-treated spayed rat (lane A), cyclic rat in proestrus (lane B), spayed guinea-pig (lane C), estrogen-treated spayed guinea-pig (lane D). For further details on treatments see Experimental. In human endometrium from different patients the 17 kDa protein was not observed (lane E). *Stomach* from: spayed rat (lane F), estrogen-treated spayed rat (lane G), male rate (lane H), human male (lane I). *Small intestine* from: spayed rat (lane J), estrogen-treated spayed rate (lane K), male rat (lane L), spayed guinea-pig (lane M), estrogen-treated spayed guinea-pig (lane N). *Skin* from: male rat locally injected with polymyxin (lane O), skin from a non-treated area of the same animal (lane P). *Nipple* from: spayed rat (lane Q), estrogen-treated spayed rat (lane R). Rat *uterus* in estrus (lane T). Rat peritoneal *eosinophils* (lane U), human polymorphonuclear *leukocytes* (7% eosinophils) from an allergic patient (lane V), human mononuclear leukocytes (lane W), rat leukocytes (lane X), preimmune serum controls for lanes T–X (lanes C_1–C_5). Rat *uterus* in estrus (lane Y). Pure human *MBP* (lane Z), preimmune controls for lanes Y and Z (lanes Y_1 and Z_1).

(A) GRYLLVRRPESFNKAQLVSRSSYEGTLA

(B) TCRYLLVRSLQTFSQAWFTCRRCYRGNLVSIH

1 10 20 30

Fig. 3. *N*-terminal sequence of the 17 kDa protein (A), aligned with the *N*-terminal sequence of the human major basic protein (B) [19]. Boldface letters indicate identical amino acids, while underlined letters indicate the amino acids that share similarities, according to the computer search criteria.

Coomassie blue staining or immunostaining of Western blots (Fig. 2, lane S). When the lysis buffer was free of urea, no 17 kDa protein was found on the gel, and when DTT was omitted, the 17 kDa protein band was not shifted, but was slightly less intense when visualized with Coomassie blue staining or Western blotting and immunostaining (data not shown).

Partial amino acid sequence

Automated Edman degradation of over 1 nmol of the 17 kDa protein revealed a free *N*-terminal residue, and allowed a 28 amino acid sequence determination (Fig. 3). The chromatogram, corresponding to 50% of the injected protein, was not blurred by the presence of other proteins, hence indicating the homogeneity of the purified protein.

The partial amino acid sequence revealed a strong homology of the *N*-terminal portion to the human eosinophil major basic protein (MBP) (19). As shown in Fig. 3, from No. 3 Arg to No. 16 Ala:

—amino acid residues are matched in 57% of the sequence (bold letters),
—and 33% additional amino acid residues share common structural properties with human MBP amino acids or are coded by closely related codons (underlined letters).

MBP was the only screened protein that had a significant homology to the 17 kDa protein.

Antiserum production

One rabbit produced antibodies against the 17 kDa protein. Its serum recognized a single 17 kDa molecular weight band when tested against uterine tissue powders from maximally induced rats, electrophoresed in 13.5% gels and transferred onto Immobilon membranes (Fig. 2). Rabbit preimmune serum did not reveal any band.

A 5–20% acrylamide gradient gel was run with uterine powders from treated or untreated spayed rats in order to visualize any high molecular weight proteins sharing common epitopes with the 17 kDa protein. From 17 to 200 kDa molecular weight, only the 17 kDa protein was stained, suggesting that the protein was not a cleavage product arising either during the estrogenic treatment or from the purification procedure (results not shown).

Western blot analysis of tissue and blood cell distribution of the 17 kDa protein

Figure 2 shows the Immobilon transfer of a few selected tissues, analyzed as described in Experimental. The 17 kDa protein was absent from rat heart, liver, striated muscle, aorta, skin and brain. It was constitutively present in rat small intestine and stomach: the staining level was comparable in male and estrogen-treated or untreated female rats. The intensity was much greater in uteri from treated rats (maximally induced). In uteri of cycling rats, a large individual variability was observed, even for the same estrous cycle phase, but the staining was always weaker than in estrogen-treated spayed rats (data not shown).

In guinea-pig uteri, the effect of estrogen treatment was not as drastic as it was in rats, however a distinguishable band appeared upon estrogen treatment of spayed guinea-pigs, while no band was detected without treatment.

No important individual variations were observed except in the cycling rat uteri.

A weak 17 kDa band was revealed by immunostaining in rat leukocytes.

A strong signal was given by rat peritoneal eosinophils (Fig. 2), and suggested that the 17 kDa protein was abundant in these cells.

Concerning the human samples, our anti-rat 17 kDa protein serum cross-reacted with a unique human polymorphonuclear leukocyte protein of slightly lower molecular weight, whereas it did not reveal any band in a human mononuclear leukocyte fraction. The antiserum cross-reacted (although weakly) with the pure native human MBP that had the same molecular weight—14 kDa—as the human polymorphonuclear leukocyte protein (Fig. 2). We found no protein cross-reacting with the anti-17 kDa protein serum in either stomach or endometrium (whatever the cycle stage).

In some tissue gels (human endometrium, rat small intestine), the Coomassie blue staining revealed a protein band in the 17 kDa molecular weight region (results not shown). However, when transferred onto an Immobilon membrane,

it was not recognized by the anti-17 kDa protein serum.

Immunohistochemical localization of the 17 kDa protein in uterus

In animals in which the 17 kDa protein was maximally induced, an intense DAB reaction product deposit was observed in the endometrium adjacent to the circular muscle layer in sections incubated with the anti-17 kDa protein serum, whereas sections incubated with preimmune serum or with no serum were unlabeled (Fig. 4). Examination at a higher magnification indicated that the staining was mainly distributed in stroma, in glandular epithelium, and in the immediate vicinity of the eosinophils surrounding the small vessels in the circular muscle layer or in the subserosal region. The staining was absent from the luminal epithelium.

No staining was seen in uteri of ovariectomized rats.

The kinetic study on the appearance of the 17 kDa protein could be detected as early as one day after estradiol benzoate implantation. This is in agreement with what was previously observed [14] with tissue powders subjected to electrophoresis. On the first day, only a few eosinophils were detected, each surrounded with a light stained zone. Then the staining spread throughout the stroma (Fig. 4d). On day 4, staining intensity was maximal, although tissue powder electrophoreses revealed that the amount of 17 kDa protein increased until day 10 [14].

When rats were sacrificed on days 0–15, after the silastic tube removal, staining could be seen in all of the samples thus indicating that the 17 kDa protein persisted for at least 15 days after the end of the treatment.

No appreciable background staining was observed when preimmune serum was used instead of anti-17 kDa protein serum (Fig. 4b, f and g). Endogenous peroxidase staining was negative (Fig. 4c), indicating that this type of tissue fixation and/or paraffin embedding destroyed uterine peroxidase activity [20].

DISCUSSION

This paper reports the partial sequencing and tissue localization of the previously described rat uterine 17 kDa protein [14]. Its partial *N*-terminal amino acid sequence revealed a strong homology to the major basic protein (MBP) from human eosinophils [19].

When uterine proteins from spayed rats were separated by SDS–PAGE, Western blotted and immunostained, no band was observed in the 17 kDa protein zone. After estrogen treatment, immunochemical analysis of uterine sections showed that staining appeared in conjunction with the eosinophil migration. These two observations suggest that our antiserum detected a protein of eosinophil origin. Moreover, the fact that our antiserum cross-reacted with pure human MBP and the clear homology of the 17 kDa protein partial amino acid sequence to the human MBP revealed that the 17 kDa protein was the rat counterpart to the human MBP.

Attempts to run the purified protein or uterine tissue proteins on 2-D gels (experiment not shown) by the O'Farrell procedure [21], or by a nonequilibrium pH gradient electrophoresis procedure [22] did not succeed, probably due to the high percentage of SDS required to keep the 17 kDa protein soluble.

The rat MBP isolated by Lewis *et al.* [23] seems to be more easily solubilized than the 17 kDa protein [14]. The differences in the protein environments could explain this: the eosinophil granule as compared to the uterine stroma. It has also been described that, in order to avoid its aggregation in solution, MBP must be stabilized by reduction with dithiothreitol and by *S*-carboxymethylation [19].

The present electrophoretic characterization indicated a 17 kDa molecular weight for the rat MBP. Our antibodies cross-reacted with a band of the same molecular weight from guinea pig uterus, and with pure human MBP. According to Lewis *et al.* [23] and Gleich *et al.* [24, 25], rat and guinea-pig MBPs have similar molecular weights. An 11 kDa molecular weight, much lower than our 17 kDa molecular weight, was determined by the electrophoretic system of Fairbanks *et al.* [26] and Reisfield *et al.* [27] for rat and guinea-pig MBPs. However, these systems showed different molecular weights when the proteins were run with different concentrations of SDS. The aberrant migration might be due to the sensitivity of extremely basic proteins to the presence of different concentrations of SDS. Furthermore, using the Laemmli electrophoretic system as in the present study, Wasmoen *et al.* [19] obtained a 13.8 kDa molecular weight for human MBP, close to the 14 kDa molecular weight observed for the human MBP cross-reacting with our antiserum. This cross-reacting protein was detected in an eosinophil-rich polymorphonuclear leukocyte fraction and

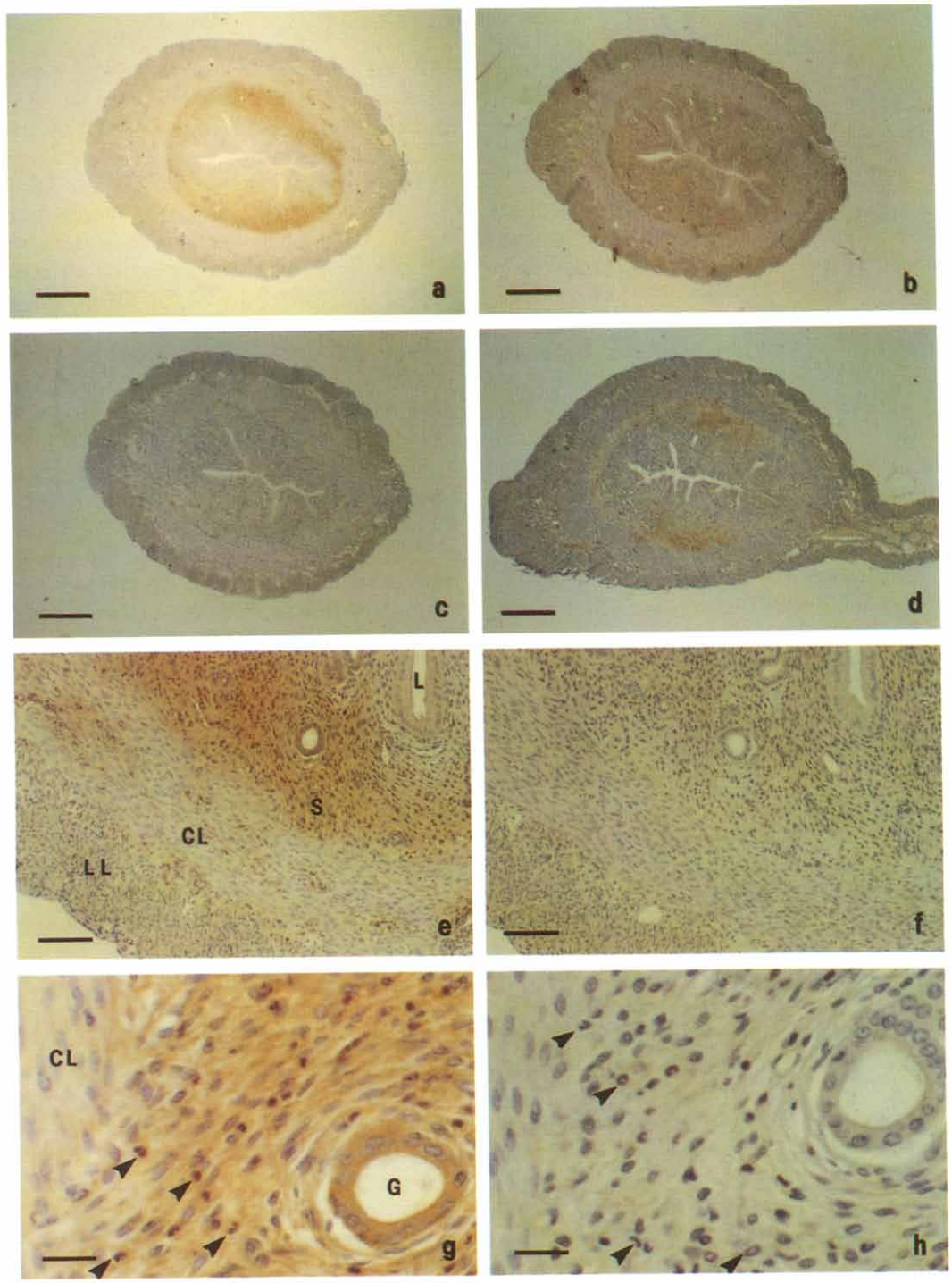

Fig. 4. Immunohistochemical localization of the 17 kDa protein in uteri from estrogen-treated spayed rats. *Estrogen treatment*. **a**–**h** (except **d**): 6 days, **d**: 2 days. *Staining*. Sections **a**, **d**, **e** and **g** were incubated with a 1:1000 dilution of anti-17 kDa protein rabbit serum, then with GAR and finally with R(PAP). A brown peroxidase product is observed. Sections **b**, **f** and **h** are preimmune serum controls. Section **c** is an endogenous peroxidase control. Sections were counterstained with Mayer's Hemalum. The 17 kDa protein is located in the stroma (S, in **e**) and the glandular epithelium (G in **g**). It is not found in the muscle layer (CL, circular layer, in **e** and **g**, and LL, longitudinal layer, in **e**), and does not reach the luminal epithelium (L in **e**). Many eosinophils are visible in the stroma (arrowheads in **g** and **h**). For **a**–**d** ($\times$ 20): Bar = 500 μm. For **e**–**f** ($\times$ 100): Bar = 100 μm. For **g**–**h** ($\times$ 1000): Bar = 10 μm.

not in a mononuclear leukocyte fraction. Rat and guinea-pig MBPs can thus be considered as having an apparent molecular weight of 17 kDa.

Uterine immunohistochemical localization of the 17 kDa protein showed that the protein accumulated in the stroma and in the deep glandular epithelium, but was never observed in the luminal epithelium or in the myometrium, except in the immediate vicinity of the eosinophils close to the myometrium vessels. In a previous paper, an incomplete scraping off of the endometrium led us to believe that the protein was located in the myometrium [14]. On day 2 of the estradiol treatment, staining was closely associated with the eosinophils but was more uniformly distributed after a 4-day treatment. This result can be compared with the localization of the uterine peroxidase imported by eosinophils under estradiol treatment [28, 20]. From these studies, the peroxidase of eosinophil origin was mainly associated with the stroma, and less with the myometrium. By contrast, there was a remarkbly exclusive localization of the 17 kDa protein in the endometrium: even at high magnification when focusing on the endometrium–myometrium junction, no staining deposit was seen in the muscle compartment. These results are consistent with those of Tchernitchin who reported that eosinophils are found predominantly in the stroma and in the connective tissue between the muscular layers [29].

In an attempt to correlate the uterine localization of the 17 kDa protein and the estrus cycle phases of the rat (data not shown), we noted great individual variations in the amounts of 17 kDa protein. Such differences may reflect major differences in uterine eosinophil content [30, 20]. After removal of the estradiol silastic tubes from spayed rats, the 17 kDa protein persisted for more than 2 weeks. This could be due to a long lifespan of the eosinophil in the tissue, or a long resident time of this eosinophil product after deposition, or a combination of both phenomena.

The 17 kDa protein was found to be constitutively present in the small intestine and the stomach from the rat and also from the guinea-pig. Estrogen treatment had a clearcut effect in guinea-pig uterus, but not as drastic as that observed in rat uterus. Thus, eosinophil proteins are not distributed identically in different rodent uteri, even under identical hormonal treatments. Differences in estrus cycle length could possibly modify uterus receptivity to the treatment, but this remains to be demonstrated. The protein was not detected in the human tissues tested: endometrium samples from five women and a stomach sample from one man. Concerning the endometrium, this result could reflect the fact that only a small number of eosinophils are usually found in the human uterus [11, Chap. 20]. Furthermore, our antiserum reactivity towards human MBP was much weaker than towards rat MBP.

The function of MBP in reproduction is unclear. Wasmoen *et al.* [31] showed an increase in the human plasma MBP level before the onset of labor; this seemed to represent active secretion of the protein from the uterine/placental interface. Immunofluorescent studies showed that MBP is localized in the poorly defined placental trophoblast X-cells [32]. It has also been hypothesized that cells adjacent to the myometrium that contain MBP play a role in the induction of labor [33]. The rat myometrium is a preferential target for the action of steroid hormones, but their mechanism of action in uterine motility remains a subject of controversy.

Considering that the 17 kDa protein is of eosinophil origin and is released from the granules after tissue eosinophil infiltration, one would expect a parallel increase in the other main eosinophil proteins upon estrogen treatment. In fact, the 17 kDa protein, which can represent up to 3% of the proteins that migrate on an acrylamide gel [14], was the only protein in that part of the gel the amount of which was modified. This might partly be due to the fact that such an eosinophil protein increase could be masked by the trophic effect of estrogens on uterine proteins. Moreover, a selective release of individual components from the granules is suggested by some *in vitro* experiments [in 11, Chap. 5]. It would be very interesting to examine, by uterine section immunostaining, whether the other main eosinophil proteins were released and in what uterine compartment they eventually accumulate. This could provide new insight to understanding the physiological relevance of the eosinophil infiltration process.

When rat skin was injected with polymyxin, the 17 kDa protein appeared in the tissue, probably with a concomitant eosinophil infiltration. The 17 kDa protein was also found in rat stomach and small intestine whatever the hormonal status. These results show that the 17 kDa protein can accumulate in other tissues than the uterus, and by a mechanism that is independent of estrogen stimulation.

In conclusion, we have purified a 17 kDa protein that was previously shown to substantially accumulate upon estrogen treatment in the uterus of spayed rats. Its partial amino acid sequence revealed a strong homology to the human MBP. To our knowledge this is the first report of a precise localization of a protein of eosinophil origin, other than peroxidase, in rat organs.

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