Local calcergy. A histological, histochemical and electronprobe X-ray analytical study

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Summary. The early stages of local calcergy in the mouse are characterized by a close focal relationship between injected lead (Pb), calcium (Ca) and phosphorus (P) and this association occurs before the latter 2 ions are detectable by conventional histochemical techniques.

The subcutaneous injection of a dilute solution of lead acetate (PbAc) is followed by calcification of the injection site in the rat[1] and a number of other species, including the mouse[2]. This reaction has been called local calcergy. Its pathogenesis is essentially mysterious and nothing is known of the fate of the calcified lesion over a prolonged time period.

In the present experiments, 46 7-week-old white mice were each given an injection s.c. of PbAc 100 µg/0.2 ml (in sterile solution) into the mid-line of the lumbar region. As a control measure, this was immediately followed by an injection of 0.2 ml of twice-deionised sterile water into the subcutaneous tissues of the interscapular skin. These injections were given using an appropriate aseptic technique.

Groups of 4 animals were killed 3, 6 and 12 h, 1, 2, 4, 6 and 38 weeks later and 14 animals were killed after 52 weeks. In each instance the injection site was removed and bisected. Half was fixed in formol-alcohol (90% formalin, 10% absolute alcohol) for 48 h, double-embedded and sectioned at 6 µm. The chloranilic acid (CA) stain[3] was applied to demonstrate the presence of calcium ions and the von Kossa (VK) technique applied to demonstrate the presence of phosphate.

The other half of the injection site was snap-frozen (−70 °C) for scanning electron microscopy (SEM) and electronprobe X-ray analysis by a Cambridge Stereoscan S-600 with a Si(Li) detector attached to a Link System 290 X-ray microanalyzer. X-ray spectra were displayed on a TV-screen and photographed. Areas chosen for analysis were studied at an SEM magnification of ×1000. An operational voltage of 15 kV and a specimen tilt of 45° was used.

Histological and histochemical examination revealed fluid accumulation in the connective tissue dorsal fascia of the skin 3 h after injection. The CA and VK reactions were negative. After 6 h, there was an acute inflammatory reaction with foci of positive CA and VK reactions adjacent to dilated capillaries. After 12 h, the inflammatory reaction was maximal. After 1 week there were extensive positive CA and VK reactions in the dorsal fascia. In the succeeding weeks, the calcified area became fragmented and surrounded by dense fibrous tissue.

At the control injection sites, there was fluid accumulation in the dorsal fascia with a mild acute inflammatory reaction occurring between 5 and 12 h after injection and subsiding thereafter.

Electronprobe X-ray analysis of the 3 h injection site revealed a variable reaction in the dorsal fascia with focal areas showing high concentrations of lead (Pb) and these were associated with concentrations of calcium (Ca) and phosphorus (P) (figure 1). Significant concentrations of Ca and P were never found in areas devoid of Pb. 6 h after injection, Ca and P were again found in the same focal areas and were more often detected; these ions were not inevitably associated with Pb.

12 h after injection essentially similar results were obtained. 1 week after injection, P and Ca only were detected in the dorsal fascia. At 4, 38 and 52 weeks (figure 2), the findings were similar. Pb was not detected at any of these times. Analyses of the control injection sites did not demonstrate the presence of Pb, Ca or P.

Previous histological and histochemical studies[2] have shown that histochemically demonstrable Ca and P were first detected 5 h after the injection of PbAc at a time when the staining reaction for Pb was maximal. These results are largely confirmed by the histological and histochemical

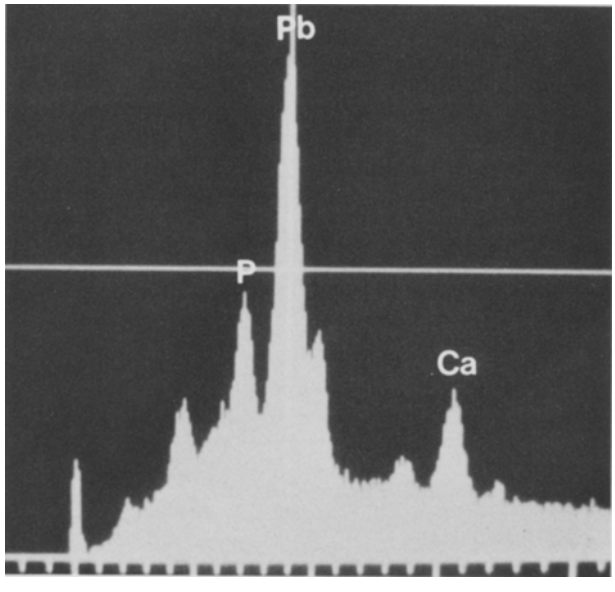

Fig. 1. Mouse skin 3 h after injection of PbAc 100 µg/0.2 ml. Electronprobe X-ray microanalysis of the dorsal fascia. Peaks indicating the presence of P, Pb and Ca are labeled.

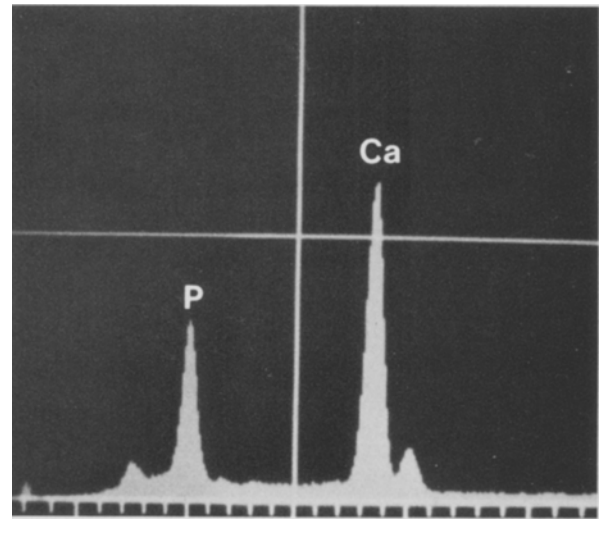

Fig. 2. Mouse skin 52 weeks after the injection of PbAc 100 µg/0.2 ml. Area of calcification in the dorsal fascia. Peaks indicating P and Ca are present.

findings of the present experiments. However, it is evident from the electronprobe X-ray analytical studies that early focal events are taking place in the dorsal fascia which represents a calcification process and that these reactions are not detectable by standard histochemical techniques.

Therefore the earliest stages in the calcification reaction of local calcergy in the mouse are represented by the close association of Pb, Ca and P ions. As the reaction progresses, Pb is not inevitably associated with Ca and P and after 1 week, it is undetectable. Therefore Pb acts as a trigger for the calcification reaction and when this reaction becomes detectable by histochemical techniques, Pb is apparently unimportant in the progression of the reaction.

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Inhibitors of the acrosomal proteinase acrosin: Human urinary trypsin inhibitor (UTI) and 4-(2-carboxyethyl) phenyl trans 4-aminomethylcyclohexanecarboxylate hydrochloride (DV-1006)

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Summary. Human urinary trypsin inhibitor (UTI) and (4-(2-carboxyethyl) phenyl trans 4-aminoethylcyclohexanecarboxylate hydrochloride (DV-1006) competitively inhibited the human acrosomal proteinase acrosin; Ki values were 1.2×10^{-8} M and 9.4×10^{-7} M, respectively.

The acrosomal proteinase acrosin (EC 3.4.21.10) is essential for sperm passage through the zona pellucida of the ovum, and is thus the key enzyme in the fertilization process[1,2]. Recently, we purified urinary trypsin inhibitor (UTI) as an apparently homogeneous protein from normal human urine, estimated its mol. wt to be 67,000 (UTI-I) by gel filtration[3-5], and succeeded in transforming it into lower molecular weight forms (mol.wt 45,000, 22,000, and $<10{,}000$) with several proteinases such as urinary enzyme[3,4], papain[6,7], and pronase[6,7]. The highly purified form of this inhibitor reacted with trypsin and to a lesser extent, with chymotrypsin and the fibrinolytic enzyme plasmin[4,8]. In this paper, we demonstrate the strong inhibitory effect of UTI (UTI-I) and of a synthetic trypsin-plasmin inhibitor 4-(2-carboxyethyl) phenyl trans 4-aminomethylcyclohexanecarboxylate hydrochloride (DV-1006)[9] on human acrosin.

Materials and methods. The following substances were used: soybean trypsin inhibitor (SBTI) and leupeptin from Sigma Chemical Co., USA; DV-1006 from Daiichi Kagaku, Tokyo; UTI, purified by affinity chromatography as reported previously[5], 1210 U/mg protein, mol. wt 43,000 by sodium dodecyl sulfate polyacrylamide gel electrophoresis (mol.wt 67,000 by gel filtration); human acrosin, purified by the method of Zaneveld et al.[1] except that Sephadex G-75 (Pharmacia, fine) was used for the gel filtration in place of Sephadex G-50 gel, 1100 mU/mg protein. Acrosin activity was measured using N^{α}-Benzoyl-L-arginine methylester (Bz-Arg-OEt; Sigma Chemical Co., USA) as substrate. Details are given in the table. 1 milliunit (mU) was defined as the amount of enzyme which caused a change in absorbance of 0.001/min at 253 nm[1].

I_{50} and Ki values for acrosin hydrolysis of Bz-Arg-OEt

Inhibitor	Inhibitor concentration for 50% inhibition (M)	Ki(M)
UTI	5.9×10^{-8}	1.2×10^{-8}
DV-1006	3.3×10^{-6}	9.4×10^{-7}
SBTI	1.1×10^{-6}	-
Leupeptin	5.0×10^{-7}	2.1×10^{-7}

Constant amounts of acrosin (13.5 mU) were incubated with increasing amounts of inhibitor in 2.0 ml of 0.1 M borate buffer containing 0.05 M $CaCl_2$, pH 8.0, for 15 min at room temperature (22-23 °C). The enzymatic reaction was initiated by the addition of Bz-Arg-OEt in deionized water; end volume: 3.0 ml. In the experiments where I_{50} values were determined, the substrate concentrations were 0.2 mg/ml.

Results and discussion. Comparison of the effects of UTI and DV-1006 with known acrosin inhibitors, SBTI[1] and leupeptin[10] were made. The concentrations of the inhibitor required for 50% inhibition (I_{50}) are shown in the table. UTI and DV-1006 were found to be very powerful inhibitors of acrosin. By the method of Dixon[11], the inhibitors were shown to be competitive and the dissociation constant (Ki) values evaluated were approximately 1.2×10^{-8} M for UTI (assuming mol. wt 43,000) and 9.4×10^{-7} M for DV-1006. The Ki value of UTI is lower than that of leupeptin ($Ki=2.1\times10^{-7}$ M), which is the most powerful inhibitor of acrosin known at present (with boar acrosin; $Ki=8.6\times10^{-8}$ M (Fritz et al.[10]).

In 1971, DV-1006 was first reported by Muramatu[9] as an excellent synthetic inhibitor of trypsin, plasmin, kallikrein, and thrombin. It is now used in Japan for the clinical treatment of gastric peptic ulcers. UTI is also a trypsin-plasmin inhibitor and is non-antigenic for humans. In the presence of serine proteinase inhibitors such as tosyllysine chloromethyl keton and diisopropylfluorophosphate, fertilization is prevented both in vitro and in vivo[1,2]. Therefore, the use of these inhibitors as antifertility agents may be possible.

The relation of the UTI molecule to other naturally occurring acrosin inhibitors is interesting; the antigenicity of UTI is identical to serum inter-α-trypsin inhibitor, which also inhibits acrosin[1] and its concentration decreases during the ovulatory cycle[12]. Schumacher and Zaneveld[12], and Wallner et al.[13] have purified other new acrosin inhibitors from human cervical mucus. Hochstrasser et al.[14] showed that the anti-inter-α-trypsin inhibitor cross-reacts with the human mucus trypsin inhibitor.

Studies on the molecular structure and physiological significance of these inhibitors are now in progress.