Distribution of Hyaluronidase in Bovine Urogenital Organs: On Immunofluorescent Study

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Introduction

Duran-Reynals (1928) reported that the extract of mammalian testis markedly enhances the infiltrating ability of vaccine and its resultant extracts accelerate the permeability in the animal tissues. Such a substance in the extract was designated as "spreading factor" by him. Since the spreading factor was later confirmed to be the identical substance to hyaluronidase by Chain and Duthie (1940), studies on hyaluronidase have been made from various angles. However, there has been no evident report on its localization and the site of its production in the testis, except the previous paper on the sheep (Nagakubo et al., 1970). In this experiment the authors pursued the localization of hyaluronidase in the bovine testis by fluorescent antibody technique.

Materials and Methods

After dissolving 10 mg of crystalline bovine hyaluronidase obtained from Worthington Biochemical Company in 1 ml of the phosphate buffer pH 7.2, an equal amount of Freund's complete adjuvant was added for complete mixing in the syringe. Then, aliquots were injected into food pads and in the thigh muscle of rabbits. 3 mg of antigen, dissolved in the same buffer as used for the initial injection, was given intravenously to each animal for the booster injection on the thirty-sixth day from the initial injection, together with the preliminary intravenous administration of 10 mg of restamin. The same procedure was repeated one week later. One week after the last injection, blood was harvested from the ear artery for the confirmation of a sufficient rise of the antibody titer. The titer of the antiserum by passive hemagglutination (Stavitsky, 1954) using 10 mg of antigen to sensitize the red cells, was 262,966.

The anti-sera were taken by the heart puncture, pooled, and the

most of the part was used for the preparation of the conjugate. The remainings were either stored in the refrigerator at 4°C or in the deep freezer at -20°C after a quick freezing. The fluorescein-conjugated antibody solution was prepared according to the modified method of Marshall et al. (1958). The molar F/P ratio of the conjugate was 1.4 (Mc Devitt et al., 1963). The specificity of antigen-antibody reaction was examined by gel-diffusion test. The bovine testis, epididymis, ductus deferens and prostate were obtained at the slaughter house.

Two types of tissue blocks were used. The small blocks of the tissues were either rapidly frozen by the dry-ice-acetone mixture or embedded in paraffin after the fixation with the various solutions described below. After the frozen blocks were sliced at 4 μ in thickness by a rotary microtome in a cryostat kept at -18°C, they were dried for a short time at room temperature and then fixed for 30 minutes with one of the following fixatives; 95% acetone, 99% ethanol, 10% ethanol in 95% acetone, 50% ethanol in 95% acetone and 1% acetic alcohol. Copper sulphate was added to each fixative as an inhibitor at the final concentration of 10^{-4} mol. The controls were examined according to the method described by Coons (1950, 1956 and 1958), Cherry et al. (1960) and by Yasuda et al. (1963). The tissue slices fixed with 10% formalin, 99% ethanol, 1% acetic alcohol, Carnoy and Bouin's solution were embedded in paraffin and kept at 4°C until occasion demanded. Zeiss fluorescent microscope was used for all the observations. Each tissue slice was stained with hematoxylin-eosine for the confirmation of the basic histological structure of the tissue used.

Results

The enzyme preparation obtained from Worthington Biochemical Company showed five antigen-antibody systems on the agar diffusion plate; a thick and strong precipitation line at the center and two weak lines at each side of the thick band (Fig. 1). The antigen had no common factor with bovine albumin nor with sheep hyaluronidase. Disc electrophoresis revealed one thick line and 4 faint lines at the cathode.

The antibody titer measured by the passive hemagglutination test was as high as 262,966. As shown in Fig. 2, specific fluorescence is strongly observed around the basement membrane and in the interstitial area in the testis.

The sites which emit fluorescence in the interstitial connective tissue do not correspond to the connective tissue cells, fibers, blood

vessels, but to the cluster of some kinds of the cells which are probably the interstitial cells, to the narrow interspace between connective tissue fibers which are distributed diffusely in the interspace with the exception of the area near the basement membrane of the seminiferous tubule, where the specific fluorescence is highly concentrated.

Within the seminiferous tubule, the specific fluorescence is generally very weak and is recognized in the basal part of the tubule. The fluorescent site corresponds to the interspace between each spermatogenic cell, and shows the irregular configuration which may partly correspond to Sertoli cell. Occasionally, the enzyme was found in the cytoplasm of the spermatogonium. The presence of hyaluronidase in the sperm (Kepp et al., 1954) could not be clearly observed in the present experiment, but the fluorescent spots probably caused by hyaluronidase lodged in the sperm were recognized at the center of the cross section of the seminiferous tubule.

In the epididymis, no fluorescence was observed in the wall of ductus epididymidis. The slight fluorescence was noticed in a mass or in a diffuse pattern, in the lumen of ductus epididymidis (Fig. 4).

The ductus deferens was divided into three portions, namely the part near the testis, intermediated portion and part close to the prostate. Any of above mentioned three portions did not show specific fluorescence.

No fluorescence could be observed in the epithelium, cavity of the gland and in the muscular layer of the prostate.

Discussion

Chain and Duthie (1940) confirmed that hyaluronidase was identical to the "spreading factor." They also found that the rise of the bacterial infectivity and diffusion of pigments were caused by the increased permeability of tissues due to a hydrolysis of the hyaluronic acid in the tissues. Having discovered that the autolysate of pneumococci acts on hyaluronic acid to decompose it to an oligosaccharide with low molecule, Meyer et al. (1937) named it hyaluronidase.

According to Hasegawa et al. (1968), hyaluronidase is said to be contained in snake venom in addition to testis, and also in pneumococci, streptococci, staphylococci and bacilli of gas gangrene. Its action seems to vary in animals in vivo and to be quite complicated. There have been various discussions on its role during fertilization, and during the bacterial infection. Hasegawa et al. (1968) have classified hyaluronidase into three types: they are testi-

cular and snake venom hyaluronidase, bacterial hyaluronidase and leech hyaluronidase. The location of testicular hyaluronidase has not been known histologically yet, except the previous report on the sheep testis (Nagakubo et al., 1970). Since hyaluronidase loses activity rapidly in a solution due to its considerable instability, its purification has been quite difficult, though Sorn et al. (1965) attempted the purification by use of column chromatography. Using relatively purified bovine hyaluronidase purchased from Worthington Biochemical Company, the authors sensitized the rabbits and, examined the localization of hyaluronidase in the bovine urogenital organs by fluorescent antibody solution. As a result, in the seminiferous tubule of the testis, fluorescence was present in the form of threads relatively near the basement membrane.

It was most abundantly found in the layer of spermatogonium, followed by spermatocytes; thus, the amount of specific fluorescence decreased toward the inner part. However, there are two possibilities in this fluorescence: it may be slight diffusion due to the extremely strong specific fluorescence in the connective tissues stated below, or it may be present in sperms (Swyer, 1947). But, in the the present study, probably due to the minuteness of the sperms no evident localization could be observed. In the layer where spermatogonia exist, the cells which are supposed to be the Sertoli cells show weak specific fluorescence. There is a considerable amount of hyaluronidase in the interstitial area which surrounds the seminiferous tubules. The specific fluorescence is present in relatively high concentrations in the interstitial cells in the interspace between each connective tissue cell or fiber and in the area around the basement membrane of the seminiferous tubules. It is presumed that hyaluronidase produced in the interstitial cells is immediately liberated into the amorphous space of the connective tissues, and then reaches the basement membrane. After permeating through the basement membrane, hyaluronidase may be taken into the Sertoli cell or may flow into the interspace between each spermatogenic cell. Then it may be taken into spermatogonium or spermatocyte and transferred to the sperms, or the enzyme taken into Sertoli cell may be transferred to the sperms.

It is not evident whether or not hyaluronidase is directly taken into sperms. This is the assumption made from the result of this study as well as from that of the preliminary report (Nagakubo et al., 1970).

As mentioned above, the fluorescent spots which might be attributable to hyaluronidase in the sperm were visible in the central area of the seminiferous tubule. But, these spots may be aggregated

sperms but not the single sperm.

Though S w y e r (1947) reported the presence of appreciable amount of hyaluronidase in the sperm, evident fluorescence was not recognized in the sperm in this study, probably because the content of hyaluronidase in a sperm was too low to be observed by the fluorescent microscope. In the epididymis, there is no hyaluronidase in the epithelium of ductus epididymidis and in the connective tissues. There was no specific fluorescence in any part of the seminal duct and the prostate. Although hyaluronidase should be present in the lumen of the seminal duct since there are many sperms, no specific fluorescence could be found, probably because the cavity was too large or because the seminal fluid had run out at the time of the experiment.

As stated above, the material used as antigen for the present study was composed of 5 immunological factors. Although there was no cross reaction between these factors and bovine albumin or sheep hyaluronidase, it is not determined at this moment whether they are truly the isozymes of hyaluronidase or not. Accordingly, it is understood that the localization of hyaluronidase reported in this study represents the localization of five factors. Further study is requested by using more purified antigen.

Conclusion

Using immunofluorescent technique, the localization of hyaluronidase was examined in the bovine urogenital organs.

Hyaluronidase was found in the interstitial cells, in the amorphous intercellular space of the interstitial connective tissue.

The enzyme was concentrated around the basement membrane.

In the seminiferous tubule, hyaluronidase was recognized in the interspace between each spermatogenic cell as well as in the cytoplasm of the Sertoli cell. The concentration of hyaluronidase decreased toward the center of the lumen of the seminiferous tubule. Small spots of the fluorescence probably caused by hyaluronidase lodged in the sperm were noticed around the central area of the seminiferous tubule.

This enzyme may be produced in the interstitial cell, infiltrate between the connective tissue elements, be accumulated once around the basement membrane, pass through the basement membrane, be taken into the Sertoli cell or flow through the interspace between spermatogenic cells, and finally be transferred to the spermatogenic cells or to the sperm. The enzyme was not present in the epididymis, in the spermatic cord and in the prostate.

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Plate

Explanation of figures

- Fig. 1. The gel-diffusion test on the Ouchterlony plate shows 5 precipitin lines; a thick line at the center and two thin lines at each side of the thick line.
- Figs. 2 and 3. Bovine testis. Hyaluronidase was found in the interstitial cells, interspace between each connective tissue element and around the basement membrane. In the seminiferous tubule, the specific fluorescence is very weak and is recognized in the basal part of the tubule. Some fluorescent spots are visible in the central area of the cross section of the tubule. $\times 250$.
- Fig. 4. The small mass of the fluorescence is noticed in the lumen of ductus epididymidis. $\times 250$.

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