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Antigenic Properties of Subcellular Fractions from Canine Pancreas: Development of a Zymogen Membrane Specific Antibody[1] (39091)

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Despite the fact that autoantibodies react with body constituents of the animals that produced them, they are best induced by immunization with cross-reacting antigen from a foreign (xenogenic) species (1, 2). We have come across a situation in which a pancreas-specific autoantibody is regularly induced in rabbits by immunization with antigenic material prepared from dog pancreatic zymogen membranes.

Materials and methods. Pancreases were obtained from dogs which had been fasted for 24 hr. These animals were anesthetized with pentobarbital; the pancreas was removed and immediately placed in ice cold buffer containing 10m*M* tris-maleate, 0.25 *M* sucrose and 5×10^{-4} *M* EGTA, pH 5.5 (TMSE buffer). The tissue was then washed and homogenized in 200 ml of cold TMSE buffer with a Potter–Elvehjem homogenizer fitted with a Teflon pestle. The homogenate was filtered through a 110-mesh cheesecloth and fractionated by differential centrifugation into nuclear, zymogen granule (ZG), mitochondrial, and microsomal subfractions. ZG membranes were prepared from ZG's by lysis in three volumes of 0.2 *M* sodium borate in 0.9% NaCl, pH 8.0 followed by centrifugation to remove soluble components, and flotation of the membranes on a discontinuous sucrose density gradient as described by Meldolesi *et al.* (3).

The various fractions were assayed for succinate dehydrogenase (SDH), trypsin, and amylase activity, and, as judged by these determinations, the membrane subfraction contained only trace amounts of nonmembrane contaminants. The ZG fraction, however, was usually contaminated by mitochondia to the extent of 10%.

Proteins of the different subfractions were compared by electrophoresis on 10% polyacrylamide gels containing 0.1% sodium dodecyl sulfate (SDS) according to the method of Lämli (4), except that the samples were treated with 1% SDS at room temperature approximately 10 min before application to the gels. Where applicable 3% β-mercaptoethanol was also added. Coomassie Brilliant Blue R was used for visualization of proteins, while the periodic acid Schiff stain (PAS) (5) was used for sugar residues.

Antibody was prepared by combining the purified ZG membrane preparation (100 μg/ml) with an equal volume of complete Freunds' adjuvant (Difco Laboratories, Detroit, Michigan); the resulting emulsion was injected subcutaneously into the nape of the neck and dorsal thoracic skin of three albino rabbits. The antigen with the addition of incomplete Freunds' adjuvant was injected at 2-week intervals for two more doses. After a rest of 3 weeks, a second series of five inoculations was applied. Final bleedings were performed 7–10 days after the last immunization and collected antiserum was stored at −20°C until use.

The antiserum was evaluated by Ouchterlony double-diffusion in 1% agarose gel. Protein content of the various antigens was routinely determined by the method of Lowry (6), using bovine serum albumin as a standard. Protein concentrations of the pancreatic subfractions were 23.5, 10.5, and 1 mg/ml, for the crude pancreatic homogenate, the ZG fraction, and the ZG membrane preparation, respectively. Thirty percent homogenates in 0.9% NaCl were prepared from various dog organs other than pancreas. These homogenates were tested against the antiserum by the Ouchterlony technique for cross-reactivity. Protein concentrations of these preparations ranged from 8.5–26.5

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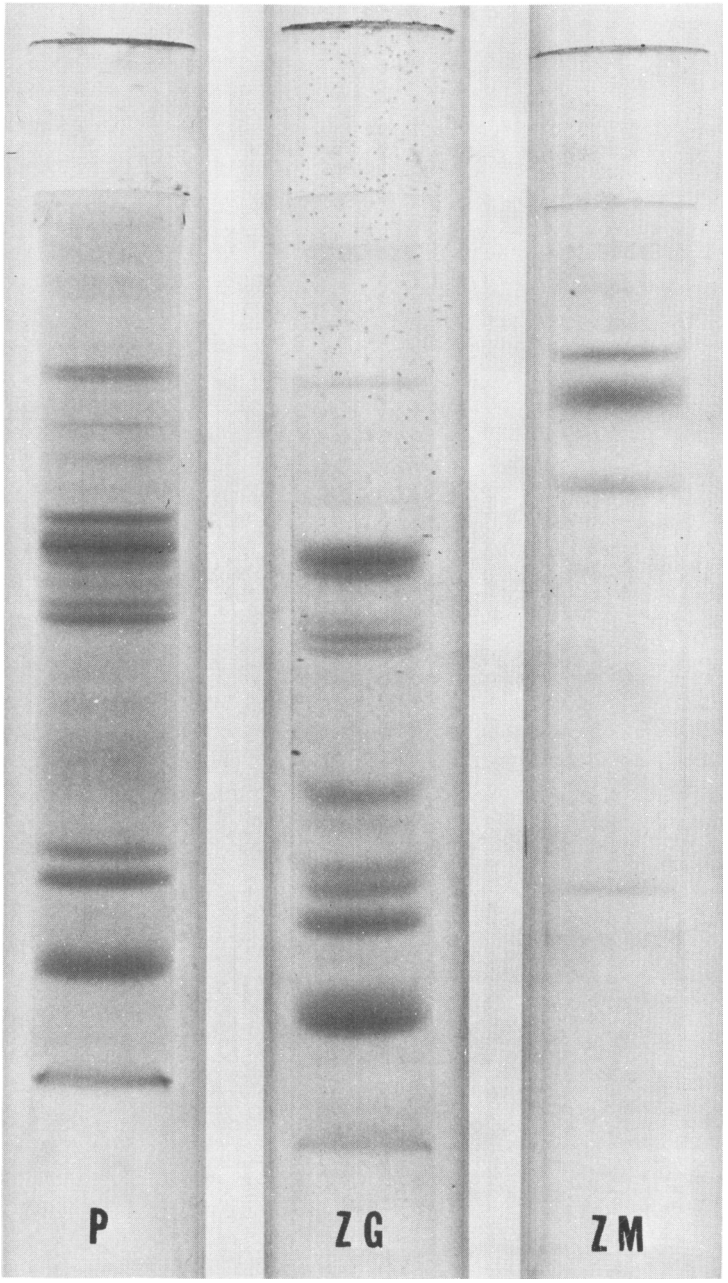

FIG. 1. Typical electrophoretograms of the proteins of crude pancreas homogenate (P), zymogen granules (ZG), and zymogen granule membrane (ZM) fraction (100 μg of protein was added per gel and run in SDS system without mercaptoethanol as described in the text).

mg/ml. All antigens were tested against the antiserum from dilutions 1:1 to 1:16.

Results and discussion. The gel electrophoretograms of the isolated fractions are shown in Fig. 1. Although the protein patterns are quite complex in both the crude homogenate and the ZG fractions, only three bands of mol wt 62,000, 79,000, and 90,000, respectively, were observed in the ZG membrane fraction with the protein

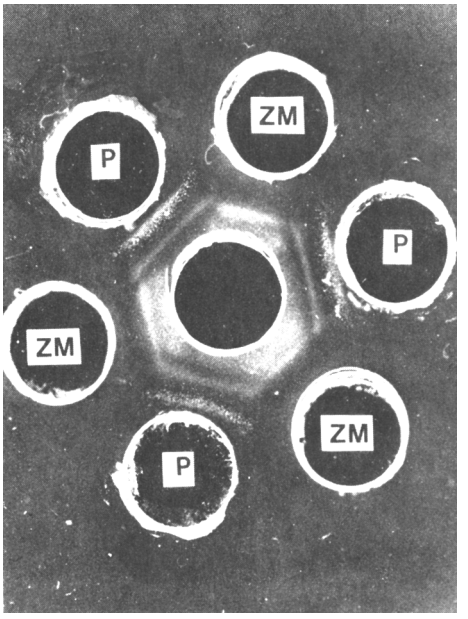

FIG. 2. Anti-ZG membrane antiserum after double-diffusion against crude pancreatic homogenate (P) and purified ZG membrane preparation (ZM). Protein concentrations were 23.5 mg/ml for P and 1 mg/ml for ZM preparations.

stain. All three of these bands were also PAS positive, indicating their glycoprotein composition. In addition a small mol wt species running just in front of the bromphenol blue tracking dye was PAS positive, though it did not stain with Coomassie blue.

The antiserum, when tested by Ouchterlony double-diffusion in agarose gel, gave a strong line of precipitation with ZG membrane preparation and a double precipitin line with crude pancreatic homogenate (Fig. 2). There were no qualitative differences observed between the antisera raised in all rabbits. When serial dilutions of the subcellular fractions were used, the antiserum reacted with the crude pancreatic homogenate at all dilutions up to 1:16, while the ZG membrane preparation gave a precipitin line up to a 1:4 dilution. Homogenates prepared from other dog organs, including heart, lung, muscle, kidney, adrenal, urinary bladder, uterus, esophagus, stomach, small intestine, colon, liver, thyroid, parotid gland, and spleen, showed no reaction in all dilutions tested. Dog gallbladder homogenates consistently gave a weak precipitation line that disappeared when 1:2 dilution of the homogenate was used. The anti-ZG membrane serum also failed to react with dog bile and blood serum. Moreover, the antiserum gave no reaction when tested against pancreozymin-cholecystokinin (PZ-CCK), secretin, chymotrypsinogen A and B, and gastrin. Normal (preimmune) rabbit serum failed to react with any of the subcellular preparations used in this study.

Having demonstrated that the antiserum contained a pancreas-specific antibody, we set out to determine whether it was species-specific or not. Pancreatic homogenates from various mammals (including rabbit, rat, guinea pig, and man) gave a clear line of precipitation with the antibody, with reaction of partial identity indicated by spur formation. In contrast the antiserum failed to react when tested against homogenates of chicken and frog pancreas. We propose to call this antibody anti-ZM antibody and the antigen it defines "ZM" antigen (ZM signifies ZG membrane subfraction).

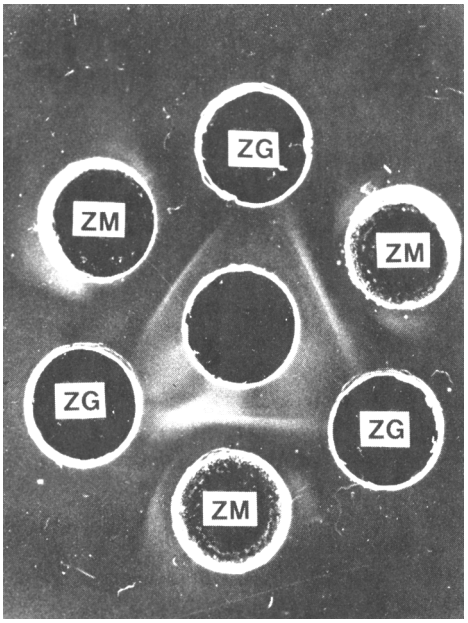

FIG. 3. Anti-ZG membrane antibody in the center well with intact zymogen granules (ZG) and ZG membrane fraction (ZM) in the peripheral wells. Protein concentrations were 10.5 mg/ml for ZG and 1 mg/ml for ZM fractions.

We also wanted to know if the antigenic properties of the ZG membrane subfraction were due to their inner or outer components. When the antiserum was tested against intact zymogen granules (ZG) and ZG membrane subfraction, a precipitation line was seen only with the membrane fraction and no reaction found with the intact zymogen granules (Fig. 3). However, when the antiserum was tested with lysed zymogen granules (after treatment with 0.2 *M* sodium borate buffer) a clear line of precipitation was seen. It is reasonable to speculate that this phenomenon represents indirect evidence that the antigenic properties of the membrane fraction used in this study are located in the inner aspect of the ZG membrane.

The reason for two precipitation lines being observed in the whole homogenate is unclear, though it is possible that a cross reaction is occurring with a similar antigen found on the surface of the plasma membrane of the acinar cells. Considering the evidence that the secretory process involves membrane fusion between the ZG and plasma membranes (7, 8), it is entirely possible that an antigen located on the inner side of a ZG membrane would then be found on the outer surface of the plasma membrane.

Summary. Immunization of rabbits with a membrane preparation from dog pancreas produced precipitating antibody against an antigen present in the pancreas of several mammals. This antigen appears to be organ-specific but not species-specific and is localized in the pancreatic zymogen granule membrane fraction. It is thought to represent glycoproteins of mol wt 70,000–90,000 as well as a smaller species of mol wt less than 10,000.

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