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Studies on Ultrastructure and Immunology of the Insulitis in Rabbits Immunized with Insulin*

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Summary. Rabbits were immunized with crystalline bovine and porcine insulin. Besides determining the blood glucose levels and antibody titers, electronmicroscopic studies were performed. The following results were obtained.

1. Immunization with bovine or porcine insulin constantly results in the formation of humoral antibodies. In addition, about 60% of the animals showed a lymphocytic infiltration of the pancreatic islets, an insulitis, after immunization with bovine insulin. In contrast, no insulitis occurred after immunization with porcine insulin.

2. The infiltrate mainly consisted of lymphocytes and immunoblasts. Insulitis can therefore be regarded as an immune response of the cellular type.

3. Lysis of the cell membranes between lymphocytes and immunoblasts as well as degenerative changes of the beta cells and phagocytosis of cell debris suggested an autoimmune process against insulin, proinsulin, C-peptide or associated proteins.

4. Antibody titer and hyperglycemia correlated within the single groups of the study. Hyperglycemic animals showed the highest antibody titers. Hyperglycemia and antibody titer did not depend on the existence and extent of an insulitis. It is therefore suggested that hyperglycemia is caused by biologic neutralization of endogenous insulin by its binding to humoral antibodies. Insulitis, on the other hand, obviously represents an independent immune response of the cellular type.

Zusammenfassung. Kaninchen wurden mit kristallinem Rinder- oder Schweine-Insulin immunisiert. Neben Blutzuckerkontrollen und Antikörpertiter-Bestimmungen wurden elektronenmikroskopische Untersuchungen am Pankreas durchgeführt. Es ergaben sich folgende Befunde.

1. Die Immunisierung mit Rinder- oder Schweine-Insulin rief in jedem Fall die Bildung humoraler Antikörper hervor. Zudem zeigten nach Rinder-Insulin-Immunisierung etwa 60% der Tiere eine Insulitis mit lymphocytärer Infiltration der Pankreas-Inseln. Die Schweine-Insulin-Immunisierung hatte dagegen keine Insulitis zur Folge.

2. Die Zellinfiltration der Inseln bestand überwiegend aus Lymphocyten und Immunoblasten. Sie entspricht damit dem Typus einer cellulären Immunreaktion.

3. Zellmembranauflösungen zwischen Lymphocyten und B-Zellen sowie degenerative B-Zellveränderungen und die Phagocytose von Zellmaterial deuten auf einen Autoimmunprozeß hin, der sich gegen Insulin, Proinsulin, C-Peptid oder andere dem Insulin assoziieret Proteine richten kann.

4. Es bestand eine enge Korrelation zwischen der Höhe des Antikörpertiters und der Höhe der Hyperglykämie innerhalb der einzelnen Versuchsgruppen. Tiere mit Hyperglykämie hatten gleichzeitig die höchsten Antikörpertiter. Dabei waren Hyperglykämie und Höhe des Antikörpertiters unabhängig von dem Vorhandensein oder Ausmaß einer Insulitis. Es wird daher angenommen, daß die Hyperglykämie auf einer biologischen Inaktivierung des endogenen Insulins beruht, welches durch humorale Antikörper gebunden wird. Die Insulitis scheint dagegen eine davon unabhängige celluläre Autoimmunreaktion darzustellen.

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The lymphocytic infiltration of the islets of Langerhans which is called insulitis (von Meyenburg, 1940) is known for some time as a specific finding in juvenile diabetics with acute onset of the disease (Le Compte, 1958). Since lymphocytic insulitis resembling the inflammatory infiltrate of the islets in human diabetes had been evoked in cows by immunization with homologous and heterologous insulin, autoimmunologic factors in the pathogenesis of diabetes mellitus have been discussed (Gepts, 1965). Besides in cows and sheep (Renold *et al.*, 1964; Federlin *et al.*, 1968) insulitis was only yet observed in rabbits immunized with bovine insulin (Toreson *et al.*, 1964). In addition, some of these animals developed hyperglycemia. The present study was undertaken to examine by electron microscopy if the experimental insulitis in rabbits is comparable with an autoimmune response of the cellular type and if insulitis also occurs in rabbits immunized with porcine insulin. Furthermore, a correlation between insulitis, hyperglycemia and production of humoral antibodies was proofed.

Materials and Methods

Fifteen rabbits (New Zealand white rabbit; weighing about 2000 g) were immunized with crystalline bovine insulin (Hoechst) in Freund's adjuvant (Difco Lab.) and ten rabbits with crystalline porcine insulin (Hoechst) in Freunds' adjuvant. The immunization was performed according to the slightly modified method of Lee *et al.* (1969). Four insulin injections were given at weekly intervals into the toe-pads of the hindlegs: In the first week, 1 mg insulin in complete Freund's adjuvant; in the second, third and fourth week, 0.5 mg insulin in incomplete Freund's adjuvant was administered. Three control animals received Freund's adjuvant only. Blood samples for determination of glucose were taken biweekly. Blood glucose was measured by the glucose oxidase method (Boehringer, Mannheim).

The animals were sacrificed 6 to 8 days after the last immunization. At sacrifice blood samples were taken for determination of glucose and antibody titer to insulin. The antibody titration was performed according to the slightly modified method of Farr (1958) for quantitative immunochemical measure of antigen-antibody complexes.[1] The titer is defined as that dilutation of sera which shows a 50% binding of 1 μg J^{127} porcine insulin/ml. By means of the antibody titer it was then possible to calculate the absolute insulin-binding-capacity (IBC) of the sera.

For light microscopic studies, portions of heart, lung, liver, kidney, spleen and lymph nodes were fixed in 10% formol solution. Pancreatic tissue was fixed in Bouin's solution. Sections were stained with hemalaun-eosin, periodic acid Schiff's reagent and Gomori's aldehyde-fuchsin.

For electron microscopic studies, pieces of pancreas were immersion fixed immediately after removal in 3% glutaraldehyde buffered with 0.1 M sodium cacodylate (pH 7.4) for two hours. After rinsing in 0.1 M sodium cacodylate buffer the cubes were postfixed in 1% osmium tetroxide buffered with 0.1 M sodium cacodylate for 90 min. The fixed tissue was dehydrated in ethyl alcohol and after passing propylenoxid embedded in Epon 812. 600–800 Å sections were cut on a Reichert ultramicrotome OM U2, double-stained with uranyl acetate and lead citrate and examined in a Philips electron microscope EM 300 at 60 kV.

Results

1. Blood Glucose Levels

The blood glucose levels of the nonfasting control animals and the animals before treatment ranged from 70 mg/100 ml to 125 mg/100 ml. The amounts of glucose in the blood of fasting animals were almost the same. During the third

1 The authors wish to thank sincerely Dr. Franz Klaus Jansen, Diabetes-Forschungsinstitut, Düsseldorf, for valuable assistance in determination of the antibody titers.

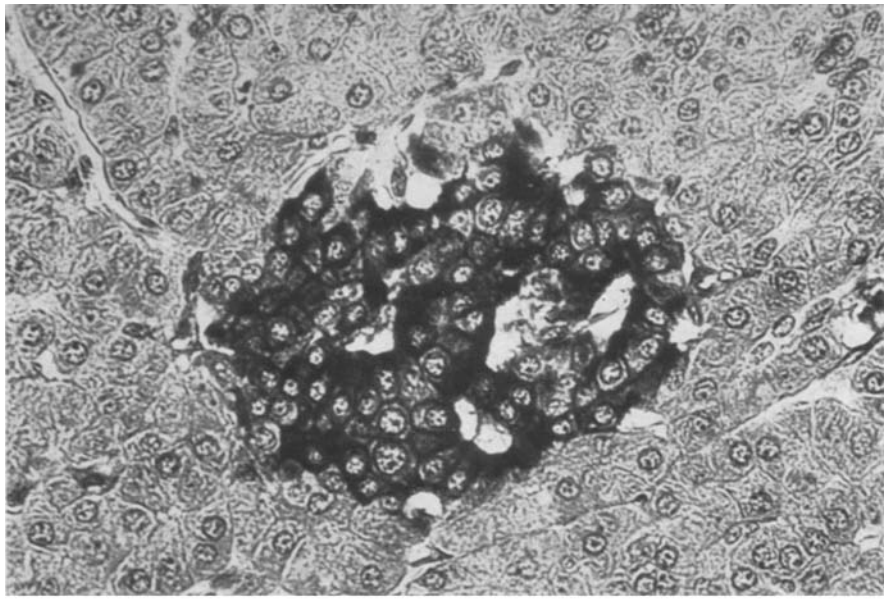
and fourth week of the study about 30% of the animals immunized with bovine or porcine insulin displayed hyperglycemic episodes lasting mostly three to seven days. The blood glucose levels in these rabbits generally ranged from 130 mg/100ml to 180 mg/100 ml (Table 2). Only single animals in both groups showed hyperglycemia up to 280 mg/100 ml. The elevation in blood glucose was independent from the presence and degree of an insulitis (Table 1). For instance, the rabbit with the most striking islet lesions remained normoglycemic. On the contrary, the rabbit with the highest blood glucose level developed no insulitis. Moreover, hyperglycemia occurred also in those rabbits which were immunized with porcine insulin and which, on the whole, revealed no insulitis.

Table 1. Frequence of insulitis, mean blood glucose levels and mean insulin-binding-capacity (IBC) in rabbits 6–8 days after the 4. weekly immunization with bovine or porcine insulin in Freund's adjuvant. IBC is calculated on that dilution of serum with 50% binding of 0.1 μg of porcine J^{127} insulin/ml. Standard deviation (S)

Group	Animal number	Mean IBC (μg/ml)	Mean blood glucose (mg/100 ml)
I. Adjuvant (control) group	3	—	94 (74–125) $S \pm 17$
II. Porcine insulin — immunized group	10		
with insulitis	—	—	—
without insulitis	10	0.2 (0.04–0.6) $S \pm 0.18$	116 (100–152) $S \pm 24$
III. Bovine insulin — immunized group	15		
with insulitis	8	3.8 (0.15–11.0) $S \pm 3.6$	110 (80–148) $S \pm 20$
without insulitis	7	4.4 (0.8–13.4) $S \pm 4.15$	120 (82–230) $S \pm 39$

2. Antibody Titer

Precipitating antibodies were demonstrated only once whereas soluble antibodies were formed in all animals immunized with bovine or porcine insulin. The antibody titers ranged from 1:1 (IBC: 0.04 μg/ml) to 1:360 (IBC: 13.4 μg/ml). In particular, the following observations were made: (1) The mean insulin-binding-capacity (IBC) of the sera after immunization with bovine insulin differed only slightly in the rabbits with and without insulitis (Table 1). The amount did not refer to the degree or even to the existence of an insulitis. Thus, the animal with the most striking insulitis exhibited a very low IBC (0.15 μg/ml) of the serum. (2) There was a correlation in elevation of the blood glucose level and the amount of the antibody titer. Hyperglycemic animals (130 mg/100 ml–280 mg/100 ml) showed on the average higher antibody titers than normoglycemic animals



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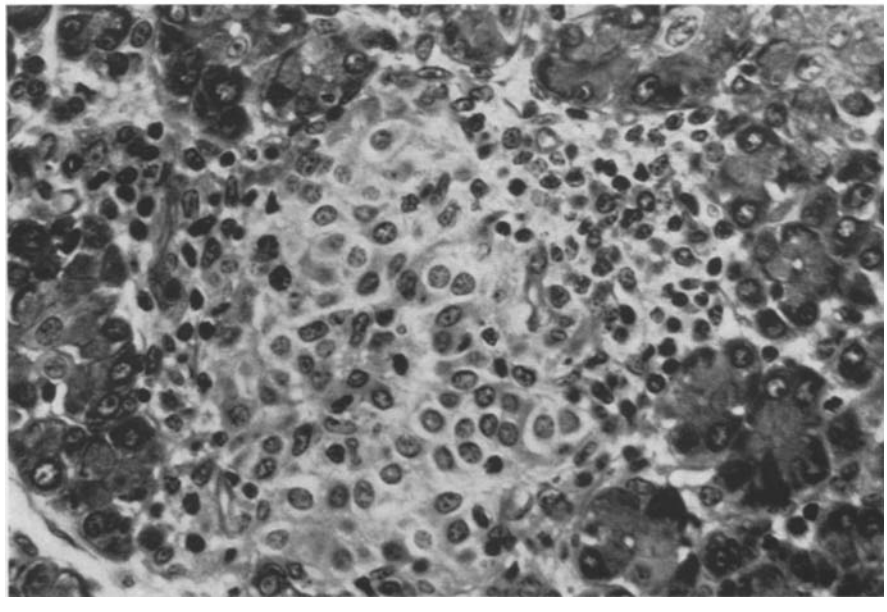

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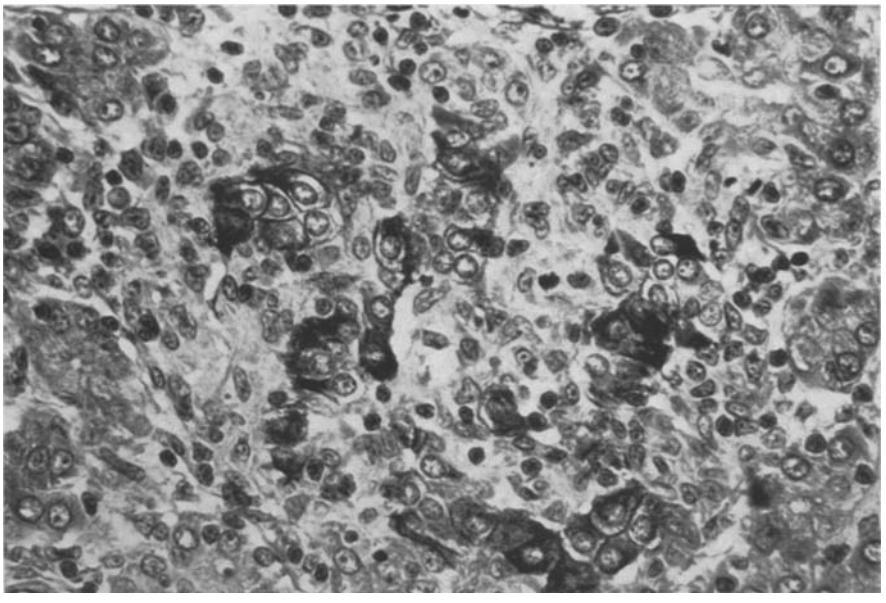
Fig. 1. a Control animal: Normal rabbit islet with distinct granulation of the beta cells. Aldehyde-fuchsin. ×500. b Rabbit pancreas after weekly immunization with bovine insulin (4. week): Poor demarcation of an islet from acinous tissue by periinsular insulitis consisting of mononuclear cells. PAS. ×500. c Rabbit pancreas after weekly immunization with bovine insulin (4. week): Marked islet destruction by peri- and intrainsular insulitis. Few clusters of beta cells with different degranulation and some alpha cells surrounded by mononuclear cells. Aldehydefuchsin. ×500



c

Fig. 1 c

Table 2. Mean insulin-binding-capacity (IBC μg/ml) in hyperglycemic and normoglycemic rabbits 6–8 days after the 4. weekly immunization with bovine or porcine insulin in Freund's adjuvant. Standard deviation (*S*)

Group	Animal number	Mean blood glucose (mg/100 ml)	Mean IBC (μg/ml)
I. Adjuvant (control) group	3	94 (74–125) $S \pm 17$	—
II. Porcine insulin — immunized group	10		
with hyperglycemia	3	142 (132–152) $S \pm 8$	0.4 (0.15–0.6) $S \pm 0.1$
with normoglycemia	7	104 (70–120) $S \pm 18$	0.04 (0.04) $S \pm 0$
III. Bovine insulin — immunized group	15		
with hyperglycemia	5	160 (130–230) $S \pm 39$	9.0 (2.64–13.4) $S \pm 5.6$
with normoglycemia	10	97 (81–125) $S \pm 16$	1.9 (0.15–4.4) $S \pm 1.6$

(Table 2). (3) Although rabbits immunized with porcine insulin sometimes developed hyperglycemia the IBC of these antisera was in general much lower (0.04–0.6 μg/ml) than the IBC of the bovine insulin antisera (0.15–13.4 μg/ml).

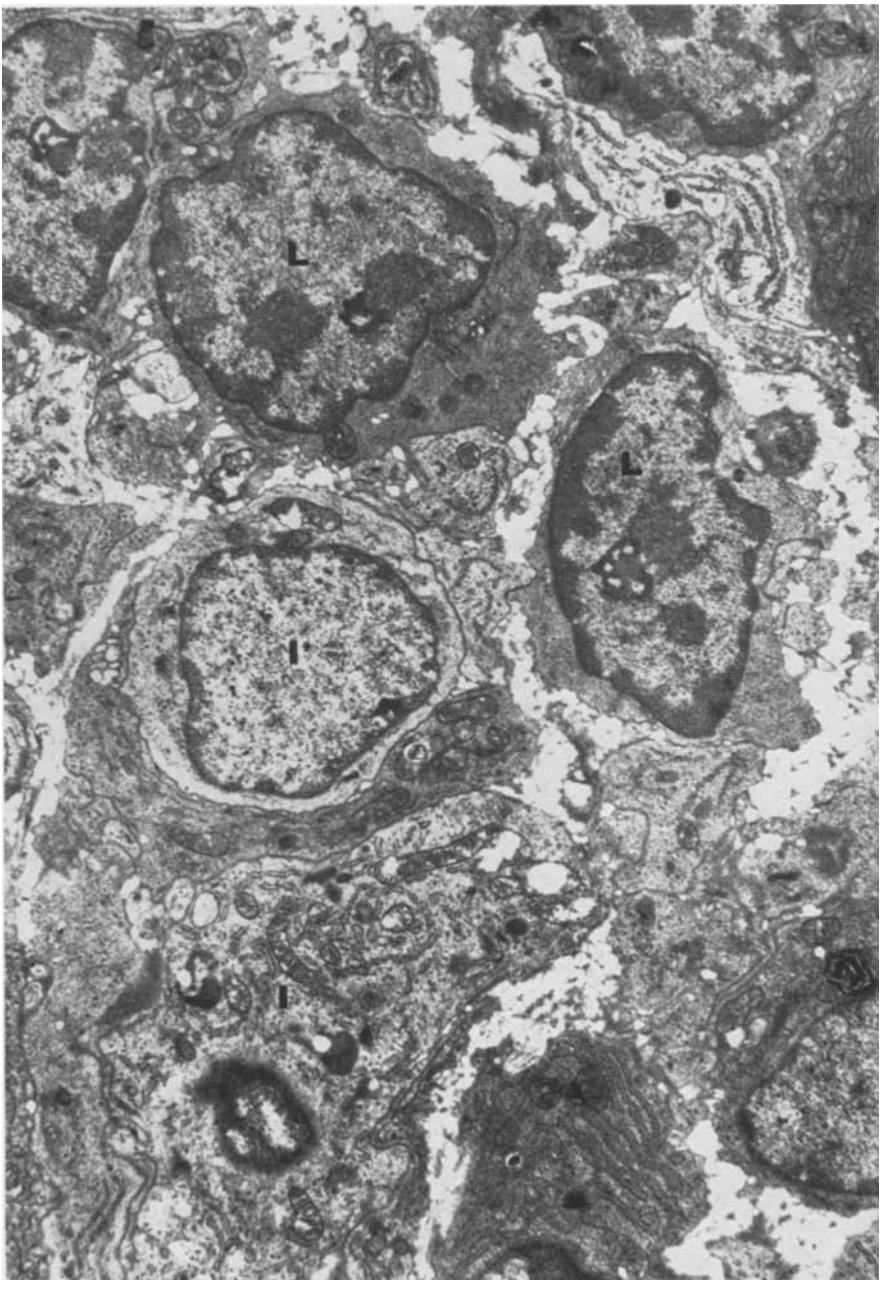


Fig. 2. Rabbit pancreas after weekly immunization with bovine insulin (4. week): Part of the periinsular infiltrate. Several lymphocytes (*L*) of the intermediate type with already well developed nucleolus and abundant ribosomes in the cytoplasm. Close by immunoblasts (*I*) with sparse granular cytoplasm and abundant cell organelles. × 7650

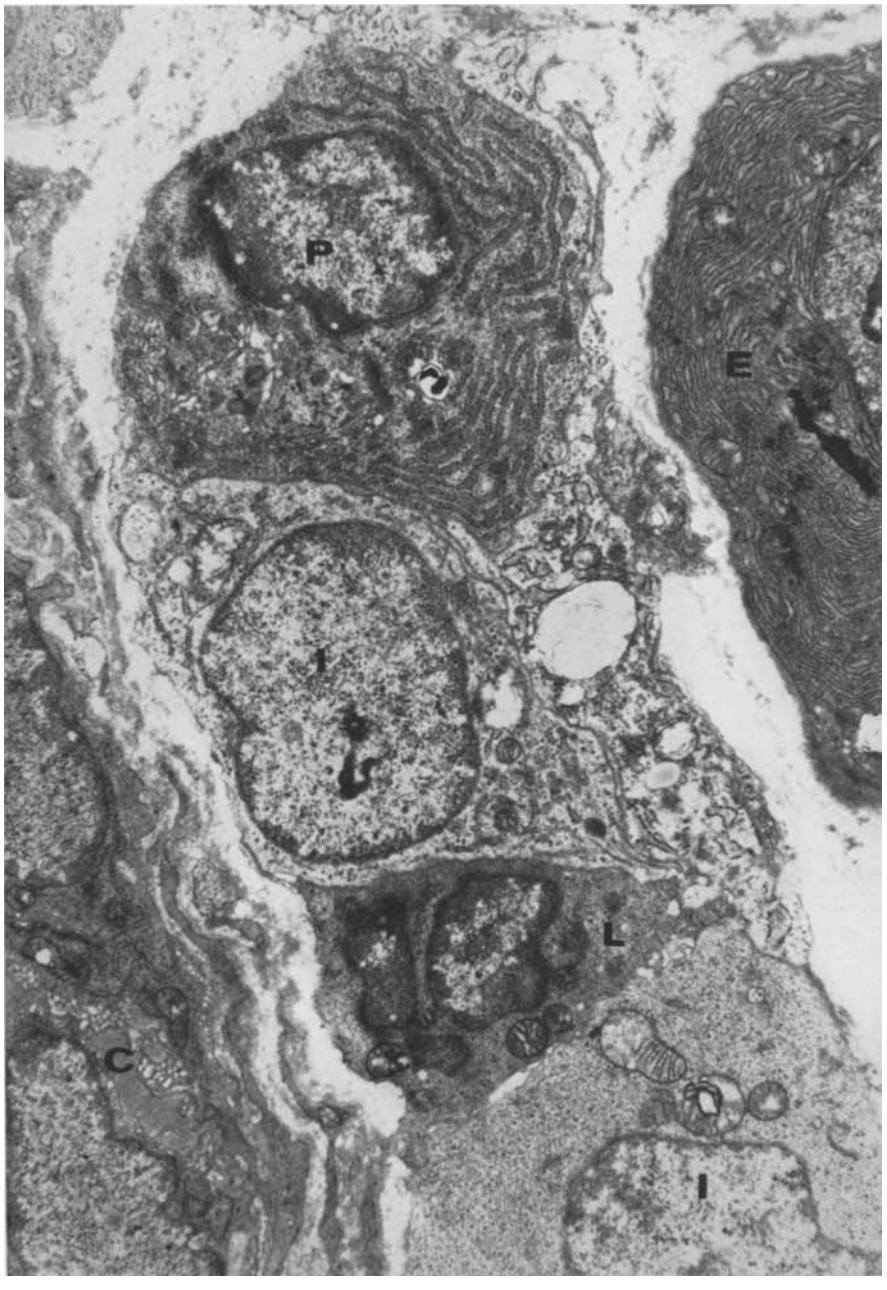


Fig. 3. Rabbit pancreas after weekly immunization with bovine insulin (4. week): Between two immunoblasts (*I*) one lymphocyte (*L*) with an increased ratio of heterochromatin to euchromatin in the nucleus. On the top a plasma cell (*P*). Exocrine cell (*E*). Capillary (*C*). × 9100

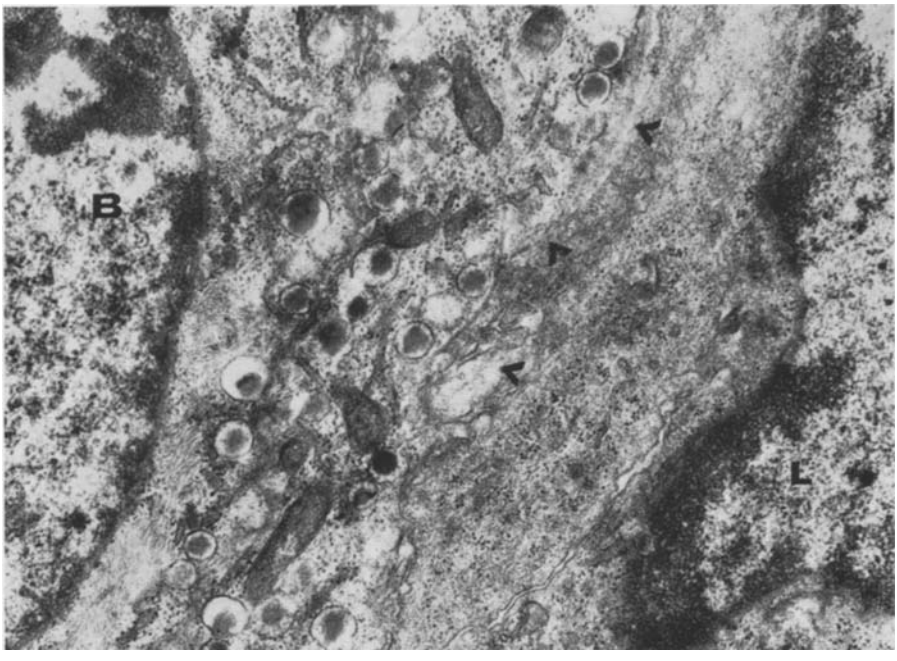


Fig. 4. Rabbit pancreas after weekly immunization with bovine insulin (4. week): Lymphocyte (L) in close contact to a beta cell (B). Circumscribed decomposition of the cell membranes (Λ). $\times$ 21500

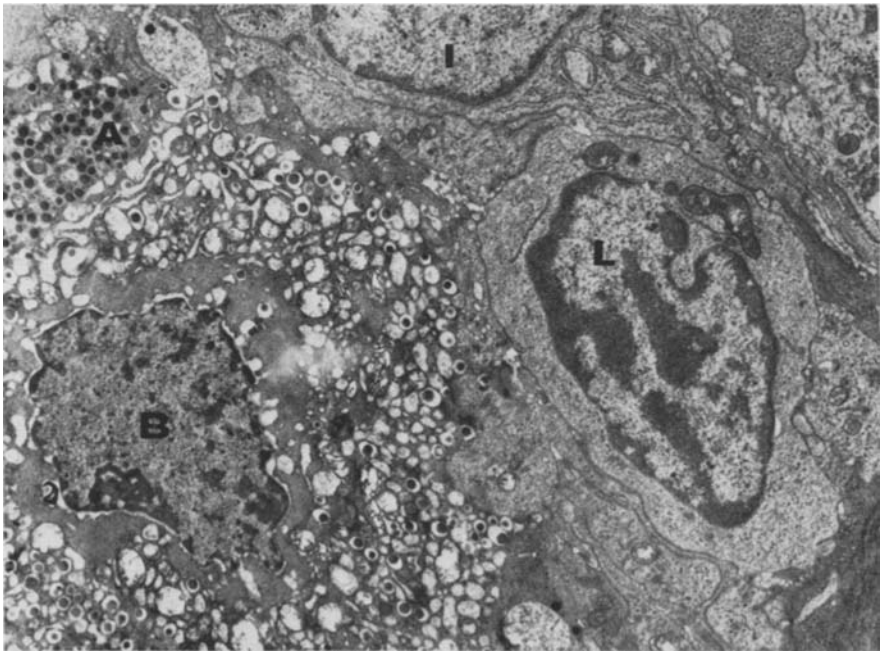


Fig. 5. Rabbit pancreas after weekly immunization with bovine insulin (4. week): Degenerative changes of a beta cell (B) with cystic dilatation of the rough endoplasmic reticulum and shrinkage of the nucleus. In the vicinity a lymphocyte (L) and an immunoblast (I). Alpha cell (A). $\times$ 6800

3. Light and Electron Microscopy

a) No histopathological changes of the endocrine and exocrine pancreatic tissue were noted in the control animals immunized with Frennd's adjuvant (Fig. 1a).

b) After immunization with porcine insulin in Freund's adjuvant no insulitis was found. By means of electron microscopy, no changes of the islets were noted too, i.e. there were neither heavy degranulations nor degenerative alterations of the beta cells.

c) After immunization with bovine insulin in Freund's adjuvant insulitis occurred in 8 of 15 rabbits.

Lightmicroscopically the islets were surrounded and invaded by mononuclear cells to a different extent (Fig. 1 b). Sometimes, the inflammatory infiltrates were only observed in single islets; sometimes, all islets were involved in the inflammatory process. They then showed only few beta cells having close contact with the surrounding mononuclear cells (Fig. 1 b). Often, the remaining beta cells appeared to be hyperactive by the marked enlargement of the nuclei and the distinct degranulation of the swollen cytoplasm as demonstrated by Gomori's aldehyde-fuchsin staining (Fig. 1 c). The alpha cells showed no changes in size or staining reaction. The inflammatory infiltration of the islets extended often into the adjacent acinous tissue. There, the largest infiltrates of mononuclear cells were found arround small veins. The spleen and the regional lymph nodes showed hyperplasia of the follicles and the paracortical zones. The remaining parenchymatous organs which were also examined lacked lymphocytic infiltrates as seen in cellular hypersensitivity.

Electronmicroscopically lymphocytes and immunoblasts could be distinguished as the main cell types of the peri- and intrainsular infiltrate in rabbits immunized with bovine insulin. The lymphocytes were characterized by a fairly high ratio of heterochromatin to euchromatin in the nucleus, by a vague nucleolus and by a cytoplasm containing only few organelles. Lymphocytes of the intermediate type show already a nucleolus and contained numerous monoribosomal particles in the cytoplasm (Fig. 2). Immunoblasts were frequently observed in different phases of development. As its common characteristics distinct nucleoli, an increased ratio of euchromatin to heterochromatin in the nucleus and an abundant cytoplasm with some organelles were observed. Besides these two cell types rarely plasma cells occurred and were generally located in the periphery of the infiltrate (Fig. 3). Granulocytes were only seen in individual cases.

Frequently, the invading lymphocytes and immunoblasts were in close contact with beta cells. The cell membranes between these lymphocytes and beta cells were found out of focus to such a degree that this observation can be regarded as limited decompositions of the adjacent cell membranes (Fig. 4). The ergastoplasm and the organelles of the affected beta cells, however, were for the rest intact. Other beta cells showed degenerative changes although the cell membranes appeared to be undisturbed. These beta cells revealed cystic dilatation of the endoplasmic reticulum and a shrinkage of the nucleus (Fig. 5). Sometimes, cellular debris was observed in immunoblasts acting as macrophages (Fig. 6). Partly, lymphocytes and immunoblasts had also close contact with alpha cells and acinous cells. In some acinous cells, focal degeneration of the cytoplasm was obvious.

The remaining beta cells were heavily degranulated. Furthermore, the hyperplasia of the rough endoplasmic reticulum and the enlargement of the Golgi

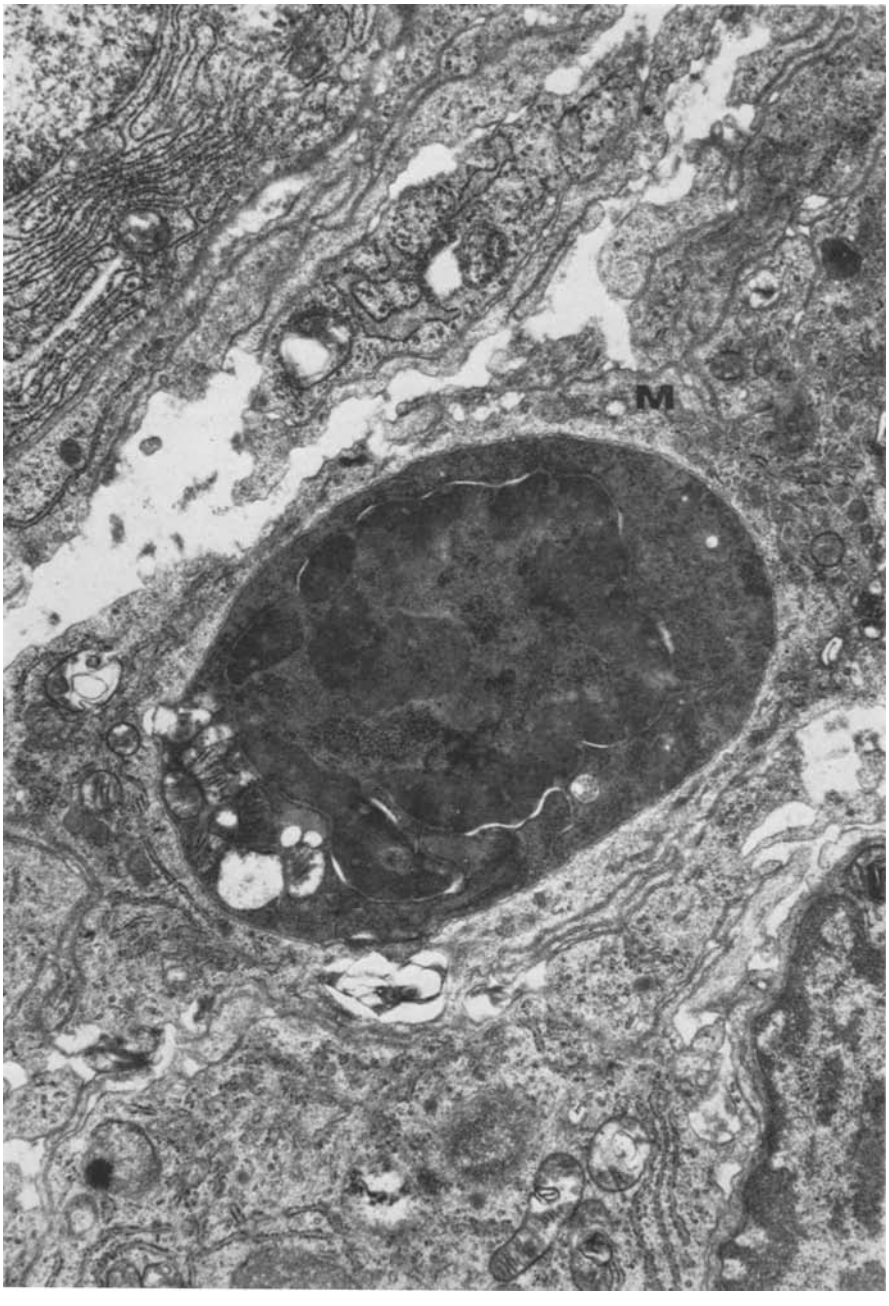


Fig. 6. Rabbit pancreas after weekly immunization with bovine insulin (4. week): Immunoblast acting as a macrophage (*M*). In the cytoplasm phagocytosed cell debris. × 9500

complexes with numerous microvesicles in the vicinity suggested a highly increased synthesis and secretion of insulin (Fig. 7). Moreover, the few granules attracted attention by its different electron density and different size of the core.

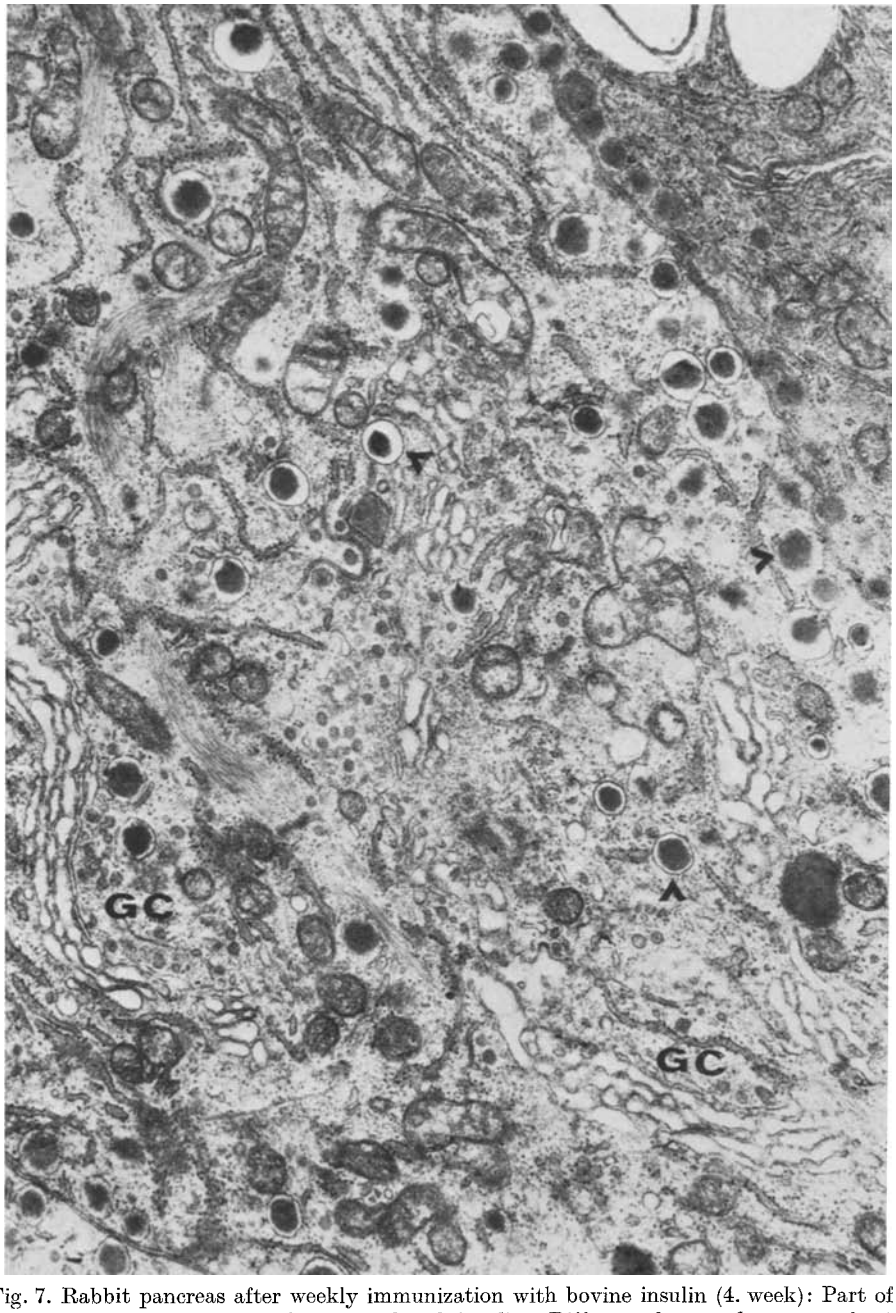


Fig. 7. Rabbit pancreas after weekly immunization with bovine insulin (4. week): Part of a strongly activated beta cell of an animal with insulitis. Different electron dense granules (Λ) and hyperplasia of the rough endoplasmic reticulum and the Golgi complexes (*GC*). In the vicinity of the Golgi complexes abundant microvesicles. × 19500

Some of these could be characterized as pregranules (Logothetopoulos, 1968). Normal beta cells could always be observed when insulitis was only slightly developed or generally lacked. In particular, neither in normal nor in hyper-

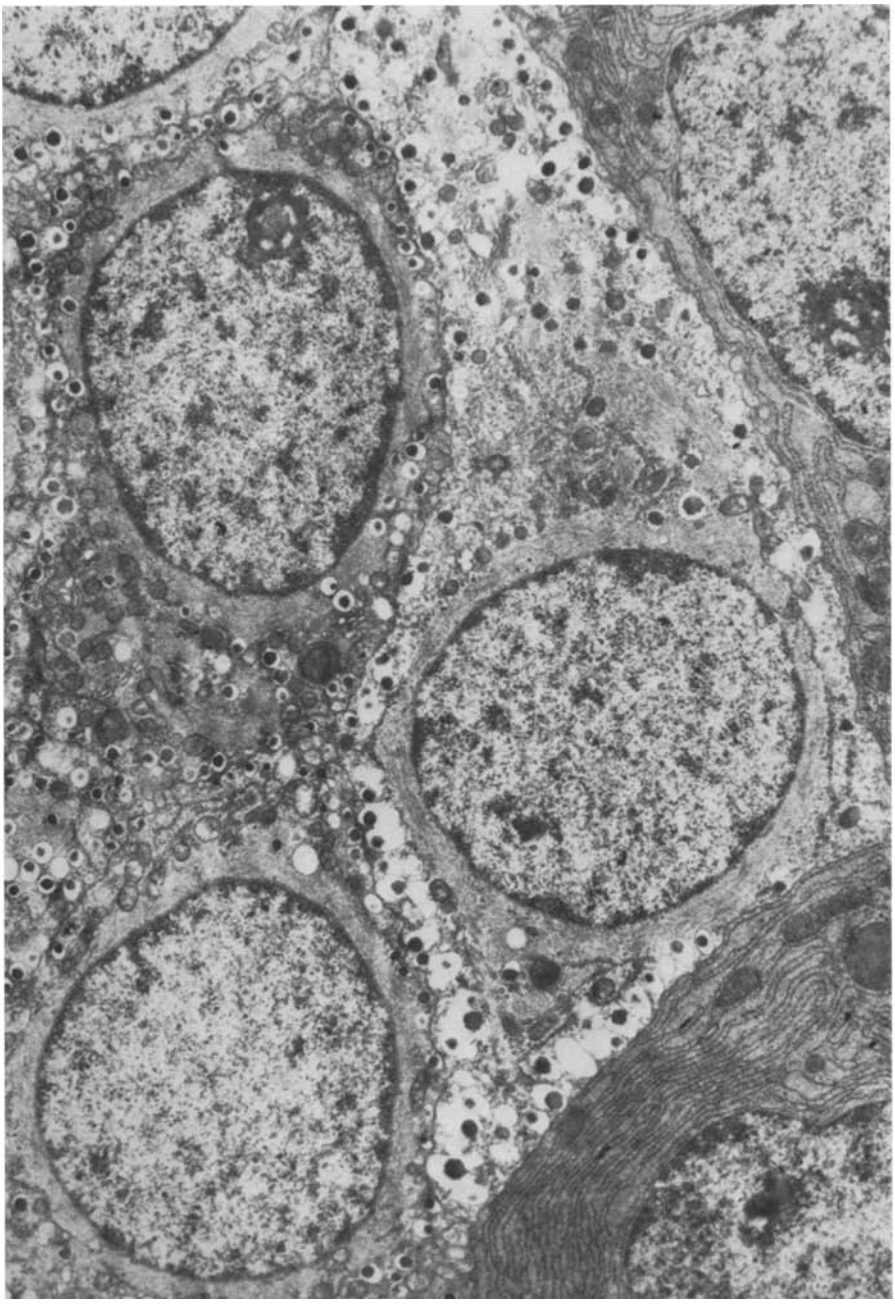


Fig. 8. Rabbit pancreas after weekly immunization with bovine insulin (4. week): Slightly degranulated beta cells of an animal without insulitis. Displacement of some granules to the cell surface indicating a momentary increased secretion. × 9500

active beta cells an increase of the lysosomes and the perinuclear fibrillar material was noted. Only the frequent displacement of granules to the cell surface in some beta cells of animals with marked hyperglycemia indicated that insulin release was temporary increased (Fig. 8). Indications for a permanent hyperactivity as noted in animals with a severe insulitis did not exist.

Discussion

Besides a production of humoral antibodies after active immunization with heterologous and homologous insulin an insulitis with lymphocytic infiltration in and around the islets of Langerhans is observed in cows, sheep and rabbits (Renold *et al.*, 1964; Toreson *et al.*, 1964; Federlin *et al.*, 1968). This lymphocytic type of insulitis resembles very much the insulitis in juvenile diabetes mellitus of short duration (Le Compte, 1958; Gepts, 1965). In rabbits, insulitis was first reported by Toreson and Coworkers (Toreson *et al.*, 1964; Toreson *et al.*, 1968; Lee *et al.*, 1969) after immunization with bovine insulin. Other investigators (Fellenberg and Rose, 1968) did not confirm these islet lesions in the same species. In our study, insulitis occured in 60% after immunization with bovine insulin. However, no insulitis was found in rabbits immunized with porcine insulin.

Since lymphocytic insulitis develops only by means of immunization with insulin in Freund's adjuvant it is likely that it represents an immune response of the cellular type. Moreover, the specific localization of the infiltrate within the islets suggests that the cellular immune response assumes autoimmune character. The immunological system seems to be unable to distinguish between exogenous and endogenous insulin or associated protein fractions as they normally can be demonstrated in crystalline insulin (Schlichtkrull *et al.*, 1970), According to the studies of Schlichtkrull *et al.* (1970) dimer insulin, proinsulin, and as yet uncharacterized proteins can be fractioned by gel filtration from the so-called monocomponent insulin. These proteins are considered very good antigens whose antibodies strongly cross react with pure crystalline insulin and neutralize its biologic effect to some extent. On the contrary, no antibodies are formed when monocomponent insulin has been administered. Under this point of view it is therefore impossible to define exactly the antigen when normal crystalline insulin is injected. In our study, it only can be suggested that immune cells of the rabbit are sensitized against antigens which are species-nonspecific and occur in the islet of cow and rabbit. Furthermore, the destruction of the normal islet architecture and the disappearance of beta cells suggest that the antigen is somehow involved in the insulin producing system and probably belongs to the fractions of proinsulin, C-peptide and associated proteins.

The electronmicroscopical studies supported the suggestion that the insulitis is based on cellular autoimmune mechanisms. The ultrastructural findings resembled those of other experimental autoimmune disorders (Lampert and Carpenter, 1965; Themann *et al.*, 1968, Karesen, 1970) or in vitro investigation of cellular immunity (Weiss, 1968). Thus, the inflammatory infiltrate of islets mainly consisted of lymphocytes, lymphocytes of the intermediate type and immunoblasts in different phases of development. Plasma cells and plasmablasts were rare and mostly localized at the periphery of the infiltrate; granulocytes,

in general, were absent. Moreover, cytotoxic changes of the beta cells could also be demonstrated, though they were only discreetly developed. As the most impressive finding circumscribed decompositions of the adjacent cell membranes between small lymphocytes and beta cells were observed without having evidence for a lysis of these cells. A similar lesion was lacking between immunoblasts and beta cells. Often, several lymphocytes were located between beta cells in such a way that it looked like as if the immune cells inserted pseudopods between or into the beta cells. The release of granules and other constituent parts of the cytoplasm into the intercellular space as it is reported by Lee *et al.* (1969) was not observed. Single degenerative changes in beta cells adjacent to immune cells and phagocytosed cell debris suggested a cellular decay. Together with a production of humoral antibodies these facts support the assumption that immune cells are sensitized against antigens occuring inside and outside of the beta cells.

The still intact beta cells within the inflammatory infiltrates often showed the characteristics of hyperactivity. Possibly, the increased release of insulin by the remaining cells is necessary in order to prevent an insulin deficiency due to the progressive inflammatory destruction of the beta cells. Considering the blood glucose levels, this suggestion seems to be possible. Thus, some of the animals, in spite of a destructive insulitis, remained normoglycemic. However, it has to be assumed that in the case of a prolonged study progressive insulitis may lead to manifest diabetes. Some observations support this suggestion (Grodsky *et al.*, 1966).

A correlation was found in the level of the blood glucose data and the antibody titers. Thus, the highest titer of insulin antibodies were found in those animals which also showed the highest blood glucose levels during the study. The correlation did not depend on the existence or the extent of an insulitis. These facts provide presumptive evidence that hyperglycemia is based on biological neutralization of the endogenous insulin by an immunological binding to the humoral antibodies (Grodsky, 1965). On the contrary, a close relation of hyperglycemia and antibody titer to insulitis as it is suggested by Lee *et al.* seems to be less probable.

It remains unclear why all animals formed humoral antibodies after immunization with insulin while lymphocytic infiltration of the pancreatic islets was a irregular finding. In particular, we do not know, why insulitis only occurred after immunization with bovine insulin while insulitis was lacking in rabbits immunized with porcine insulin. Moreover, very low titers of the antibodies to porcine insulin were measured. This low antigenicity of porcine insulin in rabbits when compared with bovine insulin can possibly be explained by the strong parallelism in amino acid sequences of porcine and rabbit insulin. The fact that hyperglycemic episodes occurred in some of these animals despite a very low antibody titer may also fit to the concept of a far reaching identity between porcine and rabbit insulin. On the basis of this theory, antibodies to porcine insulin may specially be adapted to neutralize the biological effect of rabbit insulin. On the other hand, many studies show that the differences in amino acid sequences of the different insulins only slightly account for its different immunologic behaviour (Lockwood and Prout, 1962; Berson and Yallow, 1963). To our knowledge it is yet unclear, if the primary structures of insulin precursors also play the same uncharacteristic

role in immunologic determination of the whole molecule. Furthermore, it has to be referred to the unclear importance of the secondary and tertiary structure of these proteins in immunization.

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