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Immune Insulitis and Manifest Diabetes Mellitus

Studies on the Course of Immune Insulitis and the Induction of Diabetes Mellitus in Rabbits Immunized with Insulin*

G. Klöppel, E. Altenähr, G. Freytag, and F.K. Jansen

Institute of Pathology, University of Hamburg, and Diabetes Research Institute at the University of Düsseldorf, Germany

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Summary. Rabbits were immunized in different ways with bovine insulin in order to study the influence of duration and mode of immunization on the course of experimental immune insulitis and on the induction of diabetes mellitus. 2 groups (II, III) of 10 animals each received four insulin immunizations within four weeks either with predominantly Freund's adjuvant incomplete (FAI) or with exlusively Freund's adjuvant complete (FAC). 3 groups of 10 to 12 animals each were immunized with insulin for a period of 16 weeks. Within this time group IV received four immunizations with predominantly FAI, group V received seven immunization with predominantly FAC, and group VI sixteen immunizations with predominantly FAI.

In groups II and III, 50% of the animals showed insulitis after 4 weeks independent of the mode of immunization. In groups IV, V and VI, a different frequency of insulitis was found after 16 weeks dependent on the mode of immunization (IV 10%, V and VI 50% to 70%). The extent of insulitis was, in general, smaller than in groups II and III. At the third, respectively fourth week two animals, also showing the most severe insulitis observed, developed an acute diabetes mellitus. After the fourth week no other animal showed diabetes mellitus. The B cells of the diabetic animals were heavily degranulated and exhibited poorly developed endoplasmic structures. Analogous B cell changes were not observed in animals with insulitis but without diabetes mellitus.

The data suggest that immune insulitis does not run a chronic progressive course but represent a temporary immunity phenomenon. The occurrence of diabetes mellitus at an early phase of immunization may possibly be due to transient autoaggressive mechanisms to B cells at the climax of immune insulitis.

Zusammenfassung. Kaninchen wurden auf verschiedene Weise mit Insulin immunisiert, um den Einfluß der Dauer und des Modus der Immunisierung auf den Verlauf der experimentellen Immun-Insulitis und auf die Induzierung eines Diabetes mellitus zu untersuchen. 2 Gruppen (II, III) von je 10 Tieren wurden viermal innerhalb von 4 Wochen entweder mit überwiegend Freunds Adjuvans incomplete (FAI) oder ausschließlich mit Freunds Adjuvans complete (FAC) und Insulin immunisiert. 3 Gruppen von je 10 bis 12 Tieren wurden über 16 Wochen mit Insulin immunisiert. Innerhalb dieser Zeit wurde die Gruppe IV viermal mit überwiegend FAI, die Gruppe V siebenmal mit überwiegend FAC und die Gruppe VI sechszehnmal mit überwiegend FAI immunisiert.

In den Gruppen II und III fand sich nach 4 Wochen unabhängig vom Immunisierungsmodus bei 50% der Tiere eine Insulitis. In den Gruppen IV, V und VI fand sich nach 16 Wochen in Abhängigkeit vom Immunisierungsmodus eine unterschiedliche Häufigkeit der Insulitis (IV 10%, V und VI 50% bis 70%). Das Ausmaß der Insulitis war dabei generell geringer als in der Gruppe II und III. In der dritten, bzw. vierten Woche entwickelte sich bei zwei Tieren, welche auch die stärkste Insulitis zeigten, ein akuter Diabetes mellitus. Zu späteren Zeitpunkten wurde keine weitere Manifestation eines Diabetes mellitus beobachtet. Die

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B-Zellen der diabetischen Tiere waren stark degranuliert und zeigten hypoplastische endoplasmatische Strukturen. Analoge B-Zellenveränderungen wurden bei Tieren mit Insulitis aber ohne Diabetes mellitus vermißt.

Aufgrund der Ergebnisse kann angenommen werden, daß die Immun-Insulitis keinen chronisch-progredienten Verlauf nimmt, sondern ein temporäres Immunphänomen darstellt. Das Auftreten eines Diabetes mellitus zu einem frühen Zeitpunkt der Immunisierung ist möglicherweise die Folge eines vorübergehenden autoaggressiven Geschehens gegenüber den B-Zellen auf dem Höhepunkt der Immun-Insulitis.

40 to 60% of New Zealand white rabbits immunized with bovine insulin in Freund's adjuvant develop insulitis of the lymphoidcellular type (Lee *et al.*, 1969; Klöppel *et al.*, 1972; Freytag, and Klöppel, 1973). Though the infiltration of the pancreatic islets often seems to proceede with destructive lesions of the B cells, diabetes mellitus is rarely found in these animals. Grodsky and coworkers observed diabetes mellitus in two rabbits immunized with bovine insulin (Grodsky *et al.*, 1966). In both animals the antibody titre to insulin were extremely high. Moreover, the pancreas of one of the animals exhibited insulitis. From this it was concluded that immunological mechanisms can evoke diabetes mellitus.

The present study is intended to investigate the significance of mode and duration of immunization for the course of experimental immune insulitis and induction of diabetes mellitus in rabbits immunized with bovine insulin in different ways.

Materials and Methods

Animals

A total of 58 New Zealand white rabbits with an average weight of 2500 g were used in this experiment. The rabbits were single housed in metal cages. They had free access to Altromin 1010 food and drinking water.

Imunization Procedures

Five groups (II–VI) of 10 to 12 rabbits each were immunized with recristallized bovine insulin (Hoechst Co., Frankfurt) in Freund's adjuvant complete (FAC) or incomplete (FAI) (Difco Lab.) at varying time periods and varying time intervals (see Fig. 1). In all animals the first injection consisted of 1 mg insulin plus FAC, whereas all following injections contained 0.5 mg insulin with FAC or FAI, as indicated in Fig. 1. Insulin was dissolved in 0.2 ml a.d. by adding a few drops of 0.01 N HC1, and then emulsified in an equal volume of FAC or FAI. These insulin preparations were injected into the toepads of the hindlegs. The animals of all groups, except group IV, were killed one week after the last injection. The animals of group IV were killed 13 weeks after the last injection. 5 animals (group I), receiving FAC only, served as controls.

Blood Glucose Determination

In the rabbits which fasted for at least 6 hours, once a week blood glucose was determined prior to immunization (see Fig. 1). The samples were withdrawn from the ear veins. Intravenous glucose tolerance tests (GTT) (1 g glucose/1 kg body weight) were performed at the beginning and at the end of the study. Blood glucose was determined prior to glucose injection and after 120 min. In animals suspected of diabetes mellitus blood glucose was checked before and 30, 60 and 120 min after glucose was injected. Glucose assays were done by the glucose oxidase method (Boehringer, Mannheim).

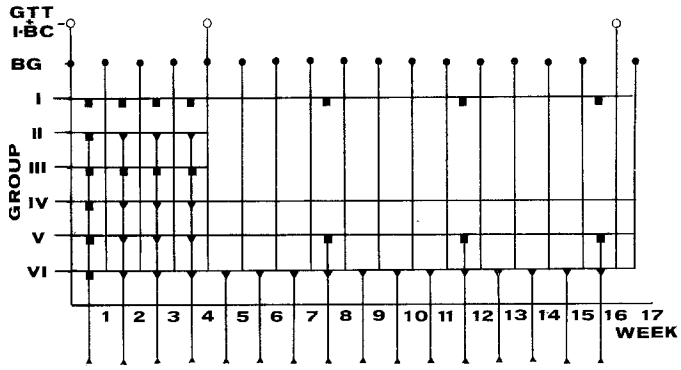

Fig. 1. Time schedule of immunization and diagnostic methods. ▲ Insulin injection. ■ Freund's adj. comp. ▲ Freund's adj. incomp. o Glucose tolerance test (*GTT*), Insulin-binding capacity (*I-BC*). • Blood glucose determination (*BG*)

Insulin Antibody Determination

Insulin antibody titres were determined from blood glucose samples collected at the beginning and at the end of the study. A radioimmunoassay was used for determination. Details of this method have been published previously (Jansen and Freytag, 1973).

Histological and Ultrastructural Methods

The pancreata of all rabbits were removed immediately after sacrifice. The main portion of the pancreas was fixed in Bouin's solution. Paraffin-embedded sections were stained with hematoxylin and eosin, period acid Schiff (PAS), phosphotungstic acid hematoxylin (PTHA) and Gomori's aldehyde fuchsin method. Portions of heart, lung, liver, kidney, spleen and lymph nodes were fixed in formol solution (10%). Sections were stained with hematoxylin and eosin.

Small pieces of pancreatic tissue were immersed in cold 3% glutaraldehyde buffered with 0.1 M sodium cacodylate (pH 7.4) for 2 hours. Following brief rinsing in cacodylate buffer the cubes were postfixed in 1% cacodylate buffered osmium tetroxide and after passing propylenoxide embedded in Epon 812. Ultrathin 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

Blood Glucose Levels

The fasting blood glucose levels of the controls (group I) and the untreated animals ranged from 60 mg% to 125 mg%.

In group II the blood glucose values were normal for all animals within the first two weeks. At the third week one of the eleven animals became acutely hyperglycemic showing a fasting blood glucose of 180 mg%. Continuation of the diabetic blood glucose level (fasting 210 mg-%) at the fourth week and a pathological GTT confirmed the diagnosis of diabetes mellitus in this animal (Fig. 2). It died suddenly at the end of the fourth week. Histological examination of the pancreas revealed severe insulitis.—At the fourth week elevated blood glucose levels were found in two other animals of this group. In one animal the antibody titre of which later turned out to be one of the highest (3.8 ng/ml I-BC) in this

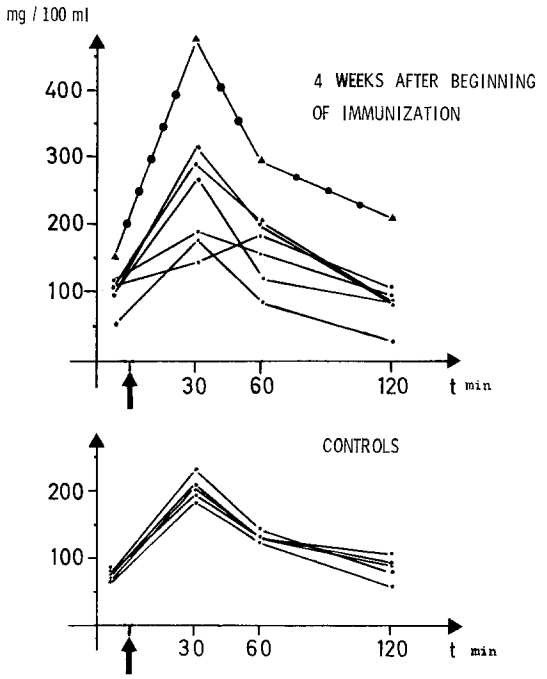

Fig. 2. Intravenous glucose tolerance test at the fourth week in rabbits immunized with insulin for a period of four weeks and in controls. Pathologic tolerance to glucose in one immunized animal, diagnostic of manifest diabetes mellitus

group, the fasting blood glucose was only slightly supranormal (140 mg%). Its GTT was within normal limits, and histological examination of the pancreas at the end of the study failed to demonstrate an insulitis. In the other animal severe hyperglycemia developed showing values between 270 mg% and 370 mg% fasting blood glucose. This rabbit originally belonged to group VI. However, since it died of diabetes mellitus already at the end of the fifth week, it was associated with group II. Its pancreas exhibited severe insulitis.—All other animals of this group with or without insulitis remained nomoglycemic.

In group III one animal showed slightly elevation of the fasting blood glucose (143 mg%) at the third week. However, at the fourth week the GTT and the blood glucose level were within normal limits. The I-BC, determined at sacrifice, was high (6.1 ng/ml I-BC), and the pancreas exhibited a moderately strong insulitis. All other animals of this group remained normoglycemic.

In groups IV, V and VI the fasting blood glucose values as well as the GTTs of all animals were normal throughout the whole period of immunization.

Insulin-Binding Capacity (I-BC)

In the controls and untreated animals no antibodies to insulin could be demonstrated.

In group II the antibody titres varied little (for single values see Fig. 3). They were in general below those observed in earlier studies (Klöppel *et al.*, 1972;

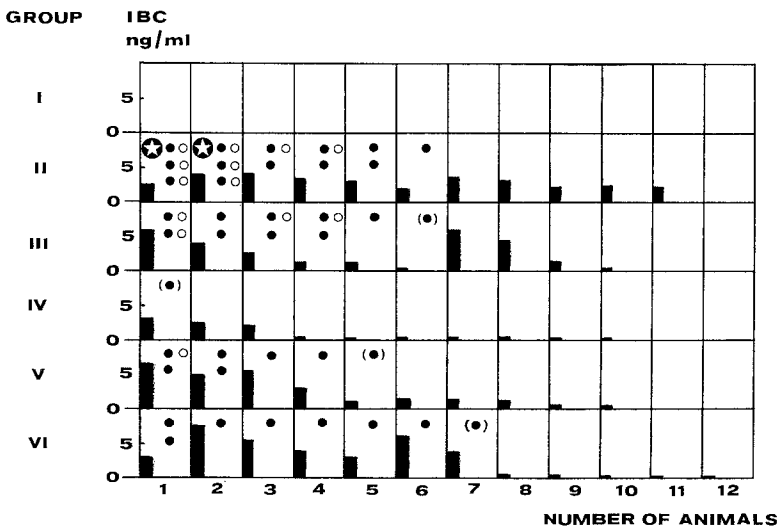

Fig. 3. Survey of results in the single animals. ■ Terminal I-BC (ng/ml). Degree of insulitis: (●) 5–10 lymphoid cells around single islets. ● 10–20, ●● 20–40 and ●●● > 40 lympoid cells around and within almost every islet. Degress of B cell degranulation: ○○○—80%, ○○—50%, ○—20%. ✪ Manifest diabetes mellitus (pathologic GTT and diabetic fasting blood glucose values)

corrigendum notice: I-BC μg/ml should be I-BC ng/ml [pp. 3 and 4, tables 1 and 2]). A possible explanation for this may be the use of new cages which provided better protection against infection and thus prevented a general unspecific stimulation of the immune apparatus by bacterial or viral agents. The I-BC of the normoglycemic animals did not correlate in height with the presence and the severitiy of insulitis. The diabetic animals with severe insulitis showed moderately strong I-BC values. One of the highest I-BC (3.8 ng/ml), as mentioned above, was determined in the animal showing no insulitis but a slight terminal hyperglycemia.

In group III the degree of I-BC was not related to insulitis (Fig. 3). As already mentioned, the animals with the highest I-BC (6.1 ng/ml) showed a slight transient hyperglycemia.

In groups IV, V and VI the I-BC differed strongly in the single animals (Fig. 3). A high I-BC was generally correlated with a chronic insulitis, whereas a low I-BC was not.

Light Microscopy

In the controls the pancreatic tissue was normal. In the extrapancreatic tissues granulomatous infiltrates were occasionally observed (liver, lung). Similar changes were also found in treated animals.

In groups II and III insulitis was observed in 6 out of 11, respectively in 6 out of 10 animals. The pattern of insulitis did not differ in this two groups. Insulitis was said to be present when more than 5 to 10 infiltrate cells were noted around and within several islets. The severity and the frequency of insu-

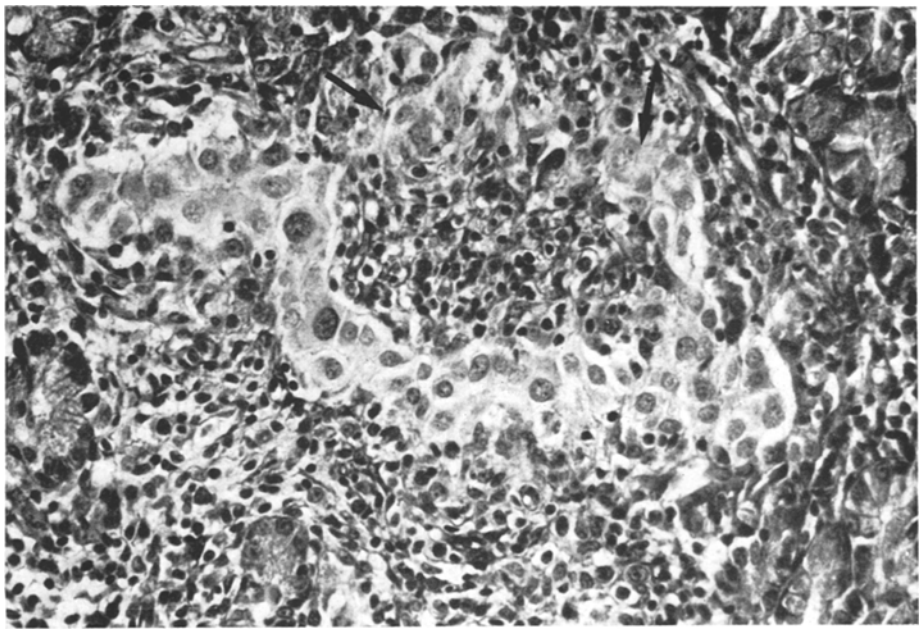

Fig. 4. Islet of a diabetic rabbit immunized with insulin (group II). Diabetes mellitus acutely developed at the third week. The islet shows severe insulitis. Numerous lymphoid cells surround and invade the islet. Destruction of separated islet cell complexes (arrow). Cytoplasmic degeneration of remaining islet cells. PAS. × 500

litis were further graded as worked out on Fig. 3. The pattern of insulitis observed in the non-diabetic animals paralled that described in detail elsewhere (Klöppel *et al.*, 1972; Freytag and Klöppel, 1973). The most severe insulitis (+++) was observed in the two diabetic animals. All islets were surrounded and invaded by masses of mononuclear cells destroying the normal islet architecture (Fig. 4). The B cells appeared to be reduced in number. There were no cytological signs of hyperactivity. The A cells were neither reduced in number nor cytologically altered. — As regards the relationship of insulitis with the aldehyde fuchsin staining of the B cells, indicating the granulation, it was found that the extent of the insulitis correlates with B cell degranulation (Fig. 3). The most severe insulitis (+++) in the diabetic animals was associated with degranulation of almost all B cells (○○○). Modest insulitis, on the contrary, generally lacked B cell degranulation.

In the groups IV, V and VI insulitis was observed in 1 out of 10, in 5 out of 10 and 7 out of 12 animals (Fig. 3). Insulitis showed a slightly different pattern in group V and IV. In group V the islet infiltrations resembled that observed after short-acting immunization. However, the extent of islet infiltration and islet destruction was smaller than in group II and III. A slight B cell degranulation in one animal was associated with a moderately strong insulitis (Fig. 3). In group VI insulitis showed the characteristics of chronic inflammation. The number of infiltrate cells was, in general, strongly reduced. The islet tissue

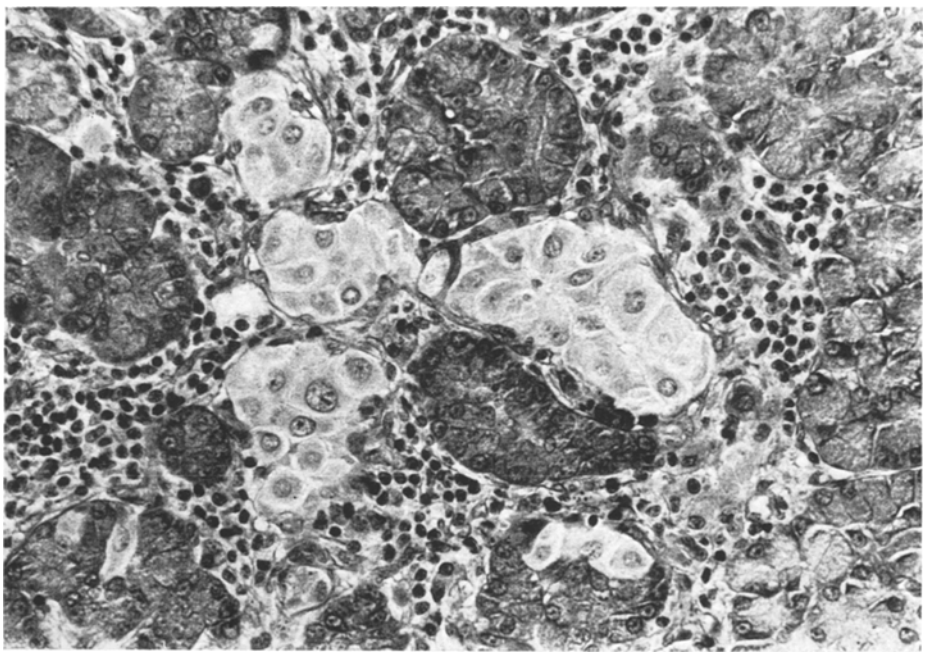

Fig. 5. Islet of a rabbit immunized with insulin over 16 weeks (group VI). The islet shows chronic insulitis. Islet tissue is split up and surrounded by small layers of fibrous tissue and by lymphocytic infiltrates. Hypertrophy of single B cells. PAS. × 500

was split in small clusters and surrounded by fine collagen bundles, suggesting that a marked process of destruction due to insulitis might have occurred at earlier times (Fig. 5). Close contacts of lymphoid cells with islet cells were rarely observed. The B cells of islets involved in chronic insulitis were well granulated and often appeared to be hyperactive. The A cells were normal.

Electron Microscopy

In the controls and in all rabbits without insulitis, no significant changes were found in the ultrastructural appearance of the islets and their cells.

In groups II and III the fine structures of the islets, the islet cells and the infiltrate cells of the insulitis animals without diabetes were similar to those already observed (Klöppel *et al.*, 1972), and will only be described in brief. The infiltrate consisted of lymphocytes and lympho-(immuno-)blasts. In addition, macrophages and rarely plasmacells were seen. These immune cells were often found closely attached to B cells, sometimes inserting pseudopod-like structures into the B cells. In moderately strong insulitis single B cells showed shrinkage of the nucleus, condensed chromatin structures and cystic dilatation of the rough endoplasmic reticulum (RER). Furthermore, these pancreata exhibited hyperactive B cells with prominent RER, frequently forming fingerprint-like structures, and with large Golgi complexes. A and D cells appeared to be unaltered.

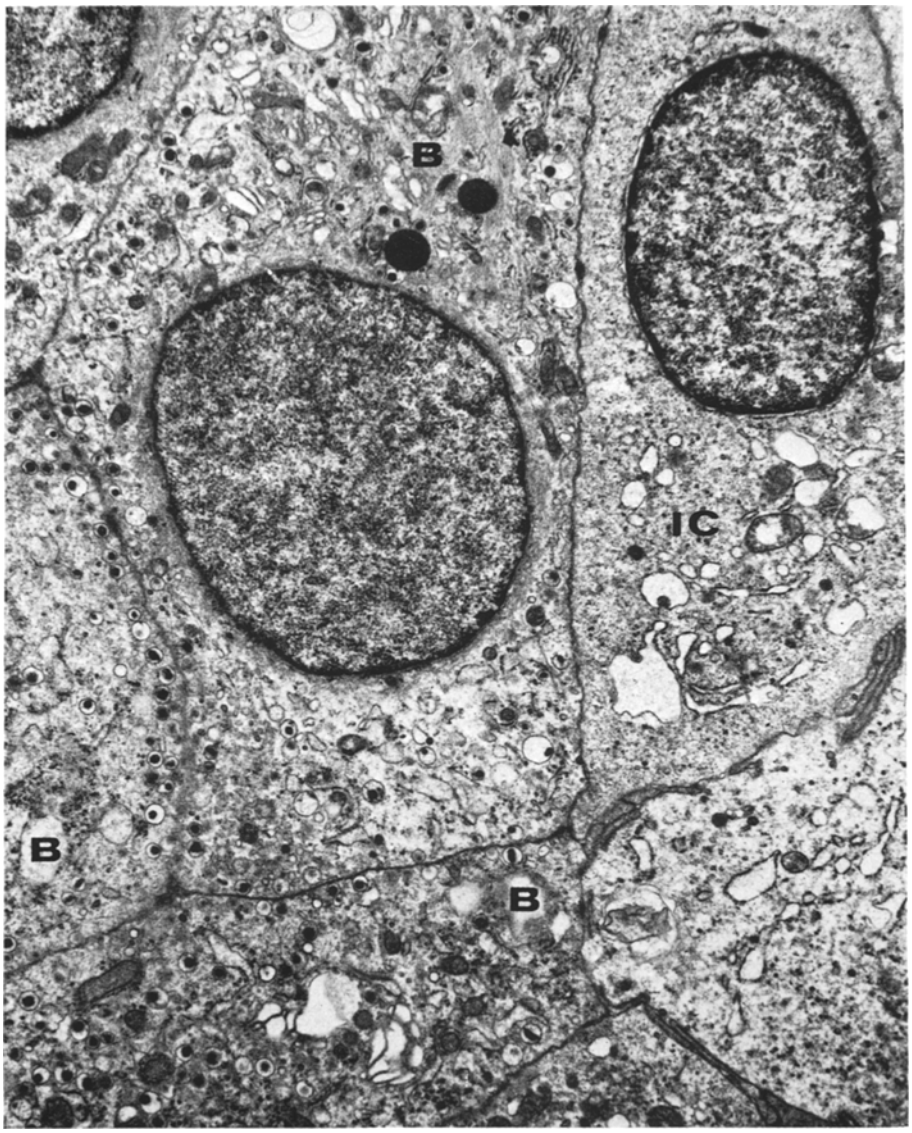

Fig. 6. Survey electron micrograph of islet cells from the rabbit with severe insulitis developing diabetes mellitus at the third week (group II). Hypogranulated B cells (*B*) in addition to completely degranulated islet cells (*IC*). × 7040

In the diabetic animal[1] small groups of B cells were tightly surrounded by lymphocytes and lymphoblasts. As a rule, all B cells were enlarged and hypo-

1 Preservation of the pancreatic tissue for electron microscopical examination was poor in the first diabetic rabbit because of its sudden unexpected death at the end of the fourth week shortly before the planed sacrifice. No ultrastructural examination could be carried out in the second animal since it also died suddenly at the end of the fifth week.

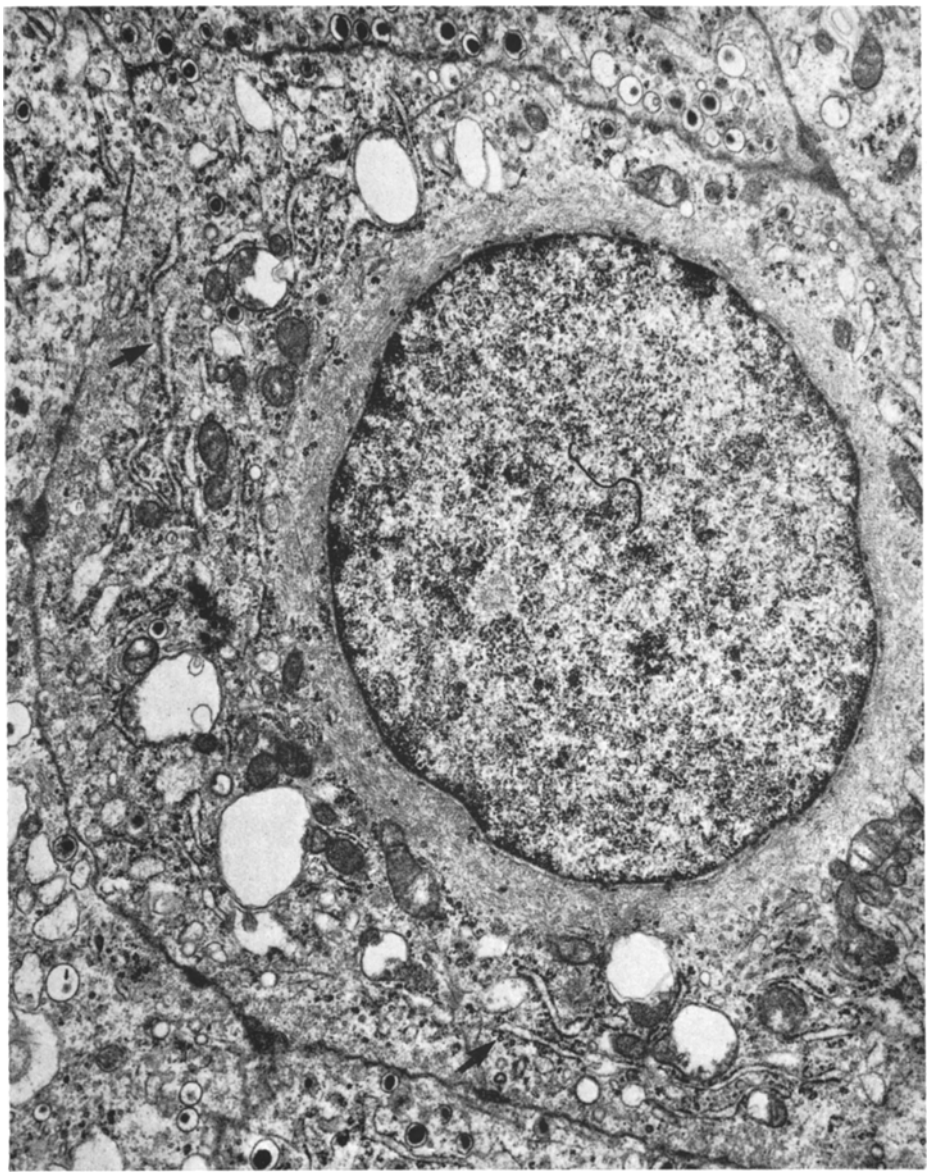

Fig. 7. Hypogranulated B cell of the diabetic rabbit. Note the poorly developed endoplasmic structures (arrow). × 12298

granulated (Fig. 6). Transitions to islet cells devoid of secretory granules were observed. The hypogranulated B cells contained only few organelles and a poorly developed RER (Fig. 7). The membranous sacs of the RER were short and dilated. They often contained varying amounts of diffuse electron dense material (Fig. 8). Glycogen particles were not observed with certainty. The Golgi com-

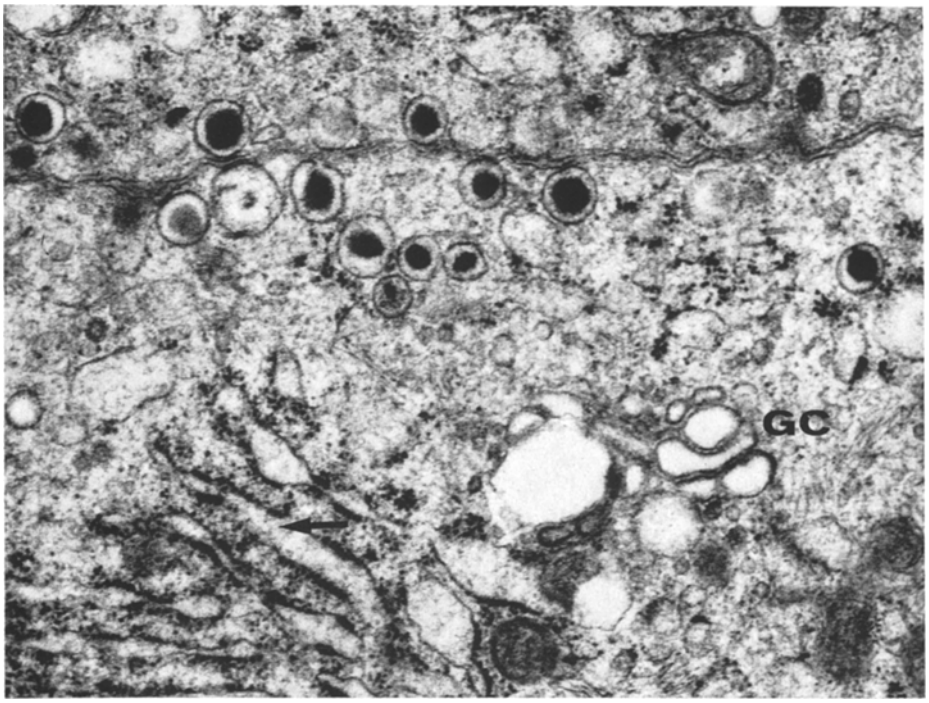

Fig. 8. Parts of two B cells from the diabetic rabbit: dilated endoplasmic sacs containing electron dense material (arrow). Small Golgi complex (*GC*) surrounded by single microvesicles. Typical beta granules at the cell surface. × 33442

plexes were small and reduced in number (Fig. 6 and 8). The few secretory granules in each B cell were typical in there structure. They were mostly lined up at the periphery of the cells. The plasma membranes were undulated and markedly interdigitated. No lysis of cell membranes was found at the contact areas between B cells and lymphoid cells. The perinuclear collar of the B cells was small. A and D cells remained unchanged.

In insulitis animals of group V the same ultrastructural changes of the islets could be observed as in non-diabetic animals of groups II and III, but to a lesser extent.

In insulitis animals of group IV and VI the islet cell complexes were surrounded by lymphocytes, lymphoblasts and macrophages (Fig. 9). The lymphoblastic cell type seemed to be reduced in favour of small lymphocytes and macrophages. Islet cells and mononuclear cells were often separated by fibroblasts and collagen fibres. Instead of close contacts between B cells and lymphocytic cells, often small intercellular spaces were observed between these cells. Sometimes cloudy electron dense material was present adjacent to macrophages. Possibly this material represents precipitated immune complexes (Klöppel *et al.*, 1971), since large amounts of precipitating antibodies to insulin could be demonstrated in some of these animals. The B cells in rabbits with marked chronic

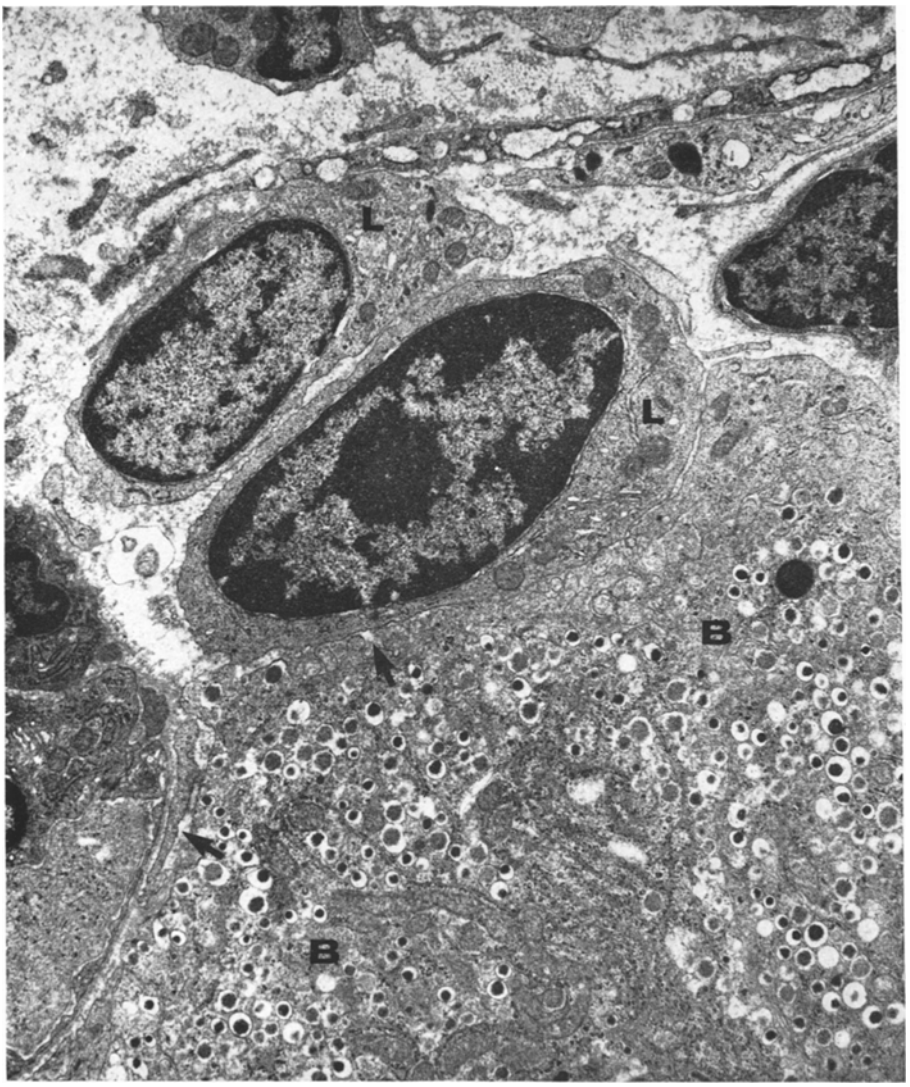

Fig. 9. Chronic insulitis in a rabbit without diabetes immunized for a period of 16 weeks (group VI): lymphocytes (*L*) adjacent to B cells (*B*), but separated by small intercellular spaces (arrows). × 7040

insulitis exhibited signs of hyperactivity, forming prominent RER structures and Golgi apparatuses. Degenerative lesions of the B cells were lacking. The A and D cells remained unchanged.

Discussion

Insulitis is thought to be diagnostic of diabetes mellitus in man (Gepts, 1965; LeCompte and Legg, 1972). Similar islet lesions can be produced in animals infected with certain viruses (Craigehead and Steinke, 1971; Müntefering *et al.*, 1971;

Münterfering, 1972), injected with anti-insulin serum (Lacy and Wright, 1965; Logothetopoulos and Bell, 1966; Klöppel *et al.*, 1971; Freytag, 1972) or immunized with insulin (Renold *et al.*, 1964; Toreson *et al.*, 1964; Federlin *et al.*, 1968). While a transient diabetes mellitus—like syndrome or permanent diabetes mellitus, resulting from the biological inactivation of insulin (Armin *et al.*, 1961), respectively from the destruction of islet cells (Craigehead and Steinke, 1971; Müntefering, 1972) are frequent in the two former models of insulitis, occurrence of diabetes mellitus in animals immunized with insulin is a rare event. Manifest diabetes mellitus was only observed with certainty in two rabbits of Grodsky's study (Grodsky *et al.*, 1966). Other species, though having severe insulitis following immunization with insulin, did not show decreased tolerance to glucose (Renold *et al.*, 1966; LeCompte *et al.*, 1966; Renold *et al.*, 1969). From the ultrastructure and histology of experimental immune insulitis one would expect that immune insulitis is frequently associated with diabetes mellitus. The inflammatory infiltration of the islets and the close contacts of lymphoid cells with B cells lead to suppose autoaggressive mechanisms, which finally may result in B cell destruction and diabetes mellitus. It was therefore attempted to examine the unclear relation of experimentel immune insulitis to the occurrence of diabetes mellitus in rabbits immunized with bovine insulin in different ways.

The results following short-acting immunization with insulin suggested that insulitis, independent of the mode of immunization, obviously reached its maximum after four weeks. Long-acting immunization over sixteen weeks led to a decrease in frequency and extent of the islet infiltrates dependent on the mode of immunization. On the basis of these data one may conclude that immune insulitis does not represent a chronic progressive inflammation of the islets of true autoimmune nature. Rather, it appears to be a temporary immunity phenomenon, clearly related to the immunization procedure. This interpretation of the nature of immune insulitis is in accordance with the fact that a diabetic state never occurred within the late phase of the long-acting immunization, as would have been expected had the destruction of B cells by immune mechanisms proceded.

Manifest diabetes mellitus occurred in two animals after short-acting immunization with insulin. The diabetes mellitus was confirmed by repeated determinations of the fasting blood glucose and the performance of a GTT. In both rabbits the insulin antibody titres were not unusally high. The pancreata showed the most severe insulitis observed with marked destruction of the islets and almost complete degranulation of the B cells. Ultrastructurally many lymphocytes were found in close contact with B cells. These B cells were generally hypogranulated and showed a decreased endoplasmic reticulum with small Golgi complexes. Analogous changes could not be observed in animals with insulitis but without diabetes mellitus. The B cells of these normoglycemic animals were only slightly degranulated and showed well developed endoplasmic structures.

The findings in the diabetic animals suggested that a manifestation of diabetes mellitus was probably caused by destructive immune insulitis. In support of this hypothesis is also the fact that the diabetic disease started at the third, respectively fourth week of the study when insulitis was already at its maximum. In the two diabetic animals observed by Grodsky following immunization with insulin (Grodsky *et al.*, 1966) diabetes mellitus also started at the end of the

third week. At sacrifice twenty-one, respectively sixty-three weeks after immunization modest insulitis was found in one animal whereas the other lacked it. The discrepancy between Grodsky's and our study concerning the presence and extent of insulitis in the diabetic rabbits may be explained by the fact that immune insulitis slowly decreases and even disappeares in long term studies. It is most likely that an initially severe insulitis induced diabetes mellitus in Grodsky's rabbits but then gradually receded.

In addition to insulitis the antibody production to insulin might also be involved in the process leading to the diabetic state. It is known that insulin antibodies can bind endogenous insulin in man and rabbits (Grodsky, 1965). Transient biological neutralization of insulin seems to occur in single rabbits which develop high antibody titres (Klöppel *et al.*, 1972). It is therefore possible that, in addition to insulitis, antibodies to insulin may also play a role in the manifestation of diabetes mellitus following immunization with insulin.

In summary, the findings in rabbits after short—and long-acting immunization with insulin suggest that experimental immune insulitis shows no progressive self-perpetuation, as it is known from true autoimmunity, However, the occurrence of diabetes mellitus in association with severe insulitis, at least in single cases, indicates evidence of autoimmune mechanisms to B cells at insulitis climax. It remains unclear why experimental immune diabetes is only a rare event, although distinct immune insulitis frequently occurs in the immunized rabbits. The significance of the hypogranulated B cells that are without morphologic evidence of increased function and are only observed in the diabetic rabbits is also unknown. Hereditary defects of the B cells or viral infections might therefore be discussed as additional manifesting factors of immune diabetes mellitus. However, there is no convincing evidence yet to support these factors.

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Dr. G. Klöppel
Pathologisches Institut
der Universität
D-2000 Hamburg 20
Martinistr. 52
Federal Republic of Germany