The Histopathology of Immune Diabetes in the Rabbit

Wilfred E. Toreson, M.D., Ph.D., John C. Lee, M.D.,
and Gerold M. Grodsky, Ph.D.

NEW ZEALAND WHITE RABBITS immunized with bovine insulin develop humoral antibody that binds and inactivates both exogenous and endogenous insulin.[1] Previously we reported the occurrence of diabetes in 2 such rabbits, with glycogen infiltration of the B cells in the islets of Langerhans and pancreatic ductular epithelium, and extensive mononuclear inflammatory infiltration of the hydropic islets.[2,3] Early vascular and cellular components of the inflammatory reaction were present 1 week after a single inoculation of insulin in Freund's complete adjuvant. Extensive cellular infiltration may affect all the islets 1 week following a second inoculation.[4] Because humoral antibody is not detected by highly sensitive radioimmunoassay until 1 week after the second inoculation, these observations are especially interesting. The diabetes exhibited by actively immunized rabbits differs pathogenetically and morphologically from transient diabetic states evoked by passive transfer of heterologous antibody to mice, rats, and rabbits.[5-9] We here extend these studies to demonstrate, by light and electron microscopy, the type and incidence of lesions of the islets of Langerhans in insulin-immunized rabbits, several of which manifested either transient hyperglycemia or overt diabetes.

Materials and Methods

A total of 25 white New Zealand rabbits were given injections of recrystallized acidified bovine insulin solution in Freund's adjuvant 1, 2, or 3 times at 1-week intervals. First we injected the toepads of the forefeet with the insulin in complete adjuvant. The second and third doses, in incomplete adjuvant, were usually into the toepads also, but in a few instances were subcutaneous into the loose tissues of the neck. Details have been published elsewhere.[1] No insulin was subsequently given to any animal.

Tissue for light and electron microscopic studies of the islets of Langerhans was provided by 15 control rabbits. Six of the 15 were inoculated according to the above schedule with Freund's complete and incomplete adjuvant without insulin in the same manner as were the insulin-immunized rabbits. These "adjuvant controls" were sacrificed 7 weeks after the first inoculation.

From the Departments of Pathology and of Biochemistry, and the Metabolic Research Unit, University of California School of Medicine, San Francisco Medical Center, San Francisco, Calif.

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Address for reprint requests: Dr. Toreson, Department of Pathology, State University of New York Medical School, The Downstate Medical Center, Brooklyn, N. Y. 11203.

Pancreases of the immunized rabbits were examined at biopsy or necropsy 1, 2, 4, 6, 7, 20, and 62 weeks after the first inoculation (Table 1). Surgical biopsy was performed following the administration of intravenous pentobarbital anesthesia. In each animal, approximately 0.5 gm. of pancreas was obtained from the splenic portion.

Part of the excised pancreatic tissue was subdivided quickly into pieces measuring 1 cu. mm. and immersed either in 1% osmium tetroxide (OsO_4) for 90 min.,[10] or in cold, distilled 3% glutaraldehyde (buffered to pH 7.4 with 0.1 M sodium cacodylate[11]) for 12 hr. followed by a 90-min. postfixation in 1% OsO_4.[12] The fixed tissues were dehydrated in ethyl alcohol and embedded in epoxy resin.[13] Thin sections were double-stained with uranyl acetate and lead hydroxide or lead citrate.[14] Electron micrographs were then made with an RCA-EMU2B or a Siemens Elmiskop I (80 kv) with a double condenser and 35-μ objective aperture. Thick sections cut from epoxy-embedded material were studied by phase microscopy after staining with a modified Goodpasture stain.[15]

Additional blocks were fixed either in 20% formol-Zenker or in 10% neutral buffered formalin and embedded in paraffin. Sections 2–4 μ thick were stained with hemalum-phloxine-saffron (HPS), chromalum-hematoxylin-phloxine (CAHP), aldehyde-fuchsin (AF), hematoxylin and eosin (H & E), and periodic acid-Schiff reagent (PAS).

Blood glucose levels in all animals were measured frequently by photometric microdetermination with glucose oxidase.[16] Daily determinations were performed on blood from rabbits of experimental Groups A and B (Table 1). For the other groups, we made weekly or biweekly determinations.

Table 1. Schedule of Examination of Pancreatic Tissue from Insulin-Immunized Rabbits

Group	Rabbits (No.)	Inoculations (No.)	Anti-insulin antibody (titer)*	Range of blood glucose levels (mg./100 ml.)	Interval between 1st inoc. & tissue exam. (wk.)
A	5	1	0	60–100	1
B	3	2	14	60–100	2
C	7	3	26	60–100	6, 20
D	4	3	39	100–174	7
E	6	3	119	133–600	4, 7, 20, 62

* Dilution of serum that binds half of 0.1 μg. (2.5 mU.) of bovine ^{131}I-insulin per milliliter. The highest titer observed in the rabbits of each group is given.

Anti-insulin antibody titrations were performed simultaneously with glucose determinations (Table 1); on several occasions we measured plasma insulin. Elsewhere, Grodsky[1] described the method used to separate antibody by salt precipitation followed by extraction and differential immunoassay of bound exogenous or endogenous insulin. Feldman *et al.*[17] have published details of the method for titration of antibody by hydrodynamic flow chromatography. For most studies utilizing this method, the titer is the serum dilution that binds half of 0.1 μg. of bovine ^{131}I-insulin. In Groups A and B, we achieved a more sensitive antibody-titering system by using 0.01 μg. (250 μU.) of insulin; in these assays, antibody could be detected even at concentrations capable of binding only 25 μg. of insulin per milliliter.[17]

Results

Normal and Adjuvant Control Groups

The pancreases of rabbits given adjuvant injections revealed no histologic abnormalities. By light and electron microscopy, the islets were

indistinguishable from those of rabbits that received no injections. The B cells showed comparable cytoplasmic granularity in CAHP- and AF-stained sections. Thick sections of epoxy-embedded, Goodpasture-stained pancreas examined by phase microscopy displayed normal granularity in both B and A cells. Electron microscopic examination of thin sections demonstrated no ultrastructural differences. For the evaluation of ultrastructural differences, glutaraldehyde-fixed tissues were remarkably superior to tissues directly immersed in OsO_4.

Immunized Groups

Group A. During the week following a single inoculation of insulin in complete adjuvant, blood sugar levels of these 5 rabbits remained normal (60–100 mg./100 ml.). Serums collected on the seventh day contained no antibody. Light microscopy revealed 1 or more dilated capillaries containing mononuclear cells in many islets of Langerhans. The mononuclear cells were predominantly lymphocytes; polymorphonuclear heterophilic and eosinophilic leukocytes were rarely evident. The leukocytes were seen exclusively within the lumens of islet capillaries (Fig. 1). CAHP- and AF-stained sections had normal cytoplasmic granularity. Pancreatic exocrine cells and ductular epithelium were normal. In 2 pancreases, there were discrete perivascular foci and diffuse periductular and interacinar areas of histiocytic and lymphocytic infiltration (Fig. 2).

Group B. Daily blood sugar values in these 3 rabbits remained normal during the 2-week observation. Serum antibody was not present on the seventh day, but measurable titers appeared by the fourteenth day; the highest titer was 14. In the pancreas of the rabbit with this titer, every islet was infiltrated with mononuclear cells. Infiltration was limited to the islets; it sometimes appeared to involve exocrine parenchyma and interstitial tissues, at which times step-serial sections usually revealed an inflamed islet immediately nearby. Intracapillary agglomerates of leukocytes tended to be inconspicuous amid the extraordinary extravascular cellular infiltration representing the predominant change in every islet (Fig. 3). The infiltrating cells were both small and large mononuclear forms, resembling lymphocytes, histiocytes, and macrophages; a few typical plasma cells were identified. Cytoplasmic granules of B cells were much reduced, according to CAHP and AF stains and by phase microscopy of Goodpasture-stained, thick epoxy sections. Changes in the tissues of the other 2 rabbits in every way resembled those in Group A (Fig. 4 and 5).

Group C. Seven rabbits, each given 3 immunizing injections of insulin-adjuvant, showed blood glucose levels no higher than 100 mg./100 ml. during 6 or 20 weeks of observation. All evidenced good antibody

response: the highest titer observed was 26. Generally the antibody titer had fallen emphatically by the seventh week. After biopsy of the pancreas on the sixth week, the blood glucose level of 1 rabbit rose to 335 mg./100 ml. Biopsy material from 2 rabbits was studied by light microscopy, from 2 others by electron microscopy. Intracapillary leukocyte aggregates and peri-insular infiltrations were observed at both 6 and 20 weeks (Fig. 6). Cytoplasmic granularity of the B cells was sparse; none of the B cells was stained by the PAS method.

Group D. Two rabbits had blood sugar levels of 110 and 174 mg./100 ml., respectively, 1 week after the second inoculation. Levels to 174 mg./100 ml. were observed in the third, fourth, and fifth weeks. The other 2 rabbits first manifested hyperglycemia 1 week after the third inoculation; the highest level was 160 mg./100 ml. At surgical biopsy, all had blood glucose levels less than 100 mg./100 ml. in the sixth and seventh weeks of the study. The highest antibody titer noted was 39. The dilated islet capillaries of excised pancreatic tissue in 1 rabbit showed aggregates of leukocytes similar to such aggregates in the normoglycemic rabbits. In the pancreases of 2 rabbits, histiocytes, lymphocytes, and plasma cells infiltrated and surrounded the islets (Fig. 7). Cytoplasmic granules were sparse in the AF-stained B cells; no cells contained glycogen. The pancreas of the fourth rabbit had tiny islets containing more A than B cells.

Group E. Six rabbits became hyperglycemic 1 week after the second or third inoculation and remained so throughout the study. We examined the pancreatic tissues 4, 7, 20, and 62 weeks following the first immunizing inoculation. The higher blood glucose levels varied from 326 mg./100 ml. to 600 mg./100 ml.; the lowest level was 133. The highest antibody titer was 119. Although titers decreased on about the seventh week, in the 1 instance where prolonged measurements were made, the titer was demonstrable after 62 weeks.

Pancreatic tissue of the rabbit examined on the fourth week after 3 weeks of hyperglycemia ranging from 392 to 510 mg./100 ml., and an antibody titer of 119, showed no beta granules. There was no glycogen in B cells or ductular epithelium. Inflammation characterized by edema, cellular exudation, and mononuclear cellular infiltration involved the islets and adjacent periductal, perivascular, and interacinar stroma. The islets were small, sometimes multilobular, and contained many A cells and few B cells.

One rabbit, examined on the seventh week, had manifested only mild hyperglycemia (152 mg./100 ml.) during the third to sixth week. In the seventh week, values of 350 and 386 mg./100 ml. were observed. The antibody titer of this rabbit was 45 in the fifth week. The other rabbit ex-

amined on the seventh week did not become hyperglycemic until 1 week after the third inoculation, when the blood glucose level rose to 326 mg./100 ml. Thereafter the level fell; it was 133 mg./100 ml. in the seventh week. This rabbit had an antibody titer of 36 in the fifth week. Pancreatic tissues of both rabbits showed heavy infiltrates of mononuclear cells in and around small islets displaying B cells with agranular or glycogen-containing cytoplasm (Fig. 8).

The remaining 2 rabbits have been described in detail in a previous publication.[3] These rabbits had glycogen infiltration of B cells and ductular epithelium, with mononuclear cellular infiltration of the islets. Cellular infiltration persisted for 62 weeks in 1; during the early period there was little circulating antibody-bound insulin. With time, antibody titer and blood glucose levels declined, while the amount of endogenous antibody-bound insulin in the serum increased. Cellular infiltration in the pancreas of the other diabetic rabbit was sparse, possibly reflecting the minute size of the residual islets and their predominantly A-cell composition.

Electron Microscopy. Electron micrographs of the islets of Langerhans in immunized animals with mononuclear cellular infiltration revealed B cells ultrastructurally similar to those of control animals receiving adjuvant or no injections. In glutaraldehyde-fixed tissues, cytoplasmic granules in B cells were reduced in number and were prominently arrayed adjacent to the cell membrane. The degree of granularity in B cells correlated well with that observed in AF-stained paraffin sections. In osmium-fixed tissues, the majority of these granules appeared as empty vesicles. Most cells contained increased numbers of single-membrane-limited dense bodies presumed to be lysosomes. Perinuclear fibrillar material frequently appeared increased.

The mononuclear cells consisted of lymphocytes and macrophages. Lymphocytes were large, with abundant cytoplasm containing many free ribosomes, a little rough-surfaced endoplasmic reticulum, and a few mitochondria. Most were aggregated about small blood vessels (Fig. 9). Some, however, were in close contact with B cells (Fig. 10 and 11). Macrophages contained the usual number of mitochondria, Golgi membranes, and rough-surfaced endoplasmic reticulum. The majority of macrophages had a number of large, single-membrane-limited inclusions containing numerous amorphous dense particles and myelin figures (Fig. 11).

Discussion

Active immunization with insulin evoked transient hyperglycemia or persistent diabetes in 10 of 25 rabbits. Some animals became diabetic

3 weeks after the first immunizing inoculation; others evidenced brief hyperglycemic episodes as late as the sixth week. Normally, surgical biopsy had little or no effect on the blood glucose levels of immunized or normal animals;[18] pancreatic tissue removed at operation equaled approximately 0.5 gm. in each instance—only 1/7 to 1/5 of the total pancreatic weight. One exceptional rabbit, still normoglycemic 6 weeks after immunization, did respond to biopsy with a rise in blood sugar; however, levels returned to normal within 2 weeks. Possibly, failure to increase the incidence of diabetes after surgical removal of part of the pancreas was due to insufficient amount of tissue removed or to regeneration.

Hyperglycemic and diabetic rabbits exhibited varying granularity of B-cell cytoplasm, and glycogen infiltration of B cells and ductular epithelium. These changes were similar to alterations encountered in diverse varieties of experimental diabetes. Glycogen-filled B cells were not detected histochemically unless hyperglycemia above a level of 250 mg./100 ml. had persisted at least 3 weeks. Such cells remained evident for many weeks thereafter.

In the rabbit examined at Week 62, sparsely granular B cells without glycogen were seen, but glycogen was still apparent in ductular epithelium. In the most severely diabetic rabbits, a reduced number of B cells was observed; only a few small islets composed of A cells and a few nongranular or glycogen-filled cells could be found in multiple sections of several tissue blocks. Loss of B cells may be attributed to the inflammatory response to immunization.

Lymphocytic and monocytic infiltration of the islets of Langerhans was the most remarkable finding in immunized rabbits. One week after a single inoculation, capillary dilatations containing aggregates of mononuclear cells became apparent. This change, somewhat resembling the phenomenon of margination, was also found after 2 or 3 inoculations and even after 20 weeks following immunization. The infiltrating leukocytes may have been transported to the islets by the blood. However, lymphocytes and macrophages also accumulated adjacent to blood vessels and ducts in the pancreatic stroma. Perhaps in-situ differentiation from foci of mesenchymal cell proliferation accounted for some of the infiltrative leukocytes.

We would like to emphasize that a very sensitive test failed to disclose anti-insulin antibody in the blood plasma at the time when capillary dilatation with intravascular mononuclear cellular aggregation first appeared and extravascular proliferations were already manifest without hyperglycemia. We cannot unequivocally exclude the presence of small amounts of circulating antibody, but it is probable that the initial effect

of immunization with insulin is related to beta-cell damage by immunologic cells infiltrating the islets. In an earlier publication, we presented other evidence that the tissue lesion may be a primary factor producing diabetes in immunized rabbits.[3]

Despite high antibody titers in diabetic rabbits, little endogenous insulin was bound to circulating antibody. This would indicate an impaired pancreatic function. In contrast, though antibody titers were lower, large amounts of endogenous insulin were present on the circulating antibody in nondiabetic immunized animals.

One week after a second inoculation, many lymphocytes and monocytes infiltrated all the islets in 1 of 3 immunized rabbits. A few plasma cells were present in the lesions, but polymorphonuclear leukocytes were rare. Nuclear pyknosis and karyolysis were also evident in the islet cells. Although antibody was detected in its blood plasma, this rabbit remained normoglycemic. The numbers of lymphocytes, macrophages, and plasma cells varied in lesions of diabetic rabbits examined at later intervals, but the cellular composition of the infiltrate remained qualitatively the same as when observed at 2 weeks.

Ultrastructural changes in the B cells of immunized rabbits included loss of specific granules, widened perinuclear collar of fibrillar material, and increased numbers of lysosomes—alterations perhaps resulting from damage to the insulin-producing cells. Macrophages containing large inclusions with myelin figures might indicate phagocytosis of damaged B cells. Abundant free ribosomes and small amounts of rough-surfaced endoplasmic reticulum in the lymphocytes are compatible with immunologic activity.[19]

Our morphologic observations indicate that inflammation of the islets of Langerhans is an important factor in the pathogenesis of immune diabetes. The extent and severity of B cell injury could directly affect insulin production and thereby influence the severity and duration of the metabolic disturbance. The slightly delayed development of the lesions, their sharp restriction to the islets, and the composition of the cellular infiltrate suggest that cellular antibody may be the prime etiologic element. Humoral antibody, appearing after the islet lesions have begun to develop, might further intensify the metabolic disturbance by limiting the availability of circulating insulin.

Intravenous and intraperitoneal injections of heterologous anti-insulin serums evoke acute transient diabetes in mice, rats, and rabbits but do not produce pancreatic lesions similar to those seen in our investigation. Moloney and Coval[5] and Kitagawa *et al.*[6] did not report histologic studies of diabetic mice, rats, and rabbits. Wright[7] stated that there are few

granular B cells in the pancreas of the diabetic rat and extraction yielded little insulin. Lacy and Wright [8] described interstitial pancreatitis in rats made diabetic by repeated injections of guinea pig anti-insulin serum. The resulting inflammation was not selective for the islets, which showed only degranulation of B cell cytoplasm. Logothetopoulos and Bell [9] gave each of the young, adult male mice they studied 2 intraperitoneal injections of a globulin fraction of anti-insulin serum every day for as long as 15 days, maintaining blood glucose levels above 250 mg./100 ml. Not only did the B cells lose cytoplasmic granules and accumulate glycogen, but monocytes and neutrophilic and eosinophilic polymorphonuclear leukocytes accumulated around the islets, while the exocrine interstitium remained free from inflammation. Lymphocytic infiltration, however, was not observed in any of these conditions.

Cellular infiltration of the islets of Langerhans evoked experimentally in cows by active immunization with homologous and heterologous insulin has also been observed by Renold and associates.[20,21] No instance of diabetes was noted. The islet lesion in immunized cows described by LeCompte *et al.*[22] resembles the inflammatory reaction in the islets of our rabbits.

Inflammation of the islets of Langerhans of juvenile acute-onset diabetic patients described by Gepts [23] is very similar morphologically to the reaction in our rabbits. Gepts found infiltrates consisting of lymphocytes, reticular cells, and a few polymorphonuclear leukocytes. Plasma cells were not seen. Some of his subjects had not received insulin, and none had been given insulin prior to a few hours or days before death.[24] Thus, an immune response at the tissue level may play a part in the etiology of some forms of human diabetes.

There is little evidence of circulating insulin autoantibody in diabetic patients not treated with insulin. Complement fixation tests [25-27] with serums of untreated diabetic and normal subjects have yielded widely differing results. Fluorescein-labeled gamma globulin prepared from the serum protein of one of 3 diabetic patients displayed fluorescence of B cells when exposed to sections of normal human pancreas.[28] A hemagglutination test, a test-tube precipitin procedure, and the Ouchterlony double-diffusion technique all failed to disclose isoantibodies to pancreatic tissue in diabetic patients.[29] Finally, no immunoglobulin capable of binding crystalline ^{131}I-insulin was found in untreated diabetic subjects.[30]

In insulin-treated chronically diabetic patients, binding and inactivation of exogenous and endogenous insulin by circulating antibody can contribute to insulin resistance.[17] That antibody evoked by insulin therapy

may modify the course of the diabetes in other ways and lead to definite variations in morphology of islet lesions remains speculative.

Summary

Lesions of the islets of Langerhans were consistently present 1 week after single immunizing inoculations of insulin in Freund's complete adjuvant were administered to New Zealand white rabbits. Anti-insulin antibody was not detectable in the serum. The lesions consisted of mononuclear leukocyte aggregates in the lumens of dilated capillaries.

One week later, after the second immunizing inoculations, there was extensive pericapillary infiltration by monocytes, lymphocytes, and plasma cells. The serums contained anti-insulin antibody, but blood glucose was normal.

The cellular infiltrates persisted in and around the islets for as long as 62 weeks, whether or not the immunized rabbit exhibited persistent normoglycemia, transient hyperglycemia, or sustained diabetes. Rabbits diabetic for several weeks evidenced loss of beta granules and/or glycogen infiltration of beta cells and ductular epithelium. Some had very small islets, with A cells outnumbering B cells.

Islet lesions in the diabetic rabbits immunized with insulin-adjuvant distinctly resemble the reported lesions of juvenile acute-onset diabetes in man and immune insulitis in cows. The lesions differ from those reported as resulting from passive immunization of mice, rats, and rabbits.

The pathogenesis of the lesions evoked in rabbits by active immunization with insulin-adjuvant may be contingent upon the participation of cellular antibody in an inflammatory reaction more-or-less destructive of insulin-producing beta cells of the islets of Langerhans.

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[*Illustrations follow*]

Legends for Figures

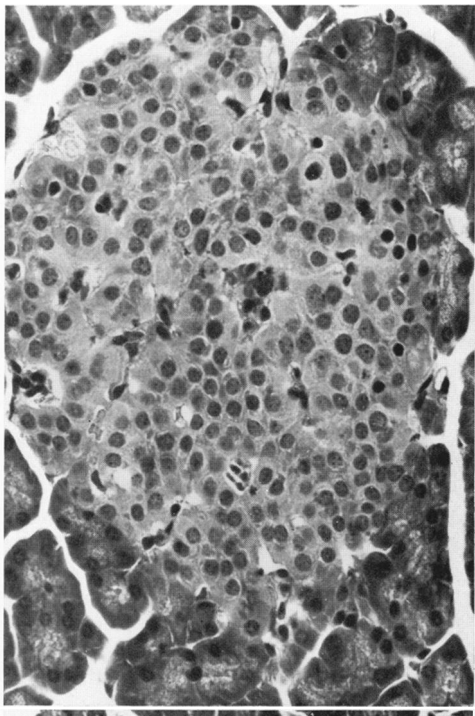
Figures 1 to 8 were prepared from paraffin sections stained with hematoxylin and eosin.

Fig. 1. Leukocytes aggregated in dilated islet capillaries of rabbit 1 week after a single inoculation with insulin-adjuvant. × 100.

Fig. 2. Diffuse mononuclear cellular infiltration of periductal and interacinar stroma 1 week after a single inoculation with insulin-adjuvant. × 100.

Fig. 3. Mononuclear cells surround an islet and divide it into small groups of cells 1 week after second inoculation with insulin-adjuvant. × 128.

Fig. 4. Leukocytes aggregated in dilated islet capillaries of rabbit 1 week after second inoculation with insulin-adjuvant. × 100.



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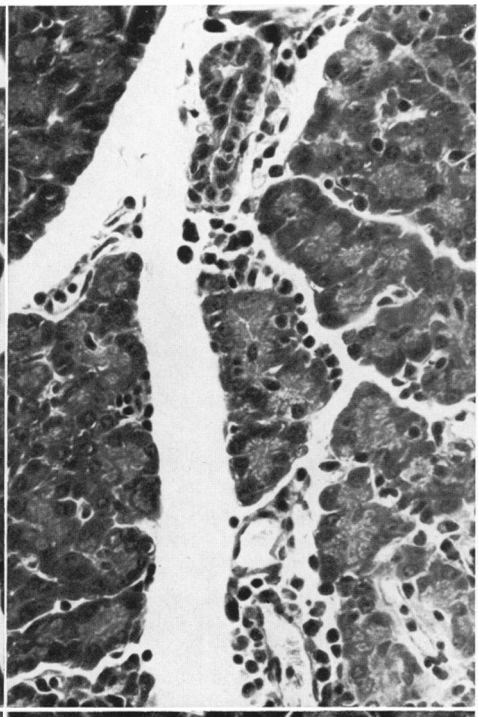

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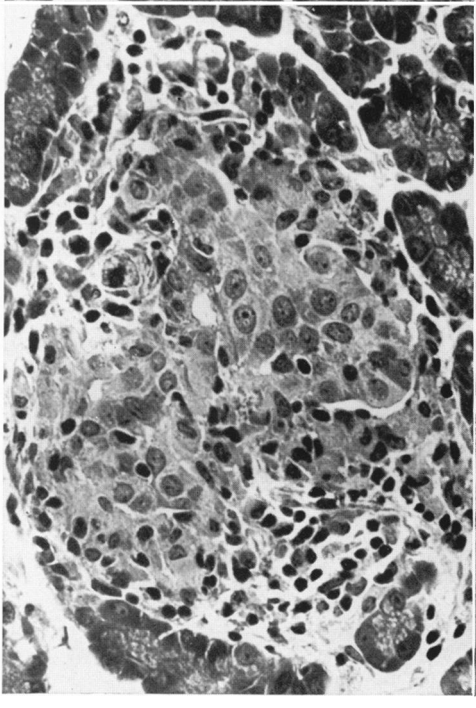

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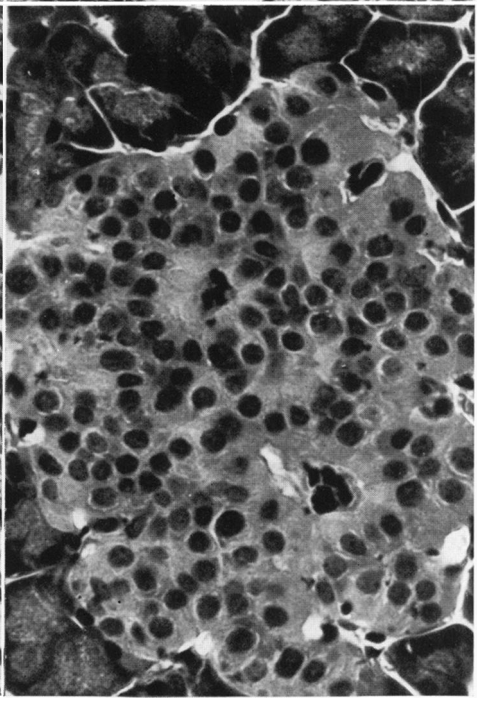

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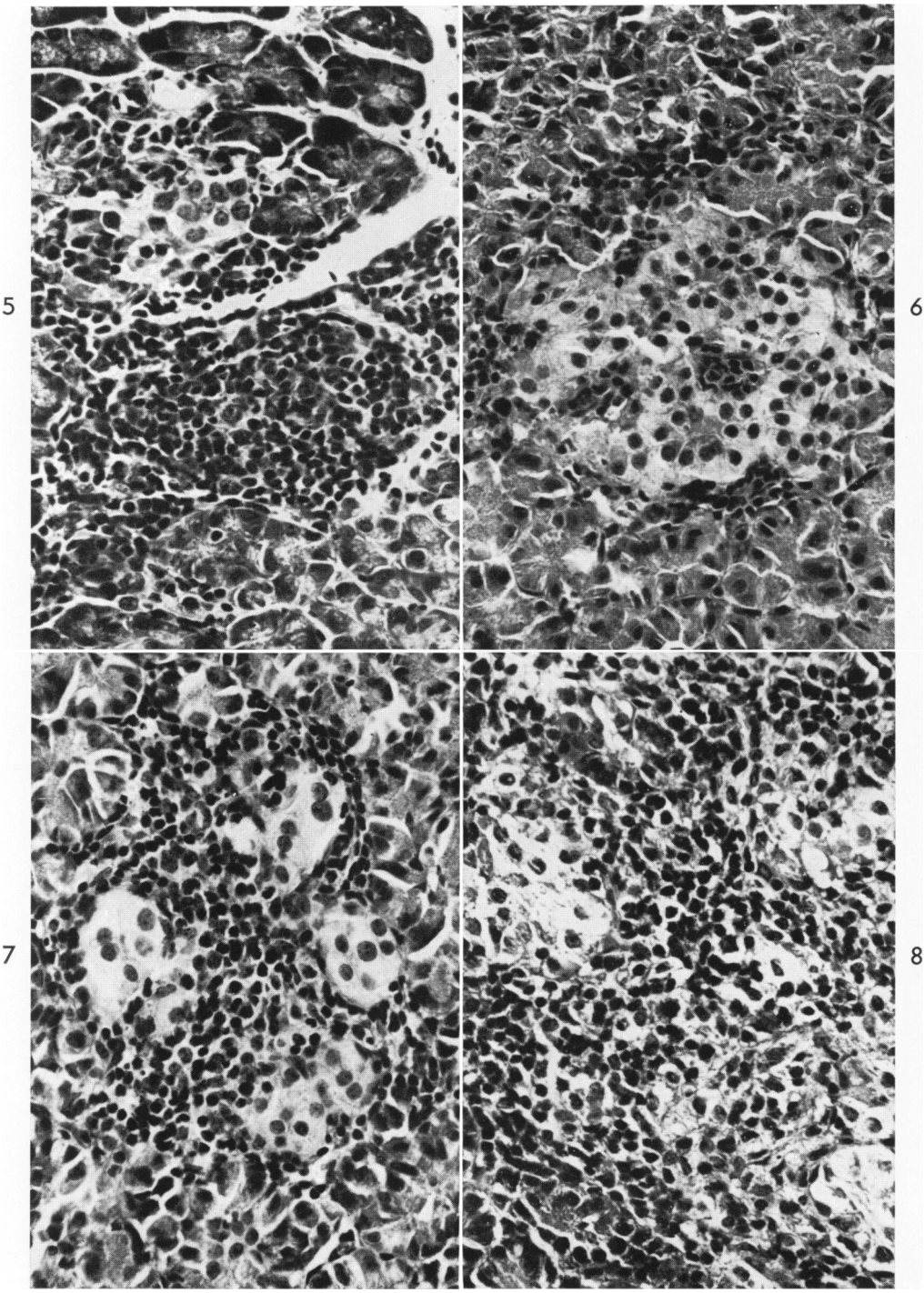
Fig. 5. Poorly delimited focus of mononuclear cells, apparently centered upon interacinar stroma; marginal areas surround and permeate clusters of islet cells (from same tissue section as Fig. 4). × 100.

Fig. 6. Dilatation of islet capillaries with intravascular aggregation and perivascular infiltration of mononuclear cells. Normoglycemic rabbit 4 weeks after third inoculation of insulin-adjuvant. × 100.

Fig. 7. Mononuclear infiltration subdividing groups of sparsely granular islet cells into small clusters. Rabbit had been hyperglycemic for 3 weeks and normoglycemic for 1 week. Biopsy performed 4 weeks after third inoculation with insulin-adjuvant. × 100.

Fig. 8. Extensive mononuclear cellular infiltration subdividing an islet into clusters of cells. Many islet cells contain glycogen; none contains beta granules. Rabbit had shown persistent moderate hyperglycemia for 5 weeks. Biopsy performed 5 weeks after third inoculation of insulin-adjuvant. × 100.



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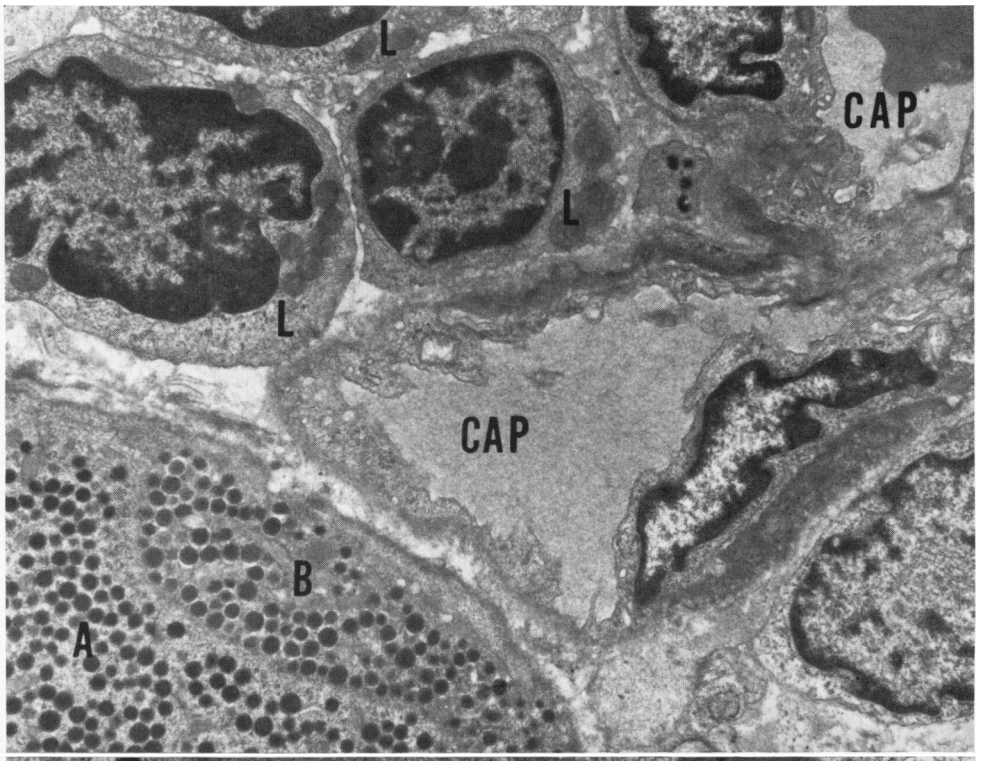

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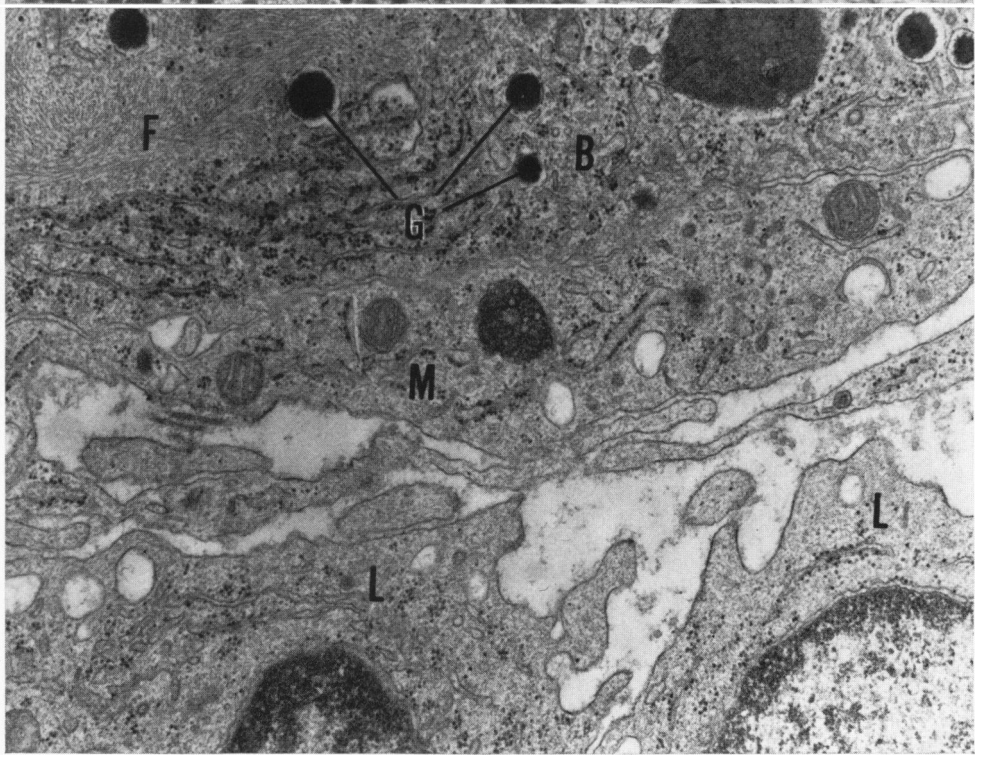

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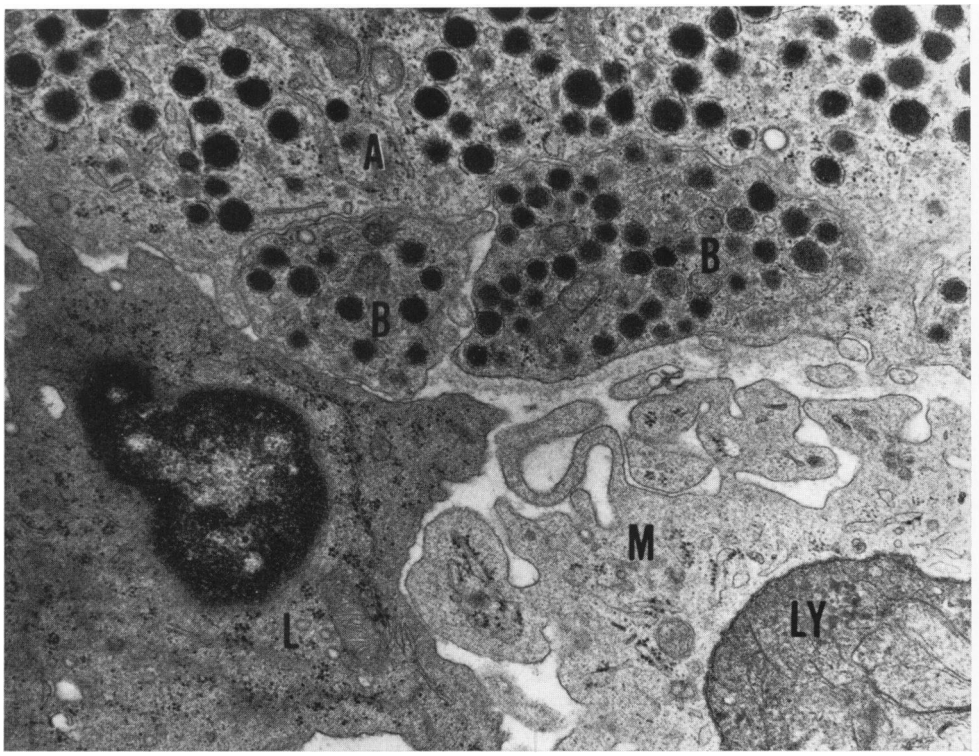

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Figures 9–11 are electron micrographs.

Key:

CAP	capillary	M	monocyte
A	alpha cell	L	lymphocyte
B	beta cell	Ly	lysosome
F	fibrillar material	G	beta granule

Fig. 9. Lymphocytic character of cellular infiltrate in immune diabetic animal, Group E. Lymphocytes are aggregated about capillaries of an islet. × 11,200. **Fig. 10.** Portion of B cell with sparse granules, large lysosome, and prominent fibrillar material. Lymphocytes and monocytes are in immediate vicinity. (Nondiabetic animal from Group B, same rabbit as in Fig. 3.) × 27,200. **Fig. 11.** Same animal as in Fig. 3 and 10, demonstrating close proximity of lymphocytes and monocytes to beta cells; portion of monocyte is shown with large inclusion containing myelin figures. × 25,000.