Experimental Immune Diabetes in the Rabbit

Light, Fluorescence, and Electron Microscopic Studies

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WE HAVE REPORTED PREVIOUSLY that immunization of New
Zealand white rabbits with bovine insulin and Freund's adjuvant pro-
duces humoral autoantibody which reacts with both exogenous (bovine)
and endogenous insulin. Some of these immunized rabbits develop hyper-
glycemia or diabetes mellitus, and the pancreas often exhibits a marked
lymphocytic infiltration of the islets of Langerhans.[1,2]

The relationship of circulating anti-insulin antibody and islet cell
lesions to the development of diabetes is not clear. This report deals
primarily with the comparative incidence and magnitude of these two
phenomena in autoimmune diabetes induced by several immunization
procedures, with particular reference to the importance of the lympho-
cyte in causing beta cell destruction.

Materials and Methods

Fifty-four female New Zealand white rabbits weighing approximately 2000 g
each were immunized by a variety of techniques and followed for periods up to 69
weeks. Initial immunization consisted of 1 mg bovine insulin in 0.1 ml 0.01N HCl
homogenized with an equal volume of complete Freund's adjuvant and injected into
the toe-pads in a divided dose. One week and 2 weeks later, the toe-pads were
again injected, but with 0.5 mg insulin homogenized in incomplete Freund's adju-
vant. Blood samples for determination of glucose and circulating antibody were
taken at 3- or 4-day intervals during the first 6 weeks of the experiment and at
weekly intervals thereafter. In some cases, rabbits were sensitized with psittacosis
agent (0.5 ml in 0.1% formalin) or paratyphoid vaccine (Lilly, 0.5 ml), adminis-
tered subcutaneously (first dose) and intraperitoneally (second dose). The agents
were administered either at weekly intervals before the immunization regimen or
on the day of insulin immunization.

In other studies, the animals were killed 1 week after the first, second, or third
standard immunization with insulin-adjuvant. Blood samples were collected 24 hr

before each immunization or sacrifice. Ten uninjected control animals were used for morphologic observations. Six additional control animals each received 3 weekly injections of Freund's adjuvant without insulin. Blood glucose measurements by the glucose oxidase method [3] and anti-insulin antibody were determined 24 hr prior to each injection. A description of the method for measurement of anti-insulin antibody by hydrodynamic flow chromatography has been published previously.[4] The titer is therein defined as that dilution of sera which binds half of 0.1 μg of bovine ^{131}I-insulin per ml.

At sacrifice, portions of pancreas were fixed in 10% Zenker-formol solution, embedded in paraffin, and stained with hematoxylin and eosin for light microscopy. Aldehyde-fuchsin staining identified beta cells.

Pancreata from 23 of the 54 experimental animals were studied by immunofluorescence. These 23 rabbits were sacrificed 1 week after one injection of insulin in Freund's adjuvant (5 animals), 1 week after two injections (5 animals), 1 week after three injections (4 animals), and 3 weeks after three injections (9 animals). All rabbits given two or more injections revealed antibody titers varying from barely detectable amounts to 19.5.

All tissue was quick-frozen in liquid nitrogen, and 6-μ sections were made with the cryostat. Sections were fixed briefly in cold acetone and stained with fluorescein isothiocyanate-conjugated goat antirabbit gamma globulin (Hoechst, Kansas City, Mo).[5] To increase sensitivity, a double-layer technique was used consisting of un-conjugated goat antirabbit gamma globulin followed by fluorescein isothiocyanate-conjugated guinea pig antigoat gamma globulin sera. The guinea pig antigoat gamma globulin sera—roughly quantitated by two-dimensional diffusion in agar [6]—produced two dense precipitin lines in 24 hr. In addition, sections of pancreas pre-treated with 3% human albumin to reduce nonspecific binding were exposed to fluorescein isothiocyanate-conjugated bovine insulin. The specificity of the goat antirabbit sera was tested by immunoelectrophoresis, revealing a single arc corresponding to the gamma globulin fraction. Known positive controls (rabbit spleen) were used with all antirabbit serum to insure its capacity to bind to globulin-containing cells. Conjugation of antisera and bovine insulin was accomplished by the method of Parker, Elevitch, and Grodsky.[7] The conjugated bovine insulin maintained its antigenic specificity to rabbit antibovine insulin antibody when measured by hydrodynamic flow chromatography.[4] In addition, rabbit antibody to bovine insulin proved to be in the gamma globulin fraction when measured by immunoelectrophoresis.

Pancreatic tissue for electron microscopy was fixed by rapid immersion in 3% distilled glutaraldehyde buffered to pH 7.4 in 0.1 M sodium cacodylate.[8] Fixation was continued overnight at 4°C, followed by 90 min postfixation in 1% osmium tetroxide [9] and then by ethanol dehydration and Araldite embedding.[10] Islets of Langerhans were localized in 0.6-μ sections after toluidine blue staining, and ultra-thin sections were double-stained with uranyl acetate and lead citrate.[11] Electron micrographs were made with a Siemen's Elmiskop IA with a 35-μ objective aperture.

Results

Table 1 summarizes the results obtained in 20 rabbits, blood glucose and circulating antibody levels of which were followed for periods up to 69 weeks after initial immunization. All animals were immunized 3 times at weekly intervals beginning on Day 1 of the initial week of the study. Twelve of the 20 animals in this group developed blood glucose levels over 150 mg/100 ml, which we consider hyperglycemic.

Table 1. Development of Antibodies and Hyperglycemia in 20 Rabbits Immunized with Insulin

Immunization	Maximum antibody titers	Time of maximum antibody (weeks)*	Maximum glucose mg/100 ml	Time of maximum glucose (weeks)*	Duration of glucose elevation (weeks) >120 mg%	>150 mg%	Time of sacrifice (weeks)*	Terminal blood glucose (mg/100 ml)
Adjuvant only (6 animals)	0	—	94–107	—	—	—	49	72–90
Insulin + adjuvant								
1	36	5	326	6	6	4.5	18	95
2	42	3	212	12	8	8	20	112
3	118	3	510	4	1.14	1.14	4	392
4	39	5	144	5	0.70	—	8	90
5	25	3	160	7	1.42	0.70	8	78
6	12·	2	117	5	—	—	6	93
7	17	5	424	5	35	35	62	175
8	7	3	292	3	2	2	10	102
Mean	37	3.6	273	4.6				
Psittacosis + insulin in adjuvant								
1	147	12	630	3	0.14	0.14	69	85
2	75	15	386	4	5	4	69	91
3	87	11	282	3	0.14	0.14	69	88
4	44	13	127	3	0.14	—	69	85
5	20	5	106	5	—	—	69	86
6	19	3	87	2	—	—	5	82
Mean	65	10	269	6.6				
Paratyphoid + insulin in adjuvant								
1	50	13	335	5	0.14	0.14	69	74
2	160	15	320	3	0.14	0.14	69	70
3	135	12	303	3	0.14	0.14	69	96
4	49	14	144	2	0.14	—	69	86
5	43	12	112	2	—	—	69	91
6	0	—	106	2	—	—	5	86
Mean	73	11	220	2.8				

* Times refer to weeks after initial immunization.

Rabbits immunized with adjuvant alone exhibited no elevation in blood glucose or detectable circulating antibody. Although as reported previously,[2] there was considerable antibody variation among animals immunized with insulin in adjuvant, titers characteristically reached peak levels at 1–2 weeks after the final immunization and declined slowly thereafter, but were always detectable in all animals regardless of the time of death. All but one of the rabbits in this first group displayed hyperglycemia, usually reaching a maximum at 1–2 weeks after peak antibody levels. Hyperglycemia was transient in most, falling below 120 mg/100 ml within 1–2 weeks. In three of the eight, however, hyperglycemia was prolonged for months; in one, diabetic blood glucose levels

were maintained until death more than a year later. As suggested in our initial small series of rabbits,[1] a decrease in hyperglycemia with prolonged time was characteristic of all animals.

Results of attempts to increase the incidence of diabetes by simultaneous or prior sensitization with psittacosis agent or paratyphoid vaccine are also summarized in Table 1. The data could be combined for the two agents, since no differences were noted between rabbits sensitized before and during immunization. In the animals treated with paratyphoid or psittacosis agents, maximum antibody tended to be higher than in those immunized with insulin alone, but required longer to develop. Maximum elevations in blood glucose still occurred within one week after final immunization even though antibodies reached their peak several weeks later. In all these animals hyperglycemia was extremely transient, usually lasting only a few days. Because of the greater incidence of prolonged hyperglycemia in rabbits immunized with only insulin plus adjuvant, this procedure was adopted for subsequent studies. In general, in the entire series, those animals with high blood glucose also demonstrated high circulating antibody.

The incidence of abnormalities in 28 additional rabbits killed after one, two, or three immunizations with insulin plus adjuvant is recorded in Text-fig 1. Initial blood glucose levels in the 28 rabbits of this series had an unusually broad range of 75–125 mg/100 ml. Lymphocytic infiltration had occurred in 4 of 10 animals by 1 week after a single immunization, although circulating antibody was not detectable at that time. Blood glucose levels were unchanged. After two immunizations, the incidence and intensity of lymphocytic infiltration increased to 4 of 8 animals and circulating antibody was detectable in all rabbits, even in those without pancreatic lesions, and was elevated in two. Blood glucose levels were still in the normal range. By the third immunization, 7 of 10 animals had lymphocytic infiltration of the islets and all had elevated circulating antibody (Text-fig 1). Hyperglycemia occurred in 2 rabbits in this series; the animals with blood glucose over 300 mg/100 ml had the highest circulating antibody and marked lymphocytic infiltration of the islets. Since all rabbits were killed before maximum antibody titers and blood glucose levels could be expected (see Table 1), the potential incidence of hyperglycemia was not determined.

Araldite sections prepared for light microscopy disclosed several important features of the islet lesions. The major component of infiltrating cells was the large lymphocyte with abundant homogeneous cytoplasm. Such lymphocytes were frequently observed in close contact with beta cells. Sometimes a single beta cell appeared to have several lymphocytes
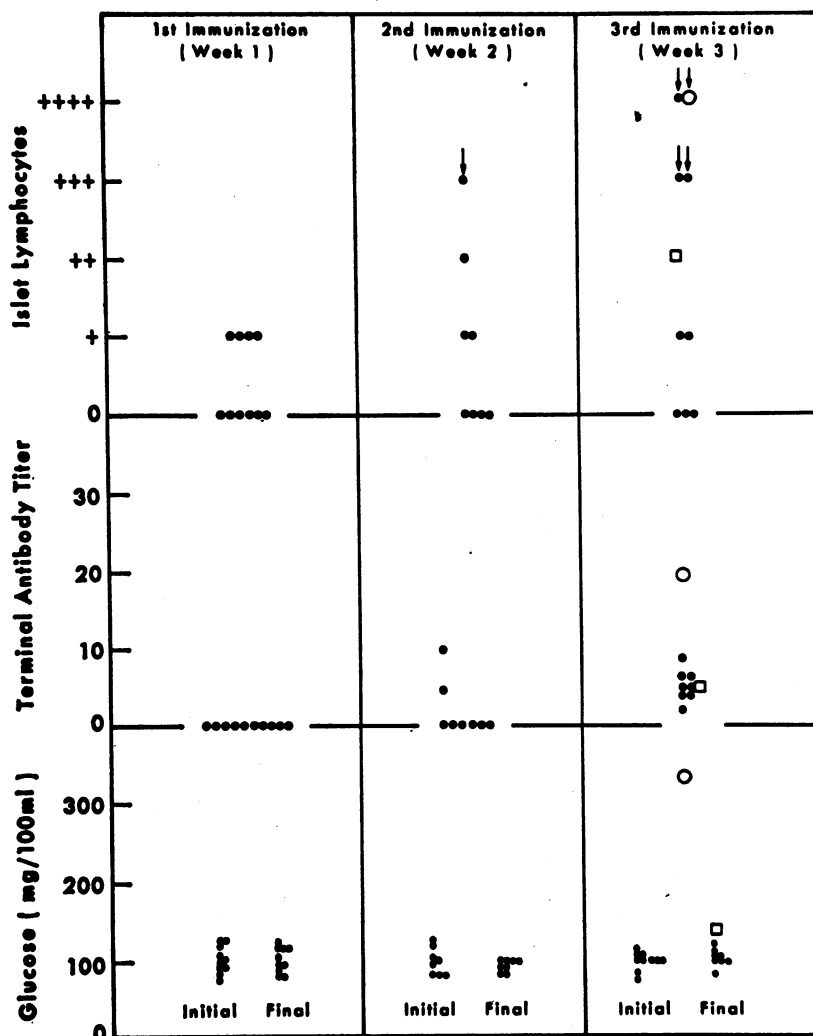
‡ = Detectable islet plasma cells.

O, □ = Animals with elevated blood glucose

TEXT-FIG 1. Effect on 28 rabbits of sequential immunizations with insulin.

attached to its peripheral membrane (Fig 7). Occasional heterophils were present. Plasma cells were observed in pancreata with + + + or + + + + lymphocytes, but not when the lesions were less well developed. Approximately one-third of the infiltrating cells consisted of large monocytes with many cytoplasmic granular incusions. None of

the lesions contained eosinophils. Lymphocytes were not seen in normal rabbits or in animals given adjuvant alone.

An exact quantitation of aldehyde-fuchsin staining of islet cells was not attempted, but staining was always reduced in animals with lymphocytic infiltration (Figs 1–6), reduction being the greatest in those with the largest amount of lymphocytic infiltration. In rabbits with antibody titer but no lymphocytic infiltration, aldehyde-fuchsin staining was identical to that in controls (Figs 1–6).

Electron Microscopy

Beta cells appeared in normal numbers in immunized animals with elevated titers but without lymphocytic infiltration of the islets. Beta granules were occasionally reduced in number, and the perinuclear fibrillar material was increased. The most constant variation from pancreata of control animals was an increase in cytoplasmic dense bodies with ultrastructural features of lysosomes (Fig 8). Within these structures, myelin figures and recognizable cytoplasmic organelles including secretion granules often were noted.

Islets of immunized animals with prominent lymphocytic infiltration always contained a reduced beta cell population, sometimes to a marked degree. The remaining beta cells usually appeared intact but with a reduction of secretion granules (Fig 9). Secretory activity was evident in viable cells with margination of granules along the plasma membrane. There was frequent close contact between infiltrating lymphocytes and beta cells. Lymphocytes often inserted pseudopods between islet cells, making close contact with two or more beta cells (Fig 10). At this contact point, many beta cells were observed to lose their cell membranes, releasing their cytoplasmic contents into the surrounding intercellular spaces (Fig 11).

Infiltrating lymphocytes were usually sizable, with large, sometimes indented nuclei containing abundant chromatin near the nuclear membrane. The cytoplasm of these cells always revealed abundant free ribosomes. Other organelles were always sparse. Large macrophages were often seen with large cytoplasmic phagosomes containing cellular debris. Plasma cells were easily verified by their abundant rough-surfaced endoplasmic reticulum and prominent Golgi apparatuses. These cells were usually localized about the periphery of the islet lesions and not in close contact with beta cells.

Alpha cells appeared in normal numbers in islets of all experimental animals regardless of lymphocytic infiltration and contained secretion

granules in expected amounts when grossly compared with the alpha cells of controls.

Fluorescence Microscopy

Frozen sections of normal rabbit spleen always displayed a brilliant yellow-green specific fluorescence of plasma cells when stained by either the single- or double-layer technique. The single-layer fluorescence was abolished easily by prior staining with unconjugated goat antirabbit globulin.

Islet cells from all experimental animals were consistently negative when exposed to fluorescein-conjugated antirabbit gamma globulin sera. Specific fluorescence never was observed in lymphocytes or in other pancreatic tissues. Plasma cells when present, however, were specifically fluorescent when stained with either the single- or double-layer technique (Fig 12). No specific fluorescence was noted in pancreatic tissue, lymphocytes, or plasma cells when sections were exposed to fluorescein-conjugated bovine insulin.

Discussion

The current studies amplify our previous observations that the immunization of rabbits with bovine insulin can induce an autoimmune hyperglycemia.[1,2] Hyperglycemia generally was transient and occurred shortly after completion of the 3-week immunization procedure. As previously noted, however, some animals remain diabetic even for periods beyond a year. Our earlier suggestion that these animals may have had an associated infection which exacerbated the reaction[1] now seems unlikely, since the phenomenon has proved reproducible (though unpredictable) under controlled conditions. Furthermore, treating animals with paratyphoid or psittacosis agents did not increase the incidence or intensity of the diabetes, although, as predicted from the studies of Wright,[12] antibody titers were elevated. Possibly the difference in sensitivity of our rabbits may be due to genetic variations among individuals.[13,14] Most animals improved with time, suggesting a functional regeneration reminiscent of that which can occur after administration of alloxan.[15]

The experimentally induced diabetes is characterized by the presence of circulating antibody which binds both exogenous (bovine) and endogenous insulin[1,2] and by prominent lymphocytic infiltration of the islets. It is obvious that circulating anti-insulin antibody may contribute to a diabetic state, and passive immunization with exogenous antibody can produce a temporary diabetes.[12,16,17] However, this temporary dia-

betes parallels the circulating level of antibody, disappears as circulating antibody is metabolized, and does not produce lymphocytic infiltration.

Previously,[1,2] we suggested that the islet lesion might be the primary factor in autoimmune diabetes induced by foreign insulin, since (1) diabetic animals had little or no endogenously secreted insulin in their circulation, although the abundant circulating antibody would normally carry excessive amounts; and (2) lymphocytic lesions were associated with marked destruction of beta cells, degranulation, and decreased pancreatic insulin. The present studies further support this concept, since (1) the advent of hyperglycemia did not correlate in time with the periods of maximum circulating antibody but occurred 1–2 weeks after the third immunization—a time when lymphocytic infiltration was highly developed; and (2) islet lesions could be seen after a single immunization, before circulating antibody was detectable. Finally, the fact that we were unable to demonstrate islet localization of rabbit antibovine insulin antibody by immunofluorescence of pancreatic tissue would indicate that antibody was probably bound in the peripheral circulation and played no active part in islet lesions.

Ultrastructural changes in beta cells of animals with anti-insulin antibody but no lymphocytic infiltration might be explained as due to increased insulin secretion.[17-19] Circulating antibody in the absence of lymphocytic infiltration did not appreciably decrease the amount of aldehyde-fuchsin-positive material in islets. The mechanism by which infiltrating lymphocytes may bring about beta cell destruction is unknown. We made frequent ultrastructural observations of the close approximation of lymphocyte and beta cell membranes with apparent beta cell lysis, but we saw no transfer of material between cells. The fine structure of the infiltrating lymphocytes with many free ribosomes suggests that this cell is specialized for protein synthesis. However, this type of structure implies that the synthesized product is to be utilized by the lymphocyte rather than exported to the environment. Lymphocytes sensitized to foreign cells or antigens, demonstrated to have a similar morphology, are capable of destroying target cells in tissue culture in the absence of humoral antibody.[20-25] The absence of anti-insulin antibodies in the lymphocytes of pancreatic tissue suggests such a mechanism in our insulin-immunized rabbits. Thoracic duct lymphocytes from immunologically stimulated animals also have similar morphologic characteristics.[20] It should be emphasized that the lymphocytes themselves may not be the primary event in the pancreas but rather a secondary reaction to an immune phenomenon.

Morphologically, monocytes and plasma cells in the islet lesions did not appear to participate in beta cell destruction. The monocyte frequently phagocytosed cellular debris but rarely made close contact with viable beta cells. Plasma cells generally appeared after the lymphocytes, and were always a minor population, usually located about the periphery of the lesion. Even though the plasma cells exhibited specific fluorescence when stained with antirabbit gamma globulin, we were unable to demonstrate that they contained specific antibody to bovine insulin.

Lymphocytic infiltration of the islets of Langerhans has been observed in human diabetics prior to insulin therapy, particularly in the acute-onset juvenile type.[26] This lesion strongly resembles that of the experimental diabetes presented here, suggesting that an immunologic mechanism may be involved. Although circulating anti-insulin antibody is usually not detected in persons not receiving insulin,[27] the recent report of Penchev, Andreev, and Ditzov [28] that precipitating antibody to insulin is found in 20% of untreated diabetics supports the possibility of an autoimmune pathogenesis.

Summary

Immunization of New Zealand white rabbits with bovine insulin in Freund's adjuvant produces circulating anti-insulin antibody that reacts with both endogenous and bovine insulins. Hyperglycemia or diabetes mellitus develops in some of these immunized animals. The pancreata of diabetic animals exhibit lymphocytic infiltration of the islets of Langerhans, while rabbits with anti-insulin antibody but without islet lymphocytes remain normoglycemic.

Immunofluorescence studies of the pancreata of immunized rabbits, both diabetic and normoglycemic, failed to demonstrate the presence of anti-insulin antibody in islets. Electron microscope studies revealed minor alterations of beta cells in immunized nondiabetic rabbits and destruction of beta cells by lymphocytes in diabetic rabbits.

This experimental model of immune diabetes morphologically resembles acute-onset juvenile diabetes in man.

References

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[Illustrations follow]

Legends for Figures

Fig 1. Normal control rabbit islet. H & E. × 370.

Fig 2. Normal control rabbit islet. Aldehyde-fuchsin. × 370.

Fig 3. Islet of immunized rabbit with anti-insulin antibody. H & E. × 370.

Fig 4. Islet of immunized rabbit with anti-insulin antibody stained with aldehyde-fuchsin compares with control (Fig 2). × 370.
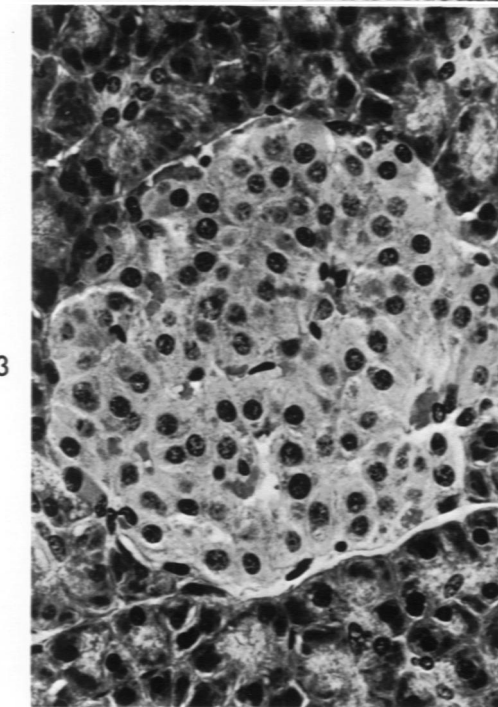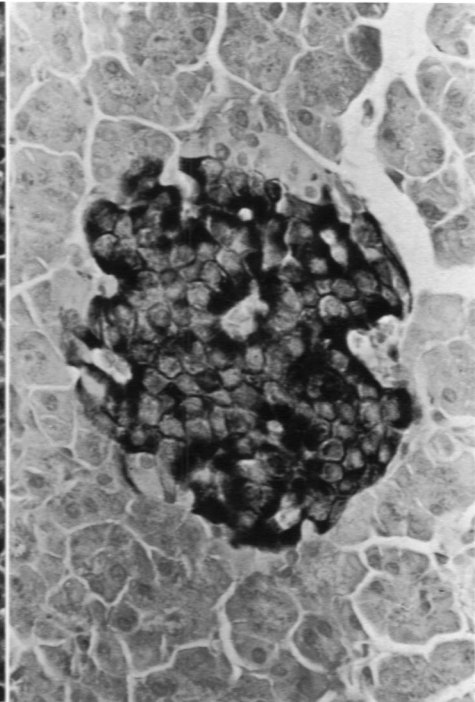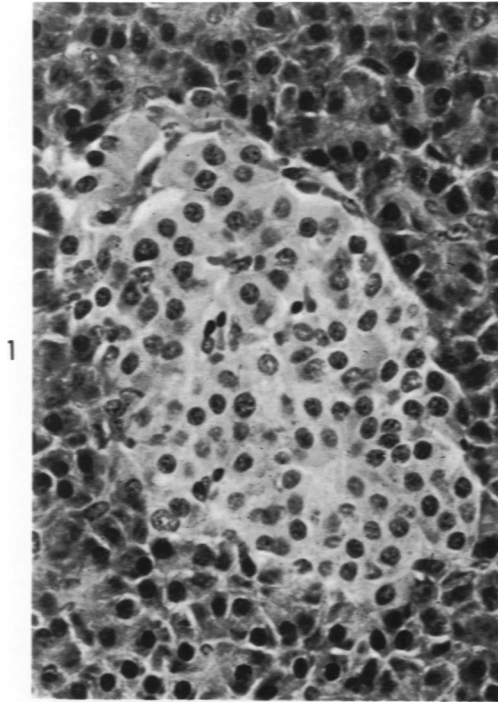
**Fig 5.** Islet of immunized diabetic rabbit displaying marked lymphocytic infiltration. H & E. × 370.

Fig 6. Same rabbit as in Fig 5, with greatly reduced aldehyde-fuchsin-stainable material. × 370.

Fig 7. Islet of immunized rabbit, heavily invaded by lymphocytes (*arrows*), which often appear attached to plasmalemma of beta cells. Araldite section stained with toluidine blue. × 460.
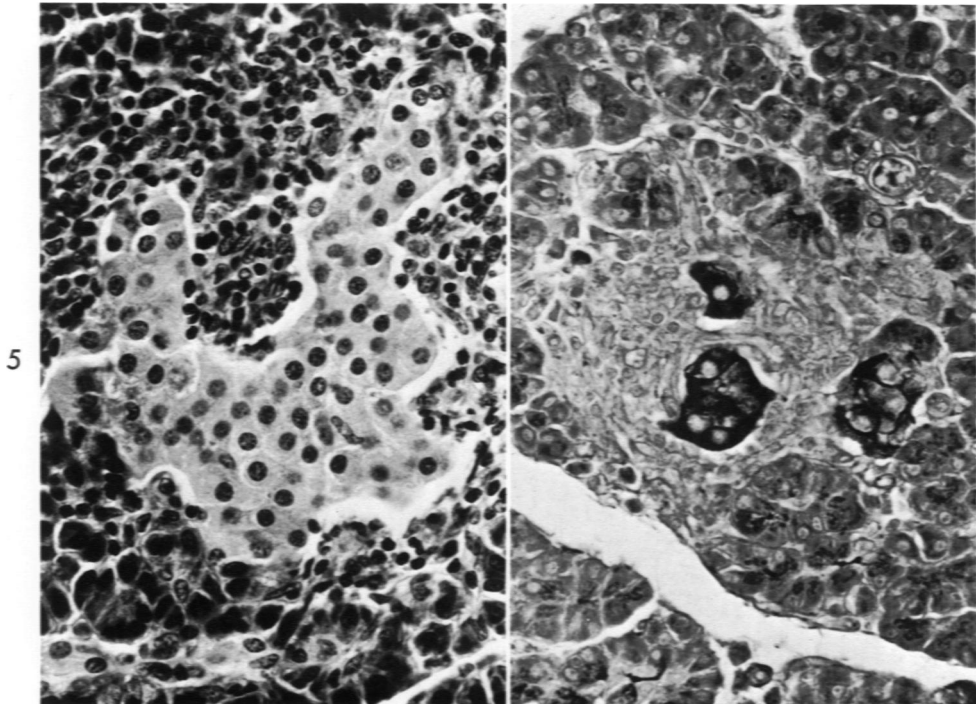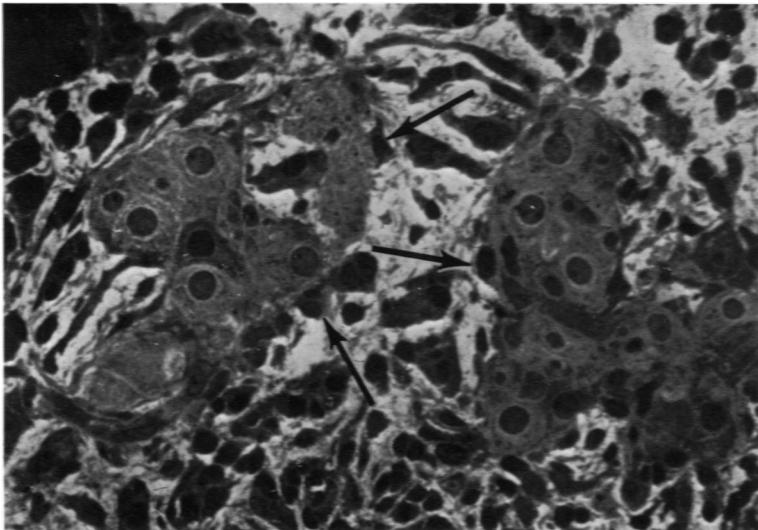
Figs 8–11 were stained with uranyl-acetate and lead citrate.

Fig 8. Electron micrograph of two adjacent beta cells (B_1, B_2) from immunized rabbit with elevated titer but no lymphocytic infiltration. Cells appear only moderately granulated but contain frequent dense bodies resembling lysosomes (*Ly*) in their cytoplasm. (*CAP*) capillary. × 13,800.

Fig 9. Adjacent beta cells (B_1, B_2) of immunized animals with prominent lymphocytic infiltration of islets. Cells appear viable with margination of insulin granules along plasma membrane (*arrows*). (*Ly*) lysosomes; (*A*) alpha cell. × 20,000.
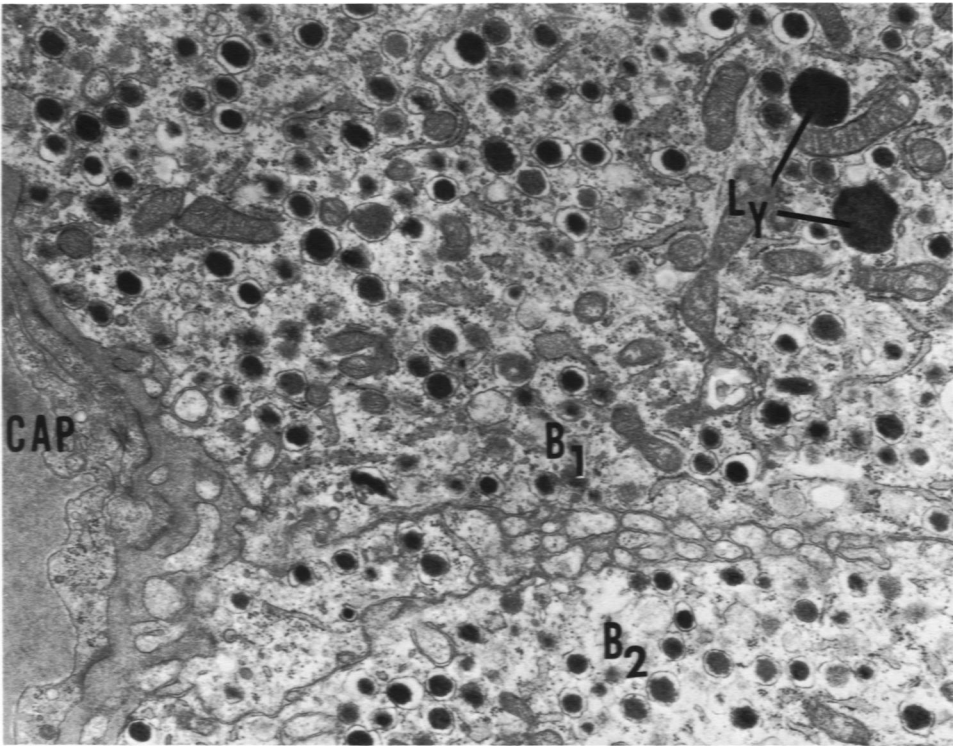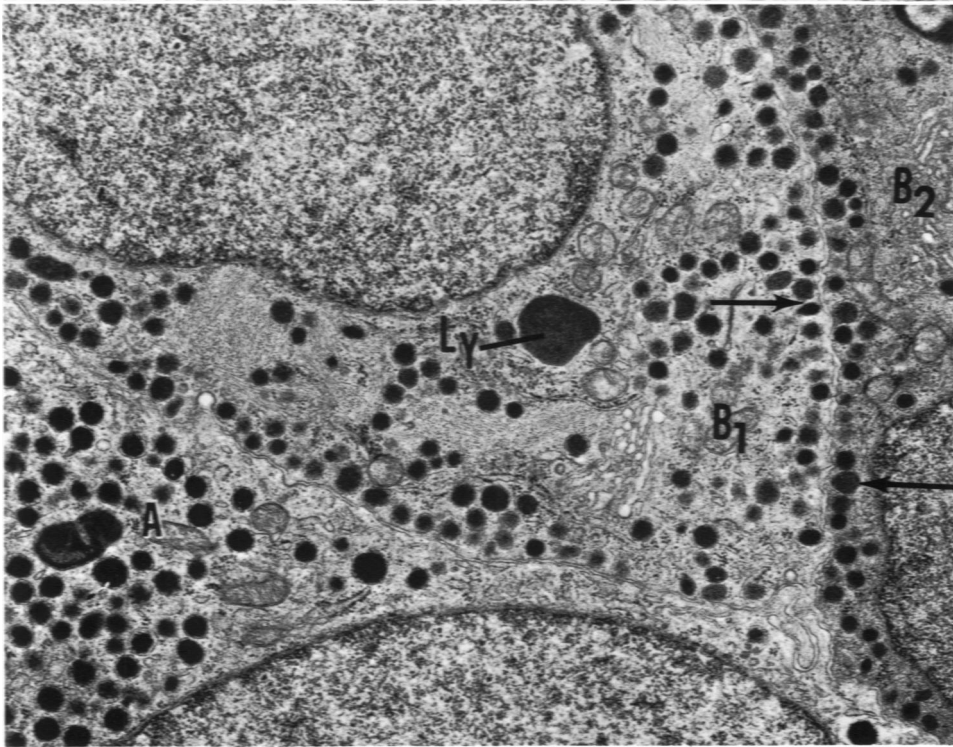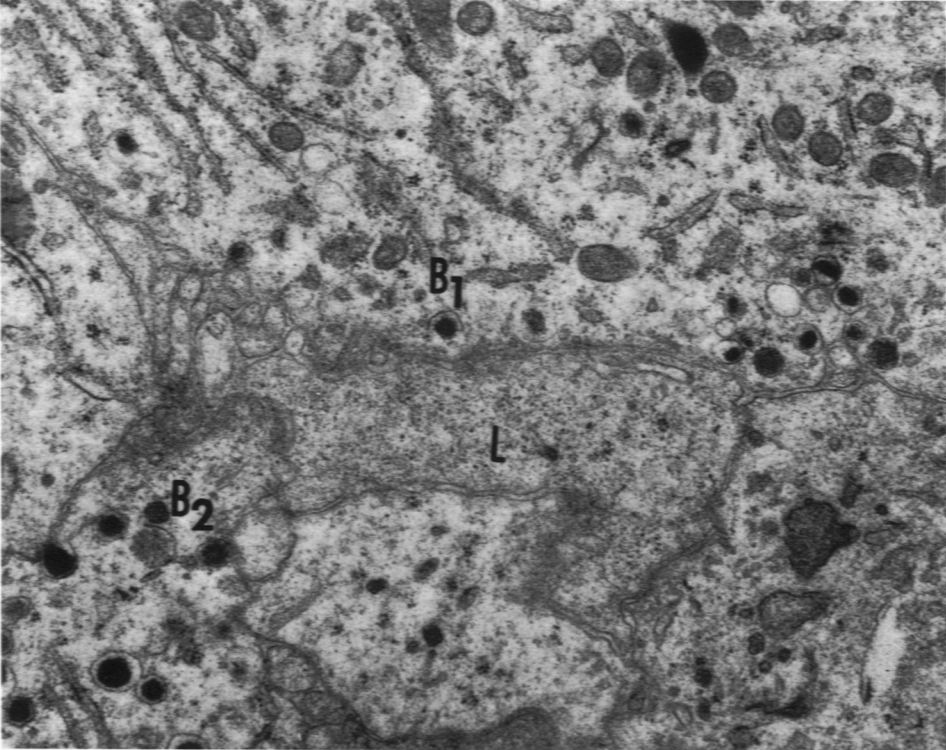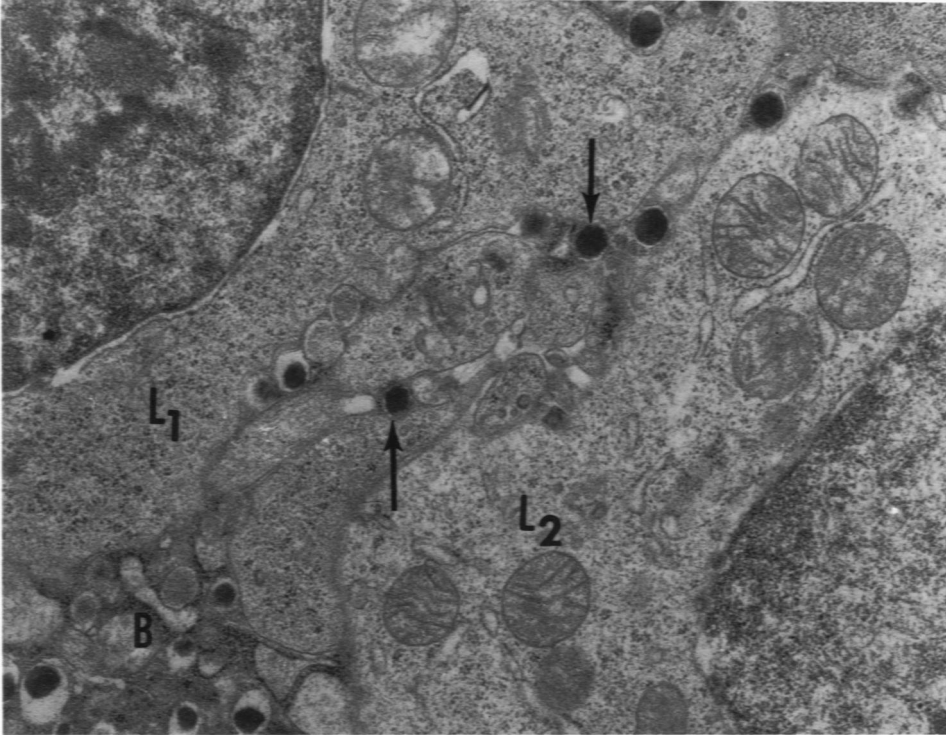
Fig 10. Pseudopod of invading lymphocyte (*L*) inserted between adjacent beta cells (*B₁, B₂*). × 19,300.
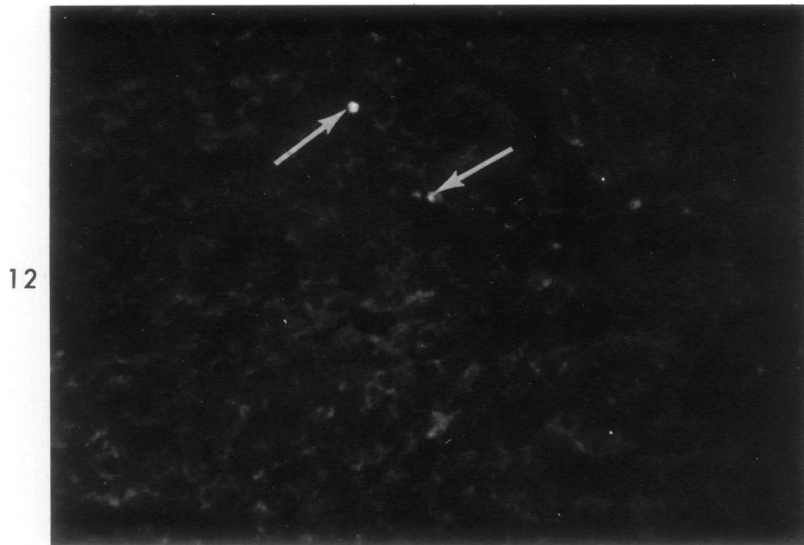
Fig 11. Invading lymphocytes (*L₁, L₂*) with disintegration of adjacent beta cell (*B*). Insulin granules have spilled into intercellular spaces (*arrows*). × 18,000.

Fig 12. Immunofluorescence of islet from immunized animal with prominent lympho-cytic infiltration, stained for rabbit globulin by double-layer technique. Only a few plasma cells (*arrows*) exhibit specific fluorescence. × 300.