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ANTI-PANCREATIC, CELLULAR
HYPERSENSITIVITY IN DIABETES MELLITUS.
EXPERIMENTAL INDUCTION OF
ANTI-PANCREATIC, CELLULAR
HYPERSENSITIVITY AND ASSOCIATED
MORPHOLOGICAL B-CELL CHANGES
IN THE RAT

By

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Previous studies have demonstrated the existence of anti-pancreatic, cellular hypersensitivity in certain patients with diabetes mellitus (10, 11). This reactivity was organ-specific and species-non-specific and was found preferably in patients with juvenile diabetes of short duration.

In this type of diabetes lymphocytic infiltration in and around the islets of Langerhans is a characteristic finding (6) and the two types of observation both support the hypothesis, that cell-mediated immune reactions are of pathogenic importance in juvenile diabetes mellitus.

It has previously been shown that immunological damage to the islets of Langerhans can be induced in experimental animals by repeated injection of large insulin doses with Freund's adjuvant (4, 7, 13) and by injection of anti-insulin antibodies

(5). In man there is no evidence in support of a role of hypersensitivity against insulin in the pathogenesis of diabetes mellitus (3). The studies of Nerup et al. (10, 11), however, suggest that pancreatic tissue contains an organ-specific component different from insulin with an antigenicity associated with human diabetes.

Experimental induction of cellular hypersensitivity against a pancreatic extract and an investigation to show if this condition is associated with organ-specific tissue damage could illustrate, whether a pathogenic role of a pancreatic auto-antigen is a relevant possibility.

In the present study we report the results of an experiment including 1) induction of cellular hypersensitivity in the rat against a preparation of fetal calf pancreas, 2) a study to detect morphological changes in the islets of Langerhans and 3) an evaluation of associated metabolic disturbances.

MATERIAL AND METHODS

Experimental Animals

Female Wistar rats of 180 g were randomly selected from an outbred strain without spontaneous occurrence of diabetes. The animals were divided in four groups (Table 1), kept in cages five by five, and nourished with food pills and water ad libitum.

Antigens

1) *Pancreatic extract* was prepared from pooled, homogenized pancreatic glands from seven calf fetuses of 16–20 weeks of gestation (11). 10 mg lyophilized extract was reliquified with 2.5 ml 0.9 per cent NaCl and mixed with 2.5 ml complete Freund's adjuvant (Difco) (CFA). The insulin concentration in the fetal calf pancreas was 5 μ g per mg dry weight.

2) *Bovine insulin* was used for the preparation of an antigen-emulsion with the same insulin concentration as that of the fetal calf pancreas emulsion: 50 μ g bovine insulin was dissolved in 2.5 ml phosphate buffer (pH 7.4), and mixed with 2.5 ml CFA.

3) *CFA*, 2.5 ml was mixed with 2.5 ml 0.9 per cent NaCl and used as a control to assess the effect of the adjuvant emulsion.

Bordetella pertussis in heat-killed suspension containing 16×10^{10} organisms per ml 0.9 per cent NaCl (kindly provided by The State Serum Institute, Copenhagen) were used as supplementary adjuvant. All antigen preparations were kept in sterile ampoules at 4° C until use.

TABLE 1
Summary of the Experiment.

Group	Number of animals	Antigen	Adjuvant	Immunized day number	Autopsies performed on day number									
A	35	-	-	-	5	8	15	15	15	15	15	15	15	44
B	55	FCP	CFA & Per	0	4	5	6	7	8	9	10	14	15	44
C	20	-	CFA & Per	0	5	8	14	14	14	14	14	14	14	44
D	55	OI	CFA & Per	0	4	5	6	7	8	9	10	14	15	44

Signatures: FCP: Fetal Calf Pancreas 100 μ g; OI: Ox Insulin 0.5 μ g; CFA: Complete Freund's Adjuvant; Per: Pertussis 1.6 $\times 10^{10}$ corpuscles.

Immunization Procedure

The immunization was performed according to the schedule shown in Table 1.

1) 55 animals (Group B) were injected intracutaneously in the foot-pad of one hind leg with 50 μ l of the pancreas CFA emulsion, corresponding to 100 μ g lyophilized calf pancreatic extract per injection. At the same time 1.6×10^{10} pertussis bacteria in 100 μ l suspension were injected subcutaneously on the dorsum of the same foot.

2) 55 animals (Group D) were injected intracutaneously in the foot-pad of one hind leg with 50 μ l of the insulin-CFA emulsion, corresponding to 0.5 μ g bovine insulin per injection. At the same time 1.6×10^{10} pertussis bacteria in 100 μ l suspension were injected subcutaneously on the dorsum of the same foot.

3) 20 animals (Group C) were injected intracutaneously in the foot-pad of one hind leg with 50 μ l CFA emulsion without antigen. At the same time 1.6×10^{10} pertussis bacteria in 100 μ l suspension were injected subcutaneously on the dorsum of the same foot.

4) 35 animals (Group A) did not receive any kind of treatment and were included in the study as controls.

Autopsy

The animals were killed by bleeding (heart puncture) in ether anesthesia with intervals as indicated in Table 1. As routine the pancreas, adrenals, kidneys, liver, thyroid and submandibular glands were removed immediately after death, fixated in 4 per cent formalin buffer, and prepared and stained with hæmatoxylin-eosin for light microscopic examination. One part of the pancreas was prepared for electron microscopy by fixation in glutaraldehyde 6.5 per cent (15) for two hours, and in osmiumtetroxide 1 per cent buffered with phosphate (12) for thirty minutes, followed by embedding in epon 812 (9). Ultrathin sections cut on an LKB Ultratome were stained with lead citrate (14) and uranyl acetate and examined in a Philips EM 300 electron microscope.

Immunological Methods

Migration inhibitory reaction (MIR). Cellular hypersensitivity was registered in vitro as the antigen-induced migration inhibition of a spleen cell suspension by means of the following technique, which is a modification of the leucocyte migration test (1):

The animal was killed by bleeding (heart puncture) in ether anesthesia and the spleen immediately isolated. Crude strains of connective tissue were removed, and the organ was homogenized by pressing and washing (with 20 ml TC 199) through a steel net (mesh size 3.4 mm). After rigorous shaking the cell suspension was left for 45 min at 37° C for spontaneous sedimentation. The cells in the supernatant were washed three times by centrifugation in 20 ml TC 199 with 10 per cent horse serum (State Serum Institute, Copenhagen) at 1800 $\times$ g for 5 min. The cells were resuspended in 6 ml TC 199 with 10 per cent horse serum and counted. The average yield of one rat spleen was 100×10^6 cells. The suspen-

sions were diluted with TC 199 with 10 per cent horse serum to a final concentration of 6×10^6 cells per ml culture medium and transferred to rubber sealed bottles of 8 ml. Antigen was added, the cell suspensions were perfused with gas (97.6 per cent O_2 and 2.4 per cent CO_2 with a flow of 0.75 l per min for 3 min) and left for incubation at $37^\circ C$. After 12 hours the cell suspensions were shaken vigorously and centrifuged at $2000 \times g$ for 10 min. The supernatants were withdrawn and set aside (see below) and each pellet was re-suspended in 250 μ l TC 199 with 10 per cent horse serum. The resulting concentrated cell suspension was transferred to capillary tubes, ending up with about 10×10^6 cells in each tube. The capillaries were closed by melting at one end and centrifuged at $4000 \times g$ for 12 min. The subsequent procedure was carried through according to the usual technique employed in the leucocyte migration test (1) with one major modification: The migration chambers were filled with the antigen-containing, preincubation supernatants (see above) and no new antigen was added. The migration cultures were measured after 24 hours, and the migration index (MI) was calculated in the usual way, values above 1.0 indicating stimulation, below 1.0 inhibition of the migration.

Extract of the fetal calf pancreas (11) was used as antigen. By titration in pilot experiments the optimal concentration was found to be 5 μ g protein per ml of the spleen cell preincubation medium. The specificity of the reaction was tested by using comparable preparations of fetal calf liver and adrenal gland in parallel control experiments (MI-values 0.82–1.06 and 0.85–1.20 respectively).

Migration inhibition experiments were undertaken in 12 animals of group A and in 5 animals of group B on days 5, 8 and 15 after start of the experiment.

Intracutaneous reactions. The intracutaneous reactivity to the pancreatic antigen was assessed in 5 animals of group A and in 5 animals of groups B and D on days 6, 9, and 14 after start of experiment. The fur was cut away from both sides of the body leaving two symmetric nude areas of 20×20 mm. On one side three injections, each of 100 μ l 0.9 per cent NaCl containing 1, 10 and 100 μ g (measured as protein) of fetal calf pancreas respectively, were given intracutaneously. At the same time similar intracutaneous tests were made on the opposite area with comparable doses of bovine insulin (0.005, 0.05 and 0.5 μ g). Lyophilized fetal calf liver extract served as control antigen in comparable intracutaneous tests. The reactions were read after 10 min and after 24, 48 and 72 hours. Only infiltration > 5 mm of diameter were considered positive.

Metabolic Investigations

All animals in groups A, B, C, and D were tested daily (with Tes-Tape®) to detect glucosuria. A glucose tolerance test was performed on day 42 in two rats from group A, four from group B, three from group C and two from group D. After 12 hours fasting 350 mg glucose in 0.7 ml water was injected subcutaneously on the dorsum of the animal. Blood was drawn from the tail vein 0, 60 and 90 min after the injection for determination of glucose (O-tolidin method).

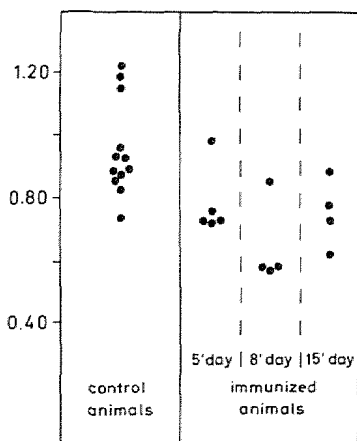


Fig. 1.

Migration inhibition reaction in rats immunized with fetal calf pancreatic extract.

RESULTS

Immunological Observations

The results presented in Fig. 1 indicate, that the immunization procedure induced a specifically altered reactivity in the animals that received injections containing fetal calf pancreatic extract. The preparation induced spleen cell migration inhibition in a majority of the animals examined. Comparison (Wilcoxon's test) of the groups displayed in Fig. 1 shows that inhibition induced by fetal calf pancreas could be observed from day 5 (control versus day 5 group: $p < 0.05$), that the most intensive reactions were present on day 8 (control versus day 8 group: $p < 0.01$), and that the migration inhibition was less pronounced in animals examined on day 15 (control versus day 15 group: $p < 0.05$). Migration inhibition was not observed in the parallel experiments with comparable extracts of liver and adrenal gland.

Positive intracutaneous reactions of immediate or delayed type were not observed with any of the antigens tested.

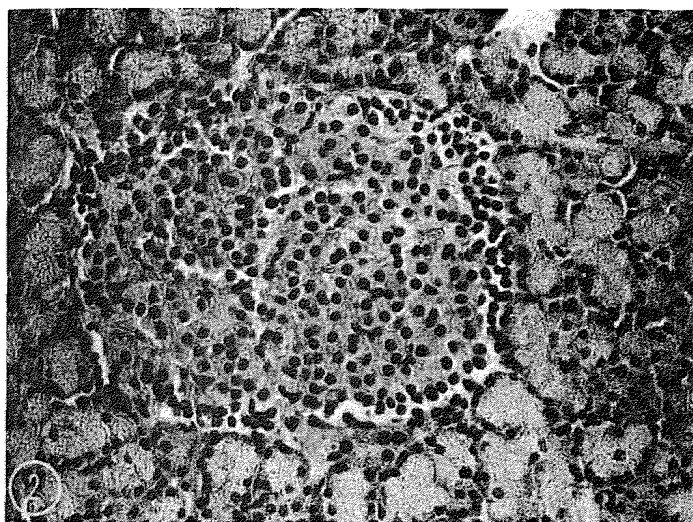


Fig. 2.

Islet of Langerhans from rat 9 days after immunization with fetal calf pancreatic extract. The islet is infiltrated with mononuclear cells mainly located at the periphery. Hematoxylin and eosin stain. 500 $\times$.

Morphological Observations

Light microscopy revealed a discrete periinsular mononuclear cell infiltration in the islets of Langerhans in some of the animals immunized with the fetal calf pancreatic extract (Group B) (Fig. 2). A similar mononuclear cell infiltration was not demonstrable in the islets of insulin-CFA injected rats (Group D), in animals injected with CFA only (Group C) or in untreated controls (Group A). Examination of the adrenals, kidney, liver, thyroid and salivary glands did not reveal any findings deviating from normal in any group of animals.

Electron microscopic visualization of the normal ultrastructure of the islets of Langerhans in the rat is presented in Fig. 3, and a normal B-cell is shown in higher magnification in Fig. 4. Two islets from each animal in all four groups were



randomly selected for electron microscopy (EM). By EM, lymphocytic infiltration, i. e. extravascularly situated lymphocytes in contact with parenchyme cells of the islets of Langerhans (Fig. 5), was found in one third of the group B animals. The lymphocytic infiltration was detectable from day 9, maximal about day 15 and had disappeared 44 days after immunization (Table 2). Lymphocyte infiltration was not found in animals belonging to groups A, C and D.

As shown in Table 2 ultrastructural changes of the parenchyme cells of the islets appeared at day 9 in animals injected with pancreas-CFA (Group B). The abnormalities were maximal on days 15-17 and had disappeared the 44th day after immunization. Two types of changes could be distinguished:

1) The islet cells were shrunk with hyperchromatic, pyknotic nuclei, loss of cytoplasmic matrix, dilatation of the perinuclear cisternae, and vacuolization and disintegration of the mitochondria. The secretory granules seemed to be intact. In islets with this type of changes, about half the islet cells were affected (Fig. 6).

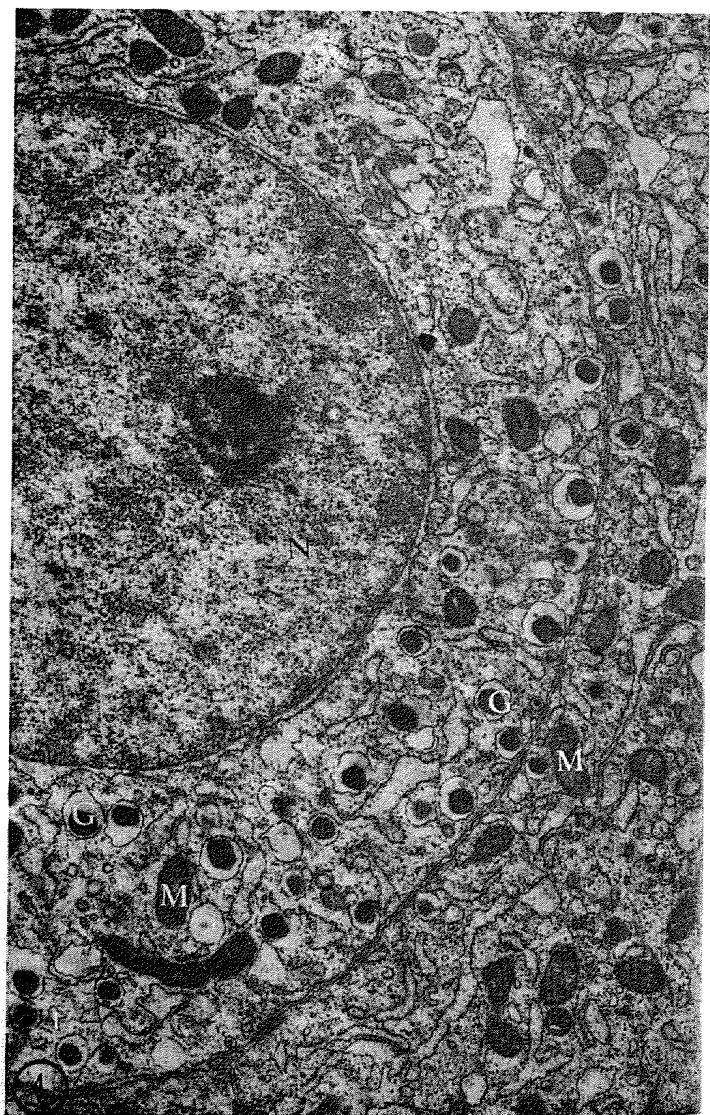
2) More often all cells of the islet were changed presenting large pale nuclei placed in an almost totally desorganized cytoplasm. The mitochondria were disintegrated and often fractured and the secretory granules had lost their membrane (Fig. 7).

The ultrastructure of islets from animals injected with insulin-adjuvant (Group D) or with adjuvants alone (Group C) was not different from the untreated control group (Group A).

The exocrine pancreatic tissue showed no changes neither by light microscopy nor electron microscopy in any group of animals except for one incidental observation: the animals of

Fig. 3.

Survey picture of a pancreatic islet from a normal, untreated rat with one alpha cell (A), and two beta cells (B). In the cytoplasm the specific granules are seen to be evenly dispersed in a matrix of moderate density. 10,500 X.





Group B showed considerably decreased staining of the endoplasmic reticulum by pyronin staining as compared to normal controls.

Metabolic observations. Glucosuria was not found in any case, neither in the pancreas-CFA treated animals (Group B), nor in the other three groups.

It was not possible by means of the subcutaneous glucose tolerance test to demonstrate decreased carbohydrate tolerance in any of the animals examined.

DISCUSSION

Several modifications of the MIR have been devised for the in vitro demonstration of cellular hypersensitivity in small laboratory animals (2). A new modification with rat spleen cells as migrating cell population was used in the present study. By this technique it was possible to demonstrate that the rats developed a state of organ-specific, anti-pancreatic, cellular hypersensitivity after injection of fetal calf pancreatic extract with CFR. The organ-specificity was ensured by the negative reactions to comparable extracts of liver and adrenal gland. The reactivity was detectable from the 5th day after immunization, and although the number of examinations is small, the results suggest that the hypersensitivity was maximal after day eight and subsequently decreasing (Fig. 1). The

Fig. 4.

Normal beta cells from rat pancreas. The cytoplasm contains 1) specific granules surrounded by a single membrane (G), 2) rod-shaped mitochondria with a well-developed internal structure (M), and 3) an abundant rough-surfaced endoplasmic reticulum. The nucleus (N) is relatively large, spherical, and poor in chromatin. 18,500 $\times$.

Fig. 5.

Langerhans islet from rat injected with fetal calf pancreatic extract 15 days earlier. Two lymphocytes (LY) are seen among the endocrine beta cells. The lymphocytes are separated from the beta cells by a narrow intercellular space, and their plasma membranes show many infoldings. 15,100 $\times$,

TABLE 2
Summary of the pathological Findings in the Islets of Langerhans of Rats immunized with Fetal Calf Pancreatic Extract (Group B).

Day number in the experiment	0-5	6	7	9	10	14	15	17	44
Number of animals examined	10	5	5	5	5	5	5	3	6
Animals with lymphocytic infiltration	0	0	0	2	0	2	2	3	0
Animals with B-cell changes	0	0	0	1	1	4	4	3	0

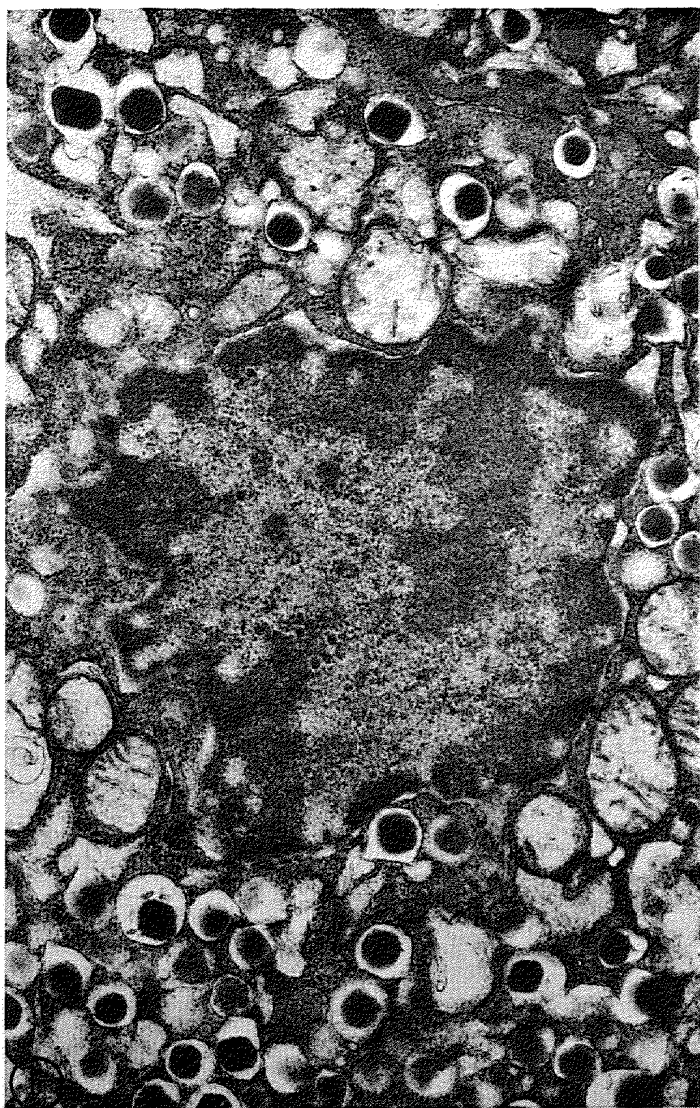
cellular hypersensitivity was not demonstrable as intracutaneous delayed type reactivity. This is in agreement with the general experience that rats do not develop delayed type cutaneous reactivity very easily, but the observation also emphasizes that the immunization procedure employed induced a comparatively low grade of hypersensitivity.

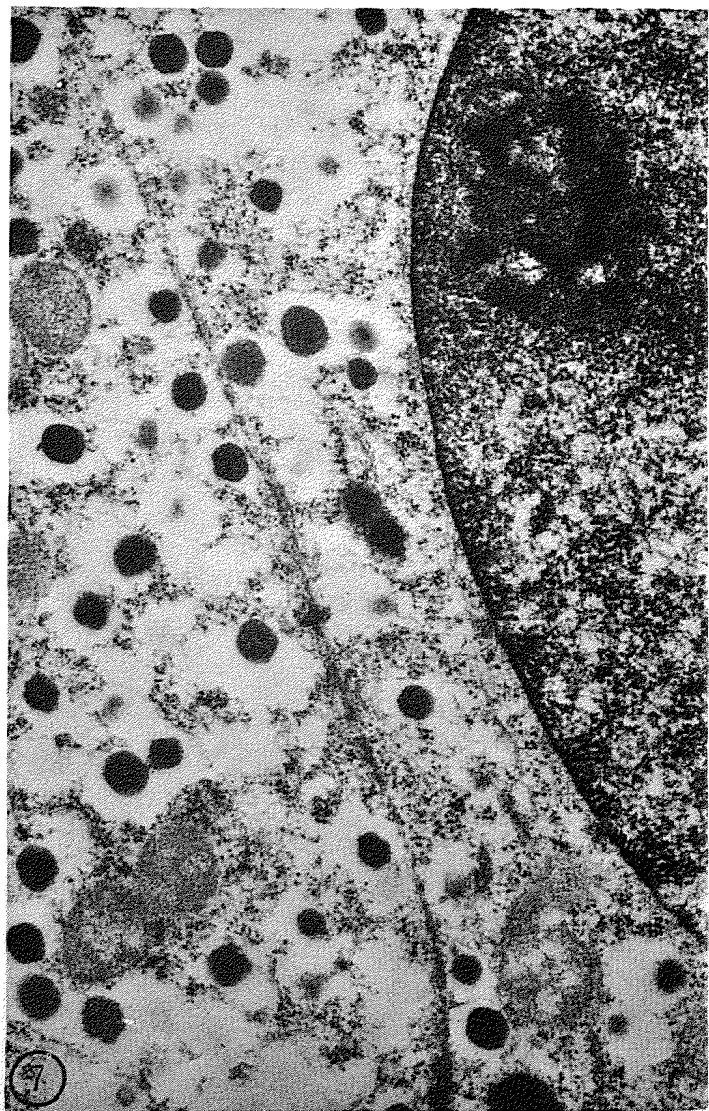
The development of anti-pancreatic, cellular hypersensitivity was associated with histopathologic changes in the pancreatic glands. The findings were discrete, consisting of mononuclear cell infiltration located at the periphery of the islets of Langerhans (Fig. 2) in some of the animals injected with fetal calf pancreatic extract and adjuvants (Group B). No abnormalities were demonstrable in the islets of animals in the other three groups. In the electron microscope lymphocytic infiltration, i.e. extravascularly situated lymphocytes (Fig. 5), could be detected in one third of the islets from animals of group B, including islets in which no light microscopic changes were found, but not in islets from any animal of groups A, C or D. As seen from Table 2 the lymphocytic cell infiltration and the degenerative changes in the B-cells developed simultaneously, being visible from day 9 and maximal on days 15-17 after immunization. The lymphocytic infiltration and the B-cell changes had both disappeared after 44 days, which is in good agreement with the general tendency for spontaneous remission of experimental autoimmune diseases. It is of interest to note that migration inhibition of the rat spleen cells in the MIR could be demonstrated earlier than the abnormalities in the islets of Langerhans, and that the islet changes became detectable about the time when the MIF was maximal.

Ultrastructural studies of the early findings in experimental

Fig. 6.

Beta cell from rat injected with fetal calf pancreatic extract 17 days earlier. Though the granules seem to be intact the cell is shrunken and the nucleus is folded and pycnotic. The mitochondria are swollen and have almost lost their internal structure. 28,000 X.





thyreoiditis (8) have shown that accumulation of mononuclear cells in the smallest vessels is prior to the extravascular presence of lymphocytes, which is the last step preceding degenerative changes in the parenchyme cells. From the present study it is not possible to establish whether the lymphocytic infiltration preceded the B-cell changes.

Spontaneous occurrence of B-cell degeneration was not observed in the strain of animals used in the experiment. Ultrastructural B-cell changes developed only in animals which had received injections of fetal calf pancreatic extract. Two different types of B-cell changes could be demonstrated, but did not occur simultaneously in the same islet or in the same animal. Apparently the changes were induced by the fetal calf pancreatic preparation, since they did not occur after comparable treatment with insulin nor with pure adjuvant emulsion. The light and electron microscopic examinations revealed that the experimental procedure induced morphologic changes in the endocrine pancreatic tissue, and similar changes in the exocrine pancreas of the experimental animals were not conspicuous. The reduced pyroninophilia of exocrine pancreatic cells in rats immunized with fetal calf pancreatic extract as compared to controls seems to indicate, however, that the immunization actually caused some cellular change also in the exocrine pancreatic tissue. The finding does not interfere with the observations and conclusions concerning the endocrine pancreas, but emphasizes the need for purified antigen preparations for future use in similar experiments.

SUMMARY

In some patients with juvenile diabetes of short duration lymphocytic infiltration in and around the islets of Langer-

Fig. 7.

Two adjoining beta cells from rat injected with fetal calf pancreatic extract 15 days earlier. The granules have lost their membranes and the cytoplasmic matrix has almost disappeared. The mitochondria (M) are swollen and contain a structureless granular material. 34,000 $\times$.

hans and anti-pancreatic cellular hypersensitivity has pointed to cell-mediated immune reactions of pathogenic importance in juvenile diabetes mellitus. In the present study groups of rats were immunized with fetal calf pancreatic extract in adjuvant, bovine insulin in adjuvant, or adjuvant alone. With a *modified leucocyte migration test on spleen cell suspension* animals immunized with fetal calf pancreatic extract exhibited a migration inhibition to the same antigen, but not to other comparable antigens. The inhibition was most pronounced 8 days after immunization. In the group of animals immunized with fetal calf pancreatic extract, discrete periinsular mononuclear cell infiltration was seen by light microscopy. Electron microscopy revealed extravascular lymphocytic infiltration in the islets of Langerhans and ultrastructural changes in the B-cells. No changes could be seen in the islets from any animal in the other groups or in the control group. The morphological changes were most pronounced 15–17 days after immunization. 44 days after immunization no morphological changes could be seen in any animal. By examinations for glucosuria and by subcutaneous glucose tolerance tests no decrease in carbohydrate tolerance could be demonstrated in any group.

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