Immunohistological Demonstration of Insulin in Islets of Langerhans after Stimulation of Insulin Secretion by Glucose, Sulphonylureas and Insulin Antibodies

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Summary. The immunohistological detection of insulin in whole pancreatic tissue and isolated islets was studied, using cryostat sections, freeze-dried material and in formalin-fixed tissue. Cryotomy produces unsatisfactory results and can only be used as a screening procedure. The freeze-drying technique followed by paraffin embedding is time consuming, but possesses an advantage in that it best guards the natural morphological relationships in cells and tissue. — The results of experiments designed to determine the immunohistologically-provable content of insulin in the islets of Langerhans of rats by means of various stimulants to insulin secretion are reported. Intravenously injected glucose in high concentration (10 g/kg) does not lead to an immunohistologically-provable decrease in the insulin content of the beta cells, after either 2, 4 or 24 h. During the first 6 h following a single intravenous medication of tolbutamide (500 mg/kg) or of glibenclamide (1 mg/kg) there is no decrease in the immunofluorescence of the beta cells. The decrease, apparent after 8 h, becomes more clear in a wide range of cells after 12 h, and has reached its climax after 24 h. At this time, nearly all of the cells of the islet are degranulated except those in the periphery. On the contrary, 5 h following intravenous injection of insulin antibodies, an almost total decrease in immunohistologically-provable insulin can be observed.

Démonstration histoinmunologique de l'insuline dans les îlots de Langerhans après stimulation par glucose, sulfonylurées et par anticorps anti-insuline

Résumé. Nous avons comparé trois techniques histoinmunologiques différentes chez le rat sur les cellules bêta du pancréas: les coupes après congélation de tissus frais, la méthode de congélation-dessiccation et la méthode avec du matériel fixé à la formaline. La méthode des coupes après congélation de tissus trouve une limitation dans la difficulté d'obtenir de bonnes préparations cytologiques. La méthode de congélation-dessiccation offre de nombreux avantages, p.ex. l'immobilisation instantanée et définitive des constituants des tissus et le fait que les changements chimiques dans les tissus sont réduits au minimum, mais elle exige beaucoup de temps. — Nous avons montré l'effet de la tolbutamide et de la glibenclamide sur les cellules bêta avec la méthode de fixation à la formaline. In vivo, l'administration intra-veineuse de glucose (10 g/kg) ne produisait pas de dégranulation des cellules B après 2, 4 et 24 h. En utilisant une seule injection de tolbutamide (500 mg/kg) et de glibenclamide

(1 mg/kg) il n'y avait pas de diminution de la fluorescence pendant les premières 6 h. Par contre, après 8 h, nous avons remarqué une faible diminution de la fluorescence qui s'accroissait pendant les 12 et 24 h suivantes. Après 24 h les îlots de Langerhans présentaient un centre complètement dégranulé et seulement quelques cellules halopériphériques réagissaient aux anticorps anti-insuline marqués à la fluorescéine. Inversement l'administration intraveineuse d'anticorps anti-insuline produisait une dégranulation complète après 5 h.

Immunhistologische Untersuchungen Langerhans'scher Inseln zum Nachweis von Insulin nach Stimulation mit Glucose und mit Sulfonylharnstoffen

Zusammenfassung. Die Arbeit schildert zunächst die verschiedenen Möglichkeiten des immunhistologischen Nachweises von Insulin im Pankreas. Als optimale Methode zur Aufarbeitung des Gewebes erwies sich die Formalinfixation mit folgender Paraffineinbettung. Die Kryostattechnik liefert unbefriedigende Resultate und eignet sich nur als Screening-Verfahren. Die Gefrier-trocknungstechnik mit folgender Paraffineinbettung ist zeitlich recht aufwendig und hat den Nachteil der limitierten Gewebsgröße. Sie bietet dagegen den Vorteil, daß sie die natürlichen Verhältnisse in Zelle und Gewebe am besten bewahrt. — Anschließend wird über die Auswirkungen von Stimulationsversuchen zur Insulinsekretion auf den immunhistologisch nachweisbaren Insulingehalt der Langerhans'schen Inseln bei der Ratte berichtet. Intravenös injizierte Glucose führt auch in hoher Konzentration (10 g/kg) weder nach 2, 4 noch nach 24 Std zu einer immunhistologisch nachweisbaren Abnahme des Insulingehaltes der B-Zellen. Nach einmaliger intravenöser Gabe von Tolbutamid (500 mg/kg) sowie von Glibenclamid (1 mg/kg) kam es ebenfalls in den ersten 6 Std zu keiner Verminderung der Immunfluoreszenz der B-Zellen. Diese zeigt sich erst nach 8 Std an einzelnen Zellen des Inselzentrums, wird deutlicher nach 12 Std in einem größeren Zellbereich und hat nach 24 Std durch Entspeicherung fast aller Zellen mit Ausnahme der Peripherie ihr Maximum erreicht. Im Gegensatz dazu tritt nach intravenöser Injektion von Insulinantikörpern eine nahezu komplette Entspeicherung von immunhistologisch nachweisbarem Insulin bereits nach 5 Std ein.

Key words: B-cells, insulin, immunohistology, freeze-drying, formol fixation, insulin secretion, in vivo- in vitro stimulation, sulphonylureas, insulin-antibodies.

I. Introduction

Histological procedures have been widely used for the staining of the islets of Langerhans. It is assumed that the staining ability of the beta-cells is correlated

with its content of insulin. For example, Pfeiffer et al. (1957) could demonstrate in calves a moderate degranulation of the beta-cells and a hypertrophy of the nuclei following oral tolbutamide application. The histological procedure, however, is disadvantageous

because cells in other organs can be stained by the same technique, casting some doubt on the specificity of the procedure. On the other hand, with the immunofluorescence technique, using a FITC-labelled anti-insulin-serum, insulin can be definitely detected in the beta-cells of the islets. Lacy and Davies (1957) demonstrated for the first time the ability of insulin to react with the labelled antibody on cryostat tissue sections. In addition to cryotomy, two other methods have been described: freeze-dried and paraffin-embedded materials are ideal for fluorescence microscopy because there is no evidence that immunological properties are altered by freezing and drying or by the short exposure to heat during paraffin-embedding. In addition, freeze-dried and paraffin-embedded material can be stored for months without any adverse effect on the immunological properties (Neumann, 1958; v. Mayersbach, 1959).

The third method is the use of formalin fixation and paraffin embedding. The reactions of formalin with tissue proteins are numerous and complex, since it can combine with a number of different functional groups. Here, formalin works not only in forming links between adjacent protein chains, but also as a polymerizing fixative. In contrast to a widely held opinion that formalin-fixed tissue is not suitable for fluorescence microscopy, the beta-cells are not significantly altered by these procedures, and insulin can be detected by immunofluorescence in formalin-fixed material (Costanzi *et al.*, 1961; Berns *et al.*, 1962).

This investigation aims at finding the best techniques for the immunohistological detection of insulin in the beta-cells and, with these techniques, studying insulin secretion, using beta-cytotropic agents and anti-insulin antibodies.

II. Materials and Methods

Animals: 53 male albino rats (Wistar, weighing between 180 to 210 g) were used for the beta-cell stimulation of insulin release *in vivo*, and 8 rats (weighing 300 g) for stimulation *in vitro*. The control group consisted of 10 rats.

A. The following substances were studied:

1. Glucose

10 animals received intravenous injections of 4 ml of a 50% glucose solution (10 g/kg). The animals were killed 90 min, 4, 8, 12 and 24 h after the injection.

2. Glibenclamide (Hb 419)

20 animals were injected with 1 mg/kg glibenclamide into the upper tail vein, according to the experiment of Kern *et al.* (1969), and killed after 90 min, 12, 24 and 48 h. Blood sugar was determined prior to the injection and at the end of the experiment.

3. Tolbutamide (Rastinon®)

0.5 g/kg tolbutamide (Lazarus *et al.*, 1967; Loubatières *et al.*, 1967; Malaisse *et al.*, 1967; Candela, 1967) was given by injection to 16 animals. The rats were killed after 90 min, 12, 24 and 48 h. Blood sugar was determined in the same manner as described above.

4. Guinea pig antiinsulin sera

7 rats received 2 ml guinea pig antiinsulin serum by continuous infusion over a 4 h period, or were injected with 0.5 ml antiinsulin serum every hour, for at least 4 h. The titres of the sera were 7290 to 500000 assayed by passive haemagglutination (Stavitsky and Arquilla, 1955), and 1:2000 to 1:500000 assayed by the cellulose adsorption technique according to Kerp *et al.* (1966). The animals were killed 5 h after the first injection or initiation of the infusion (Wright, 1959; Cunningham *et al.*, 1963; Gregor *et al.*, 1963; Logothetopoulos *et al.*, 1965; Lacy and Wright, 1965; Wright *et al.*, 1966).

B. Stimulation *in vitro* of isolated islets of Langerhans

Islets were isolated from rats according to the method described by Moskalewski (1965), Lacy and Kostianovsky (1967) with small modifications (v. Haugwitz, 1971; Huber, 1971). The islets were cultured in a medium consisting of Krebs-Ringer bicarbonate buffer, supplemented with 2 mg/ml albumin and equilibrated with 95% oxygen and 5% CO₂. Tolbutamide was used in a concentration of 500 µg/ml for 1 h at a temperature of 37°C. At the end of the culture period, the islets were transferred, with the use of a microscope, to a small test tube and freeze dried.

C. Immunohistological technique

a) Cryotomy

Rats were anaesthetized with nembutal and the pancreas was removed and immediately frozen with CO₂, using the system of Dittes — Duspiva. The tissue was sectioned at 4–6 µ, air-dried at room temperature and incubated with labelled antiinsulin globulin in a moist chamber at 37°C for 3 min. The sections were then washed 3 times in Mayer's buffer and mounted (Wallace *et al.*, 1950).

b) Preparation of the tissue by freeze drying

Small pieces of pancreatic tissue, not more than 2 mm thick, and islets, isolated by means of collagenase, were snap-frozen at –150°C in difluorodichloromethane (Frigen®) and cooled by liquid nitrogen. The frozen tissues were freeze-dried for 16–18 h at approximately 5×10^{-2} mm Hg. When drying was completed, the dried blocks were embedded in paraffin at 65°C and at a low pressure of 5×10^{-2} mm Hg. Sections at 3–5 microns were mounted without the aid of water and were then taken through xylol, 100%,

95% and 70% alcohol and phosphate-buffered saline. Staining was carried out, as described above, with labelled antiinsulin globulin.

c) Formalin fixation

The pancreas was exposed by laparotomy and the tissue was rinsed for a few seconds in normal saline to remove the erythrocytes. Small pieces (0.5 cm²) of pancreas were fixed in 4% formalin for 12–18 h,

with an emulsion consisting of complete Freund's adjuvant (for the first 4 immunizations) and 20 units recrystallized bovine insulin. Four days after each subsequent injection, the guinea-pigs were bled by cardiac puncture. The sera were stored at –20°C until used. Antiinsulin titres were determined by passive haemagglutination, according to the method described by Stavitsky and Arquilla (1955) and by the cellulose adsorption technique (Kerp *et al.*, 1966).

Fig. 1. Immunoelectrophoretic pattern of guinea-pig IgG. Anti-guinea-pig serum was placed in the troughs, normal guinea-pig serum in the upper and lower wells and purified serum containing the antiinsulin antibodies in the central well (DEAE-Sephadex chromatography)

Fig. 2. Freeze-dried islets in pancreatic tissue. Stained with FITC-labelled insulin-antibodies. Note the granular pattern of the fluorescence

dehydrated in alcohol and embedded in paraffin. Sections at 3–5 μ were mounted without the aid of water. They were then taken through xylol, 100%, 95% and 70% alcohol and water. Staining and mounting was again carried out as described above.

d) Production of antiinsulin serum

The antiinsulin sera were obtained from guinea pigs injected subcutaneously at weekly intervals

Only sera having a haemagglutination titre between 7290 and 590 000 or a 50% affinity of 1 unit ¹²⁵I-labelled insulin at a concentration between 1:2000 and 1:500 000 were used in our study.

e) Preparation of the FITC-labelled antiserum

The globulin fraction of the immune sera was obtained by DEAE-Sephadex A 50 chromatography, using phosphate buffer 0.02 molar, pH 7.4. The

fractions were concentrated by ultrafiltration to a protein content of 20 mg/ml. Immunoelectrophoresis, using veronal buffer, pH 8.6, produced one band for IgG globulin (Fig. 1). The fractions were labelled with fluoresceinisothiocyanate (FITC), according to the method of Rinderknecht (1962), using 0.1 mg FITC on celite per mg protein. The unreacted fluorescein was removed, and the optimally labelled conjugates were obtained by DEAE-Sephadex chromatography, using a phosphate buffer of 0.01–0.06 molar, pH 7.4. The dye concentration of conjugates was determined

the immunohistological procedures, only fractions with a F/P ratio of 0.8–1.5 were used.

III. Results

A. Immunohistological patterns of the islets of Langerhans, using cryotomy, freeze drying and formolfixation.

Sections obtained by cryotomy were usually of minor quality. Cell membranes and nuclei could frequently not be distinguished, and the islets were occasionally surrounded by a fluorescing ring. Even

Fig. 3. Formol fixation of pancreatic tissue. Same staining as in Fig. 2. Note the homogeneous pattern of the fluorescence
a) Untreated rat. b) 8 h after the intravenous administration of glibenclamide, 1 mg/kg. c) 12 h and d) 24 h after glibenclamide application.

by optical density at the absorption maximum of bound dye (495 nm). Protein determination was performed with the colorimetric technique of Lowry *et al.* (1951). Dye/Protein (F/P) ratios in conjugates were calculated from the values obtained, as described above, according to the formula of Nairn (1964). For

using ethanol or acetone before or after the staining procedure, the histological quality could not be improved. Freeze-dried pancreatic tissue showed almost no autofluorescence and therefore the contrast of the fluorescing islets was very intense. Cell membranes and nuclei were well separated, and the fluorescence

usually showed a more granular pattern (Fig. 2). After formol fixation the cytoplasm of the cells showed a homogeneous fluorescence. The nucleus was well defined, and the A-cells could be easily distinguished as a dark area at the islet periphery. However, we frequently found a strong autofluorescence of the pancreatic exocrine tissue and an orange autofluorescence of the erythrocytes (Fig. 3a).

2. Glibenclamide

No change in fluorescence intensity could be observed in the islets, 90 min and 4 h after the application of glibenclamide, even though blood glucose levels decreased from the normal of 120 mg% to 60 mg%. The first decrease in fluorescence became apparent after 8 h (Fig. 3b). Twelve hours after the single injections of glibenclamide, the insulin content of the

Fig. 4. 48 h after the single intravenous injection of glibenclamide (1 mg/kg). Note the increase in fluorescence (Formalin-fixed tissue)

Fig. 5. a) 5 h after the intravenous injection of 2 ml of insulin-antibodies (titre 1 : 21 810)
b) After the injection of a potent anti-insulin serum (titre 1 : 590 000). Almost complete degranulation, with a few fluorescing cells at the periphery (freeze-dried islets)

B. Immunohistological patterns of the islets of Langerhans after stimulation in vivo, using freeze drying and formalin fixation.

1. Glucose

There was no decrease in fluorescence in the beta-cells of rats, 90 min, 4, 8, 12 and 24 h after the injection of glucose.

beta-cells was clearly diminished. Areas with heavily degranulated beta cells were confined to the centre of the islets, surrounded by almost normally stained beta cells (Fig. 3c).

3. Tolbutamide

The single intravenous injection of 0.5 g/kg did not show a detectable effect on the insulin content of the

beta cells of the rats after 90 min and 4 h. Despite the decrease in blood sugar from 140–120 mg% to 80–70 mg%, there was no decrease in fluorescence. However the fluorescence was clearly diminished when examined after 12 h, and the maximum decrease in fluorescence was 24 h after the application (Fig. 3d). The increase of the content of insulin in the beta cells, as detected by immunofluorescence, could be observed after 48 h (Fig. 4).

4. Antiinsulin serum

Five hours after initiation of the infusion or after the first injection, the islets of the rats showed almost a complete decrease in fluorescence. The islets showed the same pattern of fluorescence described 12 and 24 h after glibenclamide or tolbutamide injection. The centre of the islets did not have fluorescence, and appeared in a manner almost identical to the autofluorescence seen in unstained preparations, and only a few fluorescing cells were situated peripherally. However, when very potent sera (haemagglutination titres 1 : 590 000) were used, the islets were completely dark, showing no fluorescence. Blood glucose levels in most of the rats treated with antibodies were close to 350 mg% at the end of the fifth hour, when the rats were killed (Figs. 5a and b).

pancreatic tissue. The fluorescence was as dense and homogeneous as that observed in formalin-fixed material. A decrease in fluorescence in the tolbutamide treated islets could not be observed (Fig. 6), in spite of the fact that the immunologically measurable insulin (IMI) increased in the culture media (Huber, 1971). These findings support the results obtained after stimulation *in vivo*: there is no decrease in fluorescence during the first few hours.

Discussion

The present results confirm earlier reports (Lacy and Davies, 1957) which state that the dynamics of insulin secretion on a morphological basis can be studied by immunofluorescence as well as by usual histological staining methods. Although unfixed cryotome sections have been widely employed in immunofluorescence for the demonstration of insulin in the islets of Langerhans, it must be emphasized, that the only advantage of this technique is the rapid, simple preparation of the tissue. However the sections are usually 5–7 μ in thickness and the staining of minor quality. Freeze drying is almost ideal, and the stained slides are of good quality. In this case however, the tissue pieces

Fig. 6. Isolated islet. Incubation with 500 μ g/ml tolbutamide for one hour did not reduce the fluorescence intensity (Freeze-dried islet)

C. Immunohistological patterns of the isolated islets after stimulation *in vitro*, using the freeze drying technique.

A series of cultures (40 islets in each) from 8 animals were prepared and examined subsequently. Sections for immunohistology were prepared from the cultured, freeze-dried and paraffin-embedded islets. Incubation of the sections with FITC-labelled antiserum produced a green, homogeneous fluorescence of the islets, surrounded in some instances by non-fluorescing exocrine

must be small and the technique is extremely time consuming. Costanzi *et al.* (1961) were the first to report that formalin fixation does not significantly alter the ability of the beta granules to bind with anti-insulin serum.

Formalin fixation produces a more dense and homogeneous fluorescence of the beta cells than that found in freeze dried tissue preparations, where insulin is also present in a granular form. This homogeneous fluorescence in the formalin-fixed material

may be caused by the polymerizing effect of formalin.

Mechanisms of insulin secretion have been widely studied by means of tolbutamide, using histological staining procedures. However, using an aldehyde-fuchsin staining procedure, cells other than those of the islets, e.g. in the pancreatic ducts, can also be stained. Therefore the use of purified and optimally labelled antiinsulin sera is advantageous in possessing a higher degree of specificity.

First experiments to study the secretion of insulin in rats after oral application of tolbutamide (50 mg/kg) were done by Bänder *et al.* (1957). They found that a reduction in granulation became apparent 30 min after the application, and that after 6 h the granule content had been decreased to 25% of its original value. Six hours after application of 2000 mg/kg, degranulation proceeded in the same manner, reducing the granule content to one quarter of the original. However, after a period of 24 h examination of the islets disclosed full degranulation of betacells, and a return of the islet cell system to its normal granulation density was not to be seen until 96 h after tolbutamide administration.

With the oral administration of 5 mg/kg glibenclamide in rats, Bänder *et al.* (1969) found an initiation of degranulation of the beta-cells after 4 h. The decrease amounted to about 50% of the initial content after 8 h, and was followed by an almost complete degranulation after 24 h. At this time, Kellner *et al.* (1969) still found sulphonylureas in the blood using ^{14}C -labelled glibenclamide (0.2 mg/kg). Therefore it must be postulated that this slow but progressive degranulation is the result of the continuous stimulating effect of sulphonylureas. With the electron microscopic technique the degranulation following glibenclamide application became apparent only after 6–8 h (when using 10 or 20 times the minimal effective dose) and was fully developed after 12–24 h (Kern *et al.*, 1969). The authors found the degranulation to occur most frequently in the central portion of the islets. These reports support our results obtained with the immunofluorescence technique. Even high doses of glibenclamide (5 mg/kg) were not able to produce a decrease in fluorescence in the islets after 90 min, although blood glucose levels were clearly reduced. The same results were obtained using isolated islets. After an incubation time of 90 minutes with tolbutamide, no decrease in fluorescence could be observed. These findings, however, do not exclude the possibility of quicker secretion of insulin by the beta cells. This could be demonstrated with intravenously injected antiinsulin-serum, in which instance a decrease in fluorescently stained insulin in the centre of the islets could be observed after 5 h. At this time, brilliantly fluorescing beta cells could be observed only in the islet periphery.

Previous studies (Logothetopoulos *et al.*, 1965) comparing degrees of degranulation showed a much greater loss of granules after the application of insulin

antibodies, than after infusion of glucose alone or in combination with hydroxybutyrate and acetoacetate. Thus they would suggest that circulating excess antibody will effectively bind insulin where it is present along the entire length of the sinusoids of the islets, establishing an effective diffusion gradient for insulin from the beta-cells or from extracellular space.

Our own experiments, attempting to demonstrate the injected antibody on the surface of B-cells or its binding by beta granules failed. Even FITC-labelled antibodies injected *in vivo* could not be detected in the islets. On the other hand, Blumenthal *et al.* (1964) demonstrated in chicken embryo by fluorescence microscopy that the injected antiinsulin-globulin entered the B-cells and was here bound by the beta-granules. Moreover, Lacy and Wright (1965) had observed that the intravenous or intraperitoneal injection of serum containing antibodies to bovine insulin, was followed by an acute form of allergic pancreatitis, indicating that the injected antibodies reach the insulin-producing cells and cause some form of damage. Numerous morphological examinations have shown that insulin released during the first few hours after application of sulphonylureas is too low to cause a visible loss of insulin when examined by light or electron microscopy.

These results suggest that the insulin released immediately is not stored in granules and cannot be detected by histology, immunohistology or electron microscopy. The current immunohistological studies corroborate the electron microscopic observations of Kern *et al.* (1969) that B-cells contain insulin in two different forms. One form, which may be free in the cytoplasm is released as a rapid expulsion within a few minutes. The second is released more leisurely from granules providing that the stimulating effect of the sulphonylureas is active over a longer period of time. So far, the two compartment model of Grodsky *et al.*, (1967) would find some morphological correspondence, although another experimental model with other time conditions was used. The immunohistological technique is undoubtedly of greater value for further morphological investigations employing specific antibodies against insulin precursors than is the normal histological procedure.

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