Diabetologia 9, 191–196 (1973)

Immune Reactions to Fractions of Crystalline Insulin

II. May Peri-insulitis be Produced by an Antigen Different from True Sanger Insulin?*

F.K. Jansen and G. Freytag

Diabetes Research Institute at the University of 4 Düsseldorf 18 and Pathological Institute, University of Hamburg, Germany (F.R.G.)

Received: September 7, 1972, accepted: January 12, 1973

Summary. The immunization of mice with impure, once crystallized, porcine insulin in different doses over 3 months led to a high antibody titre with 1 μg monocomponent(-MC-)porcine insulin effected only a low, antibody response after 3 months of immunization, thus proving a low immunogenicity. A single insulin adjuvant immunization differentiated between stimulated, normally reacting and tolerant animals. In stimulated animals with high titre antibodies no insulitis was found, but some animals tolerant towards insulin produced an insulitis, suggesting that this cellular immune reaction may not be induced by true Sanger insulin. The antigen responsible for insulitis may be an antigenically independent antigen contained in crystallized insulin, or an antigenically related derivative of insulin.

Key words: histological alterations, exocrine pancreas, endocrine pancreas, insulitis, periductulitis, tolerance to insulin, insulin antibodies, cellular immune reaction, crystalline insulin, chromatographed insulin, monocomponent insulin, fractions of insulin.

The active immunization of animals with crystalline insulin led to the production of insulin antibodies and lymphocytic insulitis, first described by Renold (1964) in the heifer. Toreson (1964) was able to produce insulin antibodies, insulitis and diabetes by immunizing rabbits with crystalline insulin. Federlin (1968) saw the insulitis in active immunized sheep. In this connection it is also of interest that granulocytic and monocytic infiltrates within and around the pancreatic islets, as well as in the acinar tissue, can follow the injection of antiserum against insulin and pancreatic extracts in animals (Freytag, Klöppel, 1969). Chromatographic methods for the purification of insulin (Schlichtkrull, 1969) led to a higher degree of purity of crystalline insulin, permitting the reinvestigation of the participation of contaminating antigens in the histopathological alterations induced by crystalline insulin.

An experiment designed to produce an immunological tolerance to insulin (Jansen, 1971a, b), effected with crystalline insulin and monocomponent insulin, suggested that peri-insulitis might in part be produced by impurities of the true Sanger insulin[1] (Freytag, Jansen, Klöppel, 1973). However, it could not be decided whether the responsible impurity is antigenically related to true insulin or a substance with no antigenic resemblance i.e. an independent protein of the islets of Langerhans. Therefore, in the same animals, the insulin antibody content was examined in relation to insulitis and periductulitis. The immunological part was carried out by F.K. Jansen, the histopathological part by G. Freytag.

Materials and Methods

Details concerning mice, the two insulin charges, namely 1 × crystallized porcine insulin and monocomponent (MC-) porcine insulin (kindly supplied by Dr. Schlichtkrull, Novo, Copenhagen), and histopathological methods were described in part I (Freytag, Jansen, Klöppel, 1973).

For the determination of the antibody content, 0.1 ml blood was taken from the orbit at different intervals during the immunization period (Fig. 1). This was diluted immediately in phosphate buffer, pH 7.4; 0.04 M, containing 0.5% EDTA. Blood pools of each group were diluted 1:20, individual samples 1:50, centrifuged for elimination of erythrocytes and stored at −25°C for determination of the antibody titre by radioimmunoassay. As the serum dilution after the elimination of erythrocytes could not be calculated accurately, all dilutions were related to undiluted blood.

Prior to the immunization, blood samples were taken and pooled to determine the non-specific insulin fixation of serum (Fig. 1). 10 days after the 3 months immunization period (3 × per week with 6 different doses of insulin without adjuvant), pooled blood samples were examined for antibody production. 2 days later, a single test immunization of all animals, including the controls, was performed with 100 μg 1 × cryst. insulin, in complete Freund's adjuvant, to test the immunological tolerance to insulin. The test immunization was essential to differentiate between normally reacting and tolerant animals, as only normal animals are likely to produce insulin antibodies. Blood samples were taken at 3, 5, 7 and 14 weeks after the test immunization from all animals, pooled for each group, to follow up the antibody production. 20 weeks after the test immunization, half of the animals of each group were sacrificed for histopathological examination, while the other half of each group was re-immunized subcutane-

* Published in part at the Congress of the European Association for the Study of Diabetes, Madrid 1972.

Supported by Landesamt für Forschung des Landes Nordrhein Westfalen, W. Germany.

1 True Sanger insulin = insulin corresponding exactly to Sanger's formula.

ously with 100 μg insulin in complete Freund's adjuvant in order to intensify the histopathological lesions. They were examined individually for their antibody content. The antibody titre was determined 10 days after re-immunization, these mice being sacrificed 2 weeks later.

The insulin antibody titre was determined by radio-immunoassay (Meade, 1962), utilizing ^{125}I labelled insulin (supplied by Hoechst, Frankfurt, Germany) and amberlite CG 400 I (from Serva Heidelberg, Germany). Amberlite, provided in chlorine-form, had first been separated from the chlorine with 2 N NaOH and thereafter adapted to the phosphate form at pH 7.4; 0.04 M. Samples of 1 ml from blood pool dilutions of 1/20 and 1/40 were incubated in triplicate for 18 h at 4°C with 3 μU (about 111 pg) radio-active insulin. Subsequently, 300 mg amberlite were incubated for half an hour to adsorb the free radioactive insulin, not bound by antibodies. ½ ml of the supernatant was measured in a Gamma-counter (Packard). Tubes with known antibody excess and those without antibodies served as high and low controls, thus limiting the antibody binding effect of our test system. The experimental sera were first calculated as percentages of the antibody activity between high control, set = 100% and low control, set = 0%. We then converted these values into ng antibody binding capacity — ABC 50% —, calculated for 1 ml undiluted blood at the point of 50% antigen binding. This was achieved by comparing experimental values with a dilution curve established by a pool of high titre antisera from these mice.

For determining the correlation between antibodies and histopathological findings, all values were assessed as + or — in a 4 square correlation. Antibodies of more than 0.050 ng/ml ABC 50% were regarded as +. The histopathological alterations were defined as + and — in part I (Freytag, Jansen, Klöppel, 1973).

The equation of the 4 square correlation is:

$$V = \frac{a \cdot d - b \cdot c}{\sqrt{(a+b)\,(a+c)\,(b+d)\,(c+d)}}$$

The value V varies between +1 and −1, indicating high correlations near +1 and −1, and no correlation near 0 (Lienert, 1962).

period (a) various groups of mice received different charges or doses of insulin. While stimulated animals could be recognized by their high or low antibody titre, normally reacting and tolerant animals did not produce antibodies at all. They could only be distinguished by a single test immunization of crystalline insulin in complete Freund's adjuvant (b), identical for all animals, and producing a normal antibody titre for normally reacting animals, or much fewer or no antibodies in tolerant animals.

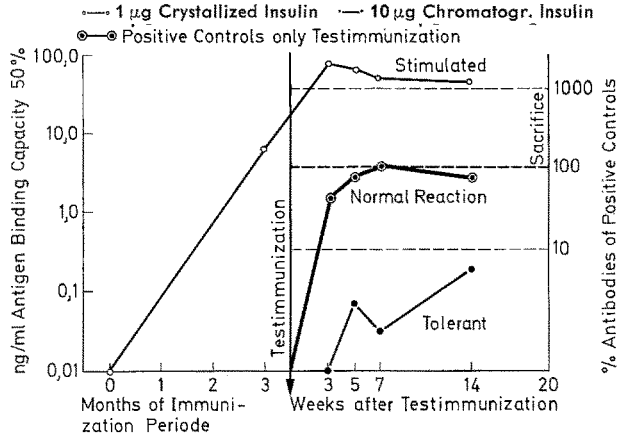

Fig. 2. Development of the antibody titre in normally reacting, stimulated and tolerant animals. The response of the positive controls getting only the test immunization is considered as a normal response and set = 100%. The dose of 1 μg crystallized insulin stimulated to much higher titres, while the dose of 10 μg chromatographed insulin produced tolerance with titres lower than 10% of the controls

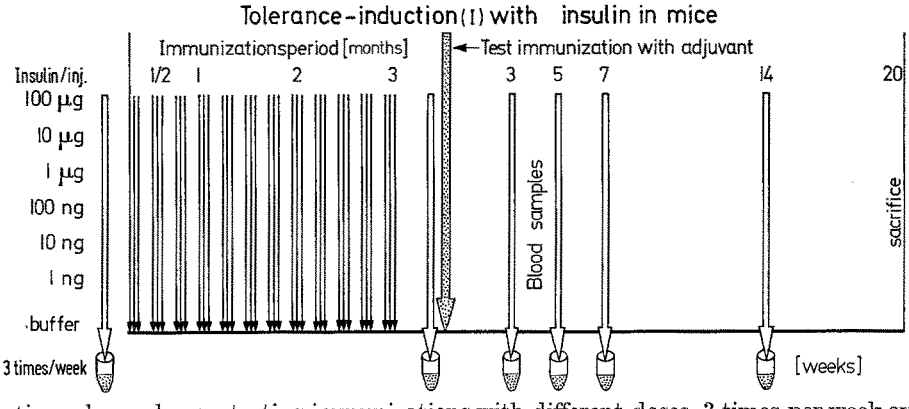

Fig. 1. Immunization scheme demonstrating immunizations with different doses, 3 times per week over 3 months, the single test immunization of 100 μg insulin in complete Freund's adjuvant 13 days later, and the different blood collections before and after the test immunization for the determination of antibodies

Results

With the immunization producing a tolerance against insulin, two different stages must be distinguished: a) the immunization period without adjuvant and b) the test immunization with complete Freund's adjuvant (Fig. 1). During the 3 months' immunization

Immunization period

In the first stage, the three months' immunization period, only one dose (1 μg) of crystallized insulin led to a high antibody production of about 5.025 ng/ml ABC, while all other doses effected no significant amount of antibodies. With chromatographed insulin, a minimal antibody production of 0.042 ng/ml ABC

50 % in the same dose range as for crystallized insulin had been stated; however, this is a very low antibody titre, as only concentrations of more than 0.010 ng/ml ABC 50 % can be determined. These antibodies are really directed against insulin, as they could be absorbed with insulin. It would have been necessary to immunize a longer time in order to obtain a higher antibody production in this dose range. The great difference in the antibody titres after a 3 months' immunization period proves that the chromatographed insulin is considerably less immunogenic.

Testimmunization

During the immunization period without adjuvant, the positive control group had not been immunized with insulin and therefore had no antibodies before the test immunization (Fig. 2). But after the testimmunization, when this control group had received the same immunization with insulin in adjuvant as the experimental animals, the antibody titre rose to a maximum within 7 weeks, remaining stable for about 20 weeks, after which all animals were sacrificed. In stimulated animals, the antibody titre rose much more rapidly, reached higher titres and decreased thereafter; yet the tolerant animals showed no antibodies or else a slow rise of the antibody titre in the first few weeks and then a rapid decline (Fig. 2). The best time for comparing the different antibody production with regard to the kinetics of their development was about 7 weeks after the test immunization, when partially tolerant animals still possessed antibodies which, however, decreased rapidly.

7 weeks after the test immunization only one dose of crystallized insulin (1 μg) was found to stimulate animals to produce a high antibody titre of about 50 ng/ml. This group is identical to the group of animals with antibodies before the test immunization (Fig. 2). Two groups showed high dose tolerance with doses of 10 μg and 100 μg. All the other doses induced a partial tolerance with intermediate antibody titres. With chromatographed insulin a dose of 10 ng effected a normal reaction, comparable with positive controls. Doses of 10 μg produced a complete high dose tolerance and the other doses a partial tolerance with intermediate antibody titres. While the stimulated group with crystallized insulin had shown a high antibody titre over more than 6 months before sacrificing, the tolerant groups (10 μg and 100 μg crystallized insulin and 10 μg chromatographed insulin) did not show any appreciable antibody content at any time. Both the stimulated and the tolerant animals are, therefore, very interesting when a comparison is made of the production of insulitis. While the determination of the individual antibody content in the stimulated animals revealed a great heterogeneity, the tolerant animals, as expected, showed no detectable antibodies. Therefore, they may be regarded as homogeneous in respect to tolerance.

Periductulitis

The comparison of insulin antibodies with periductulitis did not indicate any correlation (Fig. 3). After immunization with crystallized insulin, groups with high antibody titres (dose of 1 μg) also produced the same periductulitis as tolerant (doses 10 μg and 100 μg) and partially tolerant groups (doses 1 ng, 10 ng and 100 ng). The groups which received chromatographed MC-insulin were almost devoid of periductulitis. Independently of their antibody titre i.e. high titre antibodies, intermediate titres or completely tolerant mice, almost no periductulitis was found.

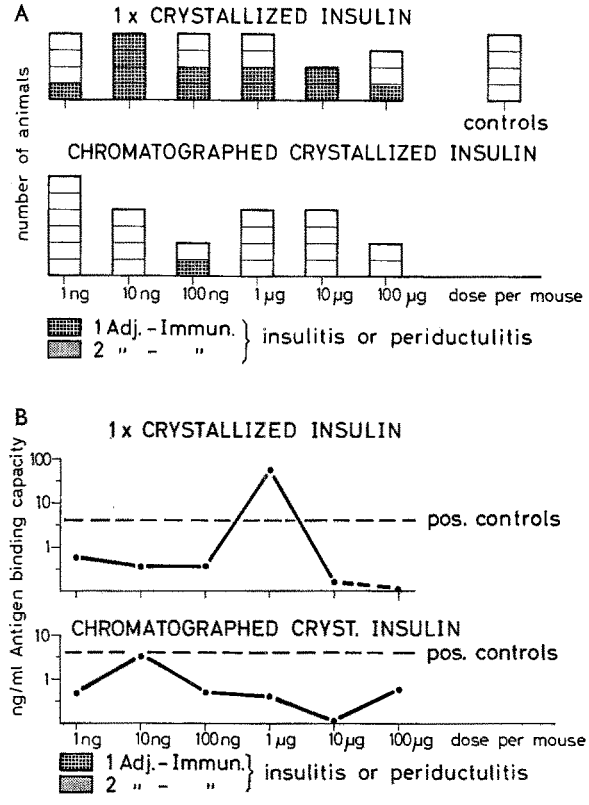

Fig. 3. Distribution of animals with periductulitis depending on the dose and the charge of insulin, i.e. whether it was impure, once crystallized, or pure, chromatographed insulin. Only animals with one test immunization were considered. A. Lymphocytic periductulitis. B. Antibodies against insulin

Accordingly, a coefficient of 0.10 was calculated in a 4 square correlation for individually examined, reimmunized mice, totalling 37 animals. 7 animals with periductulitis had antibodies. 8 with periductulitis had none, while 8 other animals without periductulitis were found to have antibodies. It is important that 3 of 5 animals tolerant to insulin (10 μg and 100 μg crystalline insulin) were able to produce the periductulitis, if only animals with one test immunization are considered.

Insulitis

Comparison of insulin antibodies with insulitis in the group of stimulated animals (1 μg cryst. insulin) possessing high titre antibodies over more than 6 months are significant because in 7 animals no insulitis resulted, suggesting that high titre antibodies against insulin are not responsible for the insulitis (Fig. 4). As the insulitis is a cellular immune reaction, it may be independent from insulin antibodies, though directed against insulin. Therefore, the behaviour of the tolerant

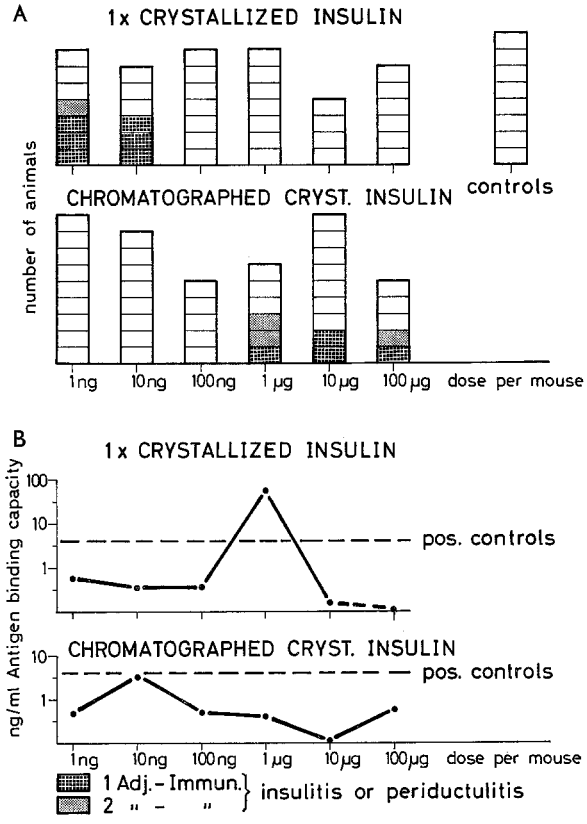

Fig. 4. Distribution of animals with peri-insulitis depending on the dose and the charge of insulin (once crystallized impure or chromatographed highly purified insulin). The antibody titres 7 weeks after the test immunization are set in relation to a normal response, as represented by the positive controls. Higher antibodies represent stimulated, lower antibody titres tolerant groups. A. Lymphocytic insulitis. B. Antibodies against insulin

animals is important. Although the tolerant groups treated with crystallized insulin had no insulitis, the tolerant group with a high dose (10 μg) of chromatographed insulin showed an insulitis in 2 animals out of 9 (=22 %).

A lack of correlation is apparent in the individually examined, re-immunized mice, which showed a heterogeneous antibody response. The statistical correlation between the individual antibody content and appearance of insulitis in a 4 square correlation revealed a coefficient of 0.001 for 37 animals, an indication that no common mechanism exists for insulin antibody production and insulitis. This is best illustrated by the insulitis found in 4 of the re-immunized animals of which 2 possessed good antibodies, the other 2 being without. 13 animals with good antibody titres had no insulitis.

Discussion

The successful production of insulitis, insulin antibodies and diabetes in the rabbit by immunization with crystalline insulin (Toreson, 1964) suggested that all these alterations were due to the insulin molecule. However, Mirsky (1966) showed that crystalline insulin is heterogeneous and Schlichtkrull (1969) demonstrated that it contains higher molecular-weight impurities, i.e. insulin dimer, proinsulin, intermediate insulin and others, which are more immunogenic than the true Sanger insulin. As immunizations with different doses of impure crystallized and pure chromatographed insulin induced the insulitis in considerably different dose ranges (part I) we proposed the hypothesis that periductulitis and periinsulitis are not induced by the true Sanger insulin molecule but by an impurity of crystallized insulin.

Periductulitis

Periductulitis and insulitis were dissociated in the experiment with chromatographed (MC-)insulin in which the insulitis-producing antigen remained while the periductulitis antigen had been eliminated, as described in part I (Freytag, Jansen, Klöppel, 1973). These two antigens may therefore be considered as antigens with different physico-chemical properties, though a weak crossreactivity cannot yet be excluded. The occurrence of periductulitis in groups tolerant to insulin is highly suggestive of the presence of an antigen different from insulin. The lack of correlation in individually examined animals confirms this belief. Two arguments are therefore in favour of the hypothesis that the antigen responsible for periductulitis is different from insulin:

1. it can be dissociated by chromatography from the antigen responsible for insulitis,
2. tolerant animals towards insulin and those tolerant towards the insulitis reaction (probably in high doses of crystallized insulin) nevertheless developed periductulitis.

Insulitis

In part I we were unable to decide whether the antigen responsible for the insulitis was perhaps an antigenically related impurity of insulin, i.e. an insulin -oligomer, -polymer, or insulin as hapten, or whether it may be a substance antigenically unrelated to insulin. The behaviour of the insulin antibody titre favoured the argument that the responsible antigen is

probably independent from true Sanger insulin. The individually examined animals showed no correlation between insulitis and insulin antibodies. In addition, animals with high antibody titres persisting over more than 5 months (1 μg crystallized insulin and 10 ng chromatographed insulin) showed no insulitis. Because in cellular and humoral immunologic reactions different mechanisms are involved — cellular reactions are effected by thymus-derived and humoral reactions by bone marrow-derived lymphocytes — a dissociation between both mechanisms may be possible. The cellular reaction may have been inhibited by the antibodies, a well known fact called enhancement phenomenon, which would have been probable in this case if the tolerant mice had not produced an insulitis. According to the concept of Weigle cellular immune reactions become more readily tolerant than the humoral antibody-producing reactions, i.e. more rapidly and with much smaller doses (Weigle, 1971). A high dose tolerance against humoral immune reactions therefore implies a tolerance against the cellular reaction. We presume that with a dose of 10 μg chromatographed insulin a high dose tolerance against insulin is achieved. Nevertheless, it produced an insulitis which favours the hypothesis that the cellular reaction of the insulitis may not be directed against the insulin molecule, but against a protein antigenically different from insulin, contained in crystallized insulin.

An objection may be raised against the interpretation of a high dose tolerance with 10 μg chromatographed insulin, as it is surprising that the higher dose of 100 μg insulin produced antibodies. But the development of the tolerance after 3 months preceded a transient antibody stimulation 2 weeks and 1 month following the beginning of the immunization period, decreasing to a partial tolerance later (Jansen, 1971 b). With the dose of 10 μg a complete tolerance is still achieved. With the dose of 100 μg it would have been reached some time later.

Two arguments are now in favour of the hypothesis that the insulitis is not produced by the true Sanger insulin:

1. the different dose-effect of pure and impure insulin (part I),
2. the appearance of insulitis in tolerant animals.

The last argument suggests, furthermore, that the insulitis antigen may be a protein of the islets antigenically different from insulin. As this antigen is difficult to separate from insulin by chromatography, it should have physico-chemical properties similar to insulin. While chromatographed insulin is almost free of the periductulitis-inducing antigen, it nevertheless contains traces of the antigen responsible for insulitis, but it is already highly purified. Thus chromatography seems to be the only way to eliminate contaminating antigens from crystallized insulin responsible for periductulitis and insulitis.

Another possible explanation for our findings would be the hypothesis that the true Sanger insulin is only slightly immunogenic with respect to insulitis but becomes more immunogenic in the presence of the crossreacting, higher molecular-weight impurities of crystallized insulin. In chromatographed insulin lacking these impurities the original low immunogenicity of the true Sanger insulin is seen. But in this case tolerance to insulin would not correspond to Weigle's hypothesis, that high dose tolerance to antibody-formation implies tolerance to the cellular reaction. Isolation and purification of the different impurities of crystallized insulin are now under investigation to elucidate the underlying mechanisms.

Acknowledgements. We wish to express our thanks to Miss H. Dorsel, G. Nahler and Mrs. R. Rusniak for their skillfull technical assistance and to Prof. Knußmann for his statistical advice.

References

Federlin, K., Renold, A.E., Pfeiffer, E.F.: Antigen-binding leucocytes in patients and in insulin-sensitized animals with delayed insulin allergy. Immunopathology Vth Int. Symposion p. 107. Ed. by P.A. Miescher und P. Graber, Basel/Stuttgart: Schwabe und Co. Publ. 1968.

Federlin, K.: Immunopathology of insulin. Monographs on Endocrinology **6**, 1—158 (1971).

Freytag, G., Klöppel, G.: Experimentelle Insulitis und Pankreatitis nach Immunseren gegen Pankreasextrakte verschiedener Reinheitsgrade. Beitr. path. Anat. **39**, 138 (1969).

Freytag, G., Klöppel, G., Howe, I.: Zur Pathogenese der experimentellen Insulitis. Verh. Dtsch. Ges. Path. **53**, 423 (1969).

Freytag, G., Menke, B.: Latenter Diabetes mellitus bei Meerschweinchen während der aktiven Immunisierung gegen Fremdinsulin. 16. Symp. Dtsch. Ges. Endokrinologie, p. 65. Berlin-Heidelberg-New York: 1970.

Freytag, G.: Immunpathologie des Diabetes mellitus. Veröffentlichungen aus der Morphologischen Pathologie. Vol. 88, 1—101 edit. by W. Giese, W. Büngler, G. Seifert und G. Peters. Stuttgart: Gustav Fischer Verlag 1972.

Freytag, G., Jansen, F.K., Klöppel, G.: Immune Reactions to Fractions of Crystalline Insulin I. Significance of Lymphocytic Infiltrates in the Endocrine and Exocrine Pancreas of Mice. Diabetologia **9**, 185—188 (1973).

Jansen, F.K.: Tolerance to high and low doses of natural crystalline insulin. Diabetologia **7**, 290—292 (1971a).

Jansen, F.K.: The ability of MC-insulin or 1 × crystallized insulin in the development of immunological tolerance in mice. Diabetologia **7**, 485 (1971b) Abstr.

Klöppel, G., Altenähr, E., Freytag, G.: Studies on ultrastructure and immunology of the insulitis in rabbits immunized with insulin. Virchows Arch. Abt. A. Path. Anat. **356**, 1—15 (1972).

LeCompte, P.M., Legg, M.A.: Insulitis (lymphocytic infiltration of pancreatic islets) in late-onset diabetes. Diabetes **21**, 762—769 (1972).

Lienert, G.A.: Verteilungsfreie Methoden in der Biostatistik. Maisenheim am Glan: Verlag Anton Hain, 1962.

Meade, R.C., Klitgaard H.M.: A simplified method for immuno-assay of human serum insulin. J. nucl. Med. **3**, 407—416 (1962).

Mirsky, I.R., Kawamura, N.: Heterogenicity of crystalline insulin. Endocrinology **78**, 1115—1119 (1966).

Renold, A.E., Soeldner, J.S., Steinke, J.: Immunological studies with homologous and heterologous pancreatic insulin in the cow. Ciba Foundation Colloquia Endocrinology **15**, 122 (1964).

Schichtkrull, J., Brange, J., Ege, H., Hallund, O., Heding, L.G., Christiansen, A.H., Jorgensen, K., Markussen, J., Stahnke, P., Sundby, F., Volund, A.: Proinsulin and related proteins. European Association for the Study of Diabetes. 5th annual meeting, Montpellier 1969.

Toreson, W.E., Feldman, R., Lee, J.C., Grodsky, G.M.: Pathology of diabetes mellitus produced in rabbits by means of immunization with beef insulin. Amer. J. clin. Path. **42**, 531 (1964).

Weigle, W.O.: Recent observations and concepts in immunological unresponsiveness and autoimmunity. Clin. exp. Immunol. **9**, 437–447 (1971).

Dr. F.K. Jansen
Diabetes-Forschungsinstitut
4000 Düsseldorf 18
Hospitalstr. 1
Federal Republik of Germany