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Quantitative Morphological Analysis of Glomerular Changes of the Rabbit Kidney on Treatment with Insulin Preparations of Different Purity

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Summary. Thirty rabbits were immunized with MC insulin and high-molecular impurities of commercial insulin preparations (a+b component) over 30, 60 and 90 days. The serum insulin antibody titer was determined in animals as the insulin binding capacity. Further, a quantitative morphological analysis of the various types of glomerular cells and of the mesangium was performed on the glomeruli as a blind study. Significant mesangial cell proliferation and an increase in mesangial matrix were found on treatment with the a+b component whereas the animals treated with MC insulin exhibited only a transient and slight mesangial activation after 30 days. There was a positive correlation between the magnitude of the insulin binding capacity and the mesangial activation. Hence, the glomerular changes which are observed after treatment with insulin which is not highly purified must be attributed to the high molecular weight contaminants. Heterologous pure insulin must be regarded as having virtually no immunological effect.

Key words: Insulin – Antibody – Glomerulus – Mesangium – Quantitative morphology.

Introduction

The diabetic metabolic state (Kuhlmann et al., 1969; Lundbaek et al., 1970; Olsen, 1971, 1972; Williamson et al., 1971; Østerby, 1972; Bloodworth et al., 1973), genetic factors (Siperstein et al., 1968; Kreines et al., 1970; Oppermann et al., 1973a, b; Salazar et al., 1973; Vracko and Benditt, 1974) and immunological processes have been discussed in considering pathogenesis of diabetic glomerulosclerosis, possibly induced by abnormal endogenous or by exogenous insulins (Burkholder, 1968; Wehner and Anders, 1970; Wehner, 1970, 1971; Fuchs et al., 1973; Klein, 1973).

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Immunological processes acquired particular importance following Schlichtkrull's (1970, 1971) success in fractionating crystalline insulin into a-, b- and c-components. According to his investigations the c-component (or main component) which constituted "pure" insulin (MC-insulin), possessed not antigenic properties. On the other hand the a- and b-components which consisted of high molecular contaminants such as proinsulin, intermediary, dimer, etc. induced marked antibody formation (Schlichtkrull, 1970, 1971).

The purpose of our investigations in rabbits was to study whether glomerular changes occur after immunization with the various insulin components. We had previously found alterations of the glomerular basement membrane whose incidence appeared to be related to the antigenicity of the components employed (Wehner et al., 1973).

Material and Methods

Experimental Animals

We carried out the investigations in a total of 52 male fully grown rabbits of similar age (4 to 5 months old) (Belgian giants, piebalds). These were allocated to the following groups:

I. Normal Animals: Seven animals remained untreated over a period of 3 months as normal controls.

II. MC Group: Fifteen animals received 20 units MC insulin (0.5 ml) subcutaneously into the cervical region three times weekly up to a period of 3 months. Treatment was carried out without Freund's adjuvant. Five animals were killed at intervals of 1, 2 and 3 months respectively.

III. a+b Component Group: Fifteen animals received 0.8 mg a+b component (0.5 ml) injected subcutaneously into the cervical region three times weekly. Five animals were sacrificed 1, 2 and 3 months respectively after the commencement of the experiments. Treatment was carried out without Freund's adjuvant.

IV. Control Group: a) Five rabbits received 0.5 ml of the solvent used for the MC insulin subcutaneously three times weekly over a period of 3 months. b) Ten animals received 0.5 ml of the solvent used for the a+b component subcutaneously three times weekly. Five animals were killed 1 and 3 months respectively after commencement of the experiments.

All animals received 5 per cent glucose solution to drink, to avoid hypoglycemic episodes in those animals treated with insulin. The animals had free access to food (Altromin-Standard).

Insulin Preparations

1. MC Insulin. This is a monocomponent insulin from porcine pancreas in neutral solution. It has a molecular weight of 6,000. Each ml of the solution contains 40 units of insulin.

2. a+b Component. This consists of partial fractions which were obtained in the purification of crystalline bovine insulin. The a-component contains high-molecular proteins (molecular weight: 25,000) and the b-component contains proinsulin, intermediary and dimer (molecular weight: 9,000–12,000). In the experimental animal the solution showed an insulin activity corresponding to 6–8 units/ml. (The insulin-preparations were generously supplied by Dr. J. Schlichtkrull, Novo Research Institute, Copenhagen, Denmark.)

Solvents

1. Solvent for MC Insulin. This consists of a neutral solution of 7 mg NaCl, 1.4 mg sodium acetate ($3H_2O$) and 1 mg methyl p-oxybenzoate in 1 ml distilled water.

2. *Solvent for a+b Component.* This consists of a solution of 0.04 mg zinc chloride, 50 mg glucose and 1 mg methyl p-oxybenzoate in 1 ml distilled water (pH 2.9). Hence all the animals received a total of 18 mg methyl p-oxybenzoate injection over the three month's experimental period.

Histological Methods

The kidneys of all animals were fixed immediately after killing in buffered 4% formalin, fixed again for 2 h in buffered 2% osmic acid and embedded in plexiglass. Sections 0.5–1 μ in thickness were then silver impregnated after Movat (Wehner, 1970).

Histometric Methods

In order to standardize the results as far as possible all three investigators evaluated the same 100 glomeruli independently. After it had been demonstrated that no significant differences existed between the results of different observers the actual investigations were carried out.

They were performed as a blind study, i.e. the investigator was not aware to which experimental group the preparations studied belonged. Classification was not carried out until the mathematical part of the investigations had been concluded. In this manner 50 different glomeruli per animal were evaluated; the following variables were determined:

1. The glomerular diameter (limited by Bowman's capsule) at ×500 magnification using a crossline micrometer (net value = 12.5 μ). The glomerular section area was calculated as a circular area in sq.μ.

2. The glomerular cell count and the counts of the different glomerular cell types, by direct counting of the nuclei in the microscopic picture. From this we calculated the percentage distribution.

3. The percentage mesangial fraction of the glomerular section area by the point counting technique, using a Reichert Visopan (object lens plan 63/0.75; 160/0.17; scale 800:1), point distance 9 μ. We calculated the absolute mesangial surface area in sq.μ from the glomerular section area in sq.μ and the percentage mesangial fraction.

4. The glomerular cell density was calculated as the cell count per 1,000 sq.μ of the glomerular area.

5. The mesangial cell density resulted from the mesangial cell count in 100 sq.μ mesangial surface area.

Antibody Determination

The insulin antibody titer was determined as the insulin binding capacity (IBC) of the serum in μg insulin/ml serum. The determination was carried out using the method of Kallee et al. (1963) with agar gel electrophoresis and autoradiography (^{125}I insulin) in the manner previously described by us (Wehner et al., 1972). The determination was carried out in each case at the time when the animals were killed, i.e. 1, 2 and 3 months respectively after commencement of immunization. In the animals treated with the solvents this was after 1 and 3 months.

Statistical Methods

Statistical evaluation of the main results was performed using Student's t-test. 5% ($p = 0.05$) was chosen as the limiting value for the error probability.

Results

I. Normal Rabbits

The following mean values were found for a normal rabbit glomerulus: With a mean section area of 7,449 sq.μ and a mean total cell count of 35.5 cells

Table 1. Morphometric glomerular parameters and insulin binding capacity in normal, MC insulin- and solvent-treated rabbits (Means ± SD)

	I normal	II MC-insulin			IVa MC-solvent
		1 month	2 months	3 months	
No. of investigated glomeruli	350	250	250	250	250
No. of total cells	35.5 ± 0.9	34.4 ± 0.3	31.2 ± 0.8	33.8 ± 1.3	33.8 ± 1.6
Epithelial cells	10.2 ± 0.3 (28.9 ± 1.1)[a]	9.9 ± 0.8 (29.2 ± 3.8)	9.5 ± 1.0 (30.8 ± 1.2)	10.4 ± 0.6 (30.9 ± 4.7)	10.2 ± 0.5 (30.3 ± 2.6)
Endothelial cells	19.5 ± 0.9 (54.8 ± 1.4)	17.8 ± 1.0 (51.7 ± 1.4)	17.3 ± 2.4 (55.1 ± 3.9)	18.2 ± 2.3 (53.8 ± 3.8)	18.1 ± 2.6 (53.4 ± 2.3)
Mesangial cells	5.8 ± 0.3 (16.3 ± 0.8)	6.5 ± 0.4 (19.0 ± 0.8)	4.3 ± 0.4 (13.9 ± 0.7)	5.1 ± 0.6 (15.2 ± 1.2)	5.4 ± 0.7 (16.1 ± 0.4)
Total glomerular area (sq.μ)	7449 ± 488	7050 ± 703	6221 ± 620	6530 ± 1140	7278 ± 638
Mesangial area (sq.μ)	666 ± 41	615 ± 61.1	533 ± 50.3	575 ± 112	644 ± 53
Mesangial area (%)	9.0 ± 0.1	8.7 ± 0.3	8.6 ± 0.2	8.7 ± 0.2	8.8 ± 0.2
Total cells/1000 sq.μ glomerular area	4.9 ± 0.3	4.8 ± 0.5	5.0 ± 0.4	5.2 ± 0.4	4.6 ± 0.4
Mesangial cells/100 sq.μ mesangial area	0.9 ± 0.06	1.1 ± 0.08	0.8 ± 0.04	0.9 ± 0.1	0.85 ± 0.07
IBC/μg insulin ml serum	–	0.17 ± 0.03	0.17 ± 0.04	0.27 ± 0.02	0.12 ± 0.02

[a] The percentage fraction of the total cell count is shown in brackets

we differentiated between 19.5 (54.8%) endothelial cells, 5.8 (16.3%) mesangial cells and 10.2 (28.9%) epithelial cells (Table 1). The mean percentage mesangial fraction of the total glomerular surface area was 9%. The glomerular cell density was 4.9 total cells per 1,000 sq.μ glomerular surface area, the mesangial cell density was 0.9 mesangial cells/100 sq.μ mesangial area (Table 1).

II. MC Group

As compared with the normal animals the animals which had received MC insulin over a period of 30 days showed a significant increase in the percentage mesangial cell fraction from 16.3 to 19%. Likewise the mesangial cell density was significantly increased from 0.9 to 1.1 cells per 100 sq.μ mesangium (Table 1). The remaining values did not show any significant differences as compared with the normal values at this time. After 2 months' immunization with MC insulin a return to normality was found as compared with the values found after 1 month, i.e. the mesangial cell density was now within the normal range (0.8 cells/100 sq.μ), similarly the mean mesangial cell fraction was now normal (13.9%) (Table 1). Thus one observes a regression of the proliferative tendency

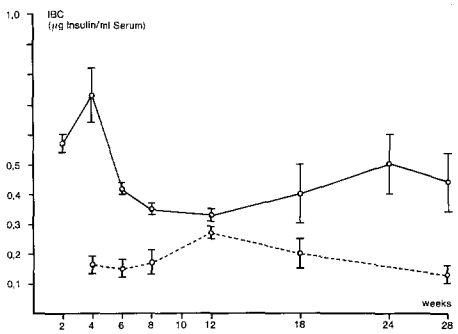

Fig. 1. Behaviour of the insulin binding capacity (IBC) in rabbits after treatment with different insulin preparations over various periods of time (Means ± SD) (a + b) component —, MC insulin ----)

of the mesangial cells after longer immunization with MC insulin. This was also evident from the values found in the animals treated for 3 months in which none of the variables studied show any significant deviation from the normal (Table 1).

The antibody titers in the animals treated for 1 and 2 months showed an IBC of 0.17 µg insulin/ml serum, and the titer in those treated for 3 months, namely 0.27 µg insulin/ml serum, was not significantly increased (Table 1, Fig. 1).

III. a + b Component Group

When compared with the normal animals the glomeruli of the animals treated for 30 days with a + b component were richer in mesangial cells, a significant increase in these cells from 16.3 to 26.9% was found. The increase in mesangial surface area to 9.7% was also statistically significant. A decrease in endothelial cells was noted (Table 2). The mesangial cell density, a reliable criterion of mesangial cell proliferation, was significantly increased when compared with normal, namely from 0.9 to 1.4 mesangial cells/100 sq.µ mesangium (Table 2). The remaining variables did not show any statistically significant changes.

After 2 months' treatment the signs of proliferation, i.e. an increase in the mesangial cells and an increase in the mesangial cell density, were still present (Table 2). However, the main change at this time was an increase in the mesangial fraction from 9 to 13.2% (Table 2). The findings in the animals which had been treated with a + b component for 3 months were practically identical. In these animals there was also an increase in mesangial cells from 16.3 to 25.4% and a significant increase in the mesangial cell density from 0.9 to 1.1 cells/100 sq.µ mesangium. Further, the mesangial fraction was significantly increased as compared with the normal animals, namely 12.5% vs. 9% and 867 sq.µ vs. 666 sq.µ respectively (Table 2).

This significant and persistant increase in mesangial cell proliferation when compared with the MC group was consistent with the insulin antibody titers. Thus, in those animals treated for 30 days with a + b component which showed the greatest mesangial cell proliferation, one also finds the highest mean antibody titer with an IBC of 0.73 µg insulin/ml serum. In the groups of rabbits treated

Table 2. Morphometric glomerular parameters and insulin binding capacity in normal, a-b component- and solvent-treated rabbits

	I normal[a]	III a+b component			IV a+b solvent	
		1 month	2 months	3 months	1 month	3 months
No. of investigated glomeruli	350	250	250	250	250	250
No. of total cells	35.5	33.7±2.5	31.7±1.0	36.0±2.5	30.6±0.9	33.9±3.2
Epithelial cells	10.2 (28.9)[b]	10.1±0.3 (30.6±2.6)	9.5±0.5 (29.8±1.2)	10.8±0.5 (30.3±1.6)	9.6±0.3 (31.4±1.2)	9.8±0.3 (28.9±2.2)
Endothelial cells	19.5 (54.8)	15.0±1.5 (43.9±1.8)	15.1±0.3 (47.7±0.9)	16.0±1.7 (44.2±1.6)	14.2±0.5 (46.5±1.5)	17.5±2.0 (51.6±2.3)
Mesangial cells	5.8 (16.3)	8.7±0.9 (26.9±1.1)	7.1±0.4 (22.4±0.7)	9.1±0.5 (25.4±0.5)	6.8±0.5 (22.1±1.0)	6.6±1.2 (19.5±1.0)
Total glomerular area (sq.μ)	7449	6657±561	5729±577	6939±604	6012±368	7251±606
Mesangial area (sq.μ)	666	636±58	750±65	867±81	613±23	780±51
Mesangial area (%)	9.0	9.7±0.3	13.2±0.5	12.5±0.4	10.3±0.6	10.7±0.7
Total glomerular cells/ 1000 sq.μ glomerular area	4.9	5.2±0.2	5.7±0.5	5.2±0.1	5.2±0.3	4.7±0.2
Mesangial cells/ 100 sq.μ mesangial area	0.9	1.4±0.03	1.0±0.05	1.1±0.05	1.1±0.04	0.85±0.07
IBC/μg insulin ml serum	–	0.73±0.09	0.35±0.02	0.33±0.02	0.12±0.01	0.12±0.01

[a] The values for SD are not shown in this case – see Table 1
[b] The percentage fraction of the total cell count

for 2 and 3 months respectively the antibody titer was lower (0.35) but significantly higher than in the MC animals. This pattern parallels the persistence of the mesangial cell proliferation and the increase in mesangial matrix observed at this time (Table 2, Fig. 1).

IV. Control Group

a) Solvent for MC. After 3 months' treatment with the solvent used for MC insulin we did not observe any significant changes in the variables studied, when compared with the normal (Table 1).

b) Solvent for a+b Component. We observe a transient and slight reaction of the mesangium in this group after 1 month, for both the mesangial cell content and the mesangial cell density were increased. In the 3 months' group we did not observe any differences in the variables studied when compared with the normal. Antibody formation was not demonstrable (Table 2).

Discussion

To summarize, it is evident that treatment of rabbits with MC insulin without Freund's adjuvant a slight and transient mesangial cell proliferation is observed after 1 month. This is only demonstrable employing morphometric methods. After treatment of longer duration the morphometric variables are completely normalized. No significant antibody formation to the MC insulin was observed.

On the other hand, in animals which had been treated with a+b component and which showed marked antibody formation, there was mesangial cell proliferation, demonstrable over the entire period of the experiments. Additionally there was an increase in mesangial matrix.

It should also be mentioned that on treatment of the animals with the solvent used for MC insulin no mesangial cell reaction was observed, whereas a transient and slight mesangial cell reaction was found in animals treated for 1 month with the solvent used for a+b component. After treatment of longer duration the morphometric variables return to normal.

One can conclude from these findings that treatment with pure insulin (MC insulin) induces practically no antibody formation whereas the impurities in insulin preparations (a+b component) provoke marked antibody formation. Further, the mesangium is shown to be a very sensitive and easily irritated glomerular structure which reacts to very slight stimuli. These changes, however, may only be demonstrable with the help of morphometric techniques. These results confirm our own findings (Wehner et al., 1973).

Freytag et al. (1973) and Jansen and Freytag (1973) have discussed a contaminating protein in crystalline insulin, and were able to induce insulitis with this on immunization of mice. This change did not occur until high doses of MC insulin were used. Schlichtkrull (1970, 1971) also considers impurities to be responsible for the antigenic properties of commercial insulin preparations. Other authors who have investigated antibody formation to insulin in diabetics found that on treatment with MC insulin antibody formation was absent or slight (Wegmüller and Fankhauser, 1970; Fankhauser and Michl, 1971; Andreani et al., 1972; Korp and Levett, 1973). However, apart from contaminants other factors also play a role in determining antigenicity, including the species (bovine, porcine) or the pH value of the solvent (Deckert et al., 1972). These authors found that acid insulin-preparations have greater antigenic properties than preparations in neutral solvent. Likewise the proinsulin contained in the b-component plays an important role in antibody formation (Chance and Ellis, 1969; Melani, 1970; Steiner, 1972). However Hinke et al. (1970) and Kerp et al. (1970) are of a different opinion, since they found only low concentrations of specific antibodies to proinsulin in the serum of insulin-treated diabetics. Although it is uncertain which of the contaminants exerts the greatest antigenic effect, our own investigations show that insulin itself is unimportant in this respect. Consequently, descriptions of previous investigations in which glomerular changes were found after immunization with heterologous insulin and in which these changes were attributed to the insulin (Grieble, 1960; Mohos et al., 1963; Ditscherlein et al., 1967; Wehner et al., 1969, 1970; Fuchs et al., 1973; Steiner et al., 1970) will have to be amended. It is evidently not the insulin

which was responsible for the immunoreaction but the impurities contained in the preparations used. The glomerular changes seen (mesangial activation) are not characteristic reactions to the high molecular impurities (a+b component), but it is possible that they appear on immunization with other high molecular weight proteins (Mauer et al., 1973). The impurities however, attain particular importance in the insulin therapy of diabetes mellitus.

As to the question of the role played by insulin in the etiology of glomerular changes (referring to glomerulosclerosis) the only possibility which remains is the formation of an abnormal endogenous insulin which possesses antigenic properties. This hypothesis is rejected by Berson and Yalow (1965). Other authors have found differences in the biological activity of insulin of healthy persons and that of diabetics (Rot et al., 1968, 1971). Penchev et al. (1968) demonstrated precipitating antibodies in the serum of untreated diabetes and Ohneda et al. (1974) found autoantibodies to insulin in a hypoglycemic woman not exposed to previous insulin therapy.

An example of the sensitivity of the mesangial cells is provided by the observation which we made on animals treated for 1 month with the solvent for the a+b component (pH 2.9). The substance methyl p-oxybenzoate belongs to the group of the allergenic p-benzoic acid esters (Christ et al., 1966; Wodniansky, 1973; Schamberg, 1967; Fiedler, 1971). Since the pH value of the solvent used for MC insulin is different from that of the solvent used for a+b component, and since we did not find any changes on treatment with the MC solvent, we would postulate that the pH value of the preparations is important. These findings are in agreement with the observations of Deckert et al. (1972).

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