On the Pathogenesis of Glomerular Lesions in the Alloxan Diabetic Rat

A Light Microscopic, Immunofluorescent and Ultrastructural Study, Including the Effects of Insulin Treatment and Immunosuppression

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ON THE PATHOGENESIS OF GLOMERULAR LESIONS IN THE ALLOXAN DIABETIC RAT

A Light Microscopic, Immunofluorescent and Ultrastructural Study, Including the Effects of Insulin Treatment and Immunosuppression

by

Erik Hägg

UMEÅ 1974
This thesis is based on the following papers:


These papers will be referred to by their Roman numerals.
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INTRODUCTION

The term "diabetic microangiopathy" is used to denote the small blood vessel abnormalities occurring in patients with diabetes mellitus, both of the juvenile and the adult type (cf. 73, 82). The lesions of the small blood vessels, i.e. arterioles, venules and capillaries, appear to be widespread throughout the body. However, the vascular changes in the kidney and retina are best known and have been extensively studied due to their great clinical importance. A common morphological denominator of this vascular disease is a periodic acid-Schiff-(PAS)-positive thickening of the vessel walls. Ultrastructurally there is an increased width of the capillary basement membrane. The concept microangiopathy also includes some paravascular changes in diabetes, e.g. the lesions in the mesangium of the kidney glomeruli. In this context it may be noted that a PAS-positive thickening of nonvascular basement membranes, e.g. of the ciliary processes in the eye (92), also have been described in diabetes.

Etiology of diabetic microangiopathy.

The etiology of the diabetic microangiopathy is still obscure. It has been proposed that the vascular disease is a genetically determined component of diabetes mellitus. Microangiopathy would then be a parallel phenomenon to the metabolic disturbance or it might actually be the primary lesion of diabetes, leading to inadequate insulin secretion (78) and/or to an impairment of the exit of insulin from the vessel lumen, thus preventing its metabolic effect in peripheral tissues (16). Siperstein et al. (79) reported increased thickness of the basement membrane of muscle capillaries in genetic prediabetics which would support this theory. However, a relatively mild, fluctuating carbohydrate intolerance cannot be excluded in such patients (44), nor other subtle metabolic abnormalities. The study of Siperstein et al. (79) can also be criticized from other aspects (53, 91), e.g. the mean age was not comparable in the control and prediabetic group. Further arguments supporting the genetic theory can be gathered from reports of individuals with typical microvascular lesions but without evidence of carbohydrate intolerance (cf. 73). Here also, there is a possibility that diabetic metabolic disturbances may have been present even though not demonstrable with the laboratory methods used at the time vascular disease was noted. Moreover,
such patients are rare. Even if the support for the genetic theory rests on a weak basis it is, nevertheless, quite possible that there may be a variable degree of genetic disposition for microvascular abnormalities in diabetic individuals (cf. 74).

Apart from possible genetic factors, there is today overwhelming evidence, as noted below, that the diabetic metabolic derangement per se is important for the development of the small blood vessel disease in diabetics.

1. According to the majority of larger clinical and histopathological studies, juvenile diabetics have, with few exceptions, no signs of microangiopathy at the clinical onset of acute diabetes, and there is an increase of the incidence and severity of the microvascular abnormalities with the duration of diabetes (cf. 73). Furthermore, most carefully and comprehensively performed quantitative electron microscopic investigations in such patients confirm these observations (46, 67, 68, 70).

2. There is a positive correlation between the progress of the vascular disease and poor control of diabetes according to the majority of clinical studies in which the influence of control to the development of vascular complications was evaluated (cf. 15, 18, 47). However, this relationship has not been definitely proven because of the limitations of the studies performed, e.g. absence of adequate control groups and reliable measurements of vascular disease and unsatisfactory conditions in measuring and supervising the degree of diabetes control. Moreover, all investigations except a few were retrospective. It must also be pointed out that the mentioned correlation does not necessarily imply that poor control causes the lesions.

3. Microangiopathy occurs in patients with long-standing secondary diabetes, e.g. haemochromatosis and chronic pancreatitis with carbohydrate intolerance (8, 34, 39). Possible explanations of the paucity of reports on microangiopathy in patients with secondary diabetes are that the patients do not live long enough to develop vascular lesions and that the lesions are mild and therefore might escape detection (34).

4. Animals with experimental diabetes, including alloxan diabetic rats, develop small blood vessel changes similar to human diabetic microangiopathy (13, 31, 66). Bloodworth et al. (13) reported nodular glomerulosclerosis and a typical retinopathy, as well as thickening of capillary basement membranes in muscle, retina and glomeruli in dogs made dia-
abetic by alloxan or growth hormone.

Pathogenesis of diabetic microangiopathy.

The sequence of events following the primary cause of the microangiopathy are also unknown. The vessel disease might be a direct consequence of metabolic disturbances (cf. 80). Furthermore, it has been suggested that the vascular lesions are mediated through an increased secretion of growth hormone (54).

Immunogenic theory. Immunological mechanisms, either secondary to the metabolic derangement or initiated in other ways, have also been proposed to be involved in the pathogenesis of diabetic microangiopathy for several reasons.

1. The demonstration of immunoglobulins, especially IgG, and complement with immunofluorescent technique in small vessel walls, including the glomerular tuft of diabetics (cf. 89).

2. Morphological similarities between the diabetic small blood vessel disease and the vascular lesions seen in certain diseases where immunological reactions are considered to be of pathogenetic importance, e.g. systemic lupus erythematosus and glomerulonephritis (14).

3. Insulin has been proposed to be a possible antigen in the supposed immunological reactions in the vessel walls of diabetics (cf. 89); diabetic subjects treated with commercial insulin regularly develop circulating insulin antibodies (II). Reports on binding of fluorescent insulin and anti-insulin sera to the same vascular structures that bind immunoglobulins in diabetes mellitus gave further support for this hypothesis (cf. 89). Moreover, glomerular lesions similar to human diabetic glomerulosclerosis could be induced in nondiabetic animals (rabbits and guinea pigs) by giving them injections of heterologous insulin (2, 14, 55, 60).

The reasons mentioned above, considered to support the immunogenic hypothesis of pathogenesis of diabetic small blood vessel disease, can, however, be subjected to criticism.

ad 1. The homogenous linear pattern of immunoglobulin localization in the glomeruli of diabetic subjects does not resemble the picture seen in immune-complex glomerular diseases where interrupted, distinctly granular deposits are found (58). However, immune complexes formed between insulin and insulin antibodies might be very small and numerous along the
glomerular basement membrane and therefore present an appearance which is
difficult to distinguish from a linear pattern (58). Eluates from diabet-
ic kidneys containing glomerular IgG did not react with normal glomerular
basement membrane (28, 89) in contrast to the findings in certain forms
of glomerulonephritis (59). These results suggest that IgG in the glomer-
uli of human diabetics may not represent specific antibodies to glomerular
basement membrane antigen. Moreover, some studies have failed to demon-
strate complement in glomerular capillary walls in human diabetes (27, 28,
58). In the study of Westberg and Michael (89) glomerular \( \beta_{1C} \) and IgG were
found only in about 50-70 per cent of the diabetics, irrespective of the
severity of the light microscopical kidney lesions.

ad 2. The proliferation of endothelial cells described by Blumenthal
et al. (14), especially in arterioles and small arteries of diabetic sub-
jects, have been questioned by other authors (cf. 85). Norton (64) per-
formed an ultrastructural study of muscle capillaries in humans with vari-
ous diseases; among others, systemic lupus erythematosus (SLE) and diabe-
tes mellitus. He found conspicuous differences between these two diseases.
The changes seen in diabetic capillaries consisted entirely of a uniform
basement membrane thickening while the capillaries in SLE demonstrated
abnormalities of all components of the walls, i.e. characteristic endo-
thelial inclusions, signs of endothelial degeneration, an irregular base-
ment membrane thickening and an increased number of pericytes. Vasculari-
ty (number of blood vessels per surface area) was normal in the diabetics
and variable in the SLE group.

ad 3. The results concerning binding of fluorescent insulin and
anti-insulin sera to small vessel walls appear to depend on the histolo-
gical technique employed. Thus, the results may differ whether formalin
or ethanol is used for fixation and whether the sections are cut from
paraffin-embedded tissue or from unfixed frozen specimens (cf. 27, 50).
Therefore, no definite conclusions can be drawn at the moment concerning
immunohistochemical investigations with conjugated insulin and anti-insu-
lin sera. Injection of heterologous insulin alone to nondiabetic animals
does not seem to evoke kidney changes similar to human diabetic nephro-
pathy (22, 33, 60, 66, 86, 87). To induce thickening of the basement mem-
brane and clear-cut nodule-like formations in the glomeruli, insulin has
to be given together with Freund's adjuvant (2, 14, 55, 60). The main ob-
jection to the hypothesis that exogenous insulin gives rise to microvas-
cular lesions in diabetes is that such changes occur both in humans and
animals never having received insulin therapy (I, II, 83). As a consequence, it has been suggested that diabetics may produce antigenically altered insulin which would give rise to insulin autoantibodies (10). However, such antibodies, circulating or cellular, have apparently not yet been unequivocally demonstrated in non-insulin treated diabetics (11).

There are also some observations in diabetes considered to support an autoimmune pathogenesis of diabetes mellitus. It may be justified to mention these in this context; in diabetic subjects an increased incidence of circulating thyroid, parietal cell and intrinsic factor antibodies has been demonstrated (cf. 45, 63). Furthermore, certain diseases considered to be of autoimmune origin, such as pernicious anaemia, myxoedema and chronic thyroiditis and idiopathic Addison's disease, have been reported to occur more frequently in diabetics than in normal subjects (cf. 63). Infiltration of inflammatory cells, e.g. lymphocytes, has been described in and around the islets of Langerhans in some patients with juvenile diabetes of short duration (29). The lesions have been designated "insulitis". Similar islets changes can be induced in animals by giving them injections of insulin (homologous or heterologous insulin emulsified in Freund's adjuvant) or anti-insulin serum (cf. 26). Moreover, by using the leukocyte migration and intracutaneous tests Nerup et al. (62) found support for cellular hypersensitivity against porcine pancreas tissue in human diabetics, including individuals not treated with insulin. There was no inhibition of leukocyte migration by porcine or bovine insulin.

Microangiopathy in experimental diabetes.

As mentioned above, microvascular lesions similar to human diabetic microangiopathy have been described in animals with experimental diabetes (cf. 20, 65). All of these reports are based on general assessments and not on quantitations of the vascular structures, except for a few studies where ultrastructural measurements of capillary basement membranes were performed. However, a firmly established thickening of vascular basement membranes in experimental diabetes appears to have been demonstrated previously in only one study using dogs (13). In alloxan diabetic rats there are 2 reports in which glomerular basement membrane thickness was measured (21, 51), but because of a limited number of investigated animals no definite conclusions can be drawn concerning differences of thickness between diabetic and nondiabetic rats (cf. II).
AIMS OF THE PRESENT STUDY

The present investigation was initiated by the observation that immunoglobulins occur in small blood vessel walls in diabetics, as demonstrated by fluorescent antibody technique (cf. 89). This was also demonstrated in experimental diabetes in 1969, when the author, in a preliminary communication, reported gammaglobulin deposition in the glomeruli of alloxan diabetic rats (35). Later, Mauer et al. (57) presented a detailed immunofluorescent study concerning the occurrence of glomerular immunoglobulin and complement in such rats.

The aims of the present investigation were to elucidate what mechanism brings about the glomerular immunoglobulin deposition and whether this deposition plays any role in the development of the glomerular lesions in alloxan diabetic rats. Various therapeutical regimens, i.e. insulin treatment and immunosuppression, were instituted for that purpose. In order to estimate the effect of these measures on the glomerulosclerosis of the diabetic rats it was necessary to select some relevant kidney parameters, morphological or functional, that could be quantitated. Since it was found that the laboratory tests used here, i.e. serum urea N, serum creatinine and urine albumin, were not suited for the mentioned intentions, interest was concentrated on morphological parameters (cf. I).

Thus, the present study comprises the following:

1. A light microscopic semiquantitative grading and ultrastructural measurements of the basement membrane thickness and a light microscopic quantitative determination of the mesangial area in the glomeruli of rats with alloxan diabetes of various durations and a comparison of the findings with those of age-matched nondiabetic controls.

2. To investigate the occurrence of glomerular immunoglobulin G (IgG) and complement (C1q) with immunofluorescent technique in alloxan diabetic and nondiabetic rats of various ages and relate these findings to the light and electron microscopic glomerular changes.

3. A study concerning the effect of insulin treatment on the glomerular lesions in alloxan diabetic rats.

4. To determine whether immunosuppression, i.e. treatment with an immunosuppressive agent, cyclophosphamide, and neonatal thymectomy, might affect the glomerular lesions in alloxan diabetic rats.
MATERIAL AND METHODS

The material and methods used will be only briefly described here. For details the reader is referred to the original articles (I, II, III, IV, V).

Animals.

Several hundred albino rats of both sexes were investigated. They belonged to a highly inbred strain, designated R-strain, kept at the Department of Cell Research, Karolinska Institutet, Stockholm. No spontaneous diabetes was observed in these animals.

Induction and control of alloxan diabetes (I).

At 3 months of age rats were given an intravenous injection of alloxan, 55 mg/kg, in order to produce permanent diabetes. The alloxan administration was made during kidney protection to prevent direct alloxan damage to the kidneys (66). The diabetic state was mainly controlled by monthly measurements of 24-hour-urine output, adjusted to weight, and tests for glucosuria with Clinitest R (Ames Company). A significant positive correlation was found between nonfasting morning blood glucose level and weighted 24-hour-urine output (r = 0.81; p < 0.001) and between the latter and 24-hour-urine glucose content (r = 0.94; p < 0.001). All diabetic rats investigated in the present series had a permanent diabetes with a mean weighted diuresis of at least 20 ml/100 g/24 h (maximum 120 ml/100 g/24 h), corresponding to a nonfasting blood glucose concentration of 200 mg/100 ml (maximum 600 mg/100 ml) and a urine glucose content of 2 g/100 g/24 h (maximum 12 g/100 g/24 h).

Nondiabetic controls.

These animals were either given a saline injection instead of alloxan at 3 months of age or were noninjected.

Treatment.

About 250 alloxan diabetic rats and almost 200 nondiabetic controls were untreated after 3 months of age.
Insulin treatment (IV). Nineteen nondiabetic controls and 17 diabetic male rats were treated with insulin from 12 to 15 months of age, corresponding to a diabetes duration of 9 to 12 months. The insulin was a commercial protamine zinc insulin (mixture of bovine and porcine insulin), 40 U/ml. It was given daily by the subcutaneous route. In the diabetic animals the doses were adjusted so that the urine volumes and glucosuria were kept at low levels.

Cyclophosphamide treatment (V). One hundred and eighty (97 males and 83 females) alloxan diabetic rats were treated with an intraperitoneal injection of cyclophosphamide (Sendoxan\textsuperscript{R}), 7.5-10 mg/kg, once a week. The therapy was started 3 weeks after diabetes induction and was continued until the animals were sacrificed or died spontaneously, up to 15 months of age. Nine (5 males and 4 females) nondiabetic controls were also given weekly injections of cyclophosphamide, 10 mg/kg, from 4 to 15 months of age.

Neonatal thymectomy (V). Seventy-two (37 males and 35 females) otherwise untreated diabetic rats had undergone neonatal thymectomy. Completeness of thymectomy was checked post mortem by naked eye inspection. Only animals with less than 50 mg of residual thymus parenchyma were included in this study.

Renal biopsies.

Renal biopsy specimens for ordinary light microscopy were taken at 4, 6, 9, 12 and 15 months of age, corresponding to a diabetes duration of 1, 3, 6, 9 and 12 months. In most cases the rats were bled by heart puncture after biopsy and killed. About 20 per cent of the rats (67 of 326 rats that underwent renal biopsy), however, were kept alive for further biopsies (1-3 repeated biopsies). In the diabetic animals, biopsied on several occasions, the diabetic state, as measured by diuresis, did not tend to change after the operative procedures.

The renal tissues used in the initial immunofluorescent studies were the same specimens employed in the ordinary light microscopy investigations. There was, however, a problem since the blood remaining within the vessel lumina interfered with the assessment of fluorescence in the tissues. Therefore, in subsequent experiments the kidneys were perfused
in situ with saline after bleeding by cardiac puncture. With this procedure, the kidneys became practically free of blood. The immunofluorescent results presented here are based solely on such perfused kidneys.

Renal tissue for light microscopy and immunofluorescence were both processed according to Sainte-Marie (76) with a slight modification. In 6 untreated diabetic rats, unfixed frozen kidney biopsy specimens were also employed for immunofluorescent studies.

In some 15 month-old rats biopsy specimens were taken for electron microscopy before the animals were killed. The tissue was fixed by immersion in glutaraldehyde, postfixed in osmium tetroxide and embedded in Epon 812 (II).

**Light microscopic methods (I).**

Sections were stained by the PAS and van Gieson techniques. All quantitative and semiquantitative studies were performed on codified PAS stained sections.

1. **Semiquantitative grading of glomerular basement membrane thickness.** All renal biopsy specimens were included. Grading was performed on an arbitrary 4 point scale (1+, 2+, 3+ and 4+). The thickness of the PAS-positive structure in the glomerular capillary walls, here designated basement membrane thickness, was graded. Each section contained about 40-80 glomeruli and the mean thickness of all glomeruli was recorded.

2. **Determination of relative mesangial area by point-counting method.** All rats aged 4 months and most of the 15 month-old animals were included in this study. The mesangial area was expressed in per cent of total corpuscular area within Bowman’s capsule. The result of each rat was the mean value of 10 glomeruli.

3. **Assessment of tubular and interstitial changes.** This was performed using an arbitrary 4 point scale (0, 1+, 2+ and 3+).

**Immunofluorescent studies (III).**

These were made on codified sections from all perfused kidneys using an indirect method. The first layer consisted of rabbit sera against rat IgG and rat β_{1C}, prepared in this laboratory. The second layer was a com-
mercial fluorescein conjugated sheep anti-rabbit immunoglobulin (Statens Bakteriologiska Laboratorium, Stockholm). All specimens were stained and examined in a Zeiss fluorescence microscope at one and the same time. Grading of the intensity of fluorescence was on an arbitrary semiquantitative 4 point scale (0, 1+, 2+ and 3+).

Electron microscopic measurements of glomerular basement membrane thickness (II).

Kidney specimens from 15 month-old rats were used. The thickness of the peripheral glomerular basement membrane was measured on electron micrographs using 2 methods; one described by Jørgensen and Bentzon (43) and the other by Weibel and Knight (88). The measurements were performed on codified glomeruli.

Laboratory investigations (I).

Quantitative blood and urine glucose content were assayed by the glucose oxidase method, using GLOX® (KABI, Stockholm). The over-all error of the method as calculated from the results of duplicate blood glucose determinations was about 4 per cent and was fairly constant for all ranges.

Conventional assay procedures were used for serum urea N and serum creatinine (38). Urine albumin was determined by the Lowry method (52), preceded by protein precipitation in trichloroacetic acid and solubilization of albumin in ethanol (49).

Statistical methods.

For calculating the arithmetic mean and standard deviation as well as the errors of the methods conventional statistical methods were applied (37, 38).

When comparing graded values between two groups the nonparametric rank sum test of Wilcoxon was used (90). Differences between two means were analysed by using Student's t-test. Differences of survival rates were tested by the chi-square method. A two-sided hypothesis was assumed in all cases. p < 0.05 was chosen as the level for statistical significance for all tests.

Correlation coefficients were calculated according to the methods of Pearson (r) or Spearman (r_s).
RESULTS

The results of the ultrastructural measurements of glomerular basement membrane thickness in 15 month-old rats showed a good numerical agreement between the two measuring methods employed (II) (Table I).

Table I. Ultrastructurally measured and light microscopically graded thickness of the glomerular basement membrane in 3 groups of 15 month-old female rats. Measuring methods according to Jørgensen and Bentzon (43) and Weibel and Knight (88). SD = standard deviation.

<table>
<thead>
<tr>
<th>Rat no.</th>
<th>Electron microscopically measured mean thickness (Å)</th>
<th>Light microscopically graded thickness (1+, 2+, 3+, 4+)</th>
<th>Mean diuresis ml/100g/24h during the period 3-15 months of age</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Jørgensen method</td>
<td>Weibel method</td>
<td></td>
</tr>
<tr>
<td>Untreated control rats</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>1950</td>
<td>2230</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>1960</td>
<td>2380</td>
<td>2</td>
</tr>
<tr>
<td>3</td>
<td>2300</td>
<td>2410</td>
<td>2</td>
</tr>
<tr>
<td>4</td>
<td>2350</td>
<td>2290</td>
<td>2</td>
</tr>
<tr>
<td>5</td>
<td>2170</td>
<td>2420</td>
<td>1</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>2150 ± 190</td>
<td>2350 ± 80</td>
<td></td>
</tr>
</tbody>
</table>

Untreated diabetic rats |
| 6 | 2930 | 3710 | 2 |
| 7 | 3510 | 3220 | 3 |
| 8 | 3220 | 3460 | 3 |
| 9 | 3180 | 3090 | 3 |
| 10 | 3180 | 3270 | 3 |
| 11 | 3060 | 3060 | 3 |
| 12 | 3020 | 3320 | 3 |
| 13 | 3600 | 3240 | 3 |
| Mean ± SD | 3210 ± 230 | 3240 ± 170 | 58 ± 18 |

Cyclophosphamide treated diabetic rats |
| 14 | 3380 | 3210 | 3 |
| 15 | 3400 | 3210 | 3 |
| 16 | 3410 | 3210 | 3 |
| 17 | 3090 | 3320 | 3 |
| 18 | 2700 | 2980 | 1 |
| 19 | 2550 | 2610 | 1 |
| 20 | 3160 | 3210 | 3 |
| 21 | 2770 | 3010 | 3 |
| Mean ± SD | 3060 ± 340 | 3030 ± 270 | 61 ± 20 |

Numbers within brackets are the mean values ± SD of the last 5 rats of the group.
Table 2. Ultrastructurally measured and light microscopically graded thickness of the glomerular basement membrane in 3 groups of 15 month-old male rats. Measuring method according to Weibel and Knight (88). SD = standard deviation.

<table>
<thead>
<tr>
<th>Rat no.</th>
<th>Electron microscopically measured mean thickness (Å)</th>
<th>Light microscopically graded thickness (1+,2+,3+,4+)</th>
<th>Mean diuresis ml/100g/24h during the period 3-12 months of age</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Electron microscopically measured mean thickness (Å)</td>
<td>Light microscopically graded thickness (1+,2+,3+,4+)</td>
<td>Mean diuresis ml/100g/24h during the period 3-12 months of age</td>
</tr>
<tr>
<td></td>
<td>Electronic microscopically measured mean thickness (Å)</td>
<td>Light microscopically graded thickness (1+,2+,3+,4+)</td>
<td>Mean diuresis ml/100g/24h during the period 3-12 months of age</td>
</tr>
<tr>
<td>Untreated control rats</td>
<td>2980</td>
<td>3</td>
<td>2890 ± 60</td>
</tr>
<tr>
<td>Untreated diabetic rats</td>
<td>3480</td>
<td>3</td>
<td>3480 ± 160</td>
</tr>
<tr>
<td>Insulin treated diabetic rats</td>
<td>3210</td>
<td>3</td>
<td>3210 ± 270</td>
</tr>
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The light microscopical grading of the thickness was fairly well correlated to the results of these electron microscopic methods ($r_s$ at least 0.60; $p < 0.005$).

The untreated nondiabetic rats of both sexes showed a significant age-related increase of light microscopically graded glomerular basement membrane thickness ($p < 0.01$) and of relative mesangial area ($p < 0.005$ for males, $p < 0.001$ for females) (1) (Table 3). At 15 months of age the males had a significantly thicker measured basement membrane than the females ($p < 0.001$) (Tables 1 and 2). When comparing control rats with untreated diabetic animals at 4 months of age (corresponding to a diabetes duration of one month) there were no differences concerning the light microscopical glomerular findings. At 15 months of age, however, the diabetic rats showed a significant increase of glomerular basement membrane thickness ($p < 0.001$ when measured ultrastructurally, $p < 0.01$ when
Table 3. Mean relative mesangial area (mesangial area in per cent of total corpuscular area within Bowman's capsule) in controls and untreated alloxan diabetic rats at 4 and 15 months of age. SD = standard deviation; n = number of rats.

<table>
<thead>
<tr>
<th>Group</th>
<th>Age 4 months</th>
<th>Age 15 months</th>
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<tbody>
<tr>
<td></td>
<td>Mean ± SD</td>
<td>n</td>
</tr>
<tr>
<td>Control rats</td>
<td></td>
<td></td>
</tr>
<tr>
<td>♂</td>
<td>8.1 ± 0.6</td>
<td>8</td>
</tr>
<tr>
<td>♀</td>
<td>8.0 ± 0.6</td>
<td>8</td>
</tr>
<tr>
<td>Diabetic rats</td>
<td></td>
<td></td>
</tr>
<tr>
<td>♂</td>
<td>8.3 ± 1.2</td>
<td>8</td>
</tr>
<tr>
<td>♀</td>
<td>8.1 ± 0.9</td>
<td>8</td>
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graded semiquantitatively in the light microscope) (Tables I and 2) and of relative mesangial area (p < 0.001) (Table 3) as compared with the controls (I, II, IV).

In about 25 per cent of the older untreated diabetic rats (12 and 15 months of age), a few glomeruli contained focal, intensely PAS-positive, homogenous deposits resembling fibrinoid caps in human diabetic nephropathy (I). However, no Kimmelstiel-Wilson-like nodules and no hyaline arteriolosclerosis were observed in the diabetic animals. Certain tubular (focal dilatation with flattened epithelium and PAS-positive thickening of the basement membrane) and interstitial changes (cell infiltration and fibrosis) were seen both in nondiabetic and diabetic rats, but they tended to be more frequent and severe among the latter animals (I).

Immunoglobulin G could be demonstrated in the glomeruli of almost all untreated diabetic rats as early as one month after diabetes induction (Fig. 1), i.e. before light microscopical glomerular lesions were detectable (III). The frequency and amount of IgG deposition did not change significantly with increasing diabetes duration up to 12 months. However, there was a positive correlation (rₜ = 0.66; p < 0.001) between the occurrence of IgG and the severity of the diabetic state, as measured by weighted diuresis. No significant correlation could be demonstrated when glomerular IgG was related to the graded and measured glomerular basement membrane thickness and to the mesangial area in 15 month-old diabetic rats. When IgG occurred in the glomeruli, it was regularly localized in the mesangium. Sometimes immunoglobulin was also
Fig. 1. Semiquantitatively assessed mesangial fluorescence for IgG in controls and untreated alloxan diabetic rats at various ages (the bars at 15 months should be added to one another).

detected in some of the glomerular capillary walls in a segmental, linear pattern. With the exception of slight amounts in some 15 month-old animals, control rats did not exhibit glomerular IgG. The difference of glomerular immunoglobulin occurrence between control and diabetic animals
was significant at all corresponding ages (p ranging between < 0.01 and < 0.05).

Specific fluorescence for $\beta_{1C}$ was not observed with certainty in any of the investigated glomeruli of the present experimental series using the paraffin embedding method (III). Staining of frozen unfixed renal tissue from 6 untreated diabetic rats (4 and 9 months of age) showed, however, in 3 cases a definite fluorescence for $\beta_{1C}$ in the mesangium. A corresponding investigation of unfixed material in 4 nondiabetic animals revealed no glomerular $\beta_{1C}$.

The mean value of serum urea N in untreated diabetic rats 2 weeks after alloxan injection was double that of the controls at 3 months of age (p < 0.001) (I). With longer duration of diabetes the mean urea N level did not change in diabetic females. In diabetic males, however, there was a significant increase (p < 0.01) of serum urea N in 15 month-old rats as compared with 3.5 month-old animals.

Serum creatinine did not change significantly after alloxan injection or with the duration of diabetes in untreated diabetic animals (I).

Urine albumin assays (I) were performed in some nondiabetic and untreated alloxan diabetic male rats. Male rats were chosen in the present study since female rats often displayed amounts of albumin too small to be determined by the method employed. These investigated male specimens showed a significant increase of urine albumin (mg/24 h) with age in non-diabetic animals (p < 0.001). Alloxan diabetic rats showed a significant decrease of albumin 2 weeks after alloxan administration compared with controls at 3 months (p < 0.01). Fifteen-month-old diabetic rats had a significantly higher mean urine albumin content than controls of the same age, but the inter-individual variation was marked in the diabetic group.

Twelve insulin treated alloxan diabetic rats survived for renal biopsy at 15 months of age (IV). These rats showed a significantly decreased (p < 0.01) incidence and quantity of glomerular IgG as compared with untreated diabetic animals (Fig. 2). There was also a tendency toward a decreased glomerular basement membrane thickness, graded as well as measured (Table 2), and mesangial area in the treated rats, but the differences were not significant between the treated and untreated group. The glomeruli of nondiabetic insulin treated animals (11 rats) did not differ from those of untreated controls.
Fig. 2. Influence of insulin administration on semiquantitatively assessed mesangial fluorescence for IgG in 15 month-old male rats.

Cyclophosphamide treated and neonatally thymectomized diabetic rats did not exhibit any significant differences concerning glomerular IgG, semiquantitatively graded and ultrastructurally measured basement membrane thickness, and of the mesangial area as compared with untreated diabetic rats (V). However, in 15 month-old cyclophosphamide treated animals, especially the females, there was a tendency toward reduced thickness of the glomerular basement membrane (Table I) without decrease of IgG in the glomeruli and of the degree of diabetes, as measured by weighted diuresis. Compared with untreated controls, cyclophosphamide treated nondiabetic rats did not show any differences in the glomerular structures at 15 months of age.
DISCUSSION

Methodological aspects.

In the present study some of the glomerular structures, i.e. basement membrane and mesangial area, known to be affected in human diabetic glomerulosclerosis, were quantitated in alloxan diabetic rats. The light microscopically graded PAS-positive layer of the glomerular capillary walls is not precisely defined anatomically. However, it was convenient to use the term "basement-membrane-thickness" and there was a positive correlation between the thickness of this structure and the thickness of the glomerular basement membrane proper as determined by the electron microscope. Thus, it has been demonstrated in this study that such light microscopic semiquantitative grading of PAS stained sections can serve as an indicator of basement membrane thickness in the glomeruli of normal and diabetic rats.

Lesions in the kidneys of nondiabetic and diabetic rats.

Applying these methods to the present study, it was found that both nondiabetic and alloxan diabetic rats developed a significant age-related increase of glomerular basement membrane thickness and mesangial area. In time, however, this increase was significantly more marked in the diabetic than in the nondiabetic group. Such age-related glomerular changes in nondiabetic and diabetic rats have been described previously (5, 9, 23, 25, 32, 40, 56, 57, 66), but these reports are based on general assessments and not on quantitations of the above-mentioned structures. As mentioned in the Introduction there appears to be only one previous study on experimental diabetes, using dogs, in which a clearly established measured thickening of capillary basement membranes has been demonstrated (13). Mesangial area does not seem to have been measured before in experimental diabetes.

There was no significant correlation between the severity of the diabetic state, as measured by weighted diuresis, and the light microscopic parameters of the glomeruli, basement membrane and mesangial area, in alloxan diabetic rats aged 15 months. However, when relating the ultrastructurally measured basement membrane thickness with the diuresis in 15 month-old diabetic female rats a significant positive correlation was found. In some of the reported studies on experimental diabetes
there was a tendency to a positive correlation between advanced glomerular lesions and a severe degree of diabetes (25) or poor diabetic control (6, 7, 31).

The glomerular lesions in rats with long-term alloxan diabetes are similar to those in human diabetic glomerulosclerosis of the diffuse type (cf. 85). It is reasonable to assume that the glomerular disease in alloxan diabetic rats is secondary to the diabetic state caused by insulin deficiency; the kidneys were protected during alloxan injection to avoid direct alloxan damage to these organs, the lesions developed after some months of untreated diabetes and were correlated to the duration of the diabetic state. Furthermore, similar glomerular changes develop in rats made diabetic by pancreatectomy (25). It is not known whether or not increased food intake may contribute to the development of the lesions in diabetic rats.

The cause of the tubular and interstitial lesions, well-known to occur spontaneously in the rat (cf. 9), is unknown. However, the moderate, and especially the severe lesions of the kidneys in this study, bore a resemblance to chronic pyelonephritis (cf. 12).

The present study confirmed the previously reported (35, 57) occurrence of glomerular IgG and $\beta_{1C}$ in alloxan diabetic rats. Complement was only demonstrated in diabetic animals when unfixed frozen specimens were employed. As in the investigation of Mauer et al. (57), the protein deposition appeared to antedate light microscopical glomerular changes. The localization of IgG and $\beta_{1C}$ was mainly mesangial in contrast to a preferably capillary wall deposition in human diabetic glomerulosclerosis of the diffuse type (27, 89). In the present study there was no tendency to any change of frequency and amount of glomerular IgG with the diabetes duration, varying from one to 12 months. There was, however, a significant positive correlation between the occurrence of IgG and the severity of the diabetic state, as measured by diuresis. Mauer et al. (57) reported an age-related increase of glomerular IgG in alloxan diabetic rats. A closer examination of their results shows a significant increase of the frequency and amount of that protein in the glomeruli from 2 to 4 months' diabetes. However, when excluding the 2 month-values, no significant change of glomerular IgG with the diabetes duration can be demonstrated. The cause of the discrepancy between the present study and that of Mauer et al. concerning the occurrence of glomerular IgG during the early phase of alloxan diabetes is unknown. The difference might be due
Serum creatinine did not change significantly after diabetes induction in the present series. Therefore, the marked elevation of serum urea N seen after a short duration of diabetes does not seem to be caused by renal insufficiency. One of the factors known to influence serum urea concentration is the oral intake of protein (I). In diabetic rats the food intake, adjusted to body weight, was found to be increased to 2-3 times that of the controls (I). Thus, increased protein intake might contribute to the elevated serum urea N of the diabetic animals in this study.

An unexpected finding was the decrease of urine albumin in rats with short-term alloxan diabetes as compared with nondiabetic rats. The cause of this phenomenon is unknown. It might possibly be due to liver damage by alloxan resulting in impaired albumin production.

**Insulin administration and diabetic glomerulosclerosis.**

Thus, the alloxan diabetic rat, representing an insulin-deficiency-type of diabetes (61), seems to be a useful model for studying the pathogenesis of diabetic glomerular disease; as in human diabetic glomerulosclerosis there is an increase of glomerular basement membrane thickness and of mesangial area which can be quantitated. Since, as mentioned above (cf. 89), insulin has been suggested to be actively involved in the development of diabetic microangiopathy, the influence of insulin treatment on the glomerular lesions in alloxan diabetic rats was studied. Previous reports on comparative investigations of glomerular structure between untreated and insulin treated diabetic animals are few in number and do not give enough information to permit any definite conclusions concerning the effect of insulin administration (17, 69, 75). Spiro and Spiro (81) found increased activity of glucosyltransferase, an enzyme considered to be an indicator of basement membrane synthesis, in the kidney cortex of alloxan diabetic rats as compared with age-matched controls. In insulin treated diabetic rats the enzyme level was normal or close to normal.

Deb and Chakravarty (19) reported several histochemical changes in the kidneys of rats with short-term alloxan diabetes. Most of the staining reactions were normal in diabetic animals given insulin.

In the present study insulin doses were adjusted so that the diabetic state of the rats was markedly improved during the period of therapy.
Treated animals exhibited practically no glomerular IgG, indicating that immunoglobulin deposition in the diabetic rats is essentially a reversible phenomenon since almost all untreated diabetic rats had IgG in the glomeruli as early as one month after diabetes induction. Furthermore, the glomerular IgG observed in the diabetic animals can hardly represent insulin antibodies. As mentioned previously, there was a significant positive correlation between the occurrence of glomerular IgG and the severity of the diabetic state, as measured by diuresis, in untreated alloxan diabetic rats. This finding is in accordance with the effect of insulin treatment and indicates that immunoglobulin deposition in the diabetic rat kidney is dependent on the severity of diabetes. The tendency to a smaller glomerular basement membrane thickness and mesangial area in insulin treated diabetic rats as compared with untreated rats supports this suggestion. Heterologous insulin administration to nondiabetic rats did not induce any glomerular changes as compared with untreated controls. As mentioned above, glomerular lesions similar to human diabetic glomerulosclerosis have been evoked in nondiabetic animals only when insulin has been given together with Freund’s adjuvant (2, 14, 55, 60). This finding together with the results of the present study concerning insulin administration to nondiabetic and alloxan diabetic rats do not support the view that the presence of insulin, either exogenous heterologous or antigenically altered endogenous insulin, is contributing to the development of diabetic small blood vessel disease.

**Immunological mechanisms and diabetic glomerulosclerosis.**

To test the hypothesis that immunological mechanisms are involved in the pathogenesis of diabetic microangiopathy, the effect of immunosuppression on the glomerulosclerosis of alloxan diabetic rats was investigated. Studies of this kind in diabetic animals have apparently not been performed previously. Immunosuppression consisted of treatment with cyclophosphamide which has well documented immunosuppressive properties, impairing both cell-mediated and humoral immunity in the rat (cf. 30). Another group of diabetic rats had undergone neonatal thymectomy causing a general defect in delayed hypersensitivity reactions in the adult rat (3). The impairment of humoral antibody responses is, however, more variable, e.g. depending on the nature of the antigen given (4). Cyclophosphamide treatment and neonatal thymectomy did not bring about any significant renal changes in diabetic animals as compared with untreated
diabetics. Drug treated diabetic rats, especially the females, showed, however, a tendency to reduced thickness of glomerular basement membrane without decrease of glomerular immunoglobulin content or of the severity of the diabetes. This tendency was abolished when the basement membrane thickness was adjusted to the body weight.

A crucial question in this context is whether the degree of immunosuppression can be considered to be sufficient in the present series. To inhibit certain experimental autoimmune diseases of the thyroid and central nervous system in the rat, cyclophosphamide was given at a minimum dose of 25-35 mg/kg per week (71, 72). In these studies the drug was administered intraperitoneally daily or 5 days a week during 16-17 days. At a dose level of 5-7 mg/kg per week, little or no suppressive effect on experimental allergic encephalomyelitis was obtained (72). This might suggest that the doses given in the present series, 7.5-10 mg/kg once a week, have been inadequate for immunosuppression. It must be pointed out, however, that the drug was administered for several months in the present investigation. A direct comparison of doses between the studies is therefore not possible to perform. Moreover, in a pilot study where higher doses of cyclophosphamide were used, the mortality was unacceptably high.

There are good reasons to believe that neonatal thymectomy performed in this study resulted in impaired immunological functions of the rats. Jankovic et al. (41) were of the opinion that thymectomy, performed neonatally, was satisfactory if less than 100 mg residual thymus was found in the adult rats. Since some thymus atrophy occurs in untreated alloxan diabetic rats (V, 36), the accepted upper limit of residual thymus weight in thymectomized diabetic animals in the present investigation was put at a lower level, viz., 50 mg. The majority of these rats had only 0-10 mg of residual thymus. The lowest thymus weight recorded among the non-thymectomized diabetic animals was 55 mg.

As mentioned before, there was in this study a discrepancy between the stationary occurrence of glomerular IgG and the progressive increase of basement membrane thickness and mesangial area in untreated alloxan diabetic rats. This finding, together with the negative results of the immunosuppression studies do not support the hypothesis that immunological reactions are important for the development of diabetic microangiopathy. Immunoglobulin and complement deposition in vascular structures in diabetes might be due to nonimmunological mechanisms, e.g. trapping of proteins in damaged vessel walls and/or altered vascular permeability.
Evidence for increased capillary permeability in diabetes mellitus has been presented both in humans (42, 48, 84) and in animals (77).

SUMMARY AND CONCLUSIONS

The finding of immunoglobulins in small blood vessel walls in diabetic individuals has raised the question whether immunological mechanisms, in which for instance insulin might participate, are involved in the pathogenesis of the diabetic microangiopathy. In order to test this hypothesis the kidneys of rats with long-term alloxan diabetes were investigated. For that purpose the main glomerular structures studied, i.e. the basement membrane thickness and the mesangial area, were quantitated. Laboratory tests reflecting kidney function turned out to be less suitable for this object.

The following results were obtained:

A significant, positive correlation was found between light microscopic assessments and ultrastructural measurements of glomerular basement membrane thickness. This indicates that such a light microscopic semiquantitative grading of PAS stained sections can serve as an indicator of glomerular basement membrane thickness in normal and diabetic rats.

Nondiabetic control rats exhibited a significant age-related increase of semiquantitatively graded glomerular basement membrane thickness and of the mesangial area.

Untreated diabetic rats had normal light microscopical glomerular structures at 4 months of age (corresponding to a diabetes duration of one month). At 15 months of age, however, these animals showed a significantly increased thickness of glomerular basement membrane and mesangial area as compared with nondiabetic controls. Lesions resembling fibrinoid caps in human diabetic glomerulosclerosis were found in some of the older diabetic rats. Otherwise, no changes similar to Kimmelstiel-Wilson nodules or hyaline arteriolosclerosis in human diabetes were noted. Certain tubular (focal dilatation with flattened epithelium and PAS-positive thickening of the basement membrane) and interstitial (cell infiltration and fibrosis) lesions were observed in alloxan diabetic as well as in
nondiabetic animals.

IgG occurred in the glomeruli of almost all diabetic rats as early as one month after diabetes induction, i.e. before light microscopical lesions could be detected. The incidence and amount of this IgG did not show any tendency to change with increasing diabetes duration. There was a significant, positive correlation between the occurrence of IgG and the severity of the diabetic state, as measured by weighted diuresis. When IgG occurred in the glomeruli, it was regularly found in the mesangium. Sometimes fluorescence was also seen along the capillary walls in a segmental, linear pattern. In nondiabetic animals no immunoglobulin was found in the glomeruli except for slight amounts in some old rats.

Complement (C1) was not detected in the kidneys when using ethanol-fixed, paraffin-embedded tissue. In a few cases unfixed frozen sections were also employed and in some of the diabetic rats a bright fluorescence for C1 could then be observed in the mesangium.

Alloxan diabetic rats treated with insulin showed a significantly lower frequency and smaller amount of IgG in the glomeruli as compared with untreated diabetic rats. There was also a tendency to reduced thickening of glomerular basement membrane and of mesangial area in the treated animals. The glomeruli of nondiabetic rats given insulin did not deviate from those of untreated controls.

Diabetic rats thymectomized at birth and those treated with cyclophosphamide did not show any significant differences as compared with untreated rats concerning glomerular IgG, basement membrane thickness or mesangial area. There was, however, a tendency toward reduced thickening of the glomerular basement membrane in the drug treated rats, especially the females.

The findings of progressive glomerular lesions similar to human diabetic nephropathy in rats with induced insulin deficiency suggest that lack of insulin is a causal factor for the diabetic glomerulosclerosis in humans.

Glomerular IgG occurs both in human diabetics and in alloxan diabetic rats. The occurrence of IgG in the glomeruli of alloxan diabetic rats is related to the severity of the diabetic state and it almost disappears
during insulin treatment. This indicates that glomerular immunoglobulin deposition in diabetics can be caused by insulin deficiency. Administration of heterologous insulin to nondiabetic rats does not induce glomerular lesions. These findings do not support the view that presence of insulin, either exogenous heterologous or antigenically altered endogenous insulin, is contributing to the development of small blood vessel disease in diabetes.

The discrepancy between the stationary occurrence of glomerular immunoglobulin and progressive increase of other glomerular lesions and the negative results of immunosuppressive measures, i.e. cyclophosphamide treatment and neonatal thymectomy, in the alloxan diabetic rats do not lend support to the hypothesis that immunological mechanisms are involved in the pathogenesis of diabetic microangiopathy.

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