Experimental Nodular "Diabetic-Like" Glomerulosclerosis in Guinea Pigs Following Long-Acting, Heterologous Insulin Immunization

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Summary. Renal lesions similar to Kimmelstiel-Wilson nephropathy have been found in guinea pigs immunized with long-acting heterologous insulin. — The animals were treated for periods ranging from 3 to 5 months, with monthly subcutaneous injections of highly purified bovine insulin in a mixture of lanolin and paraffin oil. — Kidney sections have been examined by means of light microscopy after standard and histochemical stainings, histoimmunological techniques and electron microscopy. — The following glomerular lesions have been detected: 1. PAS-positive hyaline nodules (55.5%); 2. Thickening of the basal membrane (100%); 3. aneurysmatic dilatation of the capillaries (88.8%); 4. diffuse glomerulosclerosis (61.1%); 5. increased number of mesangial cells (38.8%); 6. fibrinoid caps (44.4%); 7. capsular adhesions (33.3%). — Histochemical findings showed that there are differential characteristics between fibrinoid caps (exudative lesions) and hyaline nodular alterations: the former were rapidly digested by trypsin, whereas the nodules resisted the tryptic treatment for 6 h. — Histoimmunological staining of the lyophilized kidney sections with fluorescein-isothiocyanate-labelled anti-bovine insulin serum showed marked fluorescence of both the fibrinoid caps and hyaline nodules of the glomeruli, and also a less intense fluorescence of the basement membrane and of the intercapillary stroma. — Electron microscopy of the glomeruli demonstrated that the basal membrane was always irregularly thickened showing a patchy fibrillary structure, and that there were nodular areas and bands of "basal-membrane-like" material. Besides, dense osmophilic deposits were noted in the mesangium. — It is concluded that insulin acting as an antigen brings about a nodular Kimmelstiel-Wilson-like nephropathy in experimental animals with normal or low blood sugar and lipids, through immunological mechanisms.

Glomérulosclérose nodulaire expérimentale semblable à celle du diabète chez des cobayes après immunisation par de l'insuline hétérologue à action prolongée

Résumé. Des lésions rénales semblables à la néphropathie de Kimmelstiel-Wilson ont été trouvées chez des cobayes immunisés avec de l'insuline hétérologue à action prolongée. — Les animaux ont été traités pendant des périodes de 3 à 5 mois par des injections mensuelles sous-cutanées d'insuline bovine hautement purifiée, mélangée à de la lanoline et à de l'huile de paraffine. — Les coupes de rein ont été examinées à l'aide du microscope à lumière, après colorations standard et histochimiques, ainsi qu'à l'aide de techniques histoimmunologiques et au microscope électronique. — On a trouvé les lésions glomérulaires suivantes: 1. Nodules hyalins PAS-positifs (55.5%); 2. épaississement de la membrane basale (100%); 3. dilatation anévrysmale des capillaires (88.8%); 4. glomérulosclérose diffuse (61.1%); 5. augmentation du nombre des cellules du mésangium (38.8%); 6. capsules fibrinoïdes (44.4%); 7. adhérences capsulaires (33.3%). — Les résultats histo-chimiques ont montré qu'il y a des caractéristiques différentielles entre les capsules fibrinoïdes (lésions exsudatives) et les altérations nodulaires hyalines: les premières

sont digérées rapidement par la trypsine tandis que les nodules résistent au traitement de 6 h par la trypsine. — La coloration histoimmunologique des coupes de rein lyophilisés, avec du sérum anti-insuline de boeuf marqué avec de l'isothiocyanate de fluorescéine montra une fluorescence prononcée des capsules fibrinoïdes et des nodules hyalins des glomérules, ainsi qu'une fluorescence moins intense de la membrane basale et du stroma intercapillaire. — La microscopie électronique des glomérules a montré que la membrane basale est toujours épaissie de façon irrégulière, présentant une structure en taches, et qu'il y a des aires nodulaires et des bandes de matière semblable à la membrane basale. En outre on a noté des dépôts osmophiles denses dans le mésangium. — On conclut que l'insuline agissant comme un antigène provoque, par l'intermédiaire de mécanismes immunologiques, une néphropathie nodulaire semblable à celle de Kimmelstiel-Wilson, chez les animaux d'expérience ayant un taux normal ou bas de sucre et de lipides.

Experimentell erzeugte „pseudodiabetische“ noduläre Glomerulosklerose bei Meerschweinchen nach Immunisierung mit heterologem, protrahiert wirkendem Insulin

Zusammenfassung. Nach Immunisierung durch heterologes Insulin mit protrahierter Wirkung ließen sich bei Meerschweinchen ähnliche Nierenveränderungen wie bei der Kimmelstiel-Wilson'schen Nephropathie nachweisen. — Die Tiere erhielten monatliche subcutane Injektionen von hochgereinigtem Rinderinsulin in einer Mischung von Lanolin und Paraffinöl über einen Zeitraum von 3–5 Monaten. — Die Untersuchung der Nierenschnitte erfolgte lichtmikroskopisch mit Hilfe von Standard- und histochemischen Färbungen sowie durch histoimmunologische Verfahren und die Elektronenmikroskopie. Dabei ließen sich an den Glomeruli folgende pathologische Befunde erheben: — 1. PAS-positive hyaline Knötchen (55.5%); 2. eine Verdickung der Basalmembran (100%); 3. aneurysmatische Kapillarerweiterungen (88.8%); 4. eine diffuse Glomerulosklerose (61.1%); 5. eine Vermehrung der mesangialen Zellen (38.8%); 6. Fibrinausfällungen (44.4%); 7. Kapseladhäsionen (33.3%). — Die histochemischen Untersuchungen zeigten Unterschiede zwischen den Fibrinausfällungen (exsudative Veränderungen) und den hyalinen Knötchen: erstere wurden durch Trypsin schnell zerstört, während die hyalinen Knötchen einer Trypsinbehandlung über 6 Std. standhielten. Histo-immunologische Anfärbung lyophilisierter Nierenschnitte durch Anti-Rinderinsulin-Serum, das mit Fluoreszin-Isothiozyanat markiert worden war, ließen eine ausgeprägte Fluoreszenz der Fibrinausfällungen, sowie der hyalinen Knötchen in den Glomeruli und eine schwächere Fluoreszenz der Basalmembran und des interkapillären Grundgewebes erkennen. Durch die Elektronenmikroskopie konnte nachgewiesen werden, daß die Basalmembran immer unregelmäßig verdickt war und stellenweise eine fibrilläre Struktur aufwies. Weiter fanden sich noduläre Bezirke und Streifen von membran-„ähnlichem“ Material. Im Mesangium ließen sich ferner dichte osmophile Ablagerungen nachweisen. Es wird gefolgert,

daß Insulin als Antigen über immunologische Mechanismen bei Versuchstieren mit normalen oder sogar erniedrigten Glucose- und Lipid-Serumspiegeln eine noduläre Kimmelstiel-Wilson „ähnliche“ Nephropathie auslösen kann.

Key-words. Nodular glomerulosclerosis, Kimmelstiel-Wilson nephropathy, long-acting heterologous insulin, histoimmunology, electron microscopy.

Different methods have been devised in order to produce in animals renal lesions similar to human diabetic glomerulosclerosis. These experiments, based upon the different histopathogenetic interpretations of the Kimmelstiel-Wilson nephropathy, gave results which are used, in turn, to support the same working hypothesis. It is well-known that the common techniques of inducing diabetes in experimental animals are:

1. alloxan intoxication;
2. pancreatectomy;
3. treatment with large doses of anti-insulin hormones, principally cortisone and growth hormone.

Actual nodular nephropathy in animals made diabetic by alloxan was detected by MANN et al. (1951) in rats, and by BLOODWORTH (1965) in dogs. However after pancreatectomy, nodular lesions were found only in dogs by RICKETTS et al. (1959).

Similar renal alterations were described in normal and in alloxan diabetic animals after treatment with glucocorticoids or growth hormone [LUCKENS et al. (1946); BLOODWORTH and HAMWI (1956); BRETÁN and OBLATT (1955); BLOODWORTH (1965)], and also in man following prolonged cortisone treatment (SOMMERS and HALLEY, 1956).

However, only the electron microscopical findings of BLOODWORTH (1965) give unequivocal evidence of nodular Kimmelstiel-Wilson-like glomerulosclerosis induced by experimental techniques. According to ASHTON (1959), BENCOSME et al. (1958), MORAN et al. (1962) and WILENS and STUMPF (1955), cortisone treatment is followed only by exudative alterations of the kidney.

It is not yet established whether all these lesions are due to insulin deficiency, or, in the case of animals treated with alloxan to its toxic effect. One must also consider the treatment with heterologous insulin, in order to keep the animal alive, as a possible cause of diabetic glomerulosclerosis.

Glomerular "diabetic-like" alterations were also observed in animals treated with cortisone associated with heterologous serum (RICH et al., 1950), and similarly in subjects with nephrotic syndrome after prolonged therapy with glucocorticoid hormones (OPPENHEIMER et al., 1963).

Noteworthy, however, is the possibility of the use of heterologous insulin to cause renal diabetic-like alterations based upon the rationale of a tissue allergic mechanism (MANCINI and COSTANZI, 1961).

GRIEBLE (1960) treated 2 groups of rabbits with insulin for 24 weeks: one group received 66 injections of long-acting insulin and the other group long-acting insulin and human serum. In the first group dilatation

of the glomerular capillaries was observed, with protein clots in the lumen, fibrinoid caps and hypercellularity of the glomeruli, at times diffuse and at times focal. In the second group these alterations were more severe, and in rare instances they were accompanied by nodular hyaline glomerular lesions.

MOHOS et al. (1963) treated rabbits for a period of 6 to 7 months with subcutaneous injections of heterologous insulin mixed with the complete Freund's adjuvant, and observed diffuse PAS-positive thickening of the basal membrane, arteriolar sclerosis with endothelial proliferation, and phlogistic infiltration of the interstitial tissue. Glomerular nodular lesions were found only in one animal. No lesions were detected in animals treated with insulin alone, and only minor alterations were found in animals treated with complete Freund's adjuvant.

BLUMENTHAL et al. (1965) also produced similar kidney changes in rabbits treated with heterologous insulin and adjuvants, and found that the diseased glomeruli fix fluorescent insulin.

In these experiments 2 facts must be underlined which may prompt justified criticism: one is the use of commercial insulin not highly purified, which by itself may be the cause of an immunological reaction; and the other is the use of complete Freund's adjuvant, which may be responsible for some renal alterations.

The purpose of this paper is to describe in detail our previous experiments (ZAMPA and MANCINI, 1965), which showed nodular Kimmelstiel-Wilson-like nephropathy in guinea pigs immunized with highly purified heterologous insulin mixed with an incomplete adjuvant.

Experiments

1. Materials

Twenty five guinea pigs (weight 400–500 g) were maintained on a free diet. All animals were age-comparable, and were in the same physiological conditions.

A) a) Eighteen animals were injected subcutaneously at monthly intervals with 1 mg (22.2 units) of $6 \times$ recrystallized bovine insulin (Boots), homogenized with a mixture of lanolin and paraffin oil in equal parts (WRIGHT, 1961; ZAMPA, 1963). Twelve animals were treated for 5 months, 4 for 4 months and 2 for 3 months. The animals were killed 15 days after the last injection.

b) After 3 and 4 months treatment, three more injections of 10 units each of bovine insulin labelled with fluorescein isothiocyanate (FICT), were given intraperitoneally to 6 guinea pigs just before they were

killed (one injection every 12 h, the animals being killed 6 h after the last injection).

B) Controls

a) Five guinea pigs were injected subcutaneously every month for 5 months with a mixture of lanolin and paraffin oil in equal parts.

b) Two guinea pigs were treated only with 3 injections of 10 units of FICT labelled bovine insulin just before being killed.

2. Methods

a) *Histology and histochemistry.* Kidney fragments from all animals were sampled, some fixed in acetone, some in 10% neutral formalin, and some in Davidson's solution, and then embedded in paraffin wax. Sections were stained as showed in Table 1.

b) *Histoimmunology.* Kidney fragments from the 6 animals treated with long-acting insulin for 3 and 4 months (group Ab), and from the 2 guinea pigs of the control group (Bb) were rapidly frozen in petroleum ether at -78°C , embedded in paraffin wax under vacuum and lyophilized. Sections, after the paraffin was removed with petroleum ether, were rehydrated in a humid atmosphere, fixed in acetone for 10 min at $+37^{\circ}\text{C}$, and then treated with the following fluorescein isothiocyanate (FICT)-labelled antibodies:

1. anti-guinea pig globulins from rabbits;

2. anti-bovine insulin antibodies from guinea pigs. Sections were also observed directly under U.V. light. The following controls were carried out:

1. treatment with FICT-labelled gamma-globulins from the same non-immunized animals;

2. treatment with unlabelled sera from immunized

Table 1

Methods	Nodules	Basal membrane	Fibrinoid caps	Plasmatic glomerular deposits
Haematoxylin eosin	Eosinophil	Eosinophil	Eosinophil	Eosinophil
Mallory	Blue or yellow-blue	Blue or yellow-blue	Yellow	Yellow
Silver reticulin staining	Argiophil fibrils	Slight argiophil	Diffuse argiophil at times	
Alcian-PAS	PAS +	PAS +	PAS + +	PAS +
PTAH	Brown	Slight brown	Violet brown	Orange
Weigert fibrin	—	+	+	—
Congo red	—	—	—	—
Weigert elastic fibrils	—	—	—	—
Toluidine Blue:				
pH 4.70	—	—	+ (RED)	± (RED)
pH 5.05	—	±	+ + (RED)	+ (RED)
pH 5.30	+ (GREEN)	+	+ + (VIOLET)	+
pH 5.70	+ + (GREEN)	+	+ + (VIOLET)	+
Hyaluronidase digestion 36 h	—	—	—	—
Peptic digestion 3 h	+	+	+	+
Tryptic digestion 3 h	—	—	+	+
Tryptic digestion 6 h	±	— (±)	+	+
Electron microscopy:				
optic density	+	+	+ +	
fibrillary aspect	+ +	+ +	—	—
Histoimmunology:				
FITC-labelled anti-guineapigs globulins	+ +	+	+ +	±
FITC-labelled serum anti bovine insulin	+ +	+	+ +	—

animals and after thorough washing, treatment with the same FICT-labelled sera.

C) Electron microscopy

Kidney fragments from 5 guinea pigs treated with long-acting insulin for 5 months, and from the control group (Ba) were fixed in osmic acid (OsO₄) at pH 7.4, treated with uranyl acetate and lead, and then embedded in methacrylate (methyl-methacrylate/butyl-methacrylate 1:9; Dupont, U.S.A.).

Ultra-thin sections were made for electron microscopical examination (Electron microscope: Siemens Mod.; Electron Microscopy Centre of the University of Bologna, Italy).

Results

A) Guinea pigs treated with long-acting insulin

a) *Light microscopy.* Histological findings are summarized in Table 2.

The glomeruli were frequently enlarged and the capillaries stretched; only a few of them were collapsed.

Basal membranes were always thickened, more or less PAS-positive and some times reduplicated; the kidneys of the animals treated for 5 months showed a more marked thickening of the basal membrane. The thickened basal membrane showed spindle-shaped or nodular areas, which were intensely argyrophil, and were not digested by trypsin pretreatment. Very often more or less dilated glomerular capillaries could be seen, which at times became microaneurysmatic. The latter alteration could be related to the lesion of the basal membrane, which seems to be very marked at that level (Fig. 1). Homogeneous PAS- and Weigert-positive or hyaline material, together with some red cells, was noted in the lumen of the dilated capillaries, particularly in the animals injected with fluorescent insulin immediately before being killed. Endothelial cells and epycytes were normal.

The intercapillary stroma was thickened and intensely PAS-positive, with increased argyrophil fibrils and with enlarged and more numerous cells.

Some sclerohyaline glomeruli were found in 2 animals, and this lesion seems to start from the vascular pole, which was frequently involved.

Table 2

TREATMENT	Animal number	Injection number	Nodules	Thickening of the basal membrane	Dilated capillaries	Polynucleosis	Plasmatic glomerular deposits	Fibrinoid caps	Capsular adhesions	Mesangial sclerosis	Arterial lesions
a) Long-acting insulin immunization	1	5	+	++	++	-	-	-	-	-	±
	2	5	++	++	+	-	-	-	+	+	-
	3	5	-	++	++	-	-	+	-	-	-
	4	5	+	++	++	-	-	+	-	-	++
	5	5	+	++	++	+	-	+	-	±	-
	6	5	-	++	++	+	-	-	-	+	-
	7	5	-	++	+	+	-	+	-	+	-
	8	5	+	++	+	-	-	+	-	+	-
	9	5	-	+	+	-	+	-	+	-	-
	10	5	++	++	+	+	-	+	+	++	++
	11	5	-	++	+	-	-	-	-	-	-
	12	5	-	+	-	±	±	-	-	+	-
b) Long-acting insulin immunization + fluorescent insulin before killing	13	3	+	++	+	-	++	-	+	+	-
	14	3	+	++	+	-	+	-	+	+	-
	15	4	++	+	++	±	++	+	+	-	-
	16	4	-	+	-	++	+	+	-	-	-
	17	4	+	+	++	-	-	-	-	++	-
	18	4	-	++	+	-	-	-	-	±	-
CONTROLS a) incomplete adjuvant	19	5	-	-	±	-	-	-	-	-	-
	20	5	-	-	-	-	-	-	-	-	-
	21	5	-	-	-	-	-	-	-	-	-
	22	5	-	+	-	±	-	-	-	-	-
	23	5	-	-	-	-	-	-	-	-	-
b) fluorescent insulin before killing	24	no	-	-	-	-	++	-	-	-	-
	25	no	-	-	-	-	+	-	-	-	-

In some animals, treated or not with fluorescent insulin, the capsular space of the glomerulus showed plasma-like material. The animals treated for 5 months with long-acting insulin only, and the 2 animals treated for 4 months in the same way plus the final injections of fluorescent insulin, showed fibrinoid caps which adhere to the basal membrane of the more peripheral capillaries. These structures were homogeneous, intensely PAS- and Weigert-positive semilunar or nodular areas, which by the light microscopy seemed to be orientated toward the capillary lumen.

the periphery of the capillaries or at the centre of the glomeruli, and are of very different sizes: from small thickening of the basal membrane to much larger structures which affect a complete tuft of glomerular capillaries.

PAS-positive hyaline nodules involved 1 glomerulus in every 10, and frequently there was more than one in the same glomerulus.

Generally, peripheral nodules, at least the smaller ones, were incorporated into the basal membrane; and showed nuclei in the centre; the larger ones affected

Fig. 1. Kidney of guinea pig immunized with bovine insulin for 5 months: the basement membrane is thickened and the capillaries are dilated. A glomerular nodule, which on the slide appears blue coloured, is located in the inter-capillary stroma. (Mallory staining. $\times 400$)

Fig. 2—3. Kidney of guinea pig immunized with bovine insulin for 5 months: it shows diffuse dilatation of the glomerular capillaries, thickening of the basal membrane, and PAS-positive hyaline nodular areas showing some nuclei and inducing a proliferative reaction of the Bowman's capsule (PAS staining. $\times 400$)

PAS-positive hyaline nodules in the glomeruli were detected in 10 animals: these nodules, particularly evident in 3 guinea pigs, were localized mainly either at

the intercapillary stroma, squeezing or even obstructing the capillary lumen, and had nuclei mainly at their periphery (Fig. 2—3). The hyaline material of the

Fig. 3 (see Fig. 2)

Fig. 4. Kidney of guinea pig immunized with bovine insulin for 5 months: PAS-positive thickening of the mesangium. (PAS staining. $\times 400$)

nodules was of different density from point to point, using the Hotchkiss and Mallory staining techniques. The central nodules divaricated and squeezed without obstructing the capillaries, and their aspect was homogeneous with or without a few nuclei (Fig. 4).

Using silver reticulin staining, the nodules showed 2 different appearances: a packed argyrophil fibrillary proliferation, more or less thick, mingled together to form a star arrangement; or neoformed fibrils sticking together at the periphery of the nodule; the central area showed a slight diffuse argyrophil aspect.

Capsular adhesions were often observed in 6 of the animals.

Lesions of the glomerular efferent arterioles were detected in 3 guinea pigs: these alterations were hyaline

thickening of the medial layer, which was markedly PAS-positive; the limited internal elastic membrane was also thickened; more characteristic was the zonal proliferation of the endothelial cells forming PAS-positive bands among the cells which were fixed to the stroma of the internal layer.

The epithelial cells of the proximal convoluted tubules swelled, and showed rough granular cytoplasm; however the brush borders were normal.

Hyaline PAS-positive casts were often seen in the tubular lumen, where in one instance a calcareous deposit was noted.

Very rarely the renal stroma was infiltrated with small phlogistic areas consisting of lymphocytes and monocytes mainly in the periphery of the renal cortex.

In these areas the glomeruli frequently showed capsular lesions and diffuse sclerosis of the intercapillary stroma.

There was no relationship between interstitial infiltrations and exudative or hyaline nodular lesions of the glomerular tuft.

b) Histochemical findings. Our interest was focused mainly on the differential characters between exudative lesions, particularly fibrinoid caps, and glomerular hyaline nodular alterations, since GRIEBLE (1960) and LEVINE (1964) have claimed that renal modifications caused by heterologous insulin are exclusively of the exudative type.

As our data in Table 1 show, there existed clear-cut differential characteristics of the glomerular alterations.

The fibrinoid caps were yellow using Mallory's method, whereas the nodular productive lesions took on a blue or a yellow blue stain; the former were intensely PAS-positive, with violet-brown colour using PTAH staining, and often were also Weigert-positive, whereas the latter were less intensely PAS-positive, showing a lighter coloration with PTAH staining and were Weigert-negative.

In the exudative lesions, silver impregnation showed, at times, a diffuse argyrophil aspect, but never the presence of fibrils; the latter, on the other hand, were numerous in nodular hyaline-productive alterations.

The extinction point of toluidine blue staining was lower in the exudative than in the nodular lesions. Furthermore, the former showed a marked metachromatic coloration after this staining.

The most important differential characteristic between these two lesions lay in the different resistance to the proteolytic enzymes. Fibrinoid caps were rapidly digested by trypsin, whereas the nodular structures resisted this treatment for 6 h.

c) Histoimmunological findings. Using direct U.V. light on renal sections from animals injected with fluorescent insulin, we observed an attenuated, diffuse yellow fluorescence of the glomeruli, and that the proximal convoluted tubules showed a marked brilliant green fluorescence.

Renal sections challenged with FICT-labelled anti-guinea pig gamma-globulins showed a marked fluorescence of the altered basal membrane of the glomeruli, and also of the exudative and the hyaline-productive lesions. On the other hand, the tubules were not as intensely coloured.

The most striking results were observed with the use of FICT-labelled anti-bovine insulin serum. The findings were: intense fluorescence of the fibrinoid caps and of the glomerular hyaline nodules; a less intense fluorescence of the basal membrane and of the intercapillary stroma, and finally a slight fluorescence of the epithelial cells of the proximal convoluted tubules.

Fig. 5. Kidney of guinea pig immunized with bovine insulin for 5 months: irregular thickening of the glomerular basal membrane (B.M.) showing nodular-like areas and bands ending toward the intercapillary stroma (I.S.) with electron microscopical density similar to basal membrane. Some foot processes of the glomerular epithelium (EP) are fused and some swollen. (Electron microscopy. $\times 16000$)

Fig. 6. Kidney of guinea pig immunized with bovine insulin for 5 months: in one area of the intercapillary stroma (I.S.) there are several bands of basal-membrane-like material (B.M.L.M.) showing a net arrangement (early stage of the nodule formation). No alterations of the capillary endothelium (En) and of the glomerular epithelium (Ep). (Electron microscopy. $\times 7000$)

Fig. 7. Kidney of guinea pig immunized with bovine insulin for 5 months: in the mesangium (I.S.) there are several small bands of basal-membrane-like material (B.M. L.M.) inside and outside the cells. The cells of the intercapillary stroma (I.S.) are more numerous. The glomerular epithelium is unaltered. (Electron microscopy. $\times 10000$)

Fig. 8. Kidney of guinea pig immunized with bovine insulin for 5 months: it shows several bands and small nodules of basal-membrane-like material (B.M.L.M.) without any connection with the basement membrane (B.M.) and some of them ending in the cytoplasm of the mesangial cells. (Electron microscopy. $\times 10000$)

Fig. 9. Kidney of guinea pig immunized with bovine insulin for 5 months: the glomerular basal membrane (B.M.) is irregularly thickened, and its aspect facing the intercapillary stroma (I.S.) where there are two nuclei (N), shows a great amount of osmophilic material (↑). This material corresponds to the exudative lesions seen with the light microscope. (Electron microscopy. $\times 16000$)

All the histoimmunological tests used are highly specific.

d) *Electron microscopy.* The basal membrane was always found irregularly doubled or tripled its normal thickness. Using higher power magnification, the basal membrane did not appear homogeneous, as is the norm, but showed a patchy, finely fibrillary structure; in other areas, a dense osmiophilic material and small vacuoles were observed. On the border facing the endothelium, and mainly towards the intercapillary stroma, zonal thickening of the basal membrane was detected; these alterations consisted of small nodules and circular bands having a similar electron density (Fig. 5).

Long, irregular fibrils also left the basal membrane, infiltrated the mesangium and finally ended at times outside and at times inside the cytoplasm of the mesangial cells (Figs. 6–7). Nodules and fibrillary bands having a similar density were noted in the mesangium without any connection with the basal membrane (Fig. 8).

Besides this material, dense osmiophilic deposits of different sizes with clear-cut border were seen placed either between the basal membrane and the endothelium, or in the mesangium, but always close to the basal membrane (Fig. 9). The neoformed fibrils could also be impregnated by this dense osmiophilic substance. The epicytes showed some slight alterations.

The podocytic cytoplasm had small vacuoles and a modest increase of the lysosomes; the endothelial cells showed a regular aspect with the exception of a slight thickening of the fenestrated membrane. Finally, the mesangial cells were generally more numerous than in the normal kidney.

B) Controls

1. Guinea pigs treated only with lanolin and paraffin oil

a) *Light microscopy:* no alterations were found at kidney level. In one animal we observed the glomerular capillaries slightly dilated and in another one a very modest thickening of the basal membrane. Histochemical investigations showed normal tissue reactions.

b) *Electron microscopy:* normal renal structures

2. Guinea pigs treated only with fluorescent insulin

a) *Light microscopy:* the glomeruli were slightly enlarged with dilated capillaries filled with red blood cells, which were enclosed in a eosinophil plasma-like material; at times the capsular space was also stuffed with this material. We never detected modifications of the basal membrane nor of the intercapillary stroma.

b) *Histoimmunological findings.* Direct U.V. light examination showed a slight diffuse glomerular fluorescence of the proximal convoluted tubules.

Comments

Our experiments showed that a nodular glomerulosclerosis similar to Kimmelstiel-Wilson nephropathy, together with a diffuse renal sclerosis of the Bell type can be brought about in guinea pigs by means of long-acting immunization with purified heterologous insulin.

The more striking renal alterations, observed mainly after 5 months but also noted after 3 months immunization, were productive and exudative lesions of the glomeruli and of the efferent glomerular arterioles.

The productive glomerular lesions seen with electron microscopy (E.M.) seem to be deposits of basal-membrane-like material. This substance, at times homogeneous and at times fibrillary, infiltrated the mesangium, where probably it was also produced, inside and outside the cells, and also formed excrescences and reliefs of the basement membrane which always appeared to be markedly thickened. The more characteristic expression of the productive lesion was the sclerohyaline nodules, which, as shown by the electron microscope, initially originated from the altered basement membrane and from the neoformed mesangial material.

These nodules were detected in 55.5% of the immunized animals. The diffuse productive lesions, found in all treated animals consisted histochemically and electron microscopically of the same material; probably this substance contains glycoprotein (neutral or slightly acid mucopolysaccharides) and collagen fibrils.

The exudative alterations which were E.M. homogeneous and osmiophilic, were placed beside the basal membrane towards the endothelium or the mesangium, and under light microscopy assumed the morphological aspect of fibrinoid caps. These were made up of globulins (demonstrated by histoimmunology), fats, fibrin and probably mucopolysaccharides. It is very difficult to establish whether there are relationships between hyaline productive and exudative lesions; however, from our experiments, we think that they are histogenetically independent according to the E.M. findings.

Some aspects of diffuse glomerular sclerohyalinosis and the capsular adhesions are probably due to exudative lesions.

Proliferative alterations were very rare and very slight, consisting of an increased number of mesangial cells, which were at times focal involving only a few glomerular tufts and at times diffuse throughout the entire glomerulus. The afferent and efferent glomerular arterioles showed sclerohyalinosis affecting mainly the media and internal tunics, and endothelial proliferation with formation of glycoproteins.

All these renal lesions that we have experimentally produced have to be considered very similar to human diabetic nephropathy for the histological, histochemical and electron microscopical aspects, and different from those obtained by the experimental work of

GRIEBLE (1960) and MOHOS (1963): the former produced renal alterations similar to those found in subacute glomerulonephritis, and the latter described severe interstitial proliferative lesions of the kidney and only very rare nodular modifications of the glomerulus. The experimental nephropathy found in our animals seems to be due only to the insulin molecule activity because of the use of the extremely purified hormone and also of a mixture of inert substances, which by themselves, as shown in the control animals, do not produce renal lesions.

Besides, we can exclude that glucose and lipid metabolism are involved in inducing renal lesions, because during the treatment period our animals showed normal or slightly low blood sugar and lipids (ZAMPA and MANCINI, unpublished). Finally the hypothesis that insulin might stimulate the pituitary-adrenal axis thereby increasing corticosteroid production, which, in turn, can bring about kidney alterations, appears to be very unlikely because this mechanism is only caused by acute blood sugar drop, whereas the insulin introduced was long-acting and the blood sugar of the animals was normal too.

On the other hand the heterologous insulin can be considered an antigen causing not only anti-insulin antibody production, as we have already demonstrated (ZAMPA et al., 1961; MANCINI et al., 1961; COSTANZI et al., 1961), but also either direct renal lesions (CZERKAWSKY, 1963) or a tissue kidney immunological sensitization against the same insulin. Our former work showed that microangiopathic lesions of the glomerulus, capillaries and arterioles of diabetic patients contain gamma-globulins and fix the complement too (MANCINI et al., 1964).

The above evidence suggests that the renal lesions produced in the present experiments could be due to immunological factors, and that three possible mechanisms can be put forward:

1. formation of tissue antibodies against insulin (MOHOS, 1963);
2. production of circulating anti-insulin antibodies which link the insulin fixed to the glomerular basal membrane (GRIEBLE, 1960);
3. intracapillary binding of the antigen to the specific antibodies, and production of a damaging immunocomplex (MOVAT, 1962).

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