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Abnormal binding of negatively charged serum proteins to diabetic basement membranes is largely a systemic phenomenon

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Summary. Direct immunofluorescence employing goat anti-human IgG, IgA, IgM, C_3 component of complement, fibrinogen, albumin, and polyvalent immunoglobulins was performed on postmortem samples of gingiva, parotid gland, thyroid, kidney, and pancreas tissue of 15 diabetic and 15 control patients.

Basement membrane thickness quantification of kidney tubules, gingival capillaries, and parotid gland ducts and acini was also done utilizing a calibrated magnifier on uniformly enlarged photomicrographs which had been specially stained to highlight basement membranes.

Results revealed binding of IgG, albumin, and polyvalent immunoglobulin to kidney glomerular and tubular basement membranes and parotid ductal and acinar basement membranes in all diabetic subjects. Thyroid follicular basement membranes were positive in 8 of 15 diabetic patients for the same antisera. All gingival and pancreatic tissue from diabetic and control patients was negative for binding of all serum proteins tested.

Basement membrane thickening in kidney tubules and gingival capillaries was observed in diabetic subjects; however, there was no apparent difference between diabetic and control patients in thickness of ductal or acinar basement membranes of the parotid gland.

Key words: Diabetes mellitus – Basement membrane – Immunofluorescence

From the standpoint of decreasing mortality rate and increasing longevity, diabetic patients stand to benefit most from an understanding of the membranopathy that is associated with the secondary sequelae of their disease.

Immunofluorescence studies have been performed on a number of tissues

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by investigators hypothesizing a role for the immune system in the pathogenesis of certain aspects of diabetic membranopathy [1, 5, 14]. Although abnormal deposition of serum proteins, including immunoglobulins and C_3, has been observed in diabetic basement membranes [3, 4, 13, 14, 15], the absence of detectable anti-basement membrane antibody in sera or kidney eluates, the inability to detect insulin or insulin-antibody in the membranes, the frequent absence of a complement component, and the presence of albumin, a non-immunologically associated protein, have all been offered as evidence against an immunological mechanism for the phenomenon [3, 4, 12, 13, 15]. Other investigators have suggested that morphologic alterations in the basement membrane of the diabetic, at least in the glomerulus, could be related to the inability to clear circulating immune complexes or due to a class I immune mechanism [1].

In the present investigation, we sought to unify the study of abnormal deposition of serum proteins in diabetic basement membranes by assessing the extent of basement membrane alteration within multiple tissues of individual diabetic subjects. Previous studies have not dealt with systemic manifestation of this basement membrane abnormality within individual subjects, but have been confined to localized tissue phenomena. Parotid gland and gingival tissue have not been studied heretofore with respect to abnormal plasma protein binding to basement membranes and are of interest in view of the evidence that diabetic patients tend to have more extensive periodontal disease than non-diabetics [18], and in view of the finding of asymptomatic bilateral parotid gland enlargement in some diabetics [17], both of which could conceivably be related to aberrant basement membrane morphology.

A second objective of the present study was to determine whether basement membrane thickness, an additional parameter of diabetic membranopathy, was correlated to abnormal serum protein binding in diabetic patients in a number of different tissues.

Materials and methods

Subject population. Subjects for the investigation consisted of both Type I (insulin dependent) and Type II (insulin independent) diabetics who were presented for post mortem examination at Hennepin County Medical Center, Minneapolis, Minnesota. Criteria for inclusion in the study were a history of diabetes mellitus for a minimum of five years and a severity of the disease requiring the daily use of insulin and/or oral hypoglycemic agents. Sex and age matched control subjects consisted of decedents with a negative medical history for diabetes mellitus who were also autopsied at Hennepin County Medical Center.

Tissue sampling. Autopsies were performed within 2–18 h after death. The reliability of direct immunofluorescence on tissue obtained at autopsy has been evaluated by Sheibani et al. [19] by comparing ante mortem and post mortem renal diagnoses with the conclusion that such studies can be performed with confidence on necropsy tissue for as long as 20 h after death.

Tissue sections for study were obtained from gingiva, parotid gland, thyroid, kidney and pancreas of the subjects. Gingival specimens were obtained from the right mandibular canine and premolar area. Parotid gland specimens were obtained from the superior pole of the gland. Thyroid specimens were from the inferior pole of the right lobe, kidney specimens from the right renal cortex, and pancreatic specimens from the tail of the pancreas, in order

to sample the greatest possible number of islets. Samples were divided into two portions for immunofluorescence (IF) and light microscopy.

Sample preparation. Immediately after collection, samples for immunofluorescence were snap frozen in isopentane (K & K Laboratories, Plainview, New York), precooled in liquid nitrogen. These samples were subsequently stored in a −70° C freezer until sectioning. Tissue for sectioning was mounted from isopentane in OCT (Tissue-Tek II) commercial mounting media (Lab-Tek division of Miles Laboratories, Naperville, Illinois, 60540) and sections were cut at 2–4 μ in an International Equipment Company cryostat (Needham, Massachusetts, USA) at −20° C. The sections were washed for two minutes in acetone, after which they were subjected to three separate 15–20 min washes in phosphate-buffered saline (PBS) solution (pH 7.0–7.3) using a magnetic stirrer. The samples were then incubated with fluorescein isothiocyanate-labeled goat anti-human IgG, IgA, IgM, C_3 component of complement, fibrinogen, albumin, and polyvalent immunoglobulin (F/P ratio: 2–4 μg/mg – Meloy Laboratories, Springfield, Virginia 22151). A 1 to 5 dilution of antisera to PBS was used. The incubation period extended for 45 min after which the samples again underwent three separate 15–20 min washes in phosphate-buffered saline solution with constant stirring at room temperature. The washed sections were coverslipped with a 90% solution of glycerin and phosphate-buffered saline.

Immunofluorescence samples were examined within 18 h of incubation with antisera by epi-illumination using a Carl Zeiss Fluorescence Photomicroscope equipped with a 200 watt mercury illuminator light source, an LP 520 barrier filter, and a blue excitation filter set including a 410–490 nm. chromatic beam splitter and a 510 nm filter to direct the beam through the Zeiss Neofluor objectives. Specificity of positive fluorescence was checked by examining sections that were not incubated with fluorescent antiserum. Positive fluorescence was subjectively graded from 1 to 3 according to increasing intensity.

Samples for light microscopy were fixed in 10% buffered formalin. Basement membrane thickness quantification was achieved by the utilization of a calibrated magnifier on uniformly enlarged photomicrographs of tissues which had been specially stained by the periodic acid Schiff [10] or periodic acid silver methanamine [6] method to specifically highlight basement membranes. Measurements were accomplished according to the technique of Lin et al. [8] and Waterhouse and Squier [21]. After preliminary measurement attempts, it was judged to be appropriate to exclude the thyroid follicular and pancreatic acinar basement membranes from the basement membrane thickness investigation. In the case of the former, the vascular supply was such that vascular basement membranes closely approximated follicular basement membranes to the point of nondistinction. In the case of the latter, basement membrane definition was not distinct enough in a majority of the samples to render a valid comparison.

Statistical significance of the basement membrane thickness data was calculated using Student's *t*-test for paired observations [7].

Results

Immunofluorescence

The findings of the direct immunofluorescence phase of the study included positive fluorescence in kidney, parotid gland, and thyroid tissues (see Table 1–3 and Figs. 1–4).

Positive fluorescence in the kidney was due to the binding of antisera against IgG, polyvalent immunoglobulins (Ig's), and albumin to the glomerular and tubular basement membranes of all 15 diabetic subjects (see Table 1). Corresponding age and sex-matched control subjects displayed essentially negative fluorescence. Occasional small vessel fluorescence was noted in four diabetic and two control subjects due to binding of antisera against C_3 and fibrinogen. When it occurred, it was more intense in the diabetic subjects than in the controls (3+ vs. 1+).

Table 1. Research subjects

Subject number	*Diabetic* Age	Sex	Cause of death	Diabetic type	Subject number	*Control* Age	Sex	Cause of death
1	55	F	Micronodular cirrhosis and fatty metamorphosis of the liver	II	1A	55	F	*Diplococcus pneumoniae* bacteremia associated with hyposplenism
2	75	F	Diffuse myocardial fibrosis and ischemic myopathy	II	2A	75	F	Chronic obstructive pulmonary disease
3	81	M	Atherosclerotic heart disease	II	3A	80	M	Atherosclerotic heart disease and congestive heart failure
4	82	M	Myocardial infarction	II	4A	81	M	Septic thrombus of superior vena cava
5	61	M	Squamous cell Ca of bronchogenic origin	II	5A	62	M	Atherosclerotic heart disease
6	34	M	Acute pulmonary embolism following renal transplant	I	6A	35	M	Ruptured berry aneurysm
7	67	F	Acute myocardial rupture	I	7A	72	F	No anatomic cause of death
8	62	F	Metastatic breast Ca	II	8A	65	F	Suicidal drug overdose
9	61	M	Myocardial infarction	II	9A	62	M	Multiple traumatic injuries
10	47	M	Basal ganglia hemorrhage	I	10A	51	M	Atherosclerotic heart disease
11	46	M	Diabetic ketoacidosis	I	11A	50	M	Traumatic injury
12	41	M	Atherosclerotic heart desease	I	12A	40	M	Acute myocardial infarction
13	37	M	Gunshot wound	I	13A	31	M	Multiple drug overdose
14	32	M	Atherosclerotic heart disease	I	14A	30	M	Ruptured berry aneurysm
15	33	F	Pericardial tamponade secondary to uremic pericarditis	I	15A	44	F	Mitral valve prolapse syndrome

Table 2. Immunofluorescence findings for kidney

Experimental group	IgG	C_3	Fib	IgM	Ig's	IgA	Alb	Control group	IgG	C_3	Fib	IgM	Ig's	IgA	Alb
R-1	+++	−	+++[a]	−	+++	−	+++	R-1A	−	−	−	−	−	−	−
R-2	++	−	−	−	+++	−	++	R-2A	−	−	−	++	−	−	−
R-3	+++	−	−	−	+++	−	+++	R-3A	−	−	+[a]	−	−	−	−
R-4	+++	−	−	−	+++	−	+++	R-4A	−	−	−	−	−	−	−
R-5	++	−	−	−	++	−	+	R-5A	−	−	−	−	−	−	−
R-6	+++	−	−	−	+++	−	+++	R-6A	−	−	−	−	−	−	−
R-7	++	−	−	−	+++	−	+++	R-7A	−	−	−	−	−	−	−
R-8	++	−	+++[a]	−	++	−	+++	R-8A	+	−	+[a]	−	++	−	−
R-9	+++	+++[a]	−	−	++	−	+++	R-9A	−	−	−	−	−	−	−
R-10	++	−	−	−	++	−	+++	R-10A	−	−	−	−	−	−	−
R-11	+++	−	−	−	+++	+	+++	R-11A	−	−	−	+	−	−	−
R-12	+++	−	−	−	+++	−	+++	R-12A	−	−	−	−	−	−	−
R-13	++	−	−	−	++	−	+++	R-13A	−	−	−	−	−	−	−
R-14	+++	−	−	−	++	−	+++	R-14A	−	−	−	−	−	−	−
R-15	+++	−	++*	−	++	−	+++	R-15A	−	−	−	−	−	−	−

Except where indicated, fluorescence was localized to the glomerular and tubular basement membranes
+, ++, +++ Increasing degrees of intensity to fluorescence

[a] Fluorescence around vessels

Table 3. Immunofluorescence findings for parotid

Experimental group	IgG	C_3	Fib	IgM	Ig's	IgA	Alb	Control group	IgG	C_3	Fib	IgM	Ig's	IgA	Alb
R-1	+++	–	–	–	+++	–	+++	R-1A	–	–	–	–	–	–	–
R-2	++	–	–	–	+++	–	+++	R-2A	–	–	–	–	–	–	–
R-3	+	–	–	–	++	–	++	R-3A	–	–	–	–	–	–	–
R-4	+++	–	–	–	+++	–	++	R-4A	–	–	–	–	–	–	–
R-5	++	–	–	–	+++	–	–	R-5A	–	–	–	–	–	–	–
R-6	+++	–	–	–	+++	+++	+++	R-6A	–	–	–	–	–	–	–
R-7	+++	–	–	–	+++	+++	+++	R-7A	–	–	–	–	–	–	–
R-8	+	–	–	–	+++	++	++	R-8-A	–	–	–	–	–	–	–
R-9	++	–	–	–	+++	++	++	R-9A	–	–	–	–	–	–	–
R-10	+	–	–	–	+++	–	++	R-10A	–	–	–	–	–	–	–
R-11	+++	–	–	–	+++	–	+++	R-11A	–	–	–	–	–	–	–
R-12	+++	–	+++[a]	–	+++	+++	+++	R-12A	–	–	–	–	–	–	–
R-13	++	–	–	–	++	–	++	R-13A	–	–	–	–	–	–	–
R-14	+++	–	–	–	+++	++	+++	R-14A	–	–	–	–	–	–	–
R-15	+++	–	–	–	+++	++	+++	R-15A	–	–	–	–	–	–	–

Except where indicated, fluorescence was localized to acinar and ductal basement membranes
+, ++, +++ Increasing degrees of intensity to fluorescence

[a] Fluorescence around vessels

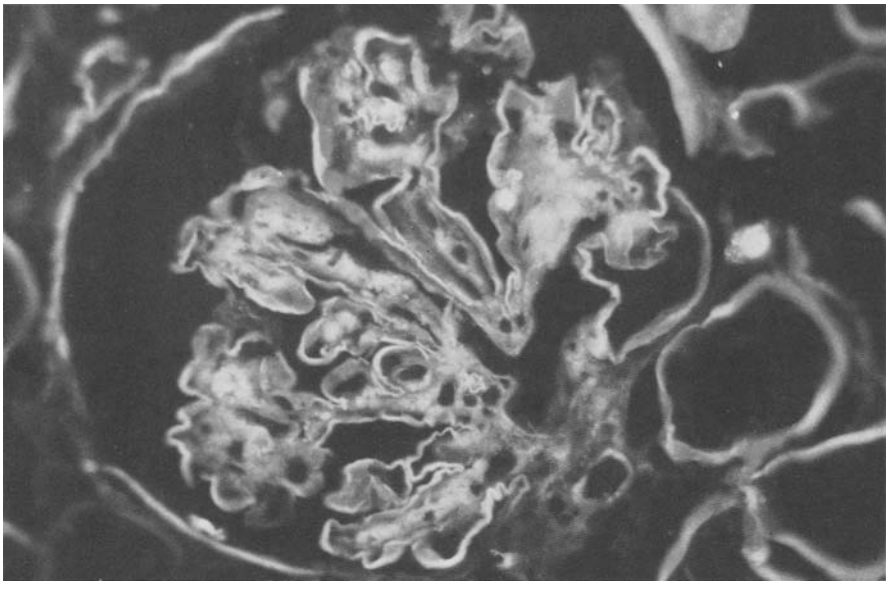

Fig. 1. Kidney glomerulus displaying fluorescence of basement membranes due to binding of antisera to I_gG

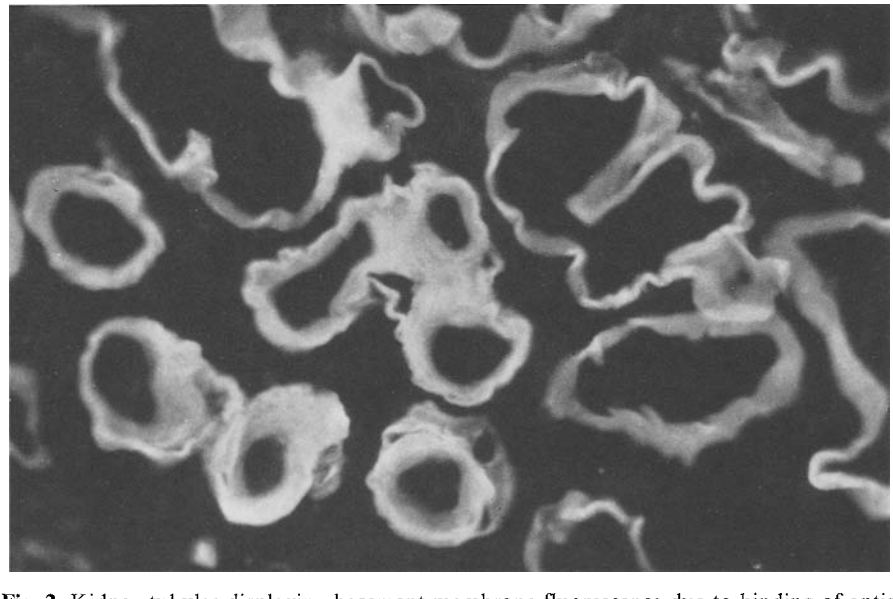

Fig. 2. Kidney tubules displaying basement membrane fluorescence due to binding of antisera to albumin

Table 4. Immunofluorescence findings for thyroid

Experimental group	IgG	C_3	Fib	IgM	Ig's	IgA	Alb	Control group	IgG	C_3	Fib	IgM	Ig's	IgA	Alb
R-1	–	–	–	–	–	–	–	R-1A	–	–	–	–	–	–	–
R-2	–	–	–	–	–	–	–	R-2A	–	–	–	–	–	–	–
R-3	–	–	–	–	–	–	–	R-3A	–	–	–	–	–	–	–
R-4	–	–	–	–	–	–	–	R-4A	–	–	–	–	–	–	–
R-5	–	–	–	–	–	–	–	R-5A	–	–	–	–	–	–	–
R-6	+++	–	–	–	+++	–	+++	R-6A	–	–	–	–	–	–	–
R-7	+++	–	–	–	+++	–	+++	R-7A	+	–	–	–	+	–	+
R-8	+	–	+	–	++	–	++	R-8A	–	–	–	–	–	–	–
R-9	+++	+	–	–	+++	–	+++	R-9A	–	–	–	–	–	–	–
R-10	++	–	–	–	++	–	++	R10A	–	–	–	–	–	–	–
R-11	++	–	–	–	+++	–	++	R-11A	–	–	–	–	–	–	–
R-12	–	–	–	–	–	–	–	R-12A	–	–	–	–	–	–	–
R-13	–	–	–	–	–	–	–	R-13A	–	–	–	–	–	–	–
R-14	++	–	–	–	+++	+	+++	R-14A	–	–	–	–	–	–	–
R-15	–	–	–	–	++	–	–	R-15A	–	–	–	–	–	–	–

Except where indicated, fluorescence was localized to follicular basement membranes
+, ++, +++ Increasing degrees of intensity to fluorescence

[a] Fluorescence around vessels

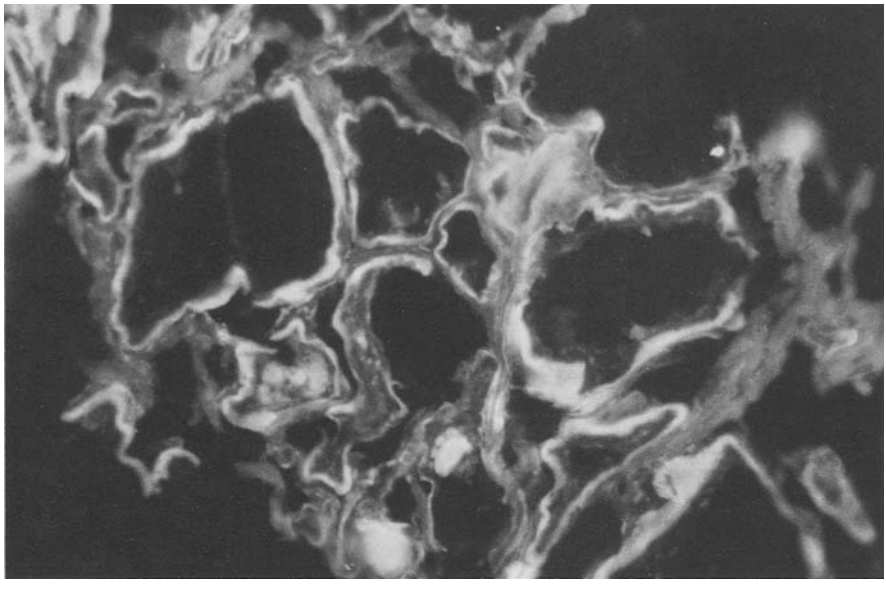

Fig. 3. Thyroid tissue displaying follicular basement membrane fluorescence due to binding of antisera to I_gG

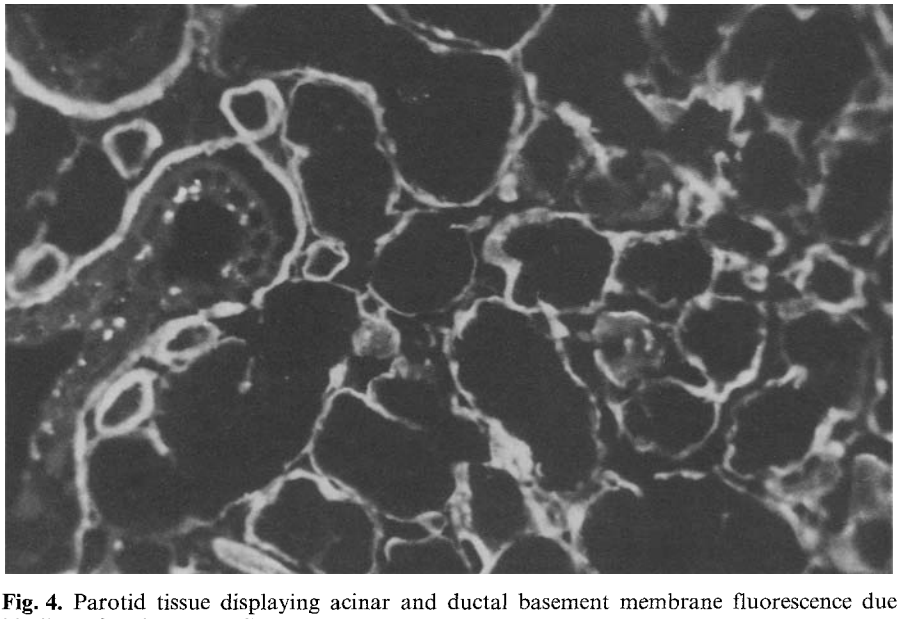

Fig. 4. Parotid tissue displaying acinar and ductal basement membrane fluorescence due to binding of antisera to I_gG

Table 5. Kidney tubules average basement membrane thickness[a]

Diabetic subject number	Basement membrane thickness	Control subject number	Basement membrane thickness
R-1	3.3	R-1A	2.0
R-2	4.0	R-2A	3.1
R-3	2.9	R-3A	1.9
R-4	7.5	R-4A	1.7
R-5	3.5	R-5A	2.65
R-6	7.8	R-6A	2.7
R-7	5.6	R-7A	2.0
R-8	6.7	R-8A	3.25
R-9	4.2	R-9A	2.8
R-10	6.6	R-10A	3.0
R-11	5.05	R-11A	1.75
R-12	6.7	R-12A	4.1
R-13	7.9	R-13A	2.0
R-14	9.25	R-14A	2.05
R-15	7.9	R-15A	2.3

[a] Thickness in millimeters as measured on uniformly enlarged photomicrographs

Positive fluorescence of acinar and ductal basement membranes in the parotid gland was similar to the kidney (see Table 2). All 15 diabetic subjects displayed acinar and ductal basement membrane fluorescence due to binding of the same antisera with the additional finding of positive fluorescence due to binding of antisera against IgA in 7 of the 15 diabetic subjects. Six of the seven diabetics displaying this additional finding had Type I diabetes. All control subject tissue was again negative.

Thyroid follicular basement membranes of 8 out of the 15 diabetic patients displayed positive linear fluorescence due to binding of antisera against IgG, polyvalent Ig's, and albumin (see Table 3). Control subjects were negative. Seven out of the eight diabetic subjects displaying positive fluorescence in this tissue had Type I diabetes.

Both experimental and control pancreatic and gingival tissues were fluorescence negative for all antisera.

Basement membrane measurements

Results of the basement membrane measurement phase of the investigation revealed statistically significant kidney tubular basement membrane thickening (see Table 5), as well as statistically significant gingival capillary basement membrane thickening (see Table 7) in diabetic subjects compared to controls. Renal tubular basement membrane thickening was significant at the $p < 0.0005$ level, and gingival capillary basement membrane thickening was significant at the $p < 0.01$ level.

Statistical analysis of parotid acinar and ductal basement membrane data revealed no statistically significant difference in the thickness of these membranes between diabetic and nondiabetic subjects.

Table 6. Parotid acini and ducts average basement membrane thickness[a]

Diabetic subject no.	Acinar B.M. thickness	Ductal B.M. thickness	Control subject no.	Acinar B.M. thickness	Ductal B.M. thickness
R-1	2.3	2.75	R-1A	1.9	2.0
R-2	1.2	2.75	R-2A	2.1	3.6
R-3	0.9	1.5	R-3A	1.0	2.6
R-4	1.4	1.7	R-4A	0.7	1.9
R-5	1.2	2.8	R-5A	1.3	2.3
R-6	1.6	3.0	R-6A	1.5	2.4
R-7	1.7	3.5	R-7A	1.0	1.5
R-8	2.75	2.9	R-8A	1.8	2.75
R-9	2.0	3.1	R-9A	1.4	2.4
R-10	1.99	2.6	R-10A	1.6	4.5
R-11	2.0	5.0	R-11A	2.0	3.65
R-12	1.75	3.8	R-12A	1.8	3.05
R-13	2.45	3.75	R-13A	1.4	4.3
R-14	2.25	2.7	R-14A	2.25	2.1
R-15	2.1	4.3	R-15A	1.65	2.75

[a] Thickness in millimeters as measured on uniformly enlarged photomicrographs

Table 7. Gingival capillaries[a] average basement membrane thickness

Diabetic Subject no.	B.M. thickness	Capillary diameter	B.M. thickness / Capillary diameter	Control subject no.	B.M. thickness	Capillary diameter	B.M. thickness / Capillary diameter
R-1	0.39	2.2	0.17	R-1A	1.15	7.1	0.161
R-2	1.25	4.9	0.255	R-2A	0.48	2.5	0.192
R-3	0.51	3.1	0.164	R-3A	0.18	1.1	0.163
R-4	1.55	3.5	0.442	R-4A	0.875	3.1	0.282
R-5	0.68	1.6	0.425	R-5A	0.52	2.3	0.226
R-6	1.225	3.2	0.382	R-6A	0.55	2.2	0.250
R-7	0.68	3.0	0.226	R-7A	0.38	2.2	0.172
R-8	0.625	1.6	0.390	R-8A	0.475	2.5	0.190
R-9	0.285	1.4	0.203	R-9A	0.285	2.6	0.109
R-10	0.55	1.3	0.423	R-10A	1.275	5.6	0.227
R-11	0.42	1.5	0.280	R-11A	1.47	5.3	0.277
R-12	0.735	3.2	0.229	R-12A	0.275	1.3	0.211
R-13	1.085	4.3	0.252	R-13A	0.70	2.8	0.250
R-14	0.575	3.4	0.169	R-14A	0.46	1.7	0.270
R-15	0.60	5.1	0.117	R-15A	0.34	3.0	0.113

[a] Basement membrane thickness and capillary diameter in centimeters as measured on uniformly enlarged photomicrographs

Discussion

The present investigation in unique in that its results reflect multi-organ basement membrane changes within individual diabetic subjects, thus substantiating a systemic nature to the process. Previous studies have not docu-

mented abnormal serum protein deposition in basement membranes of multiple tissues within a single diabetic subject.

Linear follicular fluorescence in the thyroid to IgG and albumin has heretofore been reported for only two diabetic cases [15].

A previously unreported abnormal binding of serum proteins to basement membrane in the diabetic parotid gland is demonstrated in this investigation. Interestingly, binding of IgA occurred only to parotid acinar and ductal basement membranes of the Type I diabetics in this study and binding of IgA to basement membranes of other Type I diabetic tissues studied did not occur. Secretory IgA differs from serum IgA in that its production occurs locally by plasma cells in the connective tissue stroma of the parotid gland. Dimeric IgA with complexed J chain diffuses through the interstitium, crosses the basement membrane, and enters the intercellular space where secretory component, present on lateral and basal epithelial cell membranes may serve to facilitate transport into the epithelial cell by an endocytotic process. Future studies might be directed toward determination of the presence of secretory piece. If secretory IgA is being bound to the basement membrane of the parotid in diabetic patients, as is suggested by these preliminary findings, the saliva of the diabetic may be less effective at controlling pathogens entering the body via the oral route.

A major objective of this investigation was to attempt to correlate selected serum protein deposition, as evidenced by positive immunofluorescence, with basement membrane thickening as interrelated parameters of diabetic membranopathy. Such a relationship is confirmed by this study for renal tubules. Clearly, however, no such relationship was noted in the parotid gland (Table 5).

Gingival capillary basement membrane thickening in diabetics was not associated with abnormal serum protein deposition in the present study. This negative finding in gingival capillaries is in contrast to the findings of serum protein deposition in the dermal capillaries of diabetic patients as reported by Chavers et al. [3]. The number of positive capillaries per diabetic subject in their study was modest, however, comprising approximately 8% of the capillaries per diabetic patient. This would appear to characterize the microangiopathy as being focal in nature. In the present investigation, the number of capillaries per gingival sample appeared to be fewer than in the dermal samples of Chavers et al., partially due, in all probability, to the fibrotic change observed by light microscopy in many of the samples.

The reason for the relationship between thickening and increased serum protein deposition, in some basement membranes, but not in others, is not clear. It could, however, be related to the functional and structural differences of the various tissues. For example, renal tubular basement membrane and capillary basement membrane might be considered to have more of a filtrative function, whereas thyroid follicular and parotid and pancreatic acinar and ductal basement membranes might differ due to their association with more of a secretory function. The deposition of IgA within diabetic membranes of the parotid, which has been demonstrated in this study, may

be illustrative of just such a local phenomenon based on functional tissue differences, since IgA deposition was not noted in other tissues displaying binding for IgG and albumin. Vracko's studies [20] may also shed some light on the question. In his study of diabetics, it was observed that basement membrane thickening was increased predominantly in the two sites in which basal lamina reduplication occurs normally during cell replenishment, the muscle capillaries and renal tubules.

The exact mechanism for either increase in thickness or abnormal serum protein deposition in basement membranes of diabetics is not yet established, however, an attractive hypothesis has been advanced by Rohrbach and Martin [16], where the lack of a specific proteoglycan, heparan sulfate, leaves newly formed basement membrane functionally defective, thus triggering synthesis of increased amounts of basement membrane that is defective. Heparan sulfate is postulated to function as a negatively charged shield that prevents serum anions from permeating the membrane. The aberrant basement membrane of the diabetic individual is, therefore, more cationic due to decreased amounts of this proteoglycan. Serum proteins, such as IgG_4, IgA, and albumin, with low isoelectric points, would thus be able to bind to the defective membrane [11].

Findings of the present study also tend to exclude a specific immunologic mechanism for the abnormal binding of immunoglobulin. The absence of complement binding and the similarity of findings for Type I and Type II subjects are strongly supportive of a non-immunologic mechanism for this phenomenon.

Since negative findings in some diabetic tissues may be indicative of slightly differing basement membrane morphology and composition from tissue to tissue based on functional requirements, further studies are directed toward elucidating these possible tissue differences and functional impairments.

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