Proteinuria and Activated T-Lymphocytes in Diabetic Nephropathy

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The reasons for the presence of activated T-lymphocytes (ATL) in some long-standing insulin-dependent diabetic (IDDM) patients are unknown. These cells have been implicated in the genesis of proteinuria in some forms of immune-mediated renal disease. We measured ATL in 18 IDDM patients with diabetic nephropathy, 10 with nonnephrotic proteinuria (total urinary protein excretion rate >0.5 and <3.5 g/24 h) and 8 with nephrotic proteinuria (total urinary protein excretion rate >3.5 g/24 h), and in 17 age-, sex-, and duration-of-diabetes–matched diabetic control subjects without clinical proteinuria (total urinary protein <0.5 g/24 h). T-lymphocytes purified from peripheral blood were stained by direct immunofluorescence with the fluorescein-labeled monoclonal antibody anti-HLA-DR. Absolute number and percent of DR-positive T-lymphocytes were significantly higher in patients with nonnephrotic proteinuria (median and range 162 × 10^6/ml, 40–320 × 10^6/ml; 13.9%, 8.1–19.4%) compared with nonproteinuric control subjects (81 × 10^6/ml, 2–240 × 10^6/ml, $P < .05$; 6.2%, 0–13.1%, $P < .01$). In 8 patients with nephrotic proteinuria, absolute and percent DR-positive T-lymphocytes tended to be lower (36 × 10^6/ml, 14–56 × 10^6/ml; 3.4%, 1.1–5.4%) than in nonproteinuric control subjects. An increased number of activated T-lymphocytes may be part of an immune-mediated process associated with the development of proteinuria in diabetic nephropathy. In advanced renal disease with nephrotic proteinuria, this immune process may become exhausted. *Diabetes* 37:507–11, 1988

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Received for publication 18 March 1987 and accepted in revised form 13 October 1987.

Expression of HLA-DR antigen on T-lymphocytes indicates their active involvement in an immune process (1). Although few activated T-lymphocytes are found in healthy subjects (2), significantly elevated levels are present in recently diagnosed insulin-dependent diabetic (IDDM) patients (3,4). T-lymphocyte activation tends to disappear after a few years of diabetes (4), probably the result of exhaustion of the immune process leading to destruction of the β-cell. Some long-term IDDM patients, however, maintain elevated levels of activated T-lymphocytes (4). Because most of these patients have undetectable levels of C-peptide (5), it is unlikely that residual β-cells are the cause of the persistent activation of the cellular immune system, and some other reason must be postulated. T-lymphocyte activation is reported to play a role in the pathogenesis of nondiabetic forms of renal disease (6). It is possible, therefore, that activated T-lymphocytes are also involved in the development of diabetic nephropathy.

We have studied the absolute number and percent of activated T-lymphocytes in the peripheral blood of nephropathic long-standing IDDM subjects with a wide range of proteinuria and impaired glomerular function and have compared them with those of nonproteinuric diabetic control subjects.

MATERIALS AND METHODS

Patients. Eighteen patients with persistent clinical proteinuria due to diabetic nephropathy were randomly selected from our cohort of ~70 nephropathic IDDM subjects. On the basis of at least three 24-h urinary total protein excretion measurements over the previous year, the patients were subdivided by conventional grouping into those with nephrotic proteinuria (>3.5 g/24 h) and those with nonnephrotic proteinuria (>0.5 and <3.5 g/24 h) and were matched with 17 patients similar for sex, age, and duration of diabetes but without clinical proteinuria (all with total urinary protein

<0.5 g/24 h). Their clinical features are shown in Table 1. All patients studied had a history of ketosis, with long-standing uninterrupted treatment with insulin. All 18 patients with diabetic nephropathy had clinical evidence of diabetic retinopathy (15 proliferative, 3 nonproliferative; assessed by direct ophthalmoscopy), and 15 also had evidence of both peripheral and autonomic neuropathy of varying degrees of clinical severity. The control group of 17 IDDM patients without nephropathy included 4 patients with both peripheral and autonomic neuropathy and 10 with evidence of diabetic retinopathy (8 nonproliferative, 2 proliferative). All patients were within 20% of ideal body weight (Metropolitan Life Insurance tables, 1959) and free of other major disease. Fifteen proteinuric patients, however, were treated for arterial hypertension with various drugs ranging from selective β-blockers to vasodilators, calcium antagonists, and loop diuretics. The nonproteinuric control subjects received no medication except insulin.

Methods. Twenty-four-hour urine collections were started at home during the day before admission to the hospital, and the collection was completed on arrival at the hospital, the urine being passed into clean detergent-free containers. Patients were admitted fasting to a metabolic ward between 0800 and 0900 h, having omitted insulin, tea, coffee, alcohol, and smoking since 2200 h the previous evening. After 15 min of rest, right brachial arterial pressure (phase I/V) was measured in the supine position to the nearest 2 mmHg by a single observer (J.J.B.). Blood was taken through an indwelling Teflon cannula without stasis for glycosylated hemoglobin (HbA_1), plasma creatinine, urea, electrolytes, and T-lymphocytes. An injection of 70 μCi of ^{51}Cr-EDTA was then given in the other arm, and samples were taken at predetermined regular intervals for the next 8 h for estimation of glomerular filtration rate (GFR) (7).

Preparation of T-lymphocytes. Twenty milliliters of blood were taken in preservative-free heparin. Total white blood cells (WBCs) were counted in a Coulter counter, and a blood film was prepared for differential WBC count. Peripheral blood mononuclear cells were separated within 2 h by centrifugation on a Ficoll-Triosil density gradient at 400 × *g* for 20 min at 18°C. The cells at the interface were recovered, washed three times in Hanks' balanced salt solution (Wellcome, Beckenham, UK), and resuspended at a concentration of 4 × 10^6/ml in RPMI-1640 (Gibco Europe, Uxbridge, UK) containing 2 mM L-glutamine, 200 U/ml penicillin, 100 μg/ml streptomycin, 2 μg/ml amphotericin B, and 10% heat-inactivated fetal calf serum (Gibco). Five hundred microliters of this cellular suspension were allowed to form rosettes with 500 μl of 1% neuraminidase-treated sheep red blood cells (SRBCs) at 4°C for 90 min. The rosetted T-lymphocytes were first counted to obtain the number of T-lymphocytes, then separated on a Ficoll-Triosil gradient followed by SRBC hypotonic lysis with Tris-buffered ammonium chloride solution. The resultant lymphocyte fraction contained >95% T-lymphocytes as determined by further SRBC rosetting and use of Leu-4 monoclonal antibody. The contamination of surface immunoglobulin-positive cells and monocytes, as assessed by direct immunofluorescence [fluorescein isothiocyanate (FITC) $F(ab')_2$ rabbit anti-human immunoglobulins; Dako, High Wycombe, UK] and MO2 monoclonal antibody (Ortho Diagnostics, High Wycombe, UK), was <3%. The viability of all cell preparations assessed by trypan blue dye exclusion was >95%.

To identify cell membrane activation markers, purified T-lymphocytes were stained for the presence of DR antigen by direct immunofluorescence with saturating amounts of FITC-conjugated anti-HLA-DR antibody (Becton Dickinson, Cowley, Oxford, UK). After 40 min incubation at 4°C, the cells were washed three times and examined under UV microscopy. For each experiment, at least 400 cells were counted by an observer (A.L.-Y.) unaware of the clinical details.

Other measurements. Urinary total protein concentrations were estimated by the Bradford method (8) and albumin concentration by radioimmunoassay (9). Total HbA_1 (normal range for healthy nondiabetic control subjects 5.5–7.3%) was estimated by the Corning method (Corning, Palo Alto, CA), plasma creatinine was measured by a reaction-rate method (LKB, South Croydon, Surrey, UK), and urea by standard multichannel biochemistry.

Statistics. The Kruskal-Wallis test of analysis of variance was used to test for differences among groups of patients. The Wilcoxon test was used for comparison between groups. Spearman's correlation coefficient was used to test for associations between variables.

RESULTS

Blood pressure, although treated, was higher in the proteinuric diabetic subjects; blood glucose control, as indicated by HbA_1 levels, was similar in all groups. The numbers of WBCs, total lymphocytes, and T-lymphocytes were similar in all groups (Table 1). Mean GFR was lowest and plasma creatinine and urea concentrations were highest in the diabetic subjects with nephrotic proteinuria; however, there

TABLE 1
Clinical characteristics and white blood cell counts in 3 groups of insulin-dependent diabetic patients

		Proteinuric subjects	
	Nonproteinuric subjects (*n* = 17)	Nonnephrotic (*n* = 10)	Nephrotic (*n* = 8)
Sex (M/F)	13/4	6/4	5/3
Age (yr)	41 (27–59)	46 (29–65)	39 (30–52)
Diabetes duration (yr)	23 (14–39)	26 (19–42)	24 (16–36)
White blood cells × 10^6/ml	7.57 (4.2–11.6)	6.27 (3.41–10.05)	6.34 (5.50–8.32)
Lymphocytes × 10^6/ml	2.4 (1.1–3.8)	2.1 (0.92–3.10)	2.0 (1.40–3.47)
T-lymphocytes × 10^6/ml	1.5 (0.72–2.45)	1.17 (0.50–2.25)	1.25 (0.82–2.65)

Values are means (median for white blood cells) and ranges.

TABLE 2
Measures of arterial pressure, glycemia, and renal function in 3 groups of insulin-dependent diabetic patients

	Nonproteinuric subjects	Proteinuric subjects	
		Nonnephrotic	Nephrotic
Mean blood pressure* (mmHg)	92 (83–103)	107 (83–117)†	105 (83–133)§
HbA_1 (%)	9.6 (7.6–11.8)	9.3 (6.5–17.1)	10.7 (7.4–13.8)
Glomerular filtration rate ($ml \cdot min^{-1} \cdot 1.73\ m^{-2}$)	114 (74–143)	57 (8–200)‡	34 (7–74)‖
Plasma urea (mM)	5.6 (4.1–6.7)	12.1 (4.5–34.3)‡	18.1 (6.4–47.6)‖
Plasma creatinine (μM)	90 (64–135)	193 (64–597)†	257 (96–457)†
Total urinary protein (g/24 h)	0.12 (0.01–0.30)	1.07 (0.5–3.3)	7.6 (3.9–11.1)
Albumin excretion rate (mg/24 h)	12.1 (2.8–20.4)	492 (73–2045)	2708 (759–4585)

Values are means and ranges.
*Diastolic + 1/3 pulse pressure.
†$P < .02$, ‡$P < .01$, §$P < .05$, and ‖$P < .002$ vs. nonproteinuric patients.

were no statistically significant differences between the two proteinuric groups. Total urinary protein and albumin excretion were, by selection, highest in the nephrotic patients (Table 2).

Percent and absolute number of DR-positive T-lymphocytes were significantly higher in the 10 patients with nonnephrotic proteinuria (median and range 13.9%, 8.10–19.4%; 162 × 10^6/ml, 40–320 × 10^6/ml) compared with nonproteinuric control subjects (6.2%, 0–13.1%, $P < .01$; 81 × 10^6/ml, 2–240 × 10^6/ml, $P < .05$) (Fig. 1). In the 8 patients with nephrotic proteinuria, however, percent and absolute DR-positive T-lymphocytes were found to be lower than in nonproteinuric diabetic control subjects (3.4%, 1.1–5.4%; 36 × 10^6/ml, 14–56 × 10^6/ml), although this difference did not reach statistical significance. Within each group, no significant correlation was found between numbers of DR-positive T-lymphocytes and HbA_1, total urinary protein excretion rate, plasma urea concentrations, or GFR levels.

DISCUSSION

This study shows that both the percent and absolute number of activated T-lymphocytes are significantly higher in long-standing IDDM subjects with nonnephrotic proteinuria than in those without proteinuria, suggesting an association between activation of T-lymphocytes and diabetic renal complication.

Involvement of cellular immunity in nephropathies unrelated to diabetes has been repeatedly reported. Thus, in Heymann's nephritis, an animal model of membranous nephritis, the disease is passively transferred by lymphoid cells (10). In humans, lymphocytes from patients with glomerulonephritis (11), minimal-change nephropathy (12), and membranous nephritis (13) are sensitized against antigens of the glomerular basement membrane.

The stimulus for T-lymphocyte activation in these diabetic patients is not known. It is unlikely to be the same trigger responsible for the β-cell–destructive process, because in most of these long-term patients, total destruction of β-cells with exhaustion of endocrine pancreatic function has occurred (5). Modification of glomerular basement membrane by glycosylation may confer antigenicity to the membrane, and carbohydrate residues are essential for this process (14). Treatment with periodate, which breaks the six-carbon ring structure of carbohydrates, abolishes the glomerular basement membrane immunogenicity (6). Immunogenic molecules, however, need to be seen in conjunction with proteins of the HLA class II genes to trigger T-lymphocyte activation. The mesangium is ideally suited for this function, because it contains cells that are rich in HLA class II proteins

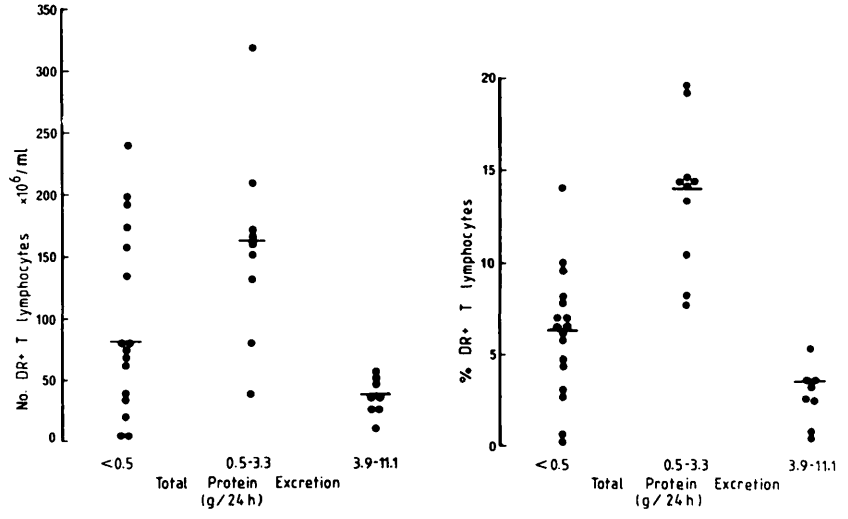

FIG. 1. Absolute number (*left*) and percent (*right*) DR-positive T-lymphocytes in diabetic patients without proteinuria (total urinary protein <0.5 g/24 h) and with nephrotic (total urinary protein 3.9–11.1 g/24 h) and nonnephrotic (total urinary protein 0.5–3.3 g/24 h) proteinuria. *Horizontal bars* represent median values.

(15) and can both present the immunogenic components of the glomerular basement membrane to the T-lymphocytes and release interleukin 1, the soluble T-lymphocyte–activating factor. Alternatively, the trigger for T-lymphocyte activation could originate in nonrenal structures, rendered similarly immunogenic by the diabetic process. This could explain the elevated levels of activated T-lymphocytes found in patients with severe autonomic neuropathy, many of whom, however, also had proteinuria (16).

Whatever the initial stimulus, a T-lymphocyte–soluble product has been proposed as the vascular permeability factor (17). In vitro studies indicate that T-lymphocyte activation can alter glomerular basement membrane through the release of endoglucosidase, an enzyme that digests heparan sulfate (18), a proteoglycan essential in providing the electrically negative charges that restrict the filtration of anionic plasma proteins such as albumin (19).

Interestingly, the level of activated T-lymphocytes in the group of patients with nephrotic proteinuria was found to be lower than in the other two groups. Cell-mediated immunity can be impaired by uremia (20). Although plasma urea concentrations tended to be higher in the patients with nephrotic proteinuria compared with those of patients with nonnephrotic proteinuria, this difference was not significant, and there was no correlation between indices of renal impairment and levels of activated T-lymphocytes. This result raises the possibility that in advanced diabetic renal disease the immune process may have become exhausted, the T-lymphocyte activation being "burned out," perhaps because of significant loss of altered glomerular basement membrane and removal of the primary stimulus to T-lymphocyte activation. Indeed, parallels for this model are found in other disease states, e.g., chronic active hepatitis, where T-lymphocyte activation may be pronounced during the active process but is reduced in inactive disease (21).

Urinary protein loss is dependent on several factors, including hemodynamic alterations and charge- and size-selectivity defects (22). Although T-lymphocyte–mediated charge-selectivity damage may be responsible for the initial development of proteinuria, factors that maintain and worsen proteinuria may be more dependent on hemodynamic and size-selectivity defects. There is evidence for this, and it is well known that diabetic proteinuria progresses from a highly selective proteinuria, in which anionic molecules like albumin are preferentially lost, to a low-selectivity proteinuria characterized by the loss of relatively greater amounts of larger neutral molecules such as IgG (23,24). These late, possibly self-perpetuating, mechanisms for the progression of proteinuria may explain the increased urinary protein loss in patients with nephrotic proteinuria in the face of an exhausted immune process, which may nevertheless have initiated the sequence of events by altering the charge-selective properties of the glomerular membrane.

The association described here does not rule out the possibility that the activation of T-lymphocytes reflects an immune response unrelated to the renal complications. Some patients were receiving antihypertensive drugs, which can lead to immune-mediated drug reactions (25). These drugs are unlikely to be responsible for the T-lymphocyte activation, however, because both groups of proteinuric patients, with high and low levels of activated T-lymphocytes, were on comparable drug regimens. An alternative possible cause for persistent T-lymphocyte activation in long-standing IDDM subjects is the administration of insulin. Receptors for insulin appear on T-lymphocytes when they become activated (26), and in culture, presence of insulin maintains the T-lymphocyte activation state (27). In our study, however, insulin does not appear to be a major activating stimulus, because all the patients were receiving insulin regardless of whether they had evidence of T-lymphocyte activation. Finally, T-lymphocyte activation does not seem to be a direct consequence of the extent of the renal-destructive process, because one would expect to find higher levels of activated T-lymphocytes in the late nephrotic stages of the disease, when structural disruption is more marked.

Our findings are therefore consistent with the view that activation of T-lymphocytes may be one of the contributory factors to the initiation of proteinuria in IDDM.

ACKNOWLEDGMENTS

We thank Professor Harry Keen for help and encouragement in this study, the Wolfson Foundation for supporting J.J.B., and Action Research for supporting A.L.-Y.

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