Autoantibodies to Autonomic Nerves Associated With Cardiac and Peripheral Autonomic Neuropathy

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OBJECTIVE — This study examines whether autonomic nerve autoantibodies (ANabs) are associated with development of autonomic neuropathy using a prospective study design.

RESEARCH DESIGN AND METHODS — A group of type 1 diabetic patients were followed prospectively with regard to autonomic nerve function on four occasions. At the third examination, 41 patients were tested for ANabs (complement-fixing autoantibodies to the sympathetic ganglion, vagus nerve, and adrenal medulla), and the results were related to cardiac autonomic nerve function (heart rate variation during deep breathing [expiration/inspiration ratio] and heart-rate reaction to tilt [acceleration and brake index]) and to peripheral sympathetic nerve function (vasoconstriction after indirect cooling [vasoconstriction index]).

RESULTS — ANabs were detected in 23 of 41 (56%) patients at the third examination. Compared with patients without ANabs ($ANabs^-$), patients with ANabs ($ANabs^+$) showed significantly higher frequencies of at least one abnormal cardiac autonomic nerve function test at the third examination (17 of 23 [74%] vs. 7 of 18 [39%]; $P = 0.03$) and fourth examination (15 of 21 [71%] vs. 4 of 16 [25%]; $P < 0.01$). In contrast, there was no similar difference at the first or second examination. The relative risk for $ANabs^+$ patients to develop cardiac autonomic neuropathy at follow-up was 7.5 (95% CI 1.72–32.80). The vasoconstriction index was more abnormal in $ANabs^+$ than in $ANabs^-$ patients at the fourth examination (median 1.40 [interquartile range 1.58] vs. 0.35 [2.05]; $P = 0.01$).

CONCLUSIONS — ANabs were associated with future development of cardiac and peripheral autonomic neuropathy in diabetic patients, implying an etiological relationship between nervous tissue autoimmunity and these diabetes complications.

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Autonomic neuropathy is a common and serious complication of diabetes and is known to be an independent risk factor for increased mortality in diabetic patients (1–6). Currently, approaches to treatment are limited by a lack of any clear understanding of the etiopathogenesis of this condition. To develop novel and evidence-based strategies for the prevention and treatment of diabetic autonomic neuropathy, it is therefore essential to establish the disease mechanisms involved in its pathogenesis.

It is often assumed that autonomic and peripheral neuropathy are similar in this context and thus that metabolic and vascular events are of major importance (7,8). However, the possibility of an autoimmune basis to autonomic neuropathy has been frequently highlighted. Lymphocytic infiltrations and small nerve fiber damage within autonomic nerve structures in diabetic patients with severe symptomatic autonomic neuropathy have been reported (9). Furthermore, diabetic autonomic neuropathy has been associated with increased levels of circulating immune complexes and activated T-cells (10). Moreover, autoantibodies to autonomic nerve structures (ANabs) have been reported independently from several different laboratories (11–14). Although some of these studies have implied a correlation between ANabs and autonomic dysfunction (15,16), this has been difficult to establish. The question would be addressed most powerfully using a prospective study design, in which patients with and without ANabs were followed with regard to development of diabetic autonomic neuropathy.

In light of this, we determined ANabs in a prospectively followed group of type 1 diabetic patients (17–19) and related the findings to autonomic nerve function at the time of blood sampling, as well as 3 and 6 years previously and 7 years after. The aim of our study was to clarify whether ANabs are associated with disturbed autonomic nerve function.

RESEARCH DESIGN AND METHODS

— In 1984–1985, a total of 58 diabetic patients, all diagnosed with type 1 diabetes at 15–25 years of age, were evaluated with regard to autonomic nerve function at the Diabetes Clinic at Malmö University Hospital, Sweden (17). After the baseline examination, all patients were invited to three subsequent follow-up examinations conducted at the same clinic over a period of 13–14 years. At the third examination (18), 6 years after the first examination, blood samples for estimation of ANabs were taken from all 41 participating patients. ANab analy-

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Abbreviations: ANab, autonomic nerve autoantibody; E/I, expiration/inspiration; ICA, islet cell antibody; LDI, laser Doppler imaging.

A table elsewhere in this issue shows conventional and Système International (SI) units and conversion factors for many substances.

Table 1—*Clinical characteristics of type 1 diabetic patients assessed for autonomic nerve antibodies*

	Examination			
	Baseline	Second	Third	Fourth
n	41	38*	41	37*
Age (years)	34 ± 9	39 ± 8	40 ± 9	46 ± 9
Men/women	25/16	23/15	25/16	23/14
Duration of diabetes (years)	13 ± 8	17 ± 8	19 ± 8	25 ± 9
Microalbuminuria	11 (42)	5 (16)	7 (26)	8 (21)
Systolic blood pressure (mmHg)	129 ± 17	128 ± 14	130 ± 18	133 ± 19
Diastolic blood pressure (mmHg)	77 ± 10	80 ± 8	79 ± 8	81 ± 10
A1C (%)	7.1 ± 1.3	6.5 ± 1.1	7 ± 1.3	7.3 ± 1
Retinopathy	16 (39)			34 (92)

Data are means ± SE or *n* (%). *At the second examination, 38 of 41 patients assessed for ANabs participated, and at the fourth examination 37 of the 41 participated. Microalbuminuria was measured in 26, 32, 27, and 37 patients at the first, second, third, and fourth examination, respectively.

ses were performed by the Department of Immunobiology, King's College, London, U.K. Data in this report are based on these 41 patients (Table 1).

Assay for complement-fixing nervous tissue antibodies

Complement-fixing autonomic nervous tissue antibodies to sympathetic ganglia, vagus nerve, and adrenal medulla were determined as previously described (20). Autoantibody assays were performed in blinded fashion by operators unaware of the clinical details of the patients. Briefly, an indirect immunofluorescent complement-fixation technique was used with monkey adrenal gland (INOVA, Birmingham, U.K.), rabbit vagus nerve, and cervical ganglia as substrates. Vagus nerve and sympathetic chain cervical ganglia were isolated from an adult New Zealand White rabbit, snap frozen, and stored in liquid nitrogen until use. Cryostat sections (5-μm thick) of vagus nerve and ganglia were allowed to dry and then fixed in acetone for 5 min. Sections were incubated with 50 μl of undiluted test serum for 30 min at room temperature. Slides were then washed in PBS (140 mmol/l NaCl, 3 mmol/l KCl, 8 mmol/l Na_2HPO_4, and 1 mmol/l KH_2PO_4, pH 7.2) for 15 min, and 50 μl of fresh normal human serum (a source of complement) was added at a 1:5 dilution in PBS. After incubation for 30 min at 37°C, slides were again washed, 50 μl fluorescein isothiocyanate-conjugated sheep anti-human C3c (Binding Site, Birmingham, U.K.) diluted 1:20 in PBS was added, and slides were incubated for 30 min at room temperature. Slides were thereafter washed, mounted in PBS glycerol, and examined under an ultraviolet microscope (Polyvar, Vienna, Austria). Unaware of clinical details, two observers independently read the slides. Positivity for ANabs was confirmed by repeated measurement using substrate from a different animal.

Islet cell antibodies

Islet cell antibodies (ICAs) were determined using a prolonged two-color immunofluorescence assay (21), and the results were expressed in Juvenile Diabetes Foundation units. In the Diabetes Autoantibody Proficiency Program no. 13, the assay performed with 100% sensitivity and specificity. ICAs >0 Juvenile Diabetes Foundation units were considered abnormal. GAD antibody was determined by a radioligand assay (22) and presented as an index. In the Diabetes Autoantibody Standardization programs, the GAD antibody assay performed with 80% sensitivity and 96% specificity. A GAD antibody index >13.4 was considered abnormal (97.5 percentile of 134 healthy control subjects 35–70 years of age).

Cardiac autonomic nerve function tests

Parasympathetic vagal nerve function (23) was evaluated by determination of the expiration/inspiration (E/I) ratio during deep breathing. Six maximal expirations and inspirations were performed during 1 min in the supine position. The R-R intervals were recorded using a continuous electrocardiogram recorder. The E/I ratio was calculated as the mean of the longest R-R interval during expiration divided by the mean of the longest R-R interval during inspiration. The E/I ratio was expressed in age-corrected values (i.e., *Z* scores in SD). Values ≤1.64 were considered abnormal (<95% CI, one-sided test) (19).

The heart rate reaction to tilt, an immediate acceleration followed by a transient deceleration, was evaluated using continuous electrocardiogram recording. After a 10-min rest, the patient was tilted (<2 s) to the upright position (head up 90°) and remained there for 8 min. The acceleration index and brake index (24) were determined using the formulas: acceleration index = A − B/A × 100 and brake index = C − B/A × 100. In the formulas, A indicates mean R-R interval before tilt, B indicates the shortest interval during the immediate acceleration, and C indicates the longest R-R interval during the deceleration. Indexes were expressed in age-corrected values (i.e., *Z* scores in SD), and values ≤1.64 were considered abnormal (<95% CI, one-sided test) (19).

Peripheral sympathetic nerve function test

At the fourth examination, cutaneous sympathetic nerve function was evaluated using laser Doppler imaging (LDI) of the skin (25). Finger skin blood flow changes were measured during constant local heating of the fourth finger to a temperature of 40°C followed by indirect cooling of the contralateral hand. A sympathetic vasoconstriction index was determined, defined as the LDI value after indirect cooling divided by the LDI value after local heating. The vasoconstriction index was expressed in age-corrected values (i.e., *Z* scores in SD), and values ≥1.64 were considered abnormal) (26).

Statistical analysis

The Mann-Whitney *U* test was used for comparing differences between groups, and Fisher's exact test was used for comparing frequencies between groups. *P* values <0.05 were considered significant. Data are presented as median (interquartile range) or *n* (%). Relative risk, sensitiv-

Table 2—*Autoantibody distribution at the third examination*

	ANabs$^+$	ANabs$^-$
n	23	18
CF sympathetic ganglia (*n*)	12	—
CF adrenal medulla (*n*)	8	—
CF vagus nerve (*n*)	6	—
CF sympathetic ganglia + CF adrenal medulla (*n*)	1	—
CF sympathetic ganglia + CF vagus nerve (*n*)	1	—
CF adrenal medulla + CF vagus nerve (*n*)	1	—
GADA (*n*)	2	5
ICA (*n*)	2	1
GAD antibodies + ICA (*n*)	7	4

CF, complement fixing.

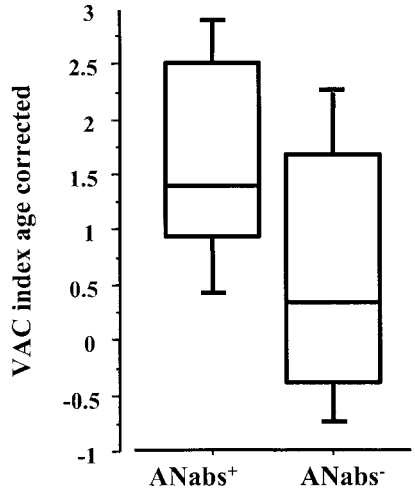

Figure 2— *The vasoconstriction (VAC) index expressed in age-corrected Z scores at examination 4. Data are presented as box and whisker plots showing the 10th–25th, 50th (median), 75th, and 90th percentiles. The vasoconstriction index was significantly* (P = *0.01) more abnormal in ANabs$^+$ versus ANabs$^-$.*

ity, and specificity, were analyzed using standard methods.

RESULTS — At the third examination, ANabs were detected in 23 of 41 (56%) patients: 12 had complement-fixing sympathetic ganglia (52%), 8 had complement-fixing adrenal medulla (35%), and 6 had complement-fixing vagus nerve (26%) autoantibodies (Table 2). ICAs were found in 21 of 41 (51%) patients, but there were no associations between ICAs and ANabs (Table 2).

Figure 1*A* shows that the E/I ratio did not differ between patients with ANabs (ANabs$^+$) and patients without ANabs (ANabs$^-$) at baseline, second examination, and third examination. However, at the fourth examination, the median E/I ratio was significantly lower in ANabs$^+$ than in ANabs$^-$ patients (−1.68 [1.66] vs. −0.87 [1.16]; $P < 0.05$). In agreement, the number of patients with an abnormal E/I ratio was significantly higher in ANabs$^+$ than in ANabs$^-$ (11 of 21 ANabs$^+$ [52%] vs. 2 of 16 ANabs$^-$ [13%]; $P < 0.02$) at the fourth examination. There was no association between complement-fixing vagus nerve and abnormal E/I ratios at the fourth examination. Abnormal E/I ratio was found in 2 of 13 patients with complement-fixing vagus nerve antibodies compared with 2 of 24 patients without complement-fixing vagus nerve antibodies.

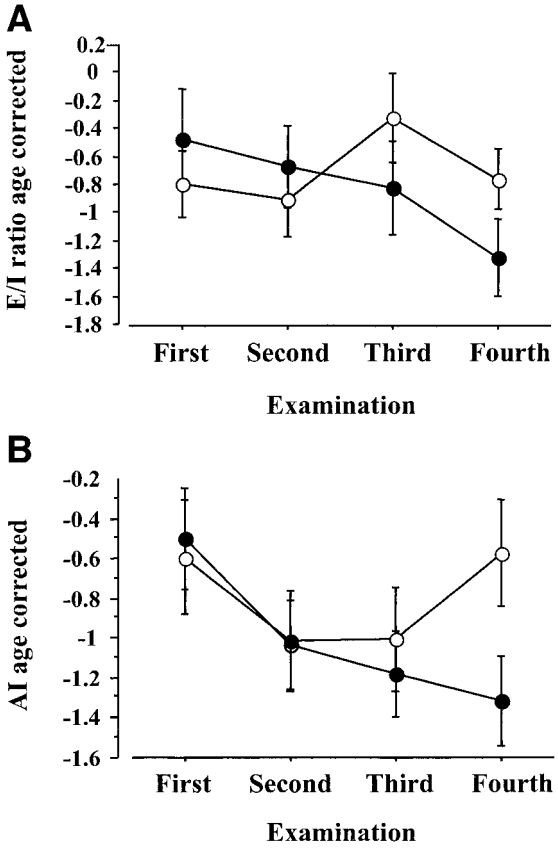

Figure 1—A*: The E/I ratio in ANabs$^+$ versus ANabs$^-$ at four examinations. The E/I ratio was significantly* (P < *0.05) lower at examination 4 in ANabs$^+$* (■) *versus ANabs$^-$* (□). B*: Acceleration index in ANabs$^+$* (■) *versus ANabs$^-$* (□) *at four examinations. The acceleration index (AI) tended to be significantly lower* (P < *0.07) in ANabs$^+$ versus ANabs$^-$ at examination 4. Results are expressed in age-corrected Z scores and presented as means ± SE.*

In agreement with the E/I ratio, there was a tendency for the acceleration index to be lower in ANabs$^+$ versus ANabs$^-$ at the fourth examination ($P < 0.07$) (Fig. 1*B*). Although there was no significant difference in the brake index between ANabs$^+$ and ANabs$^-$ patients, there was a tendency for ANabs$^+$ patients to have an increased frequency of abnormal brake index at the third examination (12 of 23 [52%] ANabs$^+$ vs. 4 of 18 [22%] ANabs$^-$; $P = 0.06$). At the fourth examination, the median vasoconstriction index was significantly more abnormal in ANabs$^+$ than in ANabs$^-$ (1.40 [1.58] vs. 0.35 [2.05]; $P = 0.01$) (Fig. 2).

Signs of cardiac autonomic neuropathy (CAN; i.e., at least one abnormal cardiac autonomic nerve function test) were as frequent in ANabs$^+$ as in ANabs$^-$ at baseline (10 of 22 [45%] vs. 11 of 18

Table 3—*Antibody status at baseline and autonomic neuropathy score 7 years later among 23 ANabs$^+$ patients*

Patient	CF adrenal medulla	CF sympathetic ganglia	CF vagus nerve	Abnormal E/I	Abnormal acceleration index	Abnormal brake index
1		+		+	+	+
2			+			
3		+				
4		+		+		
5	+			+		
6		+			+	
7		+				
8		+				
9	+			+	+	+
10			+		+	
11	+			+	+	
12	+					
13	+		+	+	+	
14	+	+		+		
15		+		+		
16	+			+		
17	+					
18			+	+		
19		+			+	
20		+		+		
21		+			+	
22			+	*	*	*
23	+			*	*	*

*Patient did not participate at follow-up. CF, complement fixing.

[61%]; $P = 0.36$) and the second examination (13 of 21 [62%] vs. 11 of 17 [65%]; $P = 0.99$). However, thereafter the frequency of CAN increased in ANabs$^+$ patients and decreased in ANabs$^-$ patients (third examination: 17 of 23 [74%] vs. 7 of 18 [39%], $P = 0.03$; fourth examination: 15 of 21 [71%] vs. 4 of 16 [25%], $P < 0.01$). With a sensitivity of 71% and a specificity of 75%, the relative risk ratio for ANabs$^+$ having CAN at the fourth examination was 7.5 (95% CI 1.72–32.80).

There were no associations between ANabs versus retinopathy, microalbuminuria, and HbA_{1c} (A1C). Compared with ANabs$^-$ patients, ANabs$^+$ patients tended to be younger (35.5 [11.0] vs. 42.5 [9.5] years; $P < 0.04$) and have shorter diabetes duration (14.0 [10.0] vs. 19.5 [8.5] years; $P < 0.05$) at the second examination.

CONCLUSIONS — Our prospective 13- to 14-year study of type 1 diabetic patients showed an association between ANabs, taken 6 years after the beginning of the study, and objective signs of autonomic dysfunction at the time of blood sampling. Moreover, a closer association between ANabs and autonomic dysfunction could be seen 7 years later, whereas no associations were shown 3 and 7 years before blood sampling. Hence, our study infers that ANabs have a primary association with the development and progression of autonomic neuropathy in type 1 diabetic patients and implies that autonomic nervous tissue autoimmunity has an etiological role in this condition.

The present study shows associations between ANabs and both sympathetic and parasympathetic autonomic dysfunction. ANabs$^+$ patients showed a deterioration in the E/I ratio (a parasympathetic test) and vasoconstriction index (a sympathetic test) compared with patients without ANabs. However, there were no obvious associations between vagal nerve antibodies and parasympathetic dysfunction or between sympathetic ganglion nerve antibodies and sympathetic dysfunction (Table 3). Hence, our study indicates that autoreactivity directed against autonomic nerves in general rather than specific antigenic targets in the parasympathetic or sympathetic nervous tissue drives the generation of ANabs. This diversity of antigenic and tissue targets could explain why previous attempts to characterize the antigenic structures against which ANabs are directed have not been successful (16,27).

ANabs were detected in more than half of our patients. The finding of a similar frequency of ANabs in other studies (11,14,15,28) strongly implies that ANabs are a frequent characteristic of type 1 diabetes. Our study is the first to show an association between ANabs and the future development of autonomic dysfunction, suggesting that the routine detection of ANabs may be of clinical relevance in the prediction and management of this condition.

We did not find any association between ANabs and islet antibodies, suggesting that ANabs are specifically related to the autonomic nervous system and do not reflect an extension of islet autoimmunity. This is also in agreement with previous reports showing a lack of association between islet antibodies and diabetic autonomic neuropathy (29–31). The fact that ANabs, once present, tend to persist (16) also favors the suggestion that ANabs are specific for autonomic nervous tissues. In contrast, islet antibodies tend to disappear with increasing diabetes duration, presumably as antigenic targets in the islets are lost due to β-cell destruction (32).

We did not find any association between ANabs and A1C, suggesting that susceptibility to ANabs formation is not related to glycated protein factors acting as neoepitopes on nerve structures. Indeed, our type 1 blood glucose levels in our diabetic patients were rather well controlled, with A1C levels close to target according to treatment standards in Sweden. The observation that ANabs$^+$ patients were younger and had shorter diabetes duration than ANabs$^-$ patients also argues against a direct effect of glycation as the cause of ANabs.

The accumulated findings from this and previous studies provide persuasive evidence that autoimmunity is a primary pathogenetic factor in the development of autonomic nervous tissue damage, as also suggested by previous reports of cellular autoimmunity (9,33), associations with iritis (34), and findings of lymphocytic in-

filtrates in postmortem specimens (9). ANabs could also account for the complement-fixing serum factor reported by Pittinger et al. (35) that might be neurotoxic in type 1 diabetic patients (36). The autoimmunity theory does not preclude the possibility that hyperglycemic and vascular factors also have a primary role in the development of these complications. Indeed, we propose a scenario in which autonomic nerve damage primarily caused by metabolic and/or vascular factors is an early event, exposing epitopes against which sufficient immunological tolerance does not exist, which leads to autonomic nerve infiltration by activated lymphocytes and the production of ANabs. In fact, the possibility that ANabs (autoimmunity) are promoters of the progression autonomic neuropathy has to be considered.

In summary, ANabs were found in half of type 1 diabetic patients in our prospectively followed cohort and were associated with progressive autonomic nerve dysfunction. We conclude that autonomic neuropathy in type 1 diabetes may have an autoimmune background.

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References

1. O'Brien IA, O'Hare JP, Lewin IG, Corrall RJ: The prevalence of autonomic neuropathy in insulin-dependent diabetes mellitus: a controlled study based on heart rate variability. *Q J Med* 61:957–967, 1986
2. Wheeler SG, Ahroni JH, Boyko EJ: Prospective study of autonomic neuropathy as a predictor of mortality in patients with diabetes. *Diabetes Res Clin Pract* 58:131–138, 2002
3. Chen HS, Hwu CM, Kuo BI, Chiang SC, Kwok CF, Lee SH, Lee YS, Weih MJ, Hsiao LC, Lin SH, Ho LT: Abnormal cardiovascular reflex tests are predictors of mortality in type 2 diabetes mellitus. *Diabet Med* 18:268–273, 2001
4. O'Brien IA, McFadden JP, Corrall RJ: The influence of autonomic neuropathy on mortality in insulin-dependent diabetes. *Q J Med* 79:495–502, 1991
5. Maser RE, Mitchell BD, Vinik AI, Freeman R: The association between cardiovascular autonomic neuropathy and mortality in individuals with diabetes: a meta-analysis. *Diabetes Care* 26:1895–1901, 2003
6. Vinik AI, Freeman R, Erbas T: Diabetic autonomic neuropathy. *Semin Neurol* 23: 365–372, 2003
7. Sugimoto K, Murakawa Y, Sima AA: Diabetic neuropathy: a continuing enigma. *Diabetes Metab Res Rev* 16:408–433, 2000
8. Tesfaye S, Malik R, Ward JD: Vascular factors in diabetic neuropathy. *Diabetologia* 37:847–854, 1994
9. Duchen LW, Anjorin A, Watkins PJ, Mackay JD: Pathology of autonomic neuropathy in diabetes mellitus. *Ann Intern Med* 92:301–303, 1980
10. Gilbey SG, Guy RJ, Jones H, Vergani D, Watkins PJ: Diabetes and autonomic neuropathy: an immunological association? *Diabet Med* 3:241–245, 1986
11. Ejskjaer N, Arif S, Dodds W, Zanone MM, Vergani D, Watkins PJ, Peakman M: Prevalence of autoantibodies to autonomic nervous tissue structures in type 1 diabetes mellitus. *Diabet Med* 16:544–549, 1999
12. Muhr-Becker D, Ziegler AG, Druschky A, Wolfram G, Haslbeck M, Neundorfer B, Standl E, Schnell O: Evidence for specific autoimmunity against sympathetic and parasympathetic nervous tissues in type 1 diabetes mellitus and the relation to cardiac autonomic dysfunction. *Diabet Med* 15:467–472, 1998
13. Stroud CR, Heller SR, Ward JD, Hardisty CA, Weetman AP: Analysis of antibodies against components of the autonomic nervous system in diabetes mellitus. *QJM* 90:577–585, 1997
14. Rabinowe SL, Brown FM, Watts M, Smith AM: Complement-fixing antibodies to sympathetic and parasympathetic tissues in IDDM: autonomic brake index and heart-rate variation. *Diabetes Care* 13: 1084–1088, 1990
15. Sundkvist G, Lind P, Bergstrom B, Lilja B, Rabinowe SL: Autonomic nerve antibodies and autonomic nerve function in type 1 and type 2 diabetic patients. *J Intern Med* 229:505–510, 1991
16. Zanone MM, Favaro E, Catalfamo E, Quadri R, Sacchetti C, Cerutti F, Perin PC, Camussi G: Autonomic function and autoantibodies to autonomic nervous tissues at follow-up in a cohort of young patients with type 1 diabetes: effects of serum from diabetic patients on human adrenergic cells. *J Neuroimmunol* 139:66–75, 2003
17. Bergstrom B, Lilja B, Osterlin S, Sundkvist G: Autonomic neuropathy in type I diabetes: influence of duration and other diabetic complications. *Acta Med Scand* 222: 147–154, 1987
18. Nilsson H, Bergstrom B, Lilja B, Juul-Moller S, Carlsson J, Sundkvist G: Prospective study of autonomic nerve function in type 1 and type 2 diabetic patients: 24 hour heart rate variation and plasma motilin levels disturbed in parasympathetic neuropathy. *Diabet Med* 12: 1015–1021, 1995
19. Forsen A, Kangro M, Sterner G, Norrgren K, Thorsson O, Wollmer P, Sundkvist G: A 14-year prospective study of autonomic nerve function in type 1 diabetic patients: association with nephropathy. *Diabet Med* 21:852–858, 2004
20. Zanone MM, Peakman M, Purewal T, Watkins PJ, Vergani D: Autoantibodies to nervous tissue structures are associated with autonomic neuropathy in type 1 (insulin-dependent) diabetes mellitus. *Diabetologia* 36:564–569, 1993
21. Olsson ML, Sundkvist G, Lernmark A: Prolonged incubation in the two-colour immunofluorescence test increases the prevalence and titres of islet cell antibodies in type 1 (insulin-dependent) diabetes mellitus. *Diabetologia* 30:327–332, 1987
22. Borg H, Fernlund P, Sundkvist G: Measurement of antibodies against glutamic acid decarboxylase 65 (GADA): two new 125I assays compared with [35S]GAD 65-ligand binding assay. *Clin Chem* 43: 779–785, 1997
23. Sundkvist G, Almer L, Lilja B: Respiratory influence on heart rate in diabetes mellitus. *Br Med J* 1:924–925, 1979
24. Sundkvist G, Lilja B, Almer LO: Abnormal diastolic blood pressure and heart rate reactions to tilting in diabetes mellitus. *Diabetologia* 19:433–438, 1980
25. Bornmyr S, Svensson H, Lilja B, Sundkvist G: Skin temperature changes and changes in skin blood flow monitored with laser Doppler flowmetry and imaging: a methodological study in normal humans. *Clin Physiol* 17:71–81, 1997
26. Freccero C, Svensson H, Bornmyr S, Wollmer P, Sundkvist G: Sympathetic and parasympathetic neuropathy are frequent in both type 1 and type 2 diabetic patients. *Diabetes Care* 27:2936–2941, 2004
27. Husebye ES, Winqvist O, Sundkvist G, Kampe O, Karlsson FA: Autoantibodies against adrenal medulla in type 1 and type 2 diabetes mellitus: no evidence for an association with autonomic neuropathy. *J Intern Med* 239:139–146, 1996
28. Schnell O, Muhr D, Dresel S, Tatsch K, Ziegler AG, Haslbeck M, Standl E: Autoantibodies against sympathetic ganglia and evidence of cardiac sympathetic dysinnervation in newly diagnosed and long-term IDDM patients. *Diabetologia* 39: 970–975, 1996
29. Sundkvist G, Velloso LA, Kampe O, Rabi-

nowe SL, Ivarsson SA, Lilja B, Karlsson FA: Glutamic acid decarboxylase antibodies, autonomic nerve antibodies and autonomic neuropathy in diabetic patients. *Diabetologia* 37:293–299, 1994
30. Muhr D, Mollenhauer U, Ziegler AG, Haslbeck M, Standl E, Schnell O: Autoantibodies to sympathetic ganglia, GAD, or tyrosine phosphatase in long-term IDDM with and without ECG-based cardiac autonomic neuropathy. *Diabetes Care* 20: 1009–1012, 1997
31. Zanone MM, Petersen JS, Peakman M, Mathias CJ, Watkins PJ, Dyrberg T, Vergani D: High prevalence of autoantibodies to glutamic acid decarboxylase in long-standing IDDM is not a marker of symptomatic autonomic neuropathy. *Diabetes* 43:1146–1151, 1994
32. Borg H, Marcus C, Sjoblad S, Fernlund P, Sundkvist G: Islet cell antibody frequency differs from that of glutamic acid decarboxylase antibodies/IA2 antibodies after diagnosis of diabetes. *Acta Paediatr* 89: 46–51, 2000
33. Gilbey SG, Hussain MJ, Watkins PJ, Vergani D: Cell-mediated immunity and symptomatic diabetic autonomic neuropathy. *Diabet Med* 5:845–848, 1988
34. Guy RJ, Richards F, Edmonds ME, Watkins PJ: Diabetic autonomic neuropathy and iritis: an association suggesting an immunological cause. *Br Med J (Clin Res Ed)* 289:343–345, 1984
35. Pittenger GL, Liu D, Vinik AI: The neuronal toxic factor in serum of type 1 diabetic patients is a complement-fixing autoantibody. *Diabet Med* 12:380–386, 1995
36. Pittenger GL, Malik RA, Burcus N, Boulton AJ, Vinik AI: Specific fiber deficits in sensorimotor diabetic polyneuropathy correspond to cytotoxicity against neuroblastoma cells of sera from patients with diabetes. *Diabetes Care* 22:1839–1844, 1999