Anti-tubulin antibodies in recent onset Type 1 (insulin-dependent) diabetes mellitus: comparison with islet cell antibodies

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Summary. Antibodies to tubulin, the fundamental protein of microtubules, were studied by radioimmunoassay in patients with Type 1 (insulin-dependent) diabetes of varying duration and in healthy control subjects. Elevated levels of anti-tubulin antibodies were found in 46% of 28 patients with Type 1 diabetes of recent onset (≤ 6 months) and in only 6.2% of 64 patients with long-standing Type 1 diabetes (duration 6–43 years). None of 34 DR3-positive normal subjects and none of 20 Type 2 (non-insulin-dependent) diabetic patients were positive for anti-tubulin antibodies. Anti-tubulin antibody levels were elevated in two out of 26 first-degree relatives of Type 1 diabetic patients. The specificity of the detection of anti-tubulin antibodies was demonstrated by (1) dilution of the sera, (2) competitive binding experiments between labelled and unlabelled tubulin, (3) immunoblotting. Antibodies to tubulin were elevated in 60% of patients with islet cell surface antibodies and there was a significant association between anti-tubulin antibodies and islet-cell surface antibodies. These antibodies, however, recognize different specificities, since adsorp-

tion of islet cell surface antibody by rat islets did not alter the anti-tubulin antibody activity. Elevated anti-actin antibody responses were found in two out of 17 and one out of 26 patients with recent onset and long-standing Type 1 diabetes, respectively. In conclusion, anti-tubulin antibodies are detected in a high proportion of patients with diabetes of recent onset, are associated with islet cell surface antibodies and like islet cell surface antibodies decrease or disappear during the course of the disease. Therefore, elevation of antitubulin antibodies could represent another 'marker' of the immunological features of Type 1 diabetes. However, these findings, together with our previous observation of an elevation of anti-tubulin antibodies in autoimmune thyroid disorders, indicate a wider involvement of autoimmunity with tubulin and suggest that similar autoimmune phenomena could develop in Type 1 diabetes and some other diseases.

Key words: Type 1 diabetes, tubulin, microtubules, anti-tubulin antibodies.

Antibodies to islets of Langerhans are commonly detected in the sera of patients with Type 1 diabetes. These antibodies are directed against cytoplasmic antigens [1]: islet cell antibodies (ICA) or cell surface antigens [2]: islet cell surface antibodies (ICSA). The antigenic molecular species or structures which react with these antibodies are still unknown. Among the numerous putative antigens are the components of the cytoskeleton composed of microtubules, microfilaments and intermediate filaments. These subcellular structures are present in β cells and likely are involved in insulin secretion [3].

In the present study, we looked for the presence of circulating antibodies directed against tubulin and actin, the major constitutive proteins of microtubules and microfilaments, in patients with Type 1 diabetes of varying duration. We studied whether the frequency of detection of anti-tubulin antibodies varied with duration and evolution of the disease, and whether there was a relationship between anti-tubulin antibodies and ICA and/or ICSA.

Subjects and methods

Subjects

Sera were collected from the following groups: *Group 1*: 28 patients with Type 1 diabetes of recent onset (0.5–6 months, 20 males, 8 females, aged 7–40 years, mean 22 years); *Group 2*: 26 patients with long-standing Type 1 diabetes (duration 6–43 years; mean 15 years; 13 males and 13 females; aged 19–70 years, mean 38 years); symptoms appeared before the age of 40 years; *Group 3*: 38 patients with Type 1 diabetes of various duration (> 5 years) divided into two subgroups, (a) 23 without neuropathy (13 males and 10 females; aged 13–79 years, mean 39 years) and (b) 15 with neuropathy (eight had symptoms of both peripheral and autonomic neuropathy, 14 males and one female, aged 21–51 years, mean 39 years). All patients in Groups 2 and 3 had normal body weight and had always been dependent on insulin for control of symptoms and prevention of ketosis. *Group 4*: 20 patients with Type 2 (non-insulin-dependent) diabetes (11 males and 9 females; aged 16–73 years, mean 52 years). All were controlled by diet and anti-diabetic drugs (sulphonylurea $\pm$ metformin); *Group 5*: 26 non-diabetic subjects, first-degree relatives of Type 1 diabetic patients, including six fathers or mothers (aged 32–49 years) and 20 brothers or sisters (aged 5–22 years) and corresponding to 17 families (11 males and 15 females; aged 5–49 years,

Fig. 1. Anti-tubulin antibody activity of the sera of Type 1 diabetic patients, control patients and control normal subjects. Control subjects: control 1 ($n=20$) age matched with group 1 (control 2 ($n=20$) age matched with groups 2, 3, 5 and 6). Group 1 ($n=28$): patients with recent-onset Type 1 diabetes. Group 2 ($n=26$): patients with long-standing Type 1 diabetes. Group 3 ($n=38$): patients with long-standing Type 1 diabetes without (3a) or with (3b) neuropathy. Group 4 ($n=20$): patients with Type 2 diabetes. Group 5 ($n=26$): non-diabetic subjects, first-degree relatives of Type 1 diabetic patients. Group 6 ($n=34$): DR3-positive normal subjects. The shaded area indicates the range of index values obtained with normal human serum defined by 3 SD above and below the mean. Mean (horizontal line) $\pm$ SEM (vertical bar) are given for each group

Fig. 2A and B Anti-tubulin antibody activity as a function of serum dilution. **A** (x) normal human sera, (○, ●, ■, ▲), sera from diabetic patients. The amounts of second antibody were 30 and 10 μ l at $1/50$ and $1/150$ serum dilution respectively. Immunoprecipitated 125 I-tubulin is expressed as percentage of total 125 I-tubulin supplied. **B** Four sera from diabetic patients: three positive in the screening test (□, △, ●) and one in the normal range (x) were tested in the presence of normal human serum to maintain a constant amount of immunoglobulins in order to standardise the second antibody precipitation reaction. Results were expressed as the ratio of the radioactivity immunoprecipitated by the patient serum to that in normal human serum alone. Each point represents the mean of triplicate incubations. The shaded area indicates the range (mean $\pm$ 3 SD) of the values obtained with normal human serum alone

mean 20 years); **Group 6**: 34 DR3-positive normal subjects (17 males and 17 females; aged 15–50 years, mean 37 years).

Sera from eight laboratory staff, 17 medical students and 15 older healthy people were tested as controls.

In the control group, 20 subjects were closely matched for age (as far as mean age was concerned) and sex with diabetic patients in Group 1: age 22–29 years (mean 25 years); sex ratio 2.5 male: female. A second sub-group of 20 control subjects was selected to match the diabetic patients in Groups 2 and 3 (aged 22–76 years; mean 41 years; sex ratio: 1.5 male: female).

Methods

Purification and labelling of tubulin

Tubulin was purified from rat brain or normal human brain (obtained at surgery) by the temperature reversible assembly-disassembly procedure followed by phosphocellulose chromatography as described previously [4]. Pure tubulin was labelled using the 125 I-labelled Bolton-Hunter reagent (Amersham France, Les Ulis, France). The specific radioactivity of 125 I-tubulin was 300–400 Ci/mmol [5].

Purification and labelling of actin

Actin was purified from rat brain [6]. The method includes the preparation of an acetone powder from which actin is purified by a polymerization-depolymerization step and Sephadex G200 gel filtration.

Purity of actin was approximately 95% as judged by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate [6]. Actin was labelled using the 125 I-labelled Bolton-Hunter reagent as described for tubulin.

Detection of anti-tubulin or anti-actin antibodies

The assay was conducted as reported previously [7]. The labelled antigen was incubated with the serum to be tested (100 μ l at a 1:30 dilution) in the presence of normal human serum (200 μ l at a 1:30 dilution) in a total volume of 500 μ l. Immune complexes were collected using a goat anti-human Ig antibody (Antibodies Inc., Davis, California, USA). Each serum was assayed in quadruplicate and most sera were tested at least twice with reproducible results (see below for assay coefficients of variation). A serum was considered positive for anti-tubulin or anti-actin antibodies if immunoprecipitated radioactivity was $>$ mean + 3 SD of the values obtained with normal human serum. An index value of antibody binding activity was calculated as the ratio (R) of the radioactivity immunoprecipitated by the unknown serum and normal human serum. Normal human serum was a pool of 10 sera from healthy blood donors. The intra-assay coefficient of variation was 7.1% (calculated from 36 sera). The inter-assay coefficient of variation, calculated from the values obtained for three sera in nine different assays, was 13.2%. In some experiments, insoluble protein A (suspension of staphylococcus aureus, Pansorbin, Calbiochem, San Diego, California, USA) was used instead of the second antibody [6].

Fig. 3 A and B. Specificity of anti-tubulin antibodies. **A** Effect of unlabelled tubulin or actin on ^{125}I -tubulin binding activity of the serum. The displacement of ^{125}I -tubulin binding was studied in four sera from diabetic patients (three with an elevated response (●, ■, ▲) and one having a normal response (□) in the anti-tubulin antibody screening-test) and in normal human serum (○). Labelled tubulin and unlabelled tubulin (2 or 20 $\mu\text{g/ml}$) or unlabelled actin (20 $\mu\text{g/ml}$) were added at the beginning of the incubation period to sera at a final dilution of 1:50. ^{125}I -tubulin binding activity was expressed as the percentage of ^{125}I -tubulin recovered in the immune precipitate. **B** Competitive binding between labelled and unlabelled tubulin. Determination of the overall affinity constant (K_a) of anti-tubulin-antibodies in a diabetic patient. Binding values (B) obtained in the presence of various amounts of unlabelled tubulin are expressed as the percentage of maximal binding (B_0) obtained in the absence of competitor. *Insert:* double reciprocal plot of the binding values versus the concentration of tubulin. Symbol and vertical bar represent the mean $\pm$ SEM of four determinations

Assays for other antibodies

ICA were detected by indirect immunofluorescence according to Botazzo et al. [1] on unfixed frozen sections of group 0 human pancreas. Sera were screened undiluted using an anti-IgG fluorescein conjugate (MFO3, Wellcome Reagents, London, UK). ICSA were identified by a method [8] derived from that of Lernmark et al. [2]. Sera were de-complemented, adsorbed on rat blood cells and incubated with isolated rat islet cells for 60 min at 4°C . Cells were washed and incubated with the anti-IgG fluorescein conjugate for 30 min at 4°C . A serum was positive for ICSA when at least 40% of cells were fluorescent. The specificity of the detection was demonstrated by pre-adsorption of positive sera on islets. In seven experiments, adsorption induced a significant decrease of the proportion of fluorescent cells from 65.6 ± 6.1 to $26.7 \pm 9.0\%$ ($p < 0.05$, Wilcoxon's test).

Immunoblotting

Tubulin was subjected to polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate and transferred electrophoretically to nitrocellulose paper (0.45 μm pore size, HAHY, Millipore, Molsheim, France), according to Towbin et al. [9]. The nitrocellulose sheet was cut into strips and incubated in a 'blocking buffer' (10 mmol/l phosphate buffer containing 154 mmol/l NaCl and 10 mg/ml bovine serum albumin, pH 7.4) for 5 h. The 'blocked' nitrocellulose transfer strips were rinsed several times in phosphate buffered saline (PBS) for about 15 min and incubated with the serum to be tested (final dilution 1:20 to 1:1000) in the presence of 10% goat serum for 14–16 h at 4°C under constant agitation. After five washings in PBS, tubulin-anti-tubulin antibody complexes on the strips were detected by one of two procedures: (1) nitrocellulose strips were incubated with horse-radish peroxidase-conjugated goat IgG anti-human immunoglobulins (Nordic Laboratories, Tilburg, The Nether-

lands) at a 1:500 dilution for 2 h at room temperature. Peroxidase reaction was carried out in 50 mmol/l Tris buffer (pH 7.6) in the presence of 0.1% (w/v) 4-chloronaphthol and 0.03% (v/v) H_2O_2 and stopped after 5 min by washing in water. The blots were dried between filter paper and stored in the dark. A dark purple reaction product was observed wherever the antibodies were bound; (2) strips were first incubated with a goat serum anti-human Ig (Antibodies Inc.) at a 1:250 dilution for 2 h at room temperature, then rinsed and further incubated with ^{125}I -labelled protein A (2×10^4 cpm/ml) in PBS-containing 10 mg/ml bovine serum albumin for 1 h at room temperature. The strips were washed in PBS, dried and processed for autoradiography using X-Omat AR Kodak film.

Statistical analysis

Statistical analysis was performed by the χ^2 test, corrected for small sample number when required.

Results

Prevalence of anti-tubulin antibodies in recent-onset Type 1 diabetes

Normal human sera (at a 1:150 dilution) immunoprecipitated between 4% and 8% of ^{125}I -tubulin. In terms of an anti-tubulin antibody response index (R), the basal range varied between 0.80–1.20. The overall prevalence of anti-tubulin antibodies in the 212 sera tested is shown in Figure 1. The 40 control sera (20 in each sub-group) gave R values within the basal range. Of the remaining

Fig. 4A and B. Identification of anti-tubulin antibodies by immunoblotting. Tubulin was submitted to 12% polyacrylamide gel electrophoresis and electrophoretically transferred to nitrocellulose paper. Nitrocellulose strips were incubated with normal human serum (lanes 1 and 4) or patient serum: R value: 1.7 (lanes 2 and 3) at a 1:200 dilution. Antibodies bound to nitrocellulose strips were revealed using the immunoperoxidase reaction (A) or binding of ^{125}I -protein A and autoradiography (B). Molecular weights of marker proteins are expressed in kilodaltons (kD). Tubulin: 55 kD

172 sera, there were 19 elevated responses. Among the group 1 patients, 13 (46%) had an elevated anti-tubulin antibody response. In contrast, only four sera out of 64 (6%) from patients with long-standing Type 1 diabetes (groups 2 and 3) were positive for anti-tubulin antibodies. Among the group 4 patients, none had an elevated anti-tubulin antibody activity. Two sera out of 26 (8%) from first-degree relatives of Type 1 diabetic patients (group 5) were positive for anti-tubulin antibodies. None of the 34 sera from DR3-positive subjects (group 6) was positive for anti-tubulin antibodies.

Group 1 diabetic patients differentiated from other control or diabetic groups by two criteria: the frequency of a positive response and the average level of anti-tubulin antibody activity. Indeed, the mean R value of group 1 was significantly higher than the mean R values of either group 2 ($p < 0.05$), groups 3a, 3b, 4, 5, 6, ($p < 0.001$) or control groups ($p < 0.001$).

The mean age and sex distribution of the 13 positive and the 15 negative sera of group 1 were similar. During the time of study, nine out of 17 patients in group 1 exhibited a transient remission; five were positive for anti-tubulin antibodies. Therefore, the same proportion of positive anti-tubulin antibody responses (about 50%) were found in patients with or without a transient remission period. The proportion of elevated anti-tubulin

antibody responses was similarly low in groups 2 and 3 and was not related to the existence of neuropathy. There was no difference between groups 3a and 3b.

Specificity of the detection of anti-tubulin antibodies

The detection of anti-tubulin antibody activity was dependent upon the presence of a precipitating system of the immune complexes: anti-human Ig antibody or insoluble protein A. Ten sera, which yielded a normal response using the second antibody procedure, gave a normal response using the protein A technique, compared with normal human sera. Eight out of 10 sera, which were positive in the second antibody procedure, gave an elevated response using protein A. Therefore, in most sera, anti-tubulin antibodies appear to be IgG. The discrepancy for few sera between the two methods can be explained by a difference in assay sensitivity or by the existence of antibodies to tubulin which do not bind protein A (IgG₃ or IgM). The ^{125}I -tubulin binding activity of sera was related to the dilution of serum (Fig. 2). A serum with a slightly but significantly elevated R value, i.e. 1.25 at a 1:150 dilution gave a R value equal to 1.45 at a 1:50 dilution. Sera eliciting an R value in the range 1.4–1.8 at a 1:150 dilution were still positive for anti-tubulin antibodies at a 1:1500 dilution. The specificity of the binding of ^{125}I -tubulin to human immunoglobulins of sera with elevated or normal anti-tubulin antibody activity is shown in Figure 3. Unlabelled tubulin not only decreased the binding of ^{125}I -tubulin in sera which elicited elevated anti-tubulin antibody responses in the screening test but also in normal sera. This latter finding is in agreement with previous reports [10–11] showing that normal human serum contains a low level of anti-tubulin antibodies. The ability of unlabelled tubulin to displace ^{125}I -tubulin binding was concentration-dependent. In contrast, actin did not exhibit any displacing activity. From competitive-binding experiments (Fig. 3B), it was calculated that the apparent overall affinity constant of anti-tubulin antibodies in sera (which elicited a supranormal activity) was approximately $0.3 \times 10^9 \text{M}^{-1}$. These values are comparable to those obtained with anti-tubulin antibodies produced in rabbits [12]. Immunoblotting was used to document further the difference in anti-tubulin antibody titre between normal human sera and sera with elevated R values. Anti-tubulin antibodies were clearly detected in the serum of a patient (R value: 1.7; Fig. 4). At the same dilution (1:200) the reaction with normal human serum was negative. However, anti-tubulin antibodies can be seen in normal human serum at dilutions ranging from 1:20 to 1:50 (result not shown).

Relation between anti-tubulin antibodies and ICA or ICSA

Thirty-nine sera (from patients in groups 1 and 2) were assayed for ICA and anti-tubulin antibodies and 23 sera

Table 1. Correlation between the presence of anti-tubulin antibodies and ICA or ICSA in the sera of patients with Type 1 diabetes

Anti-islet antibodies		Number of patients with anti-tubulin antibodies		<i>p</i> (χ^2 test)
		Normal	Elevated	
ICA	Negative	21	6	NS
	Positive	8	4	
ICSA	Negative	12	1	<0.05
	Positive	4	6	

Table 2. Comparison of the frequencies of positive sera for anti-tubulin antibodies, anti-actin antibodies and ICSA in recent-onset and long-standing Type 1 diabetes

Type 1 diabetic patients	Percentage of sera positive for		
	Anti-tubulin antibodies	Anti-actin antibodies	ICSA
Recent-onset (<i>n</i> = 17)	53 (17)	12 (17)	70 (10)
	<i>p</i> < 0.001	NS	<i>p</i> < 0.05
Long-standing (<i>n</i> = 64)	6 (64)	4 (26)	25 (12)

Numbers in parentheses indicate the number of sera tested for the presence of antibody

for ICSA and anti-tubulin antibodies. Anti-tubulin antibodies were elevated in approximately the same proportion of ICA-positive or ICA-negative sera (Table 1), thus indicating no correlation between the presence of these antibodies in the sera of diabetic patients. In contrast, anti-tubulin antibodies were elevated in six out of 10 ICSA-positive sera and in one out of 13 ICSA negative sera. There was a statistically significant association between ICSA and anti-tubulin antibodies ($p < 0.05$). This finding led us to postulate that tubulin as an intrinsic membrane component [13] could represent the antigen or an antigen reacting with ICSA. To test this hypothesis, four sera positive for both anti-tubulin antibodies and ICSA were adsorbed on isolated rat islets and assayed for residual antibody activities. Under incubation conditions that decreased the ICSA activity by 60% [8], there was no significant change in the anti-tubulin antibody activity (result not shown).

Anti-actin antibodies in Type 1 diabetes

In an attempt to determine whether elevation of antibodies directed to tubulin reflects a general anti-cytoskeleton autoimmune phenomenon, we looked for the presence of antibodies directed to another cytoskeletal protein: actin, in the same series of sera. Two sera out of 17 from group 1 patients and one serum out of 26 from group 2 patients gave an anti-actin antibody response higher than that of normal human sera (Table 2). The frequency of anti-actin antibodies was low and

there was no difference between the two groups. This is in marked contrast to that obtained with anti-tubulin antibodies; the difference in the frequencies of detection of anti-tubulin antibodies between the two groups of patients was highly significant ($p < 0.001$; Table 2).

Discussion

We have found that antibodies to tubulin are elevated in a high proportion of patients with Type 1 diabetes. These antibodies were detected using highly purified ^{125}I -labelled tubulin and the specificity of the binding of labelled tubulin to human Ig was ascertained by competitive inhibition with unlabelled tubulin. Anti-tubulin antibodies have been demonstrated using the soluble antigen in a radioimmunoassay or the immobilized antigen in the immunoblotting technique.

The finding of a higher proportion of anti-tubulin antibody-positive sera in recent-onset compared with long-standing Type 1 diabetes indicates that the prevalence of these antibodies is related to the duration of the disease. In this respect, anti-tubulin antibodies behave as the antibodies reacting with pancreatic islet cells [2, 14]. Interestingly, there was a statistically significant association between anti-tubulin antibodies and ICSA in diabetic patients. In contrast, we did not find any correlation between ICA and anti-tubulin antibodies and, in the same study, ICA were independent of ICSA. Although exhibiting similar properties (i.e. the prevalence in recent-onset and disappearance during the course of the disease) and although associated in individual sera, anti-tubulin antibodies and ICSA are different antibodies. Indeed, while ICSA activity was adsorbed on rat islets there was no change in anti-tubulin antibody activity. These data indicate that anti-tubulin antibodies could represent another marker for Type 1 diabetes. However, in the light of our previous studies on autoimmune thyroid disorders [7], it appears that anti-tubulin antibodies do not correspond to an organ specific marker comparable with ICA. We found that anti-tubulin antibody levels were increased in 40 to 50% of patients with Graves' disease or Hashimoto's thyroiditis. These findings suggest that some common immune phenomena are involved in thyroid, pancreas and, may be, other autoimmune disorders or that anti-tubulin antibody levels are altered in these diseases as a result of an alteration in immune regulation or cellular destruction. Since the association of autoimmune thyroid diseases and Type 1 diabetes is common [15, 16], especially in subclinical forms [17], we checked whether the diabetic patients have anti-thyroid antibodies. Among group 1 and 2 patients who exhibited elevated anti-tubulin antibodies, none was positive either for anti-microsomal or anti-thyroglobulin antibodies. Thus anti-tubulin antibodies may occur independently of any association between autoimmune thyroid disorders and Type 1 diabetes.

It has been reported that anti-tubulin antibody levels are increased in infectious mononucleosis and in other viral diseases [11]. Since viral infection is thought to be a pathogenic factor in Type 1 diabetes [18], it is tempting to assume that the increase in anti-tubulin antibody levels may be related to the development of the disease.

Since there is an association between Type 1 diabetes and HLA antigens, especially the DR3 specificity [19], it was of interest to study the relationship between an elevated anti-tubulin antibody activity and the presence of DR3. Anti-tubulin antibody responses of DR3-positive normal subjects were not different from those of unselected normal subjects.

In view of the association of anti-tubulin antibodies with the acute phase of diabetes, it would be interesting to study the predictive value of these antibodies in genetic predisposition to diabetes. In a preliminary study, two out of 26 non-diabetic first-degree relatives of Type 1 diabetic patients were found to have elevated anti-tubulin antibody levels.

The finding of simultaneous and temporary autoimmune responses against organ specific antigens (ICSA) and against a widespread cellular component, such as tubulin, may be of some interest in the understanding of the pathogenesis of Type 1 diabetes; this might be compatible with a transient defect of T-lymphocytes leading to a temporary increased production of antibodies. Such an hypothesis would be in keeping with the presence of low levels of anti-tubulin antibodies in normal human serum and the increase of these circulating antibodies during the development of autoimmune disease.

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