

**SCREENING FOR INSULITIS IN ADULT AUTOANTIBODY-POSITIVE
ORGAN DONORS.**

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ABSTRACT

Objective. Antibodies against islet cell antigens are used as predictive markers of type 1 diabetes but it is unknown whether they reflect an ongoing autoimmune process in islet tissue. We investigated whether organs from adult donors that are positive for autoantibodies against islet cell antigens exhibit insulinitis and/or a reduced beta cell mass.

Research Design and Methods. Serum from 1507 organ donors (age 25-60 years) was analyzed for ICA, GADA, IA-2A and IAA. Tissue from the 62 aAb-positive donors (4.1%) and from matched controls was examined for the presence of insulinitis and for the relative area of insulin-positive cells.

Results. Insulinitis was detected in two cases; it was found in 3 and 9 percent of the islets and consisted of CD3+/CD8+ T-cells and CD68+ macrophages; in one case it was associated with insulin-positive cells that expressed the proliferation marker Ki67. Both subjects belonged to the subgroup of three donors with positivity for ICA, GADA and IA-2-Ab and for the susceptible *HLA-DQ* genotype. Comparison of relative beta cell area in aAb-positive and aAb-negative donors did not show a significant difference.

Conclusions. Insulinitis was found in 2 of the 3 cases that presented at least three islet cell autoantibodies, but in none of the other 59 antibody-positive subjects or 62 matched controls. It was only detected in less than 10 percent of the islets, some of which presented signs of beta cell proliferation. No decrease in beta cell mass was detected in cases with insulinitis, or in the group of antibody-positive subjects.

Type 1 diabetes results from a specific and major loss of insulin-producing beta cells presumably through a T-cell mediated process (1-5). At clinical onset, patients present circulating autoantibodies against islet cell antigens which can appear many years before hyperglycemia is established, and are therefore used for prediction of the disease. In first degree relatives of type 1 diabetic patients, the risk for developing the disease is higher when multiple positivity is present for autoantibodies against the islet cell cytoplasm (Islet Cell Antibodies, ICA), insulin (Insulin Autoantibodies, IAA), the 65,000 Mr isoform of glutamate decarboxylase (Glutamate Decarboxylase Autoantibodies, GADA) or the insulinoma-associated protein 2 tyrosine phosphatase (Insulinoma Associated Protein 2 Autoantibodies, IA-2A) (6-12). Antibody positivity is therefore used for patient recruitment in prevention trials but it is still unknown whether, and, if so, for which combination, it corresponds to an insulinitis process in the pancreas. There are only few studies available on the histopathology of the pancreas in antibody-positive non-diabetic individuals (13,14). In the four reported cases, no leucocytic islet infiltrate or signs of beta cell damage were noticed; three of them were GADA-positive patients with polyendocrinopathy and one an IA-2A positive organ donor. In the present study we investigated the pancreas in 62 autoantibody-positive organ donors. This larger series allowed us to identify two cases with insulinitis - one of whom presenting signs of beta cell proliferation - and to correlate these histopathologic findings to the small subgroup of patients with three or four autoantibodies and a high risk genotype.

RESEARCH DESIGN AND METHODS

Collection of pancreatic tissue

Pancreas biopsies were obtained from the *Beta Cell Bank* which operates for a clinical trial on islet cell transplantation in Belgium (15,16). They were taken as part of a quality control procedure that was approved by the ethics committees of the Belgian Diabetes Registry and participating hospitals. Tissue (approx. 0.5cm³) was excised from the body region of cold-preserved (UW flushed) donor organs that were provided by Eurotransplant Foundation (Leiden, The Netherlands). It was fixed in 4% (v/v) phosphate buffered formaldehyde pH7.4 or Bouin's fixative, embedded in paraffin and then histologically analysed. Between 1989 and 2004, a total of 1507 biopsies were collected from patients age 25-60 years for whom also serum or plasma was available for islet cell antibody assays. For none of these donors was diabetes mentioned in the donor information sheets.

Analysis of donor blood for autoantibody and genetic risk markers for type 1 diabetes

Serum samples were prospectively tested for presence of ICA, IA-2A and GADA, and retrospectively for IAA (17). ICA were assessed by indirect immunofluorescence and endpoint titers expressed as JDF units. IA-2A, GADA and IAA were measured by liquid phase radiobinding assay and expressed as percent tracer bound in haemolysis-free sera. Cut-off values for antibody positivity were calculated as 99th percentile of antibody levels in 790 non-diabetic controls after omission of outlying values (minimally 12 JDF units for ICA, 0.4% for IA-2A, 2.6% for GADA, 0.6% for

IAA). The autoantibody assays were validated in successive Immunology of Diabetes Workshops (IDW) and international proficiency testing programs; all positive results were confirmed in a separate subsequent assay (17). Whole blood was haplotyped for DNA polymorphisms at the *HLA-DQAI* and *DQB1* gene loci and DQ-associated risk stratified as reported (18).

Screening for insulinitis

For detection of insulinitis, paraffin sections were immunohistochemically double-stained for leucocyte common antigen (LCA, using mouse anti LCA from Dako, Glostrup, Denmark) and the pan-neuroendocrine marker synaptophysin (rabbit anti synaptophysin from Dako). Binding was detected with biotinylated anti-mouse or anti-rabbit Ig (Amersham, Little Chalfont, UK) and, respectively, streptavidin HRP or AP complex (Dako) using diaminobenzidine or new fuchsin as substrate. Sections were also double-staining for insulin and glucagon (guinea pig anti insulin and rabbit anti glucagon were a gift of Dr Van Schravendijk, Brussels, Belgium). For each case, an average of 180 ± 16 (mean \pm SEM) islets were screened. Insulinitis was arbitrarily defined as an infiltrate of ≥ 15 LCA+ cells within the islet (central-insulinitis) or directly surrounding the islet (peri-insulinitis). This number was set after determining mean and range of the number of LCA+ cells per islet in 62 islet autoantibody-negative controls: 0.35 ± 0.04 (range 0-7) LCA+ cells per islet (1550 islets investigated); the “insulinitis” level was set at twice the maximum number encountered in these controls. While arbitrary and therefore susceptible to discussion, this definition provides a more quantitative basis than that used in other studies for comparing the

occurrence of insulinitis. Only one paper on human insulinitis has defined insulinitis on basis of the number of leucocytes per islet (ref 14 examined 14 normal controls to determine the number of leucocytes in the 10 islets with the largest mononuclear infiltrate).

Characterization of leucocytic infiltrates

Leucocytic infiltrates were immunophenotyped on paraffin sections using immunofluorescent double and triple staining with the following antibodies: rabbit anti-CD3 and anti LCA (Dako), mouse anti-CD4 and anti-CD8 (Novocastra Laboratories, Newcastle upon Tyne, UK), mouse anti-CD20 anti CD68 (Dako). Binding was visualized with anti-rabbit FITC or anti-mouse Cy3 (Jackson Immunoresearch, Soham, UK) and examined in an Axioskop M fluorescence microscope (Zeiss, Oberkochen, Germany) equipped with an Orca AG camera (Hamamatsu, Japan) and Smartcapture imaging software (Digital Scientific, Cambridge, UK). For negative controls primary antibodies were omitted, positive controls were conducted on paraffin-embedded human tonsils.

Quantification of relative beta cell area and beta cell proliferation

Relative insulin-positive cell area was measured according to Rahier et al (19) on coded slides using a 266 point counting grid in ten randomly chosen microscope fields at a final magnification of 140x. The number of points hitting insulin immunoreactive cells (Ni) and pancreatic parenchyma (Np) were counted in ten randomly chosen microscope fields per case. Relative beta cell area was expressed as percent and calculated as $(Ni/Np) \times 100$. All morphometric analyses were carried out blinded on coded slides. Two color immunohistochemistry using

antibodies for Ki67 (Dako) and insulin was used to determine the percent proliferating beta cells. Reproducibility of the point counting technique was evaluated with the formula of Weibel (19): the calculated relative error in each section was approx 11% for a mean relative beta cell area of 1.2%.

RESULTS

Screening of organ donors for risk markers of type 1 diabetes

Testing for the four islet autoantibodies resulted in 62 positive cases (aAb-pos) out of 1507 donors in the age group 25-60 years (4.1%). Most cases were positive for a single autoantibody (n=55), with only four double, two triple and one quadruple positive case(s) (Table 1). The three donors with ≥ 3 autoantibodies displayed a susceptible HLA-DQ genotype while the four cases with only two autoantibodies exhibited a neutral or protective HLA-DQ genotype (Table 1). We observed lower aAb titers in the single autoantibody cases than in the multiple aAb cases (results not shown), probably as a result of their high fraction of 'statistical positives' (1% cut-off). A control group of 62 aAb-negative (aAb-neg) donors was selected from the total donor group by matching for age, sex and BMI.

Screening of organ donors for insulinitis and histopathology of positive cases

Of the 62 Ab-pos and 62 Ab-neg donors only two cases presented islets with insulinitis as defined under Methods; they belonged to the small subgroup (n=3) with positivity for ≥ 3 autoantibodies (Table 1). Case #1 (M-59y) died ten hours after hospitalization for a subdural hematoma (plasma glycemia 6.4 mM at admission), and case #2 (F-46y) forty three hours after hospitalization for subarachnoidal

hemorrhage (plasma glycemia 8.2 mM at admission).

In case #1, five out of the 58 examined islets (9%) showed peri- or central insulinitis. Four of these islets contained both insulin- and glucagon- positive cells, while one was insulin-negative and mainly composed of glucagon-positive cells. No other insulin-negative/glucagon-positive islets were detected in this donor. In case #2, 27 out of 917 islets (3%) presented insulinitis (Fig 1a,b), all islets containing insulin- and glucagon-positive cells. Another 3 % of the islets was insulin-negative and mainly composed of glucagon-positive cells; these islets did not present signs of insulinitis (Fig 1c).

In both cases, the infiltrating cells predominantly corresponded to CD3+CD8+ T-cells (Fig 1g-i) and CD68+ macrophages (Fig 1f) with a few CD20+ B-cells and CD3+CD4+ T-cells detected (Table 3).

Beta cell surface area and proliferation in donors with high risk markers for type 1 diabetes

When the average beta cell surface area in Ab-pos donors with ≥ 2 autoantibodies was compared with that in donors with a single autoantibody or that in antibody negative controls, no significant difference was noted (Table 2). Individual values in cases with multiple autoantibodies fell within the range of the control group.

The average percent insulin-positive cells that were also positive for the proliferation marker Ki67 was very low (< 1 ‰) in the three groups. The range in aAb-negatives was 0 to 7 ‰. Only one aAb-positive case presented a value outside this range (49 ‰): this case was

characterized by insulinitis and positivity for the four autoantibodies (Case #1); the Ki67-positive cells were only noticed in islets with a leucocytic infiltrate (Fig 1d,f -Table 2).

DISCUSSION

In the present study we have screened for insulinitis in pancreatic tissue from 62 adult organ donors carrying autoantibody risk markers for type 1 diabetes. Insulinitis was detected in two out of three cases with at least three autoantibodies, be it in less than 10 percent of the islets. It was not found in any of the 59 cases with only one or two autoantibodies, or in any of the 62 antibody-negative controls. Both cases also presented a susceptible HLA-DQ genotype. There were no signs of a reduced beta cell mass in the two insulinitis cases nor in the group of autoantibody-positive donors. These data demonstrate that insulinitis is a rare phenomenon in autoantibody-positive non-diabetic adults.

Insulinitis is also a rare finding at clinical onset of type 1 diabetes above 30 years, which contrasts with its detection in all onset-cases younger than 7 years (4). Although type 1 diabetes in adults is, by definition, characterized by the presence of autoantibodies, a combination of three or more of such autoantibodies in association to a high risk genotype is infrequent, especially in LADA (20). The absence of insulinitis in most adult autoantibody-positive donors does therefore not exclude progression to the disease.

The present observations should however be interpreted with caution in terms of their possible significance for the development of type 1 diabetes. The seven donors with more than one antibody risk marker were older than 38 years, an age

that is not typical for the development of classical type 1 diabetes. Moreover, four of them presented with a protective HLA-DQ genotype that may have modulated an autoimmune response. It is nevertheless conceivable that the presence of insulinitis in two subjects with four risk markers illustrates a stage in the disease process that might eventually lead towards a sufficient beta cell loss so that diabetes develops. However, no decrease in beta cell mass became apparent after measuring relative beta cell surface area. There were also no substantial numbers of pseudo-atrophic islets (1) as a remnant of prior destructions and a sign of self-limiting insulinitis at an earlier age. It is conceivable that both subjects exhibit a low-intensity autoimmune process affecting only a small percent of the islets. The occurrence of Ki67-positive beta cells in some of the infiltrated islets raises the possibility that beta cell proliferation can compensate for any losses. Such sub-clinical autoimmune process may at a later stage result in slowly progressive type 1 diabetes or latent autoimmune diabetes in adults (LADA) (21,22). The histopathology of late-onset type 1 diabetes has not been well studied. One case has been described: a 65 year old female with positivity for two autoantibodies (GADA and IA-2A) and a HLA-DQ/DR risk profile was initially diagnosed with type 2 diabetes and then shown to present several islets with predominantly CD4+ T-cell infiltrates, without signs of beta cell destruction (23). In the presently described cases, infiltrating leucocytes mainly corresponded to CD8+ T-cells and CD68+ macrophages as was also the case in type 1 diabetic patients with insulinitis (2, 24-26).

Our data do not strengthen or weaken the significance of the detected circulating markers as predictors for type 1 diabetes. They indicate an association between triple antibody positivity with a high risk genotype and an insulinitis process in the pancreas. In elder individuals, this insulinitis process appears limited to less than 10 percent of the islets, and may thus not lead to type 1 diabetes, or only to a mild form. We can not exclude that cases with a low percentage of infiltrated islets were missed as a result of our sampling in one region (body) and of the relatively small number of analyzed islet sections (averaging 180 islets per organ); more extensive sampling was precluded by the islet isolation procedure for which these organs were harvested. For the same reason we may have missed differences in beta cell mass if these would have occurred in other regions.

Despite the limitations imposed by the small tissue specimen, the nature and extent of our study provides information with respect to the use of organs from adult autoantibody positive donors for transplantation. Absence of histopathologic changes in all 59 donors with one or two autoantibodies questions exclusion of these organs while the detection of insulinitis in triple antibody positive donors can be seen as an exclusion criterion.

In conclusion, we have screened 62 non-diabetic autoantibody-positive organ donors older than 25 years for the presence of insulinitis. Insulinitis was found in 2 of the 3 cases that presented at least three antibodies but in none of the other 59 antibody-positive subjects or 62 matched controls. Presence of one or more antibodies was not related to a decrease in beta cell mass. These observations can be used to include or exclude organs from autoantibody positive donors for transplantation in diabetic recipients. They also need consideration when recruiting adult autoantibody-positive subjects for prevention trials.

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TABLE 1. CHARACTERISTICS OF AUTOANTIBODY-POSITIVE DONORS									
Case	Age (yrs)	Sex (M/F)	BMI	Autoantibody titers				HLA <i>DQA1</i> *- <i>DQB1</i> * haplotype1/ haplotype2	HLA risk
				ICA	GAD-A	IA2-A	IAA		
Positivity for minimally two autoantibodies									
1	59	M	22	100	1067.2	2.6	1.7	<i>0501-0201/0501-0201</i>	S
2	46	F	21	50	213.5	54.6	<	<i>0301-0302/0301-0302</i>	S
3	44	M	24	100	1928.6	42.6	<	<i>0301-0302/0501-0201</i>	S
4	50	F	21	50	567.0	<	<	<i>02-0202/0501-0201</i>	P
5	54	M	30	25	163.9	<	<	<i>01-0602/0301-0302</i>	P
6	49	M	28	25	3.6	<	<	<i>01-0501/01-0501</i>	N
7	39	M	26	12	<	1.0	<	<i>0501-0301/0501-0301</i>	P
Positivity for only one of the tested autoantibodies									
n=55	46* (26-60)	33/22	25* (18-30)	(n=19)	(n=27)	(n=3)	(n=6)	NT	
Matched autoantibody-negative controls									
n=62	46* (25-60)	38/24	24* (18-31)	(n=0)	(n=0)	(n=0)	(n=0)	NT	

Autoantibody titers are expressed as JDF-units (ICA) or as percent tracer binding (GAD-A, IA2-A, IAA); < - below cut-off value. *Median (range). HLA risk haplotypes in bold. NT-not tested. N-neutral, P-protective, S-susceptible HLA-DQ genotype.

TABLE 2: INSULITIS, BETA CELL SURFACE AREA AND BETA CELL PROLIFERATION IN AUTOANTIBODY POSITIVE ORGAN DONORS.

Number of autoantibodies (aAb)	Case	Number of islets with insulitis / total islets examined	Relative Beta Cell Surface Area. % (range)	Ki67+INS+ cells / INS+ cells 0/00 (range)
n= 4 aAb	1	5/58	1.95	49*/0 [†]
n= 3 aAb	2	27/917	1.59	0
	3	0/99	1.46	0
n= 2 aAb	4	0/116	1.50	0
	5	0/137	0.82	0
	6	0/141	0.79	0
	7	0/331	1.28	0
n= 1 aAb	n=55	0/10,223	1.13 (0.50-2.80)	0.4 (0-5)
n= 0 aAb	n=62	0/10,334	1.21 (0.51-2.61)	0.4 (0-7)

*Ki67+ins+ cell number in islets with insulitis, [†]Ki67+ins+ cell number in islets with no signs of insulitis.

TABLE 3. IMMUNOPHENOTYPE OF THE INFILTRATING CELLS IN ISLETS WITH INSULITIS

Case #	# of islets with insulinitis examined	number of CD68+ cells per islet	number of LCA+ cells per islet	number of CD20+ cells per islet	CD3+		
					number of cells per islet	% CD3+CD4+	% CD3+CD8+
1	5	24.4 (4-53)	63.0 (16-101)	4.0 (0-13)	53.6 (20-84)	3.8 ± 1.2	82.2 ± 2.8
2	11	42.3 (19-78)	58.0 (26-137)	7.4 (0-34)	60.5 (18-169)	3.1 ± 1.3	70.9 ± 2.8

Results are expressed as mean ± SEM (range).

LEGENDS

FIGURE 1: Histology of the islets of Langerhans in adult organ donors with multiple (≥ 3) autoantibodies against islet cell antigens. Islets from case #2 show infiltration by mononuclear cells (A, haematoxylin-eosin staining; B, immunostaining of a consecutive section for leucocyte common antigen (LCA) and the pan-neuroendocrine marker synaptophysin (SYN)). Double staining for glucagon (GLUC) and insulin (INS) shows that some islets predominantly exist of glucagon-positive cells with only few insulin-positive cells (C, arrow). In case #1, double staining of consecutive sections for LCA and insulin (D) or Ki67 and insulin (E) shows the presence of Ki67-positive cells in insulitic islets, with some cells co-expressing both insulin and Ki67 (arrows). The leucocytic infiltrate consists of both CD68-positive macrophages (F) and lymphocytes. Immunofluorescent triple staining of an infiltrated islet for insulin, CD3 and CD8 (G-I,) shows that the infiltrate predominantly consists of CD3+CD8+ T-cells.

Figure 1 (low resolution; use separate High Res File for publication)

