Cell-mediated immune reactions against B cells and defect of suppressor cell activity in Type 1 (insulin-dependent) diabetes mellitus

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Summary. A cytotoxic effect of peripheral blood mononuclear cells from 22 out of 23 newly diagnosed Type 1 (insulin-dependent) diabetic patients against B cells of isolated rat islets was demonstrated. The addition of peripheral blood mononuclear cells from healthy subjects reduced the cytotoxic effect in 9 out of 10 patients. The addition of peripheral blood mononuclear cells from other diabetic patients was without significant effect in 14 out of 16 cases. The results indicate

functional abnormalities of peripheral blood mononuclear cells in newly diagnosed Type 1 diabetes. Beside cytotoxic effects against B cells, a defect in the suppressor function seems to exist. The activation of T-lymphocytes might be a consequence of such a defect.

Key words: Type 1 diabetes, cell-mediated anti B-cell cytotoxicity, defect of suppressor cell function.

It is commonly supposed that various cell-mediated immunoprocesses are involved in the destruction of B cells of the pancreas in Type 1 (insulin-dependent) diabetes mellitus. Morphologically an infiltration of the islets of Langerhans in juvenile diabetic patients who had died shortly after diagnosis has been known for many years [1, 2].

During the last few years differences in the percentage of T-lymphocyte subsets were found in this disorder [3–7]. These are only nonspecific findings, and the participation of these cells in inducing damage of the B cells has not been established. Direct functional effects of lymphocytes from Type 1 diabetic patients have been demonstrated by the leucocyte migration inhibition test [8] and by suppression of the stimulated insulin secretion from isolated mouse islet cells [9, 10]. Charles et al. [11] reported direct cytotoxicity on isolated islets by lymphocytes in a chromium release assay.

In previous studies, we observed morphological evidence of lyses of B cells in isolated rat and human islets by CD8⁺ lymphocytes from newly diagnosed Type 1 diabetic patients [12, 13]. To date, most authors have presumed that the cause of the activation of the T-lymphocytes in Type 1 diabetes is due to an immune stimulation by the target cell. Only few reports have discussed the possibility that a defect in the suppressor cell function could be responsible for the activation of the cytotoxic T-cells [14–17]. We have attempted to contribute to an understanding of this problem by studying cyto-

toxic and suppressor cell functions of peripheral blood mononuclear cells (PBMC) from newly diagnosed Type 1 diabetic patients.

Subjects and methods

Subjects

Twenty-three patients were investigated within 4 weeks after hospitalisation due to overt hyperglycaemia. All patients needed insulin from the time of diagnosis and also in the further course of the disease. The patients were characterized by a markedly reduced C-peptide level and were typed for their HLA-DR phenotypes. Fifteen normoglycaemic subjects without family history of diabetes served as control probands (Table 1). The diabetic patients were free of clinical signs of other autoimmune diseases or endocrinopathies. The radioreceptor assay for thyroid stimulating hormone receptor antibodies was negative in all cases (TRAK-Assay, Henning, Berlin, FRG).

Preparation of islets

Islets were isolated from the pancreas of 10 to 12-day-old Wistar rats by collagenase digestion [18]. The islets were first incubated for 2 h at 37 °C in a humidified atmosphere of 5% CO₂/95% O₂ in Eagle Basal Medium (pH 7.4) supplemented with 1.0 g/l bovine serum albumin, 2 mmol/l L-glutamine, 20 mmol/l Hepes, 5.0 g/l lactalbumin-hydrolysate, 5.5 mmol/l glucose, 2 × 10⁵ U/l penicillin G and 200 mg/l streptomycin (= EBM).

Isolation of PBMC

PBMC were prepared from 20 ml heparinized venous blood (preservative-free heparin, Gedeon Richter, Budapest, Hungary) by the Fi-

Table 1. Clinical data and results of the insulin release assay of the Type 1 diabetic patients and the control subjects. Insulin content (ng/islet per 20 h) in the culture medium after 20-h incubation of rat islets in incubation medium (EBM) = control or EBM + peripheral blood mononuclear cells of newly onset Type 1 diabetic patients ($n = 23$; upper part) and of control subjects (HP 1-15; lower part). Diabetic patients 1-10 tested in combination with peripheral blood mononuclear cells of healthy probands

Patient	Sex	Age at onset (years)	HLA (DR)	EBM (ng insulin/20 h)	EBM and peripheral blood mononuclear cells (ng insulin/20 h)	% of EBM	Tested with healthy control (n)
Type 1 diabetic patients							
1	m	20	3/4	9.7	29.6	306	HP 1
2	f	9	1/7	14.6	27.8	191	HP 2
3	m	8	3	10.5	25.6	245	HP 3
4	f	15	2/3	5.7	11.4	198	HP 4
5	f	16	3/4	4.1	8.9	218	HP 5
6	m	25	2/4	10.3	22.5	219	HP 6
7	f	39	4	16.5	34.9	211	HP 7
8	f	14	3/4	17.2	25.5	148	HP 8
9	f	15	2/3	9.6	13.6	142	HP 9/10
10	m	20	4/5	10.2	30.3	297	HP 11/12
11	m	6	4	6.5	18.1	278	-
12	f	8	3/4	13.6	20.0	150	-
13	m	9	+	6.0	11.9	198	-
14	m	13	3/4	10.3	26.2	254	-
15	m	22	3/4	15.0	22.1	147	-
16	f	28	4	12.2	11.2	92	-
17	f	31	3/4	7.1	12.1	170	-
18	m	36	4	11.4	18.4	162	-
19	m	26	4	6.7	11.2	167	-
20	m	15	4	9.2	24.2	263	-
21	m	19	4	7.1	16.3	230	-
22	m	12	4	9.8	15.9	162	-
23	f	13	4	12.8	18.9	148	-
Mean (1-23)		18.2		10.2	19.9	200	
SD				3.5	7.3	55.6	
SEM				0.8	1.5	11.6	
Control subjects							
HP 1	m	32	7	9.7	11.6	120	
HP 2	m	27	2/4	14.5	12.4	85	
HP 3	m	40	7	10.5	6.4	63	
HP 4	m	44	3/w 6	8.8	10.4	118	
HP 5	f	21	5/w 6	4.1	4.8	118	
HP 6	f	24	3/4	10.3	12.3	119	
HP 7	f	29	w 6/7	16.5	20.3	123	
HP 8	m	33	1/4	17.2	17.1	99	
HP 9	m	42	1/2	9.6	8.3	86	
HP 10	f	37	3	9.6	7.2	75	
HP 11	m	47	4/7	10.2	12.9	126	
HP 12	f	49	4/5	10.2	12.3	121	
HP 13	f	19	3	11.5	13.2	115	
HP 14	f	22	4	8.2	9.9	108	
HP 15	m	29	4	9.3	9.5	103	
Mean (NP 1-15)		33.0		10.7	11.2	105	
SD				3.3	4.0	19.6	
SEM				0.8	1.0	5.0	

+ No examination

coll Hypaque technique [19], washed three times and adjusted to a final concentration of 5×10^6 cells/ml. The PBMC were preincubated in EBM for 2 h. Viability was tested by the trypan exclusion test. Only preparations with a viability higher than 90% were used. PBMC prepared and incubated in EBM as described above did not release any insulin immunoreactivity into the medium.

Insulin release assay

Ten islets were incubated either in EBM alone (control) or together with 0.2 ml PBMC suspension in glass dishes for 20 h at 37 °C with 5% CO₂/95% O₂. After the incubation, the insulin content in the medium was determined by the alcohol precipitation method using a rat insu-

Table 2. Insulin release during incubation of rat islets with peripheral blood mononuclear cells of Type 1 diabetic patients and following insulin secretion after stimulation by 10 mmol/l arginine

Patient	PBMC effect on basal insulin release ^a	PBMC effect on arginine-stimulated insulin secretion ^a
1	306	54
2	191	47
3	245	56
4	198	56
5	218	42
6	219	60
7	211	61
8	148	66
10	297	43
13	198	70
14	254	84
17	170	47
20	263	91
Mean	224.5	59.1
SD	46.8	15.5
Control group (n = 11)	p < 0.001	p < 0.001
Mean	107.2	94.1
SD	20.1	17.8

^a Expressed in percent of control values **Fig. 1.** Insulin release from rat islets during incubation with peripheral blood mononuclear cells from healthy subjects (A), and from Type 1 diabetic patients without (B) and with (C) addition of PBMC from healthy probands - (1:4) (control in incubation medium alone = 100%) ($p < 0.001$)

lin standard (Novo Research Institute, Copenhagen, Denmark, Batch-No. 810611) [20]. The relative effect of the PBMC was calculated as percentage of insulin released in the appropriate control experiment, i.e. islets incubated in medium alone.

Measurement of insulin secretion capacity

After the 20-h incubation period, the islets were washed, preincubated in EBM for 1 h and then stimulated with 10 mmol/l arginine for 3 h.

The insulin secretion into the medium was then measured. The secretion capacity was calculated as per cent of the control value, i.e. insulin secretion during incubation for 20 h in EBM alone.

Test of suppressor cell function

In order to evaluate if the insulin release caused by PBMC of diabetic patients could be modulated by PBMC of healthy subjects, in 10 cases 1×10^6 PBMC from diabetic patients were mixed with 2.5×10^5 PBMC (4:1 ratio) from control subjects (and in a ratio of 19:1 in 6 cases). The mixture was incubated in EBM for 1 h and then used in the insulin release assay as described above. The individual combinations and HLA-DR phenotypes can be taken from Table 1. To exclude nonspecific effects of the PBMC mixture, PBMC from diabetic patients with positive cytotoxic reactions were applied.

All tests were performed in duplicate. The mean deviation from the mean value was lower than 10%.

Statistical analysis

All results are presented as mean $\pm$ SD. The significance of differences was evaluated by the Wilcoxon U test, Mann-Whitney test (control subjects - diabetic patients) or the Wilcoxon matched pairs signed-rank test (experiments with mixed lymphocytes).

Results

In 22 of the 23 Type 1 diabetic patients, an increased insulin release from rat islets after incubation with isolated PBMC could be demonstrated (Table 1). The nonstimulated insulin release in the group of PBMC preparations from the diabetic patients amounted to 200% expressed relative to the EBM medium alone (being 100%). In the subsequent insulin secretion test the secretion capacity was significantly reduced ($p < 0.001$) if the islets had been incubated with PBMC from diabetic patients (Table 2). In the tests with PBMC from control subjects no significant effect could be seen either in the nonstimulated insulin release (Table 1) or in the subsequent stimulation period (Table 2). The individual HLA types did not reveal a predominance of any HLA-DR type which enhanced insulin release (Table 1). Other clinical parameters also did not correlate with measures of insulin secretion.

The addition of PBMC from healthy subjects abolished the increased insulin release caused by PBMC from Type 1 diabetic patients in 9 out of 10 cases (Fig. 1). The reduction amounted to more than 50% ($p < 0.001$). Nearly the same effect resulted by addition of PBMC from control subjects in the ratio of 1:19 in 5 out of 6 cases (Fig. 2). The PBMC addition in a ratio of 1:200 was without any significant effect (3 cases).

The addition of PBMC from another diabetic patient (with positive cytotoxic reaction) did not result in a significant ($> 20\%$) reduction in 14 out of 16 cases (Fig. 3).

The HLA pattern of the diabetic patients showed a typically higher incidence of DR 3 and DR 4. The suppressor effect of control PBMC in the insulin release assay seems uncorrelated to the constellation of the HLA

Fig. 2. Insulin release from rat islets during incubation with peripheral blood mononuclear cells from Type 1 diabetic patients with addition of PBMC from healthy probands 1:4 (A), without addition of PBMC (B) and with addition of PBMC 1:19 (C) (control in incubation medium alone = 100%)

Fig. 3. Insulin release from rat islets during incubation with peripheral blood mononuclear cells from Type 1 diabetic patients without (A) and with (B) addition of PBMC from other diabetic patients (control in incubation medium alone = 100%)

types, as can be seen by the combinations from Table 1. The HLA types were not matched in all our experiments.

Discussion

Our results demonstrate that PBMC from newly diagnosed Type 1 diabetic patients cause an increased non-

stimulated insulin release from B cells of isolated rat islets. This is in agreement with the results of Charles et al. [11], who used a chromium-release test. The reduced insulin secretion capacity after the incubation with PBMC from diabetic patients also supports the results of Boitard et al. [9], who found decreased insulin secretion after the incubation in mouse islet cells. Boitard et al. [10] could identify this effect as a T-cell phenomenon. By morphological methods we demonstrated that B cells can be attacked by lymphocytes [12, 13] with a consecutive B-cell necrosis. The missing effect of PBMC from healthy subjects contradicts the assumption that this cytotoxic effect is a result of a xenogenic system.

The addition of PBMC from healthy probands significantly reduces the cytotoxic effect. These results cannot be explained by nonspecific interactions between the PBMC stemming from different subjects, since the addition of PBMC from healthy individuals and from newly diagnosed diabetic patients was at variance.

In both groups the individual mixtures (diabetic plus control PBMC and diabetic plus diabetic PBMC respectively) are not HLA-matched. Therefore, the differences in suppression cannot be simply the result of a PBMC interaction from individuals with more or less compatible HLA types. The incubation time of 20 h also seems too short to produce the effects of a mixed lymphocyte culture. Similar results were obtained by Topliss et al. [17] using the leucocyte migration test.

Taking these observation together with our preliminary morphological findings [12, 13] and the results of Boitard [10] and Charles [11], we suggest that cytotoxic effects of activated T-lymphocytes can induce the damage of B cells in Type 1 diabetes. The activation of these cells could be the consequence of an imbalance in the immunoregulatory system, in which a defect of activities of suppressor cells plays a primary role.

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