Alterations in lymphocyte subpopulations in Type 1 (insulin-dependent) diabetes mellitus: exploration of possible mechanisms and relationships to autoimmune phenomena

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Summary. Previous studies have shown a variety of immunological abnormalities in Type 1 (insulin-dependent) diabetes, including disturbances in peripheral lymphocytes and anti-lymphocyte antibodies. We measured subsets of T and natural killer cells with monoclonal antibodies in patients with diabetes, and also assayed for anti-lymphocyte antibodies using dual colour immunofluorescence and flow cytometry. We found a significant decrease in numbers of Leu 3a (helper/inducer) cells in Type 1 diabetic patients of recent onset and intermediate levels in patients with longer duration of the disease. Leu 4 (pan T cell) levels were reduced in Type 1 diabetic patients of more than 4 months duration. Leu 7 (natural killer cells) were increased in Type 2 (non-insulin-dependent) diabetic patients. Individual Type 1 diabetic patients of recent onset showed low levels of Leu 7 and 11a (natural killer cell) levels with normal 3a levels. Autoantibodies against Leu

3a+ cells were present in higher titres in the Type 1 diabetic patients of recent onset than in control subjects. We conclude: (1) Leu 3a cells may be decreased in Type 1 diabetic patients of recent onset and return to normal with time; (2) low numbers of Leu 7 and 11a cells with normal numbers of Leu 3a may be seen in some Type 1 diabetic patients of recent onset, which may help explain previous reports of decreased suppressor cells; (3) Leu 7 levels may be increased in Type 2 diabetes; (4) autoantibodies against Leu 3a+ peripheral lymphocytes may be seen in Type 1 diabetic patients of recent onset. These appear to be a marker of autoimmune phenomena rather than immunological mediators.

Key words: Lymphocytes, anti-lymphocyte antibodies, killer cells, cell-mediated immunity.

A variety of disturbances in cell-mediated immunity have been found in Type 1 diabetes, including cellular hypersensitivity to pancreatic extract [1], cytotoxic effects of diabetic lymphocytes against insulinoma cells [2] and alterations in subsets of peripheral blood lymphocytes [3–7]. Studies in Type 1 diabetes and other autoimmune diseases have suggested that antibodies directed against lymphocytes may play a part in the pathogenesis of these disorders [8, 9]. Warm and cold-reacting autoantibodies against T and B cells have been found in diabetic sera [10], and an IgG against lymphocytes has been identified in the BB Wistar rat [11]. Similar antibodies have been described in other autoimmune diseases but the pathogenic significance of these antibodies and their relationship to disturbances in peripheral blood lymphocyte subsets has not been de-

fined. The present study was designed to address these questions.

Subjects and methods

Subjects

Peripheral blood lymphocyte subpopulations were measured in three groups of diabetic patients and 14 normal control subjects. The patients included: (a) 15 Type 1 diabetic patients of recent onset (aged 3–23 years; within 4 months of presentation). The mean C-peptide level was 0.28 ± 0.01 nmol/l [13]; HbA₁ was $10.0 \pm 0.5\%$ [14]; seven had anti-thyroid antibodies (anti-microsomal haemagglutination and anti-thyroglobulin haemagglutination) [15]. (b) Thirteen Type 1 diabetic patients of more than 4 months duration of the disease (aged 9–49 years). Two had anti-thyroid antibodies. Mean C-peptide level was 0.08 ± 0.3 nmol/l; HbA₁ was $10.7 \pm 0.5\%$. (c) Eleven obese Type 2 diabetic patients of 4 months – 22 years duration (aged 29–69 years).

Table 1. Lymphocyte subsets and anti-lymphocyte antibodies in the patient groups studied

Group	Monoclonal antibodies for peripheral blood lymphocyte subsets					Anti-lymphocyte antibodies	
	Leu-2 a (cytotoxic/ suppressor) (%)	Leu-3 a (helper/ inducer) (%)	Leu-4 (pan T cell) (%)	Leu-7 (natural killer cell) (%)	Leu-11 a (natural killer cell) (%)	Number of Leu-2 a- positive cells	Number of Leu-3 a- positive cells
Control subjects	25.1 ± 1.5	48.3 ± 1.5	74.0 ± 1.4	9.5 ± 1.4	8.6 ± 1.0	84.7 ± 10.9	20.4 ± 2.6
Type 1 diabetic patients of recent onset (<4 months)	26.4 ± 1.3	42.6 ± 1.5 ^b	72.1 ± 1.7	6.8 ± 1.1	6.9 ± 1.3	82.1 ± 9.25	35.3 ± 6.9 ^a
Type 1 diabetic patients of longer duration (>4 months)	25.2 ± 1.4	43.7 ± 1.3 ^a	69.8 ± 1.3 ^a	9.3 ± 1.4	8.7 ± 1.8		
Type 2 diabetic patients	24.5 ± 9.2	43.2 ± 2.9	69.9 ± 3.2	20.3 ± 2.6 ^c	12.5 ± 2.2		

^a $p < 0.05$, ^b $p < 0.02$, ^c $p < 0.001$ versus control subjects

Two had antithyroid antibodies. Mean C-peptide level was 0.99 ± 1.5 nmol/l; HbA_{1c} was $10.0 \pm 0.5\%$. Written informed consent was obtained from all subjects. The study was approved by the Clinical Investigation Committee of the University of Chicago.

Preparation and staining of peripheral blood lymphocytes

Mononuclear cells were isolated from 20 ml of peripheral blood using Ficoll/Paque by standard methods [16]. The cells were stained either phycoerythrin conjugated with anti-Leu 2a or 3a or fluorescein isothiocyanate (FITC) conjugated with anti-Leu 4, 7 or 11a (Becton Dickinson, Monoclonal Center, Mountain View, California) according to established procedures [16]. These recently characterized antibodies specify cytotoxic/suppressor T cells, helper/inducer T cells, large granular lymphocytes and natural killer cells (HNK-1 antigen), and neutrophil and natural killer cell FcIgG receptors respectively [12]. Controls included cells incubated with media alone. The cells were analysed on a FACS IV.5 (Becton Dickinson, FACS Division, Mountain View, California, USA). 20,000 cells were analysed in duplicate and the mean data expressed as a percentage are reported.

Staining of anti-lymphocyte antibodies

Peripheral blood lymphocytes were isolated from three normal donors as described above and pooled. Heat-inactivated ultracentrifuged sera from Type 1 diabetic patients of recent onset or control subjects was incubated with cells for 1 h at 4°C. After washing, the cells were stained with FITC conjugated goat F_{(ab)2} anti-human IgG (F_c fragment specific) which was pre-absorbed with mouse immunoglobulin. The cells were washed and then stained with phycoerythrin anti-Leu 2a or 3a. Negative control cells included lymphocytes stained with FITC anti-IgG and phycoerythrin conjugated anti-Leu 2a or 3a. As a positive control, rabbit anti-thymocyte globulin in a 1:50 dilution was used in the place of sera. 30,000 cells were analysed for two colour immunofluorescence on the instrument noted above with gates set on lymphocyte peaks of forward and wide angle scatter. The number of dual colour fluorescent cells was calculated using a Tetrax computer (model 4025, Tetrax, Beaverton, Oregon, USA). Each serum sample was assayed on three occasions with the same cell donors and the mean number of dual colour cells is reported.

Statistical analysis

The significance of differences between patient groups and control subjects were evaluated by unpaired t-tests or Wilcoxon scores. All results are expressed as mean ± SEM.

Results

Lymphocyte subpopulations

The quantitative analysis of lymphocyte subpopulations is seen in Table 1. Leu 3a levels were significantly reduced in the Type 1 diabetic patients of recent onset ($p < 0.02$) compared with the control subjects and diabetic patients of longer duration ($p < 0.05$), and unchanged in Type 2 diabetic patients. Leu 2a levels were not different from control subjects in any of the diabetic groups. Reduced numbers of Leu 4 cells were seen in the diabetic patients of longer duration ($p < 0.05$). Although, as a group, significant differences in Leu 7 or 11a cells were not seen in the recent onset Type 1 diabetic patients, there was a clear tendency for strikingly low values in patients with diabetes of short duration. Furthermore, the levels of Leu 7 and 11a cells were significantly correlated ($r = 0.77$, $p < 0.001$), which was not seen in the control group. A strong inverse correlation was seen between changes in Leu 3a and 11a levels ($r = 0.67$, $p < 0.01$), and a positive correlation between 2a and 11a levels ($r = 0.68$, $p < 0.01$). Leu 7 levels were significantly increased in the Type 2 diabetic patients, which is unexplained by differences in glucose control as HbA_{1c} levels were similar in the three experimental groups.

Anti-lymphocyte antibodies

To determine whether anti-lymphocyte antibodies are present in the Type 1 diabetic patients of recent onset and define their possible relationship to peripheral blood lymphocyte abnormalities, we developed a method for detection of lymphocyte binding immunoglobulin using dual colour immunofluorescence. The level of background staining in the assay was $<1\%$ of cells. A positive control, rabbit anti-thymocyte globulin, bound 5163 Leu 2a+ cells and 4329 Leu 3a+ cells. Low levels

of binding were seen against Leu 2a and 3a cells with sera from both diabetic and control subjects. The levels of binding against the 3a subpopulation was significantly greater in the diabetic sera than the control ($p < 0.05$). Binding against other lymphocyte populations, as measured by a shift of the green immunofluorescence (FITC anti-IgG) in the orange (phycoerythrin Leu 2a or 3a) negative cells was not seen. The level of binding of the anti-Leu 2a+ cell and 3a+ cell immunoglobulin was unrelated to the levels of Leu 2a+ or 3a+ cells in peripheral blood. Levels of anti-Leu 3a+ cell immunoglobulin were significantly correlated with both anti-microsomal ($r = 0.60$, $p < 0.05$) and anti-thyroglobulin ($r = 0.83$, $p < 0.001$) antibodies.

Discussion

We have found a reduction in the Leu 3a (helper) population of peripheral blood lymphocytes in new onset Type 1 diabetes and normal levels of Leu 2a+ (cytotoxic/suppressor) cells. Type 1 diabetic patients of more than 4 months duration had intermediate 3a values, suggesting return of levels to normal with time. Our studies with anti-Leu 7 and 11a showed that the new onset patients may be divided into two groups: those with low Leu 3a and normal 7 and 11 levels, and those with low 7 and 11a levels with normal Leu 3a. We found a significant increase in Leu 7 levels in Type 2 diabetes which cannot be explained on the basis of glucose control alone, but suggests either a previously unrecognised immune disturbance in this disease or change acquired with hyperglycaemia.

Previous reports of peripheral blood lymphocytes in Type 1 diabetes are conflicting, describing both normal and low levels of helper and suppressor cells [3, 6, 7]. Pozzilli et al. used anti-Leu 3a to investigate peripheral blood lymphocytes in Type 1 diabetes and found decreased levels in diabetes of long standing [7]. It is possible that the differences we found in our study are due to using fresh, not frozen, cells, flow cytometry, and/or phycoerythrin conjugated antibodies which detect a higher proportion of cells.

The anti-natural killer antibodies Leu 7 and 11a have not been previously studied in Type 1 diabetes. Wall et al. found an increase in HNK-1-positive cells in patients with sub-acute thyroiditis which shares many immunological features with Type 1 diabetes [17]. The discrepancies between our data and the raised killer cells found by Pozzilli et al. [4] may reflect differences in cells isolated by rosette formation. Recent work with multicolour immunofluorescence has shown overlap between the Leu 2a, 7 and 11a subsets [12]. Considering the changes we observed in Leu 7 and 11a, it is possible that previous observations of decreased suppressor cell numbers are actually due to a decrease in the 11a+ and/or 7+, 2a+ subpopulation. The significance of the reciprocal changes in 3a and 11a is unclear at this

time. Lanier et al. [12] showed that antibody-dependent cellular cytotoxicity and natural killer activity resides primarily within the 11a+ and to a lesser degree 7+ subsets. Although it is intriguing to postulate an abnormality in antibody-dependent cellular cytotoxicity due to disturbances in both effector and inducer cells, a more basic understanding of these cellular interactions is necessary.

We have shown that levels of antibody binding against Leu 3a+ cells in patients with Type 1 diabetes of recent onset are significantly greater than those in control subjects. These differences are restricted to the 3a+ subpopulation. The absolute levels of binding against the 2a+ cells in both diabetic and control subjects was higher than to 3a+ cells, which may be due to the brightness of the phycoerythrin anti-Leu 2a antibody. The levels of binding against the peripheral blood lymphocytes was low and we were unable to demonstrate a relationship of anti-lymphocyte antibodies to peripheral blood lymphocyte levels. Thus it is unlikely that anti-lymphocyte antibodies are able to account for the quantitative disturbances in peripheral blood lymphocytes that we have found. Rather, the relationship of anti-lymphocyte antibodies to thyroid antibodies suggests anti-lymphocyte antibodies are autoimmune markers. Functional studies in the presence or absence of the antibodies will be necessary to confirm this. Further study of lymphocyte surface antigens expressed during the onset of Type 1 diabetes are likely to add greatly to our understanding of these anti-lymphocyte antibodies.

A variety of abnormalities of peripheral blood lymphocytes have been reported in Type 1 diabetes. Our data describe quantitative differences in T and natural killer cells which both corroborate [6] and are at variance [4] with earlier reports. The most likely explanation for these discrepancies is that Type 1 diabetic patients of recent onset are heterogenous in their immunological abnormalities. We have studied one possible mediator of immune disturbances and found that anti-lymphocyte antibodies are not a likely cause of peripheral blood lymphocyte disturbances, yet occur with other immune markers. However, such studies of peripheral phenomena can provide limited insight into the pathogenesis of the disease and further studies at the molecular and cellular level are needed. A more fundamental understanding of the immunological mediators is necessary before it will be possible to define the relationship between disturbances in peripheral blood lymphocytes and the pathogenesis of Type 1 diabetes.

References

1. Nerup J, Andersen O, Bendixen G, Egeberg J, Poulsen J (1971) Anti-pancreatic cellular hypersensitivity in diabetes mellitus. *Diabetes* 20: 424–427
2. Huang S, MacLaren N (1976) Insulin dependent diabetes: a disease of autoaggression. *Science* 192: 64–66

3. Horita M, Suzuki H, Onodera F, Ginsberg-Fellner F, Fauci A, Notkins A (1982) Abnormalities of immunoregulatory subsets in patients with insulin-dependent diabetes mellitus. *J Immunol* 129: 1426–1429
4. Pozzilli P, Gorsuch A, Sensi M, Bottazzo G, Cudworth A (1979) Evidence for raised K-cell levels in Type 1 diabetes. *Lancet* 2: 173–175
5. Jackson R, Morris M, Haynes B, Eisenbarth G (1982) Increased circulating Ia-antigen-bearing T cells in Type 1 diabetes mellitus. *New Engl J Med* 306: 785–788
6. Muscart-Lemone F, Delespesse G, Dorchy H, Lemiere B, Servais G (1982) Characterization of immunoregulatory T lymphocytes in insulin-dependent diabetic children by means of monoclonal antibodies. *Clin Exp Immunol* 47: 296–300
7. Pozzilli P, Zuccarini O, Iavicoli M, Andreani D, Sensi M, Spencer K, Bottazzo G, Beverley P, Kyner J, Cudworth A (1983) Monoclonal antibodies defined abnormalities of T-lymphocytes in Type 1 (insulin-dependent) diabetes. *Diabetes* 32: 91–94
8. DeHoratius R (1980) Lymphocytotoxic antibodies. In: Schwartz RS (ed) *Progress in clinical immunology*, Vol 4. Grune & Stratton, New York, pp 151–174
9. Serjeantson S, Theophilus J, Zimmet P, Court J, Crossley J, Elliott R (1981) Lymphocytotoxic antibodies and histocompatibility antigens in juvenile-onset diabetes mellitus. *Diabetes* 30: 26–29
10. Ozturk G, Terasaki P (1979) Non-HLA lymphocyte cytotoxins in various diseases. *Tissue Antigens* 14: 52–58
11. Dyrberg T, Nakhooda A, Baekkeskov S, Lernmark A, Poussier P, Marliiss E (1982) Islet cell surface and lymphocyte antibodies in the spontaneously diabetic BB Wistar rat. *Diabetes* 31: 278–281
12. Lanier L, Loken M (1984) Human lymphocyte subpopulations identified by using three-color immunofluorescence and flow cytometry analysis. *J Immunol* 132: 151–156
13. Faber O, Binder C, Markussen J, Heding L, Naithani V, Kuzuya H, Blix P, Horwitz D, Rubenstein A (1978) Characterization of seven C-peptide antisera. *Diabetes* 27: 170–177
14. Gabbay K, Sosenko J, Banuchi G, Mininsohn M, Fluckiger R (1979) Glycosylated hemoglobins: increased glycosylation of hemoglobin A in diabetic patients. *Diabetes* 28: 337–340
15. Beall G, Solomon D (1973) Immunologic features of thyroid disease. *Postgrad Med* 54: 181–189
16. Becton Dickinson Monoclonal Antibody Source Book (1983) B.D. Monoclonal Antibody Center, Sunnyvale, California
17. Wall J, Baur R, Schleusener H, Bandy-Dafoe P (1983) Peripheral blood and intrathyroidal mononuclear cell populations in patients with autoimmune thyroid disorders. *J Clin Endocrinol Metab* 56: 164–169

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