Two-Color Flow Cytometry Analysis of Activated T-Lymphocyte Subsets in Type I Diabetes Mellitus

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We addressed the question of whether newly diagnosed type I (insulin-dependent) diabetes mellitus patients showed an increased number of DR (Ia^+) T-lymphocytes compared with nondiabetic siblings and normal control subjects. Two-color flow cytometry measurements of peripheral-blood lymphocytes showed a slight but statistically significant increase in DR^+ T-lymphocytes in diabetic subjects as well as the nondiabetic sibling control compared with the normal control subjects. This difference was not present in long-term–diabetic subjects. Thus, in addition to minor changes in this lymphocyte subset in peripheral blood, the sibling data demonstrate a lack of specificity for the disease; therefore, these measurements are probably of limited diagnostic usefulness. *Diabetes* 37:792–95, 1988

Type I (insulin-dependent) diabetes mellitus is believed to have an autoimmune pathogenesis (1). The evidence for autoimmunity is supported by the presence of infiltration of the pancreatic islets with cells of the immune system (2), as well as by alterations of humoral immunity characterized by the presence of antibodies directed against the islet cells (3) and the insulin molecule (4) and changes in cellular immunity, i.e., increased number of lymphocytes expressing class II (DR) MHC antigens (5), functional abnormalities involving suppressor function (6), and the production of interleukin 2 (7).

Recent studies with immunosuppressive agents in type I diabetes suggest that, in some cases, the disease process can be slowed or even reversed (8,9). However, because the onset of type I diabetes may begin months to years before the development of acute symptoms (10), it is likely that the physician prescribing immunotherapy will most often be faced with patients whose β-cell mass is severely depleted at the time of diagnosis. Therefore, it is important to find markers that indicate active β-cell damage during the presymptomatic phase so that immunotherapy will be more effective.

It has been suggested that the presence of increased numbers of activated T-lymphocytes in peripheral blood may be such a marker (11). The aim of this study was to determine whether alterations in the subsets of activated T-lymphocytes, as assessed by a combination of two-color immunofluorescence and flow-cytometry analysis (12), could be found in patients with type I diabetes and their nondiabetic siblings.

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MATERIALS AND METHODS

We studied activated T-lymphocyte subsets in 28 children from the time their type I diabetes was diagnosed, 25 of their siblings, 13 nondiabetic control subjects, and 29 subjects who had type I diabetes for >2 yr.

All of the 28 type I diabetic subjects were tested within 8 days after the diagnosis of their disease. There were 12 males and 16 females aged 2–18 yr (mean age ± SD = 10.8 ± 4.3 yr). Twenty-five of these had been HLA-DR typed, resulting in 10 DR3, 8 DR4, and 7 DR3/4 subjects. In the siblings group, there were 12 males and 13 females aged 6–18 yr (mean age 11.7 ± 3.2 yr). In the control group, there were 3 males and 10 females aged 3–16 yr (mean age 9.4 ± 4.2 yr). In the long-term–diabetic group, there were 12 females and 17 males aged 3–20 yr (mean age 14.4 ± 5.1 yr). The mean duration of diabetes mellitus was 7.0 ± 4.0 yr. None of the subjects was known to have intercurrent illnesses at the time of the study.

Monoclonal antibodies. The specificities of the murine monoclonal antibodies (MoAbs) in this study were anti-Leu-4 (IgG1, SK 7), which recognizes the CD3 T-lymphocyte differentiation antigen (13); anti-Leu-3 (IgG1, SK 3), which

recognizes the CD4 T-lymphocyte differentiation antigen (13); anti-Leu-2 (IgG1, SK 1), which recognizes the CD8 T-lymphocyte differentiation antigen (13); and anti-DR (IgG2a, L243), which recognizes a nonpolymorphic HLA-DR determinant (14). All antibodies were kindly supplied by A. Saunders and N. Warner (Becton Dickinson Monoclonal Antibody Center, Mountain View, CA). These MoAbs were directly conjugated with fluorescein isothiocyanate (FITC) or phycoerythrin (PE) (15).

Cells. Mononuclear cells were isolated via a Ficoll-Hypaque gradient (Pharmacia, Piscataway, NJ). Cells were then washed three times in Hanks' balanced salt solution (HBSS) containing magnesium, sodium bicarbonate, and phenol red but no calcium. They were then resuspended for staining via a standard procedure (16).

Two-color immunofluorescence staining. Mononuclear cells (5×10^5) in a total volume of 50 μl of diluent were placed into 12 × 75-nm polystyrene tubes (Falcon, Oxnard, CA). One microgram of an FITC MoAb (anti-Leu-4, -3, or -2) was added to each tube. Tubes were then agitated and incubated for 30 min at 4°C in the dark. Five milliliters of diluent was added to each tube, and the samples were centrifuged at 400 × *g* for 5 min. The supernatant was removed, and 1 μg of PE anti-Leu-DR was added. Samples were then agitated and incubated for an additional 30 min. Five milliliters of diluent was added to each tube, and the samples were centrifuged at 400 × *g* for 5 min. The supernatant was removed, and the pellet was resuspended in a 1% paraformaldehyde/0.85% saline solution. Fixed cells were stored at 4°C in the dark until analysis (<1 day). All washes and dilutions were performed with HBSS containing 0.1% NaN_3. All procedures were performed in the dark. Controls consisted of unstained cells and cells stained with isotype-matched mouse MoAbs directly conjugated to FITC or PE to assess Fc-related nonspecific binding.

Two-color flow-cytometry analysis. This was performed with a FACS Analyzer (Becton Dickinson, Sunnyvale, CA). The details of analysis have been published (16). Fluorescence emission was measured at 530 nm for FITC and 575 nm for PE. Wide-angle forward light scatter, Coulter volume, and green (FITC) and red (PE) fluorescence were measured and analyzed in "list mode" by a Consort 30 computer system (Becton Dickinson FACS Division). Fluorescent measurements were in log scale. We used the MoAb anti-Leu-M3 directly conjugated with FITC to detect mature monocytes. The monocyte contamination within the gated-lymphocyte population was <1% in each sample and not statistically different in the various groups. For each sample, 10,000 cells were acquired. In determining the percentage of positive cells, a marker was set on the appropriate control histogram such that <1% of the cells were beyond the limits of this marker. From this reference point the percentage of cells in the specific MoAb-stained sample histogram was calculated. The mean and mode of the channel number and the peak and coefficient of variation were also determined and compared between the groups as an indirect measure of surface antigen density. Two-parameter data were collected into a 64 × 64 matrix and displayed as "contour maps" (Fig. 1). The relative percentage of cells in each quadrant representing singly or doubly stained cells was displayed and recorded.

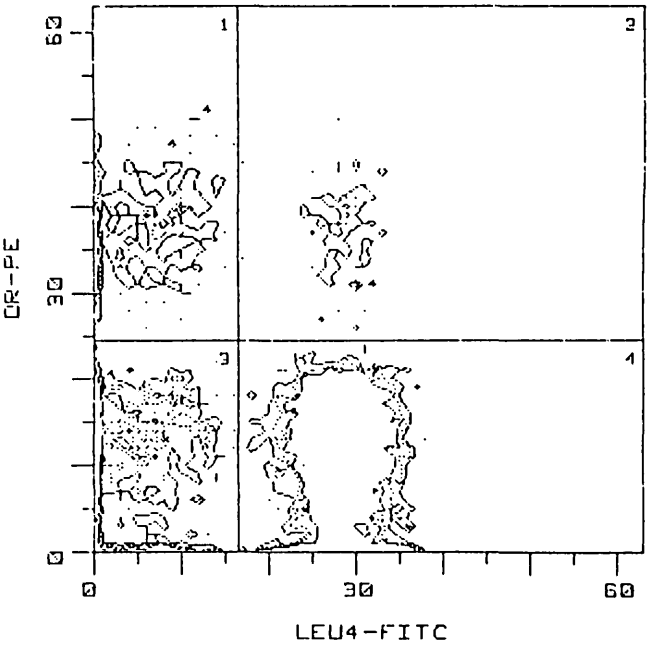

FIG. 1. Two-dimensional contour map showing subpopulation of T-lymphocytes coexpressing class II (DR) antigens found in recently diagnosed diabetic patient. *y*-Axis represents log scale of red fluorescence (anti-DR phycoerythrin; PE), and *x*-axis represents green fluorescence (anti-Leu-4 fluorescein isothiocyanate; FITC). *Quadrant* 2 shows subpopulation of doubly stained DR^+ T-lymphocytes.

Statistics. Statistical analysis of the results was done with SPSS one-way analysis of variance (ANOVA) and Student-Newman-Keuls a posteriori contrast test of means. Student's *t* tests of lymphocyte subsets between two periods were also done.

RESULTS

First, we compared relative numbers of activated T-lymphocyte subsets in newly diagnosed diabetic subjects, their siblings, and control and long-term–diabetic subjects. With Student Newman-Keuls analysis to compare these values, there was a significant difference among the four groups (Table 1).

The percentage of $Leu\text{-}4^+/DR^+$ lymphocytes was significantly elevated in newly diagnosed diabetic subjects compared with control and long-term–diabetic subjects. The percentage of $Leu\text{-}4^+/DR^+$ lymphocytes in the siblings group was also significantly elevated compared with the long-term–diabetic group. However, there was no difference between the newly diagnosed diabetic subjects and their siblings. For these two groups, there also was no difference in the fluorescence histogram mean (135 vs. 134, respectively) and mode (131 vs. 133, respectively) channel number, representing a measure of density of DR on T-lymphocytes, between the newly diagnosed diabetic subjects and siblings. However, this result shows an increase in these groups compared with the 6-mo determination and the nondiabetic control subjects who had a mean (111 vs. 114, respectively) and mode (91 vs. 102, respectively) channel number.

The percentage of $Leu\text{-}3^+/DR^+$ lymphocytes did not statistically increase in newly diagnosed diabetic subjects compared with control and long-term–diabetic subjects. There

TABLE 1
Student-Newman-Keuls analysis between means of relative numbers of activated T-lymphocyte subsets

Phenotype subset	Subjects: Newly diagnosed diabetic (n = 28)	Siblings (n = 25)	Control (n = 13)	Long-term diabetic (n = 29)	F	P
Leu-4$^+$/DR$^+$	1.7 ± 1.1*	1.3 ± 0.7†	0.8 ± 0.3*	0.8 ± 0.3*†	5.950	.001
Leu-3$^+$/DR$^+$	0.9 ± 0.6	0.7 ± 0.4	0.5 ± 0.3	0.5 ± 0.3	2.949	.038
Leu-2$^+$/DR$^+$	0.5 ± 0.4‡	0.4 ± 0.2	0.2 ± 0.1‡	0.2 ± 0.2‡	5.075	.003

Values are percents ± SD. Values sharing symbols were different at $P < .05$.

was no significant difference between siblings and the other three groups. The percentage of Leu-2$^+$/DR$^+$ lymphocytes significantly increased in newly diagnosed diabetic subjects compared with control and long-term–diabetic subjects. There was no significant difference between siblings and the other three groups.

Surprisingly, newly diagnosed diabetic subjects showed fewer peripheral-blood lymphocytes compared with control (2316 ± 825/mm^3 vs. 3191 ± 957/mm^3, t = 3.582, P = .001) and long-term–diabetic (2316 ± 825/mm^3 vs. 2757 ± 624/mm^3) subjects. Siblings showed a similar pattern compared with control (2104 ± 551/mm^3 vs. 3191 ± 957/mm^3) and long-term–diabetic (2104 ± 551/mm^3 vs. 2757 ± 624/mm^3) subjects.

Finally, in 11 type I diabetic children, the comparison of activated T-lymphocyte subsets at time of diagnosis and 6 mo later showed the abnormalities seen at time of diagnosis were corrected with time (Table 2). The values that were observed are similar to those found in the control and long-term–diabetic subjects.

DISCUSSION

Our findings confirm and extend previous observations that newly diagnosed type I diabetic subjects and their nondiabetic siblings have an increase in the percentage of activated T-lymphocytes (11,12). However, these abnormalities corrected with time, because 6 mo after diagnosis the activated T-lymphocytes in newly diagnosed diabetic subjects and their siblings were comparable to long-term–diabetic and control subjects.

The increase in activated T-lymphocytes found in newly diagnosed diabetic subjects was found in percentages of Leu-4$^+$/DR$^+$ and its major subsets, Leu-3$^+$/DR$^+$ and Leu-2$^+$/DR$^+$. This result was also seen in the siblings, in which the Leu-4$^+$/DR$^+$ lymphocytes were significantly increased compared with controls, but the small number of positive cells and the variability made the Leu-2$^+$/DR$^+$ and Leu-3$^+$/DR$^+$ subset differences less obvious.

Increased levels of activated T-lymphocytes in 14 of 15 recently diagnosed diabetic subjects and in 5 of 7 unaffected cotwins were reported by Alviggi et al. (11). In their study, increased levels of activated T-lymphocytes were present in 9 type I subjects and in 2 twins retested 6 mo after the diagnosis. Similarly, increased levels of activated T-lymphocytes in both newly diagnosed diabetic subjects (5) and first-degree nondiabetic relatives were reported by Pozzilli (12). In those studies, the percentage of activated T-lymphocytes was higher than it was in our study. This difference could be explained by the fact that the MoAbs in those studies did not define activated T-lymphocytes when tested on unpurified cells and that the number of positive cells was determined only with the fluorescence microscope, by counting several hundred cells. The two-color flow-cytometry technique we used allowed us to analyze a larger number of cells per sample (10,000) and to decrease to <1% the cells beyond the limits of the specific markers so that only lymphocytes would be gated. Hitchcock et al. (17) reported observations similar to ours in terms of a slight but significant increase in DR$^+$ T-lymphocytes in some patients, and, using a two-color flow system, they also found evidence for increased expression of DR on these cells. We also found evidence for increased DR expression, but this evidence appeared in newly diagnosed diabetic subjects as well as in the nondiabetic siblings compared with the measurement 6 mo after diagnosis or with the nondiabetic control subjects. Thus, there appeared to be little specificity to this measurement.

Although the difference in activated T-lymphocytes between newly diagnosed type I diabetic subjects and their siblings was significant compared with control and long-term–diabetic subjects, the small increase and the high variability found in each group suggest that the presence of activated T-lymphocytes may not be a good predictor of β-cell destruction, especially in the age group we studied. Furthermore, the normal levels of activated T-lymphocytes found 6 mo after diagnosis suggest that activation of T-lymphocytes may be a transient or fluctuating phenomenon not specifically marking β-cell destruction but indicating instead a general activation of the immune system in response to an acute illness or stress. Another possible explanation for the lower levels of activated T-lymphocytes found in our diabetic

TABLE 2
Activated T-lymphocyte subsets in 11 type I diabetic patients at time of diagnosis and 6 mo later

Phenotype subset	At time of type I diagnosis	6 mo after type I diagnosis	t	P
Lymphocytes/mm^3 ± SD	2095 ± 363	2188 ± 675	0.380	NS
Leu-4$^+$/DR$^+$				
Percent ± SD	2.2 ± 1.3	1.1 ± 0.6	2.470	.021
n/mm^3 ± SD	46 ± 27	25 ± 13	2.142	.043
Leu-3$^+$/DR$^+$				
Percent ± SD	1.3 ± 0.8	0.7 ± 0.4	2.402	.025
n/mm^3 ± SD	27 ± 16	14 ± 8	2.194	.039
Leu-2$^+$/DR$^+$				
Percent ± SD	0.7 ± 0.4	0.3 ± 0.2	2.846	.010
n/mm^3 ± SD	14 ± 9	6 ± 5	2.341	.029

population is the younger age and possibly the lower prevalence of DR3 subjects (35% of our diabetic population) found among younger diabetic subjects (18). Increases in activated T-lymphocytes were more frequent among DR3-typed nondiabetic first-degree relatives of type I diabetic subjects in the study of Pozzilli et al. (12). Although we have not determined the DR type of the siblings, the DR3 association remains a possible explanation for our data.

In conclusion, we have confirmed a statistically significant increase in the percentage and absolute number of activated T-lymphocytes in newly diagnosed diabetic subjects and their siblings. However, the variability, the relatively small increase, and the possibility of fluctuating numbers of lymphocytes make it unlikely that the presence of abnormally activated T-lymphocytes found with the types of markers described could effectively predict the development of symptomatic hyperglycemia in type I diabetes on a patient-by-patient basis.

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