Alterations in T-Lymphocyte Subpopulations in Type I Diabetes

Exploration of Genetic Influence in Identical Twins

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To evaluate factors influencing the alteration in subsets of T-lymphocytes, we studied 24 pairs of identical twins discordant for insulin-dependent (type I) diabetes mellitus. Subsets were assessed by monoclonal antibodies and a pure preparation of peripheral blood mononuclear cells obtained by centrifugation of heparinized whole blood with a Ficoll/Triosil gradient. In 12 pairs studied within 5 yr of diagnosis, we observed a reduction in the percentage of cells reacting with OKT8 (recognizing the CD8 antigen present on the suppressor/cytotoxic subset) ($P < .05$), but a similar level was detected in their nondiabetic cotwins. In 12 pairs studied >5 yr after the diagnosis and in whom the nondiabetic twin is less likely to develop diabetes, the percentage of cells reacting with OKT8 was reduced in both the diabetic ($P < .05$) and the nondiabetic ($P < .01$) twins. Reductions were also seen with OKT3 (recognizing the CD3 antigen present on the total T-lymphocyte population) and OKT4 (recognizing the CD4 antigen present on the helper/inducer subset), but only in the diabetic twins from the group with longer discordance. We conclude that a reduced percentage of suppressor/cytotoxic cells is associated with type I diabetes, but the reduction appears to be genetically determined. Total T-lymphocytes are also reduced but mainly in the helper/inducer subset and only in diabetic patients of long duration. Such a reduction cannot therefore be primarily genetically determined.
***Diabetes* 37:1484–88, 1988**

The aim of this study was to clarify the association between insulin-dependent (type I) diabetes mellitus and abnormalities of T-lymphocyte subsets, especially to determine whether there is any genetic influence. A number of studies with monoclonal antibodies have described differences in the subsets of T-lymphocytes, but controversy still surrounds the nature of these changes. In numerous studies, the percentage of suppressor cells was reported to be reduced (1–4). Other investigators, however, have found no difference (5–10), and some even reported an increase in this subset (11). A more consistent finding has been the reduction in the total T-lymphocyte population (2,4–7). This reduction is seen especially in diabetic patients studied years after the diagnosis (2,4,7) and appears to be predominantly a reduction of the helper/inducer subset (2,4,5,7). Other workers, however, have not been able to demonstrate any significant alteration in these subsets (8–10).

Some of the variation in the results may be accounted for by metabolic factors. Abnormalities of T-lymphocytes have been reported to be greatest in diabetic patients with poor glycemic control (12), and with improved blood glucose levels, the abnormal cell numbers can revert toward those of the controls (6,12). Other factors, e.g., a variation in either the age of the subjects or controls or differences in the specificity of the monoclonal antibodies, may also be important.

Genetic factors may also influence the T-lymphocyte population. Differences in subsets have been reported in nondiabetic first-degree relatives of type I diabetic patients (13,14), and among type I diabetic patients themselves, the percentage of particular subsets is said to differ with the possession of different HLA antigens (7).

A study of diabetes in identical twins has been in progress at Kings College Hospital for 20 yr (15) and comprises nearly 200 pairs with type I diabetes. The chances of a pair becoming concordant (both twins diabetic) decreases the longer the duration from diagnosis of diabetes in the affected twin. The majority of concordant pairs have become so within 5 yr, and we have calculated that the chances of a nondiabetic twin developing diabetes >11 yr after the diagnosis in their cotwin is <3%.

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Received for publication 18 September 1987 and accepted in revised form 7 June 1988.

In this study, we measured subsets of T-lymphocytes in two groups of identical twins discordant for type I diabetes. One group was studied within 5 yr of the diagnosis of the diabetic twin, and the other beyond 5 yr. A difference in a subset found only in diabetic twins cannot be genetically determined, whereas a difference present in both diabetic and nondiabetic twins, especially in pairs studied many years after the diagnosis, favors a genetic mechanism.

MATERIALS AND METHODS

Subjects. We studied 24 pairs of identical twins discordant for type I diabetes. Proof of their identity was established using methods previously described (15). They were divided into two groups, 12 pairs in each. Twins of group A were studied within 5 yr of diagnosis of the affected twin. They had a mean age of 17 yr with a range 7–35 yr. Seven were male. Twins of group B were studied >5 yr after the diagnosis of the diabetic twin. They had a mean duration of discordance of 14 yr (range 7–22 yr) with a mean age of 28 yr (range 15–62 yr). Seven were male. At the time of testing, all nondiabetic twins had a normal random plasma glucose of <8 mM, (glucose oxidase method, Yellow Springs analyzer) and a normal hemoglobin A_1 (assayed via electrophoretic method; Klytrac Corning) with a normal range of <7%.

Results were compared with 22 nondiabetic controls without a family history of diabetes. They had a mean age of 23 yr with a range 7–62 yr. Eleven were male. There was no significant difference between the age of either group of twins and the controls.

Methods. For identification of T-lymphocyte subsets, 20 ml of heparinized whole blood was collected. All tests were performed between 0800 and 1200 to minimize the possible diurnal variation in the T-lymphocyte population (16,17). Both twins were seen on the same day.

Peripheral blood mononuclear cells (PBMC) were obtained by centrifugation of heparinized whole blood with a Ficoll/Triosil gradient and harvesting the cells at the interphase. The cells were washed 3 times and resuspended at a concentration of 4 × 10/ml in RPMI-1640 (Gibco, Grand Island, NY) containing 2 mM glutamine, 200 U/ml penicillin, and 100 μg/ml streptomycin. Cell viability was > 98% as assessed by trypan blue exclusion. T-lymphocyte subsets were identified with monoclonal antibodies (Ortho, Raritan, NJ) via an indirect, single-color, immunofluorescence technique. The percentage of cells was determined by counting >200 cells under light microscopy with fluorescent illumination by an observer unaware of the clinical details. The antibodies used were OKT3 (which reacts with the CD3 antigen to recognize the total T-lymphocyte population), OKT4 (which reacts with the CD4 antigen to recognize the helper/inducer subset), and OKT8 (which reacts with the CD8 antigen to recognize the suppressor/cytotoxic subset). Fluorescein-labeled anti-murine antiserum (Dakopatts, Glostrup, Denmark) diluted 1:20 in phosphate-buffered saline, pH 7.4, was used as a second step to reverse cell-bound monoclonal antibodies.

The same 22 controls and 19 pairs of twins (9 from group A) were also tested for the presence of activated lymphocytes with the technique already described (18). To obtain a purified T-lymphocyte preparation, PBMC were rosetted with neuraminidase-treated sheep red blood cells (SRBC) and separated by Ficoll/Triosil gradient centrifugation. The T-lymphocytes were then freed from the SRBC by hypotonic lysis with Tris-buffered ammonium chloride solution. Activation was assessed with the fluorescein-labeled monoclonal antibody anti-DR (clone L243, Becton Dickinson, Oxford, UK) by direct immunofluorescence, and the percentage of cells was estimated as above.

Statistics. Group data are expressed as means and standard deviation. Comparison between individual twins and controls was performed with the Mann-Whitney test for unpaired data and a level of $P < .05$ was regarded as significant. Comparison between cotwins in each pair was performed with the paired Student's t test.

RESULTS

The percentage of cells reacting with each monoclonal antibody in each of the identical twins and controls is shown in Tables 1 and 2.

OKT8. Compared with controls, the percentage of cells reacting with this antibody was significantly reduced in the diabetic twins from group A, the more recently diagnosed diabetic twins ($P = .022$). This percentage was similar in the cotwins of the unaffected twins. Among the twins in group B with a longer duration of diabetes, the percentage of cells was significantly reduced in both the diabetic twins ($P = .027$) and in their nondiabetic cotwins ($P = .004$). Six of the unaffected twins in group B (nos. 1–6) were living apart at the time of testing but their level was still reduced ($P = .0078$).

OKT4. A significant reduction in the percentage of cells reacting with this antibody was seen in the diabetic twins of group A ($P = .049$). This subset was even lower in the diabetic twins of group B, in which the percentage of cells was significantly reduced compared with both the controls ($P = .011$) and their unaffected cotwins ($P = .047$). The nondiabetic cotwins in both groups had a similar percentage that was not significantly different from that of the controls.

OKT4/OKT8 ratio. This ratio was similar in both the diabetic and the nondiabetic twins from both groups, and there was no significant difference between them and the nondiabetic controls.

OKT3. The percentage of cells reacting with this antibody was reduced only in the diabetic twins in group B, those of longer duration, where the reduction was significant when compared with both the controls ($P = .008$) and their unaffected cotwins ($P = .006$). The percentage among the nondiabetic twins was similar in both groups and was not significantly different from that of the controls.

Activated lymphocytes. The percentage of activated lymphocytes was highest among the diabetic twins in group A, but the nondiabetic twins in this group had higher values than the diabetic twins from group B (differences vs. controls of $P = .002$, $P = .002$, and $P = .007$, respectively). All of these twins, however, had higher values than the long-standing nondiabetic twins from group B, whose values were not significantly different from those of the controls.

DISCUSSION

The results in these diabetic twins are similar to those of other studies in singleton diabetic patients. A reduction was seen in the percentage of the suppressor/cytotoxic subset

TABLE 1
Percentage of mononuclear cells reacting with antibodies OKT3, OKT4, and OKT8 and percentage of T-lymphocytes (IA) reacting with antibody L243 in discordant pairs of identical twins grouped by the duration from diagnosis of the diabetic twin

Pair no.	Duration of discordance (yr)	Age	Sex	OKT3 (%)	OKT4 (%)	OKT8 (%)	IA (%)
Twins discordant for <5 yr							
Diabetic twins							
1	3	20	M	62.3	38.7	28.5	12.9
2	<1	12	F	55.1	21.7	34.3	12.0
3	<1	15	M	58.1	41.2	21.0	9.9
4	<1	27	F	60.7	40.6	25.9	15.0
5	3	35	F	61.0	44.6	22.0	7.7
6	2	7	M	66.2	37.5	24.7	10.1
7	<1	11	M	58.2	31.8	25.9	15.9
8	<1	12	M	72.5	46.1	22.7	14.4
9	<1	19	M	68.3	47.4	22.3	7.5
10	2	10	F	79.4	59.0	19.6	
11	<1	24	F	79.0	46.3	34.2	
12	<1	19	M	58.4	38.5	18.9	
Mean ± SD		17		64.9 ± 8	41.1 ± 9	25.0 ± 5	11.7 ± 3
Nondiabetic twins							
1				67.1	31.1	34.3	10.6
2				52.2	37.8	33.3	12.0
3				62.7	38.8	23.6	6.4
4				66.0	35.3	30.4	12.9
5				68.5	50.8	23.1	6.6
6				58.9	32.5	21.3	9.0
7				77.3	41.4	26.3	12.0
8				63.5	42.3	18.3	11.9
9				80.3	55.1	24.5	4.3
10				68.7	56.4	14.7	
11				77.5	45.0	33.3	
12				63.2	43.0	17.8	
Mean ± SD				67.2 ± 8	42.5 ± 8	25.1 ± 7	9.5 ± 3
Twins discordant for >5 yr							
Diabetic twins							
1	10	23	M	69.0	39.5	21.5	6.0
2	20	44	M	61.5	42.9	31.7	8.1
3	20	36	M	68.3	49.5	26.9	2.9
4	22	46	F	43.8	27.6	15.3	6.9
5	12	26	F	56.6	44.6	18.9	6.3
6	17	62	F	67.0	45.6	19.5	
7	7	15	F	51.0	24.1	23.9	5.8
8	8	17	M	68.4	23.1	36.1	3.0
9	8	17	F	66.9	40.7	24.3	3.5
10	12	16	M	59.4	36.8	28.3	3.9
11	13	15	M	66.7	45.9	24.5	3.6
12	16	18	M	64.7	44.5	21.6	
Mean ± SD	14	28		61.9 ± 8	38.7 ± 9	24.4 ± 6	5.0 ± 2
Nondiabetic twins							
1				63.1	42.6	23.5	2.6
2				68.8	43.9	23.6	7.3
3				69.2	40.5	28.1	3.8
4				59.8	34.7	24.8	4.8
5				71.3	51.6	23.2	9.6
6				76.4	57.7	20.4	
7				55.2	34.9	24.5	2.9
8				74.5	42.6	28.2	2.8
9				70.9	39.6	26.4	0.8
10				62.5	38.7	22.2	2.6
11				71.6	43.6	27.1	1.9
12				69.3	53.0	16.4	
Mean ± SD				67.7 ± 6	43.6 ± 7	24.0 ± 3	3.9 ± 2.7

(1–4). There was also a reduction in the percentage of the helper/inducer subset (2,4,5,7), especially in the diabetic twins of longer duration. In these twins, the percentage of total T-lymphocytes was highly significantly reduced, which agrees with the findings by others (2,4–7).

Important in this study, however, was that the percentage of suppressor/cytotoxic cells was also significantly reduced in the nondiabetic twins from the long-standing discordant pairs. These twins were studied many years after the diagnosis of diabetes in the affected twin, and it is highly unlikely that many will now develop diabetes themselves.

Pozzilli et al. (14), using different monoclonal antibodies,

found an association between alterations in subsets and activation of T-lymphocytes in the first-degree relatives of type I diabetic patients. We could find no such correlation, and the results for the activated lymphocytes were similar to those of a larger series of twins already reported (18). We confirmed high levels in both the diabetic and nondiabetic twins studied soon after the diagnosis with the percentage of cells falling in both groups the longer the duration of diabetes. Thus the reduction in the percentage of the suppressor/cytotoxic subset is unlike the activation of T-lymphocytes in that it persists to a similar degree in both diabetic and nondiabetic twins even many years after the diagnosis.

The significant reduction in this subset in individuals with a genetic susceptibility, but now unlikely to develop type I diabetes, suggests that the association between a reduced percentage of suppressor/cytotoxic cells and type I diabetes is not a marker of immune activation or disease susceptibility and is probably genetically determined. Whether this is related to particular HLA antigens cannot be answered by this study.

The significant reduction in the percentage of the total T-lymphocyte population found only in the diabetic twins tested many years after diagnosis and the similarity found in the levels in the nondiabetic twins suggest that the reduction is not primarily genetically determined but is associated with long-standing diabetes. This study also confirms that the reduction is mainly in the helper/inducer subset, which remains unaffected in the nondiabetic twins. Whether the reduction in these subsets is due to metabolic factors or to some immunological abnormality associated with insulin administration is uncertain.

The differences in T-lymphocyte subsets found in type I diabetes in this and other studies are at variance with some reports (8–10). Differences in age cannot explain the results in our study. By necessity, the two groups of twins were not of the same age, but neither differed from the controls. Furthermore, we could not demonstrate any relationship between age and the percentage of any subset among the controls.

Some workers have suggested that the abnormalities found in T-lymphocyte subsets might be artifactual and recommend the use of whole blood rather than Ficoll separation, and analysis with fluorescence-activated cell sorter (FACS) rather than direct illumination (8). However, when the different methods of separation were actually compared, no difference in the subsets could be detected between samples obtained with whole peripheral blood mononuclear cells and cells collected from the interphase of the Ficoll gradient such as used in our study (19). The question of FACS versus direct vision was addressed in an international workshop that concluded that, although not always directly comparable, there was no significant difference, and direct vision with microscopy appeared to perform slightly better (20). Thus, there is no evidence to support a claim that alterations in subsets in type I diabetes are purely artifacts of methodology. In fact, the results from the diabetic patients in the two recent studies that claimed no alteration in subsets are very similar to those in this study (8,9); the lack of a difference is due to the lower values found in their controls, which were fewer than used in this or most other studies (2–6).

In conclusion, we have confirmed that type I diabetes is associated with abnormalities in the population of T-lymphocytes. A reduction in the percentage of cells reacting with OKT8, the suppressor/cytotoxic subset, is seen and may be genetically predetermined. A reduction in the percentage of total T-lymphocytes is mainly due to those reacting with OKT4, the helper/inducer subset, and is confined to diabetic patients, especially those of long duration. This reduction cannot be primarily genetically determined and must in some way be due to either the metabolic or immu-

TABLE 2
Percentage of mononuclear cells reacting with antibodies OKT3, OKT4, and OKT8 and percentage of T-lymphocytes (IA) reacting with antibody L243 in nondiabetic controls

No.	Age (yr)	Sex	OKT3 (%)	OKT4 (%)	OKT8 (%)	IA (%)
1	31	F	70.6	54.5	24.3	2.7
2	25	M	70.9	38.0	35.7	2.0
3	42	M	59.5	40.3	29.2	1.9
4	36	M	73.1	42.1	30.7	2.1
5	33	M	72.7	47.1	28.7	2.9
6	23	F	57.5	34.6	31.4	1.7
7	29	M	68.0	46.0	24.0	2.0
8	20	F	73.1	45.1	26.0	1.0
9	31	M	66.3	42.9	28.3	2.1
10	27	F	55.0	35.0	33.0	4.2
11	16	F	68.1	46.6	28.1	3.9
12	17	F	74.7	51.7	27.5	1.6
13	17	F	69.0	43.8	26.0	0.9
14	17	F	77.8	55.3	26.5	1.5
15	17	F	73.1	49.2	28.1	2.1
16	10	F	76.2	52.1	26.3	0.8
17	10	M	74.2	48.1	26.7	1.3
18	9	M	72.5	47.0	28.1	0.7
19	7	M	74.3	46.3	32.7	2.1
20	62	M	76.5	54.1	28.1	2.9
21	7	F	75.9	47.3	22.7	5.0
22	20	M	71.6	47.9	25.5	2.9
Mean ± SD	23		70.5 ± 6	46.1 ± 6	28.1 ± 3	2.2 ± 1

nological effects of long-term diabetes. The results of this study suggest that alterations in the subsets of T-lymphocytes are poor markers for the later development of type I diabetes.

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