Culture and Phenotype of Activated T-Cells from Patients with Type I Diabetes Mellitus

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SUMMARY

Blood T-cells from 28 patients with type I (insulin-dependent) diabetes (IDDM) of variable duration were examined for the Tac antigen by immunofluorescence, and for proliferation in the presence of interleukin 2 (IL 2). The mean percentage of Tac$^+$ cells in patients whose IDDM was of less than 2-yr duration was 6.2% compared with 2% in patients whose IDDM was of 3 or more years' duration, or in healthy controls. The percentage of Tac$^+$ cells in the patients' blood correlated positively with the amount of thymidine uptake in a 24-h culture of blood mononuclear cells and with the percentage of T-cell blasts generated in a 6-day culture. The patients' T-cell blasts stained with OKT 4 or OKT 8, suggesting that each of these subsets is present in the activated T-cell population in the patients' blood. The T-cell blasts did not show specificity for pork insulin in an antigen restimulation assay. There was no correlation between increased Tac$^+$ cells and the presence or absence of islet cell antibodies. If T-cell activation in IDDM occurs as a result of recognition of islet cell antigens, our results suggest that both HLA-DR-restricted (OKT 4$^+$) and A-, B-, and C-restricted (OKT 8$^+$) T-cell subsets contribute. DIABETES *33*:319–323, April 1984.

Evidence for autoimmunity in the pathogenesis of juvenile-onset or insulin-dependent diabetes mellitus (IDDM) includes the occurrence of anti-islet cell antibodies in most newly diagnosed patients[1,2] and the strong association with HLA DR3 and -4 antigens.[3] Jackson et al.[4] found increased percentages of Ia antigen positive, E-rosetting, blood lymphocytes in IDDM patients within 1 yr of diagnosis and speculated that these cells were activated T-lymphocytes. To explore this possibility further, we have examined blood lymphocytes from IDDM patients for another activation marker: Tac.[5] In addition, we cultured the patients' cells with interleukin 2 (IL 2) containing supernatants to permit continued proliferation of activated T-cells.[6] Response in these IL 2-supplemented cultures was measured by thymidine uptake at 24 h or by phenotyping the T-cell blasts recovered after 6-day culture. The patients' sera were also tested for antibodies to islet cell cytoplasm by immunofluorescence to look for possible correlations with blood T-cell phenotype.

IDDM patients receive injections of beef or pork insulin and might be expected to become sensitized to these proteins. Indeed, anti-insulin antibodies occur in some patients[7] and they may show binding to human insulin also. The possible specificity for insulin of activated T-cells in IDDM prompted us to explore this directly in an antigen restimulation test.

MATERIALS AND METHODS

Five to ten milliliters of blood were drawn from patients and healthy control subjects after obtaining informed consent. The IDDM patients were drawn from the Barbara Davis Childhood Diabetes Center clinic population. Thirteen were male, 15 female. Their ages ranged from 4 to 19 yr with a mean of 13 yr. The diagnosis of IDDM was based on a postprandial blood sugar >300 and glycosuria. Control blood samples were obtained from 6 healthy adults (age 21–39 yr) and 8 healthy children (age 5–16 yr). The blood was defibrinated and then centrifuged to recover serum. The cells were resuspended in Hepes-buffered Hanks' balanced salt solution (HBSS) and centrifuged on Ficoll-Hypaque to isolate mononuclear cells (MNC) as before.[8]

Cell culture and preparation of IL 2 supernatants. Patient and control MNC were washed twice in HBSS and adjusted to 10^6/ml in RPMI 1640 medium with 10% autologous serum and 10% IL 2-containing supernatant. The IL 2 supernatant was prepared by stimulating pooled tonsil lymphocytes from 2 or 3 donors with 1 μg/ml PHA in RPMI 1640 with 10%

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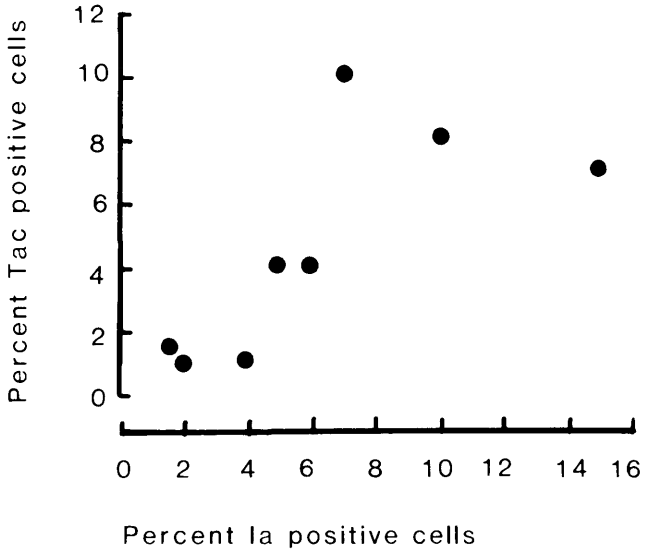

FIGURE 1. Correlation between Tac⁺ and Ia⁺ T-cells in the blood of IDDM patients.

human serum for 4 h, at which time the PHA-containing medium was removed.[9] The cells were washed once and resuspended in medium with human serum for a further 20 h at 37°C, then the supernatant was harvested by centrifugation and filtered through a 0.2-μ Millipore filter. The presence of IL 2 was confirmed by the ability of the supernatant to support the proliferation of IL 2-dependent human T-cell blasts. At a final concentration of 10%, the supernatant was not mitogenic. Cultures for measuring spontaneous thymidine uptake were in 0.2-ml volumes in Linbro 76-012-05 plates. The wells are pulsed with 0.25 μCi of tritiated thymidine (5 Ci/mmol, TRA 61, Radiochemical Centre, Amersham, United Kingdom) when the cultures were set up; cultures were harvested at 24 h.

Cultures for phenotyping were in Falcon 2057 tubes. After 6-day incubation with 10% IL 2-containing supernatant, the cells were spun down and resuspended in 60% Percoll which was overlaid with 50, 40, and 30% Percoll. The tubes were spun at 800 × *g* for 10 min to recover viable cells from the 40–50% interface.

Phenotyping by immunofluorescence. In preliminary studies, T-cells were separated from patients' blood by E-rosetting as previously described.[8] Sheep erythrocytes were lysed with ammonium chloride and the lymphocytes were incubated with monoclonal antibodies as follows. Cells (10^5) were incubated with 10 μl of antibody: anti-Tac at 1:20 (gift of Dr. T. Waldmann); OKIa; or OKT 3, 4, or 8 as previously described.[9] The cells were washed and incubated with a 1:40 dilution of fluorescein-conjugated goat anti-mouse IgG (Cappell Labs) before viewing on a Zeiss microscope with incident UV and transmitted phase-contrast optics. At least 100 cells were counted on each specimen.

Islet cell antibody test. Patients' sera were stored at −20°C and thawed immediately before testing. To avoid interference by anti-insulin antibodies,[10] sera were diluted with an equal volume of pork insulin (Actrapid 100 U/ml) before further dilution with PBS and testing. Serum (20 μl) at final dilutions of 1:2, 1:4, and 1:8 were placed on 5-μm sections of air-dried[11] human pancreas for 30 min, followed by 30 min of washing in 3 changes of PBS. The sections were then overlaid with 20 μl of a 1:32 dilution of goat anti-human immunoglobulin (Wellcome Reagents MF01) for 30 min before further washing. The slides were viewed and scored independently by two observers. Healthy control (negative) sera were included in each batch.

Antigen restimulation test. After 6-day incubation with 10% supernatant, as described above, cultures of patients' lymphocytes were centrifuged for 10 min on a Ficoll-Hypaque gradient. The cells recovered were washed in HBSS and resuspended at 10^6/ml in medium with 10% human serum. Cultures (0.2 ml) were prepared in triplicate for stimulation with 0, 0.5, 5, and 15 IU/ml of pork insulin (Actrapid, 100 IU/ml). These insulin concentrations were chosen because they were found optimal by Diem et al.[12] After 40 h, the cul-

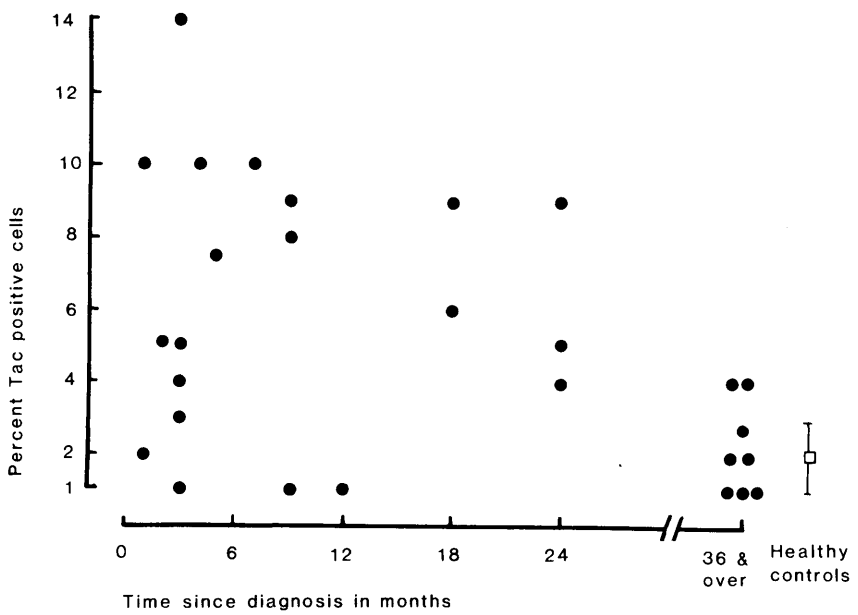

FIGURE 2. Percentage of Tac⁺ cells in blood MNC from IDDM patients (●). Control values (□) are the mean ± 1 SD for healthy children and adult laboratory workers.

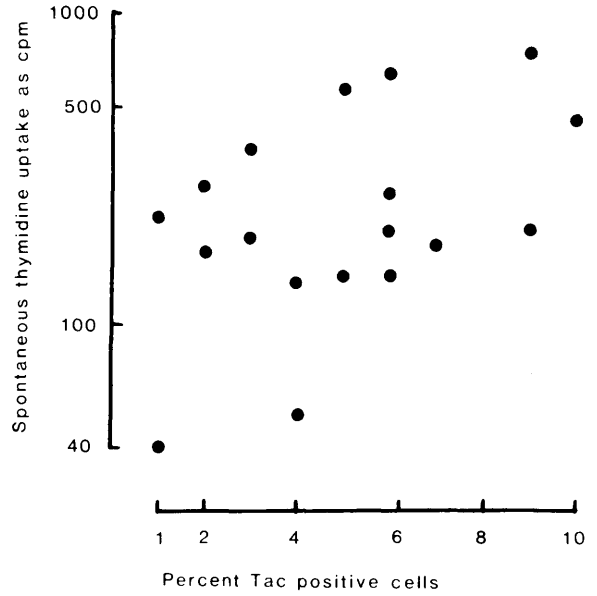

FIGURE 3. Thymidine uptake by IDDM patients' blood MNC during 24-h culture with T-cell growth factor. The counts are the mean of 12 replicate wells and are plotted against the percent of Tac⁺ cells in the patients' blood.

tures were pulsed with 0.25 μCi of tritiated thymidine and were harvested at 48 h.

RESULTS

Evidence for T-cell activation. T-cells were separated from the patients' blood by E-rosetting and stained with either OKIa for Ia antigens or anti-Tac. The percentage of cells stained with each antibody was positively correlated ($r = 0.72$, $P < 0.05$ for a two-tailed test, Figure 1). Ia-positive T-cells can only be demonstrated in cell preparations that are freed of monocytes and B-lymphocytes (which are normally Ia⁺) by a separation process such as E-rosetting. Normal monocytes and B-lymphocytes do not bind anti-Tac,[13] so a preliminary separation step is not necessary to identify Tac⁺ cells among blood mononuclear cells. For this reason, subsequent Tac estimations were performed on MNC preparations that had not been E-rosetted. Since B-cells and monocytes account for only 15–20% of blood MNC, this had a negligible effect on the percent of Tac⁺ cells.

The percentage of Tac⁺ cells in the patients' blood is plotted against time elapsed since diagnosis of IDDM in Figure 2. The highest values in the patients are seen in the first 2 yr following diagnosis. The mean value for the patients in this period is 6.2% (SE = 0.7). The mean value for IDDM patients of 3 or more years' duration was 2.25% (SE = 0.4). In the healthy adult and child controls, Tac⁺ cells were 2% (SE = 0.3). The percentage of Tac⁺ cells in the group of patients in the 2 yr following diagnosis is significantly greater ($P < 0.02$, Mann-Whitney U test) than in the healthy controls or in the patients of 3 or more years' duration.

TABLE 1
Phenotype of cells recovered from 6-day cultures with IL 2-containing medium

	N	Percent cells stained for OKT 4	OKT 8
IDDM <18 mo	15	21.8 ± 3	23 ± 3
IDDM >3 yr	6	15.5 ± 2	13.6 ± 2
Healthy controls	5	12 ± 3	11 ± 3

The Tac antigen is associated with expression of an IL 2 receptor by human lymphocytes,[5] so it was possible that the patients' Tac⁺ T-cells might proliferate in the presence of IL 2. We therefore cultured blood lymphocytes from the subsequent patients to be incubated for 24 h with 10% IL 2 supernatant, and measured thymidine uptake in 12 replicate wells over the entire culture period. Blood lymphocytes from healthy controls were run in parallel. The results (Figure 3) indicate a significant, positive correlation between the percent of Tac⁺ cells in the patients' blood and the cpm of thymidine uptake ($r = 0.47$, $P = 0.05$), though the scatter is wide. Thymidine uptake in control cultures of 8 healthy childrens' MNC was low (mean 105 cpm) and not correlated with the percent of Tac⁺ cells.

Phenotype of T-cells that proliferate with IL 2. Since IDDM patients' lymphocytes took up thymidine in the presence of IL 2 in short-term cultures, it seemed possible that T-cell blasts would be recovered from longer-term cultures. We therefore cultured the patients' lymphocytes for 6 days in medium with 10% IL 2-containing supernatants. Viable cells, including T-cell blasts, were subsequently harvested from 15 to 18 patients tested by centrifuging the cultures on a Percoll gradient. The blast-enriched preparations were phenotyped by immunofluorescence using monoclonal antibodies. The results (Table 1) indicate that the percentage of cells with T-cell antigens was consistently higher in the IDDM patients of less than 1-yr duration than in the controls. In neither the patients nor the controls were the blasts restricted to the T-4 and T-8 subset phenotype. There is a positive correlation between the sum of percentage of T-4 and T-8 blasts recovered after the 6 days of culture and the percent of Tac⁺ cells in the patients' blood at the time of drawing (Figure 4).

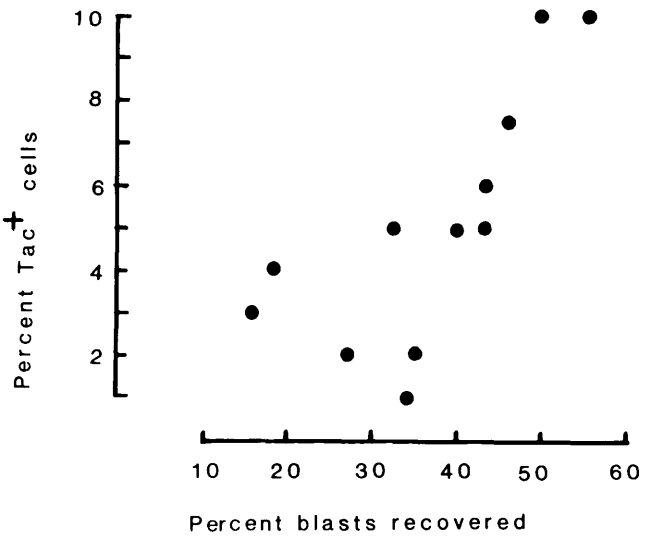

FIGURE 4. Percent of T-cell blasts (calculated as the sum of T-4⁺ and T-8⁺ cells) from Percoll gradient-fractionated cultures after 6-day culture with T-cell growth factor.

TABLE 2
Failure of soluble insulin to maintain proliferation of IDDM patients' T-cell blasts

	N	Antigen	Antigen concentration (IU or μg/ml) 0	0.5	5	15
Patients	3	SI	266 ± 52*	365 ± 63	162 ± 40	125 ± 72
Control	2	PPD	290 ± 40	626 ± 80	4940 ± 122	ND

*Thymidine uptake as cpm.
Patients' T-cell blasts were restimulated with soluble insulin (SI). The adult controls were sensitive to PPD (purified protein derivative).
ND = not determined.

Lack of specificity for insulin of T-cell blasts. A trivial explanation for the presence of activated T-cells in the blood of IDDM patients would be that they arose in response to foreign insulin injection. We consider this unlikely for two reasons. First, two of our patients with increased Tac$^+$ cells had not yet been started on insulin injections and each of three islet-cell antibody-positive siblings of other patients had increased Tac$^+$ cells (7%, 9%, and 11%) although they are not yet diabetic. Second, the T-cell blasts from 3 IDDM patients who had been treated with insulin for 4, 6, and 12 mo, respectively, did not continue to proliferate in cultures with added insulin (Table 2). Restimulation under these culture conditions is known to be both antigen- and HLA-restricted.[14] For comparison purposes, the level of counts obtained in positive responses by controls to PPD is also shown.

Relationship of anti-islet cell antibodies. Sera from patients whose IDDM was of 2-yr duration or less were tested for islet cell antibodies by immunofluorescence. No islet-cell staining was observed with any of the healthy control sera. The highest frequency of positivity for anti-islet-cell antibody (56%) was found in patients in the first year after diagnosis. Overall there was no correlation between the presence of islet-cell antibodies and raised Tac$^+$ cells (Table 3).

DISCUSSION

Our results suggest that children with IDDM have T-lymphocytes in their blood that are activated, as judged by the expression of the Tac antigen and by proliferation in IL 2-supplemented culture. This result is consistent with Jackson and co-workers' report[4] of T-cell activation in IDDM as judged by the presence of Ia antigen-positive cells in the patients' blood. The percentage of Tac$^+$ and Ia$^+$ T-cells correlated significantly in the patients we tested in parallel, although there is some scatter in the results. This scatter is to be expected, as some monocytes (which are Ia$^+$) contaminate T-cell preparations made by rosetting with neuraminidase-treated sheep erythrocytes. Furthermore, activated T-cells may express the Tac antigen before they become Ia$^+$.[15] Resting T-cells (as obtained from the blood of healthy adults) show little, if any, proliferation in cultures supplemented with IL 2 alone, without mitogen or antigen.[16] We were able to enrich cultures of IDDM patients' lymphocytes for activated T-cells by 6-day culture with mitogen-depleted IL 2-containing supernatant. When the patients' T-cell blasts were recovered from these cultures, they were found to belong to each of the commonly defined T-cell subsets, OKT 4$^+$ and OKT 8$^+$. This lack of subset specificity could be of considerable relevance to the pathogenesis of IDDM. OKT 4$^+$ cells include T-cells with helper function for B-lymphocytes[17] and they generally respond to antigen only in association with HLA-DR antigens.[18] The established association between HLA-DR3 and -DR4 and IDDM is, therefore, consistent with participation by the OKT 4$^+$ cells in abnormal immunoregulation in the patients. Abnormal immunoregulation in this context could include the provision of help for autoantibody production or help for the expansion of cytotoxic T-cell populations. The latter possibility may be the more likely, in that we found no correlation between Tac$^+$ cells and the presence of islet-cell cytoplasmic antibodies (although we did not test for islet-cell surface antibodies). OKT 8$^+$ cells include the cytotoxic subset of T-cells that is restricted by HLA-A or -B antigens.[19] The presence of OKT 8$^+$ cells among the blasts recovered from the IL 2-supplemented cultures of patients' lymphocytes might, therefore, implicate T-cell cytotoxicity in the pathogenesis of islet-cell destruction of IDDM. Although the phenotyping gives some indication of the likely HLA restriction of the activated T-cells, it gives no insight into their antigen specificity. Our own studies only excluded pork insulin as a trivial explanation for the activated T-cells in the patients. The view that the activated T-cells might have specificity for islet cell antigens is clearly tempting, but it is also possible that their presence in the circulation results from bystander activation in the pancreas during a period of T-cell attack. Further exploration of the specificity of the Tac$^+$ cells will probably have to await the development of islet-cell target antigens that are HLA matched with the responder or effector cells. Xenogeneic combinations have been used extensively with apparent demonstration of islet-cell-specific proliferative[20,21] and cytotoxic[22] responses. However, the lack of HLA restriction in this killing suggests that it is caused by non-T-cells, most likely natural killer cells that are known to be increased in IDDM.[23]

TABLE 3
Islet-cell antibodies and Tac$^+$ cells in IDDM

		Tac$^+$ cells (%) ≥5	<5
Islet-cell antibody	+	7*	3
	−	8	4

*Numbers are patients in each category.

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