Impairment of Lymphocyte-Suppressive System in Recent-Onset Insulin-Dependent Diabetes Mellitus

Correlation With Metabolic Control

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SUMMARY

Impairment of suppressor-cell activity may be important in the pathogenesis and maintenance of insulin-dependent diabetes mellitus (IDDM). In 23 recent-onset IDDM patients, lymphocyte sensitivity in vitro to theophylline was tested both in basal conditions and after improvement of metabolic control. This pharmacologic agent is mainly effective on a lymphocytic subpopulation with phenotypic and functional suppressive features. Peripheral blood lymphocytes from IDDM patients showed a loss of theophylline sensitivity, identified as inhibition of both E-rosette formation and blastogenic response to polyclonal mitogens concanavalin A (ConA) and phytohemagglutinin (PHA). An inverse relationship was demonstrated between the theophylline-induced suppression of ConA blastogenic response and blood glucose and glycosylated hemoglobin levels ($P < .01$). Metabolic control seemed to be important even in relation to lymphocyte subpopulation distribution. In IDDM patients we found a significant ($P < .05$) reduction of OKT4$^+$ lymphocytes that is correlated with blood glucose and glycosylated hemoglobin levels ($P < .01$). The improvement of metabolic control led to recovery of theophylline sensitivity. We suggest a deficiency in a suppressive system that could be involved in IDDM onset and the possible role of metabolic control in the impairment of some immunologic functions reported with this pathologic condition. DIABETES 1986; 35:1053–57.

The immune system plays a significant role in the pathogenesis of insulin-dependent diabetes mellitus (IDDM).[1] Many studies have reported an impairment in both humoral and cellular immunity. Immunologic abnormalities include *1*) the presence of various organ- and tissue-specific autoantibodies, especially against pancreatic β-cells;[2,3] *2*) circulating immunocomplexes;[4] *3*) cell-mediated immune response to pancreatic antigens;[5] *4*) depressed proliferative response to phytohemoagglutinin (PHA);[6] and *5*) reduced suppressor-cell activity.[7–10] The role of cell-mediated immunity in IDDM is further demonstrated by the presence of lymphocytic infiltration in the pancreas during an early stage of the disease.[11]

Recently, many studies have been performed on the phenotypic features of peripheral blood lymphocytes (PBLs) from IDDM patients. Although some authors reported a reduction of T lymphocytes as defined by the E-rosette test[12] or OKT_3 monoclonal antibody,[13–16] others found normal values[17,18] or a reduction only in poorly controlled patients.[19] Moreover, no agreement exists on a different distribution of lymphocyte subpopulations identified by monoclonal antibodies and often referred to as helper inducer (OKT4, Leu 3a) or suppressor cytotoxic (OKT8, Leu 7/11a). These discrepancies may be explained by methodologic considerations, patient selection, or degree of metabolic control.

Because a deficiency in different suppressor systems has been demonstrated in IDDM by several authors,[7–10,17] we assessed the E-rosette formation ability and mitogenic response after PBL treatment with theophylline. The preincubation of PBL for 120 min with theophylline can reversibly inhibit E-rosette formation. This treatment distinguishes between a theophylline-sensitive (T-TS) subpopulation that will not rerosette with sheep red blood cells (SRBC) and a theophylline-resistant (T-TR) subpopulation that will rerosette.[20] The T-TS subpopulation, which can reach elevated intracellular cAMP levels,[21] is endowed with suppressor function in many in vitro experimental models[22–24] and is enriched for cells with the suppressor-cytotoxic phenotype (OKT8$^+$, Fc γR$^+$).[25,26] Derangements of this population have been described in the elderly[27] and under several pathologic conditions.[28–30] Therefore, we investigated this lymphocyte subpopulation in recent-onset IDDM patients and its possible relations with lymphocyte subsets defined with monoclonal antibodies as well as with metabolic control.

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TABLE 1
Effects of in vitro treatment with theophylline (10 M, 120 min) on E-rosette formation and on blastogenic response to polyclonal mitogens in IDDM patients and in control subjects

		E rosette (% PBL)		ConA response (cpm × 10)		PHA response (cpm × 10)	
	N	Before	After	Before	After	Before	After
IDDM	23	59.5 ± 2.7	56.0 ± 2.5	15.6 ± 3.0	10.2 ± 2.3	38.6 ± 5.0	34.9 ± 5.2
Controls	23	69.7 ± 1.9	52.5 ± 3.0	42.1 ± 6.2	22.3 ± 4.5	54.7 ± 6.1	38.8 ± 6.6

Data are expressed as mean ± SE. Analysis of data by Student's *t* test. E rosette: IDDM vs. controls, $P < .01$; IDDM before vs. after, NS; controls before vs. after, $P < .001$. ConA response: IDDM vs. controls, $P < .01$; IDDM before vs. after, NS; controls before vs. after, $P < .001$. PHA response: IDDM vs. controls, NS; IDDM before vs. after, NS; controls before vs. after, $P < .005$. PBL, peripheral blood lymphocytes.

MATERIALS AND METHODS

Subjects. Twenty-three patients (14 men and 9 women) with newly diagnosed IDDM and 23 control subjects (13 men and 10 women) were studied. All patients gave their informed consent. The mean age of IDDM patients was 25.8 ± 6.1 yr (range 15–37 yr), and the mean age of control subjects was 27.1 ± 2.9 yr (range 23–33 yr). At the time of our study, all patients were within 2 mo of diagnosis of diabetes, based on detection of hyperglycemia or glycosuria. All patients had required insulin treatment from the time of diagnosis. The mean blood glucose at the start of the study was 177.94 ± 61.7 mg/dl (range 85–315), and the mean HbA_{1c} level was 8.68 ± 2.16% (range 4.7–13.4). Nine of these patients were reinvestigated after improvement of blood glucose control (1- to 12-mo interval).

Cell preparation. Peripheral blood lymphocytes were separated by flotation at 400 g on Lymphoprep (density 1.077 g/ml; Niegaard, Oslo, Norway), according to Böyum.[31]

E-rosette assay. This test was performed according to Aiuti et al.[32] The lymphocytes (cell concentration 10^6/0.2 ml) were mixed at 1/100 with SRBC (cell concentration 10^8/0.2 ml) and resuspended in Hanks' balanced salt solution (HBSS, Eurobio, Paris, France) containing 20% heat-inactivated, SRBC-adsorbed, fetal calf serum (FCS, Eurobio). After incubation for 10 min at 37°C, the mixture was centrifugated for 5 min at 200 × *g* and then stored at 4°C overnight. The pellet was gently resuspended with a Pasteur pipette and mounted on a glass slide. Each test was carried out twice.

Effect of theophylline on E-rosette–forming cells. Equal volumes of lymphocyte suspension and 2×10^{-3} M aminophylline (Recordati, Milan, Italy) in HBSS were mixed for theophylline treatment. The cells were incubated for 120 min at 37°C in 5% CO_2 atmosphere before the addition of SRBC. Before E-rosette assay the theophylline was removed by three washes in HBSS at 37°C for 10 min at 200 × *g*.

Analysis of surface antigens by monoclonal OK antibodies (Moabs). The reactivity of PBL with OKT3, OKT4, and OKT8 Moabs was determined by indirect immunofluorescent staining with fluorescein isothiocyanate–conjugated goat anti-mouse IgG: 50 μl of a cell suspension (5×10^5 cells) were incubated with 5 μl of monoclonal antisera (Orthoimmune Raritan, New York, NY) at 4°C for 30 min. After washing in cold culture medium, the cell pellets were resuspended in 50 μl of an optimal dilution of fluorescein-conjugated goat anti-mouse IgG 7S (Meloy, Springfield, VA) and incubated in the dark for 30 min at 4°C. After the second incubation, the suspension was washed twice in cold RPMI medium (Eurobio) resuspended in glycerol, and fluorescent-stained cells were counted under a fluorescent microscope (Orthoplan, Wetzlar, FRG).

PHA- and ConA–stimulated lymphocytes. Lymphocytes were cultured in microtiter tissue culture plates (Sterilin, Teddington, Middlesex, UK) at 37°C in 5% CO_2 air. The culture medium was RPMI-1640 (Eurobio) supplemented with 20% heat-inactivated FCS, 0.5% glutamine, and 1% penicillin streptomycin. Dilutions of PHA and ConA (Sigma, St. Louis, MO) were added to the wells in triplicate before incubation. The proliferative response was measured by pulsing cells for the final 18 h of culture with 2 μCi of methyl-[^{3}H]thymidine (Amersham, Buckinghamshire, UK). The samples were harvested on glass fiber filters by an automated multiple-sample harvester (Skatron, Flow Laboratories, Lierbyen, Norway), resuspended in a standard scintillation mixture (Instagel, Packard, Downers Grove, IL), and counted for radioactivity in a liquid scintillation spectrometer (Tricarb, Packard, Downers Grove, IL). The results are expressed in counts per minute.

TABLE 2
Lymphocyte membrane markers in IDDM patients and control subjects

	Lymphocytes			
	OKT3	OKT4	OKT8	OKT4/OKT8
IDDM patients (*N* = 23)				
%	66 ± 2.9	37.5 ± 3.4	26.4 ± 1.9	1.58 ± 0.12
Absolute values	1627 ± 52	938 ± 129	651 ± 68	
Controls				
%	71 ± 0.8	43.3 ± 0.8	29.5 ± 0.9	1.53 ± 0.06
Absolute values	2103 ± 135	1281 ± 85	877 ± 64	
P values	<.05	<.05	NS	NS

Results are expressed as means ± SE.

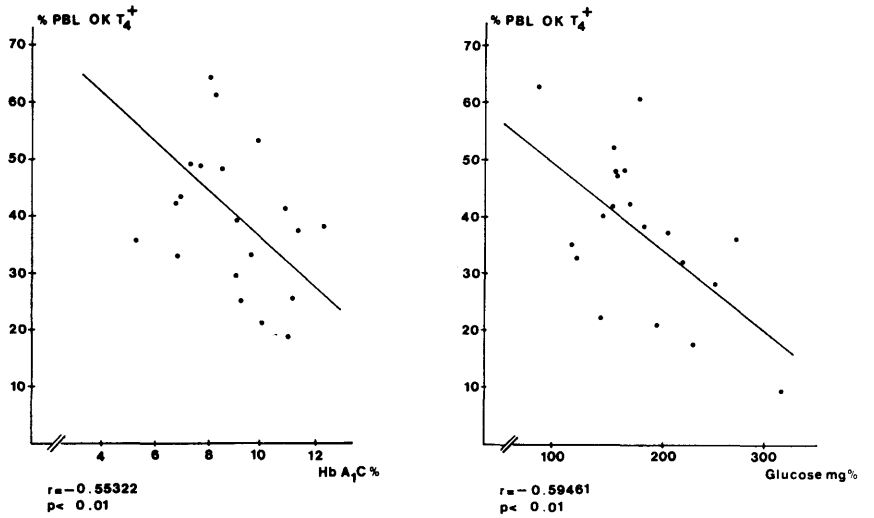

FIG. 1. Correlation between OKT4⁺ lymphocytes and metabolic control (blood glucose and HbA_{1c}) in 20 IDDM patients.

Blastogenic response of PBL after theophylline treatment. PBL of IDDM patients and healthy controls were preincubated for 120 min at 37°C with theophylline 2×10^{-3} M as for the E-rosette assay and used in the ConA and PHA systems as previously described. Suppression of thymidine incorporation was calculated by the formula:

$$\% \text{ suppression} = 1 - \frac{\text{cpm (PBL after theophylline treatment)}}{\text{cpm (PBL before theophylline treatment)}}$$

Statistical analysis. Statistical analysis was performed by the Student's *t* test and confirmed by the nonparametric Mann-Whitney *U* test.

RESULTS

Impairment of inhibitory effects induced by theophylline treatment at entry in the study. A significant reduction both in E-rosette formation ($P < .01$) and in ConA blastogenic response ($P < .01$) was found in IDDM patients compared with control subjects (Table 1). Reduction was also found in PHA stimulation but did not reach significance. Although theophylline treatment was able to significantly inhibit E-rosette formation ($P < .001$) and ConA ($P < .001$) and PHA ($P < .005$) blastogenic response in control subjects, this effect was completely abolished in IDDM patients.

Lymphocyte subpopulations. Data regarding T-cell subsets in IDDM patients are shown in Table 2. IDDM patients had a significant reduction in the proportion and absolute value of total T lymphocytes, identified by OKT3 Moab, when compared with the controls ($P < .05$). The percentage and absolute values of OKT4⁺ cells were also significantly lower in IDDM patients ($P < .05$), with a nonsignificant reduction in OKT8⁺ cells. The ratio between helper-inducer and suppressor-cytotoxic T cells (OKT4/OKT8) was not significantly different from normal controls.

Correlation between lymphocyte alterations and metabolic control. A statistically significant negative regression ($P < .01$) was found between OKT4⁺ lymphocytes and metabolic control, expressed by blood glucose values and HbA_{1c} glycosylated hemoglobin in IDDM patients (Fig. 1). An inverse relationship between the effect of theophylline treatment on PBL mitogenic response (expressed as suppression percentage) and glycemic control was also statistically significant for ConA ($P < .02$) (Fig. 2) but not for PHA-treated cells when analyzed with a linear regression test. E-rosette formation after drug treatment was not affected by metabolic control.

Regression of lymphocyte alteration after improvement of blood glucose control. Nine patients were reevaluated 1–12 mo after basal investigation (Table 3); at that time, metabolic control was improved in cases 1–7 and was worse in cases 8 and 9. In cases 1–6 a recovery of theophylline sensitivity was observed, whereas in cases 8 and 9 a reduction of theophylline sensitivity was found; overall, theophylline sensitivity correlated in 8 out of 9 cases with metabolic control. Furthermore, in case 1, four evaluations were performed during the first 12 mo; the improvement and the deterioration of glucose levels were repeatedly related to recovery and loss of theophylline sensitivity, respectively. A reduction and not an improvement of theophylline sensitivity occurred only in case 7 after reduction of glycemic levels. OKT4⁺-lymphocyte values did not show any correlation with the improvement of metabolic control in these patients.

DISCUSSION

This study shows the loss of in vitro PBL sensitivity to theophylline in IDDM patients, identified as inhibition both of E-rosette formation and blastogenic response to polyclonal mitogens. A significant reduction of IDDM lymphocyte E-rosette–forming ability is found compared with the controls ($P < .01$), the reduction being fully due to the loss of T-TS T cells; the T-TR subset (that maintains the ability of forming E rosettes) seems to be unaffected. Moreover, we observe a reduction of mitogen-induced blastogenic response and the loss of theophylline-inhibitory effects on cell proliferation in IDDM lymphocytes. These observations suggest that a deficiency in a suppressive subpopulation, such as the theophylline-sensitive subset, may exist. On the other hand, a defective suppressor-cell function has already been described in IDDM, although with different methodologic approaches and especially with generation of suppressor-cell activity after preincubation with ConA.[7,8,10,17]

Several studies on T-lymphocyte subsets in IDDM, iden-

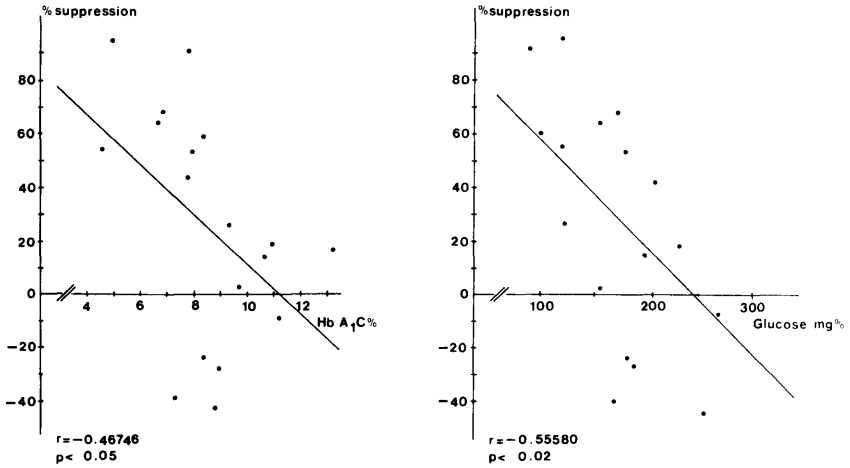

FIG. 2. Correlation between inhibitory effects of theophylline on ConA blastogenic response (% suppression) and metabolic control in 18 IDDM patients.

tified by monoclonal antibodies, have reported conflicting results.[13–18] These discrepancies could be explained by the use of different methods and different monoclonal antibodies, by the age of onset and time lapse from diagnosis, and by the patient's metabolic derangement.

We found a significant reduction of OKT3$^+$ cells ($P < .05$), in agreement with the low number of E-rosette–forming lymphocytes. The most important result, however, is a significant reduction in the OKT4$^+$ population ($P < .05$), which also appears significantly correlated with the poor metabolic control ($P < .01$).

Although the impairment of theophylline-induced suppressive activity agrees with an autoimmune pathogenesis of IDDM, how can the numerical T-lymphocyte deficit in IDDM and, in particular, the reduction of OKT4$^+$ cells be explained? Blood distribution of lymphocyte subpopulations can be quite different from that at the site of the immunologic challenge. This is the case, for instance, with active sarcoidosis,[33] with Hashimoto thyroiditis,[36] and, as in a recent report, with acute-onset IDDM, in which labeled T lymphocytes preferentially migrate to the endocrine pancreas.[35] It is therefore possible that lymphocytic traffic derangements are responsible for the low percentage of OKT4$^+$ cells in circulation.

On the other hand, it is important to assess whether our findings occur as a consequence of metabolic changes or because of a primary defect of immunologic reactivity. A significant relationship ($P < .05$) has been found in our study between metabolic control in the basal state, assessed as blood glucose and HbA_{1c} hemoglobin levels, and defective lymphocyte sensitivity to theophylline in the ConA system. An improvement or a deterioration of metabolic control was followed by recovery or by a loss of theophylline sensitivity in eight out of nine patients, which confirms the relevance of metabolic control to immunologic function. In this regard, the analysis of case 1 appears particulary meaningful (Table 3); in fact, a strict relationship between glycemic levels and theophylline sensitivity was observed in four different investigations in this patient. Therefore, it is possible that abnormalities of T-lymphocyte subpopulations can be related, at least in part, to metabolic control. Metabolic impairment may produce an in vivo alteration of some energy-providing pathway, crucial for lymphocyte functions, similar to the one obtainable in vitro in normal PBL after theophylline treatment. For this reason, theophylline would find target cells in a sort of functionally activated state and would not be able to exert the well-known inhibitory effects. It is still not clear which pathways are involved in this alteration, and further studies are necessary to better understand the role of metabolic impairment in IDDM. Nevertheless, poor metabolic control does not seem to be the only reason for the loss of theophylline sensitivity in IDDM lymphocytes, as it appears from the behavior of the E-rosette assay, which is not correlated with glycemic control.

In conclusion, these observations suggest that, given an underlying metabolic derangement, a deficiency in a subpopulation with suppressive features, as the theophylline T subset, may exist. This alteration might play an important role in the pathogenesis and maintenance of IDDM. Moreover, special attention should be directed toward carrying out phenotypic assessment on peripheral blood lymphocyte, because phenotypic features may be influenced by metabolic control and do not always reflect subpopulation distribution in target organs.

TABLE 3
Theophylline sensitivity in lymphocytes of recent-onset IDDM patients evaluated early after onset of diabetes and after metabolic improvement (or impairment)

Patient no.	Time (mo)*	Blood glucose (mg/dl)	% Suppression (ConA 16 μg/ml)
1	0	185	−26
	2	118	11
	8	220	−10
	12	117	77
2	0	254	−42
	5	110	49
3	0	271	−9
	2	106	66
4	0	178	−23
	6	94	38
5	0	198	14
	3	104	58
6	0	232	18
	2	96	38
7	0	179	52
	2	95	34
8	0	85	89
	2	163	30
9	0	120	54
	3	384	−1000

*0 = basal value.

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