Interleukin 2 and Soluble Interleukin 2–Receptor Secretion Defect In Vitro in Newly Diagnosed Type I Diabetic Patients

CARLA GIORDANO, FELICIA PANTÒ, CALOGERO CARUSO, MARIA A. MODICA, ANNA M. ZAMBITO, NUNZIA SAPIENZA, MARIA P. AMATO, AND ALDO GALLUZZO

In this study, we investigated whether an interleukin 2 (IL-2) secretion defect by peripheral blood mononuclear cells (PBMCs) after in vitro stimulation with phytohemagglutinin (PHA-M) occurs in either newly diagnosed or long-standing type I (insulin-dependent) diabetic patients and whether it is accompanied by a dysregulation of soluble IL-2–receptor (IL-2RS) production. PBMC cultures (2.5 × 10^6 cells), unstimulated or stimulated with PHA-M (25 μg/ml), from 20 type I diabetic patients (10 with time since onset <3 mo and 10 with long-term diabetes of <3 yr) and 10 control subjects were studied for the production of IL-2 and IL-2RS in their respective supernatants. No difference was found in IL-2 production in unstimulated cultures of type I patients compared with control subjects, although a significant decrease from PHA-M–stimulated cultures was seen (newly diagnosed, 1.7 ± 0.3 ng/2.5 × 10^6 cells; long-standing, 2.2 ± 0.3 ng/2.5 × 10^6 cells; $P < .001$ and $P < .05$, respectively) compared with control subjects (3.6 ± 0.4 ng/2.5 × 10^6 cells). In regard to the production of IL-2RS, no difference exists for unstimulated cultures, whereas, after PHA-M stimulation, both newly diagnosed and long-term–diabetic patients showed a decrease in the IL-2RS levels (318 ± 50 and 331 ± 62 U/2.5 × 10^6 cells; $P < .02$ and $P < .05$, respectively) compared with normal subjects (463 ± 34.2 U/2.5 × 10^6 cells). Thymus-activated cell phenotypes confirmed the T-lymphocyte activation after a 48-h culture period. The hypoproduction of IL-2 and IL-2RS in newly diagnosed patients may be the expression of the involvement of T-lymphocytes that have been activated continuously in vivo, but its presence in long-term patients suggests that the immunogenetic profile of the disease, involving immune-response genes also deputed to the control of lymphokine production levels, is such that type I diabetic patients are to be considered low IL-2 producers. *Diabetes* 38:310–15, 1989

Glucose 1 mM = 18 mg/dl

From the Laboratory of Immunology, Department of Clinica Medica, and Patologia Generale, University of Palermo, School of Medicine, Palermo, Italy.

Address correspondence and reprint requests to Dr. C. Giordano, Laboratory of Immunology, Department of Clinica Medica, University of Palermo, Piazza delle Cliniche 2, 90127 Palermo, Italy.

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In type I (insulin-dependent) diabetes the occurrence of various immunological disorders—i.e., production of islet cell autoantibodies (ICAs), qualitative and quantitative deficit of suppressor cell function, increase in activated T-lymphocytes (1,2)—is paradoxically characterized by a pronounced defect of T-lymphocytes to secrete interleukin 2 (IL-2) in response to mitogens (3–5). The mechanism of this abnormality, which occurs in some other autoimmune diseases, is unclear (6). Several investigators have proposed that an intrinsic defect in IL-2 production might lead to a dysfunction of suppressor T-lymphocytes in vivo and precede the manifestations of autoimmunity (6,7).

Furthermore, it is well known that the antigenic stimulation of T-lymphocytes results in both the production of IL-2 and expression of receptors for IL-2 (IL-2Rs) on T-lymphocytes (8). A response of T-lymphocytes to IL-2 leads to the clonal proliferation that starts the antigenic specificity of the immune response (9). Recently, a soluble form of IL-2R (IL-2RS), identified by 7G7B6 monoclonal antibody (MoAb) recognizing a distinct epitope on the human IL-2R, has been demonstrated in human serums and in in vitro culture-stimulated supernatants from human T-lymphocytes (10). The biological function of IL-2RS has not been fully clarified. However, it is accepted that IL-2RS can bind IL-2 efficiently (10,11) even if it does not inhibit IL-2–induced proliferation of T-lymphocytes or IL-2 binding to its receptor (12). We recently reported increased levels in circulating IL-2R in the serums of newly diagnosed type I diabetic patients (13). This finding on one hand seems to contradict the IL-2 defect, but on the other hand, the IL-2 defect could be explained by the fact that IL-2 hyposecretion in vitro results from an exhaustion of T-lymphocytes that have been continuously activated in vivo,

TABLE 1
Baseline characteristics in newly diagnosed and long-term–diabetic patients

	Type I diabetes	
	Newly diagnosed	Long term
n	10	10
M/F	7/3	4/6
Age (yr)	20.1 ± 1.9 (9–26)	25 ± 3.2 (10–28)
Time since diagnosis (wk)	4.6 ± 1.4 (2–10)	103 ± 12 (50–143)
HbA_{1c} (%)	8.2 ± 1 (6–12.3)	8.1 ± 0.6 (6–9.9)
Daily glycemia (mg/dl)	120 ± 8.2 (100–165)	154 ± 23 (100–230)

Values are means ± SE, with ranges in parentheses. There were no statistically significant differences between the 2 groups.

suggesting that the presence of circulating increased IL-2RS might downregulate the cellular response to IL-2.

This study addresses whether IL-2 hyposecretion is accompanied by a dysregulation of IL-2RS production by peripheral blood mononuclear cells (PBMCs) in vitro after stimulation with phytohemagglutinin (PHA-M) and whether they occur in either newly diagnosed or long-term type I diabetic patients.

MATERIALS AND METHODS

Subjects. Twenty type I diabetic patients (11 males and 9 females, mean age 22.2 ± 1.8 yr, range 10–28 yr) were studied. At the time of study, 10 were newly diagnosed with duration of symptoms <3 mo, and 10 were long-term diabetic with duration of disease >12 mo (range 24–48 mo). They were classified in accordance with the National Diabetes Data Group (14). All patients were treated from diagnosis with human insulin, usually a combination of short and intermediate acting (Actrapid and Monotard, Novo, Copenhagen), 2 or 3 times daily. In addition, all patients were on regular diet (180 g carbohydrates) and exercised regularly. No renal disease or signs of microangiopathy were detected on the basis of creatinine clearance or microalbuminuria and fundus oculi examination. Glycemic values, fasting and postprandial, were determined by strips (Glucostix, Ames Miles, Milan, Italy) and were read with reflectance method (Glucometer, Ames Miles) during the 24 h before the study. Glycosylated hemoglobin (HbA_{1c}) was determined by autoanalyzer (Daichi, Kagaku, Kyoto, Japan) on the day blood was drawn (normal range in our laboratory 5.5 ± 1.2%) (Table 1).

As a control group, 15 normal subjects (10 men and 5 women, mean age 24.9 ± 1.3 yr, range 18–29 yr) without family history of type I diabetes were studied.

Genetic profile. All the recruited patients were typed for HLA-A, -B, and -C loci with the conventional microtoxicity technique. HLA-DR antigens were detected on peripheral B-lymphocytes with the two-color immunofluorescence technique.

Humoral immunity studies. All patients were tested for insulin antibodies (^{125}I-Insuline Anticorps kit, bioMerieux, Lyon, France), ICAs, and complement-fixing-ICAs by immunofluorescence method, with type O blood group donor pancreases and other organ-specific autoantibodies, including thyroid, gastric parietal cell, and adrenal.

Cell cultures and stimulants. Cultures of adult PBMCs from type I diabetic and normal subjects, isolated by Ficoll-Hypaque centrifugation, containing 2.5 × 10^6 cells in 1 ml of RPMI-1640 medium supplemented with 10% fetal calf serum (Flow, Irvine, Scotland, UK), 100 U/ml penicillin, and 100 μg/ml streptomycin were established in individual wells of 24-well plates (Falcon, Oxnard, CA). Triplicate cultures were incubated for 48 h in a humidified atmosphere of 5% CO_2/95% air at 37°C. Cultures were either unstimulated (medium alone) or stimulated with PHA-M (25 μg/ml). Cultures were harvested by centrifugation at 550 × *g* for 7 min, and the supernatants were removed. The remaining cells were assayed for viability, enumerating them by trypan blue exclusion (dead cell percentages ≤5%), washed in phosphate-buffered saline (PBS), used for thymus-activated cell (TAC) phenotyping with anti-TAC MoAb (Techno Genetics, Turin, Italy), as previously described (15). Briefly, the cells (1 × 10^5/ml) were incubated with 10 μl of MoAb, washed and further incubated with 5 μl of fluorescein-conjugated goat anti-mouse IgG (Techno Genetics), and counted with the

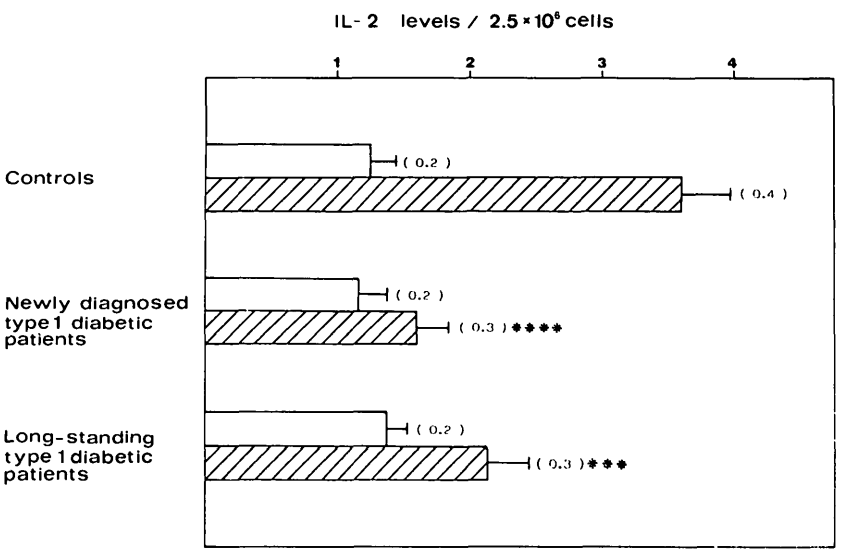

FIG. 1. Mean interleukin 2 (IL-2) levels from supernatants of unstimulated (*open bars*) and phytohemagglutinin-stimulated cultures (*hatched bars*) in 15 control subjects, 10 newly diagnosed, and 10 long-term type I diabetic patients. Numbers in parentheses are SE. ***$P < .01$, ****$P < .001$, vs. control subjects.

TABLE 2
Soluble interleukin 2–receptor levels in serum

	n	Serum soluble interleukin 2–receptor levels (U/ml)
Control subjects	15	265 ± 25.4
Type I diabetic patients		
Newly diagnosed	10	448.3 ± 31.5*
Long term	10	370 ± 149.5
All	20	412.7 ± 67†

Values are means ± SE.
*$P < .001$, †$P < .05$, vs. control subjects.

use of a Leitz microscope with incident UV and transmitted phase-contrast optics. At least 200 cells were counted on each specimen. Culture supernatants were stored at −70°C until assay for IL-2 and IL-2RS levels. The 25-μg/ml dose of PHA-M was used because previous experiments showed it yielded the optimal IL-2 production (5).

Measurement of IL-2 levels. IL-2 levels in culture supernatants were determined by radioimmunoassay method, as developed in our laboratory (5). The IL-2 reagent pack (Amersham, Little Chalfont, Buckinghamshire, UK) contained labeled IL-2 ([^{125}I]iodotyrosyl IL-2 Met^{0}, Ala^{125}) with 600 μCi/mmol sp act anti-human recombinant IL-2 antibody (rIL-2; produced in rabbits) with 90.3% cross-reactivity with natural human IL-2. Human rIL-2 (Amgen, Thousand Oaks, CA) was used to obtain a standard curve within the range 100–1000 pg/tube. Amerlex-M second antibody (Amersham) was used to separate bound from unbound human IL-2 antibody (16). The intra-assay coefficient of variation for reproducibility was 7.5–15.4% for three assays. Each supernatant sample was tested in triplicate. The values of IL-2 are given as nanograms per 2.5 × 10^6 cells.

Quantification of IL-2RS. The determination of IL-2R by enzyme-linked immunosorbent assay (ELISA) was performed in accordance with the method of Rubin et al. (11) and as previously described (13). Briefly, the method is based on a sensitive solid-phase ELISA with two MoAbs (anti-TAC and 7G7B6; 10) that recognize different epitopes of IL-2R (11). Wells of flat-bottomed 96-well microtiter plates were coated with 100 μl purified anti-TAC antibody overnight in PBS, pH 7.4. After washing, 50 μl of samples was added to antibody-coated wells in duplicate. Standard wells were prepared with human IL-2RS at concentrations of 0, 100, 400, 1600 U/ml, considering 1000 U as the amount of released IL-2R or IL-2RS present in 1 ml of a preparation of supernatant from PHA-M–stimulated PBMCs. After a 2-h incubation at 37°C, the plates were washed with Tween-PBS, and 100 μl horseradish-peroxidase–conjugated 7G7B6 antibody was put into each well. After an additional 2-h incubation, 100 μl of *o*-phenylenediamine resuspended in buffer containing 30% H_2O_2, pH 6.3, was incubated for 30 min at room temperature, and 2 N H_2SO_4 was used as stop solution. The average absorbance at 490 nm of each standard well and each patient's sample well was read with a Titertek ELISA reader (Flow, Rockville, MD). The values of IL-2RS concentrations were calculated by conversion to a numerical value of the absorbance of the test wells compared with the standard curve. The detection limit in our laboratory was 40 U/ml.

The intra-assay coefficient of variation ranged between 3.2 and 3.9%. Interassay precision was determined from the mean of the average of triplicate samples for five runs, and the coefficient of variation ranged between 10 and 12%.

Statistical analysis. All data are expressed as means ± SE; the values for the different groups were compared with Student's *t* test for paired and nonpaired values. The 95% tolerance limits in normal control subjects were calculated with the formula mean ± k1SD, where k1 value equals $t\sqrt{(n + 1)/n}$.

RESULTS

IL-2 production at 48 h by PBMCs of type I diabetic patients. In unstimulated cultures (medium alone) from normal subjects, detectable amounts of IL-2 were found in the supernatants, ranging between 0.6 and 1.8 ng/2.5 × 10^6 cells (1.25 ± 0.2, Fig. 1). In PHA-M–stimulated cultures the IL-2 level was 3.6 ± 0.4 ng/2.5 × 10^6 cells, range 2.4–5. With regard to newly diagnosed type I diabetic patients, no difference was found in IL-2 production in unstimulated cultures compared with normal subjects (1.2 ± 0.2 ng/2.5 × 10^6 cells), whereas there was a significant decrease in IL-2 production from PHA-M–stimulated cultures (1.7 ± 0.3 ng/2.5 × 10^6 cells; $P < .001$). The same phenomenon was

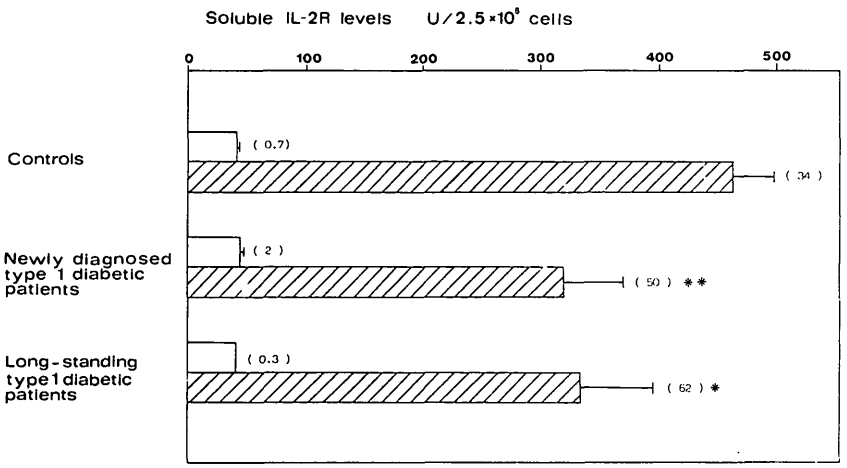

FIG. 2. Mean soluble interleukin 2–receptor (IL-2R) production from supernatants of unstimulated (*open bars*) and phytohemagglutinin-stimulated (25 μg/ml) cultures (*hatched bars*) in 15 control subjects, 10 newly diagnosed, and 10 long-term–diabetic patients. Numbers in parentheses are SE. *$P < .05$, **$P < .02$, vs. control subjects.

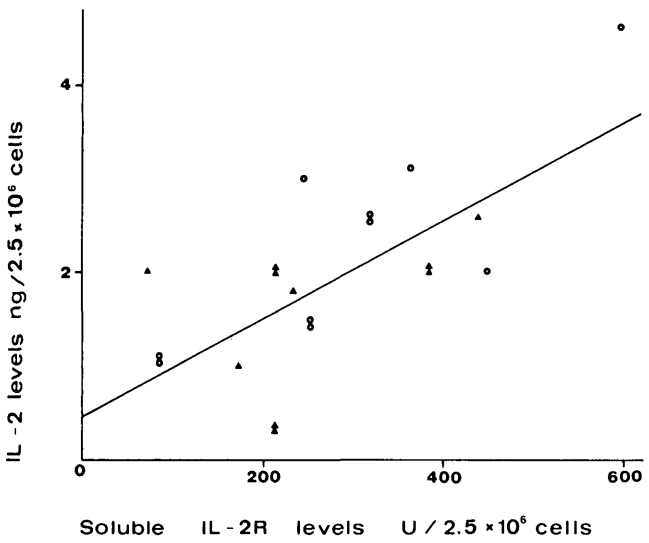

FIG. 3. Positive correlation between interleukin 2–receptor (IL-2R) and IL-2 levels produced in supernatants from type I diabetic cultures after 48-h phytohemagglutinin stimulation (25 μg/ml) in vitro (r = .701, P < .001). ▲, Newly diagnosed diabetic patients; ○, long-term–diabetic patients.

observed in long-term–diabetic patients; no IL-2 production difference was found in unstimulated cultures with respect to normal subjects (1.4 ± 0.2 ng/2.5 × 10^6 cells), but a significant deficit was detected after PHA-M stimulation (2.2 ± 0.3 ng/2.5 × 10^6 cells; P < .01).

Serum and supernatant IL-2RS generation. The mean serum levels of IL-2RS in newly diagnosed patients was confirmed to be significantly higher (448 ± 31.5 U/ml) than in control subjects (265 ± 25.4 U/ml; P < .001), whereas the long-term patients showed high mean values; however, theirs were not significantly different from those of the control subjects (370 ± 149.5 U/ml) (Table 2). Considering the 95% tolerance limits in the control subjects (upper limit 390 U/ml; lower limit 109 U/ml), 67% of newly diagnosed patients had higher levels with respect to the upper limit (390 U/ml), whereas this phenomenon was present in only 20% of the long-term patients.

In regard to the production of IL-2RS in the supernatants from unstimulated cultures (medium alone) in normal subjects, we observed very low production (40.7 ± 0.7 U/2.5 × 10^6 cells). After PHA-M stimulation, there was a mean IL-2RS production of 463 ± 34.2 U/2.5 × 10^6 cells, ranging between 315 and 770 U/2.5 × 10^6 cells. No correlation was found between IL-2RS levels and age, sex, and HLA-A, -B, -C, and -DR phenotypes in normal subjects (C.G., F.P., C.C., and A.G., unpublished observations).

Very low production of IL-2RS was similarly detected in the supernatants from 48-h unstimulated cultures from newly diagnosed and long-term type I diabetic patients (43.5 ± 2.1 and 40 ± 0.3 U/2.5 × 10^6 cells, respectively). Moreover, after a 48-h PHA stimulation, the IL-2RS amounts became detectable, but surprisingly, they decreased dramatically with respect to control subjects in both newly diagnosed (318 ± 50 U/2.5 × 10^6 cells, P < .02; range 75–530) and long-term (331 ± 62 U/2.5 × 10^6 cells, P < .05; range 85–600) patients (Fig. 2). No correlation was found between serum and supernatant IL-2RS production levels and metabolic control parameters, i.e., daily mean glycemia and HbA_{1c} levels; the variance analysis did not show any significant difference in IL-2RS levels in relation to HLA phenotypes, ICA positivity or negativity, or insulin antibody positivity. Comparison of IL-2 and IL-2RS levels showed a positive correlation (r = .701, P < .001; Fig. 3); i.e., when IL-2RS production from supernatants decreased, there was a simultaneous decrease in IL-2 levels. This phenomenon was not observed in normal subjects, because the production of IL-2RS and IL-2 were not interrelated (r = .176; NS).

TAC phenotyping. The percentages of TAC phenotypes in unstimulated cultures were significantly higher in newly diagnosed type I diabetic patients (22.1 ± 4.5%, P < .01) with respect to both long-term patients (8.5 ± 5.7) and control subjects (7.3 ± 1) (Fig. 4). After 48 h of PHA-M stimulation, TAC phenotypes of normal subjects showed 31.2 ± 8.4% positive cells, which was not significantly different from newly diagnosed patients (39.2 ± 9.2%), but the long-term patients were characterized by lesser TAC positivities (15.5 ± 3.4%, P < .05) in comparison with both newly diagnosed patients and control subjects. Note that the percentage increase in TAC phenotypes seemed to be particularly increased in newly diagnosed patients, but considering the high levels in unstimulated cultures, their increased percentages after PHA-M stimulation were less pronounced than those observed in control subjects.

DISCUSSION

These experiments show that after PHA-M stimulation, 48-h cultures from both newly diagnosed and long-term–diabetic patients are characterized by hyposecretion of IL-2 and IL-2RS compared with normal subjects. By contrast, the amounts of IL-2RS released in the serums of newly diagnosed patients are increased. The latter result confirms our earlier observations on increased IL-2RS in the serums of

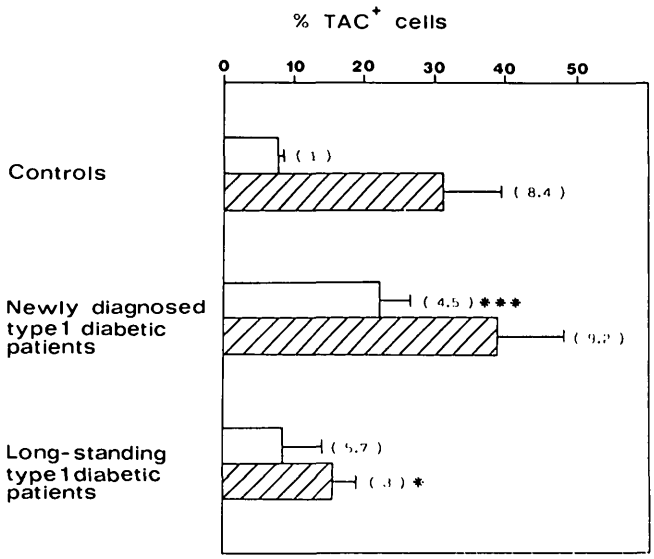

FIG. 4. Mean TAC⁺ cell percentages in 15 control subjects, 10 newly diagnosed, and 10 long-term–diabetic patients' cell cultures after 48 h. *Open bars*, cell phenotypes in unstimulated cultures (2.5 × 10^6 cells); *hatched bars*, cell phenotypes in phytohemagglutinin-stimulated (25 μg/ml) cultures. Numbers in parentheses are SE. *P < .05, ***P < .01, vs. control subjects.

newly diagnosed patients independently of metabolic control status or immunological parameters (13).

The mechanism for the IL-2R release is not clear, but there is evidence that the IL-2RS consists of a truncated form of the TAC antigen lacking the membrane-anchoring and intracellular domains (12,17–19). However, IL-2RS has been found to have epitopes reacting with anti-IL-2R and with IL-2 (11), but it seems to have low affinity for IL-2 (19). On this basis, research suggests that the release of IL-2R and IL-2RS may have immunoregulatory consequences (19,20). These observations emphasize the importance of our findings, i.e., that the capacity of circulating PBMCs from type I diabetic subjects to secrete IL-2 and release IL-2RS in the supernatants of cell cultures after stimulation with PHA-M is simultaneously reduced, although the serums of the newly diagnosed patients are characterized by elevated levels of IL-2RS. The positive correlation between IL-2RS and IL-2 levels produced in the supernatants from type I diabetic cultures after 48 h indicates that this phenomenon is a characteristic pattern of the disease, because normal subjects do not show any correlation. The absence of correlation between the two phenomena in normal subjects is in accordance with the knowledge that the expression of IL-2R precedes the synthesis and release of IL-2, the latter reaching the highest levels in vitro at 48 h (20–22).

The release of IL-2RS may only occur after these steps (20,22), which would explain the lack of correlation between IL-2 production and IL-2R release in normal subjects. Otherwise, the positive correlation found in type I diabetic patients between IL-2 and IL-2RS suggests that this pattern may be due to a low intrinsic capacity to produce IL-2 from PBMCs or to early release of IL-2RS at 48 h by preexistent activated cells in culture. In this connection, TAC^+ cells in our study confirm the activation process in type I diabetes. Confirming the literature data, TAC^+ cells are increased in the newly diagnosed cases, which clearly shows the immune-mechanism alterations in vivo involved in the destruction of β-cells in the pancreas (1). However, when the high levels of TAC cells in unstimulated cultures are considered, their increased percentage after PHA-M stimulation is less pronounced than in control subjects, so that there is no apparent difference between these two groups.

The combined data clearly establish that PBMCs from newly diagnosed type I diabetic patients in vivo are activated and have the capacity to express and release increased amounts of IL-2RS, but in vitro, with appropriate stimulation, have decreased capacity to produce and display such receptors and have defective IL-2 production.

The reasons for the apparently conflicting results, probably showing two phenomena, may be unraveled by kinetic experiments in vitro directed toward establishing whether, after a longer period of incubation, the cell cultures may increase their IL-2 and IL-2RS production. We have been performing this type of research, but our preliminary results in ICA^+ subjects have shown that the maximum peak of IL-2RS production is after 48–96 h of culture, confirming literature data in normal subjects (20–22).

The phenomenon of decreased production of IL-2 and IL-2RS in vitro is also partially present in long-term type I diabetic patients and may be of great interest. In this connection, the theory of a generic exhaustion phenomenon cannot be applied to long-term type I diabetic patients. There is no sign of activation, as indicated by the normal levels of TAC^+ cells in their unstimulated cultures and normal serum levels of IL-2RS, although we found IL-2 hyposecretion and low IL-2RS production in the supernatants from PHA-M–stimulated cultures.

When all of these data are considered, the IL-2 and IL-2RS defect in type I diabetes seem to be linked to the immunogenetic profile of the disease (23). In fact, lymphokine production by PBMCs in response to mitogen stimulation has been shown to vary with different MHC haplotypes of PBMC donors (24–26). In this light, type I diabetic patients may be considered genetically to be low producers of IL-2, although during the early phases of immune attack in the pancreas, their cells are able to be activated and to cleave IL-2RS from their surfaces. In conclusion, although the mechanism(s) of this hyporesponsiveness still remains to be elucidated, it may reflect a more general immune dysfunction in the disease.

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