Clin. exp. Immunol. (1991) **85**, 278–281

Abnormalities within CD4 and CD8 T lymphocyte subsets in type 1 (insulin-dependent) diabetes

J. ILONEN, H. M. SURCEL* & M.-L. KÄÄR† *Departments of Medical Microbiology and †Paediatrics, University of Oulu and *National Public Health Institute, Oulu, Finland*

(Accepted for publication 23 January 1991)

SUMMARY

Abnormalities in the proportions of various T lymphocyte subpopulations have been found in a number of autoimmune diseases. Monoclonal antibodies labelled with various fluorochromes were used here to define the percentages of subsets, and especially to divide $CD4^+$ (helper/inducer) and $CD8^+$ (suppressor/cytotoxic) cells into phenotypic subgroups. Blood samples were analysed from 25 patients (age $10{\cdot}1 \pm 3{\cdot}7$ years) with recently diagnosed insulin-dependent diabetes mellitus (IDDM) and 25 age- and sex-matched control subjects. The percentages of $CD4^+$ cells and $CD4^+CD45RA^+$ cells described as naive T helper cells or suppressor/inducers were increased in the IDDM patients ($P < 0{\cdot}05$ and $P < 0{\cdot}05$, Student's *t*-test, respectively), whereas the percentage of $CD4^+CD45RA^-$ cells (memory T-helper cells, helper/inducers) was similar in the patients and controls. The percentage of $CD8^+CD11b^+$ cells containing suppressor/effector lymphocytes was decreased in the IDDM patients as compared with the controls ($P < 0{\cdot}01$) but no significant difference was seen in total $CD8^+$ cells. The percentages of $CD3^+$ cells and the proportions of these simultaneously positive for HLA-DR antigen (activated T cells) were also increased in the recent IDDM patients ($P < 0{\cdot}001$ and $P < 0{\cdot}05$, respectively), while the proportion of $CD20^+$ B cells was decreased ($P < 0{\cdot}05$). The findings support the view that disturbed immune regulation occurs in IDDM and indicate that further division of T cell subpopulations may clarify our understanding of the disease process.

Keywords lymphocyte subsets type 1 diabetes

INTRODUCTION

Monoclonal antibodies can be used to differentiate between leucocyte cell surface molecules, forming a series of markers which correlate with cellular differentiation and the functional properties of the cells (Sprent, 1989). These cell membrane antigens, now called CD markers, have been used to divide lymphocytes into subsets which show characteristic fluctuations in certain immunological or infectious processes. The main dichotomy within T lymphocytes is that between cells positive for CD4 (helper/inducer) and CD8 (suppressor/cytotoxic) antigens. This functional correlation seems to exist even though CD4 and CD8 molecules primarily define whether the T cell receptor recognizes its nominal antigen in the context of either class II (CD4) or class I (CD8) MHC molecules (Swain, 1983).

The percentages of $CD4^+$ and $CD8^+$ lymphocytes and the ratio between them has been widely studied in type 1, insulin-dependent diabetes mellitus (IDDM), as in other autoimmune diseases. Controversial results have been published, as both increased and decreased CD4/CD8 ratios have been reported at diagnosis of the diseases (Gupta *et al.*, 1982; Horita *et al.*, 1982; Jackson *et al.*, 1982; Mascart-Lemone *et al.*, 1982; Buschard *et al.*, 1983; Pozzilli *et al.*, 1983; Galluzzo *et al.*, 1984; Ilonen *et al.*, 1984; Herold *et al.*, 1984; Rodier *et al.*, 1984; Quiniou-Debrie *et al.*, 1985; Hitchcock *et al.*, 1986; Pontesilli *et al.*, 1986; Hyöty, 1988). A more consistent finding is an increase in the number of activated T cells characterized by expression of the HLA-DR antigen or the IL-2 receptor on the cell surface (Jackson *et al.*, 1982; Mascart-Lemone *et al.*, 1982; Pozzilli *et al.*, 1983; Ilonen *et al.*, 1984; Rodier *et al.*, 1984; de Berardinis *et al.*, 1988).

New monoclonal antibodies and double labelling methods described recently can be used to divide still further the subsets so far defined. $CD4^+$ cells can be divided by the different molecular weight isoforms of the CD45R marker into those containing memory and/or helper/inducer cells (CD45RO) and those containing naive, virgin and/or suppressor/inducer cells (CD45RA) (Sanders *et al.*, 1988). The latter population has been found to have decreased in the blood of patients with chronic progessive multiple sclerosis, rheumatoid arthritis and systemic lupus erythematosus (Rose *et al.*, 1985; Chofflon *et al.*, 1988; Emery *et al.*, 1987; Morimoto *et al.*, 1987). Also $CD8^+$ cells can be divided by reference to the presence of CD11b marker. Suppressor/effector cells have been reported to be $CD11b^+$

Correspondence: Dr Jorma Ilonen, Department of Virology, University of Turku, Künamyllynkatu 13, SF-20520 Turku, Finland.

(Landay *et al.*, 1983), whereas cytotoxic T cells seem to be negative for this marker (Clement *et al.*, 1984).

Better definition of these overlapping populations could clarify earlier discrepancies in the studies on IDDM and help us to understand the immunopathological mechanisms involved in this disease. Here we set out to study the percentages of various lymphocyte subsets in 25 children and adolescent patients at the diagnosis of IDDM and to compare the values with those obtained in 25 age- and sex-matched control subjects. The subsets defined included $CD45RA^+$ and $CD45RA^-$ CD4 cells and CD8 subpopulations defined by CD11b marker, which separates populations containing suppressor ($CD11b^+$) and cytotoxic ($CD11b^-$) lymphocytes. In addition, the percentages of T (CD3) and B (CD20) lymphocytes and of activated T cells ($CD3^+HLA\text{-}DR^+$) and monocytes (CD14) were defined.

MATERIALS AND METHODS

Subjects

Twenty-five subjects with recently diagnosed IDDM (18 boys and seven girls, mean age (±s.d.) 10·1±3·7 years) were included in the series. All the patients were treated with insulin, and the samples were collected within a week of diagnostic admission to the hospital. The mean blood glucose concentration at presentation was 18·7±7·8 mmol/*l*, blood haemoglobin A_{1c} (HbA_{1c}) levels were 14·7±2·9% (reference range 5·5–8·4% in non-diabetic children). Twenty of the patients were ketotic at hospital admission. The age- and sex-matched control subjects (mean age (±s.d.) 10·5±4·2 years) were patients without any known infectious or immunological disease (mostly patients coming for minor elective surgery), the blood samples from whom were collected in association with routine laboratory tests.

Lymphocyte phenotyping

Peripheral blood mononuclear cells were separated with Lymphoprep (Nyegaard & Co, Oslo, Norway) gradient centrifugation, washed with phosphate-buffered saline (PBS) and diluted in RPMI 1640 medium supplemented with 5% fetal calf serum. FITC-conjugated anti-Leu 4 (CD3), anti-Leu 2a (CD8), anti-Leu 18 (CD45RA) and anti-Leu M3 (CD14), and also PE-conjugated anti-Leu 3a (CD4), anti-Leu 15 (CD11b) and anti-HLA-DR monoclonal antibodies were purchased from Becton Dickinson (Mountain View, CA). The cells in the supplemented RPMI 1640 medium were incubated for 30 min in an ice bath with an appropriate pair of FITC- and PE-conjugated antibodies and washed with PBS. The percentage of cells positive for green (FITC) and red (PE) fluorescence was calculated using flow cytometry (FACScan, Becton Dickinson).

RESULTS

Table 1 shows the percentages of different lymphocyte subpopulations defined by various combinations of fluorochrome-labelled monoclonal antibodies. Increased percentages of $CD3^+$ T lymphocytes and T cells with HLA-DR activation marker were detected in the blood of IDDM patients. $CD4^+$ T cells and $CD45RA^+$ CD4 cells described as naive or suppressor/inducer cells were also found increased but no significant difference was detected in the percentage of total CD8 cells. However, the subpopulation of CD8 cells with CD11b marker was decreased and the proportion of B cells was also lower in IDDM patients than controls. The lymphocyte markers were defined only in the cells which were shown by their light scatter parameters to be within the lymphocyte area. In addition the percentage of monocytes labelled by a CD14 (anti-Leu M3) antibody within the combined area of mononuclear cells was counted but no difference was seen between the IDDM patients (15·1±7·5) and controls (16·6±6·0).

Table 1. Percentages (means±s.d.) of lymphocyte subsets defined by various markers in recently diagnosed IDDM patients and control subjects

	IDDM	Control	
Cell subset	$n=25$	$n=25$	P (Student's t-test)
$CD3^+$	74·0±9·1	63·6±10·8	<0·001
$CD3^+HLA\text{-}DR^+$	3·5±3·9	1·7±1·1	<0·05
$CD4^+$	44·2±8·2	38·2±8·8	<0·05
$CD4^+CD45RA^+$	31·2±8·7	25·6±8·6	<0·05
$CD4^+CD45RA^-$	13·0±3·0	12·7±4·1	NS
$CD8^+$	26·3±9·5	27·7±6·5	NS
$CD8^+CD11b^+$	6·9±3·3	11·2±5·8	<0·01
$CD8^+CD11b^-$	19·4±8·8	16·4±6·6	NS
CD20	7·2±3·5	10·3±5·5	<0·05

Table 2. Ratios between the percentages of various lymphocyte subsets in IDDM patients and control subjects

	IDDM	Control	
Ratio	$n=25$	$n=25$	P (Student's t-test)
CD4/CD8	2·1±1·2	1·5±0·6	<0·05
$CD45RA^+/CD45RA^-$ (CD4)	2·6±1·2	2·3±1·3	NS
$CD11b^+/CD11b^-$ (CD8)	0·4±0·3	0·9±0·7	<0·01

The ratio between CD11b marker positive and negative CD8 cells was significantly lower in the IDDM patients than in the controls, but the ratio between $CD45R^+$ and $CD45R^-$ cells did not differ significantly. The CD4/CD8 ratio was significantly higher in the patients (Table 2).

When the lymphocyte subpopulations showing differences between patients and controls were correlated to parameters of metabolic state, only an inverse correlation between activated ($HLA\text{-}DR^+$) T cells and blood HbA_{1c} levels was seen ($-0{\cdot}429$, $P<0{\cdot}05$). No correlation was detected between other subset percentages and HbA_{1c}, blood glucose or ketone bodies at hospital admission.

DISCUSSION

The results show marked differences between IDDM patients and controls in the percentages of various lymphocyte subpopulations. The division of the CD4 and CD8 populations revealed that the increase in CD4 cells detected was concentrated in the $CD45RA^+$ 'naive' or suppressor/inducer cells and those of the

CD8 cells that decreased were those with a CD11b marker, containing suppressor/effector cells, whereas the total number of CD8 cells did not differ from the control values. The markers thus specify more exactly the subpopulations in which abnormalities can be detected and their use may thus eliminate much of the existing controversy.

One problem in the study of recent-onset IDDM patients is the possible effect of hyperglycaemia or ketosis on lymphocyte subsets. This is not, however, probable. When the indicators of metabolic state were correlated to deranged subset values, only a weak inverse correlation between HbA_{1c} level and activated T cells was noted. Also in our ongoing study on immune functions of non-insulin-dependent diabetes patients we have not seen any differences in the same lymphocyte subpopulations between patients in good metabolic balance and those showing poor balance (Syrjälä *et al.*, in preparation).

Several reports mention an increased CD4/CD8 ratio at the onset of IDDM, derived from a decrease in CD8 cells (Gupta *et al.*, 1982; Buschard *et al.*, 1983; Galluzzo *et al.*, 1984) and/or an increase in CD4 cells (Buschard *et al.*, 1983), although a normal or decreased ratio has also been reported (Horita *et al.*, 1982; Mascart-Lemone *et al.*, 1982; Ilonen *et al.*, 1984; Rodier *et al.*, 1984; Hyöty, 1988), as also in the time preceding overt disease (Al-Sakkaf *et al.*, 1989). One obvious reason for the discrepant results is the heterogeneity of the disease. In an earlier investigation we found a difference between patients with Dw3 and Dw4 as their genetic risk marker (Ilonen *et al.*, 1984). Preceding viral infections play a role as disease precipitating factors, and many of them are known to be associated with a reduced CD4/CD8 ratio (Carney *et al.*, 1981; De Waele *et al.*, 1981; Hyypiä *et al.*, 1984). Epstein–Barr virus (EBV) mononucleosis with an inverse CD4/CD8 ratio and activation of CD8 cells has been found in few patients at diagnosis of IDDM (Hyöty, 1988; Surcel *et al.*, 1988).

The division of CD4 helper/inducer cells into those positive and negative for the CD45RA marker has been recently studied in some autoimmune diseases (Rose *et al.*, 1985; Emery *et al.*, 1987; Morimoto *et al.*, 1987; Chofflon *et al.*, 1988; Sanders *et al.*, 1988), giving a typical finding of a decrease in $CD45RA^+$ cells, which has been especially clear in compartmentalized lymphocytes such as those found in the cerebrospinal and synovial fluid (Pitzalis *et al.*, 1987; Salonen *et al.*, 1989; Chofflon *et al.*, 1989). This is in accordance with the concept of chronic antigenic stimulation recruiting memory cells to the locus of the disease process. A correlation between a decreased percentage of suppressor/inducer cells and loss of functional suppression has also been reported (Chofflon *et al.*, 1988). The present finding of increased CD45RA cells in IDDM thus clearly differs from the case in autoimmune diseases described earlier and indicates that the pathogenetic mechanisms may be different. A similar finding of an increased percentage of $CD4^+CD45RA$ cells in IDDM has recently been described by Faustman *et al.* (1989), who detected it during the pre-diabetic phase and found normalization after the onset of the disease. A normal ratio between CD45RA and CD45RO cells was described also by de Berardinis *et al.* (1988). Controversial results were reported by Al-Kassab & Raziuddin (1990) who found a decrease of CD45RA cells in IDDM patients. This discrepancy may be related to the heterogeneity of the disease—patients in the study of Al-Kassab & Raziuddin were adults and not children as in our work. Faustman *et al.* (1989) suggested that the found abnormalities could either reflect stimulation of suppressor pathways or alternatively result from the blocking of differentiation of naive cells to memory cells. The loss of suppressor/effector cells characterized by the CD11b marker in the $CD8^+$ population supports the presence of deranged suppressor pathways in IDDM. More research, including a follow up of pre-diabetic subjects, is needed to show whether the differences observed between diabetic and control subjects can be used to predict the disease.

ACKNOWLEDGMENTS

This work was supported financially by a grant from the Foundation for Diabetes Research in Finland.

REFERENCES

Al-Kassab, A.S. & Raziuddin, S. (1990) Immune activation and T cell subset abnormalities in circulation of patients with recently diagnosed type I diabetes mellitus. *Clin. exp. Immunol.* **81,** 267.

Al-Sakkaf, L., Pozzilli, P., Tarn, A.C., Schwarz, G., Gale, E.A.M. & Bottazzo, G.F. (1989) Persistent reduction of CD4/CD8 lymphocyte ratio and cell activation before the onset of type 1 (insulin-dependent) diabetes. *Diabetologia,* **32,** 322.

Buschard, K., Röpke, C., Madsbad, S., Mehlsen, J. & Rygaard, J. (1983) T lymphocyte subsets in patients with newly diagnosed Type 1 (insulin-dependent) diabetes: a prospective study. *Diabetologia,* **25,** 247.

Carney, W.P., Rubin, R.H., Hoffman, R.A., Hansen, W.P., Healey, K. & Hirsch, M.S. (1981) Analysis of T lymphocyte subsets in cytomegalovirus mononucleosis. *J. Immunol.* **26,** 2114.

Chofflon, M., Weiner, H.L., Morimoto, C. & Hafler, D.A. (1988) Loss of functional suppression is linked to decreases in circulating suppressor inducer ($CD4^+2H4^+$) T cells in multiple sclerosis. *Ann. Neurol.* **24,** 192.

Chofflon, M., Weiner, H.L., Morimoto, C. & Hafler, D.A. (1989) Decrease of suppressor inducer ($CD4^+2H4^+$) T cells in multiple sclerosis cerebrospinal fluid. *Ann. Neurol.* **25,** 494.

Clement, L.T., Dagg, M.K. & Landay, A. (1984) Characterization of human lymphocyte subpopulations: alloreactive cytotoxic T-lymphocyte precursors and effector cells are phenotypically distinct from $Leu2^+$ suppressor cells. *J. clin. Immunol.* **4,** 395.

De Berardinis, P., Londei, M., Kahan, M., Balsano, F., Kontiainen, S., Gale, E.A.M., Bottazzo, G.F. & Feldmann, M. (1988) The majority of the activated T cells in the blood of insulin-dependent diabetes mellitus (IDDM) patients are $CD4^+$. *Clin. exp. Immunol.* **73,** 255.

De Waele, M., Thielemans, C., van Camp, B.K.G. (1981) Characterization of immunoregulatory T cells in EBV-induced infectious mononucleosis by monoclonal antibodies. *N. Engl. J. Med.* **304,** 460.

Emery, P., Gentry, K.C., Mackay, I.R., Muirden, K.D. & Rowley, M. (1987) Deficiency of the suppressor inducer subset of T lymphocytes in rheumatoid arthritis. *Arthritis Rheum.* **30,** 849.

Faustman, D., Eisenbarth, G., Daley, J. & Breitmeyer, J. (1989) Abnormal T-lymphocyte subsets in type 1 diabetes. *Diabetes,* **38,** 1462.

Galluzzo, A., Giordano, C., Rubino, G. & Bompiani, G.D. (1984) Immunoregulatory T-lymphocyte subset deficiency in newly diagnosed type 1 (insulin-dependent) diabetes mellitus. *Diabetologia,* **26,** 426.

Gupta, S., Fikrig, S.M., Khanna, S. & Orti, E. (1982) Deficiency of suppressor T-cells in insulin-dependent diabetes mellitus. An analysis with monoclonal antibodies. *Immunol. Lett.* **4,** 289.

Herold, K.C., Huen, A., Gould, L., Traisman, H. & Rubenstein, A.H. (1984) Alterations in lymphocyte subpopulations in type 1 (insulin-dependent) diabetes mellitus: Exploration of possible mech-

anisms and relationships to autoimmune phenomena. *Diabetologia*, **27,** 102.

HITCHCOCK, C.L., RILEY, W.J., ALAMO, A., PYKA, R. & MACLAREN, N.K. (1986) Lymphocyte subsets and activation in prediabetes. *Diabetes*, **35,** 1416.

HORITA, M., SUZUKI, H., ONODERA, T., GINSBERG-FELLNER, F., FAUCI, A.S. & NOTKINS, A.L. (1982) Abnormalities of immunoregulatory T cell subsets in patients with insulin-dependent diabetes mellitus. *J. Immunol.* **129,** 1426.

HYÖTY, H. (1988) Flow-cytometric analysis of lymphocyte subsets in relation to virus infections at the onset of type 1 (insulin-dependent) diabetes. *AMPIS*, **96,** 741.

HYYPIÄ, T., ESKOLA, J., LAINE, M. & MEURMAN, O. (1984) B-cell function in vitro during rubella infection. *Infect. Immun.* **43,** 589.

ILONEN, J., SURCEL, H.-M., MUSTONEN, A., KÄÄR, M.-L. & ÅKERBLOM, H.K. (1984) Lymphocyte subpopulations at the onset of Type 1 (insulin-dependent) diabetes. *Diabetologia*, **27,** 106.

JACKSON, R.A., MORRIS, M.A., HAYNES, B.F. & EISENBARTH, G.S. (1982) Increased circulating Ia-antigen-bearing T cells in type 1 diabetes mellitus. *N. Engl. J. Med.* **306,** 785.

LANDAY, A., GARTLAND, G.L. & CLEMENT, L.T. (1983) Characterization of a phenotypically distinct subpopulation of Leu-2^+ cells that suppresses T cell proliferative responses. *J. Immunol.* **131,** 2757.

MASCART-LEMONE, F., DELESPESSE, G., DORCHY, H., LEMIERE, B. & SERVAIS, G. (1982) Characterization of immunoregulatory T lymphocytes in insulin-dependent diabetic children by means of monoclonal antibodies. *Clin. exp. Immunol.* **47,** 296.

MORIMOTO, C., STEINBERG, A.D., LETVIN, N.L., HAGAN, M., TAKEUCHI, T., DALEY, J., LEVINE, H. & SCHLOSSMAN, S.F. (1987) A defect of immunoregulatory T cell subsets in systemic lupus erythematosus patients demonstrated with anti-2H4 antibody. *J. clin. Invest.* **79,** 762.

PITZALIS, C., KINGSLEY, G., MURPHY, J. & PANAYI, G. (1987) Abnormal distribution of the helper-inducer and suppressor-inducer T lymphocyte subsets in the rheumatoid joint. *Clin. Immunol. Immunopathol.* **45,** 252.

PONTESILLI, O., CHASE, H.P., CAROTENUTO, P., HERBERGER, M.J. & HAYWARD, A.R. (1986) T-lymphocyte subpopulations in insulin-dependent diabetes mellitus. *Clin. exp. Immunol.* **63,** 68.

POZZILLI, P., ZUCCARINI, O., IAVICOLI, M., ANDREANI, D., SENSI, M., SPENCER, K.M., BOTTAZZO, G.F., BEVERLEY, P.C.L., KYNER, J.L. & CUDWORTH, A.G. (1983) Monoclonal antibodies defined abnormalities of T-lymphocytes in type 1 (insulin-dependent) diabetes. *Diabetes*, **32,** 1.

QUINIOU-DEBRIE, M.C., DEBRAY-SACHS, M., DARDENNE, M., CZERNICHOW, P., ASSAN, R. & BACH, J.F. (1985) Anti-islet cellular and humoral immunity, T-cell subsets, and thymic function in type 1 diabetes. *Diabetes*, **34,** 373.

RODIER, M., ANDARY, M., RICHARD, J.L., MIROUZE, J. & CLOT, J. (1984) Peripheral blood T-cell subsets studied by monoclonal antibodies in Type 1 (insulin-dependent) diabetes: Effect of blood glucose control. *Diabetologia*, **27,** 136.

ROSE, L.M., GINSBERG, A.H., ROTHSTEIN, T.L., LEDBETTER, J.A. & CLARK, E.A. (1985) Selective loss of a subset of T helper cells in active multiple sclerosis. *Proc. natl Acad. Sci. USA*, **82,** 7389.

SALONEN, R., ILONEN, J., JÄGERROOS, H., SYRJÄLÄ, H., NURMI, T. & REUNANEN, M. (1989) Lymphocyte subsets in the cerebrospinal fluid in active multiple sclerosis. *Ann. Neurol.* **25,** 500.

SANDERS, M.E., MAKGOBA, M.W., SHAW, S. (1988) Human naive and memory T cells: reinterpretation of helper-inducer and suppressor-inducer subsets. *Immunol. Today*, **9,** 195.

SPRENT, J. (1989) T lymphocytes and the thymus. In *Fundamental Immunology* (ed. by W. E. Paul) p. 69. Raven Press, New York.

SURCEL, H.-M., ILONEN, J., KÄÄR, M.-L., HYÖTY, H., LEINIKKI, P. (1988) Infection by multiple viruses and major lymphocyte abnormalities at the diagnosis of diabetes in a two year old child. *Acta paediatr. Scand.* **77,** 471.

SWAIN, S.L. (1983) T cell subsets and the recognition of MHC class. *Immunol. Rev.* **74,** 129.