Diabetologia (1995) 38: 1449–1454

Diabetologia

Defective expression of the apoptosis-inducing CD95 (Fas/APO-1) molecule on T and B cells in IDDM

C. Giordano[1], R. De Maria[1], G. Stassi[1], M. Todaro[1], P. Richiusa[1], M. Giordano[1], R. Testi[2], A. Galluzzo[1]

[1] Laboratory of Immunology, Endocrinology Section, Institute of Clinical Medicine, Palermo, Italy
[2] Department of Experimental Medicine and Biochemical Sciences, University of Rome "Tor Vergata", Rome, Italy

Summary Triggering of CD95 (Fas/APO-1) cell surface receptors regulates the elimination of autoreactive T and B lymphocytes through a mechanism of cell suicide called apoptosis. Three different mutations involving CD95 or its ligand are responsible for induction of autoimmunity in susceptible mouse strains. To determine whether a defect involving the CD95 receptor is associated with human insulin-dependent diabetes mellitus (IDDM), we have studied the expression of CD95 on peripheral blood mononuclear cells from IDDM patients at different stages of the disease. Three-colour flow cytometry and mean fluorescence analysis showed that T and B lymphocytes from newly diagnosed IDDM and patients with long-standing disease, and subjects at high risk of developing the disease were highly defective in CD95 expression ($p < 0.001$), whereas monocytes from all the groups studied expressed normal amounts of CD95 molecules on their cell surface. T-cell subset analysis showed that the impairment of CD95 expression in IDDM patients and high-risk subjects involved both $CD3^+$ $CD4^+$ ($p < 0.001$) and $CD3^+$ $CD8^+$ cells (p range: < 0.01–0.001), suggesting that this alteration concerns both helper and cytotoxic T cells. Moreover, after activation in vitro with anti-CD3 monoclonal antibody, T cells from newly diagnosed IDDM patients maintained a reduced CD95 expression during the entire cell culture period (24–72 h) in comparison to the control population ($p < 0.001$). In conclusion, we found a reduced expression of the apoptosis-inducing CD95 receptor on T and B lymphocytes of individuals with clinical and preclinical IDDM. We hypothesize that this defective expression may impair the capacity of autoreactive lymphocytes to undergo CD95-mediated apoptosis, contributing to the lack of control on beta-cell specific B- and T-cell clones. [Diabetologia (1995) 38: 1449–1454]

Key words CD95, Fas/APO1, insulin-dependent diabetes mellitus, cellular immunity, apoptosis.

Insulin-dependent diabetes mellitus (IDDM) is an autoimmune disease characterized by a humoral and cell-mediated immunity against pancreatic beta-cell antigens [1–2]. The main mechanism responsible for the establishment and maintenance of self-tolerance concerns the deletion of autoreactive lymphocytes in the thymus and periphery. Physical elimination of self-reactive lymphocytes occurs through a process called apoptosis, a form of cell suicide associated with endogenous endonuclease activation and DNA fragmentation [3–4].

The CD95 (Fas/APO-1) antigen is a 45 kDa protein belonging to the tumour necrosis factor/nerve growth factor receptor family. Molecular cross linking of this protein induces apoptotic cell death through the activation of an acidic sphingomyelinase and ceramide generation [5–7]. Mouse Recessive Lymphoproliferation MRL mice homozygous for *lpr* or *gld* mutations suffer from a systemic lupus erythematosus-like autoimmune disease and die at

Received: 9 March 1995 and in revised form: 6 June 1995

Corresponding author: Dr. C. Giordano, Laboratorio di Immunologia, Istituto di Clinica Medica, Piazza delle Cliniche 2, I-90127 Palermo, Italy

Abbreviations: MFI, Mean fluorescence intensity; mAb, monoclonal antibody; ICA, islet cell autoantibody; IAA, insulin autoantibody; PE, phycoerythrin; FITC, fluorescein; Per-CP, peridinid chlorophyll protein; PBMC, peripheral blood mononuclear cells; AICD, activation-induced cell death.

around 5 months of age. These mice are characterized by the absence of Fas-induced apoptosis, as the Fas protein is very weakly expressed on resting and activated lymphocytes from MRL *lpr/lpr* mice [8–9], whereas the Fas ligand in MRL *gld/gld* mice carries a point mutation which severely affects its ability to induce apoptosis in cells expressing Fas [10]. Moreover, levels of the soluble isoform of FAS, which competes with the membrane-associated molecule for the ligand binding and inhibits Fas-mediated apoptosis, are elevated in patients with systemic lupus erythematosus, suggesting that the lack of a normal expression or function of this molecule may be critical for the occurrence of autoimmune diseases [11]. We thus analysed the expression of CD95 on lymphocytes from subjects at different phases of IDDM. We show here that CD95 expression is reduced on B and T cells from IDDM patients and that this defect seems to be present in all stages of the disease.

Subjects and methods

Subjects. Twenty-two IDDM patients, 10 women and 12 men, were studied. At the time of the study, 10 were newly diagnosed with a duration of symptoms less than 3 months ((mean ± SD) 16.2 ± 1.4 years), and 12 were long-term diabetic patients with a disease duration between 2 and 5 years (age 20.8 ± 1.9 years). Eight patients with non-insulin-dependent diabetes mellitus (NIDDM), four women and four men, with no apparent diabetic complications were also studied (age 45.6 ± 3.4 years). All patients were in good metabolic control as determined by fasting and postprandial daily blood glucose levels, (measured 24 h before the study) and by glycated haemoglobin (HbA_{1c}) evaluation. Patients were classified according to National Diabetes Data Group criteria [12].

Eight euglycaemic first degree relatives of IDDM patients (thus at high risk of developing IDDM), with islet cell antibody (ICA) positivity, (> 20 Juvenile Diabetes Foundation units), and insulin autoantibody (IAA) positivity greater than mean values + 5 SD with respect to the normal population (> 80 nU/ml), were also studied [13]. Humoral immunity tests were evaluated three times to confirm positivity. Twelve normal subjects (six women and six men) aged 19.1 ± 2.2 years, and eight (four women and four men) with a mean age of 42.1 ± 4.2 years, without a family history of diabetes, constituted the control groups. Five ICA-negative patients with Hashimoto's thyroiditis (four women and one man) treated with L-thyroxine and with normal daily blood glucose levels aged 41.7 ± 6.2 years, were also studied.

Immunofluorescence staining and flow cytometry analysis. Peripheral blood monuclear cells (PBMC) were isolated from heparinized blood by Ficoll-Hypaque density gradient centrifugation, washed twice in phosphate buffered saline containing 0.1 % sodium azide and immediately used for immunofluorescence staining. PBMC were first incubated at 4 °C for 30 min with optimal amounts of purified UB2 monoclonal antibody (mAb) (anti-CD95 IgG1; MBL International Corporation, Watertown, Mass., USA) washed and treated with phycoerithrin-(PE) labelled goat anti-mouse IgG antibodies. After a further washing, cells were incubated for 10 min with 6 % normal mouse serum, and treated at 10^7 cells/ml in a total volume of 100 µl with saturating concentrations of the following fluorescein-(FITC) or peridinid chlorophyll protein-(Per-CP) conjugated mAbs: Per-Cp Leu4 (anti-CD3, IgG1), FITC Leu3a (anti-CD4, IgG1), FITC Leu2a (anti-CD8, IgG1), FITC Leu12 (anti-CD19, IgG1), FITC LeuM3 (anti-CD14, IgG2b). Isotype matched normal, Per-CP, and FITC antibodies were used as negative controls. All FITC-, PE- or Per-CP-conjugated antibodies were from Becton Dickinson (San Jose, Calif., USA). Cells were then washed twice in cold phosphate buffered saline/azide and resuspended at 10^6 cells/ml for two or three colour cytofluorimetric analyses. Cells incubated with an irrelevant mAb of the same isotype and the conjugate served as a control. Relative fluorescent intensities of individual cells were analysed using a FACScan flow cytometer (Becton Dickinson). At least 10^4 cells were analysed. Correlated five parameter data were collected into list-mode data files by using FACScan research software and converted for analysis in Consort 30 software (Becton Dickinson). Dead cells and debris were excluded from analysis by forward angle and 90° light scatter. All measurements were made with the same instrument settings to standardize the different runs to enable identical fluorescence characteristics to be obtained on different days. Analyses of each group of patients and control subjects were conducted in parallel.

Lymphocyte culture and activation in vitro. PBMC were cultured (1×10^6 cells/ml) in RPMI 1640 medium supplemented with 10 % fetal calf serum, 2 mmol/l L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin in 24-well flat-bottomed plates (Falcon Labware, Becton Dickinson, Lincoln Park, N.J., USA). The cells were maintained for up to 3 days at 37 °C in a humidified atmosphere in 5 % CO_2. The following mAbs were used: anti-CD3ε mAb (OKT3, Ortho Pharmaceutical, Raritan, N.J., USA) or control isotype matched (5 µg/ml) mAb and goat anti-mouse IgG (5 µg/ml). At 0, 24, 48, 72 h cells were harvested and examined for CD95 expression, using FITC-conjugated UB2 (anti-CD95 IgG1; MBL International Corporation) and PE conjugated Leu1 (anti-CD5, panT, IgG_{2a}).

Statistical analysis

Data are presented as the mean ± SD. Significance was tested using the Student's *t*-test by analysis of variance to compare data from the patients with NIDDM and Hashimoto's thyroiditis and the age-matched control group, and the Student-Newman-Keuls test to perform group analysis. Critical values of q were read in tables in accordance with Harter's method [14].

Results

CD95 expression was initially evaluated on T ($CD3^+$), B ($CD19^+$) cells and monocytes ($CD14^+$) from IDDM patients and subjects at high risk of developing the disease. Since CD95 is present, although heterogeneously, on all PBMC, results were expressed as mean fluorescence intensity (MFI) ratio (the ratio between the MFI relative to the anti-CD95 staining and that due to the unspecific binding of isotype-matched control Ab). CD95 was very weakly expressed ($p < 0.001$) on $CD3^+$ cells from high-risk subjects

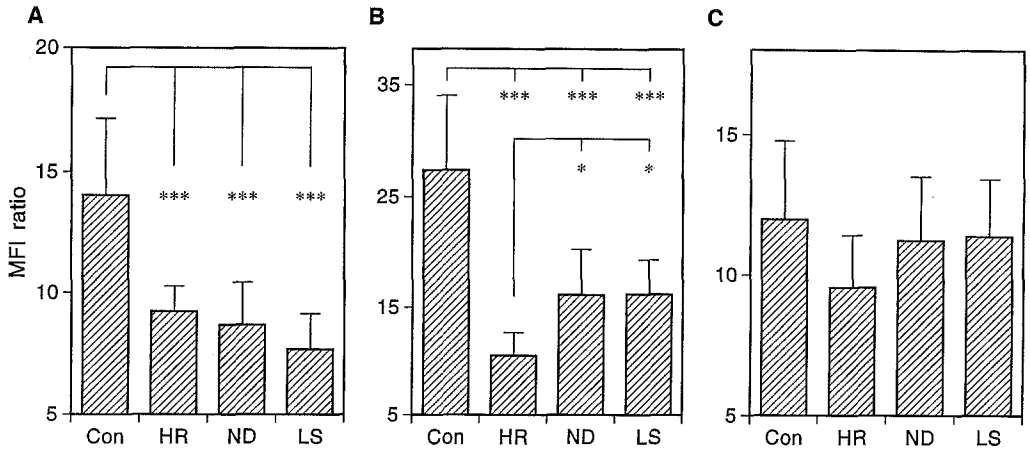

Fig. 1 (A–C). CD95 expression of T **(A)** and B **(B)** cells and monocytes **(C)**. Con, Control group; HR, high-risk IDDM group; ND, newly diagnosed IDDM group; LS, long-standing IDDM group. MFI using an irrelevant mAb of the same isotype (IgG1) on the different runs was calculated daily using the same instrument setting with a mean value of 3.8 ± 0.2 (range 3.6–4.01). Mean ± SD, *** = $p < 0.001$; * = $p < 0.05$

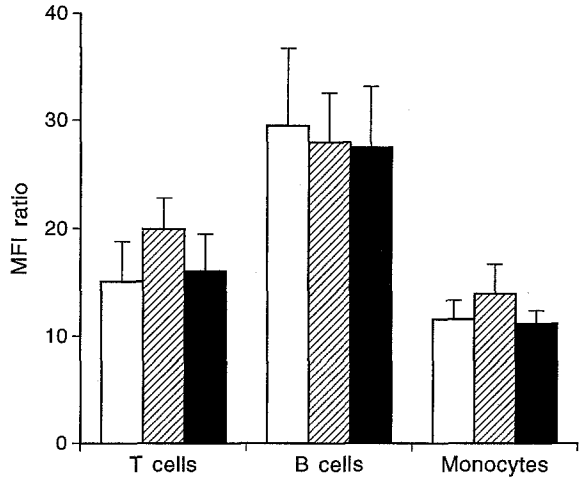

Fig. 2. CD95 expression on PBMC from control subjects (□), NIDDM patients (▨) and Hashimoto's thyroiditis patients (■). Mean ± SD

(MFI ratio 9.22 ± 1.07), newly diagnosed IDDM patients (8.71 ± 1.7) and patients with long-standing IDDM (7.68 ± 1.49), compared to the normal subjects (14.08 ± 3.13) (Fig. 1 A). Figure 1B shows that a markedly low CD95 expression ($p < 0.001$) was also present in $CD19^+$ cells from both newly diagnosed (16.02 ± 4.22) and IDDM patients of long-duration (16.15 ± 3.2) compared to the normal subjects (27.39 ± 6.57). Interestingly, the defect in CD95 expression was more pronounced in the high-risk subjects (10.54 ± 1.97), whose B cells were significantly defective not only compared to the normal subjects ($p < 0.001$) but also in comparison to the IDDM patients ($p < 0.05$). In contrast, no statistical differences in CD95 expression on $CD14^+$ cells were found between the four groups (Fig. 1C).

To exclude that diabetic metabolic derangement might be responsible for the low CD95 expression found in IDDM patients, we evaluated its expression on PBMC from NIDDM patients as compared to an age-matched control group. Moreover, to investigate whether the low CD95 expression found in IDDM patients is a general phenomenon associated with autoimmune status, we also evaluated CD95 levels on PBMC from patients with Hashimoto's thyroiditis with no apparent signs of insulitis, as demonstrated by their serum ICA negativity and normal blood glucose levels. No significant difference was found in any cell population examined (Fig. 2).

In addition, after in vitro stimulation with anti-CD3 mAb, we found that T cells from newly diagnosed IDDM patients showed a reduced CD95 expression compared to control subjects at the different cell culture times (Fig. 3).

Figure 4 shows one representative experiment from a series of five: the difference in CD95 expression in T cells from one IDDM patient with respect to one control subject at time 0 (Fig. 4A) and with anti-CD3 mAb stimulation (Fig. 4B) after a 48-h culture period is shown ($p < 0.001$).

We next analysed the distribution of CD95 on T-cell subsets from subjects with clinical and preclinical IDDM. As shown in Figure 5A, $CD3^+$ $CD4^+$ cells were highly defective in CD95 expression in both IDDM groups (newly diagnosed: 10.03 ± 2.46; long-disease duration: 11.53 ± 2.3) and in high-risk subjects (10.01 ± 1.83), as compared to the normal subjects (18.22 ± 3.83; $p < 0.001$). Defective CD95 expression was also present, although to a lesser extent, on $CD3^+$ $CD8^+$ cells (Fig. 5B). Control $CD3^+$ $CD8^+$ cells expressed an MFI ratio of 6.86 ± 1.55, whereas those from newly diagnosed and patients with long disease duration showed lower values (5.08 ± 1.31 and 4.26 ± 1.14, $p < 0.01$ and $p < 0.001$, respectively). This defect was also present in $CD3^+$ $CD8^+$ cells from the high-risk subjects (4.8 ± 1.28; $p < 0.001$).

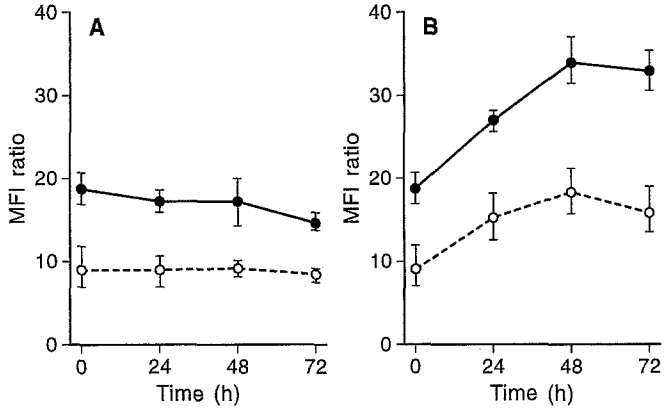

Fig. 3 (A, B). CD95 expression on PBMC from control subjects (——) and newly diagnosed IDDM patients (....) after activation in vitro with control isotype matched **(A)** or anti-CD3 **(B)** mAb after 0, 24, 48 and 72-h cell culture. (At the different times of observation IDDM patients vs control subjects $p < 0.001$). Mean ± SD

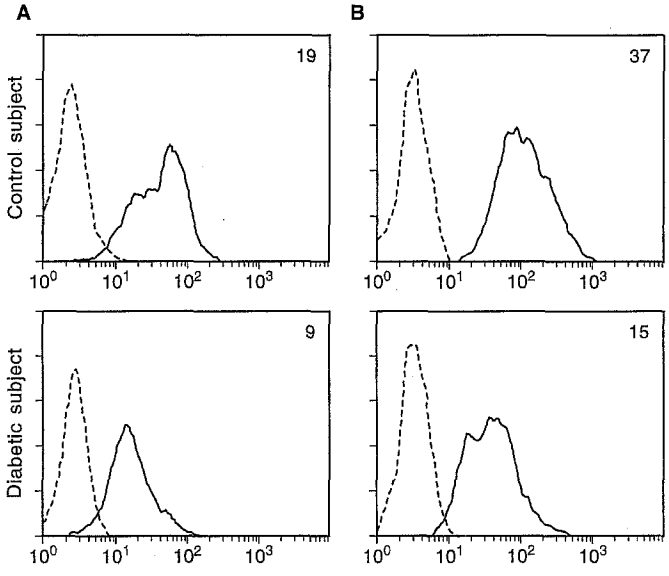

Fig. 4 (A, B). Expression of CD95 on control and IDDM PBMC at 0 h **(A)** and 48 h after CD3 stimulation **(B)**. Cells were stained with FITC- conjugated anti-CD95 (——) or control mAb (....). In each panel, the *x*-axis represents fluorescence intensity and the *y*-axis the relative cell number. One representative experiment from a series of five is shown. Numbers within the panels represent the MFI ratio (the ratio between the MFI relative to the anti-CD95 staining and that due to the unspecific binding of isotype-matched control mAb IgG1)

Discussion

We have shown the presence of a marked defect in the expression of CD95 by peripheral lymphocytes of IDDM, but not NIDDM patients. This defect is present in all phases of the disease, and involves both B and T lymphocytes.

The immune system generates an immense number of B- and T-cell receptor specificities in order to recognize and to induce a clonal response against an equivalent number of antigenic structures. However, potentially harmful autoreactive T and B lymphocytes, which must be inactivated or eliminated to protect from self-aggression, are also produced in this process [15–16].

Recent studies have suggested that peripheral clonal deletion is the major mechanism responsible for maintaining tolerance and preventing autoimmunity. The elimination of clones which have undergone the initial expansion phase is due to an apoptotic process called activation-induced cell death (AICD), which correlates with CD95 expression on activated lymphocytes [17–18]. CD95-induced apoptosis seems to be essential to preserve peripheral and not thymic tolerance, as clonal deletion is normal in mice carrying the Fas mutation and suffering from autoimmunity [18]. Hence, it appears that a decreased expression of CD95 may affect the capacity to maintain the normal levels of peripheral tolerance, essential for protection from autoimmune diseases [19]. We found that T and B lymphocytes from individuals suffering from, or at high risk of developing IDDM show a marked reduction in the number of CD95 molecules on their cell surface, suggesting that an ineffective AICD may contribute to the pathogenesis of the disease. Functional in vitro studies based on the cross-linking of the CD95 protein with mAb anti-human Fas/Apo-1 (IgM) confirm the coexistence of defective AICD in newly diagnosed IDDM patients (C. Giordano, G. Stassi, M. Todaro, unpublished data).

Defective CD95 expression appears to be restricted to IDDM, as we found that PBMC from patients with Hashimoto's thyroiditis express normal CD95 levels. Moreover, CD95 expression of human PBMC obtained from patients suffering from systemic lupus erythematosus, rheumatoid arthritis and primary Sjögren's syndrome has been shown to be higher than those of normal controls subjects [20].

A non-deletional form of T- and B-cell inactivation may also play a role in maintaining self-tolerance [18]. Indeed, the induction of peripheral anergy, mostly dependent on antigen doses, has been frequently claimed as an important mechanism to control cells potentially able to react against self-antigens [21]. However, peripheral lymphocytes from individuals with IDDM, or high risk of developing the disease, respond weakly to a variety of proliferating stimuli [22–23]. The presence of this hyporesponsive status makes it unlikely that failure of peripheral anergy contributes to the pathogenesis of IDDM.

Defective Fas/Apo-1 expression is responsible for the failure of AICD in *lpr/lpr* mice [18]. Transgenic MRL *lpr/lpr* mice in which the Fas cDNA is under the control of the T-cell specific CD2 promoter did not suffer from autoimmunity but, as the expression of the Fas molecule was not corrected in B lymphocytes, they had significantly higher levels of autoanti-

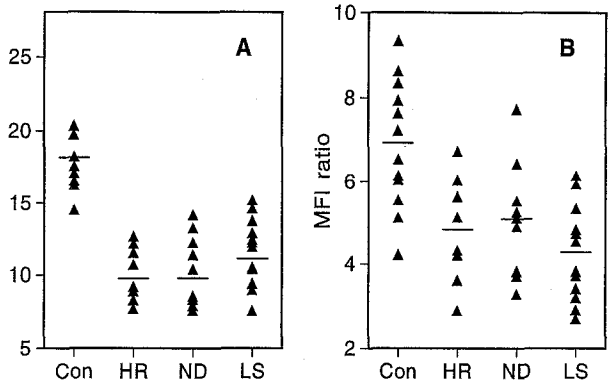

Fig. 5 (A, B). CD95 expression on $CD3^+$ $CD4^+$ cells **(A)** and $CD3^+$ $CD8^+$ cells **(B)**. Con, Control group; HR, high-risk IDDM group; ND, newly diagnosed IDDM group; LS, long-standing IDDM group. **(A)** Con vs HR, ND and LS $p < 0.001$. **(B)** Con vs HR, ND and LS p range $< 0.01 - < 0.001$

bodies compared to the normal MRL strain without the *lpr* mutation [24]. It therefore appears that normal levels of CD95 on B lymphocytes are essential for successful control of autoantibody production. This is consistent with the observation of even lower CD95 expression on B lymphocytes in subjects at high risk of IDDM whom we selected on the basis of high levels of IAA and ICA, and who have a reduced CD95 expression on B lymphocytes, compared to IDDM patients. Importantly, the defect is not modified after anti-CD3 mAb in vitro stimulation of PBMC from newly diagnosed IDDM patients during the entire observation period.

$CD4^+$ cells play a major role in initiating the autoimmune response against pancreatic beta cells [25, 26]. We found an extremely low CD95 expression on $CD4^+$ cells from IDDM patients, suggesting that CD95 evaluation of peripheral lymphocyte subsets may represent a useful immunological parameter for the screening and the prediction of IDDM.

In conclusion, we have demonstrated a strong correlation between the presence or the high risk of developing IDDM and an impaired expression of the apoptosis-inducing CD95 molecule on T and B cells. This finding suggests that loss of tolerance in IDDM may be partly due to a defective expression of the apoptosis-inducing CD95 receptor.

Acknowledgements. This work has been partially supported by CNR P.F. "Invecchiamento" – grant No 93.00386.PF40, to A. Galluzzo; and by 40% MURST 1993 Project "Fisiopatologia del diabete, pancreas endocrino e correlazioni endocrino-metaboliche" – Comitato 06, to A. Galluzzo.

References

1. Castano L, Eisenbarth GS (1990) Type I diabetes: a chronic autoimmune disease of human, mouse, and rat. Ann Rev Immunol 8: 647–679
2. Birk OS, Cohen IR (1993) T-cell autoimmunity in type 1 diabetes mellitus. Curr Op Immunol 5: 903–909
3. Krammer PH, Behrmann I, Daniel P, Dhein J, Debatin KM (1994) Regulation of apoptosis in the immune system. Curr Op Immunol 6: 279–289
4. Williams GT, Smith CA, McCarthy NJ, Grimes EA (1992) Apoptosis: final control point in cell biology. Trends Cell Biol 2: 263–267
5. Itoh N, Yonehara S, Ishii A et al. (1991) The polypeptide encoded by the cDNA for human cell surface antigen FAS can mediate apoptosis. Cell 66: 233–243
6. Oehm A, Behrmann I, Folk W et al. (1992) Purification and molecular cloning of the APO-1 cell surface antigen, a member of the tumor necrosis factor/nerve growth factor receptor superfamily. Sequence identity with the Fas antigen. J Biol Chem 267: 10709–10715
7. Cifone MG, De Maria R, Roncalioli P et al. (1994) Apoptotic signaling through CD95 (Fas/Apo-1) activates an acidic sphingomyelinase. J Exp Med 180: 1547–1552
8. Watanabe-Fukunaga R, Brannan CI, Copeland NG, Jenkins NA, Nagata S (1992) Lymphoproliferation disorder in mice explained by defects in Fas antigen that mediates apoptosis. Nature 356: 314–317
9. Drappa J, Brot N, Elkon KB (1993) The Fas protein is expressed at high levels on $CD4^+$ $CD8^+$ thymocytes and activated mature lymphocytes in normal mice but not in the lupus-prone strain, MRL *lpr/lpr*. Proc Natl Acad Sci USA 90: 10340–10344
10. Takahashi T, Tanaka M, Brannan CI et al. (1994) Generalized lymphoproliferative disease in mice, caused by a point mutation in the Fas ligand. Cell 76: 969–976
11. Cheng J, Zhou T, Liu C et al. (1994) Protection from Fas-mediated apoptosis by a soluble form of the Fas molecule. Science 263: 1759–1762
12. National Diabetes Data Group (1979) Classification and diagnosis of diabetes mellitus and other categories of glucose intolerance. Diabetes 28: 1039–1057
13. Ad Hoc Expert Committee (1990) Position statement. Prevention of type I diabetes mellitus. Diabetes Care 13: 1026–1027
14. Harter HL (1970) Tests based on range and studentized range of samples from a normal population. In: Order statistics and their use in testing and estimation. U.S. Government Printing Office, Washington, D.C.
15. Jones LA, Chin LT, Longo DL, Kruisbeek AM (1990) Peripheral clonal elimination of functional T cells. Science 250: 1726–1729
16. Hartley SB, Cooke MP, Fulcher DA et al. (1993) Elimination of self-reactive B lymphocytes proceedes in two stages: arrested development and cell death. Cell 72: 325–335
17. Arnold B, Schonrich G, Hammerling GJ (1992) Extrathymic T-cell selection. Curr Op Immunol 4: 166–170
18. Green DR, Scott DW (1994) Activation-induced apoptosis in lymphocytes. Curr Op Immunol 6: 476–487
19. Fowlkes BJ, Ramsdell F (1993) T-cell tolerance. Curr Opin Immunol 5: 873–879
20. Mysler E, Bini P, Drappa J, Ramos P, Friedman M, Krammer PH, Elkon KB (1994) The apoptosis-1/Fas protein in human systemic lupus erythematosus. J Clin Invest 93: 1029–1034
21. Michinson NA (1992) Specialization, tolerance, memory, competition, latency, and strife among T-cells. Ann Rev Immunol 10: 1–12
22. Giordano C, De Maria R, Todaro M et al. (1993) Study of T-cell activation in type I diabetic patients and pre-type I

diabetic subjects by cytometric analysis: antigen expression defect in vitro. J Clin Immunol 13: 68–78
23. De Maria R, Todaro M, Stassi G, Di Blasi F, Giordano M, Galluzzo A, Giordano C (1994) Defective T cell receptor/CD3 complex signaling in human type I diabetes. Eur J Immunol 24: 999–1002
24. Wu J, Zhou T, Zhang J, He J, Gause WC, Mountz JD (1994) Correction of accelerated autoimmune disease by early replacement of the mutated *lpr* gene with the normal Fas apoptosis gene in the T cells of transgenic MRL-*lpr/lpr* mice. Proc Natl Acad Sci USA 91: 2344–2348
25. Signore A, Pozzilli P, Gale EAM, Andreani D, Beverly PCL (1989) The natural history of lymphocyte subsets infiltrating the pancreas of NOD mice. Diabetologia 32: 282–289
26. Bedossa P, Bendelac A, Bach JF, Carnaud C (1989) Syngenic T cell transfer of diabetes into NOD newborn mice: in situ studies of the autoimmune steps leading to insulin-producing cell destruction. Eur J Immunol 19: 1947–1951