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Rapid communications

Persistent reduction of CD4/CD8 lymphocyte ratio and cell activation before the onset of Type 1 (insulin-dependent) diabetes

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Summary. Over a period of 5 years, lymphocyte subpopulations and their markers of activation were studied prospectively in 56 first degree relatives of Type 1 (insulin-dependent) diabetic probands. Lymphocytes were phenotyped using a panel of monoclonal antibodies which recognise CD3, CD4, CD8 lymphocytes, K/NK cells, HLA Class II products and IL-2 receptors (IL-2r). Twenty-six subjects were negative for islet cell antibody (ICA), 18 had complement fixing ICA (CF-ICA) and 12 only conventional ICA (ICA-IgG). The total number of observations (blood samples collected) was 386. Overall, changes in T cell data were observed in the three groups of first degree relatives compared to 70 normal subjects without a family history of diabetes. Six individuals developed Type 1 diabetes in the course of the study. They all possessed CF-ICA and five out of six showed a persistent reduction (< 1.5) of the CD4/CD8 lymphocyte ratio before the clinical onset of the disease. Activated lymphocytes were found on two occasions in two of these subjects. We conclude that imbalance of lymphocyte immunoregulatory subsets is present before the onset of Type 1 diabetes in susceptible individuals; the persistence of a reduced CD4/CD8 lymphocyte ratio may reflect the ongoing process leading to B-cell destruction.

Key words: Lymphocyte subsets, pre-Type 1 (insulin-dependent) diabetes, lymphocyte activations, genetic susceptibility.

Studies of cell-mediated immunity in patients with Type 1 (insulin-dependent) diabetes at the time of clinical diagnosis have shown significant abnormalities of lymphocyte subpopulations including a reduced percentage of CD4 (helper/inducer) and CD8 (cytotoxic/suppressor) lymphocytes [1]. Lymphocytes possessing Class II molecules and receptors for interleukin-2 (IL-2r) have been detected in peripheral blood at the time of diagnosis [2–3] and it is likely that these markers of activation reflect at peripheral level the autoimmune cell-mediated process known to take place in the pancreas against B cells.

There is now increasing evidence suggesting that the onset of Type 1 diabetes represents the final phase of a pathological event which began many years before. Specific immune disregulation occurs in this pre-clinical asymptomatic period and the presence of islet cell antibodies (ICA) [4] and of circulating activated T cells [5] has been reported in individuals genetically susceptible to the disease.

We report here the results of a five-year longitudinal investigation of lymphocyte subsets in a group of first degree relatives in our Bart's-Windsor Family Study, six of whom subsequently developed Type 1 diabetes.

Patients and methods

Family members: In 1983 a prospective study of cell-mediated immune parameters was started with the aim of evaluating peripheral lymphocyte subpopulations in unaffected members of families with at least one diabetic child. Fifty-six first degree relatives were regularly followed-up and blood taken at least twice a year for a period of 5 years. The total number of samples collected over this period was 386 and an equal number of tests (approximately 6 per subject) were performed over the period of follow-up.

The characteristics of relatives studied are listed in Table 1. Of these, six individuals developed Type 1 diabetes in the course of follow-up.

Methods

Lymphocyte phenotyping

This was performed using a panel of internationally recognised monoclonal antibodies defining CD3 (UCHT1), CD4 (Leu 3a) and CD8 (UCHT4) lymphocytes subsets, CD7 (H25), total Ia+ cells (DA2) and CD25 (IL-2r-TAC) (all kindly donated by Dr. Peter Beverley, University College and Middlesex School of Medicine, London, UK). Cells were obtained from peripheral blood following Ficoll Hypaque (Pharmacia, Milton Keynes, UK) gradient centrifugation and stored in liquid nitrogen until required. Aliquots of 10^5 cells were in-

Table 1. Characteristics of first degree relatives at the time of entry into the prospective study

Groups	No. of individuals	Sex F/M	Mean Age (years ± SD)
CF-ICA+	18	10/ 8	27 ± 11
ICA IgG+	12	4/ 8	32 ± 12
ICA-ve	26	12/14	43 ± 16

Table 2. Percentage of total T, T lymphocyte subsets, K/NK cells and activated T cells in the three groups of 1st degree relatives

	First degree relatives			
	ICA IgG+ subjects $n=12$	CF-ICA $n=18$	ICA negative $n=26$	Normal subjects $n=70$
CD3	66.3 ±8.7	59.6 ±7.9	59.9 ±8.7	65.2±2.7
CD4	36.5 ±5.9	38.1 ±6.5	35.8 ±7.1	46.6±2.0
CD8	23.2 ±4.4	22.7 ±4.4	23.7 ±5.8	23.2±1.8
CD4/CD8 ratio	1.66±0.3	1.68±0.2	1.60±0.3	2.2±0.3
CD7	12.9 ±5.7	12.5 ±4.6	11.2 ±6.3	13.2±3.1
Ia+	6.2 ±2.9	6.1 ±3.0	7.6 ±4.3	7.1±2.4
CD25 (IL-2r)	2.5 ±1.8[a]	1.2 ±1.3	1.9 ±1.9	1.1±0.8

Mean values ± SD of the total number of determinations performed over the period of follow-up in 1st degree relatives. Statistical analysis within groups of 1st degree relatives:
[a] $p<0.03$ vs CF-ICA+ but not significant vs ICA negative individuals. All other values do not differ significantly (Mann Whitney U Test).
1st degree relatives compared to normal subjects:
- CD3+lymphocytes significantly reduced in CF-ICA ($p<0.02$) and ICA negative subjects ($p<0.05$);
- CD4+lymphocytes significantly reduced in all three groups (ICA IgG+ $p<0.01$; CF-ICA $p<0.02$; ICA neg. $p<0.01$);
- CD8+lymphocytes not different in the three groups;
- CD4/CD8 lymphocyte ratio significantly reduced in all three groups ($p<0.01$);
- CD7+lymphocytes not different in the three groups;
- Ia cells not different in the three groups;
- CD25+cells significantly raised only in ICA IgG+ subjects ($p<0.01$)

cubated with 50 µl (working dilution) of the relevant antibody for 30 min at 4° C, washed twice and incubated for 45 min at 4° C with 50 µl of a rabbit anti-mouse fluorescinated antiserum (Gibco, Paisley, Scotland). Cells were washed three times in phosphate buffered saline (PBS) and 5 µl of each labelled sample were dispensed in a well of a Hamax plate (Medicell International, London, UK). Fluorescent positive cells (at least 300) were counted on an inverted UV microscope. Intra-assay variation under these conditions is 1.5%.

Other tests

All subjects were typed for HLA A, B, C antigens by standard microlymphocytotoxicity test and DR typed by soluble cell immunofluorescent tests; conventional ICA (ICA IgG) and the complement fixing subgroup (CF-ICA) were detected following the standard protocols distributed at the First International Workshop on the Standardisation of the ICA test. These specificities were assayed both at the time when the samples were collected for lymphocyte phenotyping and retested and titred during the subsequent stages of the ICA Standardisation Programme. In our laboratory, the coefficient of variation at 10 JDF units of the standard serum is 12%.

Statistical analysis

Statistical analysis was made using the Mann Whitney U test.

Results

Overall analysis

Significant changes were observed with regard to the percentage of total T lymphocyte subpopulations in the three groups of 1st degree relatives compared to a group of 70 normal subjects without a family history of diabetes [6] (Table 2). The most relevant change was the decrease of CD4+ cells (helper phenotype). As CD8+ cells were not different, the CD4/CD8 lymphocyte ratio was then significantly decreased in 1st degree relatives. When the presence of ICA was taken into account and correlated with abnormally low values of CD4/CD8 lymphocyte ratio (<1.5), 69% of CF-ICA, 46% of ICA-IgG and 38% of ICA negative first degree relatives showed on at least three occasions this abnormal parameter.

Total Ia+ lymphocytes (>15%) and IL-2+ cells (>5%) [5] were observed on one occasion in the majority of subjects independently of the presence or absence of ICA; IL-2r+ cells were detected on two occasions in five females and one male (3 CF-ICA, 2 ICA-IgG, 1 ICA negative) of whom one was HLA identical and five shared one HLA haplotype in common with the proband; total Ia^+ lymphocytes were also found on two occasions in five females and three males of whom two were HLA identical and six HLA haploidentical to the proband (2 CF-ICA, 1 ICA-IgG, 5 ICA negative). Only in two subjects (one female and one male) of whom one was CF-ICA, and the other ICA-IgG were the two markers of T cell activation present at the same time. Overall, conventional ICA+ individuals showed higher values of IL2r+ cells compared to subjects possessing CF-ICA ($p<0.03$) (Table 2).

Finally, we did not observe any correlation between HLA phenotype and T-cell data in the three groups of 1st degree relatives.

Lymphocyte abnormalities in subjects who subsequently developed diabetes

Six individuals (five males and one female, mean age 27+9 years) developed Type 1 diabetes in the course of the study. Five subjects had one HLA haplotype in common with the diabetic proband, one was HLA identical. The pattern of the CD4/CD8 ratio observed in these six individuals is illustrated in Figure 1. It is worth noting that the CD4/CD8 lymphocyte ratio was persistently low in five out of six subjects before the onset of the disease. No other subjects showed such constant abnormality during the period of follow-up.

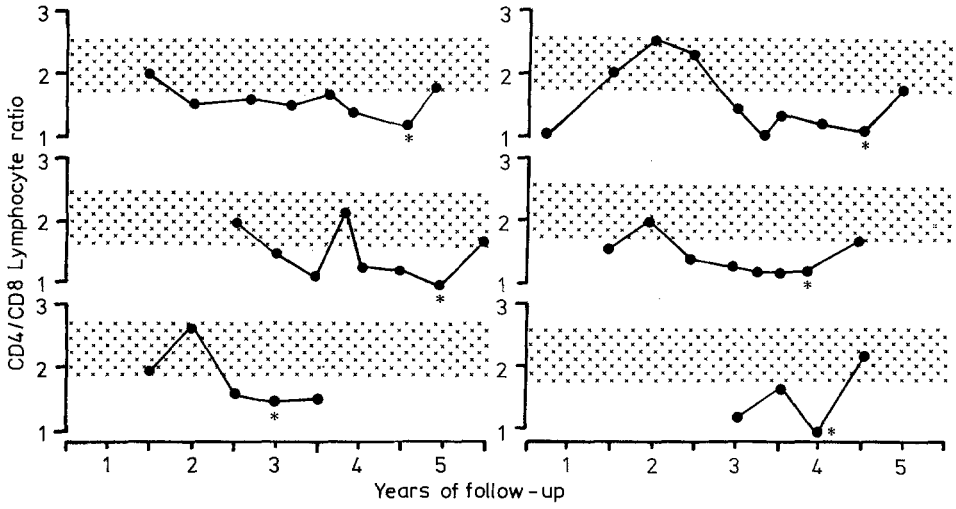

Fig. 1. The CD4/CD8 lymphocyte ratio in the course of follow-up of six subjects who developed Type 1 (insulin-dependent) diabetes (* = time of first insulin injection). Shaded area represents the normal range for CD4/CD8 lymphocyte ratio

Lymphocyte activation (high levels of Ia+ and/or IL-2r+ cells) was found on two occasions in two of these subjects. No other cell-mediated immune abnormalities were detected. All six subjects were repeatedly CF-ICA positive before the onset of clinical symptoms.

Discussion

Evidence is presented here indicating that an imbalance of immunoregulatory lymphocyte subsets is present before the onset of Type 1 diabetes in subjects genetically susceptible to the disease. There appears to be a relationship between a reduced CD4/CD8 lymphocyte ratio (< 1.5) and the presence of ICA, in particular CF-ICA, thus suggesting that an abnormal cell mediated immune response may contribute to the final mechanisms leading to B-cell destruction. It is well recognised that a reduced CD4/CD8 lymphocyte ratio (in normal subjects values are between 1.5 and 2.5) is indicative of impairment of the cell mediated immune response and subjects with such an imbalance have been shown to be more susceptible to viral infections [7]. First degree relatives have all shown on some occasions, a CD4/CD8 lymphocyte ratio below 1.5, thus confirming that immune disturbance may be independent of diabetes risk. However, the persistence of the decreased lymphocyte ratio was typical only of those subjects who later became diabetic.

A long pre-diabetic phase is now a well recognised feature of Type 1 diabetes; it might be that this is due to a genetically determined lack of effective immune recognition of certain environmental stimuli leading to chronic viral infections which may hit B cells in the pancreas. A persistent reduction of the CD4/CD8 lymphocyte ratio might favour the spread of unrecognised silent infections. Indirect evidence to support this hypothesis comes from data obtained in patients with Type 1 diabetes following vaccination with hepatitis B virus. Surprisingly, and despite good metabolic control, 31% of patients did not show a significant rise of antibody titres to the virus and 19% had only a low titre [8]. These patients also showed a reduced CD4/CD8 lymphocyte ratio.

If this is the case, how do we explain the production of ICA when helper T lymphocyte number and possible function seems to be reduced in these individuals? One possibility is that a subset within the T helper population, known to stimulate B lymphocyte proliferation [9], may be responsible for the activation of autoreactive B lymphocytes (cells which are known to be less tolerant against self-antigens) to produce ICA.

It is not unexpected that only two out of six subjects who developed diabetes showed persistent lymphocyte activation in the peripheral blood. First of all, activated lymphocytes are mainly present in the area of the damaging process and they account for the majority of infiltrating lymphocytes in insulitis. Peripheral levels may rarely reflect the overspill. In this context it is of relevance that the lowest levels of circulating IL-2r+ cells have been found in CF-ICA subjects. As previously reported [5, 10] high circulating levels of IL-2r+ cells may occur only in certain phases of the final killing process. Furthermore, it is interesting that lymphocytes with receptors for IL-2 have been observed very rarely during the pre-diabetic follow-up period and, if more than once, only in subjects with ICA in particular those with conventional ICA. In accordance with Hitchcock et al. [10] the low percentage of lymphocytes with IL-2 receptors in CF-ICA+ pre-diabetic subjects could reflect an ongoing chronic rather than acute type of autoimmune process towards B cells.

In conclusion, these results extend the observation that cell-mediated immune parameters may be found impaired in peripheral blood before the onset of Type 1 diabetes in susceptible individuals [5]. New impetus should now be given to investigate the mechanisms of "no return" in subjects genetically at risk but with persistant immune abnormalities. Functional studies of lymphocyte subsets in these individuals are bound to

spread light to this important aspect of the pre-diabetic period.

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Announcements

NIDDM - Epidemiology, Complications and Treatment

This will be the theme of the Postgraduate Course to be held at the Inter-University Center in Dubrovnik, Yugoslavia, 12–15 September 1989. The Postgraduate Course is organized by the Universities of Zagreb, Toronto and Stockholm, under the auspices of the WHO. *For further information please contact:* Professor M. Granić, The Vuk Vrhovac Institute for Diabetes, Endocrinology and Metabolic Diseases, Dugi dol 4a, YU-41000 Zagreb, Yugoslavia. Telephone: (41) 231-471; Telex: 22353 INDIAB YU; Telefax: (41) 231-515.

2nd International Symposium on Molecular Genetics of Diabetes Mellitus

The symposium will take place in Greifswald, GDR, from April 22–26, 1990. *The topics are:* The hormone genes, their structure and control of expression; polymorphisms related to diabetes mellitus; regulation of expression of transferred genes in mammalian cells. *For further information please contact:* Prof. Dr. H. Zühlke, Department of Biochemistry, University of Greifswald, DDR-2200 Greifswald, GDR.

2nd International Exhibition of Audiovisual Aids in Diabetes Information

This exhibit will be held in Ferrara, Italy, in April 1990. The exhibition will be organized by the Unit for Diabetes and Metabolic Diseases of USL (Local Health Authority) No. 31 of Ferrara and Clinica Medica B of Turin University. The exhibition will be divided into two sections, one on lantern slides and the other on films and videos. All audiovisual material completed between January 1, 1985 and December 31, 1989 will be considered for presentation at the meeting. *For further information, please contact:* Dr. Franco Tomasi, Servizio di Diabetologia, Arcispedale S. Anna, corso Giovecca 103, I-44100 Ferrara, Italy.

Joint International Meetings, Italy - Israel, 1990

Immunobiology of Normal and Diabetic Pregnancy

This meeting will be held 15–18 March 1990 in Erice, Sicily, Italy. *Deadline for abstracts:* 30 November 1989. *For further information please contact:* C.I.S.D., Via Baglivi 12, I-00161 Roma, Italy. Telephone: +(6) 8687 36.

10th International Workshop on Immunology of Diabetes

This workshop will be held 19–21 March 1990 on the Dead Sea shore in Israel. *Deadline for abstracts:* 30 November 1989. *For further Information please contact:* Kenes Congress Organizers, P.O. Box 50006, Tel Aviv 61500, Israel.

3rd International Workshop on Lessons from Animal Diabetes

This workshop will be held 21–24 March 1990 on the Dead Sea shore in Israel. *Deadline for abstracts:* 30 November 1989. *For further information please contact:* Dr. E. Shafrir, Department of Biochemistry, Hadassah University Hospital, Jerusalem 91120, Israel.