Diabetologia (1999) 42: 1080–1085

Diabetologia

Rapid communication

Cytokine secretion patterns in twins discordant for Type I diabetes

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Abstract

Aims/hypothesis. The search for T-cell reactions that are associated with disease in Type I (insulin-dependent) diabetes mellitus is severely hampered because control groups cannot be matched for relevant immune response genes. We therefore compared T-cell responses between identical twins discordant for Type I diabetes.
Methods. Pairs of monozygotic twins ($n = 17$) discordant for Type I diabetes were studied. Cultures were set up from whole blood immediately after sampling and cells were challenged with human recombinant hsp60, with the mitogen phytohaemagglutinin or with the staphylococcal superantigen. Supernatants were removed after 48 or 96 h and analysed for T-helper1 type cytokines interferon-γ, TNFα and T-helper2 type cytokines IL-4, IL-10 by sandwich-ELISA.
Results. The height of the T-helper1 type cytokine response to hsp60, phytohaemagglutinin or staphylococcal enterotoxin B did not show disease association, i.e. it was similar between discordant twins. In contrast, the production of T-helper2 type cytokines differed between discordant twins. The IL-10 response to hsp60 was higher in twins at low disease risk (islet cell antibody-negative) than in their diabetic cotwins ($p < 0.01$), as was the IL-4 response to phytohaemagglutinin ($p < 0.05$). No difference was seen in the cytokine response between islet cell antibody-positive twins and their diabetic cotwins.
Conclusions/interpretation. The data indicate an association between T-helper2 type cytokine secretion patterns and disease or disease risk. [Diabetologia (1999) 42: 1080–1085]

Keywords Twins, T-cell immunity, hsp60, cytokines, superantigen.

Monozygotic twins discordant for Type I diabetes mellitus (insulin-dependent) represent a unique opportunity to analyse T cell reactivities associated with disease. Cellular immune responses are strictly regulated by the major histocompatibility gene complex (MHC) and numerous immune response genes outside the MHC. Matching control subjects for immune response genes therefore seems mandatory. This is particularly true for Type I diabetes where a large proportion of patients is heterozygous for HLA-DR3/DR4 whereas this genotype is rare in the general population.

Surprisingly, recent studies of the proliferative response of T cells to islet antigens did not identify differences between discordant twins [1]. We now tested the hypothesis that the quality of T cell reactions, as defined by the T-helper(Th)1/Th2 type cytokine pattern expressed, is changed during clinical disease or in people at high disease risk, i.e. in twins with circulating islet cell antibodies (ICA) but no overt disease. The assumption of a disturbed cytokine balance of T cell responses in Type I diabetes was based on our

Received: 1 March 1999 and in revised form: 26 April 1999

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Abbreviations: Hsp, Heat shock protein; ICA, islet cell antibody; IFN, interferon; mAB, monoclonal antibody; PHA, phytohaemagglutinin; SEB, staphylococcal enterotoxin B; Th, T-helper; MHC, major histocompatibility gene complex.

previous observation that peripheral blood cells from Type I diabetic patients responded with a Th1 type cytokine bias to non-specific stimulation when compared with patients with Grave's disease [2]. Our present study included responses to a mitogen, a superantigen and to the heat shock protein (hsp)60. This stress protein has been suggested to be a major target antigen in several inflammatory diseases, including Type I diabetes and rheumatoid arthritis [3]. Because of the abundant expression of hsp 60 at sites of inflammation and its release during necrosis, this antigen is possibly of particular importance in driving cellular immune reactions during islet inflammation.

Subjects and methods

Subjects. Pairs of identical twins ($n = 17$, 10 males, 7 females) discordant for Type I diabetes were studied. Monozygosity was established in all the twin pairs as described previously [1]. The mean disease duration of the diabetic twins was 11.1 years (range 1–35 years). Twin pairs' age ranged from 5 to 60 years (mean 26.5 years). Twin pairs were always investigated on the same day and blood was drawn from the twins less than 30 min apart. In most instances, several twin pairs were analysed on the same day. No sign of acute infection was found after physical examination and anamnesis, full blood and white blood counts were in the normal ranges. All non-diabetic twins were tested by OGTT to exclude diabetes. No concomitant other acute or chronic disease was noted except for one diabetic twin suffering additionally from multiple sclerosis.

Cell culture. Peripheral venous blood was obtained from all subjects between 0900 and 1200 hours. Blood was collected in sterile 10 ml Ammonium-Heparin Monovette-tubes (Sarstedt, Nümbrecht, Germany). Under sterile conditions, 500 µl aliquots of whole blood were placed in 48-well tissue culture plates (Costar, Cambridge, Mass., USA). Whole blood cultures of twin pairs No. 1–14 were incubated (37 °C, 5 % CO_2) with 1 or 10 µg/ml human recombinant hsp60 (StressGen, Victoria, Canada). In control samples, 10 µg/ml polymyxin B (Sigma, St. Louis, Mo., USA) was added to exclude stimulatory effects induced by endotoxin. For stimulation with phytohaemagglutinin (10 µg/ml) (PHA, Sigma) and *Staphylococcal enterotoxin B* (SEB, 0.25 and 2.5 µg/ml, Sigma), a slightly modified culture protocol was used [2]. We seeded 50 µl of whole blood from each twin of the twin pairs No. 1–17 in tissue culture plates and diluted to a final volume of 500 µl with RPMI 1640 medium containing 10 % fetal calf serum (Gibco, Paisley, UK), 25 mg/l ampicillin, 120 mg/l penicillin, 270 mg/l streptomycin (Serva, Heidelberg, Germany), 1 mmol/l sodium pyruvate, 2 mmol/l L-glutamine, and supplemented with 10 ml/l non-essential amino acids (Gibco), 24 mmol/l $NaHCO_3$ and 10 mmol/l HEPES (Serva). Supernatants of blood cultures were collected after 48 or 96 h and stored frozen at –80 °C until further analysis.

Cytokine determination. Concentrations of interferon (IFN)γ, TNFα, IL-4 and IL-10 in the supernatants of blood cultures and in sera were determined by two-sided sandwich-ELISA as described previously [2]. The following monoclonal antibody pairs were used: mouse anti-human IFNγ monoclonal antibody (mAB) (Endogen, Cambridge, Mass., USA), mouse anti-human TNFα mAB, mouse anti-human IL-4 mAB and rat anti-human IL-10 mAB (PharMingen, San Diego, Calif., USA). Detection limits for cytokines in supernatants were 0.1 ng/ml for IFNγ or for IL-10 and 5 pg/ml for TNFα or IL-4. Detection limits for IFNγ or IL-10 in serum were 5 pg/ml and 1 pg/ml, respectively.

Sera of all the twins investigated were screened for islet cell antibodies (ICA) as described previously using indirect immunofluorescence assay on cryostat sections of blood group 0 human pancreas [4]. The lower detection limit of ICA positivity was 2.5 JDFU (Juvenile Diabetes Foundation Units).

Statistics. Statistical analysis was carried out using StatView 4.01 software package on Apple Macintosh computer. Comparison of means of log-transformed cytokine values were done by Student's paired and unpaired *t* test since log-normal distribution of cytokine concentrations was observed. Values for $p < 0.05$ were considered statistically significant.

Results

Table 1 summarises clinical data of the identical twin pairs discordant for Type I diabetes. Of the non-diabetic twins, seven (41 %) were ICA positive and thus at high disease risk [1, 5], ten ICA negative non-diabetic twins were at low-disease risk now estimated to be less than 5 % risk for each [1, 5]. Therefore the discordant twin pairs could be divided into two subsets, by the low or high diabetes risk of the healthy cotwin. In both subsets the duration of diabetes was similar in the affected twin, with a mean of 10.9 years in ICA positive subsets compared with 11.4 years in ICA negative healthy twin.

Samples of heparinised undiluted whole blood were cultured in vitro in the presence or absence of human hsp60 (10 µg/ml). At 96 h of culture, cytokine concentrations were determined in the supernatant. Most twins responded to hsp60 with substantial secretion of IFNγ (Fig. 1A), but no IFNγ was detectable in the absence of hsp60. There was substantial interindividual variation with no major differences between groups but a trend to more homogeneity between twins of a pair than between different twin pairs. The outcome for TNFα secretion in response to hsp60 was similar to that observed for IFNγ (Fig. 1B), i.e. there was no spontaneous TNFα secretion detectable but all twins responded to hsp60 with TNFα production. There was variation in the degree of the response but less within than between twin pairs.

A completely different picture emerged when the Th2 type cytokines IL-4 and IL-10 were measured. Although IL-4 was not detectable, there was substantial secretion of IL-10, both, spontaneously and in response to hsp60. Spontaneous IL-10 secretion was clearly different between subsets of twin pairs. Islet cell antibody positive healthy twins exhibited higher spontaneous IL-10 secretion than ICA negative healthy twins ($p < 0.01$) and the same trend appeared for the respective diabetic cotwins ($p = 0.07$) (Fig. 1C).

Table 1. Clinical data of the identical twin pairs discordant for Type I diabetes

Twin pair no.	Age	Sex	Duration of diabetes (years of discordance)	ICA (JDF Units) (Type I twin)	ICA (JDF Units) (non-diabetic twin)
1	43	m	6.5	–	–
2	13	m	2	40	40
3	19	m	8	10	2.5
4	29	m	14.6	10	–
5	30	m	13	40	10
6	13	f	6.9	–	–
7	31	m	13	–	–
8	9	m	1	20	–
9	15	f	9	–	–
10	7	f	4.5	–	5
11	35	f	11.5	10	–
12	23	m	10	20	2.5
13	60	m	35	–	20
14	44	f	20	2.5	–
15	8	f	6	–	–
16	5	f	3.7	–	10
17	40	m	26	–	–

JDF, Juvenile Diabetes Foundation Units

The two twin pair subsets also differed for the IL-10 response to hsp60. Islet cell antibody positive twins showed a response similar to their diabetic cotwins, with IL-10 concentrations mostly above 1 ng/ml (Fig. 1D). In contrast, ICA negative twins had a much stronger IL-10 response than their diabetic cotwins ($p < 0.01$, Fig. 1D). As a consequence, the two subsets of diabetic twins, as defined by their cotwins, differed in the IL-10 response ($p < 0.005$), confirming the trend seen for spontaneous IL-10 secretion. The same significant differences were obtained with lower hsp60 concentrations (1 μg/ml) or with shorter incubation periods (48 h) (data not shown).

Because of the substantial spontaneous IL-10 secretion in the cell cultures IL-10 concentrations were also determined in serum at the time of blood withdrawal for cell cultures. Circulating IL-10 was detectable in about half of the sera (Fig. 1E). Differences between twin subgroups were not statistically significant. The similarity to results obtained for spontaneous IL-10 production in vitro (Fig. 1C) was, however, striking. Serum IFNγ was detected only in 3 of the 17 twin pairs (not shown).

In parallel experiments the response to non-specific stimulation by the mitogen phytohaemagglutinin (PHA) (10 μg/ml) was studied (Fig. 2A). Similar amounts of the Th1 type cytokines IFNγ and TNFα were produced by the subsets analysed. As before, cytokine concentrations varied substantially between twin pairs but were usually similar within a twin pair. In contrast to the results for hsp60, IL-10 secretion was similar in all twin subsets in response to PHA. Stimulation with the mitogen PHA also led to measurable amounts of IL-4. Although ICA positive twins showed a similar response as their diabetic cotwins, ICA negative twins had higher IL-4 levels than their diabetic cotwins (Fig. 2A, $p < 0.05$). The same significant difference was obtained after 48 h as well after 96 h of culture or with the lower PHA dose (1 μg/ml) (data not shown).

As a third T cell stimulating compound, we used the superantigen SEB. In contrast to the outcome with hsp60 and PHA, there was no difference in the cytokine response between diabetic compared with ICA positive or ICA negative non-diabetic twins (Fig. 2B). Again, variation of cytokine concentrations, was much higher between twin pairs than within twin pairs.

Discussion

The present study measured the immune reactivity in unfractionated whole blood. Accordingly, we tried to approach physiological conditions of the subset composition and overall concentration of blood cells and the pattern of circulating immune mediators. Although this type of analysis provides valuable information on the immunoregulatory balance, it cannot show the contribution of individual immune cell subsets or of specific circulating immune mediators to a biased response.

There are several conclusions from our data. The Th1 type cytokine response to the three stimuli hsp60, PHA and SEB did not distinguish non-diabetic from diabetic twins of a pair or ICA positive from ICA negative healthy twins. A Th1 type cytokine response, as evident from the secretion of IFNγ and TNFα was observed to the stress antigen hsp60 in all twins. Because of the lower incidence of T cell responses to other islet antigens, these can be studied only with larger cohorts than available here. A further result emerging from the Th1 type cytokine re-

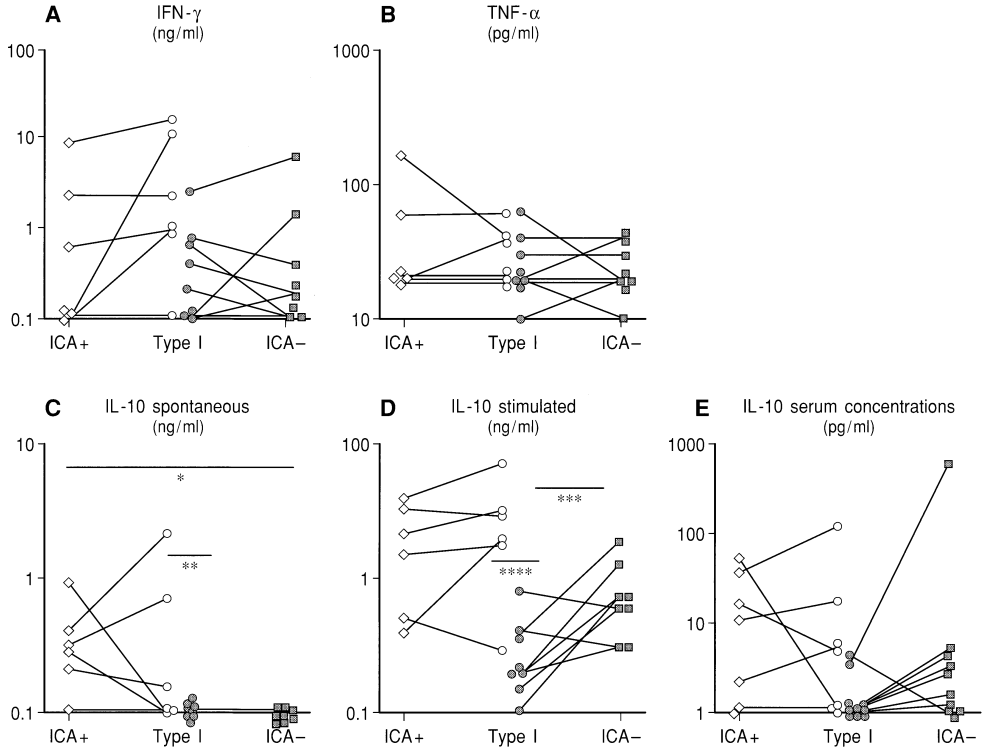

Fig. 1A–E. Spontaneous and hsp60 induced cytokine secretion patterns. In 14 of the 17 twin pairs (no. 1–14) sufficient material was available to set up whole blood cell cultures with 10 μg/ml hsp60 or medium for 96 h. Supernatants were analysed for amounts of IFNγ (**A**), TNFα (**B**), IL-10 (**C**, **D**). Serum concentrations for IL-10 (**E**) were analysed from samples obtained at the time of blood withdrawal for cell cultures. ICA positive and ICA negative twins are depicted separately, each dot represents one person. Twin pairs were indicated by a line. Concentrations of IL-4 remained below the detection limit of 5 pg/ml after stimulation with 10 μg/ml hsp60 or in control cultures, $*p < 0.01$, $**p = 0.07$, $***p = 0.008$, $****p < 0.005$

sponse to the three different stimuli was that the height of the response differed strongly between twin pairs but there was less variation in cytokine concentrations within twin pairs, i.e. between twins of a pair. This finding is particularly relevant since these were twin pairs discordant for Type I diabetes and often also discordant for islet autoimmunity. Although some of the variation between twin pairs is possibly because not all twins could be tested on the same day, it should be noted that homogeneity within a twin pair was observed for the Th1 type but not for the Th2 type cytokine response. We conclude that genetic factors determine the height of the IFNγ or TNFα response to a substantial degree and even overrule the impact of an immune-mediated disease or of metabolic derangements. The latter conclusions did not pertain to the production of the Th2 type cytokines IL-4 and IL-10. Here, statistically significant differences were observed between ICA negative twins and diabetic cotwins for two of the three stimuli. In response to hsp60, ICA negative twins had a statistically significantly higher IL-10 response than the diabetic cotwins. In response to PHA, ICA negative twins had a higher IL-4 response than the diabetic cotwins. For the same two stimuli no difference was seen in IL-10 or IL-4 responses between ICA positive and their respective diabetic cotwins.

A Th2 bias of the response to hsp60 in discordant twins of low diabetes risk is consistent with recent studies in NOD mice, where a Th1 bias of the T cell response to hsp60 was found to correlate with disease progression in mice whereas a Th2 bias was found in animals protected from diabetes development [6]. In man, previous studies of a possible association between hsp60 autoimmunity and Type I diabetes did not yield firm conclusions [3, 7, 8]. These studies did not, however, estimate the quality of the immune response as described by the cytokine profile or related variables. Hsp60 is an islet antigen in that it is expressed on the surface of beta cells during islet inflammation [9] and will be released during necrosis. It is therefore conceivable that the immune response to hsp60 is of relevance to the disease process in islets. Recent studies suggest that hsp60 is a unique autoantigen because it not only is recognised by T cells through classic rules of MHC dependent peptide presentation [6] but is also a "danger" antigen to the innate immune system. This means, macrophages, endothelial cells and other primitive immune cells are able to recognise exogenous hsp60 and secrete mediators with potent modulatory effects on subsequent T cell responses [10, 11].

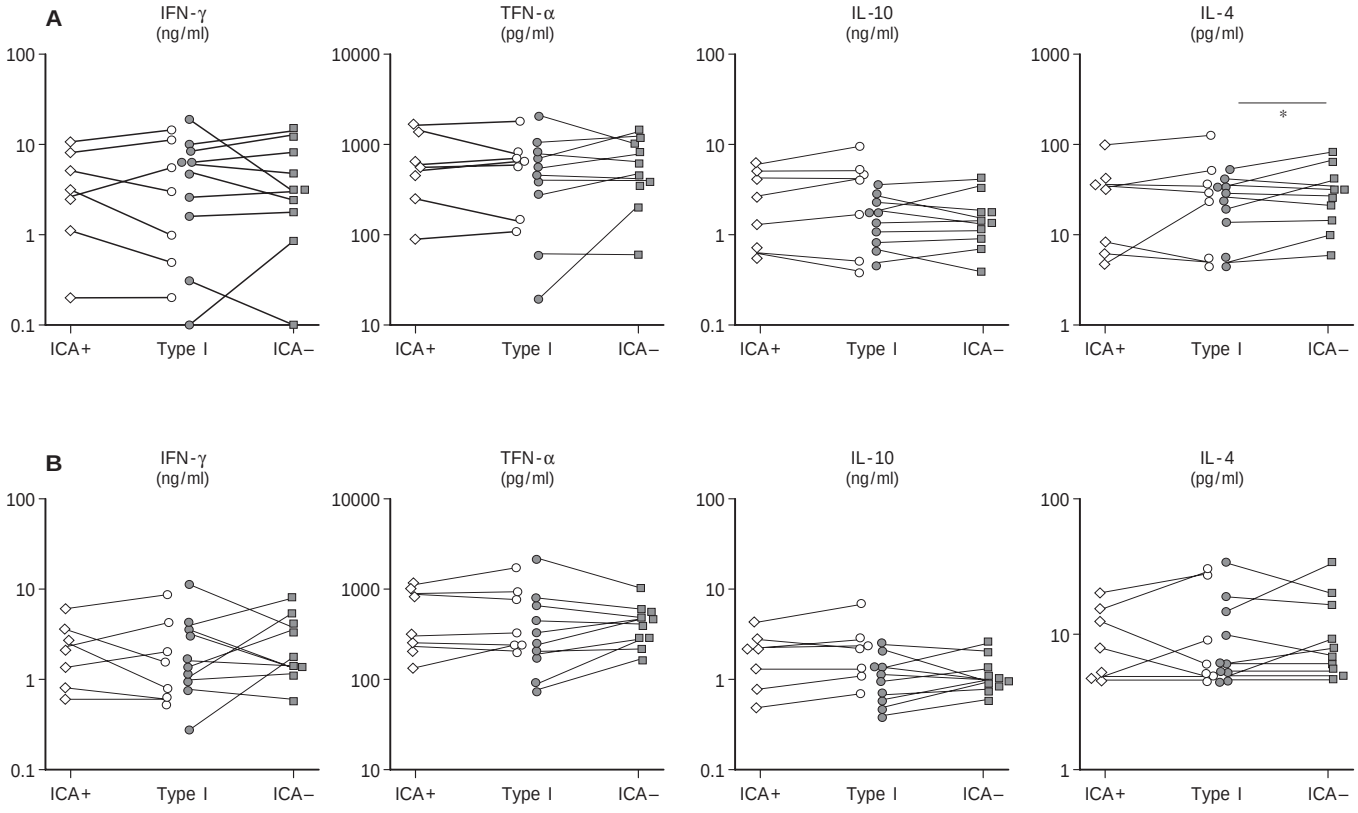

Fig. 2A, B. Cytokine profiles in reponse to (**A**) PHA or (**B**) SEB. In twin pairs 1–17 whole blood cell cultures were set up with 10 μg/ml PHA or 2.5 μg/ml SEB for 96 h. Cytokines released from leucocytes of non-diabetic twins were compared with values of diabetic cotwins. ICA positive and ICA negative twins are depicted separately, each dot represents one person. Twin pairs were indicated by a line. Control cultures without stimulus did not yield detectable concentrations of cytokines, $^*p = 0.04$

Recently, it has been reported that progression to diabetes was associated with deficient production of IL-4 by a particular subpopulation of invariant Vα24-JαQ T-cells whereas IFNγ secretion was normal. This study included monozygotic subjects discordant for Type I diabetes [12]. Although only two twin pairs and a triplet were analysed, the data fit with the concept that the Th2 type cytokine response is disease associated, even for these CD1 as restricted NK T cells, which are considered to be part of the innate immune system.

No differences in cytokine profiles were noted in response to the superantigen SEB. This may be because the superantigen interacts with a limited number of T cell receptor families only. Also, signalling through the complex of SEB, MHC and T cell receptor could be potent enough to overcome a weak Th2 type cytokine response in diabetic twins. A final, somewhat surprising, finding was that IL-10 production was different between two subgroups of diabetic twins. In response to hsp60, diabetic twins of low risk cotwins (ICA negative) had statistically significantly lower concentrations of IL-10 in culture supernatants than diabetic twins of high risk cotwins. A trend in the same direction was noted for SEB induced and spontaneous IL-10 secretion. Also, IL-10 concentrations in serum showed a similar trend. Spontaneous IL-10 production is under control of the IL-10 locus itself. It is, therefore, possible that twin pairs with either an ICA positive or an ICA negative non-diabetic cotwin differ in IL-10 gene alleles.

Our findings point to a role of IL-10 and IL-4 secretion in the disease process of Type I diabetes. Therapeutic approaches to enhance/normalise Th2-like immune reactivities may represent an alternative approach to immune intervention in Type I diabetes.

Acknowledgements. We thank M. Schulte for excellent technical assistance. This work was supported by the British Diabetes Association, the Diabetic Twin Research Trust, the German Ministry of Health, the North-Rhine-Westfalian Minister of Science and Research and by a travel grant by the European Association for the Study of Diabetes to B. A. Kallmann.

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