

Low stimulated IL-4 secretion in PBMC from patients with chronic idiopathic urticaria

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Abstract

An imbalance between the Th1 and Th2 arms of the cellular immune system has been reported in several autoimmune diseases but not in chronic idiopathic urticaria (CIU). Peak, total secretion and secretory pattern of the Th1 cytokines (IFN γ and IL-2) and Th2 cytokines (IL-4 and IL-10) were determined in resting and stimulated peripheral blood mononuclear cells (PBMC) from nineteen CIU patients, six acute urticaria patients and twelve controls. Stimulated IL-4 secretion was significantly reduced in CIU patients as indicated by their five- and three-fold lower peak levels and total IL-4 secretion, respectively. The IL-4 secretory pattern overtime was also markedly different in patients and controls. The late secretion of IFN γ at 144 h was also reduced in CIU patients. These aberrations were not detectable in AU patients. Secretion of IL-2 was lower in CIU and AU patients as compared to controls while IL-10 secretion was comparable in the three groups. Our data demonstrate for the first time a predominantly reduced IL-4 secretion in CIU patients. This is associated with reduced secretion of both IL-2 and IFN γ . These findings indicate a generalized down-regulation of both Th1 and Th2 cytokines' secretion in CIU.

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1. Introduction

Chronic idiopathic urticaria (CIU) is a common form of chronic urticaria, in which the cause is not detected after intensive clinical and laboratory investigation [1,2]. It has been suggested that CIU is an autoimmune

disease. This is based on the observation that intradermal injection of autologous serum elicits an immediate wheal and flare response in 60% of patients with CIU [3–5]. Subsequent work demonstrated the presence of anti-IgE–IgG antibodies and anti-Fc ϵ RI–IgG antibodies, in sera from most patients with CIU [6–11]. More recently we demonstrated that lymphocytes from CIU patients are characterized by aberrant p21Ras signaling adding further support to the autoimmune basis of this disease [12]. The functional classification of human CD4+ T-helper cells into two subtypes, Th1 and Th2, is based primarily on the pattern of their cytokine production [13–17]. Th1 cells are characterized by the production of interleukin-2 (IL-2), interferon gamma (IFN γ) and tumor necrosis factor (TNF). Th2 cells produce interleukin-4 (IL-4), interleukin-5 (IL-5) as well

Abbreviations: AU, acute urticaria; AUC, area under the curve; CIU, chronic idiopathic urticaria; IFN γ , interferon gamma; IL-2, interleukin-2; IL-4, interleukin-4; IL-10, interleukin-10; PBMC, peripheral blood mononuclear cells; PHA, phytohaematoagglutinin; Th1, T-helper 1; Th2, T-helper 2; SI, stimulation index.

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as interleukin-6 (IL-6), interleukin-13 (IL-13) and interleukin-10 (IL-10). Th2 cells play an important role in humoral immunity and are associated with atopic and allergic disorders. Th1 cells and the cytokines they secrete are important mediators of inflammation and cell-mediated immunity. A number of different autoimmune and/or allergic diseases in humans are characterized by an imbalance between the Th1 and Th2 arms of the cellular immune system. Rheumatoid arthritis, juvenile rheumatoid arthritis, multiple sclerosis and insulin-dependent diabetes mellitus are associated with a Th1 phenotype [18–20], while asthma is considered a prototype of Th2 mediated disease [21]. CIU has features of both autoimmune and allergic disease [22], indicating a complex underlying aberration of the immune system in these patients. It suggests that the cytokine secretory pattern of CIU patients could be dynamically skewed towards Th1 or Th2, depending on disease development and activity as recently reported in autoimmune diabetes [23]. Limited data are available regarding cytokine secretion in CIU, most of which is derived from determination of cytokines expression in biopsies of patients with active disease. Lawlor et al. demonstrated increased levels of IL-6 in blisters of pressure urticaria [24]. More recently Czametzki et al. demonstrated increased expression of MIF in several types of urticaria [25]. Later on, Hermes et al. found up-regulation of TNF α and IL-3 expression in lesional and uninvolved skin in different types of urticaria [26]. A number of different cytokines, chemokines and leukotrienes have been implicated in the activation and degranulation of mast cells [27]. One of these cytokines is IL-4, which is secreted by both mast cells and T cells and promotes their reciprocal activation. Whether any of these cytokines is involved in the pathogenesis of CIU is not known. In addition, no data are available regarding the Th1/Th2 cytokine secretory profile in lymphocytes of CIU patients, and whether there is any correlation between the cytokine pattern and other autoimmune markers in these patients. In this work we examined the overall secretion, peak levels and secretory pattern of Th1 cytokines (IFN γ and IL-2) and Th2 cytokines (IL-4 and IL-10) in stimulated PBMC of patients with CIU. We demonstrate that the stimulated IL-4 secretion is significantly reduced in CIU patients. This is associated with some reduction of both IL-2 and IFN γ secretion.

2. Results

2.1. Autologous serum injection

All CIU patients were intradermally injected with autologous serum as well as normal saline and histamine.

In all patients, skin test with histamine elicited positive results, whereas saline elicited negative results. Positive skin reactivity to autologous serum was observed in 15 out of 19 patients (84%) (Table 1). Due to the small number of patients with negative skin reactivity ($n = 4$) their experimental data are not provided separately. Nine out of the healthy controls were also injected with autologous serum, all having negative results. AU patients were not injected with autologous serum in order to avoid delay in treatment.

2.2. Th2 cytokine secretion

2.2.1. Decreased stimulated IL-4 secretion in CIU patients

Analysis of IL-4 secretion in CIU patients and controls demonstrated a significant difference between the IL-4 secretory patterns of the two groups, ($p = 0.001$). In CIU patients, the stimulated IL-4 secretion increased moderately after 24 h, remained unchanged for additional 24 h and declined to non-stimulated levels at 72 h (Fig. 1A). In contrast, IL-4 secretion in the control group increased sharply to its peak level after 24 h and declined soon afterwards reaching non-stimulated levels at 72 h. The peak level of IL-4 secretion was five times lower in patients as compared to controls: 10.9 ± 2.4 and 56 ± 20.3 pg/ml, respectively, $p = 0.012$. Consequently, the overall IL-4

Table 1
Demographic and clinical data of CIU patients

Patients no.	Age	Disease activity ^a	Duration of disease (months)	Skin test reactivity (mm) ^b
1	24	+	3	Negative
2	42	+	7	31.5
3	39	+	8	30
4	42	–/+	7	22
5	29	+	5	22
6	58	+	48	25
7	54	+	6	11
8	41	+	15	14
9	55	+	8	25.5
10	50	+	3	Negative
11	68	+	7	11.5
12	43	+	5	16.5
13	24	+	3	Negative
14	18	+	6	Negative
15	28	+	26	14
16	37	+	60	18.5
17	52	+	24	16
18	20	–	10	18.5
19	56	+	9	13
Mean $\pm$ S.D.	41		13.7	19.3
	14.3		15.7	6.5

^a Disease activity: (+) active, (–) non-active.

^b Skin test reactivity to autologous serum injection was calculated as described in Section 4.

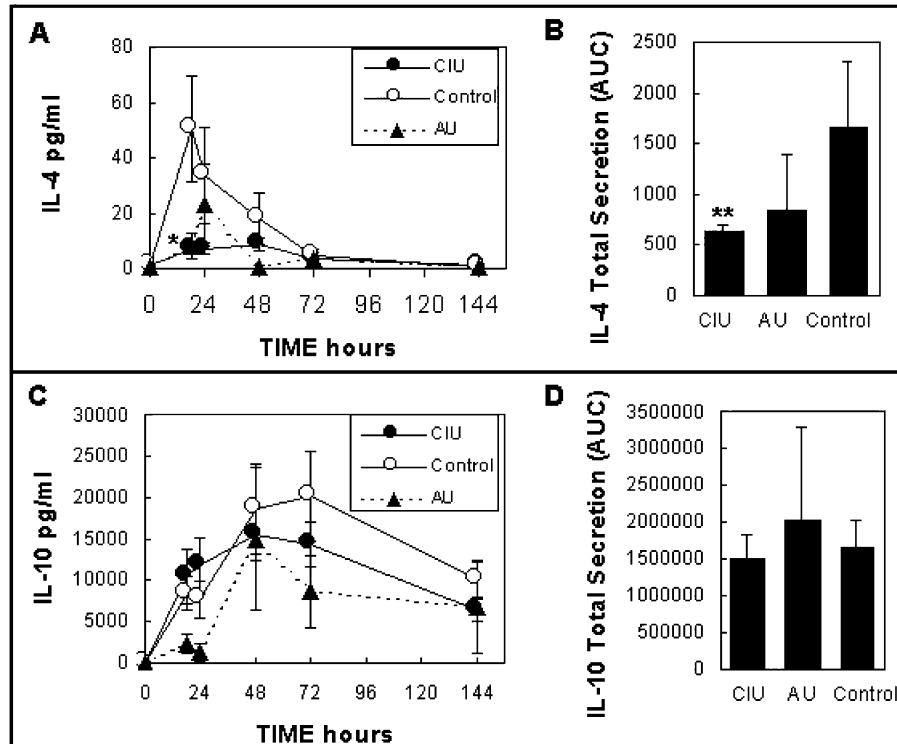


Fig. 1. Secretory profile and total secretion of Th2 cytokines of CIU, AU patients and controls. PBMC were isolated and stimulated by PHA. The PHA concentration resulting in a maximal proliferative response (10 or 20 μ g/ml) was selected for cytokine determination. Supernatants were collected at 0, 18, 24, 48, 72 and 144 h. The secretory profiles and total cytokine secretion of IL-4 (A and B) and IL-10 (C and D) were plotted. Values represent mean $\pm$ SEM of patients and controls (* secretory profile of CIU patients as compared to control, $p = 0.001$, ** total secretion of CIU patients as compared to controls, $p = 0.03$).

secretion of CIU patients was significantly reduced: 651 ± 78 and 1850 ± 690 , for patients and controls, respectively ($p = 0.03$) (Fig. 1B). The lower IL-4 secretion of CIU patients was observed following stimulation by different PHA concentrations (Fig. 2).

Total IL-4 secretion of AU patients was higher than CIU patients and lower than controls. These differences did not attain statistical significance.

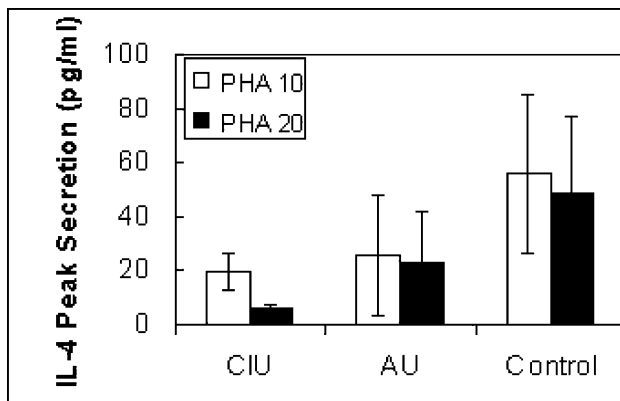


Fig. 2. Peak IL-4 secretion following stimulation with different mitogen concentrations. Peak IL-4 secretion was determined in CIU patients, AU patients and controls following stimulation with either 10 or 20 μ g/ml of PHA. Values represent mean $\pm$ SEM of patients and controls.

2.2.2. Normal IL-10 stimulated secretion in CIU patients

The IL-10 secretion in CIU patients, AU patients and controls, demonstrated a similar pattern—increasing steeply after 24 h reaching its peak at 48 h and gradually declining after 72 h (Fig. 1C). The IL-10 peak levels and its total secretion over time were comparable in patients and controls (Fig. 1D).

Taken together these data demonstrate that secretion of IL-4, but not IL-10, is reduced in CIU patients.

2.3. Th1 cytokine secretion

2.3.1. Aberrant IFN γ secretory profile in CIU patients

Analysis of IFN γ secretion pattern demonstrated a significant difference between the CIU patient and control groups ($p = 0.048$) (Fig. 3A). The IFN γ secretion increased similarly in both groups during the first 48 h after stimulation. However, IFN γ secretion in CIU patients declined after 72 h, whereas in the control group it continued to rise, reaching its peak level at 144 h. The difference between patients and controls at 144 h was significant: 5800 ± 2500 and 17700 ± 6000 , respectively, $p = 0.04$ (Fig. 3A). Total IFN γ secretion was lower in CIU patients as compared to controls.

However, this difference did not reach statistical significance. The secretory pattern and total IFN γ secretion of AU patients paralleled the control group (Fig. 3A, B).

2.3.2. Decreased IL-2 secretion in CIU patients

The IL-2 secretory profile was similar in CIU patients, AU patients and controls. IL-2 secretion increased gradually and reached its peak level after 48 h, declining soon afterwards, in the three groups (Fig. 3C). However, peak IL-2 level, was two to three times lower in the CIU and AU patients as compared to controls. This was reflected in the total IL-2 cytokine secretion, which was significantly lower in CIU and AU patients as compared to controls ($p = 0.05$ in both groups) (Fig. 3D). Taken together, these data indicate a reduced Th1 cytokine secretion in CIU patients.

3. Discussion

Our data indicate for the first time that patients with CIU are characterized by reduced secretion and altered secretory pattern of IL-4. Moreover, the pattern and/or secretion of their Th1 cytokines IFN γ and IL-2 were

also altered. These findings support the concept of an immune dysregulation resulting in cytokine imbalance underlying or associated with the development of CIU. Comparable findings were reported in a variety of other immune mediated diseases, including type 1 diabetes and systemic lupus erythematosus [18,28]. We examined the Th1 and Th2 arms of the CD4 immune system in stimulated PBMC of patients and controls for peak secretory levels, and total secretion, as well as for the pattern of secretion over time. IL-4 and IL-10 represent the Th2 cytokines whereas IFN γ and IL-2 represent the Th1 profile. IL-4 is the principal cytokine that induces naive T cells to differentiate into Th2 cells. In the absence of IL-4, the T cell precursor Th0 is exposed to the influence of Th1 directing cytokines. Once the T cell response is dominated by the Th1 phenotype it tends to develop in this direction. In our study the most striking finding was that peak IL-4 secretion from stimulated PBMC was significantly lower in patients with CIU as compared to controls. To avoid bias induced by single sampling, stimulated secretion was examined over time, demonstrating a decreased total IL-4 secretion and altered secretory pattern. The second Th2 cytokine, IL-10, also showed suppressed late secretory pattern, although the difference did not reach significance. It is

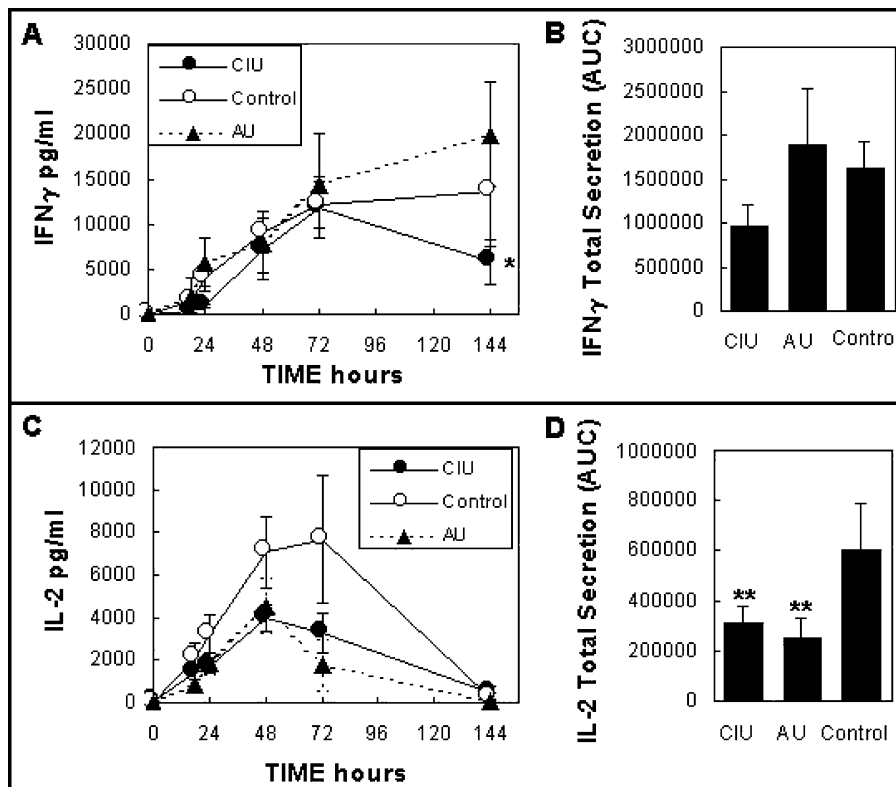


Fig. 3. Secretory profile and total secretion of Th1 cytokines of CIU, AU patients and controls. PBMC were isolated and stimulated by PHA. The PHA concentration resulting in a maximal proliferative response (10 or 20 μ g/ml) was selected for cytokine determination. Supernatants were collected at 0, 18, 24, 48, 72 and 144 h. The secretory profiles and total cytokine secretion of IFN γ (A and B) and IL-2 (C and D) were plotted. Values represent mean $\pm$ SEM of patients and controls (* CIU patients as compared to controls at 144 h, $p = 0.04$, ** total secretion of CIU and AU patients as compared to controls, $p = 0.05$).

possible that IFN γ suppressed IL-4 secretion. However, in our group of patients this is unlikely, since IFN γ and IL-2 levels were not increased. On the contrary, the secretion of IFN γ was down-regulated with a late secretory pattern significantly different from controls. In addition, IL-2 peak secretory level was also significantly lower. An acceptable explanation for this generalized suppression of cytokine secretion can be an innate aberration in T cells induced by a primary unknown environmental or genetic trigger, causing cytokine shift among other dysregulations [16]. Evidence for probable T cell dysregulation was previously reported by us, demonstrating disturbed signaling via the p21Ras pathway in lymphocytes of CIU patients [12]. p21Ras pathway has a central role in T and B cells signaling and therefore, their responsiveness to membrane receptor mediated stimulation by a variety of ligands [29]. Furthermore, T cell signaling controlled by this pathway has a key role in the thymic T cell selection essential for the induction of self-tolerance [16]. One may speculate that aberrant T cell p21Ras signaling may result in the thymic release of self aggressive autoimmune T cells, and promote autoimmune diseases via production of autoantibodies and altered cytokine secretion.

The low IL-4 secretion of CIU patients was demonstrated in the two PHA concentrations employed. Moreover, the stimulation index was comparable in CIU patients and controls (data not shown). This indicates that the different cytokine profile of these patients is not due to different proliferative response of their stimulated lymphocytes. It is also of interest that the secretory pattern and/or total cytokine secretion of the major representative Th1 and Th2 cytokines, namely IFN γ and IL-4, in acute urticaria patients were different from CIU patients. This indicates that CIU patients are characterized by a specific cytokine secretory profile not shared by other forms of urticaria.

Human basophils derived from whole blood account for most of the stimulated IL-4 production in leukocyte cell cultures [30]. It has been reported that basophils from urticaria patients are desensitized and less responsive to IgE and non-IgE stimuli [31,32]. This raises the question whether the low IL-4 production of CIU patients is due to a quantitative or functional defect of their basophils. This is, however, unlikely because the initial basophil count of our study patients and controls was normal i.e., lower than 0.5%, and almost all of these basophils are lost during the isolation of PBMC. Moreover, IL-4 secretion by basophils is predominant at the early phase after stimulation, and is significantly reduced afterwards [33], while the secretion of IL-4 by our CIU patients remained persistently low during 48 h in contrast to controls and AU patients. Nevertheless, mast cells/basophils secrete a number of relevant cytokines such as IL-4, which may influence the overall cytokine balance of CIU patients. Thus, our *in vitro*

data produced in a mast cell free environment should be interpreted with caution regarding the *in vivo* situation. Taken together, these data indicate that the reduced IL-4 secretion of CIU patients is due to lymphocytes rather than basophils.

Chronic idiopathic urticaria is already accepted as a disease with an autoimmune background [17,34]. This notion is supported by the finding of circulating anti-IgE and anti-Fc ϵ RI α antibodies and by the positive autologous serum injection test. Not surprisingly, some but not all the patients with CIU were found to have autoantibodies and only some of the patients have positive skin reaction to serum injection. This may be due to the fact that part of the autoantibodies in the serum of CIU patients are non-functional and will not cause mast cell degranulation [4,6]. Our group of patients was not different in their reaction to autologous serum injection from the groups previously described [6]. However, the aberration in cytokine secretion was found in our patients irrespective of their skin test results. Although the number of patients in our study with negative skin reactivity was limited ($n = 4$), this may indicate that aberrant cytokine secretion is a more sensitive indicator for immune dysregulation than autologous serum injection in these patients. Future studies comparing cytokine secretory patterns and the presence of anti-IgE and anti-Fc ϵ RI α antibodies as well as histamine release from basophil assay, may provide a valuable insight into the mechanisms underlying the pathogenesis of CIU.

Altogether, we provide a thorough analysis of cytokine secretion in PBMC of patients with CIU. Our data demonstrate, for the first time, a suppressed IL-4 secretion associated with aberrant pattern of IFN γ and IL-2 secretion, providing further support for dysregulation of the immune system in CIU.

4. Materials and methods

4.1. Patients

Nineteen CIU patients, mean age 40.2 years, range 18–68 years (14 females and 5 males), six AU patients, mean age 29 years (4 females and 2 males) and 12 healthy controls, mean age 40.1 years, range 29–82 years, (5 females and 7 males) participated in this study (Table 1).

Inclusion criteria for the CIU patients' group were as follows:

1. History of frequent (> 2 /week) urticarial rash for least 6 weeks.
2. Normal physical examination except for urticarial lesions.
3. Normal laboratory work-up that included sedimentation rate, CBC with normal basophil count

(<0.5%), blood chemistry, complement levels (C3, C4), thyroid stimulating hormone, anti-nuclear Abs, anti-thyroglobulin Abs, total serum IgE (<100 IU/ml), urine examination and absence of parasites in three stool samples.

4. No gingival inflammation and no vaginal infection (as judged by a dentist and a gynecologist, respectively).

The AU group consisted of six patients (2 males and 4 females) presenting at the emergency room with a first episode of acute urticaria (Table 2). Acute urticaria resolved in all patients within one week and did not recur during follow-up (mean 15 ± 3 months). Total IgE levels and CBC including basophil count were within normal limits and comparable to CIU patients.

All CIU patients stopped treatment with H1 blockers for at least 48 h before blood sampling and autologous serum injection. None of the CIU and AU patients were treated with corticosteroids or other immunosuppressive therapy at the time of venipuncture. None of the patients or controls suffered at the time of enrolment from any other significant acute or chronic disease. All patients signed an informed consent and the study was approved by the hospital ethics committee.

4.2. Autologous serum injection

Serum was obtained from venous blood samples. For serum preparation, blood was clotted at room temperature for 30 min in silicone-coated vacutainer tubes, followed by centrifugation at 550 g for 4 min. Fifty microliters of the freshly prepared serum was injected intradermally on the volar surface of the forearm. Normal saline and histamine (0.1 mg/ml) were also injected as negative and positive controls, respectively. All injection sites were scored 45 min after inoculation. The largest diameter (D1) of the wheal reaction and the diameter perpendicular to this (D2) were recorded. Results were expressed according to the formula $D1 + D2/2$. Reactions showing mean diameter of more than 5 mm were considered positive [35].

Table 2
Demographic and clinical data of AU patients

Patient no.	Age years	Cause of AU	Time to resolution days	Follow-up ^a
1	33	Penicillin	7	NR
2	19	Minocycline	5	NR
3	40	Penicillin	6	NR
4	22	Unknown	3	NR
5	54	Strawberry	2	NR
6	35	Peanut	1	NR
Mean $\pm$ S.D.		33.8 12.7		

^a NR, no recurrence.

4.3. PBMC in vitro stimulation

Thirty milliliters of blood was drawn from an antecubital vein into heparinized tubes from each patient. Blood from AU patients was drawn before commencement of treatment. PBMCs were isolated by Ficoll-Hypaque gradient centrifugation. Cells (2×10^5 cells/well) were cultured in RPMI medium supplemented with 5% heat inactivated fetal calf serum, glutamine 2 mM, penicillin 100 U/ml and streptomycin 100 µg/ml (Biological Industries, Beit Haemek Israel) in 0.2 ml round-bottomed microtiter plates. Cells were cultured for 6 days in 5% CO₂ in air humidified 37 °C incubator. Proliferative response to PHA (2.5–20 µg/ml) was assessed by ³H-thymidine uptake (20 h pulse) after 3 days.

4.4. Cytokine determination

PHA concentrations of either 10 or 20 µg/ml resulted in maximal proliferative response in all patients and controls and therefore used for cytokine determination. After cells were removed, supernatants from three to six wells were pooled at each time point and kept frozen at –70 °C for the cytokine assays.

Cytokines' concentrations were determined by enzyme-linked immunosorbent assay (ELISA). The 8D4-8 anti-IL-4, JES3-9D7 anti-IL-10 and NIB42 anti-IFN γ capture mAbs were purchased from Pharmingen, USA, and 5355.111 anti-IL-2 mAb was purchased from R&D Systems, USA. Flat bottom 96-well plates were coated with the appropriate antibodies in PBS by overnight incubation at 4 °C. Collected supernatants and standards (purified recombinant IL-4, IL-10 and IFN γ from (Pharmingen, USA) and IL-2 (R&D Systems, USA)) were added and incubated overnight at 4 °C. After washing with 0.05% Tween 20 PBS, biotinylated anti-IL-4 (MP4-25D2), IL-10 (JES3-12G8), IFN γ (4S.B3) (Pharmingen, USA) or IL-2 (BAF202, R&D Systems) were added for 45 min at room temperature followed by peroxidase-conjugated streptavidin (Jackson Immuno-Research Laboratories, Inc., PA) for an additional incubation for 30 min. Finally, the color reagent 2,2'-azino-di[3-ethyl-benz-thiazoline sulfate] (ABTS, KPL, Maryland, USA) was added and the optical density was measured by ELISA reader (Dynatech MR 5000) at 405 nm. The amount of cytokine present was determined from the standard curves of purified recombinant cytokines. The lower limits of detection were 39, 15, 31 and 15 pg/ml for IL-2, IL-4, IL-10 and IFN γ , respectively. Intra-assay variations did not exceed 10%.

4.5. Statistical analysis

Statistical analysis of the cytokine secretory curves and total cytokine production calculated as the area

under the curve (AUC) were performed by the ANOVA method of square root transformation, with repeated measures-incomplete design using the BMDP statistical software or Student *t*-test when appropriate [36].

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