

Methods for the *in vitro* determination of an individual disposition towards TH1- or TH2-reactivity by the application of appropriate stimulatory antigens

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SUMMARY

In this study we performed several methods for the determination of cytokines (RT-PCR for the demonstration of cytokine mRNA and flow cytometry for the analysis of intracellular cytokines) and compared them with a recently established test system stimulating peripheral blood mononuclear cells (PBMC) with TH1- and TH2-relevant recall antigens and analysing type 1 and type 2 cytokines by ELISA. Aim of the study was therefore to evaluate the reliability of TH1/TH2 cytokine profiles in two individuals with different types of an allergic/atopic disposition: one of them showed a strong TH1/type 1-mediated tuberculin-reaction (subject A), the other (subject B) revealed elevated IgE-levels and eosinophil counts (TH2/type 2-mediated). PBMC were incubated with the type 1-antigen purified protein derivative (PPD) and the type 2-antigen tetanus-toxoid (TT) for seven days. From the comparison of ELISA with RT-PCR and flow cytometry-analysis it became evident that all three methods allowed the definition of subject A as a 'type 1-responder'. Subject B showed a pure type 2-response in the ELISA method; PCR and flow cytometry analysis revealed the simultaneous production of type 1- and type 2-cytokines resulting in a mixed type 1/type 2-profile. Active immunization of subject A with TT at the end of the observation period of 12 months resulted in a transient shift from type 1- to a mixed type 1/type 2-profile (simultaneous PPD-induced IFN- γ and TT-induced IL-5 production). From this pilot study based on clear cut clinical criteria concerning either a humoral or cellular immunological reactivity towards allergens/antigens it is suggested that the determination of type 1/type 2-cytokines by ELISA in supernatants of PBMC stimulated with type 1/type 2-relevant antigens is a useful approach for a better classification of 'type 1-' or 'type 2-responder'.

Keywords type 1/ type 2 reactivity peripheral blood mononuclear cells
purified protein derivative tetanus toxoid TH1/ TH2 cytokines

INTRODUCTION

The detection of distinct CD4+ T helper (TH) cell subsets defined by the production of specific cytokines offered an explanation for cellular and humoral immunological reactions in different infections and in autoimmune diseases. Type 1-TH cells produce interferon- γ (IFN- γ) and tumour necrosis factor- β (TNF- β) and are involved in macrophage activation and delayed-type hypersensitivity (DTH) reactions. In contrast, type 2-TH cells produce interleukin (IL)-4, IL-5, and IL-13 and mediate antibody responses, including IgE-production [1]. These T helper cell subpopulations represent polarized forms of the specific immune

response and counteract each other. They are closely associated with resistance or susceptibility to bacterial infections but also appear to be involved in the induction and regulation of autoimmunity and hypersensitivity [2,3]. Thus, autoreactive type 1-TH cells seem to play an important role in organ-specific autoimmunity as shown in experimental encephalomyelitis (EAE) and insulin-dependent diabetes mellitus (IDDM) in animal models [4,5]. In contrast, allergic/atopic disorders are mainly triggered by type 2-TH cell responses towards common environmental allergens [6–8].

The definition of type 1- or type 2-reactivity is mainly based on the demonstration of cytokine producing lymphocytes in the target organ or the production of relevant cytokines in response to the specific antigen *in vitro* [6,7,9–13]. Cytokines can be detected by different methods and in different substrates. Thus, their secretion can be determined by ELISA in patients' sera or lymphocyte supernatants, their intracellular production can be measured by flow cytometry, or their mRNA expression by

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polymerase chain reaction (PCR). We have recently established a test system, which may allow *in vitro* the definition of an individual as a 'type 1-' or 'type 2-responder' using peripheral blood. For this purpose we incubated peripheral blood mononuclear cells (PBMC) with the type 1-specific antigen purified protein derivative (PPD) and the type 2-inducing antigen tetanus-toxoid (TT) [14,15] and measured the relevant type 1- (IFN- γ , TNF β) and type 2-cytokines (IL-5, IL-13) in the PBMC-supernatants. Thus, we could show that in patients with multiple sclerosis, a disease presumed to be type 1-mediated [4,5,16], most patients exhibited a type 1-profile. These patients had, indeed, a rather progressive course while patients with a pronounced type 2-reactivity had a rather mild clinical course [17]. Aim of the present study was therefore to compare the reliability of this 'test system' with that of other standard and well practiced immunological techniques for the distinction of 'type 1' or 'type 2'- reactivity such as flow cytometry and polymerase chain reaction (PCR). Furthermore, we wanted to analyse the stability of these cytokine profiles over an observation period of 12 months, and to see whether they are influenced by exogenous factors such as infections, allergic manifestations or immunization.

MATERIALS AND METHODS

Subjects

For the comparison of different test systems for the evaluation of type 1- and type 2-reactivity *in vitro*, peripheral blood was obtained from two individuals working in our laboratory: subject A (age 67 years, male) had a history of neurodermitis as child and hay fever later on towards grass pollen but showed a very strong tuberculin-(delayed type hypersensitivity/type 1) reaction, normal IgE-levels (<25 IU) and normal eosinophil count (<3%). The other individual, subject B (age 38 years, female) also suffered from hay fever towards grass pollen but revealed additionally a history of drug-induced allergic systemic reactions at several times [18] and showed eosinophilia and IgE-elevation at time of allergic reactions. Skin tests for tuberculin in this individual were always negative. Both had not received immunization with any vaccines within the last 12 months prior to investigation.

From both individuals peripheral blood was obtained about every 3–4 months over a period of 12 months. In subject B a flu-like episode related to an unknown viral infection with fever, cough, lymph node swelling and sore throat occurred in month four and an allergic rhinitis and conjunctivitis due to grass pollen in month 6–8. Subject A also suffered from allergic rhinitis in month 6–8.

After an observation period of 12 months, subject A was immunized with tetanus toxoid (one dose = 0.5 ml = 40 I.E. tetanus toxoid), and PBMC were isolated on day 8, after four, eight, and 12 weeks, and after six and 11 months.

Antibodies

Purified antihuman IL-4, IL-5, IL-13, IFN- γ , and TNF- β monoclonal antibodies (mAb) and the respective biotinylated antihuman mAb as well as fluorescein isothiocyanate (FITC)-conjugated antihuman IFN- γ , R-phycoerythrin (PE)-conjugated antihuman IL-4, isotype-specific antimouse IgG1 and antirat IgG1 were purchased from Pharmingen (San Diego, CA, USA). R-phycoerythrin-cyanin (PC5)-conjugated anti-CD3 mAb was obtained from Immunotech (Marseille, France).

Antigens and reagents

Tuberculin purified protein derivative (PPD 1000 TU [tuberculin units]) and tetanus-toxoid (TT, 40 IU [international units]) were purchased from Chiron Behring (Marburg, Germany). PPD and TT were used in two (50, 5 TU/ml) and three concentrations (1, 0.1, 0.01 IU/ml), respectively. Phorbol 12-myristate acetate (PMA), ionomycin, and Brefeldin A were purchased from Sigma (St. Louis, MO, USA). Pokeweed mitogen (PWM) and phytohaemagglutinin (PHA) (both obtained from Biochrom, Berlin, Germany) were used as nonspecific stimulatory agents and applied at concentrations of 10 μ g/ml and 2.5 μ g/ml, respectively.

Cell culture

PBMC were isolated from heparinized blood by centrifugation through Ficoll-Hypaque. The cells were washed two times in Hanks' salt solution and resuspended at 1 Mio cells/ml in RPMI 1640 medium supplemented with gentamycin and 25% autologous serum. PBMC were cultured with the different antigens (see above) for 7 days at 37°C, 5% CO₂ in a humidified atmosphere.

Isolation of RNA and synthesis of cytokine cDNA

After a seven-day culture, total ribonucleic acid (RNA) was isolated from PBMC according to the acid guanidium thiocyanate/phenol/chloroform extraction [19]. RNA quantity and quality was determined by absorbance at 260/280 nm.

Cytokine-specific cDNA was synthesized in a 20- μ l reaction mix containing 100 ng RNA, 1 $\times$ RT-buffer (Gibco/BRL, Bethesda, MD, USA), 0.02 μ mol dNTP-mix (dATP, dCTP, dGTP and dTTP; Promega, Madison, WI, USA), 60 pmol cytokine antisense primer (TibMolBiol, Berlin, Germany), 10 mM DTT (Gibco/BRL), 16 U RNase inhibitor (Promega) and 50 U reverse transcriptase (Gibco/BRL). The reaction mixture was incubated at 37°C for 60 min followed by heating for 5 min at 95°C to inactivate the enzyme and stored at –20°C until use.

The following antisense primer were used: IFN- γ : 5'-ACCGAATAATTAGTCAGCTT (493 bp); TNF- β : 5'-GAGA AACCATCCTGGAGGAA (162 bp); IL-4: 5'-CCGTTTCAG GAATCGGATCA (300 bp); IL-5: 5'-GCTTCTGCATTGGA GTTTGCTAGCT (293 bp) [9,10].

Amplification of cytokine cDNA by PCR

Aliquots of reverse transcribed cytokine cDNA products (2 μ l) were used as a template for amplification by polymerase chain reaction (PCR). The 50 μ l PCR mixture contained 1 $\times$ PCR-buffer (Gibco/BRL), 2.5 mM MgCl₂ (Gibco/BRL), 20 pmol of the respective sense and antisense cytokine primer, 0.01 μ mol dNTP-mix and 1.25 U Taq (Thermus aquaticus)-polymerase (Gibco/BRL). Amplification was performed by 45 cycles consisting of denaturation at 94°C for 1 min, primer annealing at 55°C for 2 min, and primer extension at 72°C for 2 min in a thermocycler (Biometra, Göttingen, FRG, Germany).

PCR products (8 μ l) were electrophoresed on 2% agarose gels, visualized by ethidium bromide staining and photodocumented (Biometra, Göttingen, FRG, Germany). The following cytokine sense primer were used:

IFN- γ : 5'-AGTTATATCTTGGCTTTTCA;

TNF- β : 5'-CCTCACACCTTCAGCTGCCC;

IL-4: 5'-CCTCTGTTCTTCCTGCTAGC;

IL-5: 5'-GCTTCTGCATTGAGTTTGCT-AGCT [9,10].

Antisense-primer were the same as mentioned above.

Intracellular cytokine analysis by flow cytometry

For intracellular cytokine analysis, PBMC were isolated and cultured as described above. On day 7, cells were restimulated with PMA (50 ng/ml) and ionomycin (1 µg/ml) in the presence of Brefeldin A (10 µg/ml) for 6 h [20,21]. Cells were washed twice in phosphate-buffered saline (PBS)/2% fetal calf serum (FCS) and stained with anti-CD3 for 30 min. Cells were then washed with PBS/2% FCS and fixed in PBS containing 4% paraformaldehyde for 20 min. After two additional washes in PBS/2% FCS containing 0.2% saponin (permeabilization buffer) cells were resuspended in 100 µl permeabilization buffer and stained with 0.2 µg anti-IFN-γ and 0.1 µg anti-IL-4 antibodies or the respective isotype controls. After 30 min cells were washed two times in permeabilization buffer and resuspended in PBS/2% FCS for analysis.

Cells were analysed by three-colour flow cytometry using a Becton Dickinson FACScan flow cytometer. A minimum of 10 000 lymphocytes were counted. Intracellular cytokine production was analysed in the CD3+ lymphocyte population using Lysis II (Becton Dickinson) software. Quadrants were set based upon the isotype controls for each antibody. Results were expressed as the percentage of IL-4, IFN-γ or IL-4 and IFN-γ producing cells from all CD3+ cells.

Measurement of cytokines by ELISA

For cytokine production 5×10^5 PBMC/ml were cultured with the different antigens in 24-well culture plates and were maintained as indicated above. Culture supernatants were collected at day 7 and kept frozen at -20°C until quantitative cytokine determination [17].

Briefly, wells of a 96-well microtitre plate were coated overnight at 4°C with 1.75 µg/ml (IL-4, IL-5, IL-13, IFN-γ, TNF-β) antihuman cytokine monoclonal antibody in hydrogenbicarbonate buffer, pH 9.6, and blocked for 1 h at room temperature with phosphate buffered saline (PBS 60 mM, pH 7.4) containing 0.5% BSA. Culture supernatants and serum were used undiluted and incubated for 2 h at 37°C; biotinylated antihuman cytokine antibodies (1.25 µg/ml for IL-4, IL-5, IL-13, IFN-γ, TNF-β) were added for 2 h at 37°C. After another incubation for 1 h at room temperature with avidin-peroxidase (2.5 µg/ml), substrate solution (0.5 mg ortho-phenylenediamine/ml citrate buffer [pH 5.0] and 0.01% H₂O₂) was added. The reaction was stopped with 25% sulphuric acid and optical density was measured at 450 nm in a microtitre plate reader. Results were related to a standard curve obtained with cytokine standards (Pharmingen, San Diego, CA, USA) in different concentrations.

Cytokine production was considered positive above concentrations of 40 pg/ml for IL-4, 100 pg/ml for IL-5 and IL-13, and 500 pg/ml for IFN-γ and TNF-β.

Definition of cytokine profiles

In a previous study we had defined a type 1-profile by a positive IFN-γ (and/or TNF-β) production after stimulation of PBMC with PPD, but no cytokine production in response to tetanus-toxoid [17]. In contrast, a type 2-profile was characterized by the detection of IL-4, IL-5 and/or IL-13 after stimulation of PBMC with TT and no reactivity to PPD. A combined reactivity of PBMC to PPD and TT, resulting in IFN-γ/TNF-β and IL-4/-5/-13 production, respectively, to the corresponding antigen was classified as a mixed type 1/type 2-profile.

RESULTS

Comparison of different methods for the evaluation of type 1- and type 2-reactivity in two individuals with different types of an allergic manifestation

Measurement of type 1- and type 2-related cytokines by ELISA in sera and supernatants of PBMC. Incubation of PBMC from subject A with PPD resulted in a strong release of the type 1-cytokines IFN-γ and TNF-β into the supernatant, while tetanus-toxoid had no effect on the production of type 1- or type 2-cytokines (Fig. 1). In contrast, PBMC from subject B responded towards tetanus-toxoid with a strong production of the type 2-cytokines IL-4 and IL-5 (as well as IL-13; not shown), but there was no reactivity with PPD (Fig. 1, Table 1).

In the absence of any antigen, PBMC did not produce any cytokines. PWM or PHA led in both subjects to the release of type 1- and type 2-cytokines in parallel (data not shown).

In the sera from the two individuals no type 1- or type 2-related cytokines could be detected (Table 1).

Measurement of type 1- and type 2- related intracellular cytokines by flow cytometry. Measurement of the intracellular cytokines IFN-γ and IL-4 by flow cytometry in PBMC incubated with PPD resulted in both individuals in an exclusive increase of IFN-γ producing CD3+ T-cells (subject A: 33%, subject B: 20.2%) when compared to the cultures without antigen (subject A: 10.7%, subject B: 14.9%).

Incubation of lymphocytes from subject B with TT led to an increase of three different types of T cells namely those producing only IL-4 (B: 7.2% vs. 0.9% without antigen), those producing only IFN-γ (B: 29.4% vs. 14.9% without antigen) as well as those producing IL-4 and IFN-γ in parallel (B: 5.1% vs. 0.3% without antigen) (Fig. 2, Table 1).

In contrast, in subject A no intracellular cytokines were observed after stimulation of lymphocytes with TT.

Measurement of type 1- and type 2-related cytokine mRNA in PBMC of the two individuals by RT-PCR. There was a strong mRNA expression of the type 1- cytokines IFN-γ and TNF-β by PBMC of subject A towards PPD but no expression of IL-4 and IL-5 mRNA in response to tetanus-toxoid (type 1-profile). In contrast, in PBMC from subject B, IL-5 mRNA could be detected after tetanus-toxoid stimulation. IL-4 mRNA was not observed. Although incubation of PBMC with PPD in this subject did not induce mRNA expression of TNF-β, it led to a slight induction of transcription of the mRNA for the type 1-cytokine IFN-γ resulting in a mixed type 1/type 2-profile (Fig. 3, Table 1).

In the absence of these antigens, PBMC of both individuals did not express any type 1- or type 2-related cytokine mRNAs (Table 2).

In summary, all methods identified subject A as a 'type 1 responder'. Subject B, was defined by evaluation of the cytokines in the supernatants after stimulation of PBMC with PPD or tetanus-toxoid by ELISA as a pure 'type 2-responder' and by PCR and flow cytometry as a mixed type 1/type 2-responder.

Follow up of cytokine production in both individuals over a period of 12 months. In both individuals PBMC were obtained every 3–4 months over a period of 12 months and incubated with the two recall antigens PPD and TT. Lymphocyte supernatants from each time point were tested for type 1- (IFN-γ, TNF-β) and type 2-cytokines (IL-4, 5) by ELISA. As shown in Fig. 4, there was a fluctuation of cytokine levels in subject B during the 12 months according to allergic (hay fever) or infectious events (flu-like

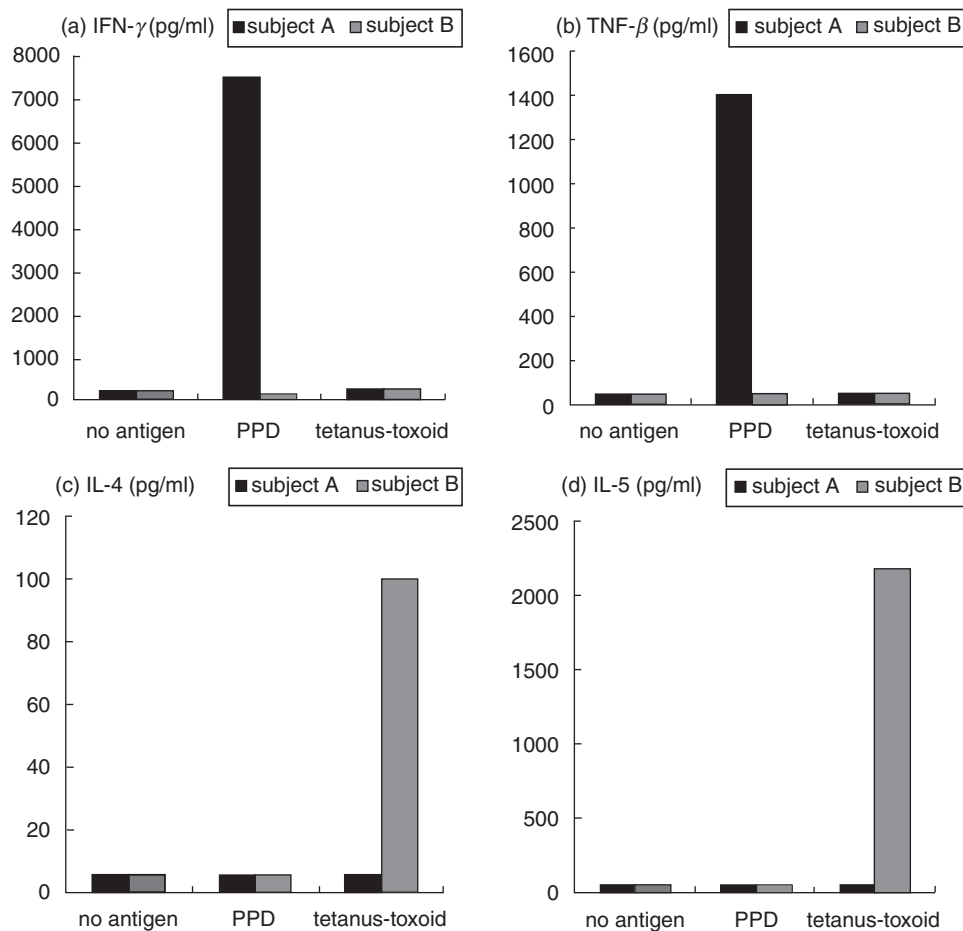


Fig. 1. Levels of type 1 (a, IFN- γ ; b, TNF β) and type 2 cytokines (c, IL-4; d, IL-5) in supernatants of PBMC stimulated with purified protein derivative (PPD) and tetanus-toxoid (TT). PBMC from subject A produce IFN- γ and TNF β after incubation with PPD but no type 2 cytokines after stimulation with TT ('type 1 profile'), while PBMC from subject B produce no type 1 cytokines after stimulation with PPD but IL-4 and IL-5 after incubation with TT ('type 2-profile').

Table 1. Comparison of different methods for the determination of cytokine profiles in three selected individuals

Method	Substrate for determination of type 1/type 2 cytokines	Cytokine production or expression of their mRNA after stimulation of PBMC with PPD/TT	
		Subject A IFN γ /IL-4 or -5	Subject B IFN γ /IL-4 or -5
ELISA	Supernatants from PBMC	+/- †	-/+ ‡
Flow cytometry	Lymphocytes	+/- †	+/+ §
PCR	Lymphocytes	+/- †	+/+ §

No cytokines could be detected in subjects' sera or in supernatants of PBMC which had not been stimulated by specific or nonspecific antigens. Incubation of PBMC with poke weed mitogen or phytohemagglutinin lead to a release of type 1 and type 2 cytokines in parallel in both individuals. † corresponds to cytokine profile 1 ‡ corresponds to profile 2 § corresponds to mixed profile type 1/type 2 Quantitative data for cytokine production are given in Figs 1, 4, and 5.

episode, presumably virus-related). In contrast, the cytokine profiles remained stable with the exception of a transient shift of type 2-profile to a mixed type 1/type 2-pattern during an infectious episode in subject B. Thus, there was a transient increase of IFN- γ production in month 4, which had again decreased in month 7 at

time of allergic rhinitis. At that time, IL-5 production strongly increased.

In subject A there was no alteration of the type 1 profile during that time despite manifestations of allergic rhinitis in month 6–8.

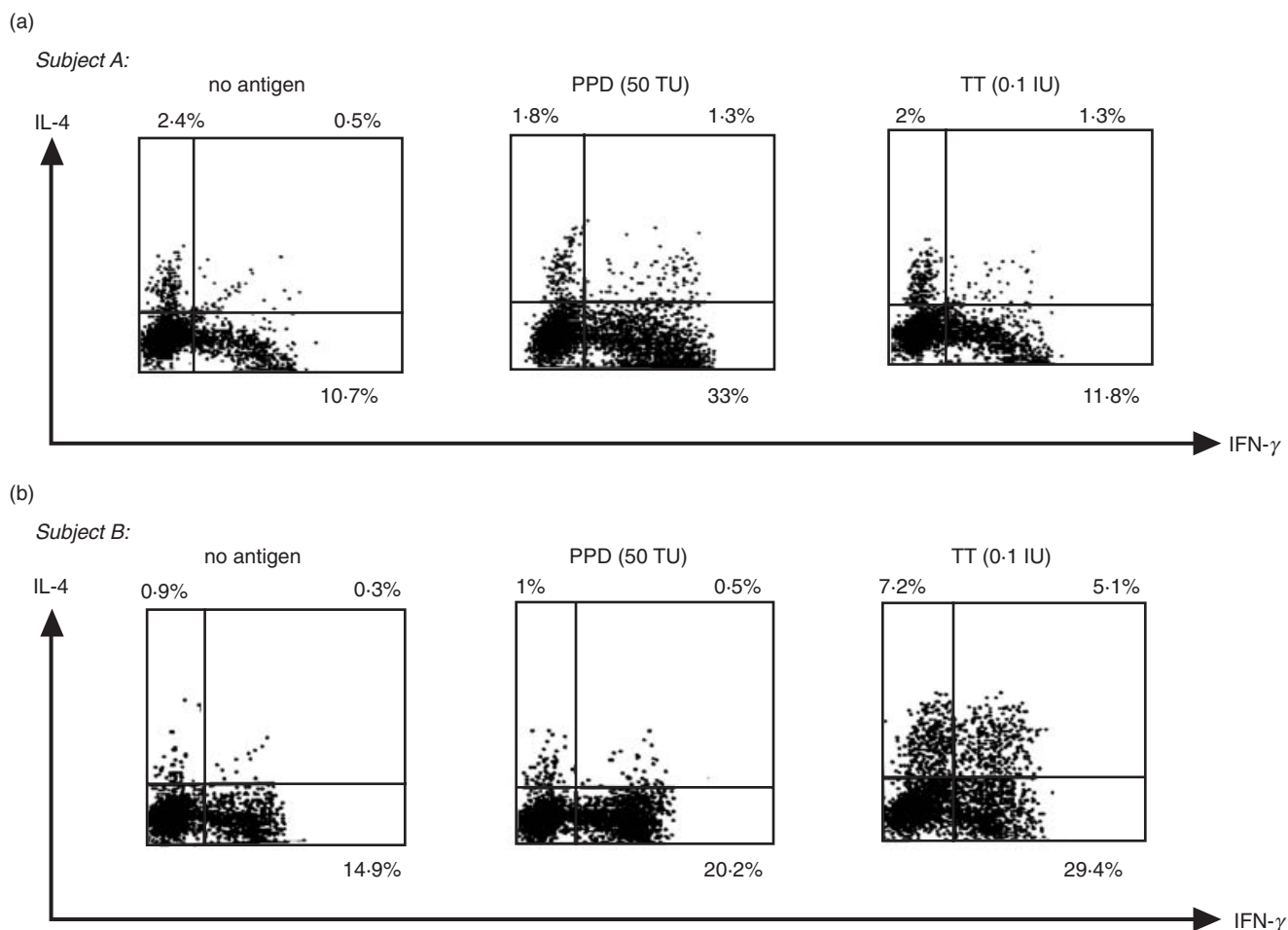


Fig. 2. Flow cytometry analysis of unstimulated PBMC and PBMC stimulated with PPD (50 TU/ml), respectively, tetanus-toxoid (TT) (0.1 IU/ml) from (a) subject A and (b) subject B. The percentage of positive CD3+ T cells producing IFN- γ only, IL-4 only and IFN- γ plus IL-4 was determined.

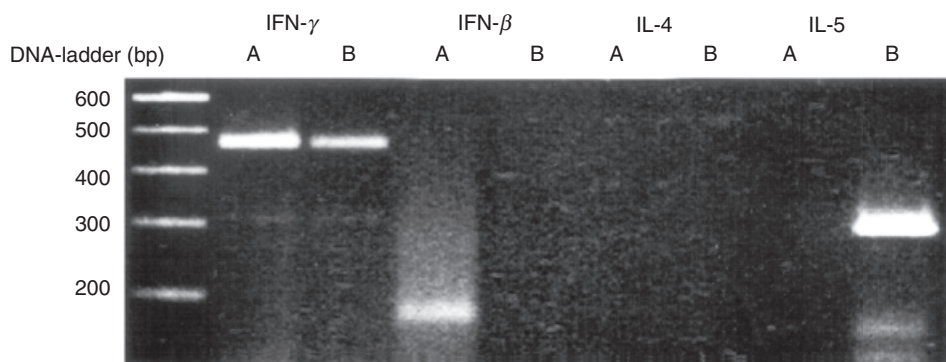


Fig. 3. Type 1- (IFN- γ , TNF β) and type 2- (IL-4, and IL-5) related cytokine mRNA expression by PBMC stimulated with PPD (50 TU/ml) and tetanus-toxoid (0.1 IU/ml), respectively, from subject A and B for seven days. Reverse transcriptase-polymerase chain reaction (RT-PCR) products were analysed on a 2% agarose gel and detected by ethidium bromide staining.

Influence of immunization with tetanus-toxoid on the type 1/type 2-balance in subject A

Subject A received immunization with tetanus toxoid. Eight days later, the PPD-induced IFN- γ production but also the IL-5 production by TT-stimulated PBMC had increased leading to a shift towards a mixed type 1/type 2-profile (Fig. 5). After a period of

about three months without further immunization the profile again had returned to a type 1-pattern.

DISCUSSION

In this study we wanted to compare different methods with

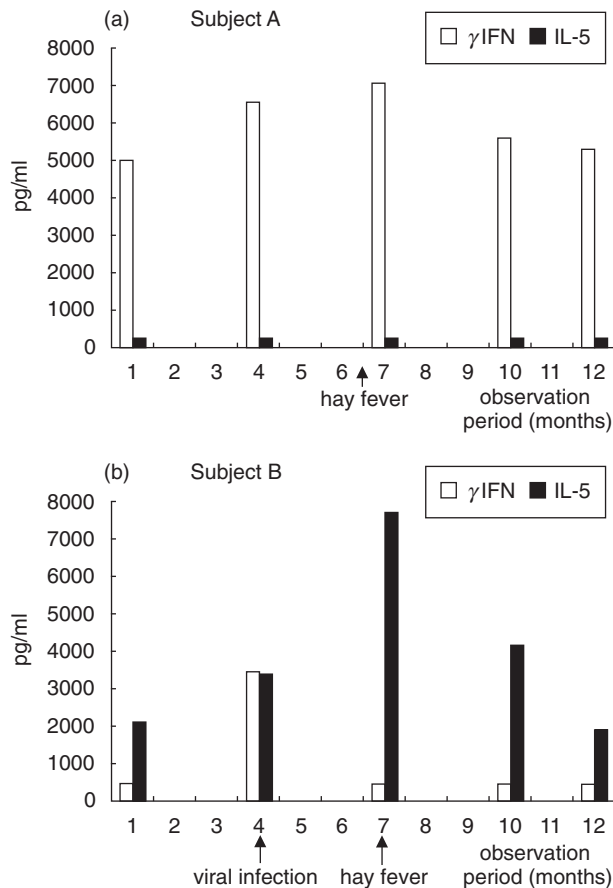


Fig. 4. Follow up of PPD-induced IFN- γ and TT-induced IL-5 production by PBMC from (a) subject A and (b) subject B over an observation period of 12 months. In subject A the cytokine profile (type 1) remained stable with slight fluctuations of PPD-induced IFN- γ levels. In subject B (type 2-responder) there was a transient slight increase of PPD-induced IFN- γ production due to a viral infection revealing a mixed type 1/type 2-profile during that time. Afterwards there was again a shift towards the type 2-profile with a strong increase of TT-induced IL-5 production due to an allergic episode.

respect to their reliability to determine 'immune profiles' in individuals, i.e. to define them as 'type 1-' or 'type 2-responder'. For this purpose we selected two subjects, with two different immunological manifestations of atopic/allergic conditions, one showing a strong tuberculin reaction (delayed type hypersensitivity, TH1/type 1-mediated; subject A), and the other elevation of serum IgE-levels and eosinophilia. Three different standard tests for the determination of type 1- and type 2- reactivity were performed: First, we measured the mRNA-expression of different cytokines by PCR after incubation of the subjects' lymphocytes with the type 1-antigen PPD and the type 2-antigen TT, second, we determined intracellular cytokines by flow cytometry, and third, we measured the secretion of cytokines by PBMC after incubation with the two antigens PPD and TT into the supernatants by ELISA. All three methods defined subject A as type 1-responder by the detection of mRNA of IFN- γ by PCR in PBMC, intracellular IFN- γ by flow cytometry and IFN- γ -secretion into supernatants of PPD-stimulated lymphocytes. Subject B revealed by the ELISA method a pure type 2-pattern while by PCR- and

flow cytometry-analysis type 1- and type 2-cytokines or their mRNA, respectively, were obtained in parallel resulting in a mixed type 1/type 2-pattern.

Analysing the cytokine profiles of both subjects within one day, no changes were observed (data not shown). Also follow up-studies of both individuals for 12 months indicated that the cytokine profiles did not change; an infectious process only transiently affected this pattern. When we examined subject A after exposure to TT, we found besides the PPD- induced IFN- γ -production an enhanced *in vitro*-secretion of IL-5 after stimulation of PBMC with TT, which began about eight days after immunization, lasted for about three months and then declined again revealing a transient switch from type 1- to a mixed type 1/type 2-pattern during that time. It is of interest that this subject mounted predominantly a type 1-reaction although he had – like subject B – a history of allergic reactions, namely neurodermitis and hay fever, which would rather suggest type 2-reactivity. This observation indicates that an allergic manifestation can be associated with type 1 and type 2 reactivity and that the magnitude and type of response to a general type 1- or type 2-inducer does not depend on previous or sensitizing exposure.

All three methods, PCR, flow cytometry and ELISA with lymphocyte supernatants are reliable and meanwhile well standardized methods for the determination of type 1- or type 2-reactivity; differences in the results are not surprising and can be readily explained by the fact that they visualize different stages within the process of cytokine-induction/production. However, detection of cytokine mRNA by PCR only confirms the gene expression but gives no information about the transcription into the protein. The observation, that expression of IL-4 mRNA was negative despite there was a secretion of IL-4 into the lymphocyte supernatants as measured by ELISA, may be related to the fact that all tests were performed on day 7; at this time point, we find all cytokines, which are not taken up again by lymphocytes (as for instance IL-2), in the supernatants while induction of mRNA expression may have become already negative. For instance, for the detection of IL-4 mRNA by PCR the optimal time point would be day 3 or 4; however, in order to simplify and unify the methodological procedure we have chosen day 7 which revealed positive results for most of the other relevant cytokines determined by ELISA and PCR. Determination of intracellular cytokines by flow cytometry gives a hint towards the transcription of the genes into proteins and allows to differentiate cytokine-producing cells by surface markers, but it lacks information whether the protein is, indeed, secreted. This method has further the disadvantage that nonspecific prestimulation of PBMC with PMA and ionomycin is required which is most likely to induce a mixed TH1/TH2-response [21]. Furthermore, this method is not suitable for the measurement of other cytokines than IFN- γ and IL-4. Therefore, the use of ELISA to measure cytokines in the supernatants of PBMC after stimulation with type 1- (PPD) or type 2- (TT) relevant antigens may be more relevant to evaluate the predisposition towards a type 1-, type 2- or mixed type 1/type 2- reactivity – also with respect to clinical manifestations – as already reported by other authors [14,15]. Stimulation with nonspecific mitogens such as pokeweed mitogen (PWM) or phytohemagglutinin (PHA) or other bacterial recall antigens (*Toxocara canis*, Pansorbin) did not allow such discrimination (data not shown), and serum samples are also not suitable for the definition of type 1- or type 2-reactivity.

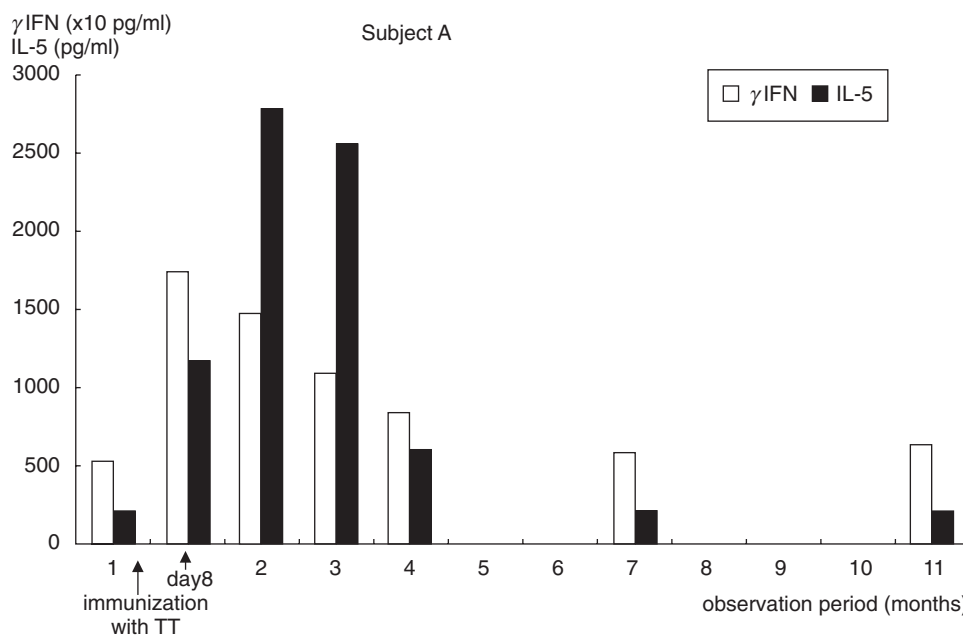


Fig. 5. Cytokine profiles and levels in subject A after immunization with tetanus toxoid (TT). Before immunization, the subject expressed a pure type 1 profile; exposure to tetanus toxoid lead to an increase of TT-induced IL-5-production *in vitro* (in addition to an increase of PPD-induced IFN- γ production) resulting in a mixed type 1/type 2 cytokine profile. Six months after immunization the cytokine-production has, again, returned to a pure type 1 profile.

In the meantime we have applied this test system to a larger group of allergic and nonallergic individuals and found that a reliable classification in type 1 or type 2-responder is, indeed, possible with this test system (manuscript submitted).

Although there is evidence that T-cell response within an individual can be greatly affected by antigen dose, i.e. the same antigen can induce TH1 or TH2-responses depending on the concentration as for instance shown for the mycobacterial antigen BCG [22 and own unpublished observation], from our experience this holds not true for the two antigens PPD and TT.

In conclusion, the presented data show that the test system analysing cytokines by ELISA in supernatants of PBMC after stimulation with type 1 or type 2 related antigens is a reliable *in vitro* method to determine 'type 1-' and 'type 2-responder'. This kind of refined immunological classification could be an important basis for the evaluation of disease activity and prognosis – as previously shown in patients with multiple sclerosis [17] – as well as for the development of new therapeutic strategies [12,23–27].

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REFERENCES

- Mosmann TR, Coffmann RL. TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. *Ann Rev Immunol* 1989; **7**:145–73.
- Mosmann TR, Sad S. The expanding universe of T-cell subsets: Th1, Th2 and more. *Immunol Today* 1996; **17**:138–46.
- Romagnani S. Th1 and Th2 in human diseases. *Clin Immunol Immunopathol* 1996; **80**:225–35.
- King C, Sarvetnick N. Organ-specific autoimmunity. *Curr Opin Immunol* 1997; **9**:863–71.
- O'Garra A, Steinman L, Gijbels K. CD4+ T-cell subsets in autoimmunity. *Curr Opin Immunol* 1997; **9**:872–83.
- Jujo K, Renz H, Abe J *et al.* Decreased interferon gamma and increased interleukin-4 production in atopic dermatitis promotes IgE synthesis. *J Allergy Clin Immunol* 1992; **90**:323–31.
- Katagiri K, Itami S, Hatano Y *et al.* Increased levels of IL-13 mRNA, but not IL-4 mRNA, are found in vivo in peripheral blood mononuclear cells (PBMC) of patients with atopic dermatitis (AD). *Clin Exp Immunol* 1997; **108**:289–94.
- Grewe M, Bruijnzeel-Koomen CAFM, Schöpf E *et al.* A role for Th1 and Th2 cells in the immunopathogenesis of atopic dermatitis. *Immunol Today* 1998; **19**:359–61.
- Yamamura M, Uyemura K, Deans RJ *et al.* Defining protective responses to pathogens: cytokine profiles in leprosy lesions. *Science* 1991; **254**:277–9.
- Rieckmann P, Albrecht M, Ehrenreich H *et al.* Semi-quantitative analysis of cytokine gene expression in blood and cerebrospinal fluid cells by reverse transcriptase polymerase chain reaction. *Res Exp Med* 1995; **195**:17–29.
- Mire-Sluis AR, Gaines-Das R, Thorpe R. Immunoassays for detecting cytokines: what are they really measuring? *J Immunol Meth* 1995; **186**:157–60.
- Ghoreschi K, Thomas P, Breit S *et al.* Interleukin-4-therapy of psoriasis induces Th2 responses and improves human autoimmune disease. *Nature Med* 2003; **9**:40–6.
- Berg PA, Klein R, Röcken M. Cytokines in primary biliary cirrhosis. *Sem Liver Dis* 1997; **17**:115–23.
- Del Prete GF, De Carli M, Mastromauro C *et al.* Purified protein derivative of *Mycobacterium tuberculosis* and excretory-secretory antigen (s) of *Toxocara canis* expand in vitro human T cells with stable and opposite (type 1 T helper or type 2 T helper) profiles of cytokine production. *J Clin Invest* 1991; **88**:346–50.
- ElGhazali GEB, Pauli S, Andersson G *et al.* Number of interleukin-4- and interferon- γ -secreting human T cells reactive with tetanus toxoid

- and the mycobacterial antigen PPD or phytohemagglutinin: distinct response profiles depending on the type of antigen used for activation. *Eur J Immunol* 1993; **23**:2740–5.
- 16 Liblau RS, Singer SM, McDevitt HO. Th1 and Th2 CD4+ T cells in the pathogenesis of organ-specific autoimmune diseases. *Immunol Today* 1995; **16**:34–8.
 - 17 Barth H, Klein K, Börtlein A *et al.* Analysis of immunoregulatory T-helper cell subsets in patients with multiple sclerosis: relapsing-progressive course correlates with enhanced TH1, relapsing-remitting course with enhanced TH0 reactivity. *J Neuroimmunol* 2002; **133**:175–83.
 - 18 Brattig NW, Diao GJ, Berg PA. The specificity of the lymphocyte transformation test in a patient with hypersensitivity reactions to pyrazolone compounds. A 10-week follow-up study before and after rechallenge. *Eur J Clin Pharmacol* 1988; **35**:39–45.
 - 19 Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 1987; **162**:156–9.
 - 20 Jung T, Schauer U, Heusser C *et al.* Detection of intracellular cytokines by flow cytometry. *J Immunol Meth* 1993; **159**:197–207.
 - 21 Schauer U, Jung T, Krug N *et al.* Measurement of intracellular cytokines. *Immunol Today* 1996; **17**:305–6.
 - 22 Krause I, Blank M, Shoenfeld Y. Immunomodulation of experimental autoimmune diseases via oral tolerance. *Crit Rev Immunol* 2000; **20**:1–16.
 - 23 Cottard V, Mulleman D, Bouille P *et al.* Adeno-associated virus-mediated delivery of IL-4 prevents collagen-induced arthritis. *Gene Ther* 2000; **7**:1930–9.
 - 24 Gran B, Tranquill LR, Chen M *et al.* Mechanisms of immunomodulation by glatiramer acetate. *Neurology* 2000; **55**:1704–14.
 - 25 Teitelbaum D, Arnon R, Sela M. Immunomodulation of experimental autoimmune encephalomyelitis by oral administration of copolymer 1. *Proc Natl Acad Sci USA* 1999; **96**:3842–7.
 - 26 Chen Y, Rosloniec E, Goral MI *et al.* Redirection of T cell effector function in vivo and enhanced collagen-induced arthritis mediated by an IL-2R beta/IL-4R alpha chimeric cytokine receptor transgene. *J Immunol* 2001; **166**:4163–9.
 - 27 Raz I, Elias D, Avron A *et al.* Beta-cell function in new-onset type 1 diabetes and immunomodulation with a heat shock protein peptide (DiaPep277): a randomized, double-blind, phase II trial. *Lancet* 2001; **358**:1749–53.