Effects of *M. catarrhalis* antigens on IFN-γ, IL-4 and IL-13 production by PBMC from children with atopic asthma or recurrent respiratory tract infections

DARIA AUGUSTYNIAK[1], GRAŻYNA MAJKOWSKA-SKROBEK[1], MARIA KRAUS-FILARSKA[2], ADAM JANKOWSKI[3]

[1]Immunology Unit, Department of General Microbiology, Institute of Genetics and Microbiology, University of Wroclaw, Poland; [2]Department of Internal Medicine and Allergology, Medical University of Wroclaw, Poland; [3]Department of Children's Immunology, Medical University of Wroclaw, Poland

Abstract

The goal of this study was to evaluate the in vitro Th1/Th2 cytokine response as well as proliferative response in M. catarrhalis antigen-stimulated PBMC from 14 atopic asthma children (AA) and 11 non-atopic children suffering from recurrent respiratory tract infections and bronchospastic symptoms (RRTI). Membrane (MA) and soluble (SA) antigens of M. catarrhalis induced PBMC to production of IFN-γ but did not induce cells to secretion of IL-4 and IL-13. The production of IFN-γ after stimulation with both MA and SA was considerably higher than spontaneous ones within each group (AA and control, P<0.001; RRTI, P<0.01). Furthermore, proliferative response of PBMC following SA stimulation between 4- and 7-day of culture showed significant increase contrary to the MA stimulation in analogous time. PBMC from RRTI group secreted significantly less IFN-γ as compared with controls (MA, P<0.05; SA, P<0.001). Release of IL-4 and IL-13 by PBMC did not differ between the three groups. Concluding, the comparable productions of IFN-γ during stimulation of PBMC with MA and SA suggest, that apart from the former also the latter may have a strong impact on the Th1-type cytokine response. We proposed also that reduced IFN-γ in RRTI group may be one of important factors in non-effective defence mechanism.

Key words: *Moraxella catarrhalis, IFN-γ, IL-4, IL-13, specific IgE, atopic asthma, recurrent respiratory tract infections, PBMC*

Introduction

Moraxella catarrhalis is a bacterium which belongs to the physiological microflora of the upper respiratory tract of human [1]. Degradation of such microflora may lead to the release of new immunogens whose long-lasting exposition, in some cases, may disturb the immunological response. In fact, infective agents together with allergic ones act on the base of positive feedback. On one hand, allergic disorders with accompanying inflammatory process in the mucosa enable microorganism to invade inside the mucus. On the other hand, bacteria by their enhancing or stimulatory effect on allergic mediator release are clinically relevant contributors to asthma exacerbation [2].

Human CD4+ T cell clones specific for bacterial antigens or allergen-specific T cell clones have been found to exhibit Th1 or Th2-like cytokine production profiles [3, 4]. IL-4 and IL-13 produced by Th2 cells are strongly implicated in the pathogenesis of atopy and allergic inflammatory diseases, whereas IFN-γ produced by Th1 cells is a potent

Correspondence: Daria Augustyniak, PhD, Institute of Genetics and Microbiology, University of Wroclaw, Przybyszewskiego 63/77, 51-148, Wroclaw, Poland. Phone: +48 71 375 62 96, fax: +48 71 325 21 51, e-mail: august@microb.uni.wroc.pl

antimicrobial agent that potentiates cellular immunity [5]. The antagonistic role of IL-4 and IFN-γ in the regulation of the IgE production in human systems is well established [6]. The influence of extracellular bacteria on IL-4/IFN-γ imbalance is known particulary in atopic dermatitis in response to *S. aureus* antigens [7], that through IL-4 induction seem to act in a manner typical for allergens. On the contrary, the participation of respiratory tract microflora antigens in inflammatory-allergic process is still controversial, and the role of such bacteria-specific IgE is still debated. *M. catarrhalis* has been chosen for this study because it colonizes the respiratory tract of human from early childhood [8], hence is more likely to be an important reservoir of pathogens during infection. Moreover, in the previous decade, *M. catarrhalis* was accepted together with *H. influenzae, B. pertussis* or *S. aureus* as a participating in allergic process microorganism. The role of *M. catarrhalis* in allergy includes: the ability to induce release of histamine, both in IgE-mediated and in lectin-mediated reactions [9, 10], the capacity to synthesize the clinically significant amounts of histamine de novo from histidine [11], and the induction of specific antibacterial IgE [12, 13].

To elucidate the in vitro cell-mediated Th1/Th2 response to *M. catarrhalis* stimulation in non-atopic children with recurrent respiratory tract infections and bronchospastic symptoms (RRTI), and in children with atopic asthma (AA), the aim of this study was to determine: 1) whether membrane and soluble antigens of *M. catarrhalis* are involved in IFN-γ, IL-4 and IL-13 production by PBMC; 2) are there significant differences in cytokine and proliferative response against *M. catarrhalis* between studied groups. IFN-γ, IL-4, and IL-13 inducers activate T cells either in a polyclonal or in a clonally restricted, antigen-specific manner. To understand the coordinate expression of Th1 and Th2 cytokines following the natural mode of T cell triggering, bacterial antigen-stimulated IFN-γ, IL-4, and IL-13 production was studied in primary PBMC cultures.

Materials and methods

Patients

Blood samples were obtained from 14 children (aged 5–15 years) with mild or moderate stage of atopic asthma (AA), 11 non-atopic children (aged 5–15 years) with recurrent respiratory tract infections and bronchospastic symptoms (RRTI), and 11 age-matched individuals of control group (C). The diagnosis of asthma was made according to such criteria as: recurrent episodes of dyspnea, chest tightness or whezzing, the atopic status of subjects confirmed by total IgE and/or positive skin prick test with common airborne allergens, family history. The RRTI group involved children who were suffering from at least 8 respiratory infections during last year including pneumonia and/or bronchitis. The control group included healthy children with negative history of asthma or other allergic, parasitic or immunologic diseases. All children were free of acute infection at the time of testing and have not been receiving any antibiotics during the preceding 3 weeks. Additionally the examination of asthma children was done during the asymptomatic period. Antibodies to *M. catarrhalis* were identified by using a whole cell ELISA assay described in detail and developed by Goldblatt et al. [14]. The seropositivity of test serum samples was confirmed by the presence of specific IgG antibodies. Results of the unknown isotype were expressed as a percentage of the relevant isotype in control pooled serum (internal standard) derived from 20 healthy children. This control serum was used to construct a standard curve on each ELISA plate. Demographic characteristics of studied populations are shown in Table 1. The study was approved by the Bioethical Commitee of Medical University of Wroclaw (no 139/2000).

Bacterial strains and growth conditions

Clinical isolate of *Moraxella catarrhalis* was collected from the sputum of the child with RRTI. Sputum sample was

Table 1. Profile of studied children

	AA	RRTI	Control
No.	14	11	11
Mean age/range	10 (5–15)	8 (5–15)	11 (5–15)
Sex (M:F)	6:8	1:10	4:7
Total IgE (IU/ml)	122 (8–900)	15 (5–70)	12 (6–48)
Specific IgG (%)*	85 (60–114)	113 (84–137)	100
IgE anty-M. catarrhalis (% of IgE-positive sera)	21	63	0

* The results of the unknown isotype in serum were expressed as a percentage of the relevant isotype in control pooled serum from 20 healthy subjects; The results of IgG represent geometric mean and range.

cultured at 37°C on Blood Agar (OXOID) containing 5% sterile sheep blood. The identity of the isolate was confirmed with standard criteria including Gram staining, oxidase and catalase production, nitrate reduction and inability to produce acid from glucose, maltose, sucrose and lactose [15]. In some experiments strain *E. coli J53* from Polish Collection of Microorganisms (PCM) was used. Strains were cultured at 37°C in brain heart infusion (BHI) broth (MERCK) and stored in BHI containing 16% glycerol at -70°C.

Preparation of bacterial antigens

Moraxella catarrhalis was routinely cultured at 37°C in BHI until stationary phase. Bacterial suspension from 100 ml of culture was centrifuged (4000 x g, 15 min., 4°C). The pellet was resuspended in 20 volumes of buffer Tris-HCl (25 mM Tris, pH 6.8) and centrifuged as above. The pellet was resuspended in 9 ml of buffer Tris-HCl-EDTA (25 mM Tris, 2 mM EDTA, pH 6.8) and sonicated for 7x60 s in 60 s intervals. The crude sonicate extract was centrifuged (10 000 x g, 20 min, 4°C) and the resulting supernatant was ultracentrifuged (100 000 x g, 1.5 h, 4°C). The pellet defined as membrane antigens (MA), whereas supernatant called soluble antigens (SA). Both membrane and soluble antigens were used in later experiments at final concentration of 5 µg/ml of bacterial protein per ml. In some experiments the formalin-killed *M. catarrhalis* and *E. coli J53* were used for stimulation of PBMC. Bacteria were prepared according to Banck&Forsgren [16] and used at a final concentration of 5×10^7 cells/ml. Prepared bacterial antigens and killed bacteria were stored in RPMI-1640 (Sigma) at -70°C until they were used as stimuli.

PBMC cultures

PBMC were separated from heparinized blood by ficoll density gradient centrifugation, washed two times in sterile Hanks medium (BIOMED-Lublin) with 18 U/ml of neomycin. PBMC were cultured at a final concentration of 10^6 cells per ml in RPMI-1640 culture medium supplemented with 15% heat-inactivated autologous human plasma, 2 mM L-glutamine and 18 U/ml neomycin at 37°C in an atmosphere containing 5% CO_2.

For cytokine production, 0.5 ml of PBMC aliquots at an initial cell density of 10^6 cells/ml was mixed with an equal volume of the RPMI culture medium and incubated in tissue culture tubes (NUNC) with 5 µg/ml of membrane or soluble antigens of *M. catarrhalis* or medium only. Cell-free supernatants were collected after 48 h of incubation and stored at -70°C pending cytokine measurements.

For proliferation assay, freshly isolated PBMC were cultured in the absence or presence of 5 µg/ml of antigens (MA or SA) or 5×10^7 cell/ml of the killed *M. catarrhalis* or *E. coli* in 96 round-bottomed microculture plates (Falcon) in three replicates (10^5 cells per well/final volume 200 µl). The microcultures were incubated at 37°C in a 5% CO_2 humidified atmosphere. Cellular proliferation was determined on day 4 and additionally for MA and SA on day 7. Sixteen hours before harvesting the cultures were pulsed with 0.4 µCi/well of [^{3}H]thymidine (Lacomed, Czech Republik). The uptake of the isotope was measured with a scintillation counter (Beckman LS 100C). Data were expressed as the stimulation index: the ratio of the mean cpm values of experimental cultures (in the presence of bacterial antigens) to the mean cpm values of the control cultures (without bacterial antigens). Viability was ensured by trypan-blue staining. In all experiments PBMC were stimulated using the optimal concentrations of tested bacterial antigens which were previously defined.

Cytokine assays

The measurements of cytokine concentrations in culture supernatants were carried out by commercially available ELISA-systems, according to the manufacturer's instructions. IL-4 was measured on a human IL-4 ELISA kit (R&D) whereas IL-13 and IFN-γ on a human IL-13 or on a human IFN-γ ELISA kit (BenderMedSystems), respectively. Sensitivities of these assays were <4.1 pg/ml for IL-4 and <1.5 pg/ml for IL-13 and IFN-γ. All plates were read with a Dynatech ELISA spectrofotometric reader at the appropriate wavelengths. All samples were run in duplicate. Cytokine production in response to antigen stimulation was calculated by subtracting cytokine levels in unstimulated cultures from cytokine levels in antigen-stimulated cultures.

Specific IgE assay

Sera samples were stored at -70°C until processed for IgE anti-*M. catarrhalis* measurements. The IgE concentrations were determined by EAST – (enzymeallergosorbent test) using AT-PLUS Allergy Test EAST/MTP kit (DDV Diagnostica) according to the manufacturer's instruction. Concentration of IgE was determined in classes from 0 to 4 on the basis of the reference sera (class 0: IgE <0.35 IU/ml (undetectable); class 1: IgE 0.35–0.7 IU/ml (low level); class 2: IgE 0.7–3.5 IU/ml (moderate level); class 3: IgE 3.5–7 IU/ml (high level); class 4: IgE >7 IU/ml (very high).

Statistical analysis

Statistical analysis was performed by nonparametric tests. The Mann-Whitney *U* test was used to compare cytokine production between the study groups, whereas the Wilcoxon's test was used to compare cytokine secretion and dynamics of proliferative response within each group. For comparison of proliferative response of PBMC to different stimuli, Walda-Wolfowitza test was used. Coefficients of correlation were calculated by the Spearman rank test. Significant differences were accepted when *P*<0.05.

Table 2. Comparative stimulation index (SI) of 4-day PBMC cultures induced by *M. catarrhalis* and *E. coli* J53 in children with AA, RRTI and control individuals

Study groups	*M. catarrhalis*		*E. coli* J53		Statistical significance
	Mean ± SD*	Range	Mean ± SD	Range	
AA (n=14)	8.15 ± 6.29	(2.32–22.66)	1.36 ± 0.58	(0.55–2.3)	*P*<0.0001
RRTI (n=11)	4.66 ± 3.96	(1.42–13.41)	1.36 ± 0.53	(0.75–2.35)	*P*<0.05
Control (n=11)	6.19 ± 2.42	(1.77–9.67)	1.86 ± 0.73	(0.72–2.84)	*P*<0.01

SI was calculated as the mean counts per minute in wells stimulated by bacteria divided by the mean counts per minute in wells without bacteria;
* – geometric mean ± standard deviation; statistics were performed with Walda-Wolfowitza test.

Table 3. Comparative stimulation index (SI) of 4-day and 7-day PBMC cultures induced by MA and SA in children with AA, RRTI and control individuals

Study groups	MA (5 μg/ml) Mean ±SD** (Range)		SA (5 μg/ml) Mean ±SD (Range)		Statistical significance
	4 day	7 day	4 day	7 day	
AA (n=14)	3.56 ± 1.96 (1.11–7.65)	3.43 ± 1.45 (1.77–6.76)	1.63 ± 0.49* (0.86–2.64)	2.77 ± 1.37* (1.13–6.54)	*P*<0.002
RRTI (n=11)	2.31 ± 1.57 (1.15–6.12)	2.58 ± 2.21 (1.03–6.87)	1.49 ± 0.39* (0.91–2.27)	2.45 ± 1.61* (0.96–6.78)	*P*<0.02
Control (n=11)	2.14 ± 1.21 (0.84–5.05)	2.48 ± 2.41 (0.92–6.1)	1.55 ± 0.72* (0.96–3.22)	3.0 ± 1.35* (0.92–5.31)	*P*<0.008

SI was calculated as the mean cpm in wells with bacterial antigens divided by the mean cpm in wells without bacterial antigens; ** – geometric mean ± standard deviation; * – statistics were performed with the Wilcoxon's test and considered the differences between 4ᵗʰ and 7ᵗʰ day in SA – stimulated proliferative responses.

Results

Differences in proliferative response of PBMC between *M. catarrhalis* and *E. coli* stimulation

To confirm the higher responsiveness of PBMC to *M. catarrhalis,* we compared the proliferative response of PBMC using optimal doses of *M. catarrhalis* and *E. coli J53* formaline-killed cells. When the ability of both bacteria to stimulate lymphocytes was tested by ³H-thymidine incorporation, it was observed that *M. catarrhalis* induced significantly higher stimulation indices than *E.coli* did, regardless of the study group (Table 2). The strongest proliferative responses of PBMC to *M. catarrhalis* were seen in AA group whereas the weaker were observed in RRTI group, however, these differences were not statistically significant.

Proliferative response of PBMC to MA and SA of *M. catarrhalis*

Dynamics of proliferative response of PBMC following the stimulation with MA showed marked differences in comparison with SA stimulation in all groups. In general MA induced stronger proliferative response of PBMC in 4-day culture, however after 7 days MA-stimulated response was similar to that induced by SA, indicating its increasing activity in future. This increasing tendency of SA-stimulated PBMC response was supported by the existence of statistically significant differences between 7ᵗʰ and 4ᵗʰ day within each group of children (Table 3).

IFN-γ production in PBMC cultures

The production of IFN-γ during a 48-hour cultivation of PBMC with MA and SA was considerably higher as compared to the spontaneous ones in all groups. An increase of IFN-γ was varied in children having from 7 up to 200-fold increase and statistically significant differences were found within each group (*P*<0.001 for AA and C; *P*<0.01 for RRTI) (Fig. 1). Furthermore, it has been demonstrated that PBMC from children with RRTI after stimulation with both MA and SA secreted significantly less IFN-γ than control individuals (33.06 pg/ml±28.43 *vs* 106.07 pg/ml±119.53 for MA, *P*<0.05 and 33.20 pg/ml±30.48 *vs* 113.24 pg/ml±61.75 for SA, *P*<0.001)
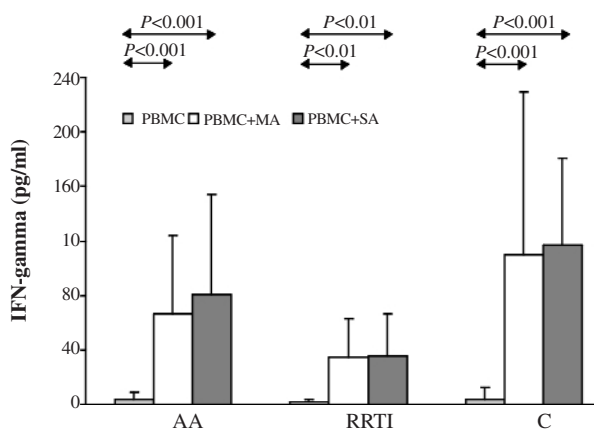
Fig. 1. Comparison of spontaneous (without antigens) and *M. catarrhalis* antigen-stimulated production of IFN-γ during 48 h PBMC cultures from AA, RRTI and control (C) children. PBMC (5×10^5 cells/ml) were cultured in the presence or absence of 5 µg/ml of MA or SA. The IFN-γ concentrations in supernatants were determined by ELISA. The results indicate geometric mean values ±SD. IFN-γ was not detected in supernetants harvested in the absence of bacterial antigens, whereas incubation PBMC with these antigens led to statistically significant amounts of IFN-γ within each group. Statistics were performed with Wilcoxon's test

(Fig. 2). PBMC from children with AA also tended to release less IFN-γ (62.37 pg/ml±55.18 for MA; 76.42 pg/ml±71.84 for SA) than controls in both cases, nevertheless, the decreased production did not reach statistical significance. No considerable differences were observed in IFN-γ concentrations within each group when stimulation of PBMC with MA to SA was compared.

IL-4 and IL-13 production in PBMC cultures

Production of IL-4 and IL-13 after stimulation with MA or SA observed in PBMC cultures, were on the similar low levels (geometric mean value: <17 pg/ml for IL-4 and <12 pg/ml for IL-13) in all studied groups of children (Fig. 3). When PBMC were stimulated with bacterial antigens, similar patterns of IL-4 expression were detected within the AA, RRTI or control children regardless of the presence of MA or SA. There were no statistically significant differences in IL-4 or IL-13 releasing between MA and SA stimulation of PBMC.

Specific antibacterial IgE

The higher titers of anti-*M. catarrhalis* serum IgE have been obtained in RRTI group, in which 7 of 11 (63%) serum samples had detectable IgE levels (class 1 – 45%, class 2 – 18% of sera) than in AA group where only 3 of 14 (21%) serum samples had specific IgE (class 1 – 14%, class 2 – 7% of sera). There were no detectable specific IgE (class 0) in 11 control individuals (Table 1). The mean negative
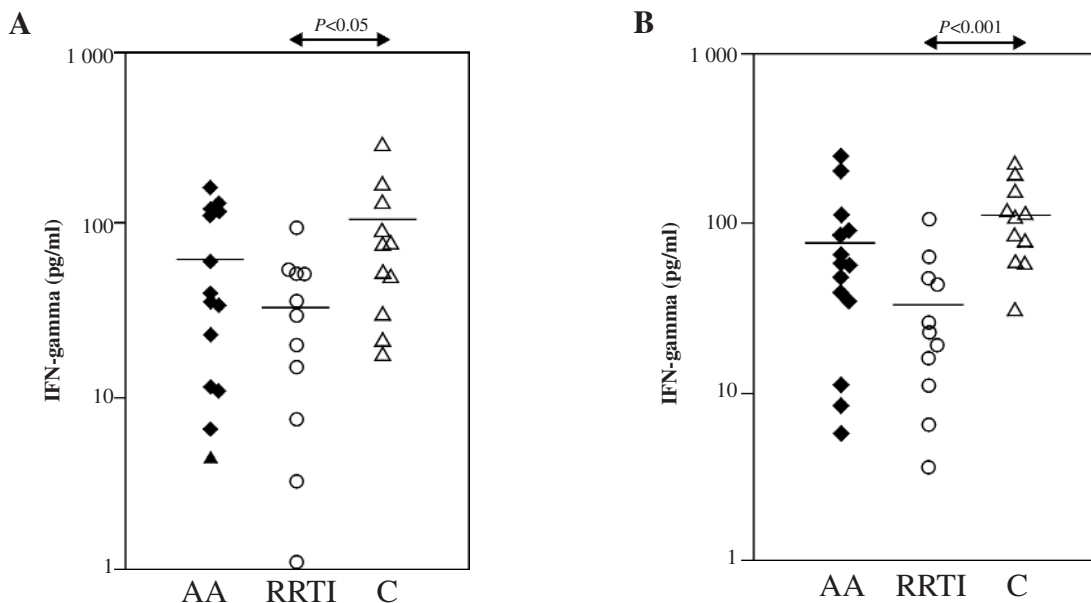

Fig. 2. Supernatant amounts of IFN-γ produced by PBMC (5×10^5 cells/ml) following stimulation with 5 µg/ml of MA (**A**) and SA (**B**) from AA, RRTI and control (**C**) group. Supernatants were collected after 48 h of culture and IFN-γ were determined by ELISA. Significantly less IFN-γ was found in RRTI than in C group regardless of the used stimuli. Bars indicate geometric mean values. Statistics were performed with Mann-Whitney U test
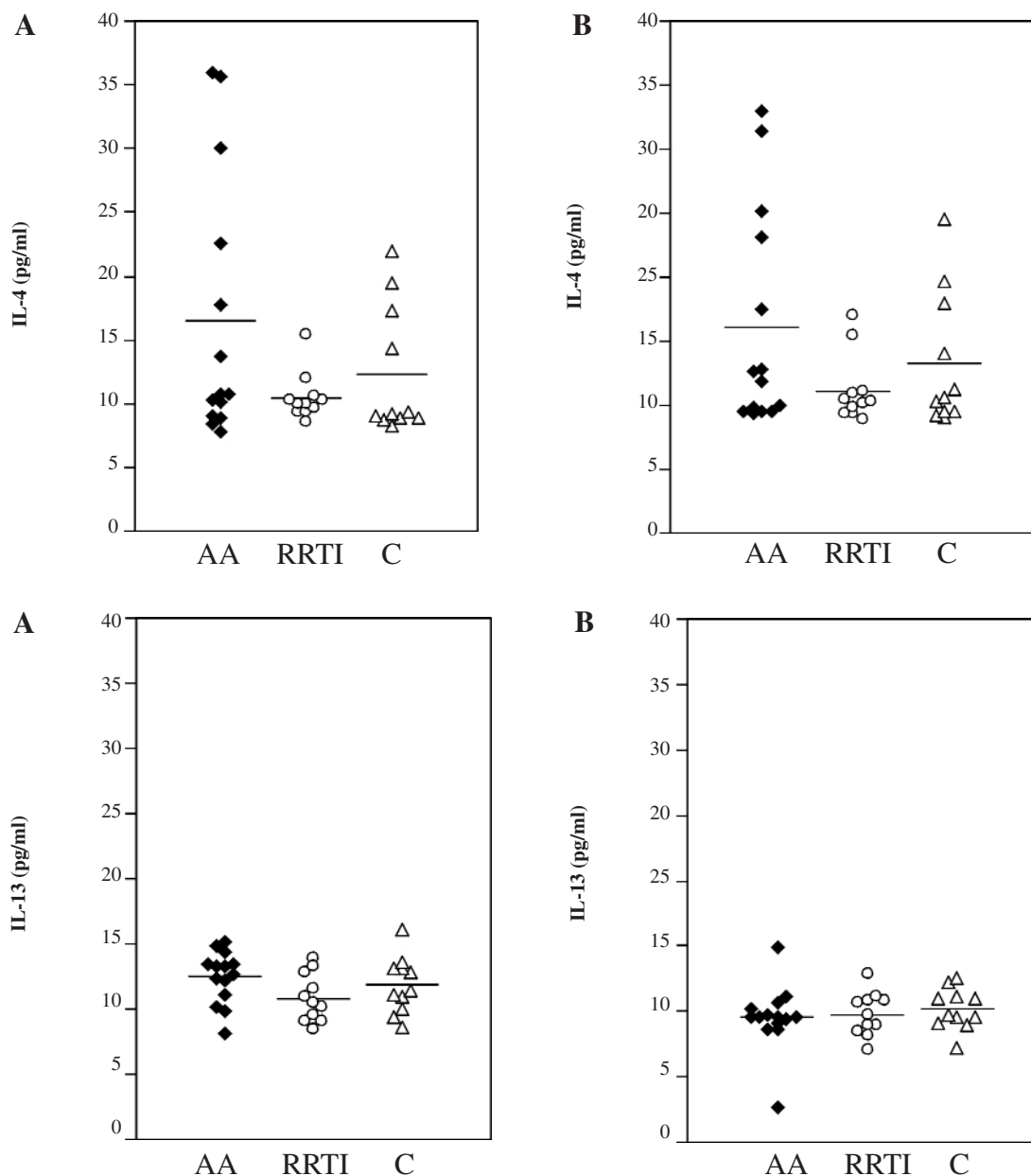
Fig. 3. Supernatant amounts of IL-4 and IL-13 produced by PBMC (5x10⁵ cells/ml) after stimulation with 5 µg/ml of MA (**A**) and SA (**B**) cultures from AA, RRTI and control (C) children. Supernatants were collected after 48 h of culture and cytokine concentrations were determined by ELISA. No statistically significant differences were shown for IL-4 or IL-13 production between study group of children. Bars indicate geometric mean values

correlation, with Spearman coefficient of correlation – 0.45, $P<0.05$, between serum concentration of specific antibacterial IgE and IFN-γ production after SA stimulation was observed.

Discussion

In this study we demonstrated that membrane and soluble antigens of *M. catarrhalis* induced PBMC to production of IFN-γ in cultures from AA, RRTI and C children (Fig. 1). Next, we demonstrated lack of marked differences in IFN-γ production after membrane and soluble antigen stimulations within each group (Fig. 2). This similar activity of studied antigens in cytokine induction as well as in 7-day proliferative response (Table 3) indicates, that although membrane antigens of *M. catarrhalis* are the main ones that trigger a protective response [1, 17], soluble

components (proteins and DNA) also may have a potent immunogenic properties. In fact, the influence of immunostimulatory activity of bacterial DNA containing unmethylated CpG motifs on IFN-γ production by PBMC [18] as well as on different human cells [19] has been recently reported. Magnitude of the IFN-γ responses varied considerably in individual blood donors, what could be due to the intensification of the prior immunization of organism by *M. catarrhalis.* Our present study revealed that the level of IFN-γ in supernatant was significantly decreased in RRTI children compared with a control group, regardless of the membrane or soluble antigen stimulation (Fig. 2). The existence of diminished IFN-γ production in children with RRTI may reflect the primary defect or the impaired function of IFN-γ synthesis by lymphocytes, as a results of their frequent activation in response to constantly repeating infections. However, it should be defined whether such phenomenon may be an important factor in the pathogenesis of RRTI. This finding was accordant with previous study in which reduced IFN-γ production by isolated CD4 and CD8 cells but in the case of response to polyclonal stimulation in nonatopic children with recurrent wheeze has been reported [20]. Decreased IFN-γ production by PBMC in atopic subjects is well known [21, 22], and is probably associated with the heterozygous IL-12R beta2 mutations in those patients [23]. Reduced IFN-γ production has been also demonstrated in children with moderate atopic asthma [24]. We demonstrated that although AA children produced less IFN-γ than control ones, the results did not reach statistical significance, probably because of mild stage of asthma in several children.

It is noteworthy that PBMC from RRTI group apart from reduced capacity to produce IFN-γ, generated also weaker proliferative response to killed *M. catarrhalis* (Table 2). More interestingly, the lowest IFN-γ levels were detected in a group, in which the highest titer of specific antibacterial IgE was observed (Table 1). Although only a limited number of children were tested, these results may lead to speculation that diminished production of IFN-γ may contribute to the higher specific IgE levels observed in RRTI children. Higher IgE amounts occur in the absence of elevated IL-4 and IL-13 secretion from PBMC, but can not exclude a local increase of these cytokines in respiratory tract. Such effect could cause the increase of local IgE that can be then detected in peripheral circulation. For now, it is still unclear whether elevated IgE concentrations in the peripheral blood reflect the local synthesis of IgE in bronchial mucosa, because of not precisely stated sites of IgE synthesis *in vivo* [25]. Negative correlation between serum IgE and IFN-γ production by PBMC has been previously reported in atopic patients [26]. Despite the fact that RRTI children were free of atopy, a mean negative correlation between specific antibacterial IgE and IFN-γ production after SA stimulation was found, so it is likely that bacterial antigens and specific anti-bacterial IgE may

play some role in the development of intrinsic asthma in such children in future, since it could be IgE mediated disease. According to Gergen et al. [27] 50% of wheezy children suffering from recurrent or chronic bronchitis is the group of risk in asthma occurence.

Exposure of PBMC to *M. catarrhalis* antigens did not reveal IL-4 and IL-13 production. The levels of both cytokines in culture supernatans were low independently, whether membrane or soluble antigens were used as stimuli (Fig. 3). The observed IL-4 and IL-13 amounts were not due to poor proliferative response of PBMC to bacterial antigens, since the levels of lymphokines did not correlate with the magnitude of the lymphocyte proliferation (data not shown). Low quantities of cytokines could be partially explained by the low frequency of antigen-specific T cells in peripheral blood, that should be rather accumulated in the upper airways where *M. catarrhalis* does exist. However, significantly increased proliferative responses of PBMC to *M. catarrhalis*, as compared to *E. coli*, (Table 2) and small amounts of IL-4 and IL-13, even when stimulation indices were very high (SI >10), suggest rather lack of production of these cytokines than absence of antigen-specific clones in peripheral blood. Although stimulation of PBMC by bacterial antigens induces predominantly Th1-type response both in atopic and normal subjects [28], there are known bacterial antigens like enterotoxins of *S. aureus* [7] or antigens of *B. pertussis* [29] that are able to stimulate IL-4 synthesis in atopic or non-atopic, respectively. On the other hand, low levels of IL-4 in children with moderate asthma in allergen inducted PBMC *in vitro* have been also reported [30]. Since no differences in Th2-type cytokine productions were observed between PBMC of patients and healthy donors, it is tempting to speculate that investigated bacterial antigens do not seem to act in a manner typical for allergens.

Concluding, the comparable productions of IFN-γ during stimulation of PBMC with membrane and soluble antigens of *M. catarrhalis* suggest, that apart from the former also the latter may have a strong impact on the Th1-type cytokine response. Moreover, the reduced IFN-γ in RRTI group may presumably be one of the important factor in non-effective defence mechanism against pathogens.

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