

T_H1/T_H2 immune response profiles differ between atopic children in eastern and western Germany

Harald Renz,^a MD, Erika von Mutius,^b MD, Sabina Illi,^b MSc, Florian Wolkers,^a MD, Thomas Hirsch,^c MD, and Stephan K. Weiland,^d MD Marburg, Munich, Dresden, and Ulm, Germany

Background: Lower prevalence rates for childhood respiratory allergies have been reported in eastern Germany than in western Germany.

Objective: Because allergic phenotypes are thought to be associated with an unbalanced T_H1/T_H2 T cell effector response, a cross-sectional investigation was carried out as part of the German ISAAC Phase II study to determine whether T_H1/T_H2 capacity might have developed in different directions during the separation between the 2 parts of the country.

Methods: In a community-based random sample of 9- to 11-year-old (n = 6399) and 5- to 7-year-old (n = 6202) children in eastern and western Germany, the prevalence of atopy was assessed. Heparinized blood samples were collected from a subgroup of children stratified according to the number of older siblings. T_H1 and T_H2 cytokine productivity was determined after stimulation with phorbol ester plus ionomycin.

Results: Individuals who grew up in eastern Germany had a marked bias toward T_H0 responsiveness (>60%), regardless of whether they were atopic. In contrast, the children of western Germany, particularly when they were atopic, showed T_H2 polarization.

Conclusions: This is the first epidemiologic survey to demonstrate regional differences in the T_H1/T_H2 response pattern and their relation to atopic disease between regions. (J Allergy Clin Immunol 2002;109:338-42.)

Key words: T_H1/T_H2 phenotype, allergy, asthma

An imbalance in the T_H1/T_H2 immune response profile has been proposed as the immunologic basis of allergy and asthma. With cells from peripheral blood and inflamed tissues of nose, airways, and skin, and using in situ and in vitro techniques, numerous clinical studies have shown T_H2 dominance with IL-4 and IL-5 overproduction in patients with hay fever, allergic bronchial asthma, and atopic dermatitis.¹⁻³ More recent data indicate

Abbreviations used

ISAAC: International Study on Allergy and Asthma in Childhood
RT: Room temperature
SPT: Skin prick test

that the T_H1/T_H2 profile is already being shaped early in life.⁴ In particular, the intrauterine environment and factors operating in the postnatal period have been proposed as contributory to shaping the phenotype of effector cells.

The fall of the Communist system, followed by German reunification in 1990, provided unique opportunities for epidemiologic research into the determinants of allergy and asthma. Several studies conducted in 1990-92 had reported lower prevalence rates of asthma, hay fever, and atopic sensitization in eastern Germany than in western Germany.⁵⁻⁷ These observations led to the question of whether the T_H1/T_H2 propensity has also developed in different directions in the 2 parts of the country and whether the relation between T_H1/T_H2 immune responsiveness and the occurrence of atopic diseases is similar under different environmental conditions.

To follow the rapid changes in lifestyle occurring in the eastern part of Germany, a large epidemiologic project was conducted in 1995-96 to investigate determinants of childhood asthma and allergies in Dresden (eastern Germany) and Munich (western Germany). With schools used as sampling units, community-based random samples of 9- to 11-year-old (n = 6399) and 5- to 7-year-old (n = 6202) children were studied through use of parental questionnaires, bronchial challenge with hypertonic saline solution, skin examination, skin prick tests (SPTs), and measurement of specific and total serum IgE with the Phase II modules of the International Study of Asthma and Allergy in Childhood (ISAAC). The aim of this analysis was to investigate the T_H1/T_H2 cytokine response pattern in the 2 parts of the country and to relate the immune response profiles to the occurrence of atopy.

METHODS

Study design

To assess the prevalence of atopic diseases, questionnaires containing the ISAAC core questions were sent to the parents for self-completion. In addition, parents were asked to give written consent to skin prick testing and blood sampling of their children. Methods

From ^aClinical Chemistry and Molecular Diagnostics, University Hospital Marburg; ^bUniversity Children's Hospital Munich; ^cthe Children's Clinic, Technical University Dresden; and ^dthe Department of Epidemiology, University of Ulm.

Supported by the German Ministry of Education, Science, Research and Technology.

Received for publication August 17, 2001; accepted October 26, 2001.

Reprint requests: Harald Renz, MD, Department of Clinical Chemistry and Molecular Diagnostics, Hospital of the Philipps University, Baldingerstraße, D-35033 Marburg, Germany.

Copyright © 2002 by Mosby, Inc.

0091-6749/2002 \$35.00 + 0 1/84/121459

doi:10.1067/mai.2002.121459

of the German ISAAC Phase II studies have been published in detail elsewhere.⁸ Only methods pertaining to this analysis are reported here. All children whose parents had consented underwent blood sampling and SPTs to a battery of common aeroallergens.⁸ A positive skin reaction was defined as a wheal size of ≥ 3 mm after subtraction of the negative control. Atopy was defined by positive skin test reactivity and/or doctor's diagnosis of any of the major atopic conditions (bronchial asthma, hay fever, atopic dermatitis).

In addition, heparinized blood samples were taken for the study of the T_H1/T_H2 cytokine response patterns. Because of the high costs of this procedure, the investigation was restricted to a subsample of all children of German nationality who participated in the blood sampling ($n = 1331$ in Munich; $n = 2068$ in Dresden).⁸ To allow investigation of the effect of family size, the samples were stratified according to the number of older siblings. Children were identified during the field work according to the following scheme: At the time of physical examination, all children with older siblings were recruited for cellular testing. As soon as a child with 3 or more older siblings was identified, he or she was included in the cytokine study. For each of these children, the next participating child with 1 or 2 older siblings and the next child with no older siblings were also enrolled into the study. This cycle was repeated whenever a child with at least 3 older siblings participated in the blood sampling. An attempt was made to identify all 3 of the children on the same day.

All blood samples were transported within a maximum of 5 hours to a laboratory in Munich or a laboratory in Dresden, where the cells were prepared immediately thereafter and cultured for 24 hours. This procedure was followed very strictly, even on weekends.

Laboratory procedures

T_H1 (IFN- γ) and T_H2 (IL-4 and IL-5) cytokine production was measured in PBMC tissue culture supernatants collected after stimulation with phorbol 12-myristate 13-acetate plus ionomycin. This stimulation protocol results in restimulation and activation of already existing effector T cells and therefore closely reflects the global T cell effector status at the time of investigation. Cells were prepared from heparinized venous blood and cultured as previously described.⁹ Briefly, PBMCs were separated by density gradient centrifugation (Lymphoprep, Life Technology) for 20 minutes at room temperature (RT). Cells from the interphase were washed 3 times (10 minutes, 3000 rpm, RT) and cultured in protein-free tissue culture medium (AimV). Then 2×10^6 PBMCs per well were stimulated with 0.5 μ g of ionomycin (Calbiochem) and 10^{-7} mol/L phorbol 12,13-bisbutyrate in 24-well time culture plates (Greiner). After a culture period of 24 hours, cell-free culture supernatants were frozen at -20°C and shipped to the laboratory of one of the investigators (H.R.), where all samples were analyzed at the same time. IL-4, IL-5, and IFN- γ were measured through use of sandwich ELISA, as previously described.¹⁰ Each sample was run in duplicate. Each ELISA plate included serial dilutions of a standard sample with known amounts of cytokine content; these were used to calculate the standard curve. Only the linear range of the standard curve was used to calculate cytokine concentrations in samples. Detection limits were 10 pg/mL for IL-4, 30 pg/mL for IL-5, and 12 pg/mL for IFN- γ . The variation coefficient for all ELISA systems used was $<7\%$.

Quality control

To ensure consistent performance in sampling and culture procedures in Dresden and Munich, an extensive quality control program was implemented throughout the entire study period. This included simultaneous training of the technical personnel from the 2 areas for 1 week in the central laboratory in Berlin under the supervision of one of the investigators (H.R.). Moreover, heparinized blood aliquots from 10 control subjects (5 atopic, 5 nonatopic) were shipped at 4 different time points throughout the

data collection period to the laboratories in Munich and Dresden. In both laboratories, cells from these samples were stimulated and frozen supernatants were returned for central analysis in Berlin according to the regular study protocol. When the measurement results were considered quantitatively, the mean values of the difference between the results of the 2 laboratories in Dresden and Munich and the average of the measurements¹¹ were 25.4 and 28.1 pg/mL for IL-4 and 627 and 455 pg/mL for IFN- γ , respectively. When dichotomous categories (below/above the detection limit) were used, the observer agreement between the 2 laboratories was 80% for the detection of IL-4 and 90% for the detection of IFN- γ .

Statistical analysis

Cytokine levels below and above the lower detection limit were rated as negative or positive, respectively, and subjects were subsequently categorized as follows:

1. Nonresponder: IFN- γ and both IL-4 and IL-5 were below the detection limit
2. T_H1 responder: only IFN- γ was detected
3. T_H2 responder: only IL-4 or IL-5 was detected
4. T_H0 responder: both IFN- γ and IL-4 and/or IL-5 were detected.

Because of the stratified sampling design, common software was not suitable for analysis of the data. Hence, SUDAAN (release 7.5.2; Research Triangle Institute) was used for all computations. This software package computes SEs in accordance with the sample design. The software package weights the actual sample of the 366 children according to the stratified sampling design. This weighted sample size is then used in further analyses. All results are thus shown as relative frequencies based on the weighted sample size, not as absolute frequencies. χ^2 tests were performed for comparisons between groups. A p value of $<.05$ was considered statistically significant.

RESULTS

Because of larger family sizes, the number of children enrolled was higher in Dresden (age 5-7 years, $n = 62$; age 9-11 years, $n = 194$) than in Munich ($n = 22$ and $n = 89$, respectively). The magnitude of cytokine levels is depicted in Table I. With the exception of IFN- γ , levels of which were significantly higher among the children of eastern Germany ($P < .01$), no significant differences were detected between the study groups. With respect to the pattern of cytokine secretion, however, marked differences were found. In both study areas, the immune response profiles were related to the age of the children, a polarization toward a T_H1/T_H2 pattern being more pronounced in the younger group (Fig 1). In Dresden (eastern Germany), a T_H0 cytokine pattern predominated in all age groups, whereas in Munich (western Germany), polarization toward T_H1 and T_H2 profiles was significantly more common in the younger group.

SPT reactivity is regarded as an objective measure of the atopic phenotype. Fig 2 presents a comparison of the distribution between the children of eastern Germany (Dresden) and western Germany (Munich) on the basis of positive SPT responses. Whereas in Munich almost one half of the children with positive SPT results presented as T_H2 responders, 77% of the children in Dresden were of the T_H0 phenotype ($P < .01$). When we stratified the results for various atopic phenotypes, significant differences again emerged between subjects living in

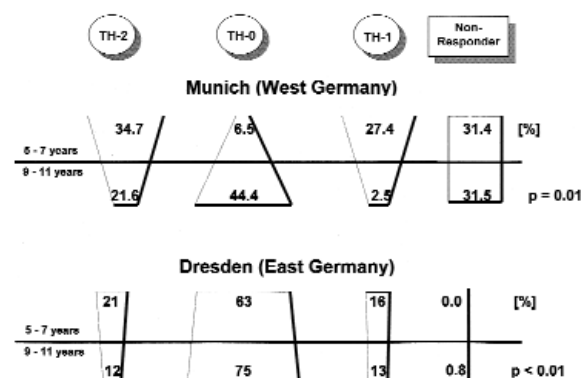


FIG 1. T_H1/T_H2 T cell polarization among 5- to 7-year-old ($n = 84$) and 9- to 11-year-old children living in Munich or Dresden. For each age group, the percentage of $T_H1/T_H2/T_H0$ responders is given. P values refer to the comparison of age groups within cities by χ^2 testing.

TABLE I. Magnitude of cytokine levels ($n = 366$)

	IFN- γ (pg/mL)	IL-4 (pg/mL)	IL-5 (pg/mL)
Minimum	0.0	0.0	0.0
1st quartile	154.0	14.0	0.0
Median	1207.5	31.0	128.0
3rd quartile	2115.0	56.0	305.0
Maximum	5319.0	258.0	2997.0

TABLE II. No effect of birth order on $TH1/TH2$ profiles

No. of older siblings	Children responding with cytokine pattern* (%)			
	T_H2	T_H0	T_H1	Nonresponder
0 ($n = 130$)	15.4	61.5	13.1	10.0
1-2 ($n = 130$)	18.5	60.8	10.0	10.7
≥ 3 ($n = 104$)	18.3	62.5	7.7	11.5

$P = .897$ (χ^2); $P = .813$ (Mantel-Haenszel). No significant differences were found between children of eastern Germany and children of western Germany.

* T_H2 , T_H0 , and T_H1 cytokine patterns are defined as described in the Methods section.

Munich and subjects living in Dresden. Among nonatopic children—ie, children who had never had a diagnosis of asthma, hay fever, or atopic dermatitis—a homogenous distribution among the T_H2 , T_H0 , and T_H1 responder groups was seen in Munich. In contrast, almost two thirds of the population in Dresden were T_H0 responders and there was an equally low frequency of T_H2 - or T_H1 -polarized children. Furthermore, a marked difference was observed between the atopic individuals who grew up in Munich and the atopic individuals who grew up in Dresden (Table I). In Munich, 31% to 45% of the subjects with asthma, hay fever, or atopic dermatitis were “classic” T_H2 responders, whereas only a small proportion (5% to 19%) of such children in Dresden showed a T_H2 cytokine profile. In contrast, 79% to 95% of atopic subjects in Dresden produced a mixed cytokine pattern and were therefore of the T_H0 phenotype. No dif-

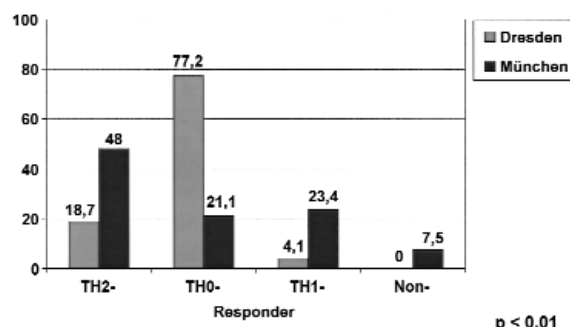


FIG 2. Comparison of the distribution of responder groups between eastern (Dresden) and western (Munich) Germany in children with positive SPT results (≥ 3 -mm wheal reaction to at least 1 allergen).⁸ P values refer to the comparison of responder groups between cities by χ^2 testing.

ference in immune response profiles was found for different numbers of older siblings (Table II).

As reported recently,⁸ Munich and Dresden no longer differed with respect to the prevalence of hay fever and skin test reactivity as of 1995-96; in contrast, the prevalence of asthma was still significantly lower in eastern Germany than in western Germany. The association of skin test reactivity with asthma, hay fever, and atopic dermatitis was of similar magnitude in Dresden and Munich (odds ratios, 2.9 and 3.7, 16.8 and 17.0, and 2.6 and 2.0, respectively). Among asthmatic individuals, there was no evidence of differences in severity between the areas (data not shown). The number of positive tests among skin test-positive children was slightly higher in Dresden than in Munich (geometric mean, 1.63 in Dresden and 1.52 in Munich; $P = .02$), but the prevalence of asthma symptoms did not differ (data not shown). Atopic children in Munich had fewer symptoms of atopic dermatitis but slightly more symptoms of hay fever than their peers in Dresden (data not shown). These findings suggest that in general, children with positive skin test results who were from Munich were not more allergic than those who were from Dresden.

DISCUSSION

This is the first epidemiologic survey to demonstrate regional differences in T_H1/T_H2 cytokine response patterns as well as differences in their respective relation to atopic diseases between regions. Individuals who grew up in Dresden (eastern Germany) had a marked bias toward T_H0 responsiveness, as defined by the coproduction of IFN γ and IL-4/IL-5, and this was true even among atopic children, suggesting that the T_H1/T_H2 paradigm is not sufficient to describe immune response patterns associated with the development of atopy.

The predominant T_H0 pattern in Dresden might be the result of different exposures early in life and might reflect earlier maturation of an immune system postnatally T_H2 -skewed⁴ toward T_H1 responsiveness. Previous studies have indicated a higher lifetime prevalence of bronchitis in areas of eastern Germany,⁵ hinting at

increased exposure to outdoor air pollution or to multiple infections in day care early in life. A larger family size (ie, a greater number of children) was also characteristic of eastern Germany, and sibship size has inversely been related to skin test reactivity in that population.¹² These epidemiologic findings resulted in the “hygiene hypothesis,” which proposes that living conditions in East Germany favored the development of effector T cells, which predominantly produce the T_H1 cytokine IFN γ . Indeed, this study is the first to demonstrate that the vast majority (more than 60%) of the children in Dresden (eastern Germany) are IFN γ producers.

In this study, we assessed the global capacity of T cell responsiveness using a well-established culture and stimulation system.^{9,10,13} This mode of activation closely mirrors the capacity of T cells to produce cytokines by avoiding induction of new effector T cells, which would be a major confounder in this study. A further advantage is its robustness, which is suitable for epidemiologic field work. In contrast, stimulation of T cells with antigens/allergens would yield a different level of information. In that case, only information about the respective antigen/allergen specific T cell subset would be obtained and the vast majority of T cells which did not respond to the respective stimuli would be ignored. Furthermore, the outcome of such a culture procedure depends on the dose and type of antigen, on the activation status, and on the frequency of antigen-presenting cells and would require a much longer culture period. The mode of activation used in our study is largely independent of these factors. However, assessment of cytokine production after antigen and allergen stimulation could provide additional important information that would have been missed in this study.

It is a surprising finding of this investigation that atopic children from eastern Germany did not present with a T_H2 -polarized immunologic phenotype; instead, atopy was associated with T_H0 responsiveness. This pattern was not only observed for the lifetime prevalence of any atopic manifestation but was also noted when the disease manifestations were split up into asthma, hay fever, atopic dermatitis, and SPT reactivity. Although the reasons are still unclear, the immunologic T_H0 phenotype of children from eastern Germany might explain both a less frequent phenotypic expression of clearly T_H2 -related diseases such as asthma⁸ and a more frequent expression of atopic eczema, which is immunologically promoted by T_H2 and T_H1 mechanisms. It is unlikely, however, that technical aspects account for this phenomenon, inasmuch as an extensive quality control program revealed no evidence of technical problems in Dresden. Furthermore, the 2 areas were similar with respect to the severity of atopic conditions. It also seems unlikely that genetic factors might have influenced the findings, given that the eastern and western populations in Germany were separated for only 2 generations and changes in disease prevalences occurred monthly after separation.¹⁴ It thus seems most likely that environmental and lifestyle conditions are important determinants in shaping the

phenotypes of T cell effectors. The development of T_H1/T_H2 effector cells represents a dynamic process that is probably regulated by numerous environmental conditions. Factors implied include the frequency and type of infections, microbial colonization of the gut, nutrition, and allergen exposure.¹⁵

Complex cellular assays were performed to investigate T helper cell functions; therefore, potential technical problems must be addressed. It was unexpected that approximately 30% of the children living in Munich would have to be regarded as nonresponders. Several reasons might account for this phenomenon. The size of the Munich study group aged 5-7 years was small. Culture conditions might not have been sensitive enough to pick up low amounts of cytokine production, and expansion of the measured cytokine panel (eg, to include IL-13, IL-10, and IL-6) might have allowed further categorization into responder groups. If the “nonresponsiveness” were due to technical factors, we would expect to find equal numbers of these cases among atopic infants and nonatopic infants. However, this was clearly not the case, inasmuch as the atopic population in Munich showed a low rate of nonresponders (Fig 2). The comparison of the 2 laboratories suggested higher readings in Dresden than in Munich. In our analyses, however, cytokine responses were categorized as dichotomous variables (ie, above/below the detection limit) and the validation study showed reasonable agreement between the 2 laboratories with this categorization. In addition, further analyses investigating potential biases due to technical problems gave very similar results regarding the differences between Dresden and Munich with respect to T_H1/T_H2 profile. Finally, in Dresden the response to the cell stimulations was very high (>95%), and there was no evidence of technical problems in the Dresden laboratory. Thus, methodologic problems cannot explain what is perhaps the most important finding of our study—namely, the marked bias toward T_H0 responsiveness among individuals who grew up in eastern Germany, even those who were atopic.

The data presented herein suggest that the association between T_H2 responsiveness and clinical entities such as asthma, hay fever, atopic dermatitis, and skin test reactivity differs between populations, being strongest in a westernized environment. The apparent strong relationship between T_H2 -type immune responses and atopic diseases described in previous studies might thus in part be attributable to some common environmental influences prevalent in westernized populations. In the absence of these factors, T cell responsiveness, even among atopic individuals, will show a mixed cytokine profile, more characteristic of a T_H0 pattern than of a T_H2 pattern. The findings of this study suggest that the pattern of T cell responsiveness will not provide an objective gold standard by which to measure asthma and allergy; rather, it will be a loosely associated phenotype with different genetic and environmental determinants.

We thank Professor Adalbert Roscher and Stephan Heyden and their colleagues in the laboratories in Munich and Dresden for their excellent cooperation.

REFERENCES

1. Humbert M, Menz G, Ying S, et al. The immunopathology of extrinsic (atopic) and intrinsic (non-atopic) asthma: more similarities than differences. *Immunol Today* 1999;20:528-33.
2. Herz U, Bunikowski R, Renz H. Role of T cells in atopic dermatitis. New aspects of the dynamics of cytokine production and the contribution of bacterial superantigens. *Int Arch Allergy Immunol* 1998;115:179-90.
3. Hamid Q, Boguniewicz M, Leung DY. Differential in situ cytokine gene expression in acute versus chronic atopic dermatitis. *J Clin Invest* 1994;94:870-6.
4. Prescott SL, Macaubas C, Smallacombe T, Holt BJ, Sly PD, Holt PG. Development of allergen-specific T-cell memory in atopic and normal children. *Lancet* 1999;353:196-200.
5. von Mutius E, Martinez FD, Fritzsche C, Nicolai T, Roell G, Thiemann HH. Prevalence of asthma and atopy in two areas of West and East Germany. *Am J Respir Crit Care Med* 1994;149(2 Pt 1):358-64.
6. Behrendt H, Krämer U, Dolgner R, et al. Elevated levels of total serum IgE in East German children: atopy, parasites or pollutants? *Allergol J* 1993;2:31-40.
7. Nowak D, Heinrich J, Jorres R, et al. Prevalence of respiratory symptoms, bronchial hyperresponsiveness and atopy among adults: West and East Germany. *Eur Respir J* 1996;9:2541-52.
8. Weiland SK, von Mutius E, Hirsch T, et al. Prevalence of respiratory and atopic disorders among children in the East and West of Germany five years after unification. *Eur Respir J* 1999;14:862-70.
9. Meissner N, Kussebi F, Jung T, et al. A subset of CD8+ T cells from allergic patients produce IL-4 and stimulate IgE production in vitro [see comments]. *Clin Exp Allergy* 1997;27:1402-11.
10. Nasert S, Burtchen N, Kussebi F, et al. Stimulation of IgE and IgA production by CD45RA T helper cells in atopic patients. *J Immunol* 1996;157:441-8.
11. Bland JM, Altman DG. Statistical methods for assessing agreement between two methods of clinical measurement. *Lancet* 1986;1:307-10.
12. von Mutius E, Martinez FD, Fritzsche C, Nicolai T, Reitmeir P, Thiemann HH. Skin test reactivity and number of siblings. *BMJ* 1994;308:692-5.
13. Murphy E, Shibuya K, Hosken N, et al. Reversibility of T helper 1 and 2 populations is lost after long-term stimulation. *J Exp Med* 1996;183:901-13.
14. Heinrich J, Nowak D, Wassmer G, et al. Age-dependent differences in the prevalence of allergic rhinitis and atopic sensitization between an eastern and a western German city. *Allergy* 1998;53:89-93.
15. von Mutius E. The environmental predictors of allergic disease. *J Allergy Clin Immunol* 2000;105(1 Pt 1):9-19.

Availability of Journal back issues

As a service to our subscribers, copies of back issues of *The Journal of Allergy and Clinical Immunology* for the preceding 5 years are maintained and are available for purchase until inventory is depleted. Please write to Mosby Subscription Customer Service, 6277 Sea Harbor Dr, Orlando, FL 32887, or call (800) 654-2452 or (407) 345-4000 for information on availability of particular issues and prices.