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Reduced memory B cells in patients with hyper IgE syndrome

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Abstract Dominant-negative mutations in STAT-3 have recently been found in the majority of patients with sporadic or autosomal-dominant hyper IgE syndrome (HIES). Since STAT-3 plays a role in B cell development and differentiation, we analyzed memory B cells in 20 patients with HIES, 17 of which had STAT-3 mutations. All but four patients had reduced non-switched and/or class-switched memory B cells. No reduction in these B cell populations was found in 16 atopic dermatitis patients with IgE levels above 1000 KU/L. There was no correlation between the reduction of memory B cells and the ability to produce specific antibodies. Moreover, there was no correlation between the percentage of memory B cells and the infection history. Analysis of memory B cells can be useful in distinguishing patients with suspected HIES from patients with atopic disease, but probably fails to identify patients who are at high risk of infection.

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Introduction

Hyper IgE syndrome (HIES) is characterized by a triad of eczema, recurrent infections of the skin and lungs and excessively elevated serum IgE levels [1]. Additional clinical features vary and include a characteristic facial appearance,

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scoliosis, retained primary teeth, joint hyper-extensibility and recurrent bone fractures following minor trauma [2]. The disease usually occurs sporadically, but families with autosomal-dominant traits have been recognized. An autosomal recessive variant of HIES, with a characteristic spectrum of clinical manifestations, has been identified as a distinct disease entity in Turkish and Mexican families [3]. The diagnosis of HIES is difficult since there are no simple laboratory tests that confirm the disease. For many years the diagnosis has therefore relied on a combination of typical clinical and laboratory findings summarized in a scoring system developed at the NIH [2]. However, this scoring system has never been prospectively evaluated and its value is limited in young children, because the characteristic findings accumulate with age.

The recent identification of mutations in STAT-3 in patients with sporadic or autosomal-dominant HIES has been a major breakthrough [4,5]. STAT-3 is a transcription factor involved in many receptor signalling pathways including several cytokine receptors [6,7]. STAT-3 is activated after binding to tyrosine kinases via its SH2 domain and requires homodimerization to develop its DNA binding activity after translocation to the nucleus [6]. This explains why heterozygous mutations can cause the disease in an autosomal-dominant fashion. Dominant-negative mutations in the SH2 domain or in the DNA binding domain of STAT-3 have been identified in 50–100% of patients with the typical features of HIES [4,5]. Thus, genetic diagnosis can now be offered to many, but not to all patients with HIES. Moreover, STAT-3 consists of 24 exons and thus the genetic diagnostic takes time, is labour intense and expensive. The identification of additional quick and reliable screening laboratory markers for the disease is therefore still needed, in particular for younger patients, where some of the typical clinical features are not yet apparent.

The discovery of STAT-3 mutations in HIES patients offers many new avenues for research on the pathogenesis of the disease including the identification of new diagnostic markers. One of these fields is the role of STAT-3 in the development and differentiation of B cells. Experiments with conditional knock-out mice suggested a role for STAT-3 in prevention of apoptosis during B cell development [8] and a B cell defect in T cell dependent IgG plasma cell differentiation and specific IgG responses [9]. Previously published observations on B cell function in patients with HIES indicated abnormalities in terminal B cell differentiation *in vitro* [10] as well as impaired antibody responses to polysaccharide and protein antigens [1,11–13]. However, the pattern of antibody deficiencies was not consistent and normal antibody responses to some antigens could be demonstrated in most patients [13,14].

Analysis of B cell differentiation is increasingly included in the immunological characterization and classification of patients with primary immunodeficiencies [15]. This is also of prognostic relevance, as for example in patients with common variable immunodeficiency (CVID), the percentage of CD27 expressing B cells correlates with susceptibility to infection [16]. Patients with HIES have not been systematically analyzed for the presence of memory B cells. In this study, we investigated a cohort of 20 patients with HIES, 16 disease controls with atopic dermatitis and excessively elevated IgE and 54 healthy donors for the presence of memory B cells in order to address the following questions:

(i) is there a lack of memory B cells in patients with HIES? (ii) is there a correlation between memory B cells and the STAT-3 genotype? (iii) do memory B cells correlate with the ability to produce specific antibodies and the patients' infection profile? (iv) could the determination of memory B cells be useful in the diagnosis of HIES?

Methods

Study subjects

Several centers involved in the care of patients with primary immunodeficiencies were contacted and a total of 29 patients with a clinical diagnosis of HIES were recruited. From this cohort, patients were included in the study, if they fulfilled the following criteria: (i) sporadic or autosomal-dominant disease, (ii) HIES score >30 points, (iii) significant susceptibility to infectious disease as defined by ≥ 3 abscesses or ≥ 2 pneumonias. Patients did not suffer from acute infection and with one exception none of the patients was receiving immunoglobulin replacement therapy at the time of study. The control group consisted of 16 atopic dermatitis patients with IgE levels above 1000 kU/L (15/16 >2000 kU/L, 2 of these >45,000 kU/L), who were recruited from the outpatient allergy clinics at the Department of Dermatology and the Children's Hospital. Participating patients and control persons or their parental guardian provided written consent for the conducted studies according to the local ethic committee requirements (Ethics protocol number 239/99).

Genetic analysis

DNA isolated from peripheral blood of patients was analyzed for STAT-3 mutations as described [4]. Details on the genetic analysis will be published in a separate report on genotype–phenotype correlations (Woellner et al., in preparation). To facilitate patient identification, the unique patient numbers (UPN) in the two studies are consistent.

Flow cytometry

EDTA blood samples were taken on site or by the local physician and shipped at room temperature for analysis within 48 h after venopuncture. PBMCs were isolated by Ficoll gradient. 2×10^5 cells were surface stained with anti-CD27 FITC, anti-IgD PE, anti-IgM Cychrome and anti-CD19 APC (all BD Pharmingen). Cells were analyzed on a FACScan cytometer using Cellquest Pro software. Isotype controls were used to define adequate instrument settings throughout this study. For comparison of the two study cohorts with healthy controls, we recruited patients from our outpatient clinics who did not have evidence for acute or chronic infectious, inflammatory, hematological or immunological disease and had blood taken for diagnostic procedures such as imaging or prior to surgery. The controls were spread throughout the pediatric and adult age range relevant to the analyzed HIES and AD patient cohorts (5–50 years).

Assays for serum antibodies

Tetanus antitoxin serum levels were determined by ELISA (Organon-Teknika, Eppelheim, Germany); values >0.1 IU/ml

Table 1 Clinical data

UPN ¹	Age (years)	Sex	Findings													
			Eczema	Abscesses	Pneumonia (pathogen ²)	Parenchymal lung changes ³	Candidia infection	Characteristic face	High palate	Retained teeth	Bone fractures	Joint hyper- extensibility	Scoliosis	Peak IgE (KU/L)	Eosinophils (max cells/ μ l)	NIH score (points)
079	7	F	Moderate	>4	>3 (SA, PA)	P	Nails	Yes	Yes	Yes	Yes	NA	No	10,000	800	53
094	7	F	Severe	>4	No	No	Nails	Mild	No	Yes	No	Yes	No	3028	360	49
005	8	M	Moderate	>3	>3 (SA,, HI)	B	No	Yes	Yes	Yes	No	No	Yes	6870	760	41
031	10	M	NA	no	2 (SA, HI)	NA	Nails	NA	NA	NA	NA	NA	NA	4493	2392	54
007	11	F	Moderate	8	8 (HI, PN)	B, BOOP	Oral/ Nails	Yes	NA	Yes	No	Yes	No	23,400	1060	53
028	11	M	Moderate	2	5 (SA, HI)	B	Oral	Mild	No	Yes	No	Yes	No	2098	152	47
032	13	M	Mild	4	4	No	Oral	Mild	Yes	Yes	No	No	No	1615	410	37
019	13	M	Severe	3–4	3	B	Oral/ nails	Mild	Yes	Yes	Yes	Yes	No	9298	<700	63
030	14	F	Moderate	>3	2	P	Oral/ nails	Mild	No	Yes	No	No	No	13,284	9270	47
081	14	F	Moderate	>4	>3	No	Nails	Mild	No	No	No	No	Mild	>2000	>800	37
071	19	M	Moderate	>3	yes (SA, HI, PA)	B,P	Oral	Yes	Yes	No	No	NA	Mild	17,683	564	35
018	21	M	Severe	>4	>3	B, P	Oral	Mild	Yes	Yes	No	No	No	43,320	>800	58
033	21	M	Moderate	4	2 (PA, SA)	P	Oral	Yes	No	No	No	Yes	NA	>20,000	300	51
017	22	F	Severe	4	>3	B, P	Nails	Yes	Yes	Yes	No	No	Mild	90,700	700–800	65
015	25	M	Severe	>4	>3	B, P	Nails	Yes	Yes	Yes	Yes	NA	Mild	3089	1090	76
070	31	F	Moderate	>3	no	No	Oral	Yes	Yes	Yes	No	Yes	Yes	21,535	544	33
029	33	F	Moderate	4	7 (SA, PN, PA,HI)	P	Oral/ nails	Yes	No	Yes	No	No	Mild	24,202	2556	64
002	35	F	Mild	no	>3 (SA)	B	Oral/ nails	Mild	NA	NA	Yes	NA	NA	32,000	2900	41
016	36	F	Severe	1–2	>3	B, P	Oral/ nails	Yes	Yes	Yes	Yes	Yes	NA	13,000	>800	68
006	52	F	Severe	>4	>3	B	No	Yes	No	No	Yes	No	Yes	>2000	<700	54

Clinical signs and symptoms in the patient cohort.

1– UPN: unique patient number, 2– SA: *Staphylococcus aureus*, PA: *Pseudomonas aeruginosa*, HI: *Haemophilus influenzae*, PN: *Streptococcus pneumoniae*, 3– P: pneumatoceles, B: bronchiectasis, BOOP: bronchiolitis obliterans organizing pneumonia, NA: not available.

were considered protective [17]. Antibody levels against measles (protective levels >200 mIU/ml [18]) and varicella (protective levels >100 mIU/ml [19]) were also measured by ELISA (Behringwerke, Marburg, Germany). Serotype-specific anti-pneumococcal IgG antibodies against the serotypes 1, 4, 5, 6B, 7F, 9V, 14, 18C, 19F and 23F were measured according to the protocol of the WHO reference laboratories at the Institute of Child Health (University College London, UK) and the Department of Pathology (University of Alabama at Birmingham, USA). A positive reference serum (89-SF, provided by Dr. Carl Frasch, FDA, Rockville, MD, USA) served as standard on each ELISA plate [20,21]. There is no clear consensus as to which antibody titer against pneumococcus can be considered protective. For the Prevnar® vaccine, a level of 0.5 µg/ml has been considered to represent an adequate vaccine response [22,23]. To evaluate the natural antibody response to pneumococcus, we therefore determined the number among 10 investigated serotypes against which antibody levels above 0.5 µg/ml had been generated.

Statistics

Statistic analysis was performed using Graph Pad Prism software (Version 4.01). The Mann–Whitney-*U*-Test was used to calculate the statistical significance of differences between groups. Calculations were based on a 95% confidence interval. $p < 0.05$ was considered significant.

Results

Clinical and genetic characterization of the patient cohort

Twenty patients fulfilled the inclusion criteria, nine patients were male and eleven were female (Table 1). The age range was 7–52 years. The HIES score was above 40 in all but 4 patients (score 33, 35, 37, 37). Only three patients had less than 3 abscesses and 3 patients had less than 2 pneumonias. Fifteen patients had documented parenchymal lung damage. Genetic analysis revealed STAT-3 mutations in all but three patients. Nine patients had mutations in the DNA binding domain, 5 patients in the SH2 domain and three patients had STAT-3 mutations outside of these protein domains. Details of the mutations will be reported in a separate manuscript (Woellner et al., in preparation).

B-cell phenotype

Four-colour flow cytometry of PBMC was used to analyze different B cell subpopulations (Fig. 1). All patients and controls had a normal percentage of CD19+ B cells among peripheral blood lymphocytes. Data on absolute B cell numbers were not available for all patients and are therefore not included. We quantified the percentage of IgD+CD27+ (non-switched memory) (Figs. 1B,C) and the percentage of IgD–CD27+ (class-switched memory) cells (Figs. 1D,E) among CD19+ B cells. Because of the known age-dependent increase in the normal values, we separately analyzed a cohort of patients aged 5–20 and a cohort of patients >20 years of age. A local healthy control cohort was analyzed under identical conditions. We have not yet analyzed these B cell popula-

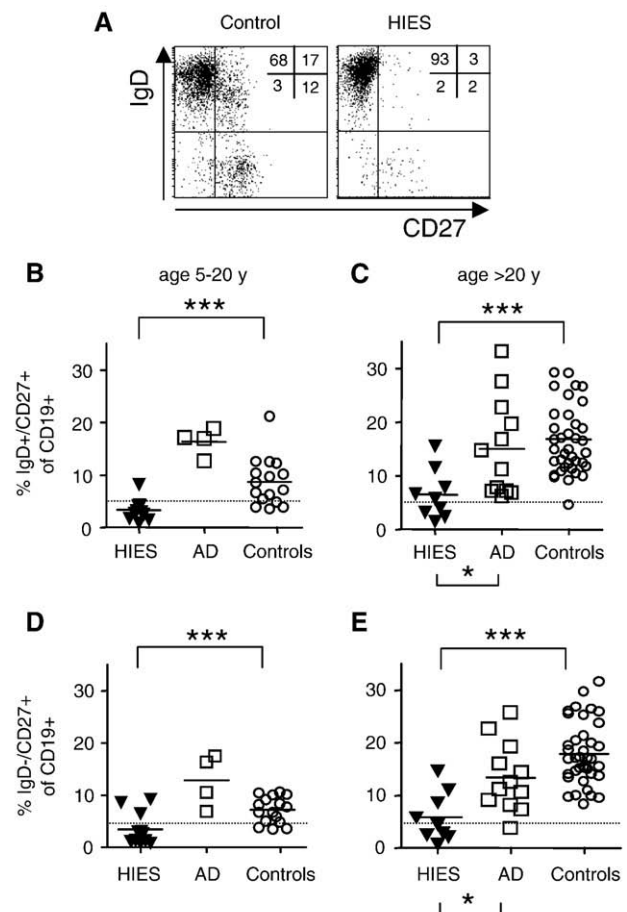


Figure 1 Reduced memory B cells in patients with HIES. (A) Representative dot plots showing the expression of IgD and CD27 on CD19+ B cells of a healthy donor (left panel) and a HIES patient (right panel). The initial gate was set on CD19+ lymphocytes. This population was then evaluated for IgD and CD27 expression. (B, C) Percentage of IgD+CD27+ cells among CD19+ B cells in patients with HIES (closed triangles), patients with atopic dermatitis (AD) and IgE levels above 1000 U/ml (open squares) and healthy donors (open circles) separately analyzed for patients aged 5–20 years (range 5–20, mean 13 years) (B) and above 20 years of age (range 21–50, mean 36 years) (C). (D, E) Percentage of IgD–CD27+ cells among CD19+ lymphocytes. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$.

tions in a sufficient number of healthy donors to define appropriate normal values particularly in the younger age group. For patients above 20 years of age, the 5th percentile of normal values at our institution is at 7% for non-class-switched and at 9.3% for class-switched memory B cells. For the purpose of this study, an arbitrary cut-off of 5% non-switched and 5% class-switched memory B cells was used as the lower limit of normal for all patients.

The percentage of non-switched memory B cells was significantly lower in patients with HIES when compared to AD patients or healthy controls. In the age group 5–20 years, 10/11 HIES patients, 0/4 AD patients and 4/16 controls had levels below 5%. This difference was also observed in the older patient cohort, where 4/9 HIES patients, but only 0/12 AD patients and 1/38 controls had levels below 5%. Similar

Table 2 Antibody responses

UPN	Age (year)	Findings			
		Anti-tetanus U/ml (Ref. >0,1)	Anti-measles mIU/mL (Ref. >200)	Anti-varicella mIU/mL (Ref. >100)	Anti-pneumococci (n of 10 serotypes >0.5 µg/ml)
079	7	<0.05 ¹	37 ¹	334	NA
094	7	0.43	288	66,946	NA
005	8	0.094 ¹	2620	7211	2/10
031	10	NA	NA	NA	NA
007	11	<0.05 ²	NA	NA	NA
028	11	NA	NA	NA	NA
032	13	NA	NA	NA	NA
019	13	0.28	310	8188	7/10
030	14	<0.05 ¹	192 ¹	510	7/10
081	14	NA	NA	NA	NA
071	19	0.29	NA	422	9/10
018	21	0.14	1470	2082	10/10
033	21	NA	NA	NA	NA
017	22	0.27	278	1965	10/10
015	25	<0.05 ¹	169 ¹	556	7/10
070	31	0.65	NA	1518	9/10
029	33	NA	NA	NA	NA
002	35	<0.05 ²	648	289	10/10
016	36	0.22	429	1156	10/10 ³
006	52	2.13	151	3125	9/10 ³

Antibody titers to tetanus, measles, varicella and pneumococci in a sporadic serum sample.

1— vaccinated, 2— vaccination status unknown, 3— PN vaccinated, NA: information not available.

results were obtained when analyzing the class-switched memory B cell population. In the younger age group, 8/11 HIES patients, 0/4 AD patients and 3/16 controls had levels below 5% and this difference was significant. The difference was more pronounced in the older age group, where 5/9 HIES patients, 1/12 AD patients and 0/38 controls had levels below 5%. Overall, only 4/20 HIES patients had both non-switched and class-switched B cells above 5%. Of these, two had a mutation in the SH2 domain, one in the DNA binding domain and one had a STAT-3 mutation outside of these two domains (UPN 018).

Antibody titers against protein antigen vaccines

Antibody titers against protein antigen vaccines were determined in a sporadic serum sample in 14 of the 20 HIES patients (Table 2). All patients had normal total IgG serum levels and IgG subclasses (data not shown). The time point of the last tetanus vaccination was not available for all patients. Protective titers against tetanus were documented in 8/14 patients and 4 patients had no response or an insufficient response to tetanus despite an appropriate booster vaccination within 5 years before this study.

Protective anti-measles antibodies were detected in 7/11 patients analyzed. One 11 year-old patient (UPN 079) with a mutation in the DNA binding domain, 4.2% non-switched and 6.5% class-switched memory B cells did not show protective titers despite 2 MMR vaccinations and she also failed to respond to the tetanus vaccine. Two further patients (UPN 030: no STAT-3 mutation and UPN 015: DNA binding domain mutation) had no protective titers to measles and tetanus despite at least 1 MMR and recent tetanus vaccinations. Both

had non-switched and class-switched memory B cells below 5%. A fourth patient (UPN 006, DNA binding domain mutation) with unknown vaccination status had no protective anti-measles titers, but a protective tetanus response. She also had non-switched and class-switched memory B cells below 5%. All four patients without protective measles titer showed a protective anti-varicella antibody response as did all 13 other patients analyzed.

Antibody titers against pneumococcal polysaccharide antigens

In the same serum sample, antibody titers against 10 pneumococcal serotypes were analyzed in 11 patients (3 children and 8 adults); 9 of these had not been immunized, 2 had been immunized with pneumovax. All but one patients had generated antibody levels above 0.5 µg/ml to at least 4/10 serotypes. Above the age of 12 years, only 2 patients had developed a response to less than 9/10 serotypes. Both of these patients had also failed to respond to the tetanus vaccine despite booster vaccination within 5 years before this study. They both had non-switched and class-switched memory B cells below 5%. One had a mutation in the DNA binding domain (UPN 015), while in the other patient no mutation was identified (UPN 030).

Discussion

This study shows that the percentage of circulating memory B cells is reduced in most patients with sporadic or autosomal-dominant HIES when compared to age-matched

healthy controls or to patients with excessively elevated IgE due to atopic disease.

The detection of mutations in STAT-3 in about 50–100% of sporadic or autosomal-dominant HIES patients (85% in our study) provides a plausible pathophysiological explanation for this observation. Two recent studies have documented a role for STAT-3 in development and differentiation of B cells using conditional knock-out mice. Postnatal deletion of STAT-3 in all bone marrow cells led to impaired B cell development and reduced B cell lineages including pro-B, pre-B, immature B and mature B cells [8]. Experiments with these mice suggested a role for STAT-3 in differentiation and prevention of apoptosis, in particular during the transition from the pre-pro-B to the early pro-B stage of B cell development. In contrast, mice with CD19 driven deletion of STAT-3, leading to STAT-3 deficiency at the pro-B stage, did not show defects in B cell development, but showed a selective defect in T cell dependent IgG plasma cell differentiation and specific IgG responses [9]. Germinal center formation, isotype switching and generation of memory B cells was normal in these mice, suggesting that STAT-3 regulates isotype-specific terminal B cell differentiation. These knock-out mouse models do not adequately mimic the complex situation in HIES patients, where dominant-negative heterozygous STAT-3 mutations affect not only B cells, but also many other cell types contributing to B cell development and differentiation [6]. However, on the basis of the murine findings, it appears plausible to assume that impaired STAT-3 signalling and perhaps mutations in HIES causing genes involved in similar pathways contribute to the reduced memory B cells and to the variable specific antibody responses in our and previously published patients [1, 11–14].

In our cohort, 17/20 patients had mutations in STAT-3. The presence or absence or the nature of the mutation did not predict the B cell phenotype. Among the four HIES patients who had levels above 5% for both non-switched and class-switched memory B cells, there were two patients with mutations in the SH2 domain, one patient with a mutation in the DNA binding domain and one patient had a mutation outside of these domains. There was also no correlation between the clinical score and the lack of memory B cells. These observations suggest that factors other than STAT-3 mutations have an additional impact on the B cell phenotype in HIES patients.

In CVID patients, reduced memory B cells appear to correlate with the ability to produce specific antibodies and with susceptibility to bacterial infection [16]. It has previously been shown in different studies reporting pre- and post-vaccination titers that the ability of HIES patients to mount specific antibody responses is highly variable [12, 13]. The abnormalities in immunoglobulin production were most pronounced when experimental booster responses to phi X174 were analyzed. Here, 5/5 patients showed defective responses, whereas deficient antibody responses to tetanus, diphtheria and pneumococcal polysaccharide vaccines were observed in some, but not all of the 11 analyzed patients with HIES [13]. Our data confirm this variability in specific antibody responses, which may be a result of the individually different levels of residual STAT-3 activity. The potential contribution of residual STAT-3 activity to the heterogeneity of many of the clinical and immunological features of HIES will be an important area of future research. Of note, none of

the four patients who had low specific antibody titers had normal memory B cells. On the other hand low memory B cells were also observed in many patients with normal antibody responses to all four analyzed antigens. A correlation between memory B cells and infection susceptibility was also not obvious. Six patients had less than three pneumonias and no parenchymal lung changes; all but one of them had reduced non-switched and class-switched memory B cells. Thus, although it is likely that reduced memory B cells contribute to the susceptibility to infection in HIES patients, the levels of circulating memory B cells neither correlate with the ability to produce specific antibodies nor with the individual infection profile.

Our data suggest that determination of memory B cells may be a useful and simple additional laboratory test for the diagnosis of HIES. Only 4/20 HIES patients, but 15/16 patients with excessive IgE levels due to atopic disease had levels above 5% for both non-switched and class-switched memory B cells. It should be pointed out that also among these 16 AD controls, there were 7 patients with IgE levels above 5000 kU/ml of whom two patients had level >45,000 kU/ml, indicating that the lack of memory B cells is not causally linked to the excessive IgE production. It is important to note that although the arbitrary cut-off of 5% was helpful in elucidating the difference between the cohorts in this particular study, it cannot be used as a generally valid cut-off for normal or abnormal values. Due to the variability introduced by differences in cell preparation (PBMC versus full blood), the type of flow cytometer and the instrument settings used, age-related normal values must be established at each center.

In conclusion, we report reduced memory B cells in most patients with HIES who were recruited from several different countries. We therefore propose the determination of memory B cells as a useful adjunct for the diagnosis of the hyper IgE syndrome. Due to the inherent variability of flow cytometric data between different laboratories, reproduction of our findings in different laboratories will be important before a reduction of memory B cells can be generally accepted as a valid diagnostic criteria of HIES.

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