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Increased expression of interleukin-13 but not interleukin-4 in CD4$^+$ cells from patients with the hyper-IgE syndrome

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SUMMARY

Hyper IgE syndrome (HIES) is a rare immunodeficiency disorder characterized mainly by high levels of polyclonal IgE in serum and recurrent staphylococcal abscesses of the skin and lungs. The raised IgE levels have led researchers to study the synthesis of cytokines that regulate switching of immunoglobulin production towards IgE such as interleukin-4 (IL-4), IL-12 and interferon-γ (IFN)-γ. However, the role of IL-13 in the disease pathogenesis has not been investigated extensively. In this study, we investigated intracellular expression of IL-4 and IL-13 in mononuclear cells and CD4$^+$ cells isolated from patients with HIES and healthy controls. Cells were stained intracellularly with antibodies directed against IL-4 and IL-13 and analysed by flow cytometry before and after activation with PMA and calcium ionophore. The mean proportion of resting or activated IL-4 and IL-13 expressing mononuclear cells were comparable in the two groups as well as the proportion of IL-4 expressing CD4$^+$ cells. In contrast, the mean proportion of IL-13 expressing CD4$^+$ cells was increased significantly in patients with HIES in both the resting and the activated state compared to healthy controls. We conclude that increased expression of IL-13 in CD4$^+$ cells from patients with HIES could account, at least partly, for raised IgE levels in those individuals.

Keywords bone metabolism FACS IL-4 IL-13 immunodeficiency diseases

INTRODUCTION

The hyper-IgE syndrome (HIES) is a rare disorder of unknown aetiology, characterized by markedly increased levels of polyclonal IgE in serum, elevated levels of blood eosinophils and recurrent staphylococcal abscesses of the skin, lungs and other sites [1]. This disorder is also associated with certain facial and skeletal features such as recurrent fractures, hyperextensible joints and scoliosis [2]. The genetic basis of HIES is still unclear since the majority of cases are sporadic. Attempts to link HIES to variants of the interleukin-4 (IL-4) receptor on chromosome 16 have failed [3], but recently a linkage was found for HIES to a region on chromosome 4 [4]. No candidate gene on chromosome 4 has been implicated but numerous genes have been mapped near the region, including *c-kit* and *VEGFR-2* [4].

The immunological features of HIES, such as elevated IgE levels, has prompted studies of IgE synthesis in this syndrome. These include studies of the elements controlling IgE production such as the cytokines IL-4, which induces IgE switching, and interferon-γ (INF-γ), which inhibits IgE switching. Studies of cytokine production in patients with HIES have given conflicting results. Decreased IFN-γ production compared to healthy controls and normal or increased IL-4 production have been reported [5–7]. Other studies have shown no difference in IFN-γ production in comparison with controls [8]. The IL-4-like cytokine IL-13 can induce IgE switching [9,10]. Whether it accounts for the elevated IgE levels in HIES rather than IL-4 is not known. A recent study using ELISA, indicated that IL-13 levels were not elevated in HIES patients when compared to healthy controls, but rather that IL-4 levels were significantly increased [7]. IL-4 and IL-13 are produced primarily by activated T cells and mast cells [11] and the genes for both of these cytokines are located in the same region on chromosome 5 [11]. The amino acid similarity between the cytokines is approximately 30% [11]. Additionally, the receptors for the cytokines are structurally similar as one type of the IL-13 receptor shares the IL-4 receptor α chain [12]. Past studies have indicated that IL-13 could not regulate T-cell differentiation because of lack of functional IL-13 receptors on T-cells [9]. However, it has now been shown that T-cells do indeed express a functional IL-13 receptor which is capable of transducing signals through signal transducer and activator of transcription 6 (Stat6) [13,14].

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The objective of this study was to investigate the expression of IL-4 and IL-13 in mononuclear cells and $CD4^+$ cells in healthy blood donors and in patients with the HIES using intracellular cytokine staining and flow cytometry.

MATERIALS AND METHODS

Reagents

Phorbol 12-myristate 13-acetate (PMA) and calcium ionophore were purchased from Sigma (St Louis, MO, USA). Monoclonal antibodies used were anti-CD4 PerCP (Becton Dickinson, San Jose, CA, USA), anti-IL-4 PE and anti-IL-13 PE, all from Pharmingen (San Diego, CA, USA). Isotype controls used were mouse IgG1, mouse IgG1 PE, rat IgG1 and rat IgG1 PE (Pharmingen).

Cells

Peripheral blood was obtained from six healthy blood donors, three males and three females, with no history of atopic disease, and from six patients diagnosed with HIES. The patients, three males and three females, had all been diagnosed with HIES at the Institute of Child Health, London, University Hospital Nijmegen,

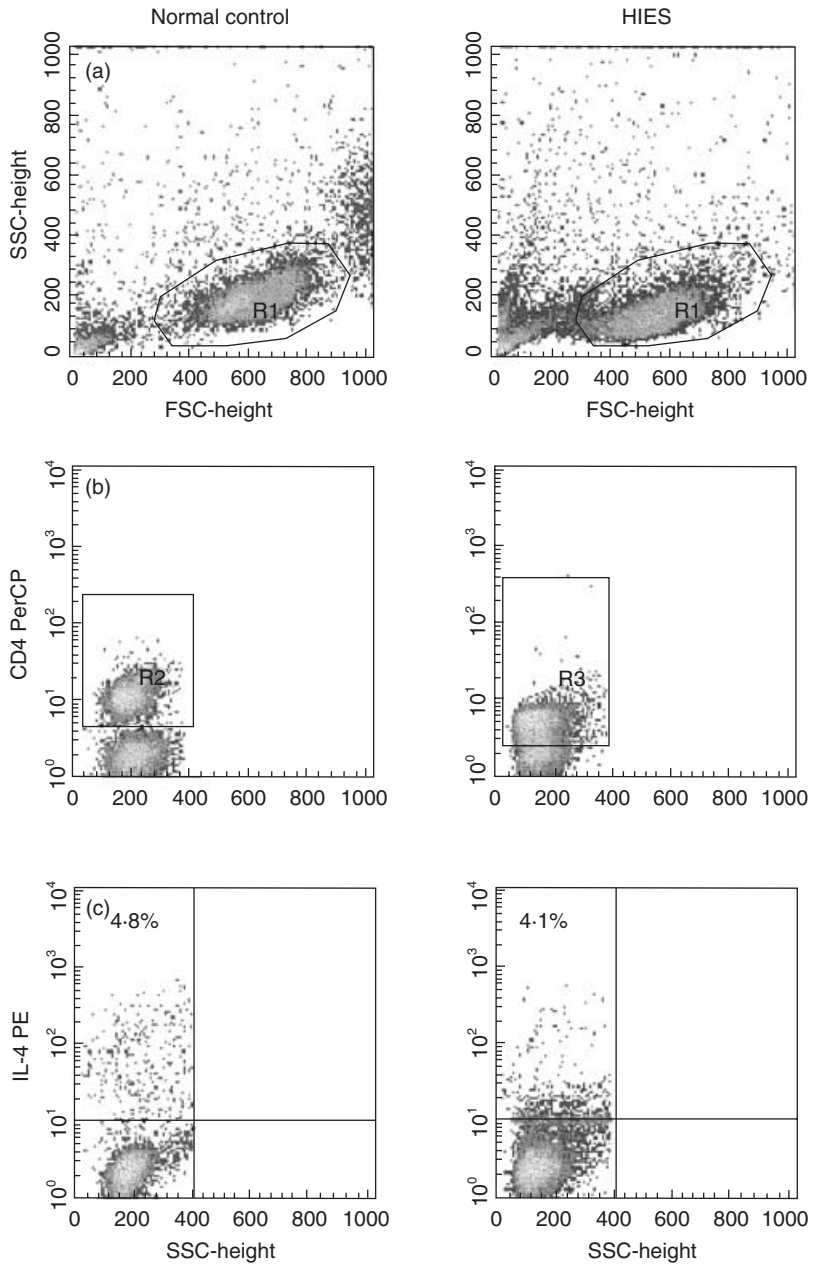

Fig. 1. Representative flow cytometric analysis of IL-4 expressing $CD4^+$ cells in a normal control and a patient with HIES. (a) FSC *versus* SSC profile of mononuclear cells (cells in gate R1). (b) CD4 positive cells from gate R1. (c) IL-4 expressing cells from gate R2 (normal control) and R3 (HIES), respectively. The percentage of cells shown in (c) was aquired by subtracting the percentage of cells staining positive for isotype controls in R2 and R3 from the percentage of IL-4-expressing cells.

and The Children's Hospital, Iceland, based on clinical symptoms including history of recurrent staphylococcal infections, facial and skeletal features associated with the syndrome and highly elevated serum IgE levels (≥20 000 IU/ml). The age of the patients at study ranged from 10 to 35 years and the healthy blood donors from 25 to 35. Mononuclear cells (MNC) were isolated by centrifugation over Histopaque-1077 gradients (Sigma, St Louis, MO, USA).

Cell culture

MNC (1×10^6/ml) were cultured in 24-well plates (Falcon, NJ, USA) for 6 h at 37°C and 5% CO_2 in AIM-V serum free medium (Life Technologies, Paisley, UK) supplemented with penicillin, streptomycin, PMA (100 ng/ml) and calcium ionophore (1 μg/ml). The protein transport inhibitor brefeldin A (Pharmingen) was included in all cultures as recommended by the manufacturer. The optimal concentration of PMA and calcium ionophore and the kinetics of IL-4 and IL-13 expression was determined by staining of MNC from a healthy individual. The optimal concentration of PMA and calcium ionophore was 100 ng and 1 μg, respectively (data not shown) and the expression of both cytokines peaked at approximately 6 h (data not shown).

Intracellular cytokine staining

Intracellular cytokine staining was performed using the Cytofix/CytoPerm Plus Kit (Pharmingen) according to the manufacturer's instructions. Cultured mononuclear cells were washed twice in AIM-V medium, Fc receptors blocked with unconjugated irrelevant mouse and rat IgG1 antibodies and then stained with PerCP conjugated anti-CD4. The cells were then fixed and permeabilized with Cytofix/Cytoperm solution and incubated with PE conjugated monoclonal anti-IL-4 and IL-13. The specificity of the antibodies was analysed in blocking experiments using unconjugated forms of the same monoclonal antibodies to IL-4 and IL-13 (data not shown).

Flow cytometry

The intracellular expression of IL-4 and IL-13 in mononuclear cells and $CD4^+$ cells was monitored on Becton Dickinson FACSCalibur flow cytometer equipped with an argon ion laser. A minimum of 20 000 lymphocyte-gated events were aquired in list mode and analysed with CellQuest software (Becton Dickinson).

Statistical analysis

The Mann–Whitney *U*-test was used to compare the proportion of IL-4 and IL-13 expressing cells from healthy control subjects and HIES patients. The calculations were made using GraphPad Prism version 3·00 for Windows, GraphPad Software, San Diego, California USA, www.graphpad.com. The difference was considered significant if $P < 0{\cdot}05$. The results are presented as mean ± standard error of the mean.

RESULTS

IL-4 expression by MNC and $CD4^+$ cells

No significant difference was found in the proportion of IL-4 expressing MNC from healthy controls and patients with the HIES, respectively. This was true both for the resting and the activated state. The mean proportion of resting IL-4 expressing MNC was 2·00% ± 0·13% in healthy controls but 0·83 ± 0·56 in patients with the HIES. In activated MNC the mean proportion was 1·62 ± 0·13 in healthy controls and 0·62 ± 0·44 in HIES. Similar to MNC, no significant difference was found in the proportion of IL-4 expressing $CD4^+$ cells from healthy controls and patients with the HIES, regardless of whether cells were resting or activated (Fig. 1). The mean proportion of resting IL-4 expressing $CD4^+$ cells was 4·04 ± 0·41 in healthy controls and 4·21 ± 1·69 in patients with the HIES. In activated $CD4^+$ cells the mean proportion was 4·37 ± 0·36 in healthy controls and 5·47 ± 2·91 in patients with the HIES.

IL-13 expression by MNC and $CD4^+$ cells

No significant difference was found in the proportion of IL-13 expressing MNC cells from healthy controls and patients with the HIES, regardless of whether cells were resting or activated (Fig. 2). The mean proportion of resting IL-13 expressing MNC was 0·12 ± 0·06 in healthy controls but 0·27 ± 0·10 in HIES. In activated MNC the mean proportion was 0·18 ± 0·04 in healthy controls and 0·30 ± 0·18 in patients with the HIES. In contrast, significantly increased expression of IL-13 was found between both resting ($P < 0{\cdot}0411$) and activated ($P < 0{\cdot}0420$) $CD4^+$ cells from healthy controls and patients with the HIES (Figs 3 and 4). The mean proportion of resting IL-13 expressing $CD4^+$ cells was 0·26 ± 0·13 in healthy controls and 2·26 ± 1·15 in patients with the HIES. In activated $CD4^+$ cells the mean proportion was 0·32 ± 0·08 in healthy controls and 2·34 ± 1·12 in patients with the HIES.

DISCUSSION

This study is the first to show that resting or activated $CD4^+$ cells from patients with the HIES express higher levels of IL-13 compared to healthy controls. In contrast, the levels of IL-4 expression were similar in the two groups, results that are in agreement with previous studies [8, 15]. IL-4 expression was similar in both the mononuclear cell population as well as the $CD4^+$ population with no differences between resting or activated cells. The reasons for the differences observed in IL-13 between HIES and controls

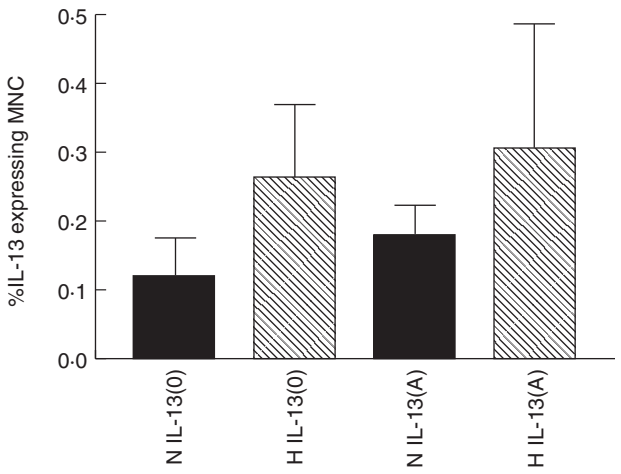

Fig. 2. The proportion of IL-13 expressing MNC from normal controls ($n = 6$) and patients with HIES ($n = 6$) before (0) and after activation (A) with PMA and calcium ionophore. The results are presented as mean ± s.e.m. N = normal controls. H = HIES patients.

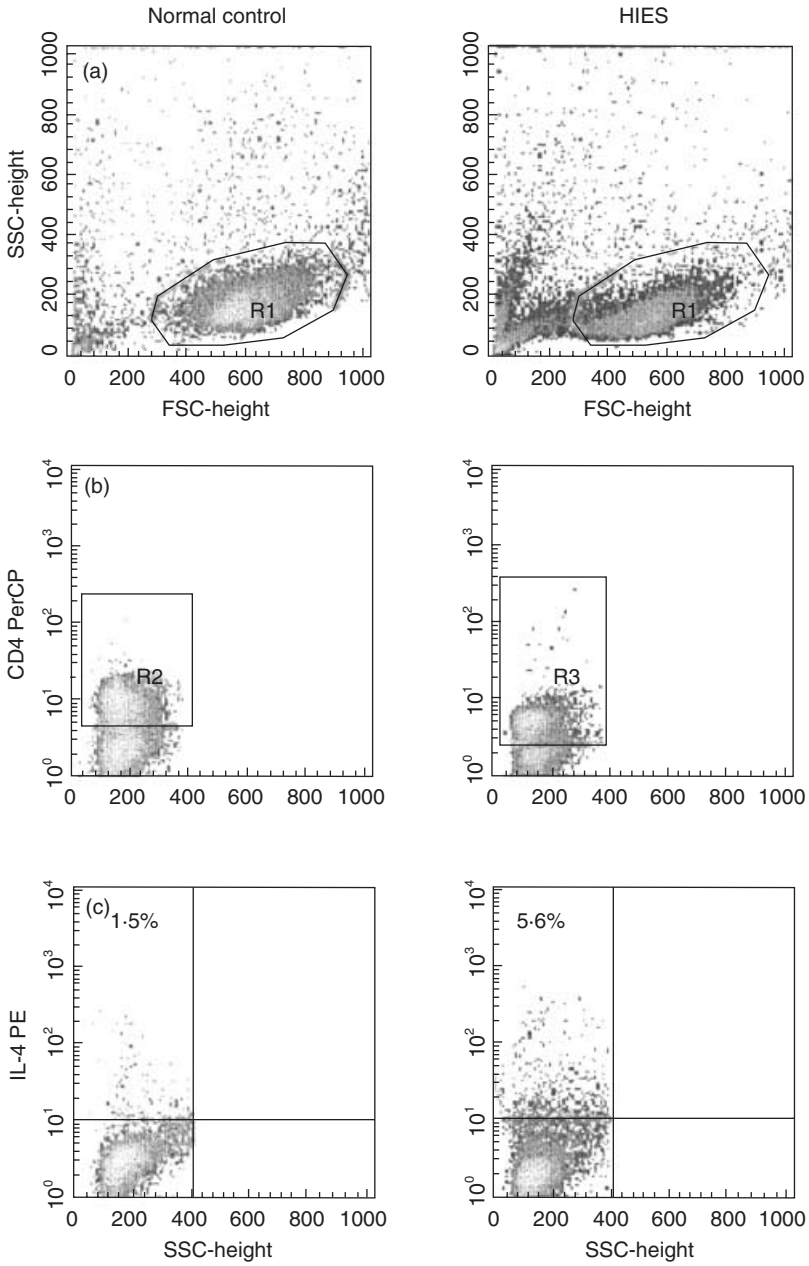

Fig. 3. Representative flow cytometric analysis of IL-13 expressing $CD4^+$ cells in a normal control and a patient with HIES. (a) FSC *versus* SSC profile of mononuclear cells (cells in gate R1). (b) CD4 positive cells from gate R1. (c) IL-13 expressing cells from gate R2 (normal control) and R3 (HIES), respectively. The percentage of cells shown in (c) was aquired by subtracting the percentage of cells staining positive for isotype controls in R2 and R3 from the percentage of IL-13 expressing cells.

are unclear, but may be related to the potential bias in the balance between Th1 and Th2 in HIES. Recent evidence suggests that enhancement of IFN-γproduction by IL-12 in patients with HIES is significantly lower compared to healthy controls [16]. This could theoretically favour naive T-cells differentiating into Th2 cells and thus have a direct effect on production of cytokines such as IL-13.

In contrast to the results obtained in our study a previous study has shown significantly higher IL-4 but similar IL-13 levels in patients with suspected HIES compared to controls [7]. In this study, ELISA was used to measure the concentration of IL-4 and IL-13 in the sera of patients. The differences between the results of our studies may, however, be methodological, as we studied intracellular expression of cytokines.

The advantage of the flow cytometer, as used in our study, is the possibility of studying cytokine-producing cells at the single cell level. It also has clear advantages over other methods used for studying cytokine production. The ELISA or ELISPOT can be difficult to interpret, mainly because of background staining and the inherent variability in counting between observers. This is particularly true where the number of cytokine-producing cells is low, as is often the case. Flow cytometric analysis also

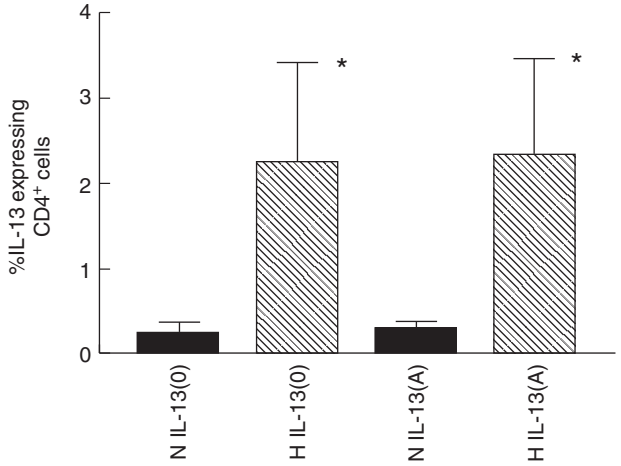

Fig. 4. The proportion of IL-13 expressing $CD4^+$ cells from normal controls ($n = 6$) and patients with HIES ($n = 6$) before (0) and after activation (A) with PMA and calcium ionophore. The difference between the proportions of IL-13 expressing $CD4^+$ cells in normal controls and patients with HIES was significant both in the resting and activated state ($^*P < 0{\cdot}05$). The results are presented as mean ± s.e.m. N = normal controls. H = HIES patients.

allows multiple parameters to be studied simultaneously, thus enabling phenotypic determination of the cytokine producing cells [17].

Determining the optimal amount of PMA and calcium ionophore for cell activation as well as the time of cell harvest is important to detect the point of maximal IL-4 and IL-13 expression. It has been shown that cell activating agents can differ in their ability to induce cytokine production, i.e. PMA and calcium ionophore induce production of Th2 cytokines such as IL-4, IL-5 and IL-10 [18]. The induction of IL-4 and IL-13 expression and production with polyclonal activation in the human system is minimal. With our culture conditions, maximal positivity was detected after approximately 4–8 h (6 h) for both IL-4 and IL-13 (data not shown).

While $CD4^+$ cells are the main source of IL-4 and IL-13 we also wanted to study the MNC population, because cells other than those that are $CD4^+$ are capable of producing IL-4 and IL-13. It has been shown recently that natural killer cells, which often fall within the MNC gate, are capable of producing IL-13 [19]. In some instances we had difficulty in gating on $CD4^+$ cells after activation with PMA and calcium ionophore because of activation-dependent down-regulation of $CD4^+$ expression, as has been reported by others [18,20]. While the decrease in CD4 expression in our studies was substantial the $CD4^+$ population remained quite distinct from the $CD4^-$ population. The mean fluorescence intensity was decreased in the activated state compared to the resting state, indicating that the density of CD4 molecules on the cell surface was reduced.

Because HIES is characterized by skeletal abnormalities it is interesting to speculate about the role of IL-13 in bone metabolism. Recent experiments have shown that IL-13 has a negative effect on the growth of osteoblasts as well as stimulating them to produce IL-6, a potent osteoclast recruitment factor [21]. Increased levels of IL-13 in HIES could therefore be a factor in the skeletal features of the syndrome.

In conclusion, our results indicate that the proportion of IL-13 expressing $CD4^+$ cells is higher in patients with HIES compared to healthy controls. A higher proportion of IL-13 expressing $CD4^+$ cells could account in part for raised IgE levels in HIES in combination with decreased IFN-γ production.

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