

Case report

Impaired type-1 activity and increased NK cells in Gleich's syndrome

Background: An altered production of cytokines has been described in Gleich's syndrome. Our aim was to study the cytokine production at the single-cell level in a patient with Gleich's syndrome and to determine whether it changed during a flare episode.

Methods: Blood samples were obtained from a 30-year-old woman diagnosed with Gleich's syndrome before and during the subsequent flare of edema of trunk and arms and weight gain. The major lymphoid subsets (CD4+, CD8+, and CD19+) and natural killer (NK) cells were measured by flow cytometry. Cytokine-producing T cells (IL-2, IFN- γ , and IL-4) were quantified in whole blood by intracellular staining with specific monoclonal antibodies and flow cytometry analysis after polyclonal stimulation with phorbol 12-myristate 13-acetate and ionomycin.

Results: Increased numbers of immature CD4+CD8+ T cells and NK cells were observed in peripheral blood during the asymptomatic period. The latter population significantly decreased during the flare. Type-1 cells were decreased in both asymptomatic and, more markedly, during the attack with respect to healthy subjects.

Conclusions: The decreased type-1 response demonstrated in this patient might be the basis of the hypereosinophilia of Gleich's syndrome. Besides, the NK cells might play a role in the pathogenesis of these inflammatory episodes.

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Among the different hypereosinophilic conditions, there is a rare syndrome first described by Gleich et al. (1). This clinical entity is associated with angioedema of the face, neck, extremities, and trunk; weight gain; hypereosinophilia (60–70%); and increased levels of serum IgM (1). This syndrome, which has a good prognosis, has no visceral organ involvement, and histologic studies have revealed only numerous eosinophils infiltrating the dermis (1–3). In 1998, Chikama et al. proposed that this syndrome could be classified into two types, one of them being an episodic (recurrent) type, as reported by Gleich et al. and the other one a nonepisodic type (4).

The pathogenesis of Gleich's syndrome is unknown, although a role of T helper (Th) cells has been assumed, since increased numbers of CD4+ T cells have been found (2, 3). Th cells can be divided into two subtypes according to the profile of cytokines they secrete: Th1 and Th2 cells (5). Th1 cells secrete typically IL-2, IFN- γ , and TNF- β , whereas Th2 cells produce IL-4, IL-5, IL-6, and IL-10 (5). The same cell can behave as Th1 or Th2 depending on environmental conditions. Thus, the Th1/Th2 equilibrium is reversible, and the cytokines produced by each type can inhibit the opposite Th subset (5). Th1 cells promote the activation of cell-mediated

immune responses and are related with a more protective immunity. On the contrary, Th2 cells are implicated in the B-cell activation responsible for humoral immunity. In particular, Th2 cells seem to participate in the production of IgE and the differentiation and activation of eosinophils (6). In this regard, increased serum levels and production *in vitro* of IL-6 have been demonstrated during the acute phase of Gleich's syndrome (7).

Recently, a new technique of flow cytometry to determine the production of Th1/Th2 cytokines at a single-cell level has been described (8). Here, we have studied the changes in the peripheral blood lymphocyte subsets and the type-1/type-2 equilibrium during the acute phase of angioedema in a patient with Gleich's syndrome with respect to the previous asymptomatic phase.

Material and methods

The patient

A 30-year-old woman suffered from recurrent episodes of pruriginous angioedema, mainly on the face, thorax, arms, and legs, associated with discomfort and weight gain (3–4 kg), hypereosinophilia (34–69%), and hypergammaglobulinemia. The monthly episodes started few days before the menstrual cycle. All symptoms resolved at the end

of each episode. The disease had begun 3 years before, with increased severity of the attacks in these years. At the admission to our service during a flare, she showed edema of the trunk and arms and a weight gain of 3 kg. Laboratory investigations revealed leukocytosis ($38\,400/\text{mm}^3$) with 69% eosinophils ($26\,596/\text{mm}^3$) and IgM 420 mg/dl (normal range: 60–250 mg/dl). The erythrocyte sedimentation rate level was normal. The patient presented a personal history of perennial rhinoconjunctivitis due to allergy to house-dust mite. The allergologic study showed positivity to *Dermatophagoides pteronyssinus* and *D. farinae* (4×3 mm) and specific IgE positive to them. Other investigations, including biochemistry study; serology for parasites; serum levels of IgA, IgG, and total IgE; rheumatoid factor; antinuclear antibody; C reactive protein; complement components (CH50, C3, C4, and C1 esterase inhibitor); serum protein electrophoresis; chest radiograph; CT scans of the thorax and abdomen; and electrocardiogram were normal. Skin biopsy obtained in an asymptomatic period showed an unspecific mononuclear perivascular infiltrate. The patient was treated with low-dose oral corticosteroids, leading to clinical remission of her symptoms and decrease of leukocyte and eosinophil counts.

Flow cytometry

Blood samples for cytometric studies were collected immediately before and during the acute phase mentioned in tubes containing potassium-ethylenediamine tetraacetic acid (EDTA), except for the measurement of cytokine-producing cells that were drawn into sodium heparin (Becton Dickinson, Plymouth, UK).

Lymphocyte subsets were studied by two-color fluorescence flow cytometry with a panel of specific monoclonal antibodies (mAbs) for the following cell surface proteins: CD3, CD4, CD8, CD16, CD19, CD20, and CD56 (Becton Dickinson, San Jose, CA, USA). The mAbs were conjugated to fluorescein isothiocyanate (FITC) or phycoerythrin (PE). Whole blood (100 μl) was incubated with a double combination of these mAbs for 30 min at room temperature. After incubation, the samples were lysed and fixed with 2 ml of FACS lysing solution (Becton Dickinson). Finally, the cells were washed twice and resuspended in 500 μl of phosphate-buffered saline (PBS) for flow cytometry analysis. At least 10^4 lymphocytes per sample, gated by the FSC/SSC parameters, were analyzed with the CellQuest software (Becton Dickinson). The cutoff for positive fluorescence was set to include < 1% of negative control mouse IgG conjugates (Becton Dickinson).

Single-cell analysis of cytokine-producing T cells

An aliquot of whole blood was first treated with 30 ng/ml of phorbol 12-myristate 13-acetate (PMA) in combination with 1 $\mu\text{g}/\text{ml}$ ionomycin (Io) for 5 h at 37°C in the presence of brefeldin A (10 $\mu\text{g}/\text{ml}$); and an aliquot of this "activated" blood was obtained for staining. First, cell-surface staining was carried out with antihuman CD3 conjugated to PerCP and antihuman CD4 coupled to PE mAbs. After incubation, red blood cells were lysed and lymphocytes fixed by adding FACS lysing solution (Becton Dickinson). Subsequently, cells were washed and incubated with a Permeabilizing SolutionTM (Becton Dickinson). After permeabilization, the cells were incubated for 30 min at room temperature with mAbs specific for human cytokines. Finally, the cells were washed twice with permeabilizing solution and resuspended for flow cytometry analysis. The following antihuman cytokine mAbs conjugated to FITC were used: mouse IgG2a antihuman IL-2, mouse IgG2b antihuman IFN- γ , and mouse IgG1 antihuman IL-4 (Becton Dickinson).

In parallel with the cytokine staining, another aliquot of activated blood was incubated with an antihuman CD69 mAb. Only those samples with > 90% of CD69+ lymphocytes were considered to be activated and their cytokine data collected.

As reference values, percentages of cytokine-producing cells were measured in 26 healthy, aged-paired subjects.

Results

Hemogram and serum immunoglobulin levels

The hemogram showed an important leukocytosis in both the asymptomatic ($33\,400$ cells/ mm^3) and the acute ($38\,400$ cells/ mm^3) phases; this was mostly due to the accumulation of eosinophils. Moreover, blood eosinophils showed a 36% increment during the outbreak with respect to the asymptomatic period (Fig. 1). The remaining leukocyte population did not demonstrate important changes during either of the phases with respect to reference values.

There were no differences in serum IgG and IgA between the asymptomatic and acute phases. However, an almost twofold increase in serum IgM was found in the acute episode (Fig. 1). In both cases, the levels of IgE were below our lower limit of detection (< 17.5 IU/ml).

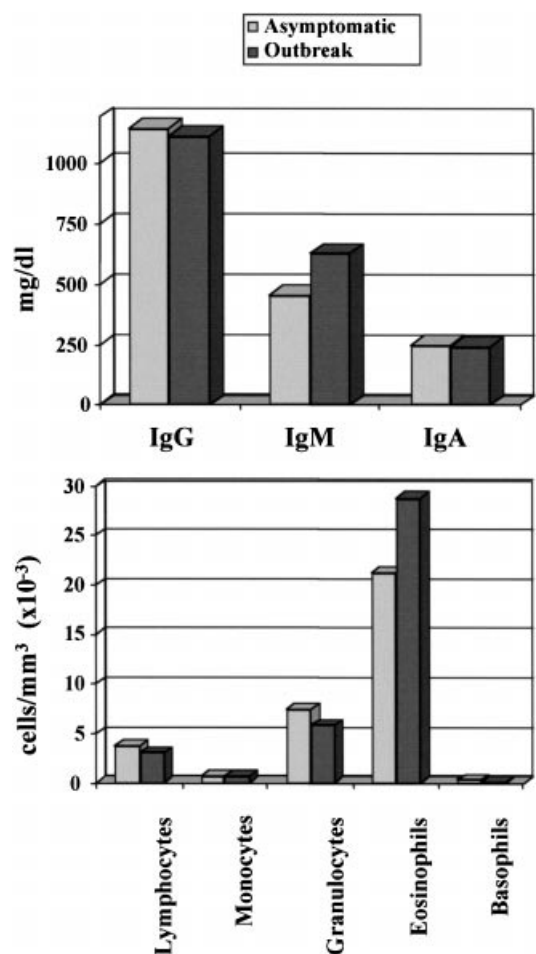


Figure 1. Serum immunoglobulin levels (upper graph) and leukocyte absolute numbers (lower graph) in patient reported here. Patient showed hypereosinophilia and increased titers of IgM in both periods, although they were higher during episode of angioedema. Reference range values: IgG (734–1486 mg/dl), IgM (41–201 mg/dl), IgA (49–401 mg/dl), cells (984–5519 cells/ mm^3), monocytes (82–1166 cells/ mm^3), granulocytes (2026–8122 cells/ mm^3), eosinophils (0–756 cells/ mm^3), and basophils (0–216 cells/ mm^3).

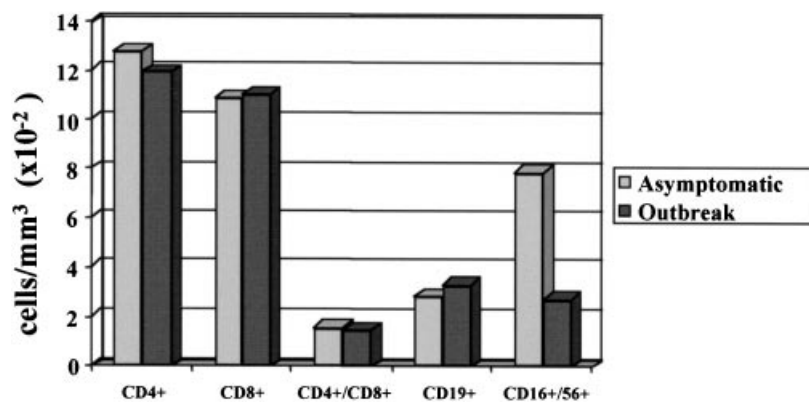


Figure 2. Absolute numbers of major peripheral blood lymphoid populations. Most impressive finding was increased numbers of immature T cells (CD4+/CD8+) and NK cells (CD16+/CD56+/CD3-). Latter decreased significantly during angioedema attack. Reference range values: CD4+ (543–1248 cells/mm³), CD8+ (356–870 cells/mm³), CD4+CD8+ (0–97 cells/mm³), CD19+ (71–375 cells/mm³), and CD16+CD56+ (50–245 cells/mm³).

With regard to IgG subclasses, an increase only in IgG1 was observed during the outbreak (from 784 to 857 mg/dl).

Changes in lymphocyte subsets during acute phase

Peripheral blood CD4+ and CD8+ T cells from the patient showed normal levels and did not change during the outbreak of the disease (Fig. 2). The blood counts of B cells, identified as CD19+ cells, were normal and increased only slightly during the outbreak (Fig. 2). Absolute numbers of T cells with immature phenotype (CD4+CD8+) were significantly increased, although they were not related to the activity of the disease, since there was no change in their number during the acute phase. The finding of significantly increased numbers of blood natural killer (NK) cells (CD16+CD56+) in the asymptomatic patient was more remarkable than the marked decrease during the outbreak of the disease when numbers were equivalent to those in healthy subjects (Fig. 2).

Measurement of type 1 and type 2 cellular cytokine responses

Fig. 3 shows the percentage of change in the proportions of cytokine-producing T cells observed during the outbreak with respect to the asymptomatic phase of the syndrome. Flow cytometry analysis after polyclonal stimulation demonstrated a marked decrease in the percentages of IL-2-secreting, CD4+ T cells with very slight changes (asymptomatic phase: 2.45%; outbreak: 0.80%), whereas the percentages of IFN- γ -secreting, CD4+ T cells were similar to those of healthy subjects and remained almost unchanged (asymptomatic phase: 9.49%; outbreak: 10.10%). However, IL-2-producing, CD8+ T cells, which were more numerous than in healthy controls, decreased during the acute episode of Gleich's syndrome (asymptomatic phase: 14.81% for IL-2; outbreak: 0.13%). The decrease for CD8+ IFN- γ + cells was not significant (asymptomatic phase: 8.20% for

IL-2; outbreak: 6.16%). However, slight changes in the frequencies of IL-4-producing, CD4+ and CD8+ T cells were detected during the outbreak (asymptomatic phase: 0.36% and 0.14%; outbreak: 0.59% and 0.11%, respectively).

Discussion

Gleich's syndrome, a rare condition with a well-defined clinical course, is distinct from other primary hyper-eosinophilic syndromes (1). Here, we described a patient exhibiting the characteristics of this syndrome: recurrent angioedema, weight gain, and malaise associated with chronic hypereosinophilia and elevated serum levels of IgM. Besides, the response to steroids was excellent with a very good prognosis. In the episodic and nonepisodic types, there is a predominance of young females (4). Moreover, to our knowledge, this is the first case in which the attacks were temporarily related to the

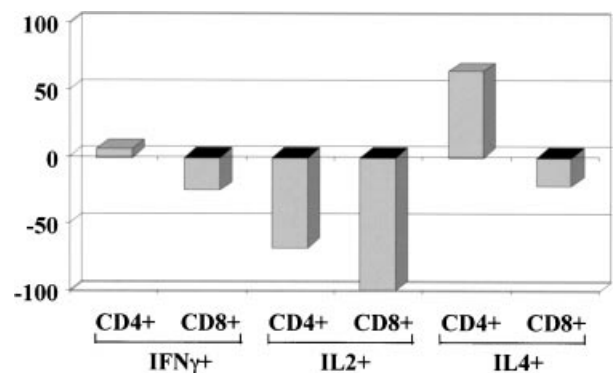


Figure 3. Percentages of variation in frequency of cytokine-producing CD4+ and CD8+ T cells during episode of angioedema with respect to asymptomatic phase. Decreased type-1 response was observed. Mean percentages $\pm$ standard deviation from 26 healthy, aged-paired subjects were as follows: CD4+ IL-2+ (28 ± 12), CD4+ IFN- γ + (11 ± 5), CD4+ IL-4+ (1.55 ± 1), CD8+ IL-2+ (4.9 ± 3), CD8+ IFN- γ + (16 ± 7), and CD8+ IL-4+ (0.4 ± 0.1).

menstrual cycle. In addition, total IgE in our patient was very low although she had allergic rhinitis, in contradiction of data from the literature (3).

Although this clinical entity is well known, its pathogenesis remains largely unknown. It is clear that eosinophils play a central role in the disease, but the primary mechanisms by which they are activated are not so clear. The favored hypothesis is based on the finding of increased T-cell activation and cytokine production (9). It has been suggested that Th cell stimulation previous to the hypereosinophilic attack induces the secretion of cytokines that act on eosinophils (9, 10). The cytokines that mainly activate eosinophils are those associated with Th2 activity (IL-4, IL-5, IL-6, IL-10, and IL-13) (11). In fact, several reports have found increased serum levels of some of those cytokines in patients with Gleich's syndrome during attacks (7, 10). Recently, a more reliable technique to characterize cytokine production has been made available (8). It consists of the quantification of the cytokine-secreting CD4+ or CD8+ T cells by flow cytometry after polyclonal stimulation and intracellular staining with specific mAbs. By using this technique, we have demonstrated that decreased type-1 activity at the single-cell level in Gleich's syndrome is more marked during the flare. We believe that the hypereosinophilia and the eosinophil activation leading to the angioedema episode are secondary to the predominance of type-2 responses due to a lesser type-1 activity. In this sense, clonal Th2 cells have been found in other hypereosinophilic conditions, such as idiopathic hypereosinophilic syndrome (12). Moreover, corticosteroid treatment induces a decrease in the IL-5 and IL-6 levels (13) and could be responsible for the remission of the symptoms in our patient.

Another relevant finding was the high counts of blood NK cells in the patient. It is well established that these cells are important *in vivo*, producers of IFN- γ which induces a polarization to Th1 response (5). Thus, the increased numbers of NK cells found in the asymptomatic phase of our patient would favor a Th1-like response, whereas the decline in their number during the outbreak would polarize toward a Th2-like response. Besides, NK cells can produce IL-5, which has been demonstrated to play a central role in various kinds of eosinophilic inflammation (14). However, the decrease in blood NK cells during the attack of Gleich's syndrome, when the eosinophilia is highest, is not in agreement with this theory. It remains to be investigated whether the changes in the number of NK cells in this syndrome are correlated with changes in the capacity to produce cytokines such as IL-5. Furthermore, the polarization of the Th1/Th2 responses observed could modify the production of cytokines by NK cells, since this has been demonstrated to be regulated by cytokines such as IL-4 and IL-12 (15). Finally, we found increased levels of CD4+CD8+ T cells in peripheral blood. Although this finding might suggest some pathogenic implication for this immature population, the fact that levels did not change during the attack rules out this possibility. It also remains to investigate the production of cytokines by these cells.

In summary, the decreased type-1 activity might be responsible for the hypereosinophilia observed during the attacks of Gleich's syndrome. Besides, the increased blood NK cells observed and the changes they undergo during attacks suggest that these cells play a relevant role in the pathogenesis of this syndrome.

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