OBSERVATION

Functional Dysregulation of Dendritic Cells in Patients With Papular Urticaria Caused by Fleabite

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Background: Papular urticaria is a chronic allergic disease caused by fleabite. The presence of eosinophils, predominance of CD4-positive T cells in lesions, and IgE response suggest a Th2 immune response to flea proteins in patients with papular urticaria caused by fleabite (PUFB). Although PUFB is defined as an allergic reaction, the immunological mechanisms and the role of dendritic cells (DCs) have not been established.

Observations: Flea body extract did not induce the maturation of monocyte-derived DCs in 10 patients with PUFB and in 10 healthy children. Simultaneous exposure of DCs to flea extract and lipopolysaccharide induced increased expression of CD83 ($P<.01$), CD86 ($P<.01$), and HLA-DR ($P<.05$), which was statistically significantly greater in patients' cells. Dendritic cells from patients stimulated with lipopolysaccharide secreted less interleukin 6 (IL-6) and IL-10 than DCs from control subjects.

Conclusions: Results of this study indicate that the involvement of DCs in an immune response produced in the disease is mediated through the altered expression of membrane molecules. This may be related to constitutive impairment in the production of regulatory cytokines such as IL-6 and IL-10 in these patients.

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PAPULAR URTICARIA, TRADITIONally defined as chronic allergic disease, is caused by exposure to ectoparasites such as fleas.[1] Clinical observations have shown that papular urticaria caused by fleabite (PUFB) is more frequent in children at approximately 1 year

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of age and ceases at approximately 7 years of age. IgE and IgG can recognize antigenic proteins from complete flea extract (26-150 kDa) in patients with PUFB, as in healthy individuals.[2] The presence of eosinophils in biopsy specimens of PUFB, predominance of CD4-positive T cells in lesions, and IgE response suggest that an immune reaction to flea proteins in patients with PUFB is predominantly Th2.[3] Atopic individuals have a persistent Th2 response accompanied by high IgE production.[4-9] The function of dendritic cells (DCs) may be altered in atopic individuals, inducing a predominantly Th2 response.[10-12]

In tissue, immature DCs (iDCs) have high phagocytic ability. Once activated by antigens, they migrate to the nearest lymphoid organ, where they activate T cells.[13] During migration, they increase expression of molecules related to antigen presentation, thereby becoming mature DCs (mDCs). Although PUFB is defined as an allergic reaction, the immunological mechanisms underlying the process have not been clearly established. The objective of this study was to determine the effect of complete flea body extract on the marker expression of and cytokine secretion by DCs derived from peripheral blood monocytes of patients with PUFB.

METHODS

SAMPLE

The sample included 10 patients aged 1 to 15 years clinically diagnosed as having papular urticaria for no longer than 5 years. From this group, 4 children reported a personal history and 9 children a family history of atopy (asthma, allergic rhinitis, and atopic dermatitis). They attended the pediatric dermatology and allergy services at the Fundación Santa Fe de Bogotá, Bogotá, Colombia. Exclusion criteria included the presentation of secondary infected lesions, immunosuppression by sys-

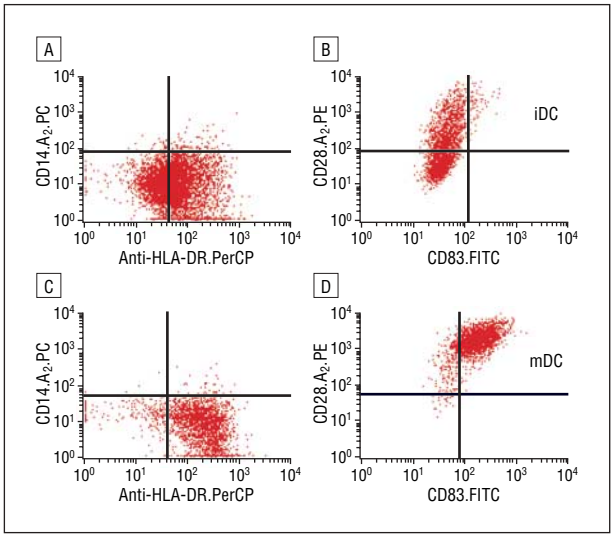

Figure 1. Plots of points representing the expression of immature dendritic cell (iDC) (A and B) and mature dendritic cell (mDC) (C and D) markers.

temic disease, treatment with immunosuppressive medication, antihistamine administration 15 days before the consultation, or treatment with flea extract. Healthy children included 10 patients from the same institution with selective surgical indication who shared the same age group and socioeconomic characteristics as the study patients. From this group, 2 children reported a family history and none a personal history of atopy. This investigation was approved by ethics committees of the Fundación Santa Fe de Bogotá and the Pontificia Universidad Javeriana.

DIAGNOSING THE DISEASE

The diagnosis of PUFB was made according to clinical characteristics. Patients had lesions that appeared usually as groups and pruritic papules that were often excoriated or crusted, appearing intermittently in a chronic course and leaving hypopigmented or hyperpigmented macules. They were located in areas where clothing fits snugly such as the socks and the waistband. In some patients, exposed areas of the extremities were also affected.

OBTAINING ANTIGEN FROM FLEAS

A complete *Ctenocephalides felis* (Greer Laboratories, Lenoir, North Carolina) flea aqueous extract (10% weight per volume) was prepared by maceration in a phosphate-buffered saline solution, with constant shaking for 2 hours at room temperature. It was centrifuged at 15 000 rpm for 15 minutes at 4°C and was filtered through a 0.22-µm membrane. The protein concentration, determined by Bradford technique, was 1.3 mg/dL. The extract was aliquoted and stored at –70°C.

OBTAINING AND STIMULATING DCs

After obtaining informed consent from the child's legal guardian, 10 to 15 mL of heparin-anticoagulated blood was drawn from each child. Peripheral blood mononuclear cells were obtained using Ficoll-Hypaque gradients.

Monocytes were separated with anti-CD14 monoclonal antibodies coupled to magnetic pearls using a commercially available system (MiniMACS; Miltenyi Biotech, Auburn, California). The cells obtained were washed in base medium (RPMI 1640) with 2% fetal calf serum. Viability was evaluated using trypan blue stain, and then cells were counted. The purity of the population was determined by flow cytometry using an anti-CD14.PE antibody. CD14-positive cell populations demonstrated greater than 94% purity in all cases.

CD14-positive cells were cultured in complete medium (RPMI 1640, antibiotics, nonessential amino acids, sodium pyruvate, and 10% fetal calf serum) in 48-well plates at a density of 5×10^5/mL in the presence of 1000-U/mL interleukin 4 (IL-4) and 50-ng/mL granulocyte-macrophage colony-stimulating factor (GM-CSF) (R and D Systems, Minneapolis, Minnesota) for 7 days to obtain iDCs. In the last 48 hours, 1- µg/mL lipopolysaccharide (LPS) was added to obtain mDCs or 10 µg of flea extract in the presence or absence of LPS to evaluate the effect of the flea extract.

A DC culture was exposed to flea extract in the presence or absence of polymyxin B sulfate at concentrations inhibiting LPS activity to establish the presence of small amounts of LPS in the flea extract that might have altered cell behavior.[14] No difference was found regarding marker expression or cytokine secretion (data not shown). All reagents used in the culture were negative for detectable LPS levels (*Lymulus* species amoebocytes kit; BioWhittaker, Walkersville, Maryland), with a sensitivity of 0.1 endotoxin unit per milliliter.[14]

FLOW CYTOMETRY

The presence of mDC markers was evaluated by flow cytometry with anti-CD14.APC (BD Biosciences, San Jose, California), anti-CD83.FITC (Pharmingen, San Diego, California), anti-CD86.PE (Pharmingen), and anti-HLA-DR.PerCP (BD Biosciences) antibodies with IgG1.FITC (Pharmingen), IgG2b.PE (Pharmingen), and IgG2a.PerCP (BD Biosciences) isotype controls. A kit (Cytometric Bead Array, BD Biosciences) was used for quantifying cytokines in supernatant using pearls having different fluorescence intensity with peridinin chlorophyll protein, covered with capture antibodies fluorescent with R-phycoerythrin, for IL-1β, IL-6, IL-8, IL-10, IL-12p70, and tumor necrosis factor (TNF) α. Concentration was calculated using different cytokine patterns in known concentrations. Data were acquired using a flow cytometer (FACSCalibur; BD Biosciences) and were then analyzed using commercially available software (Cell Quest; BD Biosciences).

ANALYZING THE RESULTS

The results are presented as mean ± SE. Statistically significant differences between means were established using the Mann-Whitney test.

RESULTS

OBTAINING DCs

HLA-DR and CD86 molecule expression was found in cells exposed to IL-4 and GM-CSF at the end of the culture by day 7 (**Figure 1** A and B). Adding IL-4 and GM-CSF to peripheral blood monocyte cultures induced the loss of CD14 molecule expression, indicating that monocytes were differentiated to DCs (Figure 1A and C). Cells exposed to LPS increased HLA-DR and CD86 expression and expressed the mature CD83 marker (Figure 1C and D). Culture conditions led to obtaining iDCs and mDCs. The percentage of marker expression was similar in cells obtained from patients and from healthy control subjects (**Figure 2**).

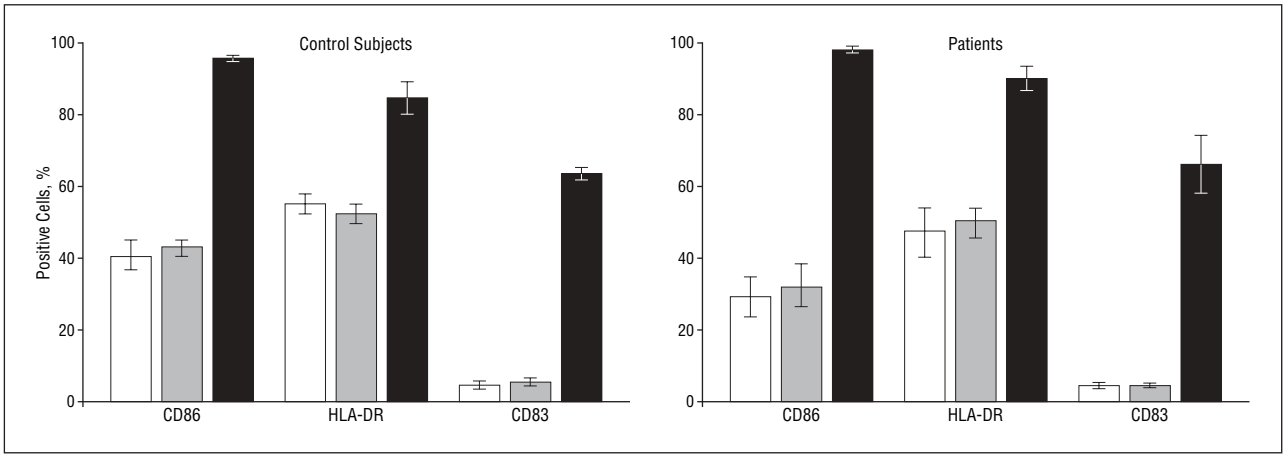

Figure 2. CD83, CD86, and HLA-DR expression in immature dendritic cells (white), in immature dendritic cells exposed to fleas (gray), and in mature dendritic cells (black) from healthy control subjects and from patients. Data are given as mean±SEM.

EFFECT OF FLEA EXTRACT ON DC CULTURES

There was no statistically significant difference between patient and control DCs exposed to flea extract, as both showed a phenotype similar to that of iDCs (Figure 2). Flea extract alone did not induce monocyte-derived DC maturation.

EFFECT OF FLEA EXTRACT PLUS LPS ON DC CULTURE

Simultaneous stimulation with flea extract and LPS increased the levels of CD83 ($P<.01$), CD86 ($P<.01$), and HLA-DR ($P<.05$) in patients' DCs compared with those of healthy controls. These results are shown in **Figure 3**.

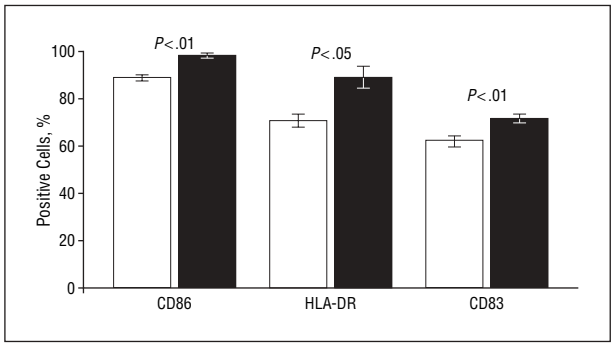

Figure 3. CD83, CD86, and HLA-DR expression in dendritic cells exposed to lipopolysaccharide and flea extract from healthy control subjects (white) and from patients (black). Data are given as mean±SEM.

CYTOKINES SECRETED BY DCs

Cytokine secretion by iDCs in culture medium alone showed no difference compared with cytokine secretion by iDCs exposed to flea extract, indicating that in the extract used, no molecules were able to induce functional changes in cells. Patients' mDCs showed a statistically significant reduction in IL-6 and IL-10 ($P<.05$ for both) compared with cells obtained from healthy controls (**Figure 4**). The IL-1β, IL-8, and TNF-α levels did not show statistically significant differences in any of the conditions studied. There was a reduction of IL-12p70 levels in patients compared with controls when cells were exposed to LPS or flea extract, although this difference was not statistically significant.

COMMENT

There are many important factors in determining a Th1/Th2 response, including antigen type and dose, the exposure route, the host's genetic background, the microenvironment of the cytokines found during antigen presentation, and the type of DCs involved and its interaction with the T cells and with the costimulatory molecules expressed.[13,15] Flea body extract did not induce the maturation of iDCs by itself, and this inability to induce reactivity has been previously proposed; however, when oral antigens are combined with Freund complete adjuvant, hypersensitivity is induced.[1,16] Therefore, flea oral secretion seems to contain a particular substance able to induce hypersensitivity in the presence of an adjuvant, and molecules having allergenic potential can be found in a complete extract.

This effect revealed by Freund complete adjuvant in in vivo experiments was similar to that observed in vitro when DCs exposed to flea extract in the presence of LPS increased expression of molecules related to antigen presentation such as HLA-DR, CD83, and CD86. The adjuvant action of LPS has also been observed with aeroallergens. Low inhaled LPS levels are needed for inducing a Th2 response to inhaled antigens in a murine model of allergic sensitization involving DC activation.[17] Lipopolysaccharide may not be a relevant factor at the moment of the bite. Although not demonstrated, it is probable that some molecules on the skin such as collagen in the presence of flea antigen may act as adjuvants for a susceptible individual to develop hypersensitivity.[1,16]

The expression of CD86 in DCs is important for the induction of a Th2 response.[18] Mice with this molecule blocked that were exposed to albumin aerosols did not develop an allergic reaction.[19] In atopic dermatitis, the use of anti-CD86 antibodies inhibits the proliferation of T cells stimulated with mite extract.[20] In addition, increased expression of CD86 in patients with allergic

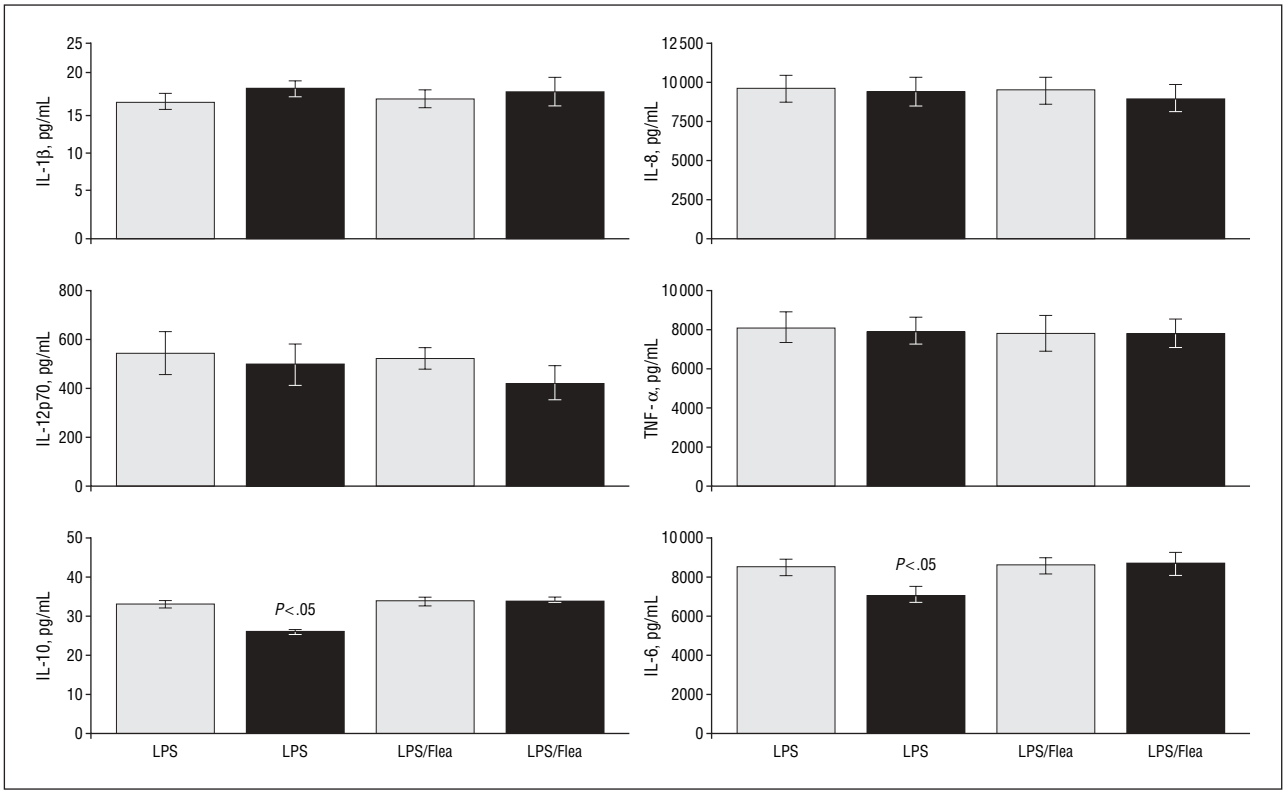

Figure 4. Cytokine levels produced by mature dendritic cells from healthy control subjects (gray) and from patients (black) exposed to lipopolysaccharide (LPS) with or without flea extract. Data are given as the mean ± SEM response of 10 individuals. IL indicates interleukin; TNF, tumor necrosis factor.

asthma has been demonstrated,[21] which is related to IgE synthesis.[22] The statistically significant increase in the expression of CD83, CD86, and HLA-DR observed in DCs obtained from patients experiencing PUFB demonstrated the specific effect of flea extract on patients' DCs compared with cells from healthy controls.

Interleukin 10 is considered to be an anti-inflammatory molecule because of its ability to inhibit the production of a large number of cytokines such as IL-2, IL-3, IL-12, TNF, GM-CSF, and interferon gamma,[23] and IL-10 lessens allergic inflammation because of its ability to inhibit the synthesis of proinflammatory cytokines such as IL-1, IL-4, IL-5, IL-6, and TNF-α. The effect of IL-10 on allergic disease has also been shown, as it has a tolerance-inducing effect on allergens by T cells,[24] inhibiting eosinophil survival[25] and IgE synthesis.[26] A statistically significant reduction of IL-6 and IL-10 was found in our patients' DCs with a maturing stimulus such as LPS, indicating a functional change in cells from patients compared with those of healthy controls. Such reduction may lead to an increase in CD86 expression and could favor the Th2 response. Findings from recent studies[27,28] indicate that allergen-specific T cells are detected in healthy patients and in allergic patients; however, the proportion of Tr1 to Th2 populations varies between them. Although higher frequencies of Th2-specific T cells are found in allergic individuals, T cells from healthy individuals predominantly secrete IL-10 and correspond to regulatory Tr1 cells.[27] Furthermore, evidence indicates that Tr1 cells, whose activity is mediated by the secretion of IL-10, are generated in vivo in human patients through allergen-specific immunotherapies.[28]

The differences found regarding surface molecule expression and levels of secreted cytokines by DCs in patients with PUFB indicate that these cells may play an active role in immunological mechanisms on which the development of the disease is based. The results show that the specific involvement of DCs in the immune response of papular urticaria is mediated by the altered expression of membrane molecules such as CD86 and HLA-DR. This finding may be related to a constitutive impairment in the production of regulatory cytokines such as IL-6 and IL-10 in patients with PUFB. Although this effect is abrogated with flea extract in the presence of LPS in vitro, this does not mean that the patients would not have active disease, because the inflammatory process involves the activation of not only the regulatory response but also the effector response. This modulating effect in the immune response generated by an adjuvant coupled with an antigen in vitro has been shown with other molecules.[29]

Dendritic cells from patients with PUFB manifest a dysregulated immune response similar to that of other allergic diseases such as asthma, allergic rhinitis, and atopic dermatitis. Therefore, we hypothesize that the immunological response of PUFB has an allergenic origin.

The immunopathologic mechanism of PUFB may be summarized by considering the following findings. The activation of the skin's DCs takes place under the influence of mediators secreted by local microenvironment cells. In atopic individuals, these mediators induce a functional change that affects not only the skin's resident cells but also the type of cytokines secreted by T cells. Accordingly, based on results of this research, patients with papular urticaria have increased expression of mol-

ecules related to antigenic presentation and lesser levels of regulatory cytokines. This scenario may favor the secretion of Th2 proinflammatory cytokines that contributes to the generation and maintenance of allergic reaction in skin caused by fleabite during childhood.

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Author Contributions: Ms Cuéllar had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. *Study concept and design:* Cuéllar, García, Rodríguez, Halpert, and Gómez. *Acquisition of data:* Cuéllar, García, Rodríguez, and Halpert. *Analysis and interpretation of data:* Cuéllar, García, Rodríguez, Halpert, and Gómez. *Drafting of the manuscript:* Cuéllar, García, Rodríguez, Halpert, and Gómez. *Critical revision of the manuscript for important intellectual content:* Cuéllar, García, Rodríguez, Halpert, and Gómez. *Statistical analysis:* Cuéllar, Rodríguez, and Halpert. *Obtained funding:* Cuéllar. *Administrative, technical, and material support:* Cuéllar and García. *Study supervision:* Cuéllar, García, and Gómez.
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