LETTER TO THE EDITOR

CD4+CD25+Foxp3+ T REGULATORY CELLS ARE NOT INVOLVED IN ORAL DESENSITIZATION

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Oral tolerance has been related to generation of T regulatory cells (Treg) or clonal anergy/deletion, respectively by administering low and high doses of fed antigens. CD4+CD25+ regulatory T cell clones can be induced by the antigen in Peyer's patches of animal models. We selected ten subjects (mean age: 89.4 ± 36.21 months; group A) with severe cow's milk allergy. They underwent oral desensitization (OD) according to the current protocols. In six months they reached a tolerance of 50 ml of cow's milk. CD4+CD25+Foxp3+ T(reg) blood levels were measured at the beginning of OD (A) and after 6 months (A'), but almost the same values were obtained: A = 0.36 ± 0.11%; A'= 0.59 ± 0.15%. These results were compared with a control group (C) of non-atopic children. Naturally outgrowing cow's milk allergy can be related to high blood levels of CD4+CD25+Foxp3+ T(reg), as previously reported in children. On the other hand, a forced oral desensitization through a progressive intake of the antigenic food seems not to be related to an enhancement of CD4+CD25+Foxp3+ T(reg) levels in peripheral blood, making the role of long-lasting systemic immunologic changes unlikely.

Nowadays, oral desensitization (OD) is a useful procedure to treat children who are still suffering from severe food allergy by the age of three-five years (1). Several studies correlated oral tolerance with T regulatory cells (T reg) or clonal anergy/deletion, respectively, in response to low or high doses of fed antigens (2). In particular, CD4+CD25+ regulatory T cell clones have resulted inducible by the antigen in Peyer's patches of animal models (3). Since a natural outgrowth of cow's milk allergy has been associated with high blood levels of CD4+CD25+Foxp3+ T (reg) in children (4), we studied the role of this cell subset in ten selected children, before and after reaching 50 ml of cow's milk tolerance. The results obtained were compared to a control group of ten sex- and age-matched healthy children.

MATERIALS AND METHODS

Study population

We selected ten subjects (8 males and 2 females, mean age: 89.4 ± 36.21 months; group A) with severe cow's milk allergy (mean wheal: 6.84 ± 3.71 mm; mean specific IgE: 31.4 ± 32.21 KUA/L). All children from group A had a history of anaphylactic reaction to cow's milk in the previous 6 months. Patients from the control group (C) were 7 males and 3 females, non atopic children, with mean age of 70.5 ± 30 months. Patients from group A underwent oral desensitization according to the current protocols (5). They became tolerant to 50 ml of cow's milk in 6 months. In their follow-up visits 8 patients reached a

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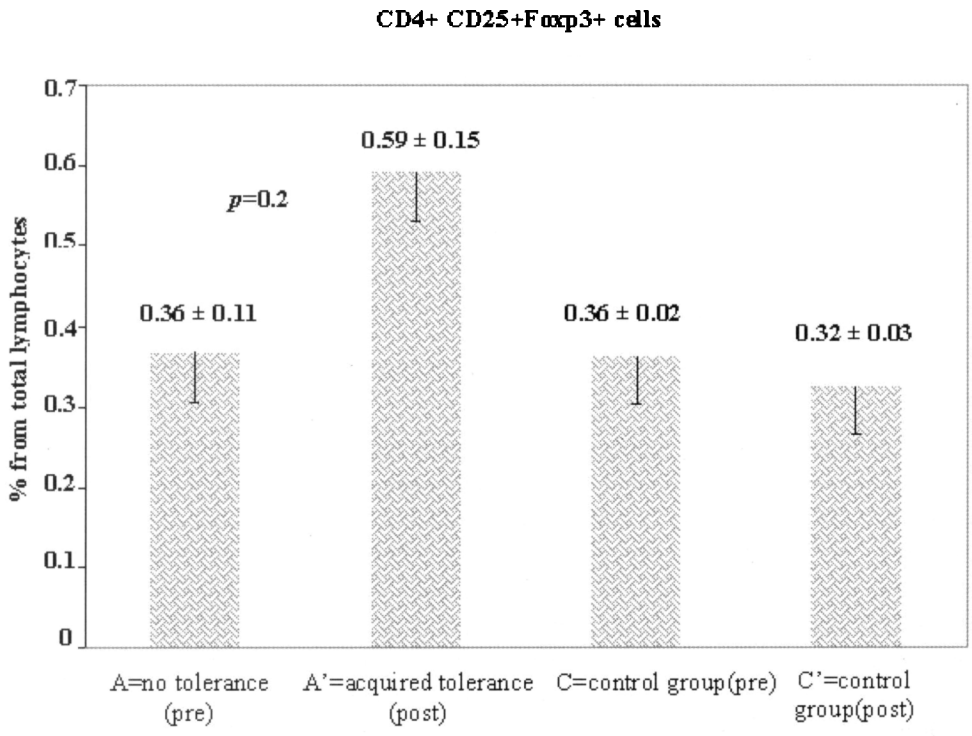

Fig. 1. *T regulatory cells before and after oral desensitization to milk. (Percentage of CD4+ CD25+ Foxp3+ cells obtained from the total amount of lymphocytes).*

full tolerance (150 ml of cow's milk) in 12 months, and 2 patients reached a partial tolerance (less than 100 ml of cow's milk), though continuing to show mild local reactions (oral allergy syndrome, mild urticaria).

Monoclonal antibodies

The panel of mAbs consisted of fluorocrome-conjugated anti-human CD4-peridinin-chlorophyll-protein complex, anti-human CD25-allophycocyanin monoclonal antibodies (CD4-PerCP, CD25-APC, Becton Dickinson, BD, San Diego, CA) for surface staining and Alexa Fluor 488 anti-FOXP3 monoclonal antibody for intracytoplasmatic staining (BioLegend, San Diego, CA).

Cell staining

Peripheral blood mononuclear cells (PBMCs) were isolated from 5 ml of heparinized blood over a Ficoll gradient (Lymphoprep separation medium, Eurobio, France), before and after OD (A,A') as well as before and after 6 months for the control group (C,C'). The cells were firstly stained directly with preconjugated Abs, then fixed and permeabilized with BioLegend's FOXP3 Fix/Perm solution and BioLegend's FOXP3 Perm buffer, respectively, and finally incubated with Alexa Fluor 488 anti-FOXP3 monoclonal antibody. PBMCs were then analyzed by FACSCanto or FACSAria flow cytometer with FACS DIVA software (Becton Dickinson).

Statistical analysis

Values for surface molecule expression were expressed as percentage of the total amount of lymphocytes. The results from the two groups were compared using the *Student's t-test*.

RESULTS

CD4+CD25+Foxp3+ T (reg)

The mean absolute numbers of circulating CD4+, CD25+ and Foxp3+ cells did not significantly differ between the two study groups before (A;C) and after OD (A') or after six months (C'): C *vs* A and

C' *vs* A' (p=0.9 and p=0.1 respectively) (Fig. 1). In particular, the percentages of CD4+CD25+Foxp3+ T (reg) blood levels were almost overlapping before (A) and after (A') OD: A = 0.36 ± 0.11%; A'= 0.59 ± 0.15%. Differences between the two timings show a trend, but the frequency of T regulatory cells (CD4+CD25+Foxp3+) did not significantly differ: A *vs* A' (p=*0.2*).

In group C, we found approximately the same T (reg) levels by measuring them before and after a few months (C= 0.36 ± 0.02 %; C'=0.32 ± 0.03).

DISCUSSION

Several studies have reported a quick loss of tolerance after a period of food avoidance, suggesting that OD is mostly dependent on a daily ingestion of the fed antigen (6). To date, there is little evidence of immunologic changes occurring during human forced tolerance: skin prick tests values significantly reduced after 18 months; the reduction of specific IgE levels and progressive increase of IgG4 were visible in a few months (7-8).

In our study, CD4+CD25+Foxp3+ T(reg) levels in peripheral blood are not significantly changed during OD. On the other hand, a natural outgrowth of cow's milk allergy has been previously related to high blood levels of CD4+CD25+Foxp3+ T(reg), in children. Further studies are needed to better understand the cellular mechanisms regulating OD. We can assume that a forced induction of tolerance is a temporary condition, making unlikely long-lasting immunologic changes.

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