

CD28 expression is increased in venom allergic patients but is not modified by specific immunotherapy

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Summary

Background Recognition of antigen bound to the major histocompatibility complex by the T cell receptor is insufficient to lead to T cell proliferation and effector function, which require co-stimulatory signals, such as those resulting from the interaction of CD28 expressed on T lymphocytes and CD80/CD86 expressed on APCs. Lack of interaction between these accessory molecules during antigen stimulation leads to a state of antigen-specific lymphocyte unresponsiveness. Previous studies have shown that rush venom immunotherapy decreases venom-specific T cell proliferation very early after the initiation of the rush.

Objective In order to see whether this hyporeactivity was associated with a down regulation of accessory molecules, we studied CD28 surface expression on T lymphocytes from 10 non-atopic controls and from 10 non-atopic patients undergoing rush venom immunotherapy.

Methods Peripheral blood samples were collected before the rush (day 0), at the end of the rush (day 1), at day 15 and at day 45. CD28 expression was analysed using flow cytometry with double labelling of the CD4⁺ and CD8⁺ lymphocyte subpopulations.

Results At baseline CD28 was expressed at a higher level on T lymphocytes from allergic patients than from control subjects ($P < 0.04$), and in particular on the CD8 subset ($P < 0.01$), reflecting a decrease in the suppressive CD8⁺CD28⁻ subpopulation. No changes were found in the percentages of total CD28⁺ T cells, CD4⁺CD28⁺ or CD8⁺CD28⁺ cells at the different time points after the initiation of immunotherapy.

Conclusion These results suggest that the CD28 pathway is probably involved in the development of allergic reactions, but at least at the phenotypic level, CD28 expression remained unchanged after rush venom immunotherapy.

Keywords: rush venom immunotherapy, tolerance, CD28, T lymphocyte

Clinical and Experimental Allergy, Vol. 26, pp. 1119–1124. Submitted 8 November 1995; revised 8 March 1996; accepted 24 April 1996.

Introduction

To induce an antigen-specific response, T lymphocytes must receive two signals from the APC. The first signal, the interaction between the TCR complex and antigen associated with MHC, is necessary but not sufficient for T cell activation. The outcome of TCR engagement is

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determined by additional signals, termed co-stimulatory, provided by cell surface molecules expressed on APCs, which are critical for the induction of maximal T cell proliferation, cytokine secretion and effector function. Therefore, when antigen is presented without appropriate co-stimulation, functional unresponsiveness (anergy) of antigen-specific T lymphocytes is induced and may represent a mechanism of non-deletional tolerance *in vivo* [1].

One such critical co-stimulatory signal is the CD86/CD80:CD28/CTLA4 ligand-receptor pair expressed on APC and T lymphocytes respectively [2–4]. This complex prevents the induction of antigen-specific tolerance [5] and its blocking induces *in vitro* antigen-specific hyporesponsiveness [6,7] and *in vivo* long lasting tolerance to human xenoantigen [8]. CD28 is expressed on all CD4⁺ and around 50% of CD8⁺ human T lymphocytes, and also on Th1- and Th2-type clones in mice as well as in humans [9,10]. Signalling via CD28 enhances cytokine gene transcription and mRNA stabilization of Th1-type cytokines (IL-2, IL-3, GM-CSF, IFN- γ) [11] but also enhances the secretion of Th2-type cytokines [12–15] and help for IgE synthesis [16]. CD28 knock-out mice show impaired lymphokine secretion after T cell stimulation and also defects in humoral immune response [17].

Specific immunotherapy has been used for years on empirical bases. In the last decade, double-blind placebo-controlled studies have shown the efficacy of this form of treatment in some types of allergic reactions. This is the case for venom immunotherapy where the protection achieved in allergic patients is around 96% [18]. Several studies have shown that immunotherapy decreases specific antigen-induced T cell proliferation [19–22]. Recent studies have shown that venom immunotherapy, using an accelerated schedule, strongly inhibits venom-specific T lymphocyte proliferation in the first weeks after the beginning of the rush [23,24,25].

In order to see if this hyporesponsiveness was related to a down regulation of co-stimulatory molecules, we

studied CD28 expression on peripheral T lymphocytes from patients undergoing rush venom immunotherapy before and at different time points after the initiation of the treatment.

Materials and methods

Subjects

Ten non-atopic venom allergic patients (age, 19 to 70 years) were included in the study. All patients had a history of systemic anaphylactic reactions after Hymenoptera stings, positive intradermal tests and RASTs. Nine were sensitive to *Vespula germanica* and 1 to *Apis mellifera*. Table 1 presents the main characteristics of the subjects. Clinical reactions were classified according to Mueller classification. Ten non-atopic controls, sex- and age-matched (19 to 70 years) were also included. They had no history of allergic disease, negative skin prick tests to inhaled allergens, negative intradermal tests to venom and normal IgE levels.

Immunotherapy regimen

The method used for treatment was the ultrarush method as described by Birnbaum and coworkers [26]. Briefly, at day 0, increasing doses of venom were injected every half an hour for 3 h 30 min to reach a final dose of 40 μ g (cumulative dose: 100 μ g). At day 15, and every month afterwards 100 μ g of venom were injected subcutaneously.

Table 1. Patients characteristics

Donor	Age	Sex	Atopy	Clinical score*	Skin test reactivity†	RAST‡
1	31	M	–	5	10 ⁻³	4
2	69	M	–	5	10 ⁻³	1
3	19	F	–	4	10 ⁻³	3
4	70	M	–	4	10 ⁻¹	1
5	70	F	–	5	10 ⁻²	3
6	30	M	–	4	10 ⁻²	1
7	57	F	–	4	10 ⁻³	4
8	45	F	–	4	10 ⁻³	3
9	27	M	–	4	10 ⁻³	3
10	64	F	–	4	10 ⁻¹	2

* The clinical reactions were classified as follows: (0) local reaction; (1) large local reaction; (2) slight general reaction; (3) general reaction: any of the above plus two of the following — angioneurotic oedema, constriction of chest, abdominal pain, nausea, vomiting, diarrhoea; (4) Severe general reaction: any of the above plus two of the following — dyspnoea, dysphagia, hoarseness; (5) Shock reaction with fall in blood pressure and/or unconsciousness.

† Venom concentration (μ g/ml) giving a positive skin test (wheal $\geq$ 5 mm).

‡ Specific IgE are expressed as RAST score 0–4.

None of the patients included in this study had adverse systemic reactions during the rush or the maintenance doses.

Monoclonal antibodies

Anti-CD28 antibody conjugated with PE was purchased from Coulter Immunology (Hialeah, FL). FITC-conjugated anti-CD4 and -CD8 antibodies were obtained from Becton Dickinson (Mountain View, CA).

Blood preparation and antibody staining

Five millilitres of peripheral blood were collected in EDTA Vacutainer tubes (Becton Dickinson, Meylan, France) before the first injection of venom (day 0), half an hour after the last injection of venom on the same day (called day 1), then at days 15 and 45 before the maintenance dose injections. One hundred microliters of whole blood diluted 1:2 in phosphate-buffered saline (PBS) were incubated with 2–5 µg antibody for 10 min at room temperature and washed twice with PBS. Incubation with the second labelled antibody was performed under the same conditions. Then, the cells were treated with 1 ml of lysing detergent and fixed in 250 µl of fixative solution (Coulter) and analysed by flow cytometry.

Two-colour flow cytometric analysis of lymphocyte subsets

Data were collected on 10 000 cells/sample using an Epics C cytofluorograph equipped with the manufacturer's filters for fluorescein (525 nm band-pass) and phycoerythrin (575 nm band-pass), photomultiplier tubes (run between 950 to 1100 V) and argon ion laser (488 nm). Logarithmic amplification was used for fluorescence measurement on 1024 channels for one-colour analysis and 64 × 64 channels for two-colour fluorescence analysis. Calibration was checked each day using 10 µm grade II fluorescent beads (Coulter). Stained cells were gated on live lymphocytes as determined by forward scatter, which is proportional to cell size to exclude any dead cells, and right angle scatter. Non-specific staining by irrelevant isotype-matched antibodies was <2% and subtracted from raw data. Fluorescence histograms were further analysed by using cytological software (Coulter) after normalization to 10 000 stained cells.

Analysis of data

In a previous study we showed that there was no significant variation in lymphocyte counts during rush venom immunotherapy over a period of 73 days [27]. Using an ultra rush schedule with a low cumulative dose

(100 µg) does not affect the yield of peripheral blood mononuclear cell recovery, contrary to other protocols where a decrease in lymphocyte counts has been observed immediately after rush [25]. Therefore, results are given as percentages of single or double-stained lymphocyte subsets. Comparisons between untreated patients and controls were performed using an unpaired *t*-test (*n* = 10 subjects in each group). One-way analysis of variance was used to compare time-related variations (for seven patients since three patients were missing at day 45).

Results

CD28 expression by peripheral T lymphocyte sub-populations in untreated venom allergic patients and non-atopic controls (Table 2)

The percentage of CD28⁺ T lymphocytes was higher in the untreated non-atopic venom allergic patients than in the non-atopic controls (*P* < 0.04). Within the T cell population, essentially all CD4⁺ T lymphocytes co-expressed CD28, both in allergic patients and in controls. Within the CD8 subset, both CD28⁺ and CD28[−] cells were observed; however, the percentage of double stained CD8⁺CD28⁺ cells was higher in the allergic patients than in the non-atopic controls (*P* < 0.01).

Evolution of CD28 expression during rush venom immunotherapy (Table 3)

No changes were found in the percentage of total CD28⁺ T lymphocytes at the different time points after the initiation of immunotherapy, by comparison with the pre-treatment values (ns using one way analysis of variance). Within the CD4⁺ T lymphocyte sub-population,

Table 2. Basal levels of percentages of CD28 positive cells in allergic patients and controls

	Patients	Controls	<i>P</i>
Number	10	10	
Age (year ± SEM)	48.2 ± 6.3	46.4 ± 5.9	ns
Sex (M/F)	5/5	5/5	
CD28 ⁺ (%)*	65.3 ± 3.3	57.9 ± 2.3	<0.04
CD4 ⁺ CD28 ⁺ (%)	96.7 ± 1.1	96.6 ± 0.7	ns
CD8 ⁺ CD28 ⁺ (%)	65.3 ± 5.4	50.3 ± 3.1	<0.01

Patients and controls were sex- and age-matched.

*Results are given as percentages of single or double-stained lymphocyte subsets.

ns: not significant.

Table 3. Percentages of expression of the CD28 marker within the different T cell sub-populations in patients undergoing rush desensitization

T cell sub-populations*	Day 0	Day 1	Day 15	Day 45
CD28 ⁺	65.3 ± 3.3	69.9 ± 2.5	64.0 ± 3.4	62.7 ± 3.3
CD4 ⁺ CD28 ⁺	96.7 ± 1.1	97.7 ± 0.7	96.6 ± 1.4	96.8 ± 1.3
CD8 ⁺ CD28 ⁺	65.3 ± 5.4	66.7 ± 5.7	56.8 ± 5.2	60.2 ± 6.6

* Results are given as percentages of single or double-stained lymphocyte subsets.

the double stained CD4⁺CD28⁺ population remained remarkably stable during the course of immunotherapy (ns).

Within the CD8⁺ T lymphocyte sub-population a small decrease of the CD8⁺CD28⁺ population was observed by day 15 which did not reach statistical significance using one way analysis of variance (for 7 patients).

Discussion

T cell modifications induced by immunotherapy have been described as early as in the 1970s. Reduction of antigen-specific proliferation was the first to be specified [20] and since has been confirmed in other studies [21,22]. In a recent study we also observed that rush venom immunotherapy decreased antigen-specific T cell proliferation [23]. The striking point was that this effect was observed early at day 15 of the rush. Another recent study has even shown that a decrease in venom-specific proliferation was observed immediately after the rush (day 1) [25] thus correlating with the efficacy of rush venom immunotherapy which is rapid, since patients can tolerate high doses of venom at the end of the rush.

CD28 has been associated with allergen-specific lymphocyte hyporesponsiveness of activated human CD4⁺ clones anergized with high doses of specific peptide [28]. In the latter study, the induction of anergy was associated with a profound down-regulation of CD28, both at the level of mRNA and surface expression of the mature protein. Therefore, this *in vitro* model of anergy induction with high doses of peptide in Th2-type clones is theoretically very similar to the induction of lymphocyte hyporesponsiveness by the high doses of venom injected *in vivo* during venom immunotherapy.

We chose to study whole T cells rather than allergen-specific clones because of the advantage of evaluating the entire repertoire of allergen-specific responses and of allowing sequential studies to look at effects of therapy. On the other hand, the increased expression of the

CD28⁺ T cell population in allergic patients is consistent with the recent report of the involvement of CD28 in the up-regulation of human IgE synthesis [16], and in the enhancement of Th-2 type cytokine secretion. Indeed, an increase in IL-5, IL-10 and IL-13 [12–15] has been reported, as well as in IL-4 (through the induction of CD40L) [12], this production being preferentially co-stimulated by CD86 (B7-2) [29,30]. However, we did not observe changes in the cell surface expression of CD28 on CD4⁺ cells during immunotherapy. This does not completely rule out a possible role for the CD28 pathway at the level of CD4⁺ cells.

Different explanations can be suggested. First, kinetics experiments showed that CD28 surface expression in activated CD4⁺ T cells decreased by 12 to 24 h after ligand binding to CD28, leading to a state of unresponsiveness to CD28 signalling. CD28 expression returned to initial or higher levels by 48 h although T cells remained unresponsive [31]. In our work CD28 expression was studied before the rush, at the end of the rush, i.e. 4 h after the initiation, and at the first maintenance dose (day 15). We could therefore have missed the possible transient down-regulation of CD28 on CD4⁺ cells. Secondly, the CD28 co-stimulatory pathway involves several other molecules such as CTLA-4 [32] and their ligands CD86 (B7-2) and CD80 (B7-1), which may also be involved in antigen-specific hyporesponsiveness. Indeed, antigen presentation in the absence of CD86/CD80 co-stimulation leads to T cell anergy [3,7].

The absence of changes in CD28 expression might also reflect the fact that only a small part of the T cell repertoire was concerned, i.e. allergen-specific T cells. However, although ubiquitous, an increase in CD28 expression was observed in non-atopic venom-allergic patients compared to non-atopic non-venom allergic controls. Moreover, changes induced by immunotherapy and concerning only venom-specific T cells have been observed at the level of the whole T cell population, such as an increase in IFN- γ and a decrease in IL-4 mRNA expression and secretion [23,24,25].

Within the CD8⁺ sub-population, the CD8⁺CD28⁻ subset is present in normal adult human peripheral blood (50.6% of CD8⁺ cells) and is thought to represent a type of memory population [33]. Previous studies have implicated the CD8⁺CD28⁻ T cells in the suppression of antigen-specific proliferation of human T cell clones [34]. In our study, this sub-population was clearly of smaller size in the untreated allergic patients than in the non-atopic controls, suggesting a lack of negative regulation in allergic patients. However, rush immunotherapy did not induce significant size variations in the CD8⁺CD28⁻ subpopulation.

In conclusion, the up-regulation of CD28 expression in allergic patients implicates this pathway in the development of allergic reactions. However, at least at the phenotypic level, CD28 expression remained unchanged after rush venom immunotherapy.

Acknowledgements

This work was supported by INSERM U 416. The authors would like to thank the nurses for their help in collecting the blood samples: Mrs Nadia Fernez, Sabine Quandalle, Laurence Capy and Bernadette Thiébaud.

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