

Concomitant augmentation of CD4⁺ CD45R⁺ suppressor/inducer subset and diminution of CD4⁺ CDw29⁺ helper/inducer subset during rush hyposensitization in hymenoptera venom allergy

L. TILMANT, J. P. DESSAINT, A. TSICOPOULOS, A. B. TONNEL & A. CAPRON *Centre d'Immunologie et de Biologie Parasitaire, Unite Mixte INSERM U167-CNRS 624, Institut Pasteur. 1, rue du Pr. A. Calmette, B.P. 245, 59019 Lille Cedex, France*

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SUMMARY

A longitudinal study of patients undergoing rush hyposensitization by honey-bee or yellow jacket venom revealed significant changes of the immunophenotypes until the optimal dose was reached, and a progressive reversion to pre-treatment values in the following months. The activation markers CD23 on B cells and CD25 (IL-2 receptor) on T and B lymphocytes decreased. Although there was little variation of the major CD4 and CD8 lymphocyte populations, CD45R⁺ cells increased whilst CDw29⁺ lymphocytes diminished. This inverse variation was associated with a peak of CD4⁺ CD45R⁺ cells with concomitant decrease in CD4⁺ CDw29⁺ cells showing an inverse effect of the treatment on the reciprocal subsets of CD4 lymphocytes. This indicates a shift in the suppressor/inducer to helper/inducer cell ratio early during rush hyposensitization which may also suggest reversion into a less mature stage of CD4⁺ cells, associated with the transition from a highly allergen-reactive state to progressive unresponsiveness.

Keywords allergy immunotherapy lymphocyte subsets lymphocyte markers

INTRODUCTION

Our current understanding of the immunopathogenesis of allergy mainly relies on studies of the cellular events that control the production of IgE antibody, demonstrating the essential role of T cells in the regulation of the allergic response (Leung & Geha, 1986; review by Ishizaka, 1988), and abnormality of T cell helper or suppressor function has often been considered as the main etiological factor.

Hyposensitization, an effective, specific treatment for allergic disease, may provide a model of choice to investigate the mechanisms that underly allergic response. Immunological changes triggered by the allergen administered at suboptimal doses may indeed be considered to be responsible for the reversion of the hypersensitivity state.

Recent studies have unravelled the complexity of the interactions among distinct T lymphocytes, each with distinct functions and distinguishable from other T cells on the basis of differences in the expression of surface antigens and secretion of interleukins. The major T cell population expressing the CD4 molecule, initially defining helper cells, can be further divided in subsets based on the relative expression of one or more

additional cell-surface markers recognized by monoclonal antibodies. The human helper/inducer T cell subset has thus been characterized by its expression of the CDw29 marker initially defined by the monoclonal 4B4 antibody (Morimoto *et al.*, 1985a), but also by reaction with other antibodies such as UCHL1 (Smith *et al.*, 1986) or WR19 (Moore & Nesbitt, 1987). The reciprocal subset of CD4⁺ cells bears the high molecular weight isoforms of the leucocyte common antigen clustered as CD45R which are detected by monoclonal antibodies such as 2H4 (Morimoto *et al.*, 1985b) or WR16 (Moore & Nesbitt, 1987), and contains mainly cells endowed with suppressor/inducer function. Thus, the reciprocal expression of these markers on CD4⁺ lymphocytes identifies two subpopulations with counteracting immunoregulatory functions. Immunophenotypes therefore not only allow us to analyse changes of T lymphocyte subsets in disorders of immune regulation, but also provide indications on the functional significance of these variations.

Only a few studies have investigated changes in the distribution of peripheral blood lymphocyte markers during specific hyposensitization, and they were limited to analysis of the major T lymphocyte populations (i.e. CD4 and CD8) (Neiburger, Neiburger & Dockhorn, 1978; Dominguez *et al.*, 1983; Hsieh, 1985; Lesourd *et al.*, 1987; Moneret-Vautrin *et al.*, 1987). Using a battery of subset-specific monoclonal antibodies, we show

Correspondence: A. Capron, Centre d'Immunologie et de Biologie Parasitaire, Unite Mixte INSERM U167-CNRS 624, Institut Pasteur, 1, rue du Pr. A. Calmette, B.P. 245, 59019 Lille Cedex, France.

reciprocal changes in CD4⁺ lymphocyte subsets with opposing regulatory function during rush hyposensitization in Hymenoptera sting allergy.

MATERIALS AND METHODS

Study subjects

The subjects were 10 patients (aged 6–67, mean 42 years) with a typical clinical history of life-threatening systemic anaphylaxis (7/10) and/or large local reactions (7/10) after Hymenoptera stings. None were sensitized to common allergens; five were sensitized to *Vespula germanica* and five to *Apis mellifera* in terms of positive skin tests (9/10) and/or positive radioallergo-sorbent test (9/10). Total serum IgE level was within the normal range. Rush hyposensitization was started, 3–7 months after the last sting, as described by Nataf, Guinépain & Herman (1984) using venom preparations supplied by Laboratoire des Staller-gènes (Fresnes, France). Briefly, the appropriate allergen was administered with a regimen of hourly injections for 3 days of increasing doses to reach 100 µg. Then, the 100 µg dose was maintained in a stepped progression of the injection interval to 4 weeks.

Peripheral blood specimens were collected in 5 ml EDTA Vacutainer tubes (Becton Dickinson, Meylan, France) before the first injection of venom (day 0), then at day 1 and day 3 (during the dose-increment period) and at days 10, 24, 45, and 73 (during the time-increment period of the treatment). A complete blood count and differential cell count was performed.

Twenty healthy laboratory personnel, who donated a peripheral blood specimen according to the same schedule, served as controls in this longitudinal study.

Monoclonal antibodies

The T1 monoclonal antibody to the cluster of differentiation (CD) 2; T4 (anti-CD4); T8 (anti-CD8); B1 (anti-CD20); MO2 (anti-CD14); 2H4 (anti-CD45R); 4B4 (anti-CDw29), obtained from Coulter Immunology (Hialeah, FL); and IOT14 (anti-CD25/interleukin-2 receptor) (Immunotech, Marseille, France) were either conjugated with fluorescein isothiocyanate (FITC) or a phycoerythrin derivative (RD-1). IOB8 (anti-CD23; Immunotech) and Leu8 (anti-p80; Becton Dickinson, Mountain View, CA) were used unlabelled.

Blood preparation and antibody staining

One-hundred microlitres of whole blood diluted 1:2 in phosphate-buffered saline (PBS) were incubated with 1–5 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 reagent and fixed in 250 µl of fixative solution (Coulter) and analysed by flow cytometry.

For an unlabelled monoclonal antibody, after incubation with this first step reagent the cells were stained for 10 min with F(ab')₂ goat anti-mouse IgG FITC conjugate (Silenus, Hawthorn, Australia), then exposed to RD-1 conjugated monoclonal antibody.

Table 1. Longitudinal variation of percentages of lymphocytes, monocytes, and their subsets during rush hyposensitization

Markers	Honey-bee group					Yellow jacket group				
	Pre-treatment values		Longitudinal change			Pre-treatment values		Longitudinal change		
	Mean ± s.e.	P value*	Variation† (%)	Time interval	P value§	Mean ± s.e.	P value	Variation‡ (%)	Time interval	P value
CD2	72.4 ± 1.79	NS			NS	76.5 ± 7.56	NS			NS
CD4	34.8 ± 2.87	<0.02			NS	44 ± 2.35	NS	−9.8	D1–D3	<0.05
CD8	23.1 ± 3.4	NS			NS	22.1 ± 4.2	NS			NS
CD14	52.2 ± 3.3	NS			NS	40.2 ± 7.1	NS			NS
CD20	9.9 ± 1.98	NS			NS	7.1 ± 0.27	NS			NS
CD23	3.3 ± 1.01	NS			NS	3.3 ± 0.87	NS	−44.8	D3–D10	<0.001
CD25	1.5 ± 0.20	NS	−10.1	D1–D3	<0.001	2.8 ± 0.68	NS	−10.2	D1–D3	<0.001
CDw29	80.1 ± 2.95	NS	−10.5	D1–D3	<0.001	83 ± 4.03	NS	−6.6	D1–D3	<0.001
CD45R	50.1 ± 5.46	NS	+20.2	D1–D3	<0.005	46.9 ± 5.4	NS	+39.2	D1–D3	<0.005
p80	62.3 ± 1.7	<0.01			NS	63.6 ± 0.59	<0.01			NS
CD20 ⁺ CD23 ⁺	0.5 ± 0.26	<0.01			NS	1.37 ± 0.52	NS	−26.3	D1–D3	<0.001
CD4 ⁺ CDw29 ⁺	30.8 ± 4.81	NS	−7.7	D1–D3	<0.001	39.2 ± 4.05	NS	−19.7	D3–D10	<0.001
CD4 ⁺ CD45R ⁺	10.2 ± 1.23	<0.01	+60.6	D1–D3	<0.005	12.7 ± 3.71	NS	+54	D0–D1	<0.005
CD4 ⁺ p80 ⁺	26.3 ± 1.9	NS	+23.5	D3–D10	<0.001	34 ± 0.63	<0.005	+18.6	D3–D10	<0.001
CD8 ⁺ CDw29 ⁺	20.3 ± 0.8	<0.01	−2.5	D0–D1	<0.05	19.5 ± 0.36	<0.01	−49.8	D1–D3	<0.05
CD8 ⁺ CD45R ⁺	17 ± 0.8	<0.02	+8.5	D1–D3	<0.05	18 ± 0.29	<0.001	+34	D0–D1	<0.05

* Comparison with normal values for each marker and combinations established in our laboratory.

† For each individual, a percentage of variation during the time interval was calculated according to the formula: 100 × (preceding—present values)/preceding value. These percentages were used to obtain a group mean for each time interval or each group of patients.

‡ Mean percentage of variation for the time interval of maximum variation, indicated only when significant in comparison with longitudinal variation in the group of healthy individuals who were studied in parallel (0.6–5.6% according to the markers in the controls).

§ By analysis of variance or Wilcoxon's test.

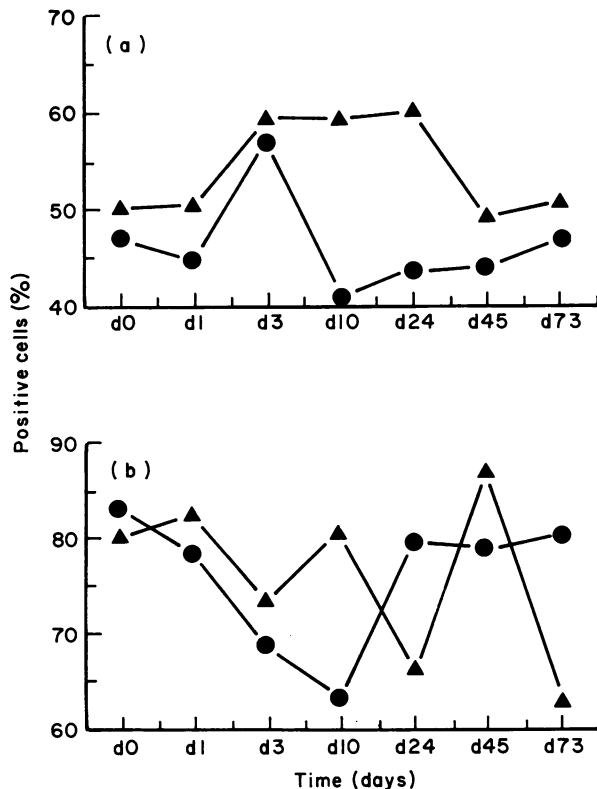


Fig. 1. Longitudinal variation of the percentage of peripheral blood lymphocytes expressing the CD45R or CDw29 marker during rush hyposensitization by yellow jacket (●) or honey-bee (▲) venom respectively. CD45R⁺ cells, as detected by 2H4 monoclonal antibody (a). CDw29⁺ cells, as detected by 4B4 monoclonal antibody (b). For both markers, standard error ranged from 8.1 to 9.0%.

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), respectively, 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 fluorescence analysis and 64 × 64 channels for two-colour fluorescence. 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 cytologic software (Coulter) after normalization to 10 000 stained cells.

Analysis of data

Patients were classified into two groups according to their susceptibility to, and subsequent treatment by, honey-bee versus yellow jacket venom. Since no significant change in lymphocyte and monocyte counts over the study period was observed, results are given as percentages of single or double-stained cell subsets. A mean and standard deviation were calculated for lymphocytes, monocytes, and their populations

collected on the same day of the hyposensitization schedule. Two-way analysis of variance was used to compare the two groups of patients and the control subjects and time-related variations. Variations were further analyzed by the distribution-free Wilcoxon's signed rank test.

RESULTS

Pre-treatment values

The percentage and absolute counts of CD4⁺ cells were decreased in honey-bee susceptible patients, this being mainly attributable to a reduction in the CD4⁺ CD45R⁺ subset. The CD20⁺ CD23⁺ subset of B cells was reduced in honey-bee venom sensitized subjects (Table 1).

T and B lymphocytes, and CD4/CD8 populations over time during hyposensitization

No significant variation in the cell phenotypes CD20, CD2, and CD8 was found, indicating little variability over time in the treated patients. In contrast, a small but significant decrease in the CD4⁺ population was found between day 1 and day 3 of hyposensitization by yellow jacket venom, but the counts reverted to basal values on day 10 and remained stable at normal values afterwards (data not shown).

Variation of activated B and T cells in patients undergoing rush hyposensitization

CD25-bearing lymphocytes decreased progressively during the first 10 days of the treatment (Table 1). Two-colour analysis indicated that these changes were in both T (CD4⁺) and B (CD20⁺) cells (data not shown).

Marked variations were also observed between day 1 and day 3 in the CD23-stained lymphocytes in patients receiving yellow jacket venom injections (Table 1). The values became stable at this low level afterwards. Less pronounced variability was found in the group of patients treated by honey-bee venom, but comparison of mean fluorescence intensities between day 1 and day 3 indicated a drop from mean channel 43 to 31 in the 64 × 64 channel histograms, reflecting a significant decrease ($P < 0.001$) in the cellular expression of CD23 by B cells in these patients also.

Inverse variations in reciprocal CD4⁺ CD45R⁺ and CD4⁺ CDw29⁺ lymphocyte subsets during rush hyposensitization

Among the immunophenotypes investigated, greatest variability was found in the expression of CD45R and CDw29 in treated patients. A significant increase peaking at day 3 in CD45R⁺ lymphocytes was observed in the two groups of patients, which persisted longer in honey-bee-venom-treated individuals (Fig. 1a). Conversely, levels of CDw29⁺ lymphocytes decreased progressively below normal values during the first 10 days of yellow jacket venom-administration and reverted to pre-treatment counts during the following 2 weeks. Similar variations of CDw29⁺ cells, although with marked oscillations, characterized the honey-bee venom-treated patients (Fig. 1b).

CD45R and CDw29 are expressed on both CD4⁺ and CD8⁺ populations, and both double-bearing subsets were found to vary. Inverse variations (Fig. 2) were observed between CD4⁺ CD45R⁺ and CD4⁺ CDw29⁺ subsets. CDw29 and CD45R are markers of two major, essentially reciprocal CD4⁺ subsets and the reciprocal changes induced by venom hyposensitization,

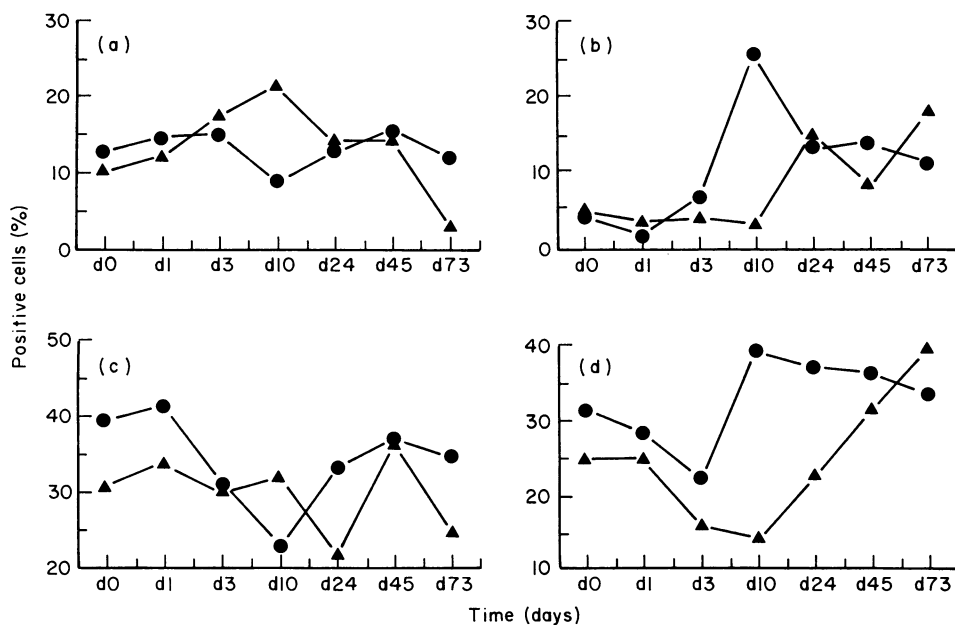


Fig. 2. Two-colour analysis of variation of CD4⁺ cells during rush hyposensitization by yellow jacket (●) or honey-bee (▲) venom respectively. CD4 and CD45R double-bearing cells (a). CD4⁺ CDw29⁻ cells (b). CD4 and CDw29 double-bearing cells (c). CD4⁺ CD45R⁻ cells (d). For each subset, standard error was below 9%.

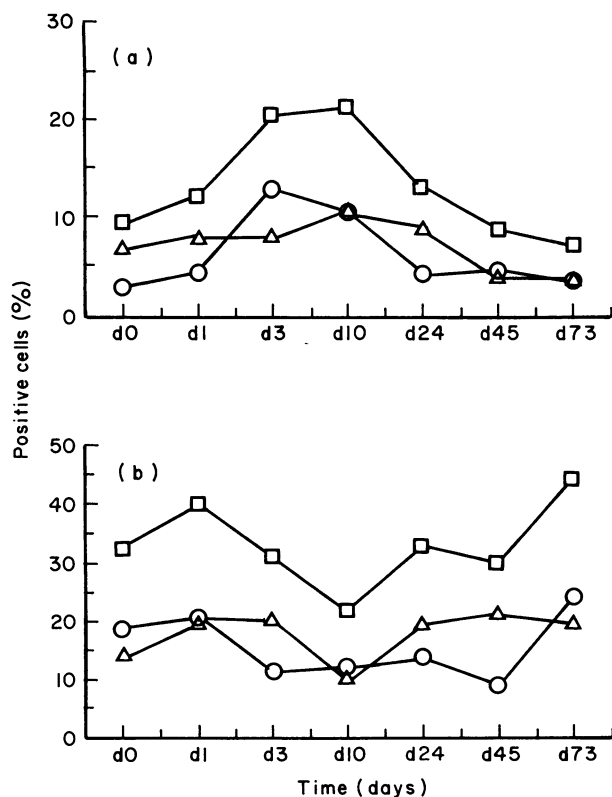


Fig. 3. Contribution of the high- (○) or low-expressing (Δ) CD45R or CDw29 subsets to variation of CD4⁺ CD45R⁺ or CD4⁺ CDw29⁺ cells during rush hyposensitization. Two peaks, one of low fluorescence and one of high fluorescence can be distinguished in each CD45R⁺ and CDw29⁺ population. 'Total' (□) refers to the sum of the two peaks of fluorescence CD4 and CD45R double-bearing cells (a). CD4 and CDw29 double-bearing cells (b).

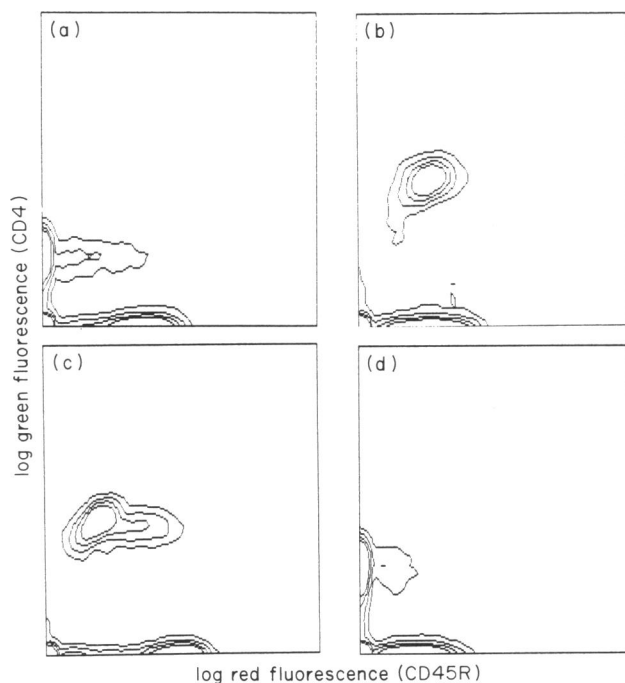


Fig. 4. Two-colour cytometric analysis of the variation of CD4⁺ CD45R⁺ lymphocytes during rush hyposensitization by honey-bee venom. Compared to double-stained cells before the treatment was initiated (a), the increase in cell counts at day 3 (b) and day 10 (c) is made of high and low CD45R-expressing cells. A reversion to pre-treatment values is shown at day 45 (d). Gated and stained lymphocytes (normalized to 10 000 live cells) were plotted on 64 × 64 channel two-colour histograms by using a weighted 9-point smoothing, to plot 1–61% respectively of peak height of the histograms. High level of CD45R expression on CD4⁺ lymphocytes varied from 3.6% (day 0) to 37.4% (day 3) and 7.3% (day 20) to revert to 1.1% at day 45.

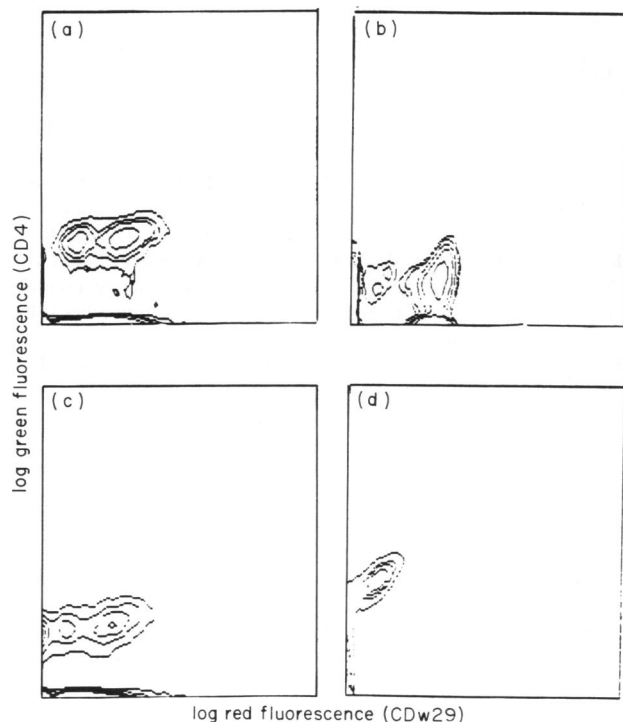


Fig. 5. Two-colour flow cytometric analysis of the variation of CD4⁺ CDw29⁺ lymphocytes during rush hyposensitization by honey-bee venom. Contour plots correspond to 2–75% of the two-colour histogram. In one representative patient, the decrease in CD4⁺ CDw29⁺ double-bearing cells was due mainly to the diminution of lymphocytes expressing low levels of CDw29 (16.1% at day 0, (a) 4.2% at day 3, (b) when cells bearing high levels of this marker did not vary significantly (26.7% to 24.0%). In another representative patient, the decrease in double-bearing cells affected mainly the high CDw29-expressing subset (24.0% at day 0, (c); 2.0% at day 3, (d)).

therefore, do not alter this pattern of expression. Indeed, the increase in the CD4⁺ CD45R⁺ subset (Fig. 2a) was reflected by elevation of the CD4⁺ CDw29⁺ cells (Fig. 2b), and converse changes were found for CD4⁺ CDw29⁺ and CD4⁺ CD45R⁺ subsets.

The expression of CD45R and CDw29 markers is bimodal (Morimoto *et al.*, 1985b; Sanders *et al.*, 1988). Thus, one important parameter for each molecule is its relative high or low expression on the positive subset. Analysis of the CD4⁺ population revealed that increase in CD4⁺ CD45R⁺ cells was due mainly to elevation of cells with high expression of CD45R (Figs 3a and 4). A reciprocal decrease affected either the high- or the low-expressing CDw29⁺ subset of CD4⁺ cells (Figs 3b and 5).

Development of the CD4⁺ p80⁺ (Leu 8⁺) subset was marked by an increase beginning at day 10, therefore delayed after the peak of CD4⁺ CD45R⁺ cells.

DISCUSSION

A variety of immunologic modifications have been shown during rush hyposensitization (Kemeny & Lessof, 1987), which may in part be responsible for the observed reduction in susceptibility to the allergens and reflect quantitative or qualitative alterations of lymphocyte functions, the rush schedule

being likely to induce rapid changes in lymphocyte responsiveness.

Yet only minor alterations in the major lymphocyte populations (CD20, CD4, CD8) were detected, confirming previous reports in this or other models (Neiburger *et al.*, 1978; Dominguez *et al.*, 1983; Hsieh, 1985; Lesourd *et al.*, 1987; Moneret-Vautrin *et al.*, 1987).

However, when these populations were analysed according to the expression of other markers (e.g. CD23, or CD45R and CDw29), marked alterations were observed during the dose-increment period, with progressive normalization afterwards (except for CD23, which remains steadily decreased). These changes, which peak up to 60% variation between 2 or 3 days, when day to day changes in the control group do not exceed 5–6%, are all the more remarkable since the patients differ in several respects (age and severity of sensitization), and they can therefore be related to the hyposensitization treatment. This allows us to distinguish two periods during hyposensitization, despite some differences in the intensity and timing of the changes attributable to differences in composition of yellow jacket and honey-bee venom.

An inverse variation was observed in CD4⁺ lymphocytes co-expressing either the CD45R or the CDw29 marker. Thus, the reciprocal pattern of expression of these markers is preserved, suggesting that the corresponding function of the positive subset is also maintained. The increase from abnormally low counts of CD4⁺ CD45R⁺ cells to normal values, peaking at day 3 to 10, reflects a normalization of the suppressor/inducer subset, while the converse decrease in CD4⁺ CDw29⁺ lymphocytes indicates a diminution in the helper/inducer subset with counteracting immunoregulatory function.

These variations cannot be attributed only to the proliferation of antigen-specific clones restricted to CD45R expression, since the frequency of peripheral blood lymphocytes which respond to venom allergens is low (Lomnitzer & Rabson, 1986; Prinz *et al.*, 1987). The specific treatment induces progressive unresponsiveness and non-antigen-specific influences on lymphocytes. Venom-responsive lymphocytes may thus enhance the differentiation of CD4⁺ CD45R⁺ cells or induce recruitment of cells of the same phenotype. Progressively these changes become more and more restricted to the allergen-specific cells, leading to the observed progressive normalization of cell phenotypes. The important consequence is a shift in the suppressor/inducer to helper/inducer cell ratio early during rush hyposensitization. The delayed and progressive elevation of CD4⁺ p80⁺ (leu8⁺) lymphocytes supports this view, since unprimed precursors of suppressor/inducers express the CD45R marker (Morimoto *et al.*, 1986) when the CD45R⁺ p80⁺ subpopulation contains secondary inducers (Damle, Childs & Doyle, 1987).

Functional studies in rodents have also established that the CD4⁺ helper/inducer T cell subset contains clones designated Th2 which secrete interleukin 4 (IL-4) (Mossman *et al.*, 1986; Killar *et al.*, 1987) and which potentiate antibody synthesis of particular isotypes, especially IgE, whereas Th1 cells are inhibitory (Coffman & Carty, 1986; Snapper, Finkelman & Paul, 1988; Stevens *et al.*, 1988). In humans, some CDw29⁺ CD45R⁺ clones were found equivalent to murine Th2 (Salmon *et al.*, 1988; Umetsu *et al.*, 1988) and non-antigen-specific help to IgE production is attributed to IL-4 release (Maggi *et al.*, 1988). Our observation of the reduction in the CD4⁺ CDw29⁺ subset

can therefore be related to regulatory influences which ultimately lead to progressive depression of IgE antibody production.

Repetitive exposure *in vivo* to tetanus toxoid results in the subsequent development of specific anergy for T cell responses (Saxon & Barnett, 1984). Loss of CD45R and gain of CDw29 reactivity is linked to ultimate maturation of CD4⁺ cells (Akbar *et al.*, 1988; Sanders *et al.*, 1988; Serra *et al.*, 1988). The reverse switch has not been observed *in vitro*, but these experiments were done with optimal stimulation, whereas during hyposensitization the specific allergen is deliberately administered at suboptimal doses. Our observation of a shift towards high CD45R expression and concomitant decrease in CDw29-bearing lymphocytes suggests that induction by hyposensitization of clonal anergy in venom-responsive T cells is associated with reversion into a less mature stage of CD4⁺ cells, reflected by a retrograde switch in the expression of T cell differentiation markers.

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