

Immunohistochemical analysis of late local skin reactions during rush venom immunotherapy

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During rush venom immunotherapy (VIT), about 65% of patients develop large local reactions (LLR) at the application site that last for at least 24 h. However, LLR subside during long-term treatment. To learn more about the provenance of infiltrating cells in late, local skin reactions during VIT, we analyzed the skin infiltrates of 23 Hymenoptera venom (HV)-allergic patients. Punch biopsies were obtained 24 h after s.c. injection of HV allergens from 23 HV-allergic patients and five nonallergic controls. Seven patients did not show LLR at the beginning of VIT. Ten patients had LLR when the dose of HV allergens was increased. Six patients showed reduced LLR after long-term treatment. Immunoenzymatic labeling of the cryostat sections with a panel of monoclonal antibodies was performed by the APAAP method. S.c. application of HV allergens induced a perivascular and periadnexial cutaneous mononuclear cell infiltrate consisting mainly of CD4⁺, CD45RO⁺, and HLA-DR⁺ cells in patients without clinically apparent LLR. In contrast, LLR were associated with a significant increase in total cells, CD4⁺ cells, CD8⁺ cells, CD11c⁺ cells, EG2⁺ cells, NP57⁺ cells, HLA-DR⁺ cells, CD45RO⁺ cells, CD45RA⁺ cells, CD23⁺ cells, and CD25⁺ cells ($P < 0.001$). Decreased LLR after long-term VIT was correlated with a significantly reduced recruitment of CD4⁺ cells, EG2⁺ cells, and CD23⁺ cells as compared to LLR in the course of dose increases ($P < 0.05$), whereas the number of CD8⁺ cells, CD11c⁺ cells, NP57⁺ cells, and CD25⁺ cells remained high. Our data suggest that s.c. injections of HV allergens attract CD4⁺ helper T cells, of both the naive (CD45RA⁺) and memory (CD45RO⁺) phenotypes, to the allergen application site. LLR represent delayed allergic rather than toxic reactions to HV components and might be relevant to the development of clinical protection during VIT.

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Although allergen-specific hyposensitization (HS) has been used successfully for several decades in the treatment of insect venom allergy, the mechanism of action is still not fully understood. It was long believed that successful HS is associated with the formation of “blocking” IgG antibodies binding firmly to allergen entering the body, thus preventing its reaction with sensitized mast cells. However, neither the increase in allergen-specific IgG antibodies (1) nor the other manifold immunologic changes observed in the peripheral blood of hyposensitized patients (2–9) are clearly correlated with therapeutic success (10). This could indicate that other lymphocyte changes are relevant to clinical

protection. Recent studies have shown that the production of IgE (11) and the differentiation of important effector and regulator cells in the allergic response (12–14) are largely dependent on the ratio between the cytokines interleukin (IL)-4 and interferon-gamma (IFN- γ). IL-2 and IFN- γ , which are mainly secreted by T_{H1} cells, are responsible for the IgG-dominated immune response and delayed-type hypersensitivity. In contrast, T_{H2} cells, which secrete IL-4 and IL-5, are involved in the production of IgE antibodies and eosinophilia (15). One explanation of the mechanism of VIT is a shift of the cytokine secretion pattern of allergen-specific T cells toward a T_{H1}-dominated immune response

(16–18). However, the exact mechanism by which VIT affects cytokine profiles remains to be investigated.

This study focuses on the large local reactions (LLR) of the skin during rush VIT. Whereas low HV concentrations of 0.01–5 µg are not associated with local skin reactions 24 h after application, approximately 65% of patients develop LLR when the dose of HV is increased (19). Similar reactions are quite common after field stings. In contrast to the immediate local response to HV, LLR start 2–4 h after the challenge and peak within 6–12 h. It has been hypothesized that erythema and swelling occurring within 2–24 h and extending to the elbow or the whole extremity (Fig. 1) are probably the result of late-phase IgE reactions, whereas the induration that remains beyond 24 h and in some patients persists for as long as 7 days is probably cell-mediated in origin (20). However, the intensity of LLR usually significantly subsides after several years of VIT (19). Furthermore, s.c. injections of the diluent of the extracts are not usually associated with clinically apparent skin reactions. Therefore, we suggested that LLR not only are elicited by the pharmacologic activity of HV components (21), but also represent cell-mediated reactions that may play a major role in the mechanism of VIT. Thus, the aim of the present study was to learn more about the provenance of infiltrating cells occurring at the allergen application sites 24 h after s.c. application of HV allergens.

Material and methods

Study groups

Twenty-three insect-venom-allergic subjects (10 male and 13 female), ranging in age from 14 to 73 years, were selected on the basis of a history of severe systemic allergic reactions according to Müller (22) or Ring & Meßmer (23), a positive intracutaneous skin test to freshly prepared dilutions of honeybee (HB) or yellow jacket (YJ) venom allergens (Venomil, SmithKline Beecham, Munich, Germany) at a concentration of $<10^{-2}$ g/l, and venom-specific serum IgE Abs measured by the CAP System (Pharmacia, Uppsala, Sweden). Patients were grouped into three categories by their local reactions in response to rush VIT (Table 1):

- Group I: seven patients with an immediate reaction but no LLR at the beginning of VIT
- Group II: 10 patients with pronounced LLR (indurations >15 cm and erythema >50 cm) after application of HV doses of 5–50 µg.
- Group III: six patients who had already been hyposensitized for at least 2 years, and who had



Fig. 1. Large local reaction (LLR) on left upper arm extending to elbow 24 h after s.c. insect venom allergen application.

a greatly reduced LLR in comparison to earlier injections.

Biopsies from the skin of five healthy nonallergic subjects served as controls.

Rush VIT

Rush VIT was performed under intensive care conditions, as described previously (24). An aqueous HB or YJ extract in albumin diluent (Venomil, SmithKline Beecham, Munich, Germany) was administered s.c. in the upper arms. Over 1–2 weeks, an initial venom dose of 0.01 µg was followed by increasing concentrations until a maintenance dose of 100 µg venom was reached. The following 100-µg boosters were given on days 7, 14, 28, 42, 63, and 84. Thereafter, the interval between maintenance injections was extended to 4 weeks.

Tissue samples

Skin biopsies from hyposensitized patients were taken 24 h after s.c. venom application from the

Table 1. Clinical characteristics, time of tissue sampling, skin test results, and venom-specific serum IgE

		Sex	Age (years)	Time of tissue sampling: 24 h after application of	I.c. test: maximum dilution leading to positive results (g/l) BV/YV		Cap BV (kU/l)	Cap YV (kU/l)
Group I (without LLR)	1	M	64	5 µg YV	—	0.01	<0.35	1.26
	2	M	61	4 µg YV	—	0.001	<0.35	20.6
	3	M	60	10 µg YV	—	0.01	<0.35	84.9
	4	M	43	7 µg YV	0.1	0.01	<0.35	15.8
	5	F	47	4 µg BV	0.001	—	38.4	1.17
	6	F	38	10 µg YV	0.01	0.001	2.02	12.5
	7	F	59	5 µg YV	—	0.001	<0.35	37.0
Group II (LLR)	8	M	39	60 µg YV	0.1	0.01	1.56	14.4
	9	M	59	50 µg BV	0.01	—	20.2	0.41
	10	M	74	5 µg YV	—	0.01	4.29	> 100
	11	M	33	50 µg BV	0.01	—	6.2	<0.35
	12	F	53	70 µg YV	—	0.001	<0.35	2.83
	13	F	20	50 µg YV	—	0.01	12.0	15.7
	14	F	63	40 µg YV	—	0.01	1.50	2.79
	15	F	30	30 µg YV	—	0.001	0.35	39.2
	16	F	47	4 µg YV	—	0.01	<0.35	1.89
	17	F	67	40 µg YV	0.1	0.01	4.7	24.4
Group III (reduced LLR after long-term treatment)	18	M	49	100 µg YV	—	0.01	<0.35	11.9
	19	M	52	100 µg BV	0.01	—	3.73	<0.35
	20	F	41	100 µg YV	—	0.001	<0.35	3.48
	21	F	67	100 µg YV	—	0.01	<5.80	10.4
	22	F	44	100 µg YV	—	0.01	0.35	<3.13
	23	F	52	100 µg YV	—	0.1	<0.35	5.74

LLR: large local reaction; BV: bee venom; YV: yellow jacket venom.

center of the injection sites with a 4-mm disposable punch. Biopsies from nonallergic controls were obtained from clinically and histologically normal, contralateral skin of patients with essentially localized skin lesions such as nevi. Biopsy specimens were immediately snap-frozen in liquid nitrogen-cooled isopentane and stored at -70°C until used. Sections of 5 µm were cut on a cryostat, mounted on SuperFrost Plus microscope slides (Menzel, Braunschweig, Germany), air-dried for 1 h, fixed in acetone for 10 min, and stored at -20°C before immunostaining.

Immunohistochemistry

For immunohistochemical analysis, a panel of different monoclonal antibodies (mAb), specific for well-defined, cell-surface differentiation antigens, was selected (Table 2) by the alkaline phosphatase antialkaline phosphatase (APAAP) method (25). Negative controls included omission of primary antibodies, and omission of second- and third-step reagents and reactions with fast red TR alone.

Quantitation

Slides were encoded and counted blindly. Five visual fields with the greatest dermal infiltrate ($\times 40$

on a Zeiss microscope) were quantified. Adnexae were excluded. The results were expressed as the number of cells per high-power view (HPV $\times 400$). Staining for ICAM-1 and VCAM-1 was assessed by counting positively stained vessels, and the staining intensity was graded as absent (0), weak (1), moderate (2), or strong (3). The variation for repeated measurements by various observers ($n=2$) was less than 5%.

Statistical analysis

Statistically significant differences of the densities of the cellular infiltrates among the different groups were analyzed by the Mann-Whitney U test. *P* values of less than 0.05 were regarded as significant.

Results

Hematoxylin and eosin (HE) staining

Patients without visible LLR (group I) showed a significant increase in the total cell number at the injection site 24 h after s.c. administration (161 ± 16 cells/HPV), as compared to healthy nonallergic controls (67 ± 22 cells/HPV) (Fig. 4). Patients with LLR at the injection site (Group II) showed a

Table 2. Monoclonal antibodies used in study

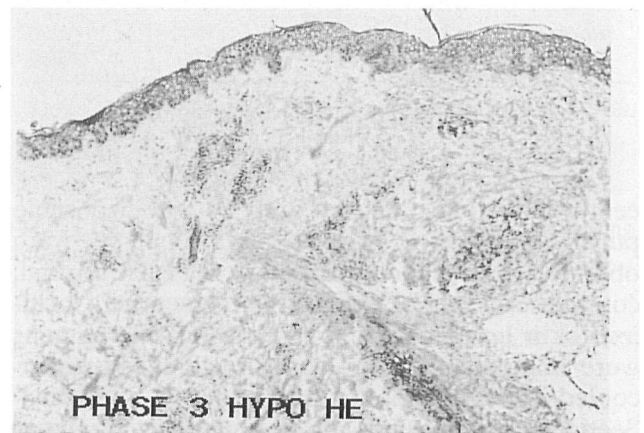
Antibody	CD code	Specificity in cutaneous infiltrate	Source	Titer used
BL-6	CD1a	Langerhans' cells, dendritic cells	Dianova	1:50
13B8.2	CD4	Helper T cells	Dianova	1:25
B9.11	CD8	Suppressor/cytotoxic T cells	Dianova	1:25
KB90	CD11c	Monocytes, macrophages	DAKO	1:50
L26	CD20	B cells	DAKO	1:50
9P.25	CD23	Low-affinity IgE receptor	Dianova	1:25
M-L25/2E5	CD23	Low-affinity IgE receptor	II-LMU	1:25
ACT-1	CD25	Interleukin-2 receptor	DAKO	1:10
UCHL1	CD45RO	Memory T cells, activated macrophages	DAKO	1:25
ALB11	CD45RA	Naive T cells, B cells	Dianova	1:100
84H10	CD54	ICAM-1	Dianova	1:25
1G11	—	VCAM-1	Dianova	1:10
NP57	—	Neutrophil elastase	DAKO	1:300
EG2	—	Activated eosinophils	Pharmacia	1:200
B8.12.2	—	HLA-DR	Dianova	1:50

DAKO: Dakopatts; II-LMU: Institut für Immunologie der Ludwig-Maximilians-Universität München.

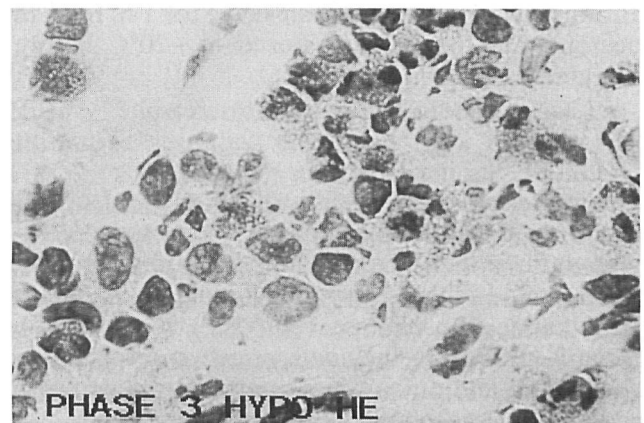
marked perivascular inflammatory reaction of the upper and middle dermis (267 ± 32 cells/HPV) with large cells, pleiomorphic nuclei, and many eosinophils (Fig. 2). Apart from the occasional injection site, the epidermis was fully normal. In patients receiving VIT for several years (group III), the total cell count was significantly reduced with a marked interindividual variation (174 ± 97 cells/HPV). Although obviously all patients had a greatly reduced late-phase reaction after some years of VIT in comparison to earlier, some patients still showed marked pseudolymphoma-like infiltrates with large lymphoid cells, resembling in appearance the activated lymphocytes seen *in vitro* during the lymphocyte transformation test.

Immunohistology

Nonallergic controls. The small perivascular infiltrate (67 ± 22 cells/HPV) consisted mainly of CD4⁺ cells (31 ± 5 cells/HPV), CD8⁺ cells (12 ± 5 cells/HPV) (Fig. 5), CD1a⁺ cells (13 ± 4 cells/HPV), and CD11c⁺ cells (9 ± 5 cells/HPV). Only occasional NP57⁺ cells (3 ± 1 cells/HPV) and EG2⁺ cells (2 ± 2 cells/HPV) were observed (Fig. 5). CD20⁺ cells were absent. CD45RA⁺ cells were below 15% (8 ± 1 cells/HPV), whereas a considerable part of the infiltrate (23 ± 4 cells/HPV) expressed the CD45RO antigen (Fig. 6). In addition, most infiltrating cells were activated as characterized by HLA-DR (40 ± 10 cells/HPV) and IL-2-receptor α -chain (CD25) (13 ± 6 cells/HPV) expression (Fig. 7). Cells expressing the low affinity receptor for IgE (CD23) were scarcely observed (2 ± 3 cells/HPV) (Fig. 7). The numbers of infiltrating cells from some nonallergic patients were quite high compared with other studies of



A



B

Fig. 2. HE stainings of tissue sample from patient with LLR obtained 24 h after s.c. insect venom allergen application. A) Overall view of perivascular and periadnexial cutaneous mononuclear cell infiltrate; B) detail with many eosinophils between mononuclear cells.

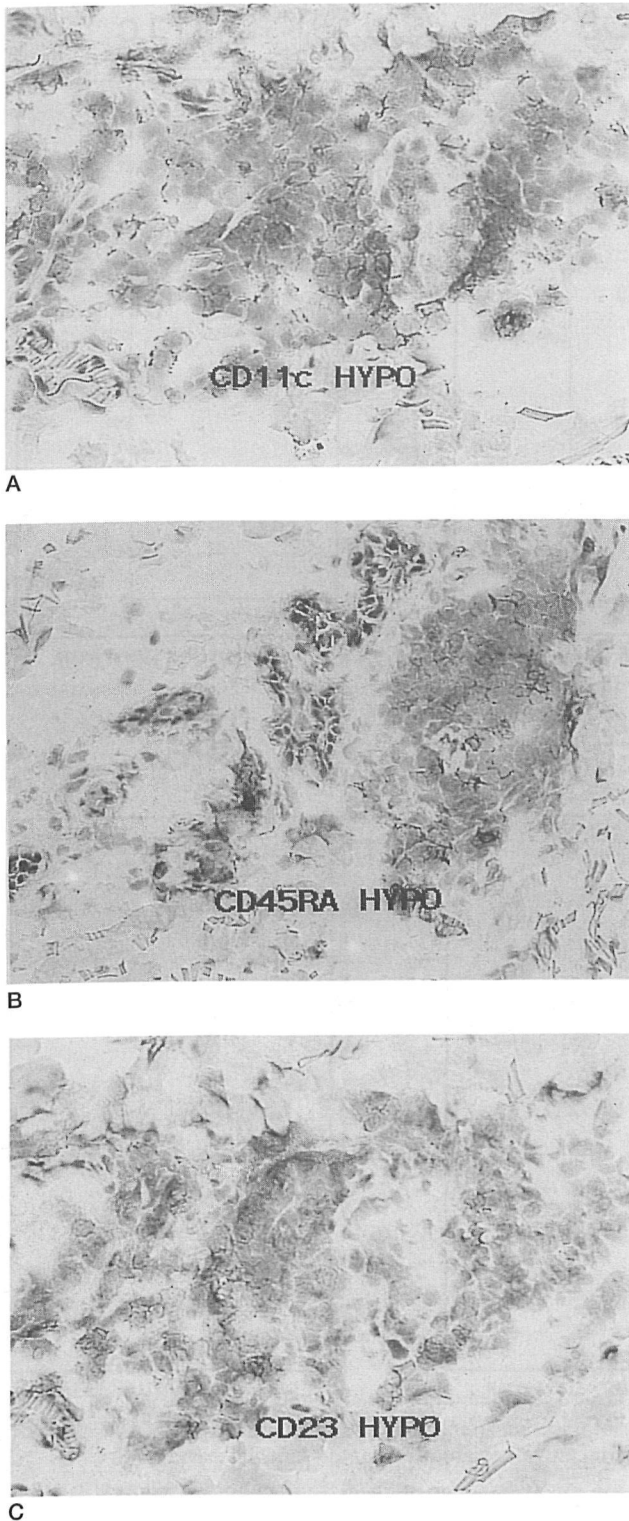


Fig. 3. APAAP staining of tissue samples from patients with LLR with monoclonal antibodies against CD11c (A), CD45RA (B), and CD23 (C) counterstained in hematoxylin.

normal skin (26, 27). Since skin biopsies from nonallergic controls were taken from clinically normal, contralateral skin of patients with essentially localized skin lesions such as nevi, these cells

Total cell count

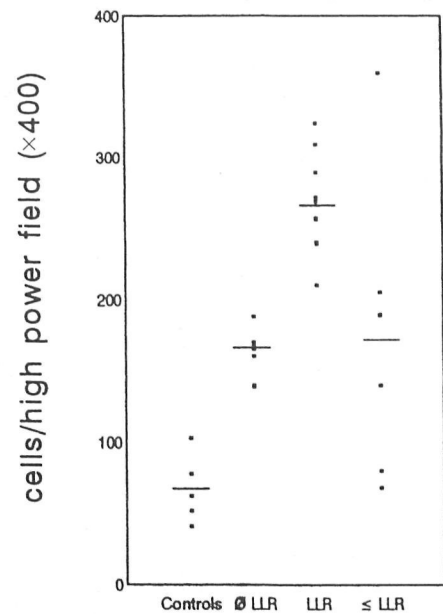


Fig. 4. Total cell counts in dermis at injection site 24 h after s.c. administration of HV allergen. Results are expressed as cells/HPV. Arithmetic mean values are represented by solid bars.

may represent a small, concomitant, nonallergic skin infiltrate of benign skin lesions.

Patients receiving rush VIT without LLR (group I). Cell counts of skin biopsies from patients receiving rush VIT obtained 24 h after s.c. HV allergen application were compared with nonallergic controls. A significant increase in CD4⁺ cells, CD11c⁺ cells (Fig. 5), and HLA-DR⁺ cells (data not shown) ($P < 0.001$) was observed even in patients without clinically evident LLR. However, unlike the infiltrates in LLR (148 ± 31 cells/HPV or $55.8 \pm 11.3\%$ of total cells), only a minority of the infiltrating dermal cells (47 ± 7 cells/HPV or $29.2 \pm 4.3\%$ of total cells) in this group expressed the CD45RO antigen (Fig. 6). In contrast, many cells (30 ± 7 cells/HPV or $19.0 \pm 5.2\%$ of total cells) bore CD45RA (Fig. 3) and/or CD45RB (112 ± 23 cells/HPV or $69.0 \pm 11.7\%$). As a consequence, the CD45RO/RA ratio (1.6 ± 0.3) was markedly decreased compared to the controls (2.9 ± 0.3) or patients with LLR (2.4 ± 0.5). Correspondingly, the number of cells expressing the IL-2-receptor α -chain (CD25) was significantly reduced (1 ± 1 cells/HPV vs 13 ± 6 cells/HPV) (Fig. 7). CD23⁺ cells occurred only sporadically (2 ± 3 cells/HPV) (Fig. 7). CD4/8 ratios were not significantly elevated (3.9 ± 0.7) compared to nonallergic controls (2.5 ± 0.6).

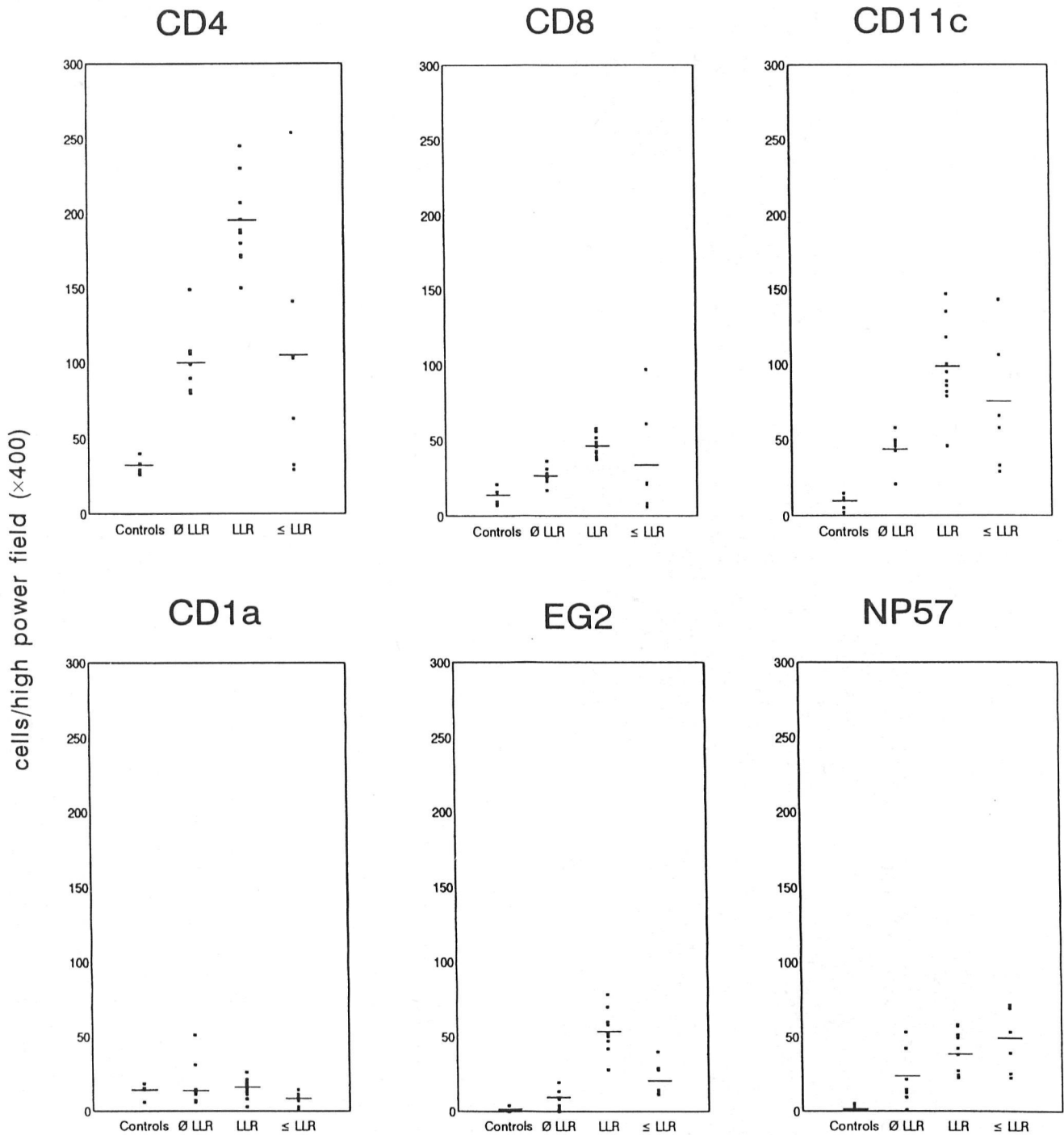


Fig. 5. CD4⁺, CD8⁺, CD11c⁺, CD1a⁺, EG2⁺ and NP57⁺ cells in dermis at injection site 24 h after s.c. administration of HV allergen. Results are expressed as cells/HPV (×400 magnification). Arithmetic mean values are represented by solid bars.

Patients receiving rush VIT with LLR (group II). In contrast, only the cutaneous infiltrates of patients exhibiting LLR during VIT were characterized by a significant increase in the number of CD8⁺ cells, EG2⁺ cells, and NP57⁺ cells ($P<0.001$) (Fig. 5). Apart from that, CD25 expression and CD45RO/RA ratios were significantly increased ($P<0.001$) compared to the results from patients with-

out clinically apparent LLR, reaching the values of healthy controls (21 ± 9 cells/HPV and 2.4 ± 0.5). Correspondingly, infiltrating cells from patients with pronounced LLR expressed significantly higher ($P<0.001$) numbers of the low-affinity receptor for IgE (CD23) (56 ± 18 cells/HPV) (Figs. 3 and 7), although CD20⁺ B cells were only thinly represented (below 0.5%). CD11c⁺ cells were again significantly

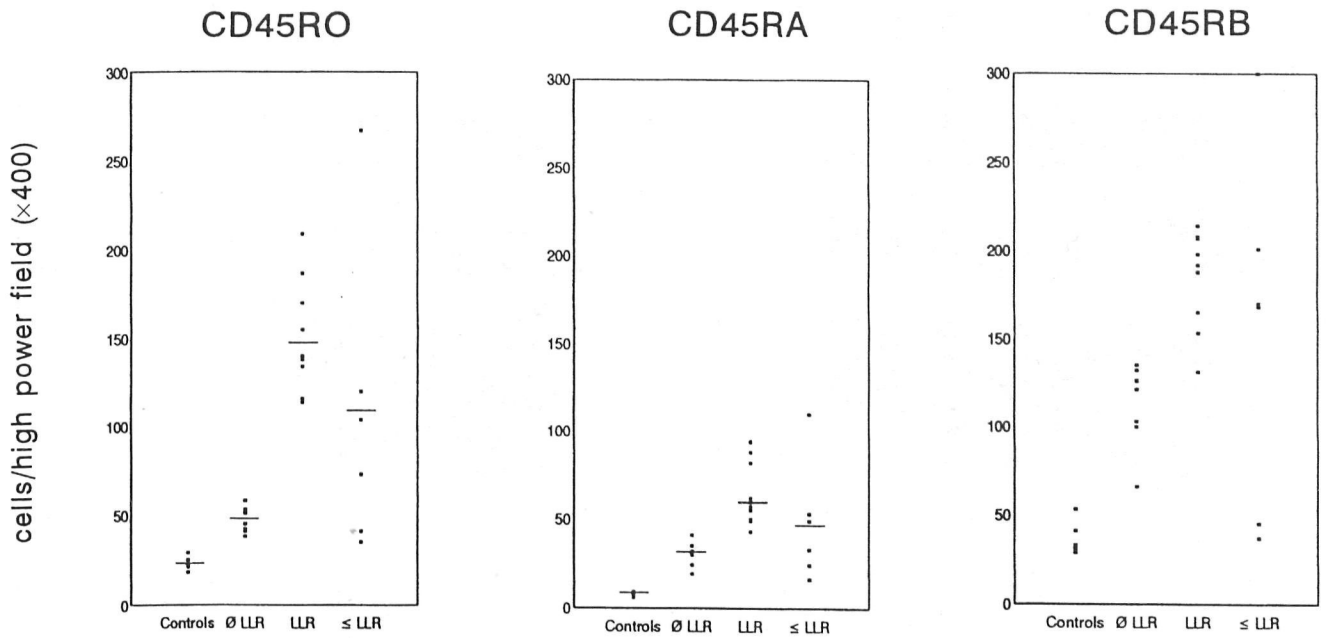


Fig. 6. CD45RO⁺, CD45RA⁺, and CD45RB⁺ cells in dermis at injection site 24 h after s.c. administration of HV allergen. Results are expressed as cells/HPV (×400 magnification). Arithmetic mean values are represented by solid bars.

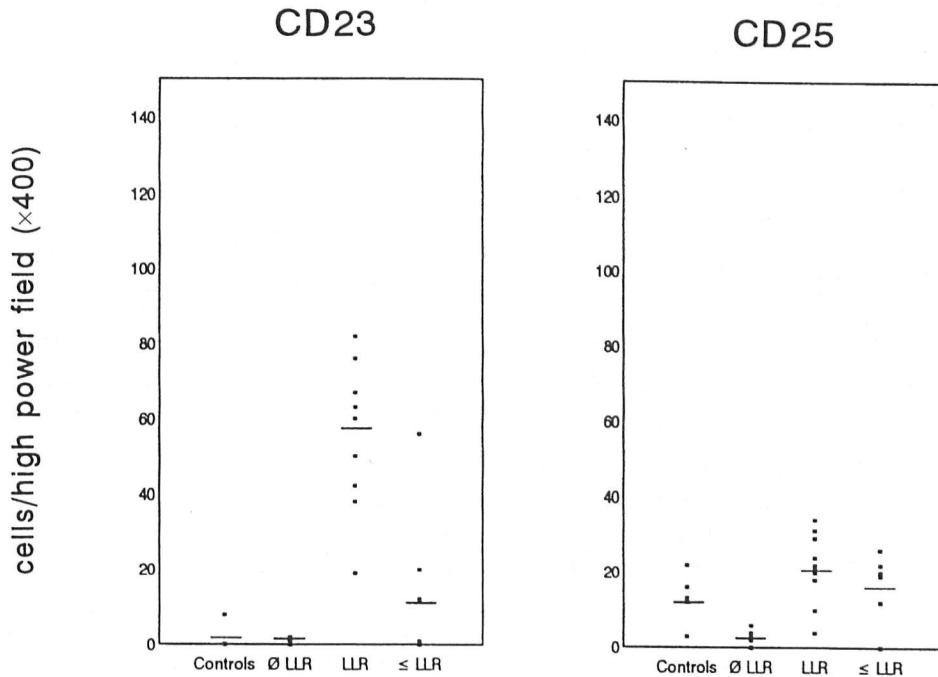


Fig. 7. CD23⁺ and CD25⁺ cells in dermis at injection site 24 h after s.c. administration of HV allergen. Results are expressed as cells/HPV (×400 magnification). Arithmetic mean values are represented by solid bars.

increased (98 ± 28 cells/HPV) compared to the results from patients without clinically apparent LLR (Fig. 3), whereas the numbers of CD1a⁺ dermal dendritic cells (15 ± 6 cells/HPV) were comparable to the results of healthy nonallergic controls (13 ± 4 cells/HPV). Furthermore, there was a trend to increased ICAM-1 and VCAM-1 expression (Fig. 8).

Patients receiving long-term treatment (group III). Cell counts of patients who had undergone hyposensitization for more than 2 years, and who had a much lower local late-phase reaction than earlier were compared with the results from patients with pronounced LLR during rush VIT. A decreased LLR of patients with long-term VIT was

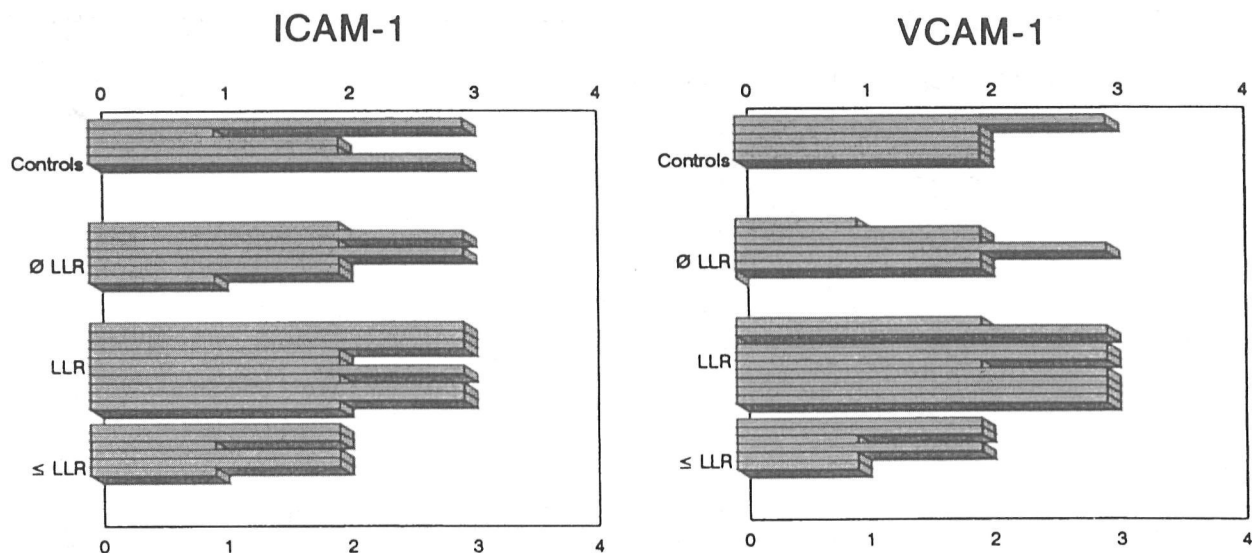


Fig. 8. ICAM-1⁺ and VCAM-1⁺ vessels in dermis at injection site 24 h after s.c. administration of HV allergen. Staining intensity of dermal vessels was graded semiquantitatively as either 0 (absent), 1 (weak), 2 (moderate), or 3 (strong).

associated with a significant reduction in the recruitment of CD4⁺ cells (104 ± 78 cells/HPV vs 193 ± 27 cells/HPV) (Fig. 5), CD23⁺ cells (15 ± 20 vs 56 ± 18 cells/HPV) (Fig. 7), and EG2⁺-activated eosinophils (22 ± 11 vs 53 ± 13 cells/HPV) (Fig. 5) ($P < 0.05$). Furthermore, there was a nonsignificant trend toward a reduction in CD1a⁺ cells (8 ± 4 cells/HPV), CD45RO⁺ cells (107 ± 78 vs 148 ± 31 cells/HPV), and CD45RA⁺ cells (48 ± 31 vs 64 ± 17 cells/HPV) (Fig. 6); ICAM-1 expression (2 vs 3); and VCAM-1 expression (2 vs 3) (Fig. 8). In contrast, the total cell infiltrate in those on long-term treatment was not significantly decreased compared to those with LLR (174 ± 97 vs 267 ± 32 cells/HPV), because some patients still showed marked pseudo-lymphoma-like infiltrates. There were no significant differences in the cellular response to allergen with regard to CD8⁺ cells, CD11c⁺ cells, and NP57⁺ cells (Fig. 5). Furthermore, CD25 and HLA-DR expression remained relatively stable at high levels (17 ± 9 cells/HPV and 120 ± 75 cells/HPV) (Fig. 7).

Discussion

Our data demonstrate that the development of large, late, local skin reactions (LLR) during rush VIT is associated with a highly significant increase in total cells, CD4⁺ cells, CD8⁺ cells, CD11c⁺ cells, EG2⁺ cells, NP57⁺ cells, HLA-DR⁺ cells, CD45RO⁺ cells, CD45RA⁺ cells, CD23⁺ cells, and CD25⁺ cells ($P < 0.001$) when compared with biopsies taken at corresponding times after s.c. injections without LLR. Furthermore, subsiding of LLR after long-term VIT is correlated with a significantly reduced

recruitment of CD4⁺ cells, EG2⁺ cells, and CD23⁺ cells ($P < 0.05$), whereas CD8⁺ cells, CD11c⁺ cells, NP57⁺ cells, and CD25⁺ cells remain stable at high levels.

By demonstrating significant changes in the pattern of cells infiltrating injection sites during the course of VIT, this study confirms our initial hypothesis that LLR are induced not by pharmacologically active but by allergenic components of the HV extracts. S.c. injection of the extract solvent was not associated with clinically apparent skin reactions. Furthermore, CD4⁺ and CD45RO⁺ memory T cells, together with EG2⁺-activated eosinophils, are regarded as characteristic of allergen-induced late-phase cutaneous reactions (28). Migration of eosinophils into the skin is largely dependent on the cytokines IL-4 and IL-5, which are mainly secreted by activated T cells (29), whereas CD23 expression on both lymphocytes (30) and monocytes (13) is mainly upregulated by IL-4. Moreover, the decrease of CD23 and EG2 in association with subsiding large, late, local skin reactions after long-term VIT is inconsistent with an undesirable toxic side-effect of components of the HV extracts. Furthermore, similar late-phase cutaneous reactions are also found in patients hyposensitized to other allergen extracts, such as grass or birch pollen extracts.

For several reasons, our data suggest that allergen-induced late-phase cutaneous reactions might play an important role in the mechanism of VIT. First of all, in tissue samples from patients at the start of VIT, when no LLR were yet clinically evident, the most surprising finding was a high proportion of infiltrating CD45RA⁺ cells in close connection

to CD11c⁺ monocytes/macrophages. Since CD20⁺ B cells, which also express the high-molecular-weight isoform of the CD45 molecule, occurred only sporadically, CD45RA could be used as a marker of the recruitment of naive T cells, i.e., immature T cells not having met the specific antigen in an MHC II context (31). The low number of activated T cells at the beginning of VIT was further confirmed by a significantly reduced CD25 expression in biopsies taken at corresponding times after s.c. injections without LLR when compared to healthy skin of nonallergic controls.

In contrast, a significant increase of CD45RO⁺ cells, the phenotype of memory T cells (32, 33), the CD45RO/RA ratio, and the activation markers CD23, CD25, and EG2 was observed only in skin infiltrates from patients with marked LLR. These findings, which are consistent with recent studies that have characterized infiltrating T cells of most inflammatory dermatoses as predominantly of the memory phenotype (34, 35), suggest a virgin to memory transition of dermal T cells in response to the *in situ* antigenic stimulus.

Because of the close connection between naive T cells and CD11c⁺ monocytes, the absence of CD20⁺ B cells, and the lack of an apparent change in the number of CD1a⁺ dermal dendritic cells, other studies should investigate whether the demonstrated CD11c⁺ monocytes can present the injected HV allergens to specific T cells. Such a finding would demand careful consideration, since naive T cells may be more susceptible than well-differentiated cells to signals affecting cytokine secretion. For example, the persistent increase in CD8⁺ cells, which agrees with a recently observed induction of T-suppressor activity by VIT (36), may be particularly relevant to the development of clinical protection. Furthermore, a recent study provides some evidence that allergen-induced IL-4 synthesis by CD4⁺ T cells from allergic donors is significantly decreased when monocytes are used as antigen-presenting cells (37). S.c. injections of the relevant allergen could lead to direct allergen contact with monocytes in the deeper dermis, avoiding contact with mast cells and dendritic cells located in the upper dermis. This predominant antigen presentation by monocytes both in the dermal infiltrate and the local lymph nodes could explain the shift in the cytokine profile of allergen-specific T cells toward a T_{H1}-dominated immune response, which was recently demonstrated in peripheral blood lymphocytes from patients hyposensitized with HV extracts (16–18).

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