The Journal of Immunology

Direct In Vivo Monitoring of Acute Allergic Reactions in Human Conjunctiva

Maaret Helintö, Risto Renkonen, Timo Tervo, Minna Vesaluoma, Heikki Saaren-Seppälä, Tari Haahtela and Juha Kirveskari

J Immunol 2004; 172:3235-3242; ;
doi: 10.4049/jimmunol.172.5.3235
http://www.jimmunol.org/content/172/5/3235

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Direct In Vivo Monitoring of Acute Allergic Reactions in Human Conjunctiva[1]

Maaret Helintö,[2]*§¶ Risto Renkonen,†§¶ Timo Tervo,* Minna Vesaluoma,* Heikki Saaren-Seppälä,* Tari Haahtela,‡ and Juha Kirveskari†§‖

Immediate allergic reactions are initiated by allergen-induced, specific IgE-mediated mast cell degranulation and involve leukocyte recruitment into the inflamed site. We compared conjunctival signs, symptoms, and in vivo leukocyte rolling and extravasation into sites of inflammation in five patients allergic to birch pollen and in 10 nonallergic controls who received a challenge to birch allergen or histamine. Both the specific allergen in allergic patients and histamine, both in patients and in healthy controls, induced symptoms and signs of an immediate allergic reaction together with leukocyte rolling within the conjunctival blood vessels. However, only allergen, not histamine, caused leukocyte extravasation into the site of inflammation in the allergic patients. Allergen also increased expression of endothelial P-selectin in conjunctival vessels and slowed the rolling of leukocytes which is required for their extravasation from blood circulation into the target tissue. Finally, i.v. heparin strongly reduced the number of slowly rolling cells during allergen- or histamine-induced reactions and this can probably hinder the leukocyte extravasation after allergen exposure. These findings suggest that slow rolling is required for leukocyte extravasation in acute allergic reactions, and it can be inhibited by heparin in vivo in therapeutically relevant conditions. ***The Journal of Immunology,*** **2004, 172: 3235–3242.**

The eye is a common site of allergic inflammation; more than half of all acute conjunctivitis is of allergic origin (1). Allergic diseases of the eye are mostly confined to the conjunctiva and are classified as acute allergic conjunctivitis, seasonal hay fever, perennial allergic conjunctivitis, vernal conjunctivitis, and atopic conjunctivitis (1). Acute allergic conjunctivitis is an acute hypersensitivity reaction caused by an exposure to allergens and seasonal hay fever is relatively mild form of allergic conjunctivitis often associated with rhinitis. Perennial allergic conjunctivitis is a mild, chronic form of allergic conjunctivitis. Vernal and atopic keratoconjunctivitis are severe, bilateral inflammations affecting the conjunctiva and cornea, and chronic diseases. In this study, we have concentrated on acute allergic conjunctivitis.

Allergic conjunctivitis is a type-I hypersensitivity reaction initiated by allergen cross-linking of specific IgE molecules on mast cells within the conjunctiva of sensitized patients (1–6). Release of mast-cell mediators such as histamine, bradykinin, platelet activating factor, and leukotrienes trigger an inflammatory reaction including local itching, redness, chemosis, and leukocyte extravasation (7). This leukocyte traffic from the blood circulation into inflamed tissues is mediated by a complex cascade of interactions between leukocytes and the endothelium lining the inner surface of the vasculature: it involves initial tethering, rolling, activation by chemoattractants, and finally, transendothelial migration (8–13). P-selectin is released from the storage granules, the Weibel-Palade bodies, onto the endothelial surface within minutes after cascade initiation, (14) and participates in the two first steps of leukocyte extravasation.

We have recently introduced a new, noninvasive application of confocal reflected light microscopy enabling direct and repeatable quantitative analysis of conjunctival inflammation in human patients (15, 16). The human conjunctiva is useful in these analyses, as it is semitransparent and normally devoid of leukocytes, which greatly facilitates the identification of newly emigrated leukocytes during the inflammatory reaction. We used the conjunctival allergen challenge model, as it reproduces the signs and symptoms of allergic conjunctivitis (17).

This study was set up to analyze and compare the clinical signs, symptoms, and the in vivo leukocyte rolling and extravasation into sites of inflammation occurring in either birch allergen- or histamine-induced conjunctival reactions, both in patients with clinical birch pollen allergy and in healthy controls.

We show here that both the specific allergen in allergic patients as well as histamine in both patients and normal controls induced symptoms and signs of an acute allergic reaction, together with leukocyte rolling within the conjunctival blood vessels. Only the allergen challenge in allergic patients, but not histamine, caused leukocyte extravasation into the site of inflammation. Allergen also enhanced expression of endothelial P-selectin in conjunctival vessels and the slow rolling of leukocytes in allergic patients which is required for their extravasation from the blood circulation into tissue. Finally, i.v. heparin strongly reduced the number of slowly rolling cells during allergen- or histamine-induced reactions and may hinder leukocyte extravasation after allergen exposure.

Materials and Methods

Subjects

The study was performed according to the Declaration of Helsinki, and the study protocol was reviewed and accepted by the Committee on Ethics of the Helsinki University Eye and Ear Hospital (Helsinki, Finland). Allergic

*Department of Ophthalmology, †Helsinki University Central Hospital Laboratory Diagnostics, and ‡Department of Allergy, Helsinki University Central Hospital, Helsinki, Finland; §Department of Bacteriology and Immunology, Haartman Institute, University of Helsinki, Helsinki, Finland; ¶Rational Drug Design Program, Biomedicum Helsinki, University of Helsinki, Helsinki, Finland; and ‖MediCel, Helsinki, Finland

Received for publication July 7, 2003. Accepted for publication December 22, 2003.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported by grants from the Academy of Finland (to R.R.), the Technology Development Center of Finland (to R.R.), the Sigrid Juselius Foundation (to R.R.), the Rector's Grant, Helsinki University (to T.T.), Research Funds from the Helsinki University Central Hospital (to T.T. and R.R.), the Friends of the Blind (to T.T.), the Eye Foundation (to M.H.), Mary and George C. Ehrnrooth Foundation (to M.H.), the Allergy Research Foundation (to M.H.), the Eye and Tissue Bank Foundation (to M.H), and the Paulo Foundation (to M.V.).

[2] Address correspondence and reprint requests to Dr. Maaret Helintö, Department of Ophthalmology, Helsinki University Central Hospital, P.O. Box 220, 00029 Helsinki University Central Hospital, Finland. E-mail address: maaret.helinto@helsinki.fi

0022-1767/04/$02.00

patients gave their informed consent. Five volunteers (all women; mean age 29.4 years, range 24–39) suffering from ocular allergic symptoms during the birch pollen season were included in the study. These allergic patients were selected on the basis of a symptom history of ocular involvement and a skin-prick test positive for birch allergen (Solu-Prick, 10 SQ, ALK, Hørsholm, Denmark). Their reaction was considered positive if the allergen caused a weal of over 3 mm, and the positive control (histamine dihydrochloride 10 mg/ml) and negative control (solvent) gave the expected results (18). All of the allergic patients had rhinitis together with their ocular symptoms; otherwise they were healthy. Ten healthy nonallergic volunteers (5 women, 5 men; mean age 29.1 years, range 26–33) were enrolled to the study and served as control subjects. Exclusion criteria for both the allergic patients and healthy controls were: any symptoms or signs of clinical activity of allergic ocular involvement, any other eye disease, any use of anticoagulative or immunosuppressive drugs, use of topical ophthalmic solutions, and use of anti-inflammatory or anti-allergic drugs at least 2 wk before the study.

Conjunctival allergen challenge

The conjunctival challenges were all done out of pollen season. One drop of birch allergen (100,000 SQ-U/ml; ALK) or histamine diphosphate (HIST)[3] (0.5 mg/ml) was applied (1 ml standard tuberculin injection syringe) into the conjunctival sac of the eye, after which the eye was kept closed for 30 s. The medial canthus was compressed to prevent the flow of allergen into the lacrimal channel. Allergic symptoms (redness, tearing, chemosis, and itching) were scaled according to Abelson et al. (17) before the challenge and 15, 30, and 60 min afterward. Confocal microscopy of the lateral bulbar conjunctiva venules for analysis of leukocyte trafficking was performed before the conjunctival challenge and 15, 30, and 60 min afterward. Healthy controls were challenged only once with HIST or birch allergen. Birch-allergic patients were challenged twice, first with HIST, and at least 2 wk after the first challenge with birch allergen.

In vivo confocal microscopy, leukocyte-rolling, and histological image parameters

A tandem scanning confocal microscope was used (TSCM, Model 165A; Tandem Scanning, Reston, VA). The objective lens of the microscope (cone tip ×24, gel contact, numerical aperture 0.6, working distance 0–1.5 mm, including a floating tip retraction mechanism, Tandem Scanning) was adjusted to give an en face view of the bulbar conjunctival vessels. The set-up and operation of the confocal microscope have been described previously (15, 19, 20).

Vessel diameters, mean centerline flow velocity, and number and velocity of rolling cells were counted for all vessels. Mean centerline flow velocity was counted for three to five freely moving bright cells by measurement of average movement in the four subsequent frames. Vessels with flow above 500 μm/s were included in the analysis. Number of rolling cells was counted for each vessel with continuous flow and a sharp image from those cells passing an imaginary horizontal line in the vessel, which was fixed in one of the local landmarks in the vessel area to eliminate the effect of small movements. The number of extravasated leukocytes in the focal plane of the conjunctival stroma was counted for an area adjacent to the vessels where rolling had been analyzed.

Conjunctival biopsies and immunohistochemistry

Conjunctival biopsy specimens were taken from two control subjects 60 min after conjunctival challenge with histamine and one birch-allergic patient 60 min after conjunctival challenge with birch allergen. The conjunctiva was locally anesthetized with obucain (Oftan Obucain; Santen, Tampere, Finland) eye-drops, and the specimen was taken from the lateral bulbar conjunctiva. The conjunctival specimens were formalin-fixed and paraffin-embedded. The glycan epitopes on L-selectin ligands were identified by mAbs (mAbs) 2F3, HECA-452, and MECA-79. Both mAbs 2F3 (5 μg/ml; BD PharMingen, San Diego, CA) and HECA-452 (15 μg/ml, kindly provided by S. Jalkanen, University of Turku, Turku, Finland) are anti-sLex mAbs, requiring both the presence of α2,3 sialylation and α1,3 fucosylation of the lactosamine. MECA-79 (1:100 culture supernatant, also from S. Jalkanen) requires 6-sulfation of the core 1 *O*-glycan decoration of L-selectin ligands. We also used the anti-VCAM-1 mAb (10 μg/ml, 1.4C3; Novocastra Laboratories, Newcastle, U.K.). A polyclonal Ab was used against P-selectin (0.5 mg/ml; BD PharMingen), E-selectin (0.1 mg/ml; HyCult, Uden, The Netherlands), and ICAM-1 (0.1 mg/ml; HyCult). Anti-CD 34 class II (QBEND 10; DAKO, Glostrup, Denmark) served as the positive control for vascular endothelium. CD3 Ab (1:100, NCL-CD3-PS1; Novocastra Laboratories) was used to count activated T cells. Isotype-matched mouse and rat IgG, IgM, and rabbit polyclonal Ab served as negative control reagents with the same concentration as the specific Ab. The mAb 7C7 (also from S. Jalkanen) served as the negative control for 2F3, and TIB146 (1:100 culture supernatant, also from S. Jalkanen) was a negative control for HECA-452 and MECA-79. Immunohistochemistry was performed according to the relevant Vector ABC Elite Kit (Vector Laboratories, Burlingame, CA) protocols. We used pretreatment in citrate buffer 2 × 5 min, pH 6 (mAbs CD 34 class II, 2F3, HECA-452, MECA-79, and CD3) and pH 3 (P- and E-selectin, and ICAM-1). Incubation of primary Ab was overnight at 4°C. A separate protocol for the anti-VCAM-1 mAb was used exactly according to manufacturer's instructions with incubation of primary Ab overnight at 4°C. The reactivity of mAbs was evaluated by J.K., who had no knowledge of the pathological diagnosis of the specimens, and the total number of positive vessels was determined from each biopsy.

Heparin treatment

Two healthy controls and two allergic patients participated also in the experiment with heparin treatment. The analytical set-up was identical with nonheparin treated ones. They each received a single i.v. bolus of heparin (2500IE, Heparin Leo; Leo Pharmaceutical Products, Ballerup, Denmark) 3 min before a conjunctival challenge with HIST (controls) or birch allergen (allergic patient). Confocal microscopy of the lateral bulbar conjunctiva venules was performed before the heparin treatment and 15, 30, and 60 min after conjunctival challenge. The activated partial thromboplastin time (APTT) clotting assay was taken three times during the experiment: before heparin treatment and 15 and 60 min after heparin bolus injection.

The same two healthy controls also participated in another heparin treatment experiment. The conjunctival challenge with HIST was performed as before. A single heparin i.v. bolus (2500IE, heparin; Leo Pharmaceutical Products) was given 40 min after the conjunctival challenge. Confocal microscopy of the lateral bulbar conjunctiva venules was performed before and then 15, 30, 45, 50, 55, and 60 min after the challenge. The APTT clotting assay was taken twice during the experiment, before heparin treatment, and 20 min after heparin bolus injection.

Statistics

Statistical analyses were performed with SPSS 9.0 for Windows (Microsoft, Redmond, WA). The one-way ANOVA served to determine the significance between time points and groups. Spearman's ρ served in calculating correlation between the tissue-emigrated cells and very slowly rolling leukocytes. Results are expressed as mean ± SD, and differences were considered significant at $p < 0.05$. Statistical analyses were not performed for heparin or immunohistochemistry data because of the small patient number.

Results

Allergic symptoms were scaled before and during both challenges. Whereas birch pollen allergen induced no symptoms in normal control individuals, it had marked effects on the allergic patients (Table I). Histamine challenge caused similar symptoms in both the patients and controls. The in vivo leukocyte rolling and extravasation was analyzed so that the number of vessels, vessel diameter, analysis time, and leukocyte velocities between the four groups and different time points were similar (Table II). Thus the differences were interpreted to result from the birch allergen or histamine challenges to the conjunctiva, rather than from sampling errors.

Characteristics of birch allergen-induced leukocyte traffic in conjunctiva

In the allergic patients, the birch allergen challenge induced a strong increase in number of rolling cells: 0.4 ± 0.5 vs 78.8 ± 40.0 (mean ± SD of rolling leukocytes/min before and at 60 min after the challenge, respectively. Value of $p = 0.011$, Fig. 1*A*). It did not cause significant rolling of leukocytes in the controls (Table III and Fig. 1*A*).

The birch allergen also significantly reduced the mean rolling velocity of the leukocytes in allergic patients, from 221 ± 210 μm/s before to 26 ± 17 μm/s at 15 min after the challenge ($p =$

[3] Abbreviations used in this paper: HIST, histamine diphosphate; APTT, activated partial thromboplastin time.

Table 1. *Comparison of allergic symptoms (mean ± SD) scaled at each time point between study groups*

Patients	Challenge	*n*	Time Point (min)	Hyperemia (0–4)	Chemosis (0–3)	Itching (0–4)	Total Score (0–10)
Allergic	Allergen	5	0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
		5	15	2.1 ± 0**	2 ± 0**	2.3 ± 2.1*	5.5 ± 2.1**
		5	30	2.3 ± 0.4**	2 ± 0**	0 ± 0	4.3 ± 0.4**
		5	60	1.3 ± 0**	1.8 ± 0*	0 ± 0	3 ± 0
Control	Allergen	5	0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
		5	15	0 ± 0	0 ± 0	0 ± 0	0 ± 0
		5	30	0.4 ± 0.4	0 ± 0	0 ± 0	0.3 ± 0.4
		5	60	0.3 ± 0.4	0 ± 0	0 ± 0	0.3 ± 0.4
Allergic	Histamine	5	0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
		5	15	2 ± 0**	1.6 ± 1.4**	1.2 ± 0*	5 ± 1.4**
		5	30	2 ± 0**	1 ± 0*	0.8 ± 0	4 ± 0**
		5	60	1.4 ± 0**	1 ± 0*	0 ± 0	2 ± 0**
Control	Histamine	5	0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
		5	15	2 ± 0**	1 ± 0	0 ± 0	4 ± 0.7**
		5	30	2.2 ± 0**	1 ± 0	0.2 ± 0	3.5 ± 0**
		5	60	1.9 ± 0**	1 ± 0	0 ± 0	3 ± 0**
Allergic	Prophl.	2	0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
	Heparin+	2	15	2.5 ± 0	1 ± 0	1 ± 0	4.5 ± 0
	Allergen	2	30	2.5 ± 0	2 ± 0	0 ± 0	4.5 ± 0
		2	60	2 ± 0	2 ± 0	0 ± 0	4 ± 0
Control	Prophl.	2	0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
	Heparin+	2	15	2.3 ± 0	1 ± 0	0 ± 0	3.25 ± 0
	Histamine	2	30	1.5 ± 0	1 ± 0	0 ± 0	2.5 ± 0
		2	60	1.5 ± 0	1 ± 0	0 ± 0	2.5 ± 0

* $p < 0.05$ compared with baseline.
** $p \leq 0.01$ compared with baseline.

0.023, Table III, Fig. 1*B*). Since only very slow rolling leukocytes can adhere to and migrate through the endothelium, we further analyzed this fraction of the slowest rollers. Before the challenge no leukocytes had a rolling velocity below 9 μm/s, but the number of these cells increased in a time-dependent manner up to 72.4% at 60 min after application of allergen to the conjunctiva (Fig. 1*C*).

In the allergic patients, birch allergen had already induced a significant increase in the number of tissue-emigrating leukocytes within 15 min (Table III). This effect was most pronounced at 30 min, when the number of leukocytes within the conjunctival tissue had risen from 0 ± 0 to 764 ± 181 cells/mm^2, $p < 0.001$). A strong time-dependent correlation appeared between number of tissue-emigrated cells and the number of adherent or very slowly rolling cells ($p = 0.001$, Spearman′s ρ). In nonallergic controls, the birch allergen did not induce transmigration of leukocytes (Table III).

Effects of histamine challenge in human conjunctiva

A direct histamine challenge was chosen to bypass the mast-cell degranulation effect induced by the allergen in vascular endothelial cells. This histamine challenge caused hyperemia, itching, and chemosis in allergic patients as well as in healthy controls; symptoms and signs were essentially identical with those caused by the allergen in the birch-sensitized patients (Table I). Histamine also induced a strong increase in number of rolling cells in both allergic patients and controls (Table III). The peak in number of rolling leukocytes/min occurred 30 min after the histamine challenge in both groups. The number of rolling leukocytes/min was 0.9 ± 1.3 and 80.4 ± 21.4 (mean ± SD, $p = 0.001$, Fig. 1*A*) before and 30 min after the challenge. No statistically significant difference appeared in number of rolling leukocytes/min between the controls and allergic patients during the histamine challenge. In contrast to

Table II. *Comparison of hemodynamic parameters (mean ± SD) measured at each time point between study groups*

Patients	Challenge	*n*	Time Point (min)	No. of Vessels	Vessel Diameter (μm)	Analysis Time (s) (range)	Leukocyte Velocity (μm/s)	Total Rolling Cells Analyzed (sum)
Allergic	Allergen	5	0	2.8 ± 1.9	31.1 ± 4.3	31 (15–61)	910 ± 363	2
		5	15	3.4 ± 1.1	32.1 ± 9.1	78 (34–118)	944 ± 77	164
		5	30	2.6 ± 1.1	28.5 ± 12.4	64 (15–140)	811 ± 111	154
		4	60	4.3 ± 1.5	28.1 ± 6.4	67 (24–100)	852 ± 88	210
Control	Allergen	5	0	2.6 ± 1.9	25.9 ± 3.9	46 (18–131)	995 ± 115	2
		5	15	3.4 ± 1.5	27.9 ± 8.1	41 (29–65)	972 ± 73	1
		5	30	3.4 ± 1.5	27.3 ± 5.9	42 (33–65)	1002 ± 80	6
		5	60	2.8 ± 1.3	28.9 ± 4.8	38 (21–79)	986 ± 127	2
Allergic	Histamine	5	0	3.2 ± 1.3	25.9 ± 6.3	34 (10–59)	875 ± 113	4
		5	15	2.8 ± 1.3	29.9 ± 4.0	48 (17–65)	897 ± 119	147
		5	30	3.0 ± 1.2	30.9 ± 1.7	32 (18–44)	773 ± 125	137
		5	60	3.2 ± 1.3	38.6 ± 14.6	58 (29–112)	828 ± 142	208
Control	Histamine	5	0	4.0 ± 1.6	26.6 ± 3.1	53 (22–123)	927 ± 149	10
		5	15	2.0 ± 1.0	38.2 ± 8.9	72 (28–154)	987 ± 205	247
		5	30	2.2 ± 1.1	38.0 ± 11.4	72 (32–189)	1024 ± 234	186
		5	60	2.4 ± 0.9	42.5 ± 6.4	84 (43–189)	984 ± 202	232

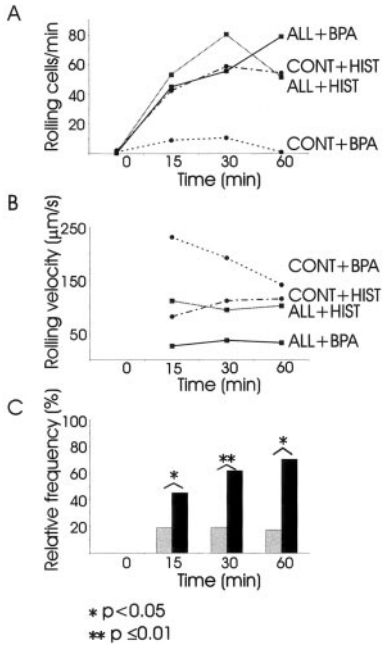

FIGURE 1. In vivo analysis of rolling leukocytes before and after histamine (HIST) and birch allergen challenges (BPA). *A*, Number of rolling leukocytes in conjunctival venules. *B*, Velocity of rolling leukocytes. *C*, Relative frequency of leukocytes rolling very slowly (0–9 μm/s) in allergic patients (ALL) during histamine and birch allergen challenges. ■, Birch allergen; □, histamine challenge. Significance values were calculated with ANOVA, *, $p < 0.05$ and **, $p < 0.01$.

the allergen-induced reduction in leukocyte rolling velocity, the histamine challenge did not affect velocity at all (Table III, Fig. 1, *B* and *C*). Consequently, and in striking contrast to the allergen challenge, the histamine challenge did not induce leukocyte transmigration in either group (Table III).

Endothelial adhesion molecules participating in leukocyte rolling and extravasation after allergen or histamine challenge

We were able to obtain conjunctival biopsy specimens after confocal microscopy, at 60 min after the challenge from one patient and two controls. Of the molecules participating in the leukocyte trafficking, we analyzed the endothelial expression of P- and E-selectin, ligands for L-selectin, as detected by HECA-452, 2F3, and MECA-79, ICAM-1, and VCAM-1. Although a small fraction of conjunctival vessels had already expressed endothelial P- selectin before the challenge, the number of P-selectin-positive vessels was further enhanced by the allergen in an allergic patient, but not with histamine after 60 min. Concomitantly, neither the E-selectin nor ICAM-1 was expressed in resting endothelium but both were induced by histamine and even more strongly by the allergen (Fig. 2). We detected no expression of the ligands for L-selectin or VCAM-1 in any of the conjunctival biopsies analyzed, although the internal positive control for the assay was always reactive. In allergic patient 60 min after allergen challenge, there were 221 polymorphonuclear leukocytes in the conjunctival tissue and only two of them (0.9%) were CD3 positive.

Effect of heparin on allergen-induced leukocyte traffic and symptoms

Of the major molecules mediating leukocyte rolling in our settings only P-selectin was present. Heparin has been suggested to be an effective inhibitor especially of P- and to a lesser extent of L-selectin-mediated rolling and extravasation (21–26). In allergic patients during allergen challenge, the numbers of rolling cells, with or without heparin prophylaxis were identical (Fig. 3*A*). However, mean rolling velocity, however, was markedly increased with the prophylactic heparin bolus: 26 ± 17 without and 135 ± 76 μm/s with heparin (Fig. 3*B*). Furthermore, although the prophylactic bolus also caused a 45-min delay in transendothelial migration, eventually leukocyte extravasation took place in these patients also (Fig. 3*C*). All of these alterations in leukocyte rolling and extravasation were present only when the prophylactic heparin bolus also caused a significant anti-coagulative effect, prolongation of the APTT (APTT 145 s, with normal values being 24–40 s). Despite the fact that the prophylactic heparin bolus almost completely abolished slow rolling and caused a significant delay in leukocyte extravasation, it had no effect on allergic symptoms or signs (Table I).

Table III. *Comparison of inflammatory parameters (mean ± SD) measured at each time point between study groups*

Patients	Challenge	*n*	Time Point (min)	No. of Rolling cells/min	Rolling Velocity (μm/s)	No. of Transmigrated leukocytes/mm^2
Allergic	Allergen	5	0	0.4 ± 0.5	221 ± 210	0 ± 0
		5	15	45.0 ± 27.1*	26 ± 17*	177 ± 193
		5	30	55.5 ± 35.7*	37 ± 17*	764 ± 181**
		4	60	78.8 ± 40.0**	32 ± 15*	642 ± 81**
Control	Allergen	5	0	1.1 ± 2.4	113 ± 0	0 ± 0
		5	15	9.0 ± 20.1	231 ± 0	0 ± 0
		5	30	10.6 ± 19.5	192 ± 55	14 ± 31
		5	60	1.0 ± 2.2	141 ± 0	0 ± 0
Allergic	Histamine	5	0	0.9 ± 1.3	110 ± 113	6 ± 13
		5	15	53.1 ± 39.8*	111 ± 36	0 ± 0
		5	30	80.4 ± 21.4**	94 ± 27	0 ± 0
		5	60	51.4 ± 11.2*	102 ± 19	0 ± 0
Control	Histamine	5	0	2.1 ± 3.4	112 ± 45	0 ± 0
		5	15	42.4 ± 16.4	81 ± 25	8 ± 19
		5	30	58.8 ± 28.1**	111 ± 41	0 ± 0
		5	60	54.4 ± 27.2*	115 ± 44	0 ± 0

* $p < 0.05$ compared with baseline.
** $p \leq 0.01$ compared with baseline

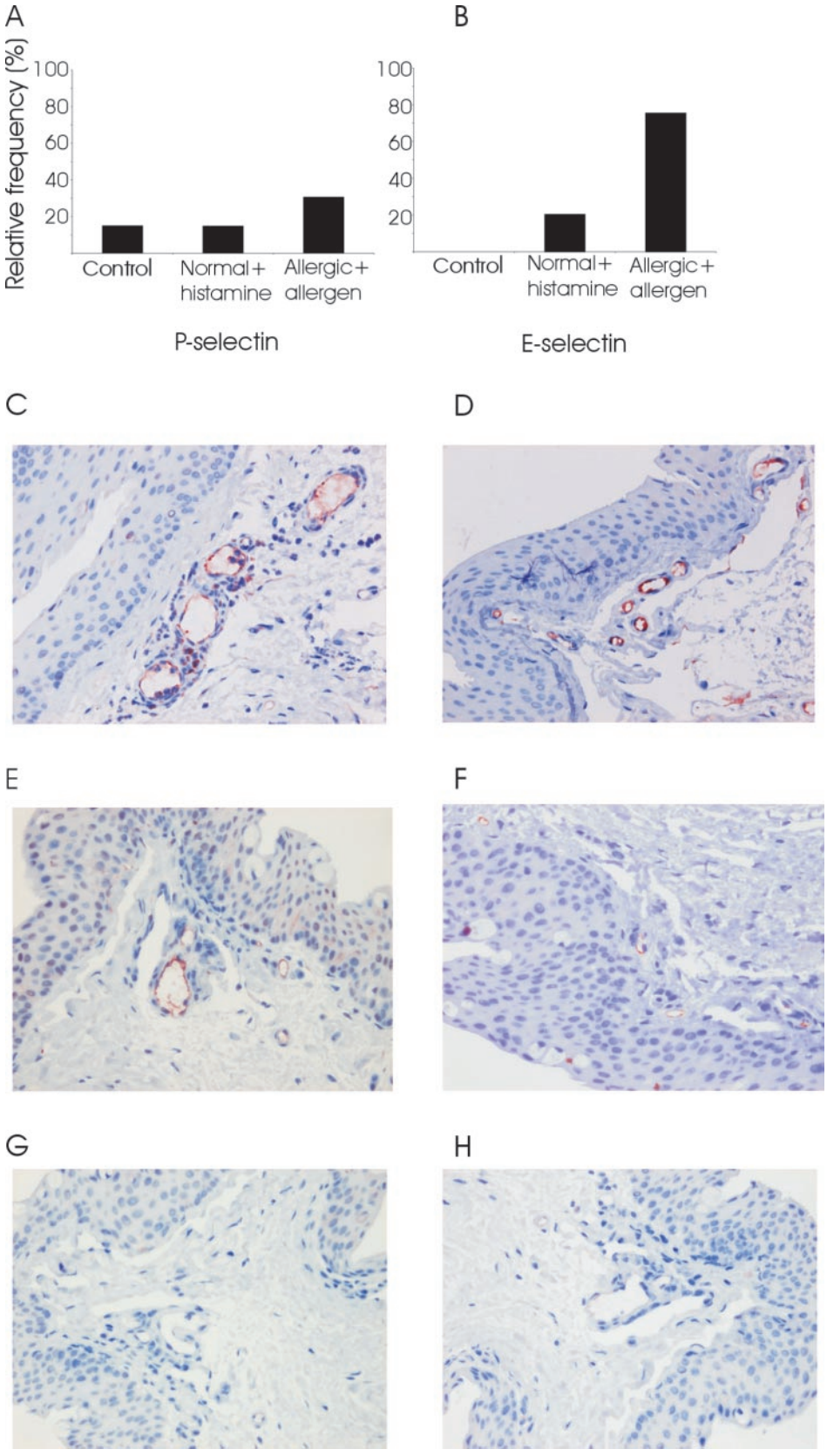

FIGURE 2. Analyses of endothelial expression of P- and E-selectin in conjunctival venules in healthy control 60 min after histamine challenge, and birch-allergic patient 60 min after birch allergen challenge. Percentage of conjunctival venules expressing P-selectin (*A*) and E-selectin (*B*). Staining in specimen (brown) with anti-P-selectin (*C*) anti-E-selectin (*D*) Abs in allergic patient. Staining with anti-P-selectin (*E*) and anti-E-selectin (*F*) in control subjects specimen. Isotype-matched background control for anti-P-selectin (*G*) and anti-E-selectin (*H*) Abs showed essentially no reactivity. Original magnification, ×200.

Effect of heparin on histamine-induced leukocyte traffic and the clinical picture

In this setting, we analyzed healthy subjects, because histamine-induced rolling had been shown to be similar in both allergic patients and healthy controls (Table III, Figs. 1, *A* and *B*). In contrast to the allergen challenge, the prophylactic heparin bolus given before the histamine challenge markedly reduced the number of the rolling cells from 58.8 ± 28.1 without to 18.9 ± 20.1 with heparin (Fig. 4*A*). Concomitantly with heparin′s maximal anti-coagulative effect (APTT was 148 s at 15 min), also the mean leukocyte rolling

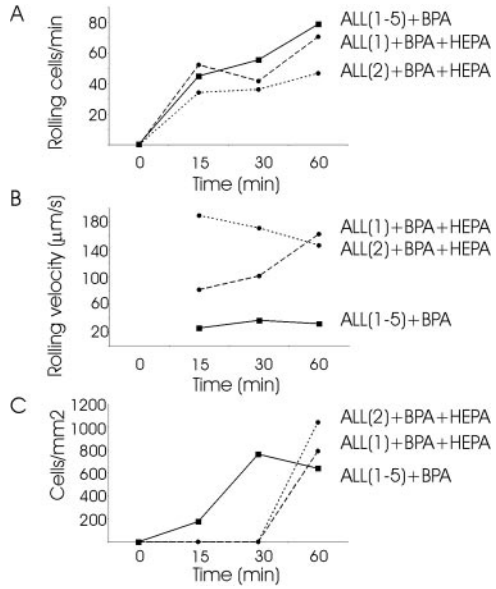

FIGURE 3. Effect of heparin prophylaxis in allergic patients (ALL) before and after birch allergen (BPA) challenge. *A*, Number of rolling cells in conjunctival venules. *B*, Mean rolling velocity. *C*, Number of extravasated leukocytes. Birch allergen challenge with prophylactic heparin (HEPA) treatment marked of the both patients are shown with dotted lines (ALL (1) and ALL (2)) and the mean of the five ALL without heparin treatment with unbroken line (ALL (1–5)).

velocity increased (Fig. 4*B*). Once again, even though the prophylactic heparin bolus significantly modified leukocyte rolling behavior, it had no effect on clinical symptoms or signs (Table I).

Finally, to monitor the capacity of heparin treatment to modify the already ongoing leukocyte rolling in conjunctival vessels induced by the histamine challenge, we gave an i.v. heparin bolus at the time-point of maximal rolling at 40 min after histamine challenge. This intervention promptly and significantly reduced the number of rolling leukocytes (Fig. 5*A*) and increased their velocity, indicating that heparin not only prevents but also modifies ongoing trafficking (Fig. 5, *B–E*). Once again, modified leukocyte rolling was evident only when the APTT measurements rose to anti-coagulative levels.

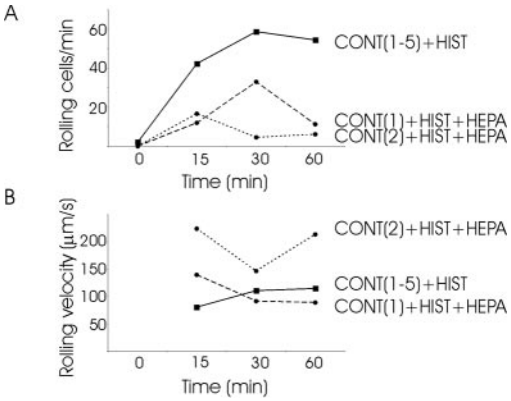

FIGURE 4. Effect of heparin (HEPA) prophylaxis in healthy controls (CONT) before and after histamine (HIST) challenge. *A*, Number of rolling cells in conjunctival venules. *B*, Mean rolling velocity. Histamine challenge with prophylactic heparin treatment marked of the both control subjects are shown with dotted lines (CONT (1) and CONT (2)) and the mean of the five control subjects without heparin treatment with unbroken line (CONT (1–5)).

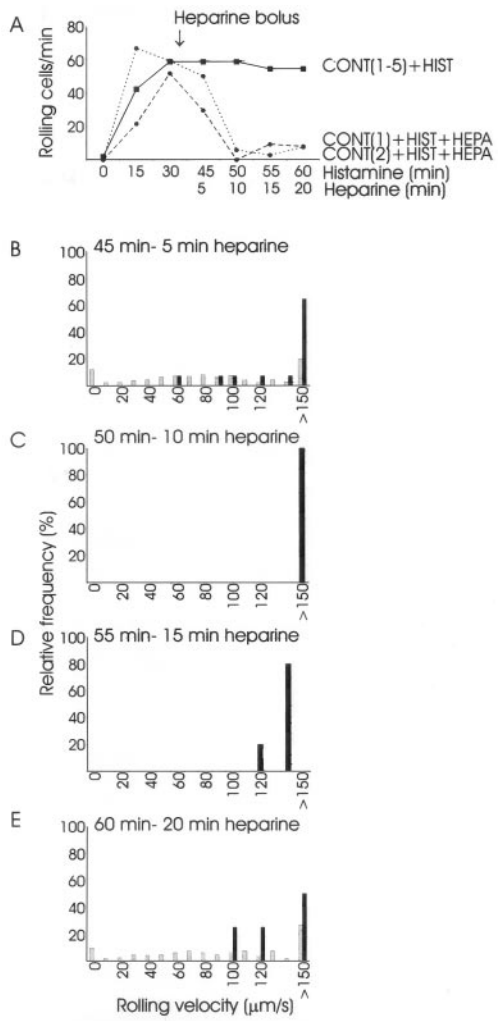

FIGURE 5. Effect of heparin (HEPA) treatment in healthy controls after histamine (HIST) challenge. The heparin bolus was given 40 min after histamine challenge. *A*, Number of rolling cells in conjunctival venules. Histamine challenge with prophylactic heparin treatment marked of the both control subjects (CONT) are shown with dotted lines (CONT (1) and CONT (2)) and the mean of the five control subjects without heparin treatment with unbroken line (CONT (1–5)). *B–E*, Relative frequencies of leukocyte rolling velocities. ■, Histamine challenge with heparin treatment; □, without heparin.

Discussion

We analyzed for the first time allergen- and histamine-induced leukocyte rolling and extravasation into sites of acute allergic inflammation in human patients. When applied to the human conjunctiva, both birch allergen and histamine significantly raised the number of rolling cells within only 15 min after the challenge. Surprisingly, only allergen, not histamine, induced a time-dependent reduction in the velocity of rolling cells in the allergic patients, followed by the extravasation of leukocytes into the conjunctival tissue. Allergen had no effect on nonsensitized controls.

Atopic allergy to environmental proteins is related to the development of highly polarized Th2 cells, with high type 2 cytokine response (IL-4, IL-5, and IL-13) but without simultaneous production of the type 1 cytokine IFN-γ. These highly polarised Th2 cells induce B cells to switch to IgE production. In the normal subjects low levels of IFN-γ block the IgE switch induced by IL-4 or IL-13. The biased polarization of the response toward Th2 cells in atopics is generalized due to enhanced levels of serum IgE, and in some of

the patients it appears to be inherited. The inflammatory late-phase response in allergic rhinitis and asthma, and probably conjunctivitis, is causally related to the high expression of IL-5 that promotes recruitment and activation of eosinophils leading ultimately to local tissue damage (27).

After this primary sensitization phase allergic subjects have allergen-specific IgEs that are bound to FcϵRI on mast cells and basophils. Multivalent allergens then aggregate these IgEs and initiate a signaling cascade leading to mast cell degranulation and release of multiple potent inflammatory mediators. Of those mediators, histamine has a major role in the clinical symptoms. In clinical trials with anti-IgE, the levels of FcϵRI on the cell surface decreased the amount of circulating IgE that has therapeutic efficacy. The focus of this study is in these previously sensitized individuals (28). In our model, clinical symptoms had been consistent with those of previous challenge studies, (1, 17) and the kinetics, if not the magnitude, of the clinical response was similar in all patients studied.

Allergen-induced leukocyte rolling has been studied with intravital microscopy in an animal model, (29) and histamine-induced leukocyte rolling has been extensively studied with intravital microscopy in rat mesentery models (30–32). As suggested in animal (33–35) and human studies, allergen challenge induces strong leukocyte recruitment, as has been detected in tissue biopsies, (36–38) although histamine alone cannot induce any extravasation (30, 36, 39). Previous results are in line with our data that histamine is sufficient to induce leukocyte rolling, but endogenous mediators other than histamine are likely to participate in the reduction in velocity of the rolling leukocytes and in the actual extravasation during the generation of acute allergen-induced inflammation (7).

We tried to elucidate the potential molecular mechanisms for the induced leukocyte rolling and alterations in the rolling velocities after allergen and histamine perturbations. First, in our immunohistochemical analysis of the few biopsies from some of the same individuals analyzed by in vivo confocal microscopy, only a small proportion of vessels expressed P-selectin before any challenge. Expression of P-selectin as well as of E-selectin and ICAM-1 did increase within 60 min after the allergen or histamine stimulus. In vitro and animal studies indicate that all these molecules are upregulated by mast cell activation (40–43). Histamine liberated from mast cells in particular has promoted leukocyte rolling via increased expression of endothelial P-selectin in vitro (44) and in vivo (30–32).

P- and L-selectin-dependent leukocyte traffic has been shown to be sensitive to heparin treatment, whereas E-selectin-dependent traffic is not. Our own results that heparin effectively inhibited leukocyte traffic in human subjects may mean that one mechanism mediating allergen-induced leukocyte traffic in vivo is P-selectin-dependent. However, more specific tools, such as anti-P-selectin Abs, P-selectin glycoprotein ligand 1, or derivates thereof are needed to pinpoint the exact molecular mechanisms (45, 46).

Because we used heparin only to elucidate putative molecular mechanisms, only a single small bolus was administered in a safe therapeutic window. This assumption was based on in vitro and animal model data suggesting that heparin concentrations markedly below the anti-coagulative level were sufficient to block P- and L-selectin-mediated rolling (22–26, 47). Our results argue against these suggestions, as we could document no modifications in leukocyte rolling or extravasation without concomitant alterations in the anti-coagulative status monitored with APTT. We used traditional unfragmented heparin, but anti-coagulative and anti-inflammatory activities have been shown to be located within different domains of the heparin molecule. As these anti-coagulative domains can be successfully modulated, preserving anti-inflammatory activity, (48) it may be possible to dissect the effects of two kinds of heparin. Our prophylactic heparin bolus was sufficient to cause a marked delay in transendothelial migration after allergen challenge, but probably due to the rapid elimination kinetics of heparin, its concentration was insufficient to block all slow rolling and transmigration after one hour. Alternatively or in addition, induction of E-selectin may compensate for inhibition of P-selectin-dependent rolling at this stage (40).

We demonstrate some unique features of the leukocyte trafficking in allergy-caused inflammation in a relevant human conjunctivitis, which challenge the role of leukocytes in the very acute phase of the allergic reaction. First, clinical symptoms began before significant leukocyte extravasation took place, often within 1 min from the topical inoculation of birch allergen, whereas no significant changes in numbers of rolling leukocytes were evident in the initial screening experiments before 5 min (data not shown). This occurred both with allergen and with histamine. Secondly, histamine is a major mediator of a full range of clinical symptoms (1) and has promoted leukocyte rolling in animal models (30–32). We show that it promotes leukocyte rolling also in human subjects, but it is insufficient to induce leukocyte extravasation. Finally, heparin was almost completely able to inhibit histamine-induced slow leukocyte rolling and allergen-induced slow rolling and extravasation, but not to modulate clinical symptoms or signs. These results argue against the direct role of local cellular inflammation in acute allergic reactions in human conjunctiva within the minutes after allergen or histamine challenge. In animal models of more chronic diseases such as delayed-type hypersensitivity, inhibition of lymphocyte, or eosinophil adhesion to the vascular endothelium has reduced inflammation (49, 50). It is possible that a longer detection period of more chronic forms of allergic inflammation may also show improved clinical outcome or the differences may be due to different effector cell populations. Although heparin did not modulate clinical outcome in our setting within the first hour after the challenge, it showed a marked potential to inhibit or completely block leukocyte traffic at routine clinical doses. Heparin treatment can inhibit cancer metastasis in animal models (21). It is part of the treatment of acute myocardial infarct and may prevent ischemia-reperfusion injury by inhibiting leukocyte traffic (48). The threshold for adequate inhibition of leukocyte traffic may differ among inflammatory processes and may vary over time, but our initial findings warrant further studies in humans.

Acknowledgments

We thank Eriika Wasenius for excellent assistance with histological specimens.

References

1. Abelson, M. B., and K. Schaefer. 1993. Conjunctivitis of allergic origin: immunologic mechanisms and current approaches to therapy. *Surv. Ophthalmol. 38(Suppl.):115.*
2. D'Amato, G., and G. Liccardi. 1994. Pollen-related allergy in the European Mediterranean area. *Clin. Exp. Allergy 24:210.*
3. D'Amato, G., F. T. Spieksma, G. Liccardi, S. Jager, M. Russo, K. Kontou-Fili, H. Nikkels, B. Wuthrich, and S. Bonini. 1998. Pollen-related allergy in Europe. *Allergy 53:567.*
4. Fonacier, L., J. Luchs, and I. Udell. 2001. Ocular allergies. *Curr. Allergy Asthma Rep. 1:389.*
5. Fountain, D. W. 2002. Pollen and inhalant allergy. *Biologist 49:5.*
6. Cardaba, B., I. Cortegano, F. Florido, E. Civantos, V. del Pozo, S. Gallardo, M. Rojo, P. Palomino, and C. Lahoz. 2002. Update in the understanding of genetic predisposition to olive pollen sensitization. *Allergy 57:41.*
7. Thorlacius, H., J. Raud, S. Rosengren-Beezley, M. J. Forrest, P. Hedqvist, and L. Lindbom. 1994. Mast cell activation induces P-selectin-dependent leukocyte rolling and adhesion in postcapillary venules in vivo. *Biochem. Biophys. Res. Commun. 203:1043.*
8. Ley, K. 2002. Integration of inflammatory signals by rolling neutrophils. *Immunol. Rev. 186:8.*
9. Butcher, E. C., M. Williams, K. Youngman, L. Rott, and M. Briskin. 1999. Lymphocyte trafficking and regional immunity. *Adv. Immunol. 72:209.*

10. Hemmerich, S., and S. D. Rosen. 2000. Carbohydrate sulfotransferases in lymphocyte homing. *Glycobiology 10:849*.
11. Johnston, B., and E. C. Butcher. 2002. Chemokines in rapid leukocyte adhesion triggering and migration. *Semin. Immunol. 14:83*.
12. Kunkel, E. J., and E. C. Butcher. 2002. Chemokines and the tissue-specific migration of lymphocytes. *Immunity 16:1*.
13. Lowe, J. B. 2001. Glycosylation, immunity, and autoimmunity. *Cell 104:809*.
14. McEver, R. P., J. H. Beckstead, K. L. Moore, L. Marshall-Carlson, and D. F. Bainton. 1989. GMP-140, a platelet α-granule membrane protein, is also synthesized by vascular endothelial cells and is localized in Weibel-Palade bodies. *J. Clin. Invest. 84:92*.
15. Kirveskari, J., M. H. Vesaluoma, J. A. Moilanen, T. M. Tervo, M. W. Petroll, E. Linnolahti, and R. Renkonen. 2001. A novel noninvasive, in vivo technique for the quantification of leukocyte rolling and extravasation at sites of inflammation in human patients. *Nat. Med. 7:376*.
16. Kirveskari, J., M. Helintö, J. A. Moilanen, T. Paavonen, T. Tervo, and R. Renkonen. 2002. Hydrocortisone reduced in vivo, inflammation-induced slow rolling of leukocytes and their extravasation into human conjunctiva. *Blood 100:2203*.
17. Abelson, M. B., W. A. Chambers, and L. M. Smith. 1990. Conjunctival allergen challenge: a clinical approach to studying allergic conjunctivitis. *Arch. Ophthalmol. 108:84*.
18. Sub-Committee on Skin Tests of the European Academy of Allergology and Clinical Immunology. 1989. Skin tests used in type I allergy testing position paper. *Allergy 44(Suppl. 10):1*.
19. Li, H. F., W. M. Petroll, T. Moller-Pedersen, J. K. Maurer, H. D. Cavanagh, and J. V. Jester. 1997. Epithelial and corneal thickness measurements by in vivo confocal microscopy through focusing (CMTF). *Curr. Eye Res. 16:214*.
20. Petroll, W. M., J. V. Jester, and H. D. Cavanagh. 1996. Quantitative three-dimensional confocal imaging of the cornea in situ and in vivo: system design and calibration. *Scanning 18:45*.
21. Lever, R., and C. P. Page. 2002. Novel drug development opportunities for heparin. *Nature 1:140*.
22. Borsig, L., R. Wong, R. O. Hynes, N. M. Varki, and A. Varki. 2002. Synergistic effects of L- and P-selectin in facilitating tumor metastasis can involve non-mucin ligands and implicate leukocytes as enhancers of metastasis. *Proc. Natl. Acad. Sci. USA 99:2193*.
23. Wang, L., J. R. Brown, A. Varki, and J. D. Esko. 2002. Heparin's anti-inflammatory effects require glucosamine 6-*O*-sulfation and are mediated by blockade of L- and P-selectins. *J. Clin. Invest. 110:127*.
24. Varki, N. M., and A. Varki. 2002. Heparin inhibition of selectin-mediated interactions during the hematogenous phase of carcinoma metastasis: rationale for clinical studies in humans. *Semin. Thromb. Hemost. 28:53*.
25. Norgard-Sumnicht, K. E., N. M. Varki, and A. Varki. 1993. Calcium-dependent heparin-like ligands for L-selectin in nonlymphoid endothelial cells. *Science 261:480*.
26. Nelson, R. M., O. Cecconi, W. G. Roberts, A. Aruffo, R. J. Linhardt, and M. P. Bevilacqua. 1993. Heparin oligosaccharides bind L- and P-selectin and inhibit acute inflammation. *Blood 82:3253*.
27. Kapsenberg, M. L., C. M. Hilkens, E. A. Wierenga, and P. Kalinski. 1998. The role of antigen-presenting cells in the regulation of allergen-specific T cell responses. *Curr. Opin. Immunol. 10:607*.
28. Kinet, J. P. 2002. Allergy and hypersensitivity. *Curr. Opin. Immunol. 14:685*.
29. Broide, D. H., D. Humber, S. Sullivan, and P. Sriramarao. 1998. Inhibition of eosinophil rolling and recruitment in P-selectin- and intracellular adhesion molecule-1-deficient mice. *Blood 91:2847*.
30. Asako, H., I. Kurose, R. Wolf, S. DeFrees, Z. L. Zheng, M. L. Phillips, J. C. Paulson, and D. N. Granger. 1994. Role of H1 receptors and P-selectin in histamine-induced leukocyte rolling and adhesion in postcapillary venules. *J. Clin. Invest. 93:1508*.
31. Thorlacius, H., J. Raud, X. Xie, P. Hedqvist, and L. Lindbom. 1995. Microvascular mechanisms of histamine-induced potentiation of leukocyte adhesion evoked by chemoattractants. *Br. J. Pharmacol. 116:3175*.
32. Kubes, P., and S. Kanwar. 1994. Histamine induces leukocyte rolling in post-capillary venules: a P-selectin-mediated event. *J. Immunol. 152:3570*.
33. Spicer, B. A., P. A. Hatt, S. M. Laycock, and H. Smith. 1986. Effect of drugs on the increase in cell numbers in the peritoneal cavity of the actively sensitised mouse after intraperitoneal challenge with antigen. *Int. Arch. Allergy Appl. Immunol. 81:81*.
34. Hom, J. T., and T. Estridge. 1994. Antigen-induced recruitment of eosinophils: importance of $CD4^+$ T cells, IL5, and mast cells. *Clin. Immunol. Immunopathol. 73:305*.
35. Kaneko, M., Y. Hitoshi, K. Takatsu, and S. Matsumoto. 1991. Role of interleukin-5 in local accumulation of eosinophils in mouse allergic peritonitis. *Int. Arch. Allergy Appl. Immunol. 96:41*.
36. Atkins, P., G. R. Green, and B. Zweiman. 1973. Histologic studies of human skin test responses to ragweed, compound 48–80, and histamine. *J. Allergy Clin. Immunol. 51:263*.
37. Bacon, A. S., P. Ahluwalia, A. M. Irani, L. B. Schwartz, S. T. Holgate, M. K. Church, and J. I. McGill. 2000. Tear and conjunctival changes during the allergen-induced early- and late-phase responses. *J. Allergy Clin. Immunol. 106:948*.
38. Bacon, A. S., J. I. McGill, D. F. Anderson, S. Baddeley, S. L. Lightman, and S. T. Holgate. 1998. Adhesion molecules and relationship to leukocyte levels in allergic eye disease. *Invest. Ophthalmol. Vis. Sci. 39:322*.
39. Issekutz, A. C. 1981. Effect of vasoactive agents on polymorphonuclear leukocyte emigration in vivo. *Lab. Invest. 45:234*.
40. Kanwar, S., D. C. Bullard, M. J. Hickey, C. W. Smith, A. L. Beaudet, B. A. Wolitzky, and P. Kubes. 1997. The association between α_4-integrin, P-selectin, and E-selectin in an allergic model of inflammation. *J. Exp. Med. 185:1077*.
41. Klein, L. M., R. M. Lavker, W. L. Matis, and G. F. Murphy. 1989. Degranulation of human mast cells induces an endothelial antigen central to leukocyte adhesion. *Proc. Natl. Acad. Sci. USA 86:8972*.
42. Leung, D. Y., J. S. Pober, and R. S. Cotran. 1991. Expression of endothelial-leukocyte adhesion molecule-1 in elicited late phase allergic reactions. *J. Clin. Invest. 87:1805*.
43. Meng, H., M. G. Tonnesen, M. J. Marchese, R. A. Clark, W. F. Bahou, and B. L. Gruber. 1995. Mast cells are potent regulators of endothelial cell adhesion molecule ICAM-1 and VCAM-1 expression. *J. Cell Physiol. 165:40*.
44. Jones, D. A., O. Abbassi, L. V. McIntire, R. P. McEver, and C. W. Smith. 1993. P-selectin mediates neutrophil rolling on histamine-stimulated endothelial cells. *Biophys. J. 65:1560*.
45. Leppanen, A., S. P. White, J. Helin, R. P. McEver, and R. D. Cummings. 2000. Binding of glycosulfopeptides to P-selectin requires stereospecific contributions of individual tyrosine sulfate and sugar residues. *J. Biol. Chem. 275:39569*.
46. Hicks, A. E., A. Leppanen, R. D. Cummings, R. P. McEver, P. G. Hellewell, and K. E. Norman. 2002. Glycosulfopeptides modeled on P-selectin glycoprotein ligand 1 inhibit P-selectin-dependent leukocyte rolling in vivo. *FASEB J. 16:1461*.
47. Koenig, A., K. Norgard-Sumnicht, R. Linhardt, and A. Varki. 1998. Differential interactions of heparin and heparan sulfate glycosaminoglycans with the selectins. Implications for the use of unfractionated and low molecular weight heparins as therapeutic agents. *J. Clin. Invest. 101:877*.
48. Wan, J. G., J. S. Mu, H. S. Zhu, and J. G. Geng. 2002. N-desulfated non-anticoagulant heparin inhibits leukocyte adhesion and transmigration in vitro and attenuates acute peritonitis and ischemia and reperfusion injury in vivo. *Inflamm. Res. 51:435*.
49. Vancheri, C., C. Mastruzzo, F. Armato, V. Tomaselli, S. Magri, M. P. Pistorio, M. LaMicela, L. D'amico, and N. Crimi. 2001. Intranasal heparin reduces eosinophil recruitment after nasal allergen challenge in patients with allergic rhinitis. *J. Allergy Clin. Immunol. 108:703*.
50. Lever, R., J. R. Hoult, and C. P. Page. 2000. The effects of heparin and related molecules upon the adhesion of human polymorphonuclear leucocytes to vascular endothelium in vitro. *Br. J. Pharmacol. 129:533*.