

Mast cells are critically involved in serum-mediated vascular leakage in chronic urticaria beyond high-affinity IgE receptor stimulation

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Keywords

chronic urticaria; endothelial cells; high-affinity IgE receptor; mast cells; vascular leakage.

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Abstract

Background: Chronic urticaria (CU) is one of the most common skin disorders whose pathogenic mechanisms are not fully clarified. Autoimmune aetiology can be ascribed to 45% of patients with CU, and basophil histamine release is positive in 40% of cases. Our aim was to use a novel approach to evaluate the serum permeabilizing effect to identify the mediators of endothelial cell (EC) leakage and to define the role of mast cells (MCs) in the process.

Methods: Permeabilizing activity of sera from 19 patients with CU and 11 healthy blood donors was evaluated by measuring serum-induced degranulation of two MC lines, expressing (LAD2) or lacking (HMC-1) the IgE receptor. Mast cell supernatant (SN) was then incubated with an EC monolayer, and endothelial permeability was evaluated by Fluorescein isothiocyanate-bovine serum albumin leakage in a transwell system.

Results: All 19 patient sera failed to induce direct EC leakage, but 15/19 and 17/19 promoted degranulation of HMC-1 and LAD2, respectively. Interestingly, 85% of autologous serum skin test-negative sera were able to cause MC degranulation. Also, 17/19 SNs from HMC-1 and all SNs from LAD2 incubated with CU sera increased endothelial permeability. Endothelial cell leakage remained unchanged after Ig depletion and was prevented by antihistamine, platelet-activating factor or leukotriene antagonist.

Conclusions: Our study shows that CU sera are able to degranulate MCs through an IgE- and IgG-independent mechanism. The nature of histamine-releasing factors involved is still unclear, but our finding opens new ways to the understanding of the pathogenesis of CU, particularly in patients not showing circulating auto-antibodies to FcεRI or IgE.

Chronic urticaria (CU) is characterized by spontaneous recurrent bouts of wheals and pruritus with or without accompanying angio-oedema, which persist for at least 6 weeks and is associated with severely impaired quality of life. Chronic urticaria affects at least 0.1% individuals in the general population, and women are more involved than men. No correlation has been found between exogenous allergens and the onset of CU, and the pathogenic mechanisms respon-

sible for the development of CU are still not fully elucidated. The observation that CU is often associated with thyroid diseases (1), most frequently Hashimoto's thyroiditis (2), led to hypothesize an autoimmune aetiology for CU, although there is no evidence that the two clinical conditions are pathogenically related. This suspect was further corroborated by the detection of antibodies to the alpha chain of FcεRI and to IgE in 35–40% and 5–10% of patients, respectively (3). These

antibodies have been implicated in the induction of histamine release by basophils and mast cells (MCs) (4–6), although not all antibodies are functionally active, suggesting that other as yet unidentified factors are responsible for the increase in the vascular permeability leading to the development of wheals and often angio-oedema (6).

Mast cells represent an important component of the cell infiltrate in the skin lesions of CU and favour the formation of wheals with the release of vasoactive substances, in particular histamine, detected at increased levels in the lesional skin (7). Besides histamine, serotonin, proteases, proteoglycans, tumour necrosis factor (TNF)- α and leukotrienes (LTs) are also released by MCs, and they may all be directly or indirectly involved in the development of wheals (8). Moreover, MC-derived mediators such as interleukin-6 (IL-6), vascular endothelial growth factor (VEGF) (9) or platelet-activating factor (PAF) (10) could be involved in inducing and maintaining CU symptoms. While autoantibodies to Fc ϵ RI and IgE have been shown to induce histamine release from MCs (5), the mechanism of cell stimulation by the sera from patients lacking these antibodies is still undefined, although some of the activating factors may derive from the cells infiltrating and surrounding the dermal postcapillary venules in the wheal (11, 12).

In this study, we have evaluated the presence of permeabilizing factors in the sera of patients with CU by a different approach using an *in vitro* assay of vascular permeability. Endothelial cells (ECs) have received very little attention in studies addressing the mechanisms of wheal formation in patients with CU despite the fact that they are the target of the vasoactive molecules released by MCs and possibly by other cells present in the infiltrate. The presence of adhesion molecules on the surface of these cells in biopsy specimens of patients with CU and the increase in adhesion molecule expression by MC SN stimulated with serum from patients with CU (13) suggest that ECs are activated in the lesional skin.

Our aim was to investigate the permeabilizing effect of the sera obtained from patients during the active phase of the disease and the contribution of MCs. Moreover, we sought to identify the factors responsible for the increase in vascular leakage using specific antagonists of the main vasoactive molecules released by MCs. We felt that this approach may represent a valuable tool to investigate the pathogenesis of CU in both autologous serum skin test (ASST)-negative and the ASST-positive patients with CU.

Methods

Patients

Serum and EDTA-plasma samples were collected from 19 patients with CU and from 11 sex- and age-matched healthy donors. Chronic urticaria was diagnosed based on the recurrence of spontaneous wheals with or without angio-oedema for more than 6 weeks. Patients with physical urticaria were excluded. At the time of blood sampling, disease activity was estimated according to the number of wheals and pruritus present, following the urticaria activity score (UAS) recommended by the recent EAACI guidelines as reported in

Table 1 Urticaria activity score

Score	Wheals	Pruritus
0	None	None
1 (mild)	<20 wheals/24 h	Present but not annoying or troublesome
2 (moderate)	20–50 wheals/24 h	Troublesome but does not interfere with normal daily activity or sleep
3 (intense)	>50 wheals/24 h or large confluent areas of wheals	Severe pruritus, interfering with normal daily activity or sleep

Table 2 Clinical characterization of patients

Patient no.	Sex	Age (years)	Clinical manifestation	ASST	UAS
1	F	38	U	+++	2
2	F	36	U	++++	1
3	F	38	U	+	1
4	M	40	U	–	1
5	F	43	U + AE	+	3
6	M	74	U + AE	–	1
7	M	76	U	–	1
8	F	20	U	++	2
9	F	76	U + AE + A	++++	6
10	F	62	U + AE	+++	5
11	F	35	U + AE	++	2
12	F	36	U	++	2
13	F	29	U	–	2
14	M	48	U + AE	+++	3
15	F	47	U + AE	–	2
16	F	48	U	–	0
17	M	64	U	–	1/2
18	F	54	U + AE	++++	6
19	F	43	U + AE	+++	6
Median (range)		48 (20–76)			2.5 (0–6)

F, female; M, male; U, urticaria; AE, angioedema; A, asthma; ASST, autologous serum skin test; UAS, urticaria activity score.

Table 1 (14). No patient was taking, nor had been taking, LT receptor antagonists, or PAF inhibitors. At the time of their visit, to perform the ASST, patients were off antihistamines for at least 5 days, and their clinical features are shown in Table 2. The study was performed following the ethical guidelines of the Declaration of Helsinki, and all subjects gave an informed consent.

Autologous serum skin test

Autologous serum skin test was performed following the method of Sabroe et al. (15), by intradermal injection of 0.05 ml of fresh autologous serum and reading the wheal and flare reaction at 30 min. Intradermal injection of saline solution (0.9% NaCl) was performed as negative control and skin prick test with 10 mg/ml histamine as positive control. Patients

showing a red wheal with a diameter of at least 1.5 mm greater than the control saline solution were considered positive.

Reagents and antibodies

Fluorescein isothiocyanate (FITC)-conjugated bovine serum albumin (BSA), histamine, loratadine (LOR) and LT D4 were purchased from Sigma-Aldrich (Milan, Italy). Platelet-activating factor receptor antagonist CV-3988 (CV) was from Biomol (Plymouth Meeting, PA, USA) and Montelukast (ML; Lukasm) from Sigma-Tau (Rome, Italy).

Primary cells and cell line

Endothelial cells were isolated from human umbilical vein (HUVEC) by collagenase digestion (16) and from human adult dermal microvasculature (ADMEC) of normal skin biopsy obtained after written consent from patients undergoing reductive plastic surgery as previously described (17).

The human MC line HMC-1, established from a patient with MC leukaemia, was kindly provided by L. Macchia (University of Bari, Italy). Cells were cultured in Iscove's medium supplemented with 10% Fetal Calf Serum (FCS), 1.2 mM monothioglycerol, 100 U/ml penicillin, 100 µg/ml streptomycin and 2 mM glutamine.

The human MC line LAD2 was kindly provided by A. Kirshenbaum (NIH, Bethesda, MD, USA). The cell line was established from bone marrow aspirates of patient with MC sarcoma leukaemia and is closely related to hMCs (18). LAD2 cells were grown in serum-free medium StemPro-34 (Invitrogen, Carlsbad, CA, USA) containing 2 mM glutamine and 100 ng/ml human stem cell factor (Peprotech, London, UK) and were periodically tested for c-Kit and FcεRI expression on the cell surface by flow cytometry (FACScan; Becton Dickinson, San Diego, CA, USA).

Degranulation assay

HMC-1 and LAD2 cells were suspended at 2×10^6 /ml in Tyrodes/BSA 0.05% and incubated with patient or healthy donor sera (1 : 100) for 30 min. The enzymatic activity of the β-hexosaminidase released was evaluated as previously described (19). Positive control was obtained by cell stimulation with 1 µM ionomycin for 30 min.

Assessment of vascular permeability

The *in vitro* assay was previously published (16). Briefly, 2×10^4 ECs were seeded onto polycarbonate inserts of a 24-well Transwell system (Costar, Cambridge, MA, USA) and used 5 days after plating. The stimuli were added to the upper chamber together with FITC-BSA, and the fluorescence was evaluated in the lower chamber at various time intervals by an Infinite200 (TECAN Italia S.r.l., Milan, Italy).

Basophil histamine release (BHR) assay

Leucocyte suspension containing 7×10^4 basophils was prepared by dextran sedimentation of 0.01 M EDTA-treated

human blood, and net histamine release was calculated as previously reported (20).

IgG depletion

Briefly, sera were diluted in binding buffer (20 mM sodium phosphate buffer, 0.15 M NaCl, pH 7) and incubated with γ-Bind plus Sepharose (GE Healthcare, Waukesha, WI, USA) for 1 h at room temperature. After centrifugation, the SN was used to induce MC degranulation, and the results were compared with those of the same sera prior to Ig depletion.

Statistical analysis

The results were expressed as means ± SDs of triplicate determinations of three different experiments and analysed using the SAS Institute's StatView 5.0 statistical package (Cary, NC, USA). The data were compared by ANOVA test using the post hoc analysis for paired multiple comparisons with the Fisher protected least significant difference test (21). *P* values of <0.05 were considered statistically significant.

Results

Evaluation of the permeabilizing effect of CU sera

In the initial experiments, we tested the permeabilizing effect of sera from both ASST-positive and ASST-negative patients with CU. The results were compared with the data obtained with histamine used as a positive control. The effect of serum was evaluated at different time points from 15 to 60 min using transwell inserts coated with HUVEC monolayers. As expected, histamine caused prompt leakage of FITC-BSA within 15 min from the start of the experiment, whereas the sera from patients were totally ineffective at all time points examined giving results comparable with the data of sera from healthy blood donors. Figure 1A shows the results obtained by incubating the sera with HUVEC for 30 min. Similar results were obtained using plasma samples from the same patients (data not shown). Based on the clinical observation that skin vessels are involved in the development of urticaria, we next analysed the effect of sera from patients and controls on ADMEC. As shown in Fig. 1B, the overall response of ADMEC was higher than that of HUVEC, but again, the leakage induced by the sera was negligible after 30 min of incubation and remained unchanged after 1 h (data not shown), suggesting failure of the sera from patients with CU to exert a direct permeabilizing effect on ECs.

Stimulatory effect of CU sera on MCs

Because sera from patients with CU did not induce any leakage of ECs, we analysed the stimulatory effect of both patient and control sera on human MCs. To this end, we used two human MC lines: (i) the cell line HMC-1, which exhibits a phenotype similar to that of human MCs (22), but lacks surface expression of the FcεRI because of the absence of the alpha- and beta-chains of the IgE-receptor complex

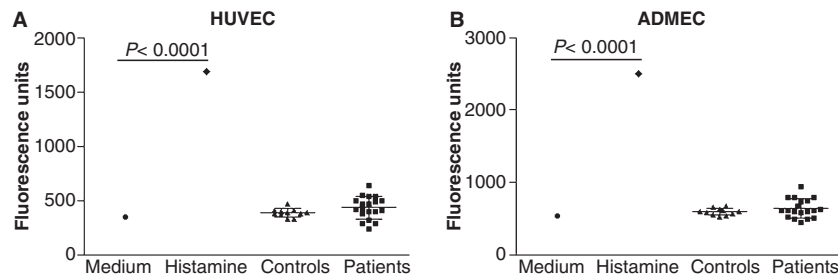


Figure 1 Serum-induced Fluorescein isothiocyanate-bovine serum albumin leakage. Permeability of human umbilical vein endothelial cell (HUVEC) (A) or adult dermal microvasculature (B) induced by serum obtained from 19 patients with chronic urticaria or by serum

from 11 representative blood donors as controls or histamine (10^{-6} M) or culture medium as negative control. Horizontal bars indicate geometric means $\pm$ SD.

and does not degranulate in response to IgE-dependent signals (23); and (ii) the human LAD2 cells, which closely resemble CD34⁺-derived human MCs, have functional Fc ϵ RI and Fc γ RI receptors (18) and release β -hexosaminidase following both Fc ϵ RI-dependent and Fc ϵ RI-independent activation (24). As shown in Fig. 2, both cell lines responded to ionomycin stimulation with the release of $50 \pm 5\%$ of their β -hexosaminidase content, whereas the values obtained with the control sera did not exceed $18 \pm 3\%$ for the HMC-1 and $10 \pm 2\%$ for the LAD2 cells.

Chronic urticaria sera elicited a stimulating effect on both HMC-1 ($32 \pm 6\%$) and LAD2 cells ($25 \pm 5\%$) significantly higher ($P < 0.005$) than that of the control sera. Chronic urticaria sera failed to induce degranulation of rat MCs *in vitro* (data not shown), suggesting that the stimulating factors in these sera are species specific. This hampered the analysis of these sera using a previously published *in vivo* model of vascular permeability (16, 17).

MC-dependent EC leakage

To determine whether the serum-induced stimulation of MCs resulted in the release of vasoactive molecules, we next investigated the ability of the SNs of serum-treated MCs to cause vascular permeability using a Transwell model system. Supernatants from both MC lines were tested to assess the contri-

bution of Fc ϵ RI that is thought to play a pivotal role in the autoimmune CU (3). As shown in Fig. 3A,B, all SNs of LAD2 and 16 of 19 SNs of HMC-1 (except numbers 4, 7, 12) induced BSA leakage in an *in vitro* assay established in our laboratory (16, 17). For comparison, 11 SNs (A–M) collected from MCs stimulated with control sera were used, and they resulted unable to induce any EC leakage reaching the value of the medium alone. The increase in EC permeability varied with different samples, but overall, ECs responded more effectively to LAD2 SN. To find out the contribution of autoantibodies specific for IgE or Fc ϵ RI, the sera of three patients (numbers 9, 10 and 14) selected based on their good response in the BHR assay were IgG depleted and used to stimulate the degranulation of MC and to induce EC leakage. The complete removal of IgG was checked by western blot (data not shown). Sera from patient number 9 and 10 presumably contained anti-IgE autoantibodies because the histamine-releasing activity was reduced by lactic acid stripping of membrane-bound IgE (from 10% to 4% and from 22% to 6%, respectively); conversely, serum from patient number 14 presumably contained anti-Fc ϵ RI autoantibodies because its histamine-releasing activity was enhanced following basophil treatment with lactic acid (from 5.4% to 16.6%). A similar procedure for IgG depletion was adopted with the serum from one patient (number 12), which was found to be negative in the BHR assay and was used as con-

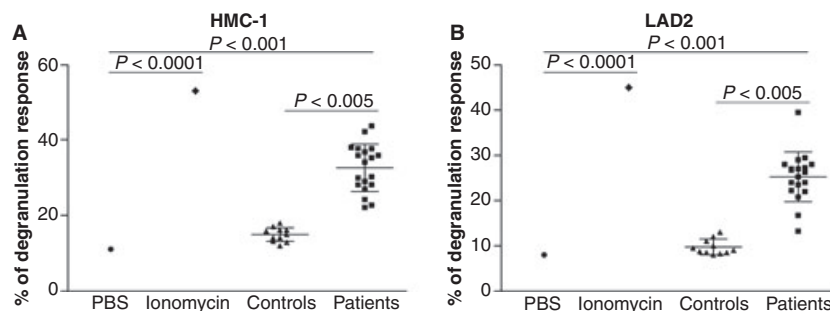


Figure 2 Serum-induced mast cell degranulation. HMC-1 (A) and LAD2 (B) cells were incubated with the patient with chronic urticaria and healthy donor sera, and the percentage of degranulation

response was evaluated. Ionomycin was used as positive control and medium as negative control. Horizontal bars indicate geometric means $\pm$ SD.

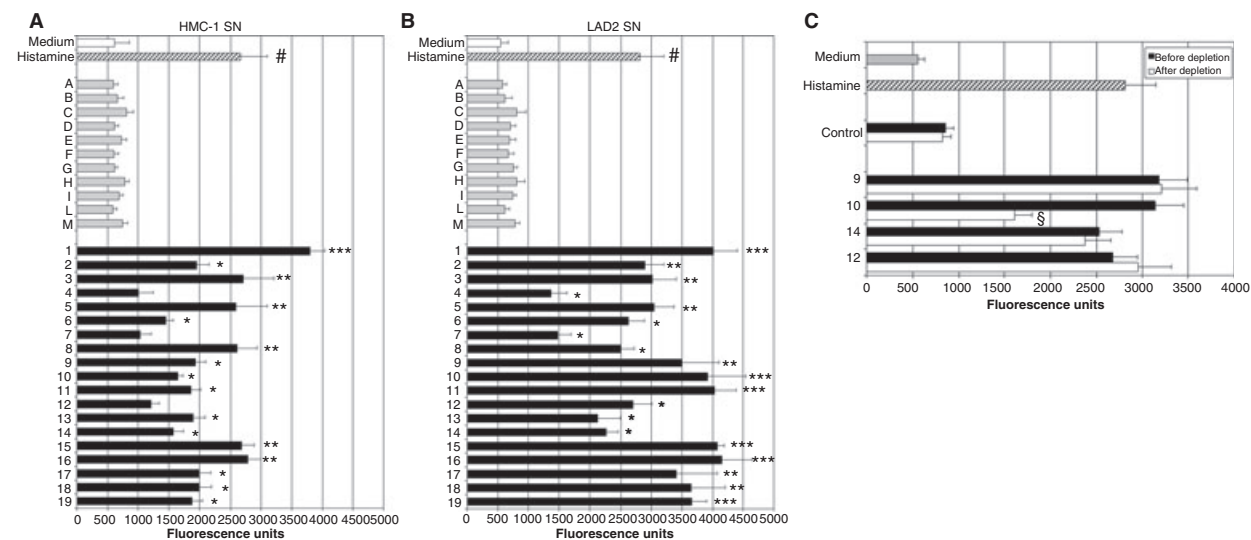


Figure 3 Endothelial leakage is mediated by chronic urticaria (CU) sera stimulation of mast cells. Supernatant (SN) of HMC-1 (A) and LAD2 (B) MC line stimulated with the 19 patients with CU and 11 healthy donor sera (A–M) induced adult dermal microvasculature monolayer leakage. Histamine (10^{-6} M) was used as positive con-

trol and medium as negative control. (C) Endothelial leakage evaluation using SN of LAD2 cells stimulated with sera before and after IgG depletion. # $P < 0.001$ vs medium, * $P < 0.005$ vs controls (A–M), ** $P < 0.001$ vs controls (A–M), *** $P < 0.0001$ vs controls (A–M), § $P < 0.001$ vs before depletion.

trol. As shown in Fig. 3C, serum-induced EC permeability following IgG depletion was markedly decreased in one serum (number 10), although not to the levels of the control sera, but remained unchanged in the positive sera (numbers 9 and 14) and in the negative control (number 12).

Notably, 7/19 ASST-negative sera (numbers 4, 6, 7, 13, 15 and 17) from patients with CU were positive in the EC

leakage assay using the SN of LAD2 cells, and five of these sera (numbers 6, 13, 15, 16 and 17) were positive in a similar test performed with the SN of HMC-1 cells (Table 3). No correlation was observed between the BSA-FITC leakage and the degranulation response of both HMC-1 and LAD2 cells (Fig. 4). Basophil histamine release was performed on the sera from 12 patients (numbers 4, 9–16 and

Table 3 Correlation among ASST, degranulation and permeability assays

Patient no.	ASST	Degranulation HMC-1	Permeability HMC-1	Degranulation LAD2	Permeability LAD2
1	+++	+	+	+	+
2	++++	+	+	+	+
3	+	+	+	+	+
4	–	–	–	+	+
5	+	–	+	+	+
6	–	+	+	+	+
7	–	–	–	+	+
8	++	+	+	+	+
9	++++	+	+	+	+
10	+++	+	+	+	+
11	++	+	+	+	+
12	++	+	–	+	+
13	–	+	+	+	+
14	+++	+	+	+	+
15	–	+	+	–	+
16	–	+	+	+	+
17	–	+	+	+	+
18	++++	+	+	+	+
19	+++	–	+	–	+

ASST, autologous serum skin test.

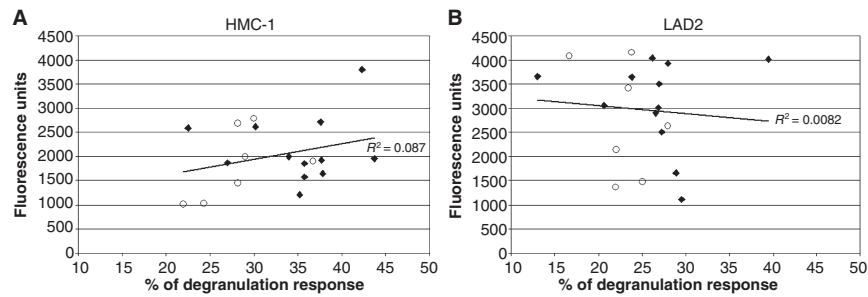


Figure 4 No correlation between permeability assay and degranulation response. A correlation curve was built among endothelial leakage and degranulation response for HMC-1 (A) and LAD2 (B)

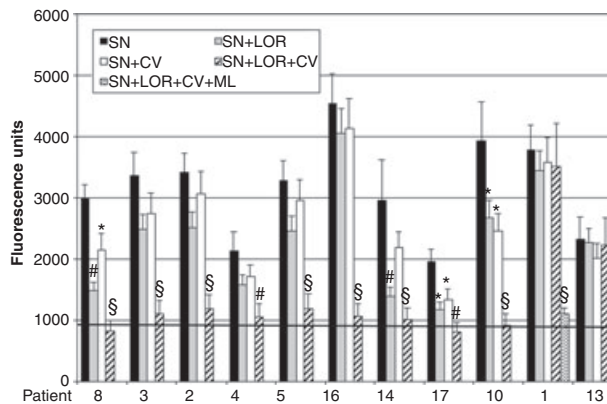


Figure 5 Inhibitory effect of endothelial permeability. Adult dermal microvasculature monolayer was treated for 10 min with loratadine (5×10^{-5} M), CV-3988 (3×10^{-5} M) and montelukast (10^{-4} M) followed by 30-min incubation with patient sera-stimulated LAD2 cell supernatant (SN). Black line represents mean of healthy donor values. * $P < 0.005$ vs SN, # $P < 0.001$ vs SN, \$ $P < 0.0001$ vs SN.

18–19) and found to be positive in four cases (numbers 9, 10, 11 and 14). No correlation was found between BHR and degranulation of HMC-1 or LAD2 cells (data not shown).

Contribution of different mediators released by MC to EC leakage

The recommended first-line treatment for CU is antihistamines, but for patients who do not respond, a second-line therapy should be added. In this case, the recommended therapy consists in the use of LT antagonists or, finally, cyclosporine (25). Based on this observation, to mimic patient treatment approaches, we analysed the effect of drugs currently used to control EC permeability. ADMEC was treated with antagonists of histamine, PAF and LTs and then incubated with LAD2 SN stimulated by 11 sera from patients with CU during acute phase. The drugs were incubated with ECs for 15 min prior to addition of SN, and FITC-BSA leakage was evaluated after 30 min. The endothelial leakage

was partially inhibited by loratadine (numbers 8, 14, 17 and 10) or PAF receptor antagonist CV-3988 (numbers 8, 17 and 10) used alone, but better controlled by the mixture of both drugs (Fig. 5). CV-3988 and LOR failed to prevent the permeabilizing effect of 2 SN (number 1 and 13), which required the addition of ML for the inhibition of the endothelial leakage.

Discussion

Increased vasopermeability leading to extravascular leakage of fluid and proteins is responsible for the formation of wheals, flare and angio-oedema, which represent the main clinical features of CU (3, 26).

In this paper, we used a transwell model system employed in our previous publication (17) to demonstrate the permeabilizing effect of plasma from patients with angio-oedema associated with C1 inhibitor deficiency. We were looking for serum-induced leakage of FITC-BSA through the endothelium monolayer, but we failed to detect this activity in the CU sera. The fact that both serum and plasma from patients with CU were also ineffective might be suggestive of the presence of preformed or newly formed permeabilizing factors that are released by cells that are going between CU serum and ECs.

Searching for cellular sources of factors that may be involved in the serum-mediated vasopermeability, we focused our attention on MCs as a likely candidate to release the permeabilizing factor/s following stimulation with CU serum. MCs that are present in the perivascular infiltrate, together with other inflammatory cells, and appear degranulated as a result of activation in lesional skin of patients with CU, probably play a more important role in promoting vascular leakage (7, 27, 28). Our *in vitro* data showing degranulation of MCs induced by CU sera but not by control sera are in line with these observations. Two well-characterized human MC lines were used in this study to discriminate between antibody-dependent and antibody-independent stimulatory effect of the patient sera. The cell line HMC-1 is similar to human tryptase-positive MCs (22), but lacks the α and β chains of Fc ϵ RI complex (23). Conversely, the human LAD2 cells closely resemble CD34⁺-derived human MCs expressing functional Fc ϵ RI and Fc γ RI receptors (18). Our finding that

both cell lines degranulate in response to CU sera suggests that MC activation occurs through both IgE receptor-dependent and IgE receptor-independent manner and supports the observation made by Eckman et al. that there is not a correlation between the detection of autoantibodies and clinical CU symptoms (29). Furthermore, MCs are widely recognized to express a plethora of membrane receptors which can be differently and selectively triggered by several ligands, such as IgG, peptides, microbial derivatives, fragments of activated complement acting as pro-inflammatory and/or regulatory stimulus for these cells depending on the different physiological context (30). Depending on the nature of the stimulus, MC activation can lead to selective response in term of quality and quantity of released mediators. Therefore, it is possible to induce cytokine production without degranulation and vice versa. This could explain the lack of correlation between the increased vasopermeability and the degree of degranulation induced by the same sera, suggesting the contribution of other mediators to serum-mediated vascular leakage besides those released with the granules. The lack of permeability effect of SNs from MC stimulated by control sera (Fig. 3) supports the specificity of the assay for patients with CU. The fact that the permeabilizing activity of two of three sera was unchanged following Ig depletion and was reduced but not completely abrogated in the third sample analysed suggests that serum factors other than autoantibodies to IgE or FcεRI play a critical role in the onset of CU. The presence of non-IgG histamine-releasing factor/s acting on dermal MCs has been suggested by Sabroe and Greaves in 5–10% of patients with CU, but their nature remains to be elucidated (31). In this context, our data, showing a lack of correlation between degranulation of HMC-1 or LAD2 and BHR, are in line with the presence of different activating factors in sera from patients with CU. In the light of these considerations, although serum of patient number 19 did not induce histamine release from MC lines, we cannot exclude that any permeabilizing factors other than histamine are secreted. Several therapeutic evidences support this hypothesis. Urticaria episodes associated with the release of high quantities of IL-6 are resistant to anti-H1 treatment (32). Furthermore, although the majority of urticarias are sensitive to anti-H1, symptom amelioration is achieved only with steroids or with a combination of antileukotrienes and anti-H1 drugs (33). The patients who are resistant to antihistamines may be treated with immunosuppressive drugs, such as cyclosporine, that prevent cytokine synthesis. This has important therapeutic implications because there is a consensus on the use of sec-

ond-generation antihistamines as first-line symptomatic drug to treat patients with CU. The rationale for using these drugs is supported by our finding that antihistamine can substantially reduce serum-induced EC permeability in the 4/11 patients analysed in the inhibition experiment. However, it is important to emphasize that the combination of antihistamines and PAF antagonists was more effective than antihistamines alone and resulted in total abrogation of BSA leakage, suggesting that a combination therapy can be more appropriate than the use of a single drug. Of interest is the observation that the serum of two patients exhibited a permeabilizing activity neutralized exclusively by an antileukotriene drug, which has been used in patients with CU resistant to antihistamine treatment (25).

In conclusion, we have provided evidence for a novel and direct role of MCs in CU. We stress that CU is a multifaceted disease with different still unknown factor(s) leading to MC activation and EC permeability, and we underline the need of an *in vitro* test, herein described, that allows to personalize the therapy. The combined degranulation and leakage assays proved to be more sensitive than ASST being positive, though to a different degree, in all patients with CU examined as opposed to 40–60% of ASST (34). Nevertheless, our data open new insights into the role of MCs in the onset and amplification of immune response in CU disorder.

Author's contributions

FB performed all the experiments on ECs. BF performed all the experiments on MC lines. FB, BF and OR performed the analysis of the results. MC, AT, PR and RA followed the patients and collected the serum samples. FB, BF, FT and CP contributed to the design of the research project and the writing of the manuscript. OR, MC, AT, PR and RA contributed to write and revise the manuscript.

Conflict of interest

FB, BF, OR, MC, AT, PR, RA, FT and CP declare no competing financial interest.

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