Original article

Novel pathogenic mechanism and therapeutic approaches to angioedema associated with C1 inhibitor deficiency

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Background: Activation of bradykinin-mediated B2 receptor has been shown to play an important role in the onset of angioedema associated with C1 inhibitor deficiency. This finding has led to the development of novel therapeutic drugs such as the B2 receptor antagonist icatibant. However, it is unclear whether other receptors expressed on endothelial cells contribute to the release of kinins and vascular leakage in these patients. The recognition of their role may have obvious therapeutic implications.

Objective: Our aim was to investigate the involvement of B1 and gC1q receptors in *in vitro* and *in vivo* models of vascular leakage induced by plasma samples obtained from patients with C1 inhibitor deficiency.

Methods: The vascular leakage was evaluated *in vitro* on endothelial cells by a transwell model system and *in vivo* on rat mesentery microvessels by intravital microscopy.

Results: We observed that the attack phase plasma from C1 inhibitor–deficient patients caused a delayed fluorescein-labeled albumin leakage as opposed to the rapid effect of bradykinin, whereas remission plasma elicited a modest effect compared with control plasma. The plasma permeabilizing effect was prevented by blocking the gC1q receptor–high-molecular-weight kininogen interaction, was partially inhibited by B2 receptor or B1 receptor antagonists, and was totally prevented by the mixture of the 2 antagonists. Involvement of B1 receptor was supported by the finding that albumin leakage caused by attack phase plasma was enhanced by IL-1β and was markedly reduced by brefeldin A.

Conclusion: Our data suggest that both B1 receptor and gC1q receptor are involved in the vascular leakage induced by hereditary and acquired angioedema plasma. (J Allergy Clin Immunol ■■■■;■■■:■■■-■■■.)

Key words: *Angioedema, C1 inhibitor, complement, endothelial cells, vascular leakage, B1R, B2R, gC1QR, bradykinin, leakage assay*

Angioedema associated with C1 inhibitor (C1-INH) deficiency is an inherited (hereditary angioedema, HAE) or acquired (acquired angioedema, AAE) clinical condition that affects approximately 1:50,000 subjects in the general population and is characterized by episodic, self-limiting, often recurrent local edema of the subcutaneous and submucosal tissues.[1,2] The inherited deficiency is caused by mutations in the C1-INH gene, whereas the acquired form is induced by autoantibodies to C1-INH and is associated with lymphoproliferative disorders, solid neoplasia, and autoimmune and infectious diseases.[3-5] The hallmark of the disease is an increased vascular permeability that leads to massive subcutaneous and/or submucosal edema and becomes life-threatening if it occurs in the larynx.

Bradykinin released from high-molecular weight-kininogen (HK) as a result of enzymatic cleavage by kallikrein deriving from prekallikren activated by membrane-bound prolylcarboxypeptidase or factor XIIa[6,7] is currently considered the main mediator of angioedema episodes.[8,9] HK circulates in plasma as a complex with factor XII (FXII) and prekallikren and binds to cytokeratin 1, the globular head of C1q receptor (gC1qR), and the urokinase plasminogen activator receptor on endothelial cells (ECs).[10-14]

Two distinct membrane receptors for bradykinin and the degradation product des-Arg^9-bradykinin have been identified.[15,16] B1 receptor (B1R) is induced in ECs by the proinflammatory cytokines IL-1β or TNF-α[17] and interacts with des-Arg^9-bradykinin and Lys-des-Arg^9-bradykinin[18] released from circulating bradykinin after cleavage by kininase I in plasma and carboxypeptidase M on cell membranes.[19] Although the role of B1R is still unclear, evidence is accumulating suggesting the involvement of B2 receptor (B2R) in the onset of C1-INH–related angioedema. Treatment of C1-INH–deficient mice with HOE 140, a selective antagonist of B2R, reduced the vascular leakage observed in these animals.[20] The B2R antagonist HOE 140/icatibant has also been evaluated in recently completed phase III clinical trials in patients with angioedema with satisfactory results, and regulatory approval has been obtained in Europe. However, although patients experienced symptom relief within 15 to 180 minutes after treatment, complete resolution was achieved only in several hours,[21] and a

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Abbreviations used

AAE:	Acquired angioedema
ADMEC:	Human adult dermal microvascular endothelial cell
APL:	Attack phase plasma
B1R:	Type 1 bradykinin receptor
B2R:	Type 2 bradykinin receptor
C1-INH:	C1 inhibitor
CPL:	Control plasma
EC:	Endothelial cell
FITC:	Fluorescein isothiocyanate
FXII:	Factor XII
gC1qR:	Receptor for the globular head of C1q
HAE:	Hereditary angioedema
HK:	High-molecular-weight kininogen
HUVEC:	Human umbilical vein endothelial cell
RPL:	Remission phase plasma

similar response to plasma-derived C1-INH and Dx88, which reduces bradykinin release, was obtained.[22-24] These results suggest that, in addition to B2R, B1R may be involved in the development and maintenance of angioedema in patients with HAE and AAE.

To test this hypothesis, we used *in vitro* and *in vivo* models of vascular permeability to analyze the effect of attack phase plasma (APL) and remission phase plasma (RPL) collected from patients with angioedema. The specific aims were to evaluate the contribution of B1R, B2R, and gC1qR to the onset of angioedema and to assess the efficacy of receptor antagonists and of an antibody to gC1qR to prevent plasma-induced vascular leakage.

METHODS

Patients

EDTA plasma samples were collected from 15 patients with HAE or AAE during the attack and remission phases of angioedema and from 15 blood donors as control plasma (CPL) and kept at –80°C. Four patients had HAE and 11 AAE caused by antibodies to C1-INH associated with lymphoplasmacytoid lymphoma in 1 patient, monoclonal gammopathy in 2, and no associated disease in all the other patients. APL samples were obtained during facial and/or abdominal angioedema, 5 to 10 hours from the beginning of symptoms. RPL samples were obtained at least 15 days apart from angioedema symptoms.

Reagents and antibodies

Fluorescein isothiocyanate (FITC)–conjugated BSA was purchased from Sigma-Aldrich (Milan, Italy). Lys-des-Arg^9-bradykinin and the B2R antagonist HOE 140 (DArg[Hyp^3, Thi^5, DTic^7, Oic^8]bradykinin) were provided by Dr W. Neugebauer (University of Sherbrooke, Quebec, Canada), and the B1R antagonists R715 (AcLys[DβNal^7, Ile^8]des-Arg^9-bradykinin) and R954 (AcOrn[Oic^2, (αMe)Phe^5, DβNal^7, Ile^8]des-Arg^9-bradykinin) were prepared by F.G., one of the authors. Goat IgG to IL-1β was purchased from Santa Cruz (Santa Cruz, Calif). Two anti-gC1qR mAbs (clones 74.5.2 and 60.11) recognizing distinct epitopes of the molecule were previously reported.[25]

Primary cells and cell line

Endothelial cells were isolated from human umbilical vein (HUVECs) by collagenase digestion.[26] ECs were also isolated from human adult dermal microvasculature (ADMECs) of normal skin biopsy obtained after written consent from patients undergoing reductive plastic surgery and seeded on fibronectin (20 μg/mL; Roche, Milan, Italy) following Kraling et al.[27] The rat basophilic leukemia cell line (RBL-2H3) was cultured and stimulated as previously described,[28] and the activity of β-hexosaminidase was measured as a degranulation marker.[29]

Assessment of vascular permeability

The *in vitro* assay was performed as previously described in detail.[30]

The *in vivo* experiments were performed in male Wistar Kyoto rats (250-270 g) under the guidelines of European (86/609/EEC) and Italian (D.L.116/92) laws and were approved by the Italian Ministry of University and Research and by the University Institutional Committee. The permeability of mesenteric vessels was analyzed by intravital microscopy as previously described.[30]

Statistical analysis

The results were expressed as means ± SDs of triplicate determinations of 3 different experiments. Data were compared by ANOVA test using the *post hoc* analysis for paired multiple comparisons with the Fisher protected least significant difference test.[31] Analysis was performed by using the SAS Institute's StatView 5.0 statistical package (Cary, NC). P values of <.05 were considered statistically significant.

RESULTS

Analysis of the permeabilizing effect of plasma samples from patients with angioedema

Initially we tested the permeabilizing effect of APL and RPL from 1 patient with HAE and 1 with AAE who had circulating antibodies to C1-INH and no associated disease. APL samples were collected within 7 hours from the onset of facial angioedema and RPL samples 15 days after the angioedema attack. CPL samples from 2 blood donors and bradykinin served as negative and positive controls, respectively. The effect of plasma was evaluated at different time intervals by using transwell inserts coated with HUVEC monolayers (Fig 1, *A*). As expected, bradykinin caused a rapid leakage of FITC-BSA within 5 minutes of incubation that slightly increased at 30 minutes. The value of RPL was essentially similar to that of CPL at 5 and 15 minutes and was slightly higher at 30 minutes. Conversely, the permeabilizing activity of APL samples was comparable to that of bradykinin, but the effect was delayed, reaching the maximal value after 30 minutes of incubation.

Because the angioedema episodes occur preferentially in the skin, we analyzed the effect of plasma from patients and controls on ADMECs. As shown in Fig 1, *A,* ADMECs were more responsive than HUVECs to the permeabilizing effect of APL and RPL from 2 patients, suggesting that plasma-dependent vascular leakage was not restricted to APL but was also seen with RPL, although the effect was less marked. These results were confirmed by using plasma samples from a total of 15 patients and 15 blood donors (Fig 1, *B*).

We also evaluated the *in vivo* effect of topical administration of either APL or RPL (200 μL) on the ileal mesentery of rats by intravital microscopy. Because the endothelium *in vivo* is activated on the luminal side, we ascertained that APL was still able to induce cell permeability if added to the abluminal side of endothelial cells (see this article's Fig E1 in the Online Repository at www.jacionline.org). We also excluded stimulation of rat peritoneal mast cells by APL ruling out their contribution to plasma-induced BSA leakage (see this article's Fig E2 in the Online Repository at www.jacionline.org). The amount of FITC-BSA extravasated into the perivascular areas in the *in vivo* model was monitored for 30 minutes after topical application of stimuli. The data presented in Fig 2, *A and B,* show that APL caused extravasation of FITC-BSA within 10 minutes, whereas RPL failed to induce vascular leakage. The leakage kinetics of plasma-induced

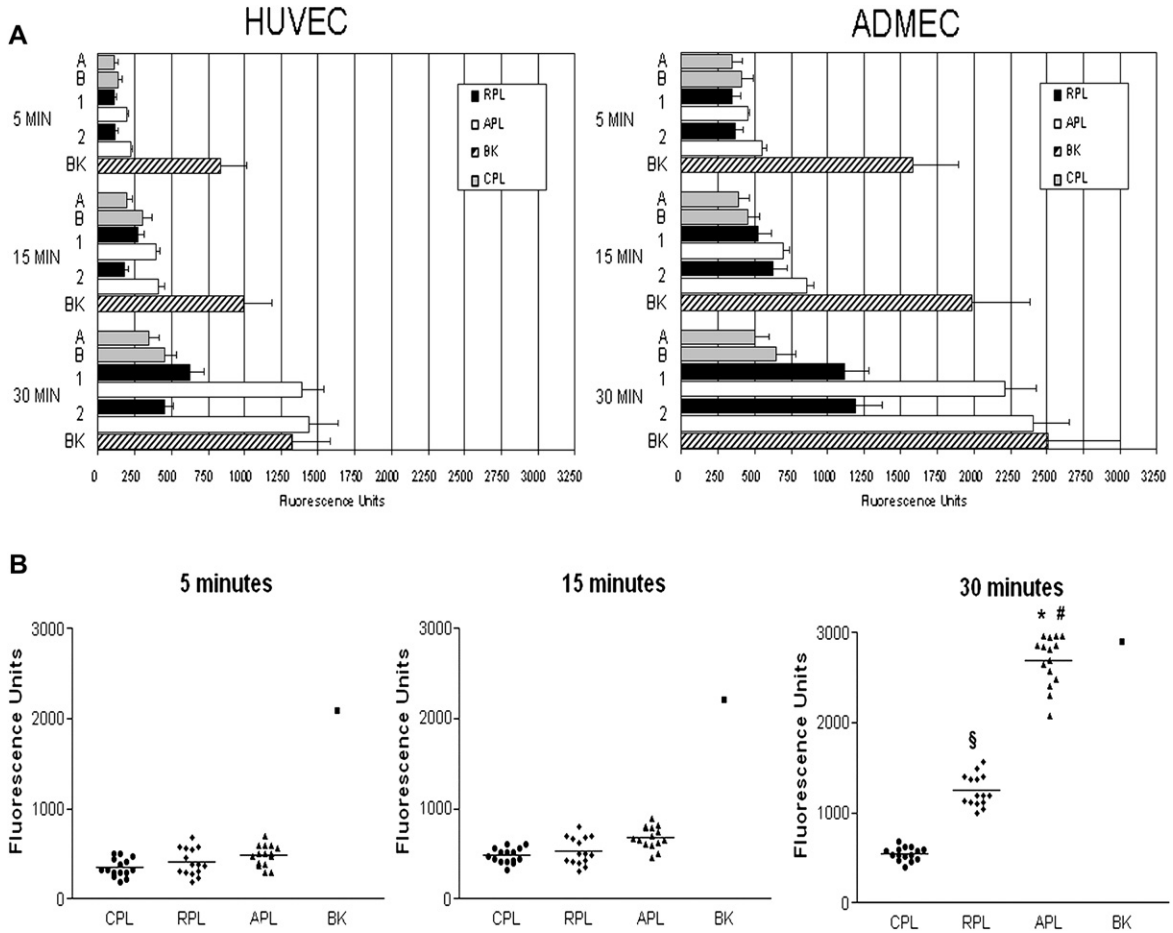

FIG 1. A, HUVEC permeability induced by APL *(white bars)* or RPL *(black bars)* from a patient with HAE *(1)* or AAE *(2)*, 2 blood donors *(A, B; gray bars)*, and bradykinin (*BK*; 10^{-6} M). **B,** ADMEC permeability induced by RPL or APL from 15 patients and by plasma samples from 15 blood donors. *Horizontal bars* indicate geometric means.*$P < .001$ vs CPL; #$P < .01$ vs RPL; §$P < .01$ vs CPL.

increased permeability *in vivo* was faster than that observed *in vitro* and paralleled the rapid effect of bradykinin seen within 2 minutes.

gC1qR and kinin receptors contribute to plasma-dependent BSA leakage

Because gC1qR expressed on ECs provides a binding site for the complex HK-prekallikren-FXII leading to the generation of bradykinin, we investigated its involvement in plasma-induced BSA leakage. ADMECs were incubated with mAb 60.11 or mAb 74.5.2 to gC1qR or medium alone for 15 minutes before exposure to APL from 3 patients for a further 30 minutes and evaluation of BSA leakage. As shown in Fig 3, *A*, mAb 74.5.2, which recognizes an epitope involved in HK-gC1qR interaction,[12] significantly inhibited the increased permeability induced by plasma, whereas mAb 60.11, which recognizes the putative binding site for C1q at the N-terminal domain of gC1qR,[25] was ineffective.

To analyze the mechanism of action of kinins released during the incubation of plasma with ADMECs, the cells were treated with different antagonists of kinin receptors for 10 minutes before addition of APL and measurement of FITC-BSA leakage. The plasma permeabilizing effect was partially inhibited by HOE 140, or the B1R antagonist R715, and was more strongly controlled by R954, another antagonist of B1R (Fig 3, *B*). The difference in the inhibitory activity of the 2 B1R antagonists is compatible with the higher affinity of R954 compared with R715. The leakage induced by APL was totally prevented by the mixture of R954 and HOE 140, suggesting the contribution of both receptors to the onset of angioedema symptoms (Fig 3, *B and C*).

Because B1R is not constitutively expressed on ECs, but is induced by proinflammatory cytokines,[32] we sought to investigate whether the plasma-dependent permeabilizing activity was enhanced by treating ADMECs with IL-1β. The selective agonist of B1R Lys-des-Arg^9-bradykinin failed to induce BSA leakage on untreated cells, but caused a prompt effect on IL-1β−treated cells within 5 minutes of the application (Fig 4, *A and B*). Treatment of ADMECs with IL-1β resulted in a marked increase in APL-induced albumin leakage compared with untreated ECs, although the effect of plasma, unlike that of Lys-des-Arg^9-bradykinin, was delayed, reaching the maximal value at 30 minutes (Fig 4, *A and B*). Interestingly, the permeabilizing effect of RPL on IL-1β–treated cells was similar to that observed with APL on untreated cells, whereas the activity of CPL on stimulated ADMECs remained unchanged (Fig 4, *B*).

To prove that plasma was acting through a newly expressed cell surface receptor, ADMECs were incubated with brefeldin A,

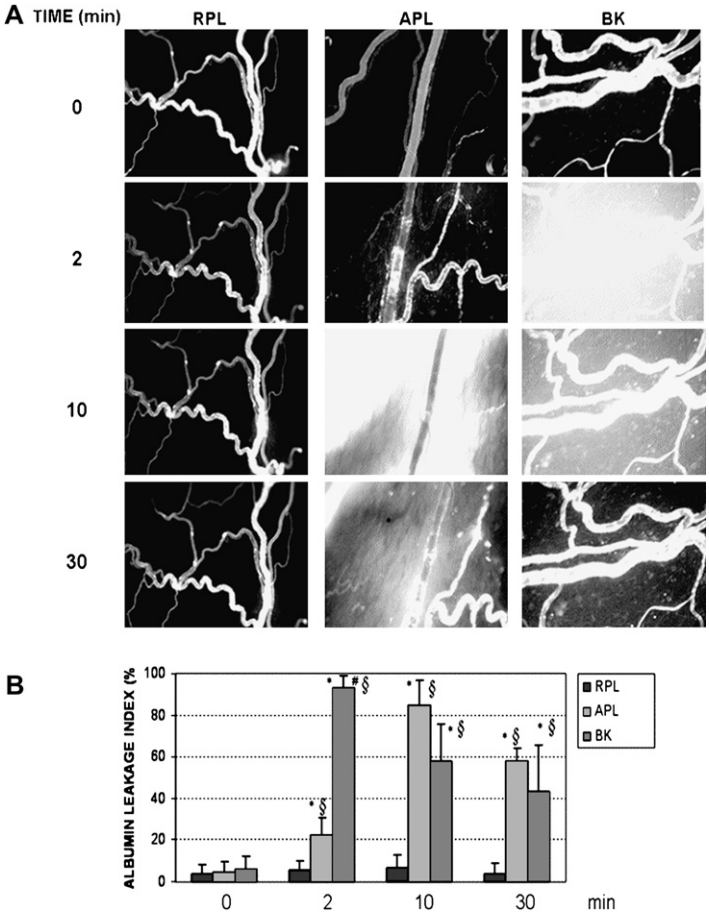

FIG 2. *In vivo* analysis of plasma-induced vascular leakage. Rat ileal mesentery was superfused with 200 μL APL, RPL, or bradykinin (*BK*; 10^{-7} M), and the extravasated FITC-BSA was monitored for 30 minutes. **A,** Images from 1 representative experiment. **B,** Means ± SDs of albumin leakage index. **P* < .01 vs time=0; §*P* < .01 vs RPL; #*P* < .01 vs APL.

a protein trafficking inhibitor, for 4 hours before exposure to APL. This treatment resulted in complete abrogation of the permeabilizing activity of plasma, indicating that the delayed effect of plasma is compatible with the slow appearance of B1R (Fig 4, *B*). Incubation of APL with solid phase-bound IgG anti–IL-1β led to a marked decrease in BSA leakage in half of the plasma samples tested, but was ineffective in the other half (see this article's Fig E3 in the Online Repository at www.jacionline.org), suggesting that besides IL-1β, other proinflammatory molecules, such as TNF-α or LPS,[17,33,34] may induce the expression of B1R.

The effect of B2R and B1R antagonists was also tested in an *in vivo* model. R954 was more effective than HOE 140 in inhibiting APL-induced FITC-BSA leakage, which was totally abrogated by the mixture of the 2 antagonists (Fig 5, *A*). R954 elicited an inhibiting effect not much different from that of HOE 140 at 10 minutes, but was definitely more active at 30 minutes (Fig 5, *B*).

DISCUSSION

Bradykinin acting through B2R expressed on ECs has been implicated in the development of angioedema associated with C1-INH deficiency, as suggested by the increased level of bradykinin found in plasma of C1-INH–deficient patients[4] and by the therapeutic efficacy of HOE 140 both in patients[21,35] and in knockout mice for C1-INH.[20] The current data show that B1R and gC1qR are also involved in the pathogenic mechanism leading to vascular leakage induced by plasma samples from C1-INH–deficient patients during the attack phase of angioedema.

The transwell system proved an invaluable tool to analyze plasma samples from patients with HAE and AAE for their ability to cause increased endothelial permeability. Unlike bradykinin, which exhibited a rapid permeabilizing effect, APL incubated with ECs caused a delayed leakage of FITC-BSA that was initially seen at 15 minutes and increased substantially at 30 minutes. This was not surprising because, despite the increased concentration of bradykinin reported in patients with both HAE and AAE,[36] this peptide is rapidly hydrolyzed in circulation, becoming biologically inactive.[37] This conclusion is further supported by the loss of bradykinin permeabilizing activity after 30 minutes of incubation with control plasma at 37°C (data not shown).

The delayed effect of APL is compatible with newly formed kinins released as a result of plasma interaction with ECs. The potential to form the permeabilizing factor is also present in the RPL provided that its effect is tested on ADMECs rather than on HUVECs. The increased sensitivity of ADMECs may be a result

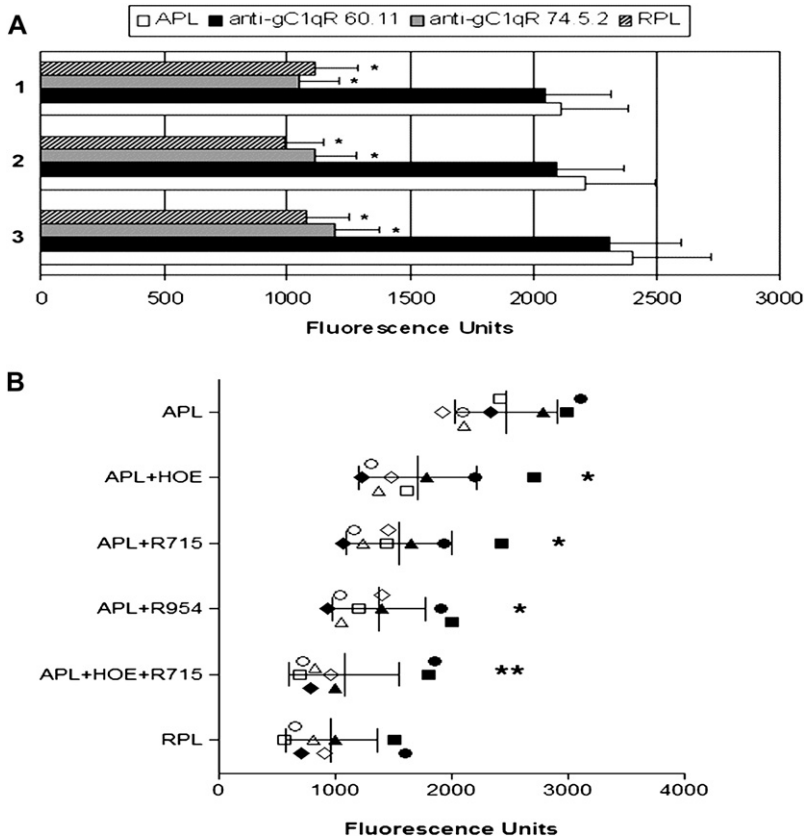

FIG 3. A, Permeability of ADMECs incubated with mAb 60.11 or 74.5.2 anti-gC1qR (20 μg/mL) for 10 minutes before addition of APL from 3 patients for further 30 minutes. $*P < .01$ vs APL. **B,** Effect of HOE 140 (10^{-5} M), R715 (10^{-5} M) or R954 (10^{-5} M) on ADMECs evaluated as in *A*. Patients with HAE, *open symbols*; patients with AAE, *solid symbols*. $*P < .05$; $**P < .01$ vs APL.

of a higher expression of receptors involved in the release and/or binding of kinins, but this issue remains to be elucidated.

The finding that APL induced increased permeability of rat mesenteric vessels suggests that the effect of plasma was not species-specific. To avoid excessive dilution of plasma samples infused intravenously and inactivation by circulating proteases that may substantially reduce their permeabilizing activity, we superfused the ileal mesentery to allow sufficient time for the plasma permeabilizing factor to exert its local effect without being washed away. *In vitro* experiments proved that plasma was still active if added to the lower chamber of the transwell, suggesting that it was also effective on the abluminal side of the EC monolayer. In addition, APL samples failed to induce *in vitro* degranulation of rat mast cells, ruling out their contribution in our *in vivo* model through the release of vasoactive factors acting on ECs.

We found that gC1qR plays a critical role in the release of kinin induced by the patients' plasma, providing a binding site for the HK-prekallikren-FXII complex followed by activation of prekallikren into kallikrein and cleavage of HK in bradykinin.[38] Because binding of HK to gC1qR is a primary step in the generation of kinins, it is not surprising that an antibody that blocks gC1qR-HK interaction completely prevented the BSA leakage induced by APL from patients with HAE or AAE. The effect of this antibody is specific because we failed to obtain similar inhibition with an antibody to another epitope involved in the binding of the globular head of C1q.[25]

The contact activation system can also be controlled at the level of kinin interaction with the receptors on ECs, as indicated by the decrease in APL-induced vascular permeability obtained in the presence of HOE 140, an antagonist of B2R. This is in line with the beneficial effect of HOE 140 in C1-INH–deficient mice and also with the symptom relief observed in patients treated with the B2R antagonist icatibant. However, it is important to note that the addition of the B2R antagonist in our *in vitro* and *in vivo* models caused only partial inhibition of BSA leakage and that a B1R antagonist was required for its complete prevention, suggesting that B1R was also implicated in this process.

The hypothesis that bradykinin acting through B2R may not be solely responsible for the APL-dependent increased vascular permeability is based not only on the partial response to HOE 140 and the inhibitory effect of the 2 antagonists of B1R, R715 and R954, but also on the slow response of angioedema episodes to drugs acting on bradykinin. Treatment of angioedema attacks with drugs that function as B2R antagonists (icatibant) or restore the control of bradykinin formation through the inhibition of kallikrein (Dx88 and plasma-derived C1-INH) results in immediate relief, but complete resolution of symptoms is rarely obtained in less than 12 hours.[21] On the basis of our results, we suggest that the permeabilizing effect of bradykinin acting on B2R in an angioedema attack caused by C1-INH deficiency is amplified by IL-1β or other proinflammatory cytokines that induce the expression of B1R.[32] This receptor responds to des-Arg[9]-bradykinin, the first catabolite of

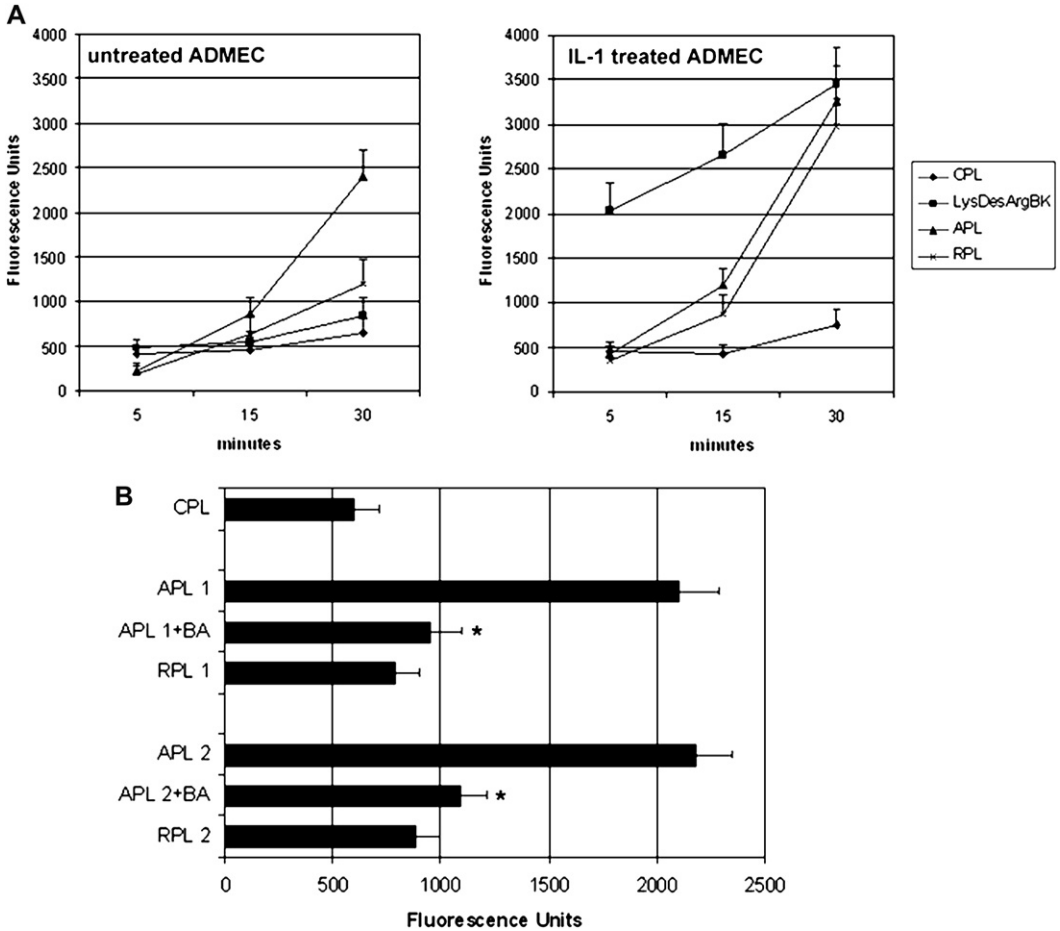

FIG 4. Modulation of B1R expression by IL-1β and brefeldin A *(BA)*. **A,** ADMEC monolayer, stimulated with IL-1β (0.1 ng/mL) for 60 minutes at 37°C, was exposed to 10% APL or RPL from 1 representative patient, or Lys-des-Arg[9]-bradykinin (*BK*; 10^{-6} M). **B,** ADMEC monolayer, treated for 1 hour with brefeldin A (0.5 μM) and then with APL (10%) from 2 representative patients for 30 minutes. $*P < .01$.

bradykinin, with a long half life (300 minutes compared with 30 seconds of bradykinin),[39] which may explain the late phase of angioedema attacks. Han et al,[20] by using C1-INH–deficient mice, reached the conclusion that angioedema is mediated by bradykinin via B2R after the observation that the vascular permeability was substantially reduced in knockout mice for C1-INH and B2R. It is important to emphasize that C1-INH knockout mice have an ongoing spontaneous increased vascular permeability that can be reversed, restoring normal C1-INH levels or antagonizing B2R. However, these mice do not develop angioedema attacks and therefore cannot be used to test the additional role of B1R.

The data presented in Fig 5, *B,* showed that the B2R antagonist HOE 140 exhibited a faster effect than the B1R antagonist R954, being more active in the inhibition of plasma-induced vascular permeability at 10 minutes and less effective at 30 minutes. This finding is consistent with the constitutive expression of B2R, whereas B1R is an inducible receptor, as suggested by the reduced BSA leakage caused by plasma on ADMECs treated with brefeldin A, which blocks transport of newly synthesized B1R to the plasma membrane. The factors in APL that induce the expression of B1R is unknown, but IL-1, shown by Sardi et al[34] to upregulate the contractile response of human umbilical veins to des-Arg[9]-bradykinin, is a possible candidate.

It is interesting to note that RPL elicited a permeabilizing effect similar to that of APL on IL-1–treated ADMECs, suggesting that this cytokine may potentially contribute to the onset of the attack phase through the induction of B1R. This possibility is also supported by the finding that removal of IL-1β from APL substantially reduced BSA leakage to levels obtained with RPL, although this was not observed in all the plasma samples examined, suggesting that other factors may also be involved. The role of IL-1β in sustaining angioedema attacks provides an explanation for the clinical observation that infections represent a well recognized condition triggering angioedema in patients with HAE.[1]

In conclusion, we propose that the interaction between the HK-prekallikren-FXII trimolecular complex and gC1qR leading to the formation of bradykinin and des-Arg[9]-bradykinin and the expression of B1R represent critical steps in the development of angioedema in patients with HAE and AAE (see this article's Fig E4 in the Online Repository at www.jacionline.org), and that the blockage of gC1qR or both B2R and B1R may provide novel therapeutic tools to control better the symptoms of the acute attack in patients with angioedema.

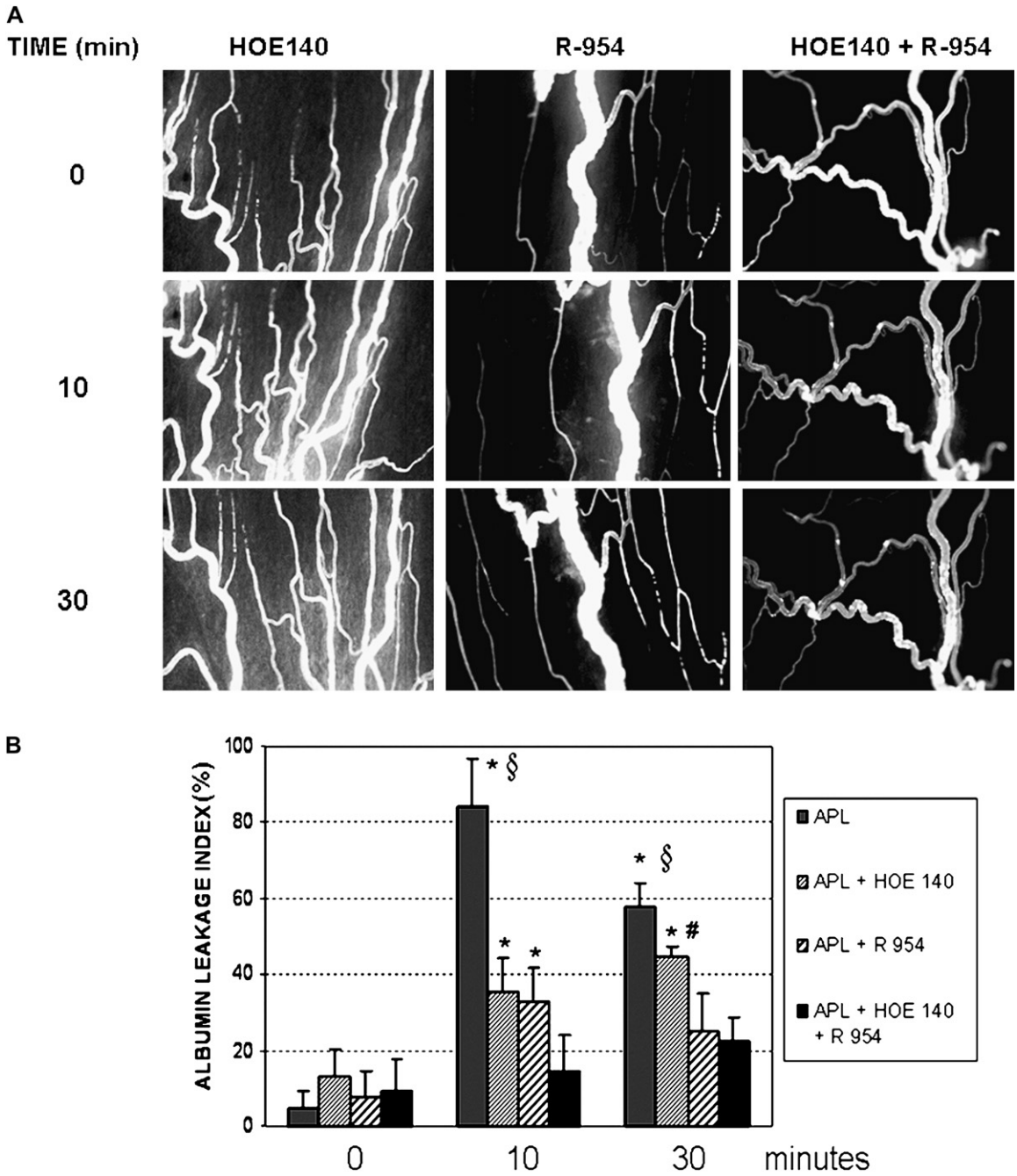

FIG 5. *In vivo* effect of kinin receptor antagonists. Rat ileal mesentery was superfused with HOE 140 (10^{-5} M), R954 (10^{-5} M), or both before the addition of APL (50 μL). **A,** Images from 1 representative experiment. **B,** Means ± SDs of albumin leakage. *$P < .01$ vs time=0; §$P < .01$ vs HOE 140 and/or R954; #$P < .05$ vs R954 and HOE 140 + R954.

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Clinical implications: On the basis of our findings, we propose B1R and gC1qR as novel therapeutic targets for angioedema associated with C1-INH deficiency.

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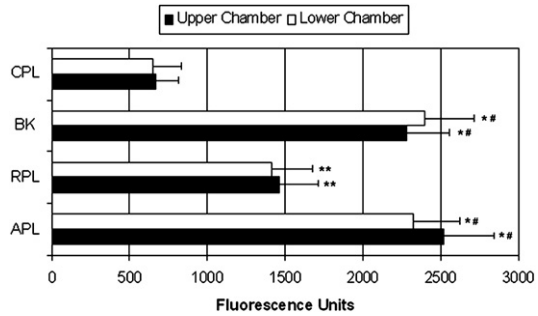

FIG E1. Endothelial leakageis induced by the stimulation on luminal or abluminal side of the monolayer. ADMEC monolayer was incubated with 10% CPL, APL, or RPL obtained from 1 representative patient, or bradykinin (*BK*; 10^{-6} mol/L) as a positive control into the lower chamber (abluminal side) or the upper chamber (luminal side) of the transwell system for 30 minutes at 37°C, and the FITC-BSA leakage was quantified by Infinite 200 (Tecan, Switzerland). Values are expressed as means ± SDs of duplicate determinations of 3 different experiments. $*P < .01$; $**P < .05$ vs CPL; $\#P < .05$ vs RPL.

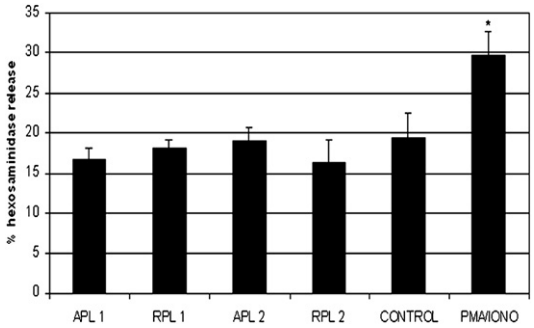

FIG E2. Permeabilizing factors promoting vascular leakage *in vivo* do not stimulate rat mast cells. RBL cells 5×10^5 were treated for 30 minutes with 2 μL plasma collected from 2 patients during the attack or remission phase diluted 1:2 in PBS, with 2 μL PBS as a negative control or phorbol 12-myristate 13-acetate and ionomycin *(PMA/IONO)*, respectively, at 10 ng/mL and 400 ng/mL final concentration, as a positive control. The percentage of β-hexosaminidase release, indicating the positive response of RBL, was calculated. Values are expressed as means ± SDs of duplicate determinations of 3 different experiments. $*P < .05$ vs control.

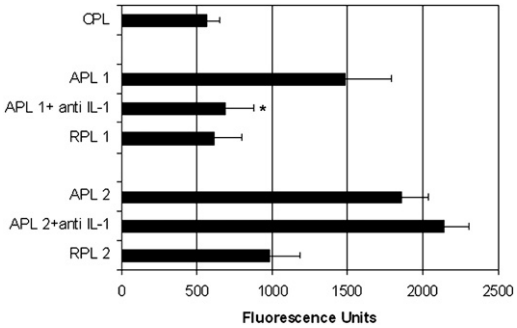

FIG E3. Removal of IL-1β may inhibit APL- induced leakage. 10% APL was incubated in a 96-well plate coated with goat polyclonal anti–IL-1β (1:30) for 45 minutes at room temperature before adding to the Traswell system (Corning, NY). The leakage was then monitored as previously described. Values are means ± SDs of duplicate determinations of 3 different experiments. $*P < .01$ vs APL.

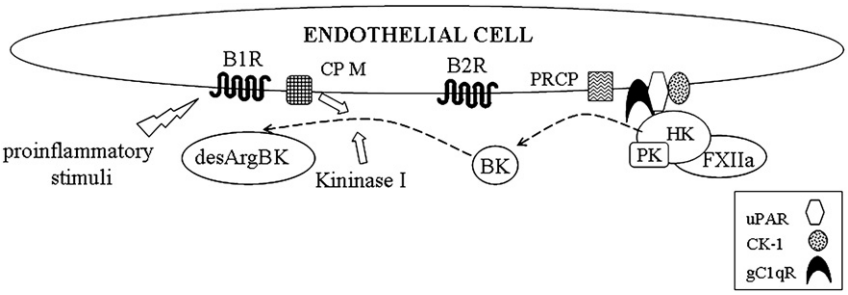

FIG E4. Model of kinin release during angioedema attack and contribution of B1R and gC1qR. *BK,* Bradykinin; CP M, carboxypeptidase M; PRCP, prolylcarboxypeptidase; uPAR, receptor for urokinase plasminogen activator; CK-1, cytokeratin 1.