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Mucosal Allergic Sensitization to Cockroach Allergens Is Dependent on Proteinase Activity and Proteinase-Activated Receptor-2 Activation

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We have shown that proteinase-activated receptor-2 (PAR2) activation in the airways leads to allergic sensitization to concomitantly inhaled Ags, thus implicating PAR2 in the pathogenesis of asthma. Many aeroallergens with proteinase activity activate PAR2. To study the role of PAR2 in allergic sensitization to aeroallergens, we developed a murine model of mucosal sensitization to cockroach proteins. We hypothesized that PAR2 activation in the airways by natural allergens with serine proteinase activity plays an important role in allergic sensitization. Cockroach extract (CE) was administered to BALB/c mice intranasally on five consecutive days (sensitization phase) and a week later for four more days (challenge phase). Airway hyperresponsiveness (AHR) and allergic airway inflammation were assessed after the last challenge. To study the role of PAR2, mice were exposed intranasally to a receptor-blocking anti-PAR2 Ab before each administration of CE during the sensitization phase. Mucosal exposure to CE induced eosinophilic airway inflammation, AHR, and cockroach-specific IgG1. Heat-inactivated or soybean trypsin inhibitor-treated CE failed to induce these effects, indicating that proteinase activity plays an important role. The use of an anti-PAR2 blocking Ab before the sensitization phase completely inhibited airway inflammation and also decreased AHR and the production of cockroach-specific IgG1. PAR2 activation by CE acts as an adjuvant for allergic sensitization even in the absence of functional TLR4. We conclude that CE induces PAR2-dependent allergic airway sensitization in a mouse model of allergic airway inflammation. PAR2 activation may be a general mechanism used by aeroallergens to induce allergic sensitization. The Journal of Immunology, 2011, 186: 3164–3172.

A number of environmental allergens from diverse sources have proteinase activity, which has been suggested to skew the immune response toward the Th2 phenotype (1, 2) and allergic sensitization. Such proteinase activity may mediate allergic sensitization to the proteinases themselves (3) or other Ags encountered by the mucosal immune system at the same time (4).

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Abbreviations used in this article: ABP, activity-based probe; AHR, airway hyperresponsiveness; AMMC, 7-amino-4-methylcoumarin; BAEE, N-benzoyl-L-arginine ethyl ester; BAL, bronchoalveolar lavage; Bio-PK-DPP4, biotin-linker-Pro-Lys-diphenyl phosphonate; CE, cockroach extract; 2-IL, 2-furoyl-LIGRLO-NH2; HCE, heat-inactivated cockroach extract; i.n., intranasally; KNRK, Kirsten murine sarcoma virus-transformed normal rat kidney cell line; KO, knockout; PAR2, proteinase-activated receptor-2; PBST, isotonic PBS with Tween 20; Penh, enhanced pause; QAR-AMC, butoxycarbonyl-Gln-Ala-Arg-AMC-HCl; SBTI, soybean trypsin inhibitor; WT, wild-type.

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A number of cockroach proteins are potent environmental aeroallergens. There is evidence that early life exposure (5) to cockroach allergens leads to allergic sensitization to cockroaches, which has been shown to have a strong correlation with the incidence of asthma (6) and asthma exacerbations (7, 8). Childhood sensitization to cockroach allergens also has been associated with an increased risk for persistent asthma and bronchial hyperresponsiveness and with a greater loss of lung function (9). In general, cockroach allergens are an important cause of asthma exacerbations in many parts of the world (10, 11).

Although most cockroach allergens are not serine proteinases, they are inhaled along with a variety of cockroach-derived proteins from the gastrointestinal tract and feces that contain serum proteinase activity. Interestingly, cockroach extract (CE) activates airway epithelial cells in a proteinase-activated receptor-2 (PAR2)-dependent fashion (12). German cockroach (Blattella germanica) extract induces PAR2-mediated activation of airway epithelial cells, which leads to the release of TNF (13) and IL-8 (14). However, it is not known whether specific cockroach allergens activate PAR2 or whether this activation is due to other cockroach-derived proteins inhaled at the same time as the allergens. At least one of the cockroach allergens, Per a 10, has trypsin-like serine proteinase activity (15) and therefore has the ability to activate PAR2.

PAR2 is a prime candidate to sense environmental exposure to serine proteinases and has been implicated in the development of inflammatory reactions (16–18). Previous reports have linked PAR2 to human asthma (19, 20). The PAR2 gene is located in Sq13, a genomic area that is linked to the development of allergy (21), and PAR2 expression is increased in the airways of asthmatic...
individuals (19). Moreover, PAR2−/− mice develop lower levels of airway hyperresponsiveness (AHR) and airway inflammation than normal mice (20). In addition, we have shown that PAR2 activation in the airways leads to allergic sensitization to concomitantly inhaled Ags (22) and also to increased allergen-mediated airway inflammation and AHR development in sensitized mice (23).

In our previous studies (22, 23), we have activated PAR2 with PAR2-activating peptides administered together with OVA and analyzed allergic sensitization or airway inflammation as a result of Ags without proteinase activity. We propose that environmental allergens with proteinase activity and PAR2 activation by such allergens function as an adjuvant and mediate allergic sensitization and inflammation. To test this hypothesis and study the role of PAR2 in sensitization to common allergens, we developed a murine model of mucosal sensitization using CE. Using our model, we provide evidence that supports the hypothesis that PAR2 activation by CE is involved in the development of allergic sensitization and inflammatory changes in murine airways and that this PAR2-dependent adjuvant activity of CE is present, although to a lesser extent, even in the absence of functional TLR4.

### Materials and Methods

#### Animals

Male BALB/c and C57BL/6NcrBir mice were purchased from Charles River Laboratories and were used when they were between 6 and 8 wk of age. Male C3-TLR4+/− mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and used at the same age. Male PAR2−/− mice on a C57BL/6 background were obtained from The Jackson Laboratory and bred at the animal facility at the University of Calgary. Wild-type and PAR2−/− C57BL/6 mice, for which breeding pairs were obtained as a gift through the courtesy of Dr. Patricia Andrade-Gorton (Johnson & Johnson Pharmaceutical Research & Development, Spring House, PA) (24), also were bred in-house and used interchangeably for comparison with the mice purchased from The Jackson Laboratory. All of the mice were housed in virus- and Ab-free conditions and maintained on a 12 h light/dark cycle. All of the experiments described were approved by the University of Alberta Health Sciences Laboratory Animal Ethics Committee (Edmonton, Alberta, Canada).

#### Cockroach extract

Lyophilized frozen whole-body German cockroach (Blattella germanica) extracts were obtained from Greer Laboratories (Lenoir, NC). Extracts were resuspended in 0.9% sterile saline solution at a concentration of 6 mg/ml of total protein. The trypsin-like activity of CE was determined with the fluorogenic peptide substrate butoxy carbonyl-Gln-Ala-Arg-7-amino-4-methylcoumarin (AMC)-HCl (QAR-AMC; Bachem, King of Prussia, PA). The activity of the cockroach powder was equivalent to 0.75–0.90 IU/mg using QAR-AMC as the substrate.

Inhibition of CE proteinase activity with soybean trypsin inhibitor

The ability of soybean trypsin inhibitor (SBTI) to inhibit the proteolytic activity in CE was monitored by measuring the hydrolysis of the substrate described previously (QAR-AMC) in the absence and presence of increasing concentrations of SBTI (Sigma-Aldrich Canada, Oakville, Ontario, Canada). The endotoxin content of CE was evaluated using a Limulus amoebocyte lysate assay kit (Sigma-Aldrich, St. Louis, MO) with a sensitivity of 0.05 endotoxin units per milliliter. CE at a concentration of 100 mg/ml tested positive in this test, indicating that it contains >0.05 endotoxin units per milliliter.

***Intranasal administration of whole CE and anti-PAR2 Ab***

After light anesthesia with ketamine (75 mg/kg) and acepromazine (2.5 mg/kg), mice were given intranasally (i.n.) 50 µg whole CE dissolved in 25 µl of 0.9% sterile saline according to the protocol shown in Fig. 1A. Control mice were given saline solution only (saline/saline group).

To investigate the role of PAR2 activation in the airways during allergic sensitization with CE, we administered i.n. a rabbit polyclonal receptor-blocking anti-PAR2 Ab, B5 (25 µl of a 1:500 dilution of antiserum) 30 min before i.n. administration of CE. B5 is known to have blocking activity in vivo (25). Control mice received the same amount of normal rabbit serum before CE administration. We also used the mouse anti-PAR2 mAb SAM-11 (Santa Cruz Biotechnology, Santa Cruz, CA). In this case, we administered i.n. 10 ng SAM-11 or an isotype control (mouse IgG2a) dissolved in 25 µl saline 30 min before CE administration.

#### Evaluation of AHR and allergic airway inflammation

Twenty-four hours after the final i.n. CE administration, we measured enhanced pause (Penh) to increasing doses of methacholine by noninvasive whole-body plethysmography (Buxco Electronics, Wilmington, NC) to determine AHR. Conscious mice were placed in whole-body plethysmographic chambers, and after 10 min of stabilization, dose–response curves to aerosolized methacholine (2–32 mg/ml) were generated.

Mice were euthanized 48 h after the last challenge. After blood collection by cardiac puncture, the trachea was exposed and intubated with a polyethylene catheter. Lungs were lavaged five times with 1 ml isotonic PBS (pH 7.4), and 5 ml of this bronchoalveolar lavage (BAL) fluid was collected. The BAL fluid was centrifuged at 300 × g for 5 min. Total cells were counted, and then cytospins of 5000 cells were prepared and stained with Diff-Quick (Fisher Scientific, Kalamazoo, MI). Airway inflammation was assessed by counting the number of inflammatory cells in the BAL fluid as described previously (26).

#### Detection of cockroach-specific IgG1

Cockroach-specific IgG1 in mouse serum was measured by ELISA. Briefly, CE (200 µg/ml in 0.1 M sodium carbonate buffer (pH 9.5)) was added onto 96-well Nunc MaxiSorp plates (Corning Life Sciences, Lowell, MA) and incubated overnight at 4 °C. Plates were washed three times with 0.05% Tween 20 (Fisher Scientific, Fair Lawn, NJ) in isotonic PBS (pH 7.4) (PBST), blocked with PBS/10% FBS (Life Technologies Invitrogen, Grand Island, NY), and incubated for 1 h at 24 °C. Plates were washed three times with PBST. Mouse serum diluted 1:200 with PBS was added and incubated for 3 h at 24 °C. Plates were washed three times, and a 1:5000 dilution of biotin-labeled rat anti-mouse IgG1 (BD Pharmingen, Mississauga, Ontario, Canada) in PBS/10% FBS was added. Plates were incubated for 1 h at 24 °C and then washed three times, followed by a 1:1000 dilution in PBS/10% FBS of HRP-conjugated streptavidin (Jackson ImmunoResearch Laboratories, Burlington, Ontario, Canada), and incubated for 30 min at 24 °C. After incubation, plates were washed three times with PBST. Tetramethylbenzidine (BD Pharmingen) was added as a substrate and allowed 20 min at 24 °C to develop. The reaction was stopped by the addition of 2 N H2SO4, and the absorbance was measured at 405 nm on a Power Wave XS (BioTek Instruments, Winooski, VT) ELISA reader. Results are shown as absorbance units.

#### Detection of total IgE

The total concentration of IgE in mouse sera was measured by sandwich ELISA using the Mouse IgE ELISA set (BD Biosciences, Franklin Lakes, NJ) according to the manufacturer’s instructions. The total concentration of IgE in sera was calculated from a recombinant mouse IgE standard curve and expressed as nanograms per milliliter.

#### Serine proteinase activity-based probe

To detect trypsin-like enzymes in the cockroach extracts, we employed a strategy described previously for the identification of serine proteinases using biotinylated diphenyl phosphonate probes (27). On the basis of the use of probes with proline-lysine or asparagine-lysine sequences in the P2 and P1 enzyme target positions (28), we elected to synthesize the biotinylated probe with the PK sequence as the tryptic target (so-called compound 4, biotin-linker-Pro-Lys-diphenyl phosphonate, Bio-PK-DPP4).

The synthesis of the biotinylated probe was achieved by an expedited solid-phase approach that we have detailed recently for chymotrypsin-like and elastase-like serine proteinases (29). The utility of the probe was verified by its high reactivity with trypsin.
Active serine proteinases in CE were labeled covalently with a biotin-tagged serine proteinase-targeted activity-based probe (ABP) (28), as done previously for murine colon extracts (30). In brief, CE (1.5 U/ml trypsin-like activity) was pretreated or not with 0.1 or 1.0 µM SBTI for 10 min at 24°C, followed by the addition of the ABP, Bio-PK-DPP4 (28). CE was reacted with 100 µM ABP (dissolved in DMSO) and incubated for 60 min at 24°C in 50 mM Tris-HCl (pH 7.4) supplemented with 0.2% (v/v) Nonidet P-40 and 1.5 mM CaCl₂ in a reaction volume of 20 µl. The reaction was terminated upon the addition of 20 µl of 2× electrophoresis sample buffer containing 2-ME, and the reaction products were heat-denatured for 3 min at 92°C. Proteins in the ABP-labeled CE samples were resolved by SDS-PAGE and transferred to polyvinylidene difluoride Hybond-P membranes (GE Healthcare, Piscataway, NJ). The membranes were blocked with 2% ECL Advanced blocking reagent (GE Healthcare) and 0.1% PBST (pH 7.4), washed in PBST, and reacted with VectaStain (Vector Laboratories, Burlington, Ontario, Canada) in PBST to detect ABP-biotinylated enzyme. The membranes were washed and treated with ECL Plus detection reagents (GE Healthcare), and ABP-biotinylated enzyme was detected by fluorescence emission using the Storm 860 imaging system (GE Healthcare). The blot signals visualized were quantified using ImageQuant software (GE Healthcare). The membranes were washed and treated with ECL Plus detection reagents (GE Healthcare, and ABP-biotinylated enzyme was detected by fluorescence emission using the Storm 860 imaging system (GE Healthcare). The blot signals visualized were quantified using ImageQuant software (GE Healthcare). A disappearance of the Western blot signal was taken as evidence for the neutralization of serine proteinase activity in CE by SBTI.

PAR₂-mediated calcium signaling triggered by CE

Calcium signaling via PAR₂ activation was monitored using a rat PAR₂-transfected Kirsten murine sarcoma virus-transformed normal rat kidney cell line (KNRK) cell line, as described previously (31, 32) using a fluo-3 dye calcium indicator, KNRK cells expressing rodent PAR₂ were grown to confluency in 75 cm² T flasks and split with trypsin-free dissociation medium. Disaggregated cells were pelleted by centrifugation and resuspended in 1 ml of DMEM/10% FCS for loading with the intracellular calcium indicator fluo-3-acetylmethyloxymethyl ester (Molecular Probes, Eugene, OR) at a final concentration of 22 µM (25 µg/ml). Indicator uptake was established over 20–25 min at 24°C in the presence of 0.25 mM sulfinpyrazone, after which time cells were washed twice by centrifugation and resuspended with the buffer described below to remove excess dye. Fluo-3–loaded cells then were resuspended to yield a stock suspension (~6 × 10⁶ cells/ml) in a buffer of the following composition: 150 mM NaCl, 3 mM KCl, 1.5 mM CaCl₂, 20 mM HEPES, 10 mM glucose, and 0.25 mM sulfinpyrazone. Fluorescence measurements, reflecting elevations of intracellular calcium levels in response to CE (pretreated or not with SBTI), trypsin, or PAR₂-activating peptide, were conducted at 24°C using a fluorescence spectrometer (PerkinElmer Life Sciences, Boston, MA), with an excitation wavelength of 480 nm and an emission recorded at 530 nm. Cells (~2 ml of 3 × 10⁶ cells per milliliter) were maintained in suspension in a stirred (magnetic bar) thermostatted cuvette (total volume, 4 ml), and CE, trypsin, or PAR₂-activating peptide stock solutions were added directly to the suspension to monitor agonist-induced changes in fluorescence. Because CE caused a slow PAR₂-independent elevation of intracellular calcium levels after a 3–5 min time period, the spike increase in fluorescence monitored during the first 2 min after agonist addition was taken as the magnitude of stimulation due to PAR₂ activation. CE (1.5 U/ml of trypsin-like activity) was treated or not with 1 or 10 µM SBTI for 10 min prior to its addition to the cells for measurements of PAR₂-triggered calcium signaling. Signals generated by CE in PAR₂-expressing KNRK cells were compared with the effects of CE on KNRK cells transfected with vector alone. Calcium signals generated by PAR₂ activation were expressed as a percentage of the calcium signal generated in the same cells by 2 µM of the calcium ionophore A23187. To verify that the calcium signals triggered by CE were due to PAR₂ activation, a cross-desensitization protocol was used (33). Cells first were desensitized with the PAR₂ agonist 2-furoyl-LIGRLO-NH₂ (2-fLI, two exposures of 10 μM), and the disappearance of the calcium signal generated by CE was monitored.

Statistical analysis

Values are expressed as mean ± SEM. Statistical differences in the mean values among treatment groups were determined using a one-way ANOVA.
and Tukey’s multiple comparison test. The paired Student t test was used to compare the mean between two groups. Differences in AHR were determined by F test analysis, which compares values over the entire curve between each treatment group. From this F score, a value for p was generated. In all of the cases, a p value < 0.05 was considered statistically significant.

Results
Intranasal administration of CE to BALB/c mice induces allergic sensitization

To study the role of proteinases in mucosal allergic sensitization, we developed a model for mucosal allergic sensitization using i.n. exposure to CE, which is known to have serine proteinase activity. Mice received i.n. 50 µg CE on five consecutive days (sensitization phase), rested for 5 d, and then received a daily i.n. challenge for four consecutive days with 50 µg CE (challenge phase) (Fig. 1A). Mice were assessed for AHR using whole-body plethysmography (Penh) 24 h after the last challenge and for allergic airway inflammation according to the analysis of the inflammatory cell content of the BAL fluid 48 h after the last challenge. Mice sensitized and challenged with CE developed AHR in response to methacholine (Fig. 1B) and showed increased total cell numbers (Fig. 1C) and total eosinophil numbers (Fig. 1D) in the airways compared with those of mice that received saline during the sensitization phase and were challenged with CE or those of mice that received saline during both the sensitization and the challenge phases. CE-treated mice also exhibited increased levels of cockroach-specific IgG1 and total IgE Abs (Fig. 1E, 1F, respectively). We then tested the dose-dependency of CE administered to the mice during the sensitization phase. In three separate groups of BALB/c mice, we administered 2, 10, or 50 µg CE daily during the sensitization phase and then challenged all of the animals with 50 µg CE daily for four more days as in the previous protocol. All three groups of mice showed similar levels of airway inflammation (total cells and eosinophils) in the BAL fluid (data not shown). This result strengthens our assumption that the first 5 d of CE administration function as a sensitization phase and not as an inducer of allergic inflammation, because the dose of Ag used during this phase did not affect the degree of inflammation.

CE induces allergic sensitization in the absence of TLR4

CEs that we used have measurable LPS activity (data not shown), and it is known that low amounts of LPS can mediate allergic sensitization to inhaled Ags through TLR4 activation (34). Furthermore, recent work illustrates cooperation between TLR4 and PAR2 in terms of signaling via NF-κB (35). To assess a possible role for TLR4 in terms of the action of LPS present in the extract or via cooperativity with PAR2, we used TLR4 knockout (KO) mice and BALB/cJ control mice with the same genetic background.

TLR4 KO mice showed increased numbers of total cells and increased accumulation of eosinophils in the BAL fluid after mucosal sensitization and challenge with CE (Fig. 2A, 2B, respectively) compared with those of mice that received only saline. Total cell and eosinophil accumulation in TLR4 KO mice was significantly lower than accumulation seen in wild-type (WT) mice. TLR4 KO mice also developed cockroach-specific IgG1 Abs after mucosal sensitization and challenge with CE (Fig. 2C). The difference in levels between KO and WT mice was also statistically significant. These results indicate that CE has adjuvant activity distinct from that of LPS, which participates in the development of allergic sensitization and allergic airway inflammation, although the presence of TLR4 is required for the full effect of mucosal sensitization to CE.

Allergic sensitization to CE is proteinase-dependent

It is known that the proteinase activity of Ags participates in the development of mucosal allergic sensitization (4). Thus, we examined whether mucosal allergic sensitization to CE is dependent on proteinase activity. To this end, we neutralized CE proteinase activity using two different approaches: 1) heat inactivation and 2) treatment with SBTI.

We first heated CE for 30 min at 65°C. This procedure decreased trypsin-like proteinase sp. act. by >90% (from 29 to 1.8 BAEE units per milligram trypsin-like activity). Mice that were sensitized and challenged with heated CE (HCE; 50 µg) showed significantly lower numbers of total infiltrating cells in the BAL fluid (Fig. 3A) with dramatically lower eosinophilic infiltration (Fig. 3B) compared with those of mice sensitized and challenged with CE. Cockroach-specific IgG1 in animals sensitized and challenged with heat by HCE showed a trend toward lower levels compared with those of mice sensitized and challenged with CE (Fig. 3C).

To verify that these differences were the result of inhibiting the trypsin-like proteinase activity of CE, we repeated the experiment while supplementing the trypsin-like activity in the HCE with trypsin. We added 0.2 µg trypsin to 50 µg HCE to reconstitute the serine protease activity to the same level as that contained in 50
μg CE that was not heat-inactivated. This supplemented HCE induced the same level of eosinophilia in the airways as the native noninactivated CE (Fig. 3D).

For our second approach, the trypsin-like activity in CE was inhibited efficiently by SBTI, because low concentrations of SBTI (0.1–1 μM) reduced enzyme activity by >95%, indicating a $K_i$ well below 0.1 μM for inhibition of the cleavage of the substrate, QAR-AMC (Fig. 4A). In parallel, using the activity-based serine proteinase probe reagent Bio-PK-DPP4, we were able to identify three biotin-labeled enzymes with serine proteinase activity in the molecular mass range between 32 and 36 kDa in CE (Fig. 4B); the labeling of all three of these enzymes was inhibited by SBTI (0.1–1 μM; Fig. 4B). To neutralize CE to be administered in our in vivo studies, we used 200 μM SBTI, two orders of magnitude greater than the $K_i$ for enzyme inhibition. CE was treated or not with 200 μM SBTI for 30 min at 37˚C prior to i.n. administration to mice.

In keeping with the results obtained with HCE, mice sensitized and challenged with SBTI-neutralized CE showed a marked decrease (50%) in the total infiltrating cell counts (Fig. 5A) and an even greater (70%) decrease in eosinophils (Fig. 5B) compared with those of mice sensitized and challenged with CE. There was, however, no difference in the IgG1 levels between animals treated with either CE or SBTI-neutralized CE (Fig. 5C). SBTI admin-
istered i.n. without Ag had no effect on the development of inflammation or the presence of cockroach-specific IgG1 (Fig. 5A–C). These results indicated that the presence of heat-sensitive and SBTI-sensitive serine proteinase activity in CE plays an important role in the development of allergic airway inflammation, particularly in terms of eosinophil infiltration.

**CE activates calcium signaling via PAR2, an effect blocked by SBTI**

Given the trypsin-like activity in CE and the ability of other CEs to activate PAR2 (12), we hypothesized that the CE that we were using would activate PAR2. We therefore tested the ability of enzymes contained in CE to signal (calcium transients) via PAR2 and to be neutralized by SBTI. In the absence of SBTI, CE caused a robust increase in calcium signaling in the PAR2 KNKR cells (Fig. 6A and left tracing in Fig. 6B). Increasing amounts of SBTI blocked the calcium signal (Fig. 6A, 6B). This calcium signal was absent in receptor-expressing cells that had been PAR2-desensitized initially by exposure to an excess of the PAR2 agonist 2-fLI (Fig. 6C, compare middle and right tracings with left tracing).

**Decreased effect of CE in PAR2−/− mice**

To study whether PAR2 activation plays a role in allergic sensitization after i.n. exposure to CE, we repeated the previous experiments using PAR2−/− mice and compared mucosal sensitization of PAR2−/− mice to sensitization of WT mice. The genetic background of PAR2−/− mice was C57BL/6, so the WT mice used for these experiments were also C57BL/6. PAR2−/− mice showed lower Penh after sensitization and challenge with CE compared with that of WT animals (Fig. 7A). PAR2−/− mice sensitized and challenged with CE showed increased total cell numbers (Fig. 7B) and total eosinophils (Fig. 7C) in the BAL fluid compared with those of mice sensitized and challenged with saline. However, the increase in total cells and in eosinophilic inflammation seen in PAR2−/− mice was lower than those seen in similarly challenged WT mice (Fig. 7B, 7C). CE sensitized and challenged PAR2−/− mice also generated cockroach-specific IgG1 (Fig. 7D), but the levels of the Abs were lower than those in WT mice. In conclusion, PAR2 is required for efficient mucosal sensitization to CE, although a lower degree of sensitization can develop in the absence of PAR2.

**Allergic sensitization to CE is PAR2-dependent**

We have shown that CE activates PAR2, as also has been shown previously (12, 13). We also have shown that PAR2 activation at the time of an encounter with an Ag leads to mucosal sensitization instead of immune tolerance (22). We sought to determine whether PAR2 activation is the mechanism used by CE to induce allergic sensitization and allergic airway inflammation. To block the ability of serine proteinases in CE to activate PAR2, we used a polyclonal receptor-blocking anti-PAR2 Ab (B5) and a mAb (SAM11). They both bind PAR2 at the serine protease cleavage site and have been shown to inhibit the ability of serine proteinases to activate PAR2 (25).

Thus, we administered i.n. the B5 anti-PAR2 Ab (25 μl at a 1:500 dilution of antiserum) or normal rabbit serum (25 μl at a 1:500 dilution) 30 min before administering CE to mice daily for the 5 d of sensitization. Mouse groups were challenged as described for previous experiments. Mice sensitized i.n. in the presence of rabbit serum developed AHR in response to methacholine challenge comparable to that of mice given only CE (data not shown). Mice sensitized to CE after pretreatment with B5 showed lower AHR than that of mice sensitized to CE in the presence of rabbit serum (Fig. 8A). The anti-PAR2 Ab reduced the total cell accumulation in the airways (Fig. 8D) and completely inhibited the accumulation of eosinophils (Fig. 8C). Our results also showed a significant decrease of IgG1 levels in the sera of animals receiving the anti-PAR2 Ab compared with those in the mice receiving normal rabbit serum (Fig. 8D, p < 0.001).

**FIGURE 6.** CE activates calcium signaling via PAR2 in KNKR PAR2 cells. KNKR cells expressing rodent PAR2 were loaded with the intracellular calcium indicator fluo-3 (22 μM), and fluorescence measurements reflecting elevations of intracellular calcium levels in response to CE pretreated or not with SBTI, trypsin, or PAR2-activating peptide were conducted using a fluorescence spectrometer. A and B, Increasing amounts of SBTI blocked the initial calcium signal (A and middle and right tracings in B). C, CE-induced calcium signaling in previously untreated PAR2 KNKR cells (C, left tracing) was absent in cells that had been PAR2-desensitized initially by exposure to an excess of the PAR2 agonist 2-fLI (C, middle and right tracings).
suggesting that allergic sensitization to CE is dependent or modulated by PAR2 activation. A similar polyclonal Ab from the serum of a different rabbit (A5) and a mAb (SAM-11) showed similar results (data not shown). Altogether, these data indicate that cockroach allergens use the PAR2 receptor system to mediate allergic sensitization.

**Discussion**

We have shown that i.n. administration of whole CE leads to the development of allergic sensitization in mice. CE is able to induce mucosal allergic sensitization even in the absence of functional TLR4, indicating that LPS, known to function as an adjuvant for mucosal allergic sensitization (36), is not required for sensitization to cockroach allergens. Furthermore, we have shown that allergic sensitization to CE and the resultant allergic airway inflammation depend on the ability of the extract to activate PAR2.

Cockroach frass already has been used for mucosal sensitization (37, 38), although in those studies frass was administered intratracheally whereas we administered i.n. cockroach allergens. The results from such studies are similar to ours except that, unlike our study, the authors of those studies were unable to sensitize C57BL/6 mice using cockroach frass. Although the two models have many other differences, it is possible that different proteinases or different amounts of allergens contained in the two preparations may explain this difference. A comparative study between cockroach frass and whole-body CE may allow us to identify proteinases that are important for allergic sensitization.

The role of proteinase activity in the development of allergic sensitization has been shown for cockroach frass (37), house dust mite (39), pollen, and fungal proteinases (4, 40), but the exact mechanisms of this effect were not studied. Our data indicate that PAR2 activation by cockroach allergens may be the mechanism. A recent paper also has proposed that PAR2 activation is important for sensitization to cockroach allergens after intratracheal administration (38). We propose that natural aeroallergens with serine proteinase activity induce PAR2 activation, which functions...
as an adjuvant and alters the immune response from immune tolerance to allergic sensitization. The adjuvant activity that leads to allergic sensitization is not known, although we have shown previously that PAR2 activation in the airways may act as a mucosal adjuvant through the release of TNF (22). We (41) and others (42) also have shown that PAR2 activation of the epithelium leads to the release of GM-CSF, which has the potential to activate dendritic cells. PAR2 activation also induces CCL20 release (43), which through activation of CCR6 can attract immature dendritic cells to mucosal surfaces (44). Each one of these pathways may be important for the development of allergic sensitization to cockroach allergens through PAR2 activation.

One issue that has impeded the elucidation of the role for PAR2 in vivo is the lack of potent and specific small molecule inhibitors of PAR2 activation. Unfortunately, we found that the PAR2 inhibitor ENMD-1068 (45) exhibits trypsin-inhibitory activity (data not shown), and this compound was therefore not of use for our study of trypsin-like PAR2-activating activity in CE. Thus, to assess a role for PAR2 activation, we used three different PAR2-targeted Abs, two separately raised polyclonal Abs (A5 and B5) and a mAb (SAM11), to inhibit PAR2 activation as has been done both in vitro (46) and also in vivo (45) in a model of arthritis. In our experiments, we administered i.n. the PAR2 Abs during the sensitization phase. The Ab administered this way was restricted to the nasal cavity, and inhibition of PAR2 activation should take place in that area. A small percentage of the Ab might have reached the lower airways and inhibited PAR2 activation in that area. However, because we did not use systemic administration of the Ab, we suggest that the effects were due to local action within the nasal cavity or the lower airways. We postulate that PAR2 expressed on nasal and airway epithelial cells could have been the main targets of the Ab and that it is likely that factors released from these epithelial cells are the source of the “adjuvant activity” induced by PAR2 activation. However, direct activation of nasal or airway mucosal dendritic cells (47), activation of alveolar macrophages (48), or even activation of mast cells (49) also may be part of the mechanism.

Both heat-inactivating CE and inhbiting serine proteinases with SBTI significantly decreased allergic sensitization but did not completely abolish it. Furthermore, supplementing the HCE with trypsin fully restored the allergenic activity of the extract. However, because neither of the two approaches were able to completely inhibit the serine proteinase activity in the extract, it is possible that the remaining activity was enough to induce the small effect seen. Hence, incomplete inhibition of the extract may explain our results, because significantly lower amounts of CE (2 μg) than those used in this study (50 μg) are sufficient to induce sensitization.

It is possible that other mechanisms in addition to PAR2 activation are responsible for the allergenic effect. For example, low LPS activity of the extract may be enough to induce allergic sensitization, although not to the levels seen when sensitization occurs in the presence of PAR2 activation. It is interesting that CE can induce eosinophilic airway inflammation and cockroach-specific IgE Abs in TLR4 KO mice, although at significantly diminished levels as compared with those of normal mice. It is known that i.n. administration of innocuous Ags normally leads to immune tolerance (50) and that LPS can act as an adjuvant and mediate TLR4-dependent allergic sensitization under the same conditions (34). Our previous work suggests that PAR2 activation in the airways may have adjuvant effects (22) similar to those seen with LPS. A recent article has shown that PAR2 activation leads to TLR4 transactivation and TLR4− and MyD88-dependent NF-kB activation (35). Also, PAR2 expression remained elevated with simultaneous exposure to LPS and PAR2 agonists in airway epithelial cells, demonstrating that there is coordinated action of two important mediators of inflammation, PAR2 and TLR4, which contributes to the enhancement of inflammatory signaling in airways (51). Our data indicate that PAR2 activation can act as an adjuvant for immunization without the need for TLR4 signaling.

If PAR2 is important for allergic sensitization to natural aeroallergens, it would be expected that changes in its expression or polymorphisms of the receptor could contribute to the development of allergy and/or asthma. There is evidence that 5q13, the genomic area where F2RL1, the gene that encodes PAR2, is located, may be linked to the development of allergy (21). Further work in this area will shed light on the role of PAR2 in human allergic diseases.

Although the presence of PAR2-activating proteinases in CE has been described (13), we have shown for the first time, to our knowledge, that several enzymes that may be able to activate PAR2 are present in CE. Whether the ABP-labeled constituents represent different molecular forms of the same enzyme (e.g., glycosylated forms or catalytically active proteolytic fragments) or different enzymes remains to be determined. Any of the three enzymes may be able to induce the allergic response, because all three are SBTI-neutralized, as is the allergenic response, and may be capable of activating PAR2 (data not shown). The identity of the three enzymes labeled by the ABP probe remains a topic for future work. Many different cockroach allergens have been isolated, and at least one of them, Per a 10, from the American cockroach is known to have trypsin-like activity (15). It is possible that one of the three proteins that we have identified is Per a 10, and at least one of them, from the American cockroach, is important for the development of allergic sensitization to cockroach allergens through PAR2 activation.

In conclusion, we provide evidence indicating that the adjuvant activity of CE, which allows it to induce mucosal allergic sensitization, is at least in part, a direct effect of PAR2 activation. Other allergens may share common features regarding PAR2 activation, which can account for their allergenic potential.

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Disclosures

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