Allergic potency of recombinant Fel d 1 is reduced by low concentrations of chlorine bleach

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Background: Sodium hypochlorite (NaOCl), the primary component of household bleach, has been shown to alter the purified mouse allergen Mus m 1, such that antibody recognition, or immunogenicity, is lost. Results of initial experiments suggest that antibody recognition is lost at lower concentrations of NaOCl than those required to fragment Mus m 1.
Objective: We sought to determine whether NaOCl had similar effects on recombinant (r)Fel d 1 and whether the loss of antibody recognition correlated with the loss of biologic activity, as measured with a basophil histamine release assay.
Methods: Recombinant Fel d 1 was treated with increasing amounts of NaOCl, and the product of the reaction was analyzed by using SDS-PAGE, Western blotting, and ELISA. The biologic activity of NaOCl-treated rFel d 1 was analyzed with a basophil histamine release assay.
Results: The protein fragmented at an NaOCl/rFel d 1 molar ratio of 7000, whereas cat-specific IgG recognition was lost at a lower molar ratio of 560. Basophil histamine release assays were performed to determine the effect of NaOCl on the biologic activity of rFel d 1. An NaOCl/protein molar ratio of 70 caused a significant reduction in histamine release from basophils of subjects with cat allergy. A molar ratio of 140 further inhibited histamine release by rFel d 1, suggesting a dose-response relationship between NaOCl and loss of biologic activity.
Conclusions: NaOCl modifies rFel d 1, resulting in loss of immunogenicity and attenuation of biologic activity, as measured by its ability to stimulate basophil histamine release. (J Allergy Clin Immunol 2003;111:396-401.)

Key words: *Fel d 1, sodium hypochlorite, basophil histamine release*

Abbreviation used
NaOCl: Sodium hypochlorite

Although there are effective methods of reducing exposure to dust mite allergen,[1] there have been few effective methods for eradication of animal and cockroach allergens. Removal of fur-bearing pets and cockroach extermination result in decreases in the respective allergens, but this decrease occurs several months after the intervention. After removal of a cat from the home, it might take 6 months or longer for cat allergen levels to decrease to levels found in homes without cats.[2] After cockroach extermination, it takes 6 months for cockroach allergen levels to decrease by 70% to 90%, thus leaving substantial levels of residual allergen. Standard housecleaning was thus only partially effective in removing residual cockroach allergen.[3,4]

A promising reagent for indoor allergen removal is sodium hypochlorite (NaOCl), the active ingredient in household bleach. NaOCl alters proteins through chlorination of amide nitrogens, followed by oxidation to imines and hydrolysis of the imine.[5] NaOCl has been shown to render HIV noninfectious and to eliminate the antigenicity of hepatitis B surface antigen.[6,7] We have previously demonstrated that allergens can be fragmented by low concentrations of NaOCl and that at even lower concentrations of NaOCl, allergens lose their immunogenicity.[8] Initial studies focused on the effects of NaOCl on purified Mus m 1. NaOCl also reduced the immunogenicity of other allergens, such as Bla g 1 and Fel d 1, but these experiments were conducted with protein extracts rather than purified or recombinant allergens. By using purified or recombinant allergens, the effects of NaOCl on any one particular allergen can be quantified more accurately because there is no extraneous protein present in the reagent that would modify the effect of NaOCl on the allergen. Moreover, the effect of NaOCl on the biologic activity of allergens has yet to be studied. We thus examined the effect of NaOCl on the immunogenicity of recombinant (r)Fel d 1 and characterized its effects on the biologic activity of rFel d 1 with a leukocyte histamine release assay.

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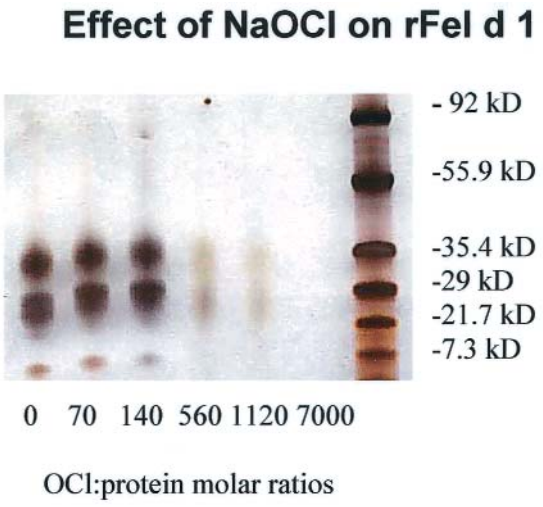

FIG 1. SDS-PAGE and silver staining of NaOCl-treated rFel d 1. NaOCl is expressed as NaOCl/rFel d 1 molar ratios. Recombinant Fel d 1 begins to disappear at an NaOCl/rFel d 1 molar ratio of 560, and no protein is detected at a molar ratio of 7000. All 3 bands dissipate at the same concentration of NaOCl.

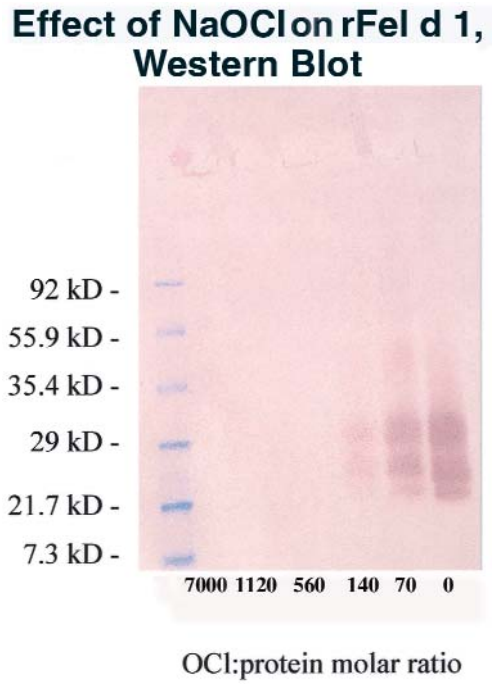

FIG 2. Western blot of NaOCl-treated rFel d 1. The detection antibody is monoclonal anti-Fel d 1 IgG. NaOCl is quantified by using NaOCl/rFel d 1 molar ratios. A distinct loss of antibody binding occurs at an NaOCl/rFel d 1 molar ratio of 140. No antibody binding is detected at a molar ratio of 560.

METHODS

Materials

NaOCl (5.25%) was obtained commercially and was standardized for free available chlorine by means of sodium thiosulfate titration.[9] Recombinant Fel d 1 (Indoor Biotechnologies) was used in all experiments, and a molecular weight of 36 kd was used to calculate NaOCl/rFel d 1 molar ratios. Blood samples were obtained from volunteers with cat allergy for basophil histamine release assays.

SDS-PAGE and Western blotting

SDS-PAGE and Western blotting was conducted according to the method of Laemmli.[10] Four percent to 12% precast tris-bis gels (Invitrogen) were used for protein electrophoresis. Samples were analyzed under reducing conditions by boiling them in gel-loading buffer containing β-mercaptoethanol for 4 minutes, electrophoresing at 200 V for 30 minutes, and visualizing with silver stain (Biorad). Gels for Western blots were electrophoretically transferred to a 0.22-μm NC membrane at 30 V for 1 hour. Membranes were blocked with BSA overnight at 4°C. Membranes were washed for 5 minutes at 25°C and then probed with 1:1000 biotinylated anti-Fel d 1 (3E4). Membranes probed with biotinylated anti-Fel d 1 were developed with 4-chloro-1-naphthol (Opti-4CN, Biorad).

ELISA

Fel d 1 was quantified by using the method of Chapman et al.[11] Polystyrene microtiter plates (Dynex Technologies Inc) were coated with monoclonal anti-Fel d 1 IgG (kindly provided by Dr Martin Chapman) at 4°C overnight. The plates were washed twice and then blocked with BSA for 1 hour at 25°C. The test samples, along with a standard curve and internal standard, were added to the plate for 1 hour at 25°C. The plates were washed twice, and then biotinylated monoclonal anti-Fel d 1 IgG (3E4) was added to the wells and incubated for 1 hour at 25°C. The plate was developed with streptavidin peroxidase and read at 405 to 495 nm. The detection limit of the assay in our laboratory is 0.07 ng/mL.

Basophil histamine release

Blood was obtained from volunteers with cat allergy after consent was obtained. The blood-drawing protocol was approved by the Johns Hopkins Joint Commission on Clinical Investigation. Basophil histamine release assays were performed according to a previously published method.[12] Forty to 60 mL of venous blood was obtained in a heparinized syringe and allowed to sediment over dextrose-EDTA-dextran for 60 to 90 minutes at room temperature. The leukocyte supernatant was used as the source of basophils. Fifty microliters of cells were added to untreated rFel d 1 or rFel d 1 treated with an NaOCl/rFel d 1 molar ratio of 70 or 140 and incubated at 37°C for 45 minutes. The samples were centrifuged for 2 minutes at 1000*g*, and the supernatant was analyzed for histamine content with an autoanalyzer. Background histamine release was determined in control samples that contained cells and buffer only. Total cellular histamine level was determined by lysing the cells with 8% $HClO_4$. Controls with NaOCl alone were performed to ensure that the NaOCl would not interfere with the measurement of histamine. Results are net histamine release compared with total histamine content.

RESULTS

Effect of NaOCl on protein content of rFel d 1

Recombinant Fel d 1 was incubated with increasing concentrations of NaOCl and analyzed by using SDS-PAGE and silver staining. Untreated recombinant Fel d 1 appears as 3 bands after silver staining: a 33-kd band likely representing the intact homodimer, a diffuse 22- to 28-kd band

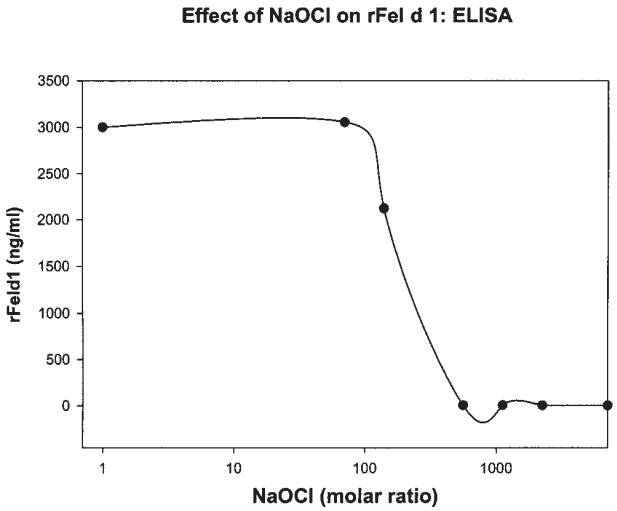

FIG 3. ELISA of NaOCl-treated rFel d 1. Recombinant Fel d 1 is expressed in nanograms per milliliter. NaOCl is expressed in terms of the NaOCl/rFel d 1 molar ratio. Decreases in the quantity of rFel d 1 detected are seen at an NaOCl/rFel d 1 molar ratio of 140, and less than 10 ng/mL rFel d 1 is detected at a molar ratio of 560.

likely representing the heterodimer, and a 5- to 6-kd band representing chain 1. Silver-stained protein was preserved at lower NaOCl/rFel d 1 molar ratios (70 and 140) but began to dissipate as the molarity of NaOCl was increased to a ratio of 560 and was undetectable at a NaOCl/rFel d 1 molar ratio of 7000 (Fig 1). Although rFel d 1 protein qualitatively remained intact when treated with NaOCl at molar ratios of both 70 and 140, all 3 bands of the hypochlorite-treated rFel d 1 demonstrated altered electropheretic mobility compared with that of untreated rFel d 1. The hypochlorite-treated rFel d 1 appears as 3 bands of slightly higher molecular weight than those of untreated rFel d 1. These results were confirmed in repeated protein gels and Western blots and had been noted previously in the experiments conducted with purified Mus m 1.[8]

Effect of NaOCl on immunogenicity of rFel d 1

After treatment with increasing concentrations of NaOCl, rFel d 1 was analyzed by means of Western blotting with monoclonal anti-Fel d 1 IgG. The mAb recognized the 33-kd homodimer and the 22- to 28-kd heterodimer of the untreated rFel d 1 (Fig 2). Cat-specific IgG binding was decreased at an NaOCl/protein molar ratio of 70, almost absent at a molar ratio of 140, and undetectable at a molar ratio of 560. The lower-molecular-weight protein bands did not appear to lose IgG binding at lower doses of NaOCl than the homodimer. All protein bands lost cat-specific IgG binding at the same treatment dose of NaOCl.

Hypochlorite-treated rFel d 1 was also analyzed by means of ELISA to quantify the effect of NaOCl on the immunogenicity of rFel d 1. Results verified the qualitative decrease in immunogenicity that were seen on Western blots. Anti-Fel d 1 antibody binding decreased with increasing concentrations of NaOCl; there was no substantial loss of IgG binding at a molar ratio of 70, but significant binding was lost at an NaOCl/rFel d 1 molar ratio of 140 (Fig 3). The untreated sample contained 3000 ng/mL rFel d 1, as measured by means of ELISA. When treated with NaOCl at a molar ratio of 70, measured rFel d 1 remained the same at 3054 ng/mL. However, at a molar ratio of 140, rFel d 1 concentration decreased by 29% to 2122 ng/mL. Substantial loss of IgG binding occurred at an NaOCl/rFel d 1 molar ratio of 560, which resulted in a final rFel d 1 concentration of 6 ng/mL. These ELISA results confirm the dose-response relationship between NaOCl and the loss of Fel d 1–specific antibody binding.

Effect of NaOCl on biologic activity of rFel d 1

Recombinant Fel d 1 was treated with the same increasing concentrations of NaOCl, and its biologic activity was quantified by using the basophil histamine release assay. Four of 4 donors who released histamine in response to untreated rFel d 1 had a decrease in leukocyte histamine release induced by hypochlorite-treated rFel d 1. Mean histamine release curves were constructed with data from the 4 donors. A total of 7 experiments were performed with blood from the 4 donors. Treatment of rFel d 1 with increasing molar ratios of NaOCl resulted in progressively decreasing histamine release in a dose-dependent manner (Fig 4). Maximal histamine release was defined as maximal histamine release by untreated rFel d 1. At an NaOCl/rFel d 1 molar ratio of 140, 50% of the maximal histamine release was not achieved. Instead, the highest histamine release achieved with rFel d 1 treated with this higher molar ratio of NaOCl was 30% of the maximal histamine release (Fig 4). When treated with NaOCl at an NaOCl/rFel d 1 molar ratio of 70, a log increase in the rFel d 1 dose was required to achieve 50% of the maximal histamine release. Attenuation of the biologic activity of rFel d 1 by NaOCl appears to occur at doses of NaOCl similar to those required to decrease immunogenicity, as assessed by means of Western blotting and ELISA.

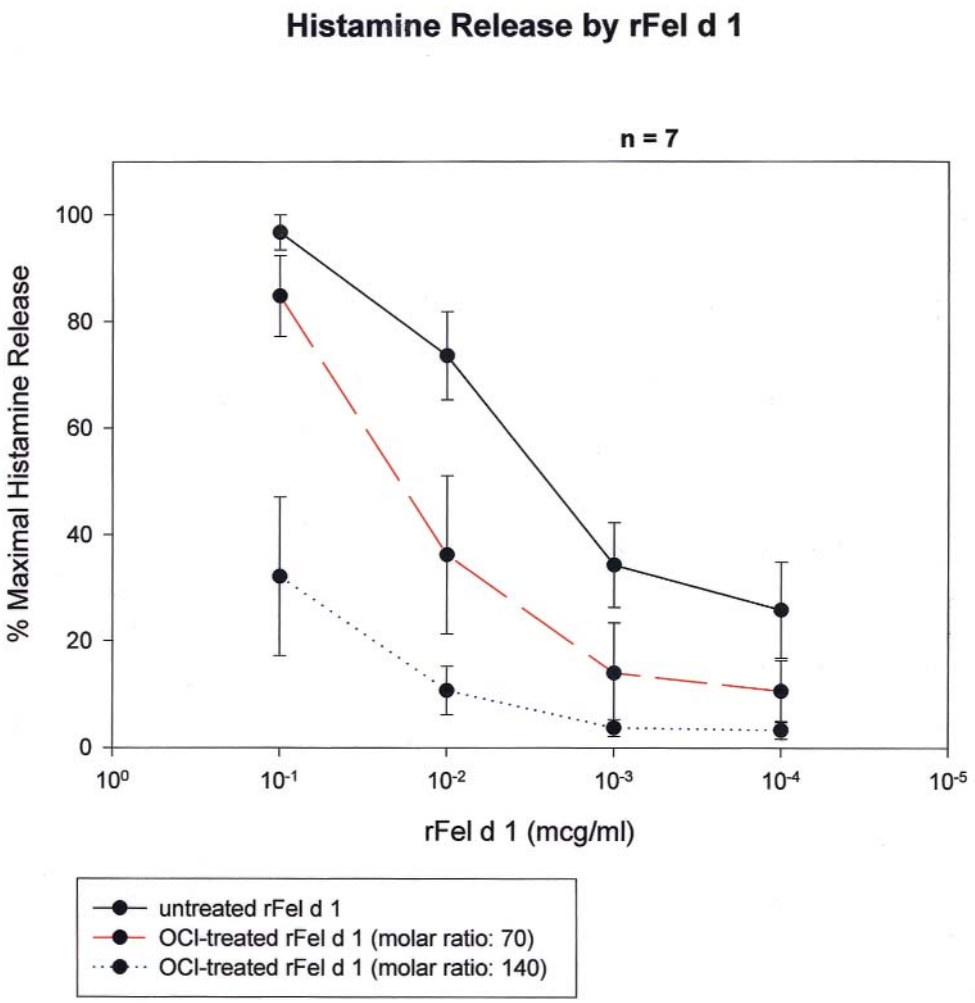

FIG 4. Leukocyte histamine release by rFel d 1. Recombinant Fel d 1 was treated with increasing amounts of NaOCl expressed as an NaOCl/rFel d 1 molar ratio. Mean curves were constructed from 7 experiments. *Bars* represent 1 SD. Histamine release is expressed as a percentage of the maximal histamine release obtained with untreated rFel d 1. Treatment with increasing amounts of NaOCl resulted in significantly decreasing release of histamine.

Results with cat hair extract

The effect of NaOCl on Fel d 1 in cat hair extract was also examined. Qualitatively similar results were found by means of SDS-PAGE, Western blotting, and histamine-release measurement. NaOCl resulted in loss of antibody binding to Fel d 1 at lower concentrations than those required for degradation of the protein (data not shown). As was found for rFel d 1, histamine release was affected in a dose-response manner by NaOCl treatment. Because of the large amounts of extraneous protein in the extract, higher amounts of NaOCl were required to affect antibody binding and biologic activity of Fel d 1. An NaOCl/Fel d 1 molar ratio of 12,000 was required to significantly decrease antibody binding, and a molar ratio of 24,000 resulted in a substantial loss of protein, as assayed with Coomassie Blue staining.

DISCUSSION

We have demonstrated that NaOCl in low concentrations reduces the immunogenicity of rFel d 1 and that the biologic potency of rFel d 1 is also reduced. The same effects were seen when cat hair extract was treated with NaOCl, although higher concentrations of NaOCl were required to achieve the same reduction.

Recombinant Fel d 1 appears as 3 bands on SDS-PAGE after silver staining. Purified Fel d 1 has been described in previous reports as a homodimer of approximately 36 kd.[13] The homodimer is noncovalently linked and composed of 2 heterodimers. Each heterodimer consists of a light chain (chain 1) and a glycosylated heavy chain (chain 2) and appears as an 18- to 22-kd band on SDS-PAGE gels.[14] Recombinant Fel d 1, expressed in *Pichia pastoris*, appears as 2 bands at 18 and 22 kd[15] in the same range as

for purified Fel d 1 from house dust.[14] The relative distribution of purified Fel d 1 as homodimers, heterodimers, or separate chains varies depending on the source material. Recombinant Fel d 1 can also appear as both homodimers and heterodimers.[15] The 33-kd band that we demonstrated by means of gel electrophoresis most likely represents the homodimer, whereas the diffuse 22- to 28-kd band likely represents the different glycosylated forms of the chain 1 and chain 2 heterodimer. We also demonstrated a 6-kd band that is consistent with chain 1.

Molar ratios were calculated by using the molecular weight of the homodimer of Fel d 1. Because the effects of NaOCl on a particular protein are dependent on the ratio of moles of hypochlorite to protein, we anticipated that we would demonstrate loss of low-molecular-weight components of rFel d 1 at lower concentrations of NaOCl than those required to affect the higher-molecular-weight components. In fact, Fel d 1 behaved as a single molecule when treated with NaOCl in solution: all 3 bands disappeared at the same NaOCl treatment dose. These results corroborate the previously published finding that Fel d 1 elutes as one major peak of homodimer.[11]

On Western blotting, the 3 bands also disappeared at the same treatment dose of NaOCl. However, the dose of NaOCl that was required to significantly affect immunogenicity was much less than that required to fragment the protein. The bands seen on Western blotting included a 30- to 33-kd band, the homodimer, and 22 and 28 kd bands, likely representing different glycosylated forms of the heterodimer. Chain 1 was not detected by means of Western blotting. This finding suggests that NaOCl affects the epitopes of rFel d 1 at lower concentrations than those required to break peptide bonds.

Previous studies[8] examining the effects of NaOCl on purified Mus m 1 demonstrated findings similar to ours: antibody binding by Mus m 1 was lost at an NaOCl/protein molar ratio of 100, whereas protein was fragmented at a molar ratio of 1000. The molar ratios that resulted in similar effects on rFel d 1 were comparable: an NaOCl/rFel d 1 ratio of 140 was required to substantially decrease antibody recognition, and an NaOCl/rFel d 1 molar ratio of 1120 was required to fragment the protein.

Although immunologic and biochemical protein studies examining the effects of NaOCl have been performed, this is the first published report of the effect of NaOCl on biologic potency of an allergen. Not surprisingly, the biologic potency of rFel d 1, as measured by histamine release, was decreased when treated with NaOCl. As increasing doses of NaOCl were used to treat rFel d 1, histamine release decreased significantly in a dose-dependent manner. The effects of NaOCl on the immunogenicity and allergenicity of rFel d 1 appear to result in a dose-dependent decrease in allergic potency.

To put this in context, household bleach products contain 5% to 6% NaOCl, and bathroom cleaning solutions contain 1.8% NaOCl. These correspond to 857 and 257 mmol/L NaOCl, respectively. These concentrations would fully fractionate concentrations of 1.3 mg/mL and 390 μg/mL rFel d 1, respectively. These concentrations of Fel d 1 are considerably higher than those found in homes. These experiments were all conducted in a laboratory setting, and therefore it is difficult to draw conclusions about concentrations that would be effective in domestic settings. Indeed, our experiments conducted with cat hair extract and previous experiments conducted with Mus m 1 indicate that the presence of extraneous protein reduces the efficacy of NaOCl.[8] However, a 1.8% (257 mmol/L) solution of NaOCl would be of sufficiently high concentration to overcome extraneous protein in the home environment. For example, when commercial allergen extracts, which contain very high concentrations of protein, were applied to surfaces and allowed to dry, a 0.3% (43 mmol/L) solution of NaOCl eliminated the allergens.[8]

Although these findings might have important practical applications, many questions remain about the feasibility of use of household bleach for allergen removal. One recently published study examined its efficacy in cockroach allergen removal after cockroach extermination.[16] Intervention homes were exterminated, and study participants in these homes were provided with cleaning materials, including household bleach. Control homes had neither extermination nor cleaning. Although Bla g 1 levels in settled dust decreased in the intervention homes compared with that in control homes, the decline in cockroach allergen levels was similar to that seen in homes in other studies that were visited by exterminators but not treated with bleach. Although this study suggests that NaOCl is not effective in removal of Bla g 1, some aspects of the study design make it difficult to draw firm conclusions. For example, the study does not directly compare extermination plus hypochlorite treatment to extermination alone. In addition, participants' compliance with the cleaning procedures is questionable. Certainly, the reduction in allergic potency of cat allergen suggests that NaOCl would be an effective allergen removal or allergen modification agent. Future studies examining the efficacy of NaOCl in allergen removal, modification, or both in a more controlled setting before field trials might prove most helpful.

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