Effect of premedications in a murine model of asparaginase hypersensitivity.

Christian A. Fernandez, Colton Smith, Seth E. Karol, Laura B. Ramsey, Chengcheng Liu, Ching-Hon Pui, Sima Jeha, William E. Evans, Fred D. Finkelman, and Mary V. Relling

Department of Pharmaceutical Sciences, St. Jude Children’s Research Hospital, 262 Danny Thomas Place, Memphis, TN 38105, USA (C.A.F., C.S., S.E.K., L.B.R., C.L., W.E.E., M.V.R.); Department of Oncology, St. Jude Children’s Research Hospital, 262 Danny Thomas Place, Memphis, TN 38105, USA (S.E.K., C.P., S.J.); Department of Medicine, Cincinnati Veterans Affairs Medical Center; the Department of Internal Medicine, Division of Immunology, Allergy and Rheumatology, University of Cincinnati College of Medicine; and the Division of Immunobiology, Cincinnati Children's Hospital Medical Center, Cincinnati, OH 45267, USA (F.D.F.).
Nonstandard Abbreviations:

PAF: Platelet activating factor

Mouse mast cell protease 1: mMCP-1

Acute lymphoblastic leukemia: ALL

Area under the curve: AUC

Dexamethasone: dex
Abstract

A murine model was developed that recapitulates key features of clinical hypersensitivity to *E. coli* asparaginase. Sensitized mice developed high levels of anti-asparaginase IgG antibodies and had immediate hypersensitivity reactions to asparaginase upon challenge. Sensitized mice had complete inhibition of plasma asparaginase activity ($P = 4.2 \times 10^{-13}$) and elevated levels of mouse mast cell protease 1 (mMCP-1, $P = 6.1 \times 10^{-3}$) compared to non-sensitized mice. We investigated the influence of pretreatment with triprolidine, cimetidine, the PAF receptor antagonist CV-6209, or dexamethasone on the severity of asparaginase-induced allergies. Combining triprolidine and CV-6209 was best for mitigating asparaginase-induced hypersensitivity compared to non-pretreated, sensitized mice ($P = 1.2 \times 10^{-5}$). However, pretreatment with oral dexamethasone was the only agent capable of mitigating the severity of the hypersensitivity ($P = 0.03$) and partially restoring asparaginase activity ($P = 8.3 \times 10^{-4}$). To rescue asparaginase activity in sensitized mice without requiring dexamethasone, a 5-fold greater dose of asparaginase was needed to restore enzyme activity to a similar concentration as in non-sensitized mice. Our results suggest a role of histamine and PAF in asparaginase-induced allergies and indicate that mast cell-derived proteases released during asparaginase allergy may be a useful marker of clinical hypersensitivity.
Introduction

Asparaginase is one of the integral components of combination chemotherapy regimens used in the treatment of acute lymphoblastic leukemia (ALL) and lymphoma. The mechanism of action of asparaginase is not completely understood, but the active enzyme depletes asparagine and possibly glutamine systemically (Wu et al., 1978; Asselin et al., 1989; Chan et al., 2014). Common adverse events to asparaginase include allergic reactions, often accompanied by the development of anti-asparaginase IgG antibodies (Pieters et al., 2011). Serum anti-asparaginase IgG levels have been found to increase before the onset of allergic reactions (Liu et al., 2012), suggesting that high antibody titers are required to induce clinical hypersensitivity. Furthermore, lower serum asparaginase activity was found in patients with anti-asparaginase antibodies compared to patients without detectable antibodies, and the enzyme activities were inversely correlated with the antibody levels (Liu et al., 2012). Anti-asparaginase IgG antibodies have been proposed to directly neutralize asparaginase activity (Albertsen et al., 2002; Pieters et al., 2011), and lower systemic exposure to asparaginase is associated with lower exposure to concomitantly administered dexamethasone and an increased risk of CNS relapse (Yang et al., 2008; Kawedia et al., 2012), although some studies showed no associations between anti-asparaginase IgG antibodies and ALL outcomes (Cheung et al., 1986; Larson et al., 1998; Asselin, 1999; Woo et al., 2000; Panosyan et al., 2004).

Classical allergies involve cell-associated antigen-specific IgE antibodies and require low doses of antigen, low titers of circulating antibody, and are mediated by the release of histamine (Finkelman et al., 2005). An alternative pathway for allergy, that appears to play a role in asparaginase-induced reactions (Liu et al., 2012), requires repeated exposure to the antigen, high antigen-specific IgG antibody levels, a large antigen dose, and the release of platelet activating...
factor (PAF) (Finkelman et al., 2005; Finkelman, 2007). Immunological studies have shown that antihistamines or PAF receptor antagonist can block the symptoms of an allergic reaction depending on the mechanism of allergy induced by the antigen (Strait et al., 2002). Understanding the pathway of asparaginase allergy will inform strategies for ameliorating the severity of hypersensitivity reactions and can help identify possible markers for detecting sensitized patients before receiving the offending drug.

In order to investigate therapeutic strategies for mitigating allergies and maintaining plasma concentrations of asparaginase, we created a murine model of asparaginase allergy. The model recapitulates several features of clinical hypersensitivity reactions developed to *E. coli* asparaginase. Our results indicate the involvement of both histamine and PAF in asparaginase-induced allergies and support the importance of monitoring asparaginase activity if pretreatment of patients with antihistamines or glucocorticoids is used to prevent allergy.
Materials and Methods

Asparaginase sensitization protocol

8-week-old female BALB/c mice received 10 μg i.p. doses of *E. coli* asparaginase (BioVendor Laboratory Medicine Inc., Candler, NC, > 96.0% purity as determined by RP-HPLC and SDS-PAGE) formulated with aluminum hydroxide adjuvant (Imject Alum, Thermo Scientific, Rockford, IL) on days 0 and 14 of treatment (Figure 1A) in order to be sensitized to asparaginase. Control (non-sensitized) mice received i.p. doses of adjuvant with vehicle alone (normal saline). Asparaginase allergies were induced in sensitized mice by challenging with a 100 μg i.v. dose of *E. coli* asparaginase on day 24 of treatment. The onset of hypersensitivity was detected by monitoring decreases in rectal temperature using a digital thermometer (model BAT-12, Physitemp Instruments, Clifton, NJ) for 2 hours following the asparaginase challenge. Pre-challenge plasma samples for determining anti-asparaginase antibody levels were collected on day 23 of treatment by retro-orbital puncture (Figure 1A), and post-challenge samples were collected by cardiac puncture at the end of the experiment for measuring antibody levels, asparaginase activity, and mouse mast cell protease 1 (mMCP-1) levels. The area under the temperature versus time curve was calculated using the trapezoidal rule. Lower AUC values indicate more severe reaction (drop in rectal temperature) and differences in the severity of asparaginase-induced allergies between treatment groups was determined by comparing the AUC values of different groups. Mice were housed in American Association of Laboratory Animal Care accredited facility and treated using institutional animal care and use committee (IACUC) approved protocols in accordance with National Institutes of Health guidelines.

Pretreatment protocol
Four different agents were investigated for their ability to mitigate asparaginase-induced allergic reactions: the antihistamines cimetidine (H2 receptor antagonist, Sigma, St. Louis, MO) and triprolidine (H1 receptor antagonist, Sigma, St. Louis, MO), the PAF receptor antagonist CV-6209 (Santa Cruz Biotechnology, Santa Cruz, CA), and dexamethasone (American Pharmaceutical Partners, Inc., Schaumburg, IL). As described in Figure 1B, antihistamine (cimetidine and triprolidine, 200 μg, i.p.) and CV-6209 (66 μg, i.v.) were given 30 and 5 minutes before the asparaginase challenge on day 24, respectively. Five different regimens of dexamethasone were investigated as described in Figure 1B-C. Single dose dexamethasone was given 2 hours before the asparaginase challenge on day 24 (2 mg/kg, i.p.), and multiple dose dexamethasone was given 2 hours before each asparaginase dose on days 0, 14, and 24 (2 mg/kg, i.p.). Pretreatment with oral continuous dexamethasone (4 mg/L in drinking water with a consumption rate of 5 mL/day yields an approximate dose of 0.8-1.0 mg/kg/day for the mice) starting 7 days before, same day (day 0), or 7 days after the initial asparaginase immunization dose was also evaluated for its influence on the severity of asparaginase-induced allergies. This regimen of dexamethasone achieves similar plasma dexamethasone concentrations as those achieved clinically (10-200 nM) in pediatric ALL patients given doses of 8 mg/m²/day (Yang et al., 2008; Yang et al., 2009; Kawedia et al., 2011).

Asparaginase activity determination

The asparaginase activity in plasma samples was determined by monitoring the enzymatically-coupled oxidation of reduced nicotinamide adenine dinucleotide (NADH) to NAD⁺ in a 96-well format, as described previously (Fernandez et al., 2013). Briefly, 10 μL of plasma from each sample was added to a 96-well microplate (Corning, Lowell, MA), and an enzyme reaction
mixture (ERM) containing asparagine, $\alpha$-ketoglutaric acid, glutamic oxalacetic transaminase, $\beta$-NADH, and malic dehydrogenase was added to each sample. A BioTek EL x 808 IU microplate reader (Winooski, VT) was used to determine the asparaginase activity. All asparaginase standards used for calibration purposes were prepared using mouse plasma, and samples with values over the standard curve were diluted with mouse plasma to get their enzyme activities within the linear range of the assay.

Determining the ex vivo asparaginase activity inhibition of samples from sensitized mice

The neutralization of asparaginase activity by post immunization plasma was determined by adding 0.2 IU/mL of asparaginase to plasma samples collected after the asparaginase challenge (day 24). Plasma from sensitized mice, non-sensitized mice, or non-sensitized mice after addition of 5 $\mu$g/$\mu$L of rabbit polyclonal anti-asparaginase IgG antibodies (Abcam, Cambridge, MA) were tested for residual asparaginase activity as described above.

Detection of anti-asparaginase IgG antibodies

An ELISA-based method was used to detect anti-\textit{E.coli} asparaginase IgG antibodies, as described previously (Wang et al., 2000; Liu et al., 2012). Plasma samples from non-sensitized mice were used as negative controls, and rabbit polyclonal anti-asparaginase IgG antibodies were used as positive controls. Anti-mouse IgG (Sigma, St. Louis, MO), anti-mouse IgE (Thermo Scientific, Rockford, IL), and anti-rabbit IgG secondary antibodies (Sigma, St. Louis, MO) were used for detection of antibodies in samples or controls.

Determining mouse mast cell protease 1 (mMCP-1) concentrations
Concentrations of mMCP-1 were determined by ELISA using the Ready-SET-Go commercial kit from eBioscience (San Diego, CA).

Statistical analysis

Differences between rectal temperature AUC, asparaginase activity, anti-asparaginase IgG levels, and mMCP-1 concentrations were determined using a pairwise Wilcoxon rank sum test. All statistical analysis was performed with the R statistical software (version 2.13.2).
Results

Mice were sensitized to *E. coli* asparaginase using the adjuvant aluminum hydroxide to create a murine immune response similar to clinical hypersensitivity reactions to asparaginase (Figure 1A and Figures 2A-E). Sensitized mice challenged with 100 μg of asparaginase (22.5 IU or approximately 1,125 IU/kg per mouse) had immediate hypersensitivity reactions characterized by a sudden drop in rectal temperature (Figure 2A) and a decrease in physical activity, whereas non-sensitized mice did not experience changes in rectal temperature (Figure 2B, P = 3.3 x 10^{-10}) or activity upon challenge. Compared to non-sensitized controls, sensitized mice experienced a peak drop in rectal temperature > 6°C within 30 minutes of the asparaginase challenge and had negligible asparaginase activity in samples collected 2 hours after the challenge (Figure 2C, P = 4.2 x 10^{-13}). The loss of enzyme activity is likely due to high levels of anti-asparaginase IgG antibodies detected before the challenge on day 23 relative to non-sensitized mice (Figure 2D, red open circles, P = 1.1 x 10^{-7}). The antibodies bind to asparaginase upon challenge and result in a drop in the measurable antibody titer (Figure 2D, P = 6.7 x 10^{-5}, comparing black to red open circles of sensitized mice), presumably due to the formation of immune complexes that lead to a decrease in asparaginase activity. Using an *ex vivo* activity inhibition assay, we found that plasma samples from sensitized mice neutralize > 30% of the added asparaginase activity (0.2 IU/mL), with the degree of activity inhibition correlated with anti-asparaginase IgG levels (Supplemental Figure 1A-B, R^2 = 0.161, P = 0.04). Consistent with the reports of elevated levels of mouse mast cell protease 1 (mMCP-1) in various pathways of anaphylaxis (Strait et al., 2002), the mMCP-1 concentrations measured in sensitized mice were elevated nearly 35-fold compared to non-sensitized mice (Figure 2E, P = 6.1 x 10^{-3}).
Having established a model of asparaginase-induced allergy, we next tried to develop pretreatment strategies to mitigate the severity of the reactions. The initial pretreatment agents we selected were based on receptor antagonists of known chemical mediators of allergy (Figure 1B-C). We found that cimetidine, triprolidine, CV-6209, or the three agents used in combination all mitigated the severity of the reaction compared to no pretreatment (Figure 3, P < 1.3 x 10^-3). Antihistamines and CV-6209 mitigated the drop in rectal temperature, but pretreatment with the combination of antihistamines and CV-6209 reduced the severity of the allergic reaction compared to no pretreatment (Figure 3, P = 1.2 x 10^-5), antihistamine alone (Figure 3, P = 1.6 x 10^-4), and single agent CV-6209 (Figure 3, P = 3.2 x 10^-4). It was unclear whether triprolidine and/or cimetidine were responsible for reducing the severity of asparaginase-induced allergy; therefore, we tested each antihistamine as a single agent in sensitized mice. Cimetidine showed little effect compared to non-pretreated sensitized mice, and this group had more severe reactions compared to mice pretreated with a combination of cimetidine and triprolidine (Figure 3, P = 1.1 x 10^-3). In contrast, mice pretreated with single agent triprolidine showed a decrease in the severity of allergies compared to non-pretreated, sensitized mice (Figure 3, P = 0.02) and their rectal temperature AUC was similar to that of mice pretreated with cimetidine and triprolidine in combination (Figure 3, P > 0.05). These results suggest that triprolidine but not cimetidine is required for mitigating asparaginase-induced allergies in our murine model.

PAF receptor antagonists such as CV-6209 are not approved for clinical use in most countries. Therefore, we wanted to test whether dexamethasone could achieve similar effects as CV-6209 on asparaginase-induced allergies. Five dexamethasone pretreatment strategies were investigated: single dose dexamethasone (Figure 1B, 2 mg/kg, i.p.) given 2 hours before the asparaginase challenge, multiple doses of dexamethasone (Figure 1C, 2 mg/kg, i.p.) given
hours before each immunization dose (Figure 1C, day 0 and day 14) and before the challenge (day 24), and oral dexamethasone (Figure 1C, 4 mg/L in drinking water) starting at 7, 0, or -7 days before the initial asparaginase immunization dose. Pretreatment with single or multiple doses of dexamethasone was also evaluated with concomitant triprolidine. We found that mice pretreated with any of the dexamethasone regimens showed a decrease in the severity of the reaction compared to non-pretreated, sensitized mice (Figure 3, $P \leq 0.03$), except for mice receiving oral dexamethasone 7 days after the initial asparaginase immunization. We found no difference in rectal temperature AUC between single dose dexamethasone pretreatment (with or without concomitant triprolidine), multiple dose dexamethasone pretreatment (with or without concomitant triprolidine), and mice receiving oral dexamethasone (Figure 3, $P > 0.05$). Furthermore, all mice pretreated with any regimen containing dexamethasone had more severe reactions compared to mice pretreated with antihistamines and CV-6209 in combination (Figure 3, $P \leq 0.06$).

Asparaginase activity was determined in samples collected after the asparaginase-induced hypersensitivity to determine if pretreatment was able to restore activity (Figure 4). Only oral dexamethasone given 0 or 7 days before the initial asparaginase was able to partially rescue activity compared to non-pretreated, sensitized mice (Figure 4, $P = 4.8 \times 10^{-2}$ and $8.3 \times 10^{-4}$, respectively). All other pretreatment strategies resulted in similar activity levels as non-pretreated, sensitized mice (Figure 4, $P > 0.05$). Nevertheless, mice receiving oral dexamethasone 0 or 7 days before the initial asparaginase had lower asparaginase activity than non-sensitized mice (Figure 4, $P = 3.8 \times 10^{-5}$ and $1.9 \times 10^{-6}$, respectively), suggesting that other methods of lowering anti-asparaginase IgG antibody levels may be required to completely rescue asparaginase activity. Similar pretreatment experiments with antihistamine, CV-6209, and
dexamethasone were also tested in mice that were not sensitized to asparaginase to confirm that there was no direct influence of premedication on asparaginase activity (Supplemental Figure 2). These results indicate that H1 and PAF receptor antagonists mitigate allergic reactions but do not influence the asparaginase activity.

The inability of pretreatment strategies to fully rescue asparaginase activity is likely due to high anti-asparaginase IgG levels that are unaffected by the pretreatment strategies we used. Mice immunized with asparaginase all developed high levels of anti-asparaginase IgG antibodies in pre-challenge samples (day 23) compared to non-sensitized mice (Figure 5A, $P < 0.04$), including mice that received multiple doses of dexamethasone. However, mice pretreated with oral dexamethasone on the day of or 7 days before the initial asparaginase immunization showed diminished antibody levels compared to sensitized, non-pretreated mice (Figure 5B, $P = 1.0 \times 10^{-3}$ and $4.1 \times 10^{-4}$, respectively) in samples available on day 17 of treatment (Figure 1A). These results indicate that oral dexamethasone was able to partially restore asparaginase activity due to its effect on anti-asparaginase IgG antibody levels.

We hypothesized that many of the pretreatment strategies were masking the severity of the hypersensitivity reactions rather than blocking the release of the chemical mediators of allergy. In order to confirm this, we measured concentrations of mMCP-1 in samples that were collected after the asparaginase-induced hypersensitivity. All sensitized mice showed elevated levels of mMCP-1 compared to non-sensitized mice (Figure 6, $P \leq 0.02$), except for mice receiving a single dose of dexamethasone (with or without concomitant triprolidine, $P > 0.05$). Interestingly, this observation was not observed with any other dexamethasone pretreatment regimen. It is possible that the pretreatment with a single dose of dexamethasone mitigated the reactions by inhibiting the release of histamine during the allergic reaction, whereas other dexamethasone
regimens masked the hypersensitivity through other mechanisms that likely affect antibody production. Similar data have been previously reported suggesting that dexamethasone can inhibit the release of histamine from mast cells and basophils (Schleimer et al., 1982; Berenstein et al., 1987; Schleimer et al., 1987).

We next investigated if increasing the dose of asparaginase used during the challenge could overcome the high antibody titers and restore asparaginase activity without requiring dexamethasone. In the absence of pretreatment, the severity of asparaginase-induced reactions increased in a dose-dependent manner, and all groups challenged with higher doses of asparaginase had more severe reactions compared to sensitized mice challenged with the standard 100 μg dose (Figure 7A, $P \leq 2.1 \times 10^{-4}$). Furthermore, dose increases $\geq$ 5-fold (5X and 10X) were able to restore enzyme activity to concentrations similar to or higher than that of non-sensitized mice (Figure 7B). While increasing the dose of asparaginase used during the challenge from 1-fold to 10-fold showed a trend toward lower antibody titers in samples collected after the challenge (data not shown), the trend failed to reach statistical significance. mMCP-1 levels were elevated in a dose-dependent manner and correlated to the severity of the reactions (Figure 8, $R^2 = 0.577$, $P = 3.0 \times 10^{-16}$).

Our pretreatment studies showed that triprolidine and CV-6209 were the best for mitigating the severity of asparaginase-induced allergies, and our dose adjustment experiments indicated that a 500 μg dose of asparaginase per mouse was required to rescue asparaginase activity in sensitized mice. Therefore, we combined both strategies to determine if we could restore activity while simultaneously mitigating the severity of the allergy. We found that the pretreatment, even at the higher dose of asparaginase, was capable of blocking the allergic reaction compared to sensitized, non-pretreated mice (Figure 7A, $P = 9.8 \times 10^{-4}$) and the dose adjustment restored
asparaginase activity to similar concentrations as non-sensitized mice (Figure 7B, P > 0.05). The anti-asparaginase antibody levels and mMCP-1 concentrations measured for pretreated and non-pretreated mice given a 5-fold increased dose were similar (P > 0.05, data not shown), suggesting that mice in both groups had similar potential for developing hypersensitivity reactions of comparable severities.
Discussion

Clinical immune responses to asparaginase during the treatment of leukemia almost exclusively occur upon re-exposure to asparaginase after an initial exposure (Albertsen et al., 2002; Pieters et al., 2011; Fernandez et al., 2014a), implying that these patients were sensitized to asparaginase administered initially. Reactions in sensitized patients are usually characterized by an immediate allergic response after administration of asparaginase, by the development of high levels of anti-asparaginase IgG antibodies, and by low serum asparaginase activity concentrations (Peterson et al., 1971; Spiegel et al., 1980; Muller et al., 2001; Panosyan et al., 2004; Pieters et al., 2011; Liu et al., 2012). The sensitization protocol we used in mice (Figure 1A-B) resulted in the development of an asparaginase-mediated immune response with a similar phenotype as clinical hypersensitivity to *E. coli* asparaginase.

Our results have several implications for improving use of asparaginase. Our data support that an H1 histamine receptor antagonist (triprolidine) used in combination with a PAF receptor antagonist (CV-6209) is best for mitigating asparaginase-induced reactions (Figure 3). Although we did not measure IgE antibodies, these response data are consistent with a role for both anti-asparaginase IgG (through FcγR dependent release of PAF) and IgE antibodies (through FceR1 dependent release of histamine) being involved in eliciting asparaginase hypersensitivity reactions. However, pretreatment with these agents did not rescue plasma asparaginase activity (Figure 4). These experiments clearly demonstrate that the symptoms and signs of asparaginase allergies can be completely masked by pretreatment and yet result in sub-therapeutic drug concentrations, thus emphasizing the importance of monitoring asparaginase activity after pretreatment with antihistamines, glucocorticoids, or any other medication used for mitigating the severity of allergic reactions. In addition, the low asparaginase activity measured in
sensitized mice is likely due to an accelerated clearance and antibody neutralization, as the maximum \textit{ex vivo} inhibition we were able to induce using rabbit anti-asparaginase IgG antibodies (5 μg/μL) was only 50% (Supplemental Figure 1). Similar neutralization results were previously reported (Peterson et al., 1971; Baechtel and Prager, 1973), suggesting that anti-asparaginase IgG antibodies cannot completely neutralize asparaginase activity and that other mechanisms are involved in the rapid \textit{in vivo} disappearance of enzyme activity. This is further supported by the > 50\% loss of asparaginase activity (Figure 4) seen in mice pretreated with dexamethasone 7 days before the initial asparaginase immunization dose even though they had negligible antibody levels (Figure 5B) compared to other pretreatment groups. This is similar to the accelerated asparaginase clearance seen in pediatric patients with relapsed ALL receiving \textit{E. coli} asparaginase and negative for anti-asparaginase IgG antibodies (Panetta et al., 2009).

We found that dexamethasone could be used to mitigate the reaction, although at the dosage schedules we studied it was not as effective in masking the allergic reaction as pretreatment with triprolidine and CV-6209 in combination (Figure 3, \( P \leq 0.06 \)). However, pretreating mice with oral dexamethasone before exposure to asparaginase lowered anti-asparaginase IgG levels (Figure 5B) and partially maintained asparaginase activity (Figure 4). These results are similar to recent clinical findings that showed a lower incidence of asparaginase hypersensitivity in patients that received dexamethasone 4 days before asparaginase administration during remission induction and support the use of glucocorticoids before beginning asparaginase (Pui, 2013; Vora et al., 2013).

Our dose adjustment studies indicate that strategies to lower anti-asparaginase IgG antibody titers (by overcoming their inhibitory effects by presenting a higher concentration of antigen) can restore enzyme activity. However, the effect of large asparaginase doses on treatment-related
toxicities is unknown. The dose adjustment experiments also show that the severity of asparaginase-induced reactions increases in a dose dependent manner (Figure 7A). Thus, when challenging patients after a suspected reaction, a lower dose of asparaginase might be expected to result in a less serious reaction, but may not overcome the antibody levels and result in lower serum asparaginase activity levels.

Histamine and PAF are considered primary chemical mediators of allergic shock by acting on smooth muscle endothelial cells (Finkelman, 2007). However, histamine or PAF are unlikely to be useful biomarkers of asparaginase-induced allergies due to their short serum half-life. Histamine concentrations reach peak levels within 5 minutes of the onset of symptoms and have a serum half-life of a few minutes (Ferrer et al., 2010), whereas PAF has a serum half-life < 15 minutes (Vadas et al., 2008). Several studies have shown that elevated levels of tryptase can be used as a biomarker of clinical hypersensitivity (Ordoqui et al., 1997; He et al., 2004; Payne and Kam, 2004; Fernandez et al., 2014b), and in accord with those studies, we found that mMCP-1 was elevated in sensitized mice from samples collected 2 hours after the asparaginase challenge (mMCP-1 is a mucosal mast cell granule-specific β-chymase that is released during basophil or mast cell degranulation in mice similar to tryptase in humans (Caughey, 2007)). Furthermore, mMCP-1 concentrations were correlated with the severity of allergic reactions (Figure 8), and previous studies have shown that increases in plasma mMCP-1 levels may be induced by either IgG or IgE-mediated allergies (Strait et al., 2002). Unlike histamine and PAF, tryptase reaches peak concentrations approximately 30-60 minutes after allergy onset, has a serum half-life of 90 minutes, and exhibits low serum concentrations in normal, non-allergic individuals (Laroche et al., 1991). While other studies have suggested that histamine levels may be a more useful immediate (e.g. 10 minute) indicator of the onset of allergies compared to the release of mast
cell proteases in mice (Nabe et al., 2013), a biomarker of allergy that is elevated and stable for several hours after an allergic reaction may be more useful as an eventual clinical biomarker of asparaginase-induced allergies. Taken together, our results suggest that tryptase may be a possible clinical biomarker for confirming episodes of hypersensitivity to asparaginase.

Aluminum hydroxide adjuvant was required for mice to develop immune responses to asparaginase, as mice immunized with asparaginase without adjuvant did not develop allergic reactions when challenged (data not shown). Selection of an adjuvant can play a large role in determining the characteristics of the immune response generated (e.g., antibody isotypes, subclass, affinity, and titer) (Hadjipetrou-Kourounakis and Moller, 1984; Kenney et al., 1989; Muller and Boos, 1998). Anti-asparaginase antibodies in ALL patients have been reported to be predominantly IgG1 and IgG4 (Cheung et al., 1986; Korholz et al., 1987), which is consistent with a TH2 response (Visciano et al., 2012). Immunization with aluminum hydroxide generally elicits a TH2 response with the development of antigen-specific IgG1 and IgE antibodies (Kenney et al., 1989). In accordance with these reports, asparaginase sensitized mice developed anti-asparaginase IgG1 antibodies, but no IgE antibodies were detected (data not shown). Therefore, clinical hypersensitivity reactions to asparaginase that are associated with the development of anti-asparaginase IgG antibodies and the asparaginase-induced allergic reaction in our murine model both appear to be TH2 driven. Nevertheless, there are several similarities and differences between the murine and human immune system that may influence the translation of our results to human allergies. Both species produce IgE antibodies that bind with high affinity to the FceRI receptor, and cross-linking the receptor results in the release of chemical mediators of anaphylaxis, including histamine and PAF (Finkelman, 2007). Both
species also produce IgG antibodies that induce the release of PAF by binding to the FcγRIII receptor, and both can mediate allergic reactions by the activation of complement through the production of anaphylatoxins. In contrast, the human and murine immune systems differ in the ability to activate complement through their respective IgG isotypes (Snapper and Finkelman, 1999), in the cells that express FceR1 (Bieber et al., 1992; Maurer et al., 1994; Maurer et al., 1996; Kinet, 1999), and in the quantity of granules within basophils, which influences the role of chemical mediators of allergy during hypersensitivity reactions (Lee and McGarry, 2007; Tsujimura et al., 2008). It is also possible that other chemical mediators of allergies besides PAF and histamine, such as cysteinyl leukotrienes and prostaglandin D2, play a role during human allergic reactions to asparaginase (Ono et al., 2009).

In conclusion, we created the first murine model of immediate hypersensitivity reaction to asparaginase. Several previous studies have sensitized mice to asparaginase and demonstrated the development of anti-asparaginase antibodies (Roberts et al., 1966; Baechtel and Prager, 1973; Goldberg et al., 1973; Uren and Ragin, 1979; Kamisaki et al., 1981; Yagura et al., 1981; Fernandes and Gregoriadis, 2001; Cantor et al., 2011) and demonstrated delayed hypersensitivity reactions to asparaginase (Kawamura et al., 1985; Gaspar et al., 1996). Our model is the first to show the development of acute symptomatic allergies in sensitized mice and to identify methods of reducing the severity of the symptoms and restoring asparaginase activity. We demonstrate that pretreatment with H1 receptor antagonists, PAF receptor antagonist, or dexamethasone can all partially mitigate the severity of asparaginase-induced allergic reactions to a similar extent; however, only dexamethasone was able to partially maintain plasma asparaginase activity. This may be particularly important for selecting a pretreatment agent in patients that are at a high risk
of developing glucocorticoid-induced osteonecrosis (Vora, 2011). Our results also support the need for a clinically available PAF receptor antagonist for mitigating immune responses (Roberts et al., 1988; Hozawa et al., 1995; Kingsnorth et al., 1995), and emphasize that it may be important to monitor asparaginase activity if pretreatment medications are used. Furthermore, our results indicate that strategies that can lower anti-asparaginase antibodies might be useful to restore enzyme activity in a similar manner as our dose escalation approach.
Author contributions

Participated in research design: Christian A. Fernandez, Colton Smith, Fred D. Finkelman, and Mary V. Relling.

Conducted experiments: Christian A. Fernandez.

Performed data analysis: Christian A. Fernandez and Colton Smith.

Wrote or contributed to the writing of the manuscript: Christian A. Fernandez, Colton Smith, Fred D. Finkelman, Mary V. Relling, Ching-Hon Pui, William E. Evans, Sima Jeha, Chengcheng Liu, Laura B. Ramsey, and Seth E. Karol.
References


Ferrer M, Nunez-Cordoba JM, Luquin E, Grattan CE, De la Borbolla JM, Sanz ML and Schwartz LB (2010) Serum total tryptase levels are increased in patients with active


Footnote

The study was supported by the National Cancer Institute [CA 21765, CA 142665, CA 36401] and by the American Lebanese Syrian Associated Charities.
Figure Legends

Figure 1. Schedule for sensitization to *E. coli* asparaginase and sample collection. (A) Mice received intraperitoneal (i.p.) injections of *E. coli* asparaginase (10 μg or 2.25 IU per mouse, green arrows) formulated with aluminum hydroxide adjuvant (1 mg, white arrows) on day 0 and day 14 of the immunization schedule in order to sensitize mice to asparaginase. On day 24, mice were challenged with an intravenous (i.v.) dose of *E. coli* asparaginase (100 μg or 22.5 IU per mouse, green arrow) to induce hypersensitivity reactions. Samples collected on day 23 (red arrow) were used to assess anti-asparaginase antibody titers before the asparaginase challenge on day 24. For a subset of mice, including those receiving oral dexamethasone, pre-challenge samples were only available on day 17 of treatment (red arrow, n = 39). Mice received pretreatment on day 24 (blue arrow) with CV-6209, antihistamine (triprolidine, cimetidine), or a single dose of dexamethasone (i.p.) in an attempt to mitigate the severity of the allergic reaction. (B) These pretreatment agents were administered 5 minutes, 30 minutes, or 2 hours before the asparaginase challenge, respectively. (C) Additional mitigation strategies included pretreatment with multiple doses of dexamethasone (i.p.) on day 0, 14, and 24 of treatment, and pretreatment with continuous oral dexamethasone starting at 7 days before, same day, or 7 days after the initial asparaginase immunization dose. Samples collected 2 hours after the challenge on day 24 (black arrow) were used to measure asparaginase activity, antibody titers, and mouse mast cell protease 1 (mMCP-1). Data in subsequent figures are color-matched accordingly to indicate the sample used for the laboratory value (black corresponding to day 24 samples [post challenge dose of asparaginase] and red to day 23 or day 17 samples [pre-challenge]).

Figure 2. Mice sensitized to *E. coli* asparaginase developed asparaginase-induced hypersensitivity reactions when challenged. (A) The rectal temperature of sensitized (○) and
non-sensitized (□) mice was monitored for 2 hours after i.v. administration of *E. coli* asparaginase (100 μg) at day 24 (as per Figure 1A). (B) Compared to non-sensitized controls, sensitized mice with asparaginase-induced allergies experienced a drop in rectal temperature that was quantified by the AUC of the rectal temperature versus time curve (P = 3.3 x 10⁻10). (C) Sensitized mice had low to no detectable asparaginase activity (P = 4.2 x 10⁻13), and (D) developed high anti-asparaginase IgG antibody titers compared to non-sensitized mice (red open circles, P = 1.1 x 10⁻⁷). Sensitized mice showed a drop in antibody levels when comparing the pre-challenge and post-challenge samples at day 23 versus day 24 (red versus black open circles, P = 6.7 x 10⁻⁵), suggesting the formation of asparaginase-anti-asparaginase IgG immune complexes. (E) Sensitized mice with asparaginase-induced allergies had elevated levels of mMCP-1 compared to non-sensitized mice in samples collected 2 hours after the challenge (P = 6.1 x 10⁻³).

**Figure 3.** Pretreatment with triprolidine, CV-6209, or dexamethasone (dex) mitigates the severity of asparaginase-induced allergies. Mice sensitized to *E. coli* asparaginase were pretreated with triprolidine, cimetidine, the PAF receptor antagonist CV-6209, single dose dexamethasone, or a combination of the agents as per Figure 1B; multiple dose dexamethasone or oral dexamethasone were given as per Figure 1C. All pretreatment agents except for cimetidine and oral dexamethasone given 7 days after the initial asparaginase immunization dose (at day 0) were able to mitigate the severity of the reaction compared to non-pretreated, sensitized mice (P < 0.03); however, the combination of antihistamine and CV-6209 showed the best ability to reduce the severity of the reaction compared to non-pretreated, sensitized mice (P = 1.2 x 10⁻⁵).
Figure 4. Oral dexamethasone given 7 days before asparaginase sensitization can partially restore asparaginase activity. Non-pretreated mice sensitized to asparaginase showed low to no detectable asparaginase activity compared to non-sensitized mice ($P = 3.2 \times 10^{-14}$). Pretreatment with antihistamine, CV-6209, or i.p. dexamethasone (dex) was not able to rescue asparaginase activity relative to non-pretreated, sensitized mice ($P > 0.05$). However, mice receiving continuous oral dexamethasone starting 0 or 7 days before asparaginase sensitization had partially restored asparaginase activity compared to non-pretreated, sensitized mice ($P = 4.8 \times 10^{-2}$ and $8.3 \times 10^{-4}$, respectively).

Figure 5. Dexamethasone effects the development of anti-asparaginase IgG antibodies. (A) On day 23 (before pretreatment with i.p. single dose dexamethasone (dex), triprolidine, cimetidine, or CV-6209), all sensitized mice had elevated anti-asparaginase IgG levels compared to non-sensitized controls ($P < 0.04$). Mice that received pretreatment with multiple doses of parenteral dexamethasone (with or without triprolidine) on day 0 and 14 before the day 23 blood collection had similar antibody levels as sensitized, non-pretreated mice ($P > 0.05$). (B) Mice receiving continuous oral dexamethasone starting 0 or 7 days before the asparaginase sensitization had lower measured anti-asparaginase IgG levels compared to sensitized, non-pretreated mice ($P = 1.0 \times 10^{-3}$ and $4.1 \times 10^{-4}$, respectively) in samples available on day 17 of treatment (before the asparaginase challenge).

Figure 6. Elevated levels of mouse mast cell protease 1 (mMCP-1) were detected in sensitized mice after asparaginase-induced allergies. Elevated levels of mMCP-1 were detected in all sensitized mice after asparaginase-induced allergies ($P < 0.02$), except for mice pretreated with a single dose of i.p. dexamethasone (dex) or a single dose of i.p. dexamethasone in combination with triprolidine.
Figure 7. Asparaginase dose adjustments restored activity levels in sensitized mice. Sensitized mice were challenged with 2, 5, or 10-fold higher asparaginase doses. (A) The severity of the reaction (drop in rectal temperature) showed a dose-dependent relationship (P < 1.0 x 10^{-3} compared to 1X, sensitized), and mice pretreated with triprolidine and CV-6209 receiving a 5-fold higher dose were able to mitigate the reaction relative to non-pretreated mice (P = 9.8 x 10^{-4}). (B) Furthermore, dose adjustments ≥ 5-fold were able to restore asparaginase activity in samples collected 2 hours after the challenge compared to non-sensitized mice (P > 0.05).

Figure 8. The severity of asparaginase-induced allergies was correlated with mouse mast cell protease 1 (mMCP-1) concentrations. The lower the rectal temperature AUC, the more severe the allergic reaction, and the higher the mMCP-1 concentrations from samples collected after the challenge on day 24 for non-sensitized mice (●) and for sensitized mice receiving 100 μg (●), 200 μg (●), 500 μg (●), or 1 mg (●) of asparaginase per mouse during the challenge (R^2 = 0.577, P = 3.0 x 10^{-16}).
Figure 1.

A. Sample Collection
   Pretreatment
      Adjuvant
      Asparaginase

B. Sample Collection
   CV-6209
   Antihistamine
   Single Dose
   Dexamethasone
   Asparaginase

C. Multiple Dose
   Dexamethasone (ip)
   Day -7 Dexamethasone Schedule (oral)
   Day 0 Dexamethasone Schedule (oral)
   Day 7 Dexamethasone Schedule (oral)
   Asparaginase
Figure 2.

A. Temperature (°C) over time (min) for non-sensitized and sensitized groups.

B. Rectal temperature AUC for non-sensitized and sensitized groups.

C. ASNase activity (IU/mL) for non-sensitized and sensitized groups.

D. Anti-ASNase IgG OD (490nm) for non-sensitized and sensitized groups.

E. mMCP-1 (ng/mL) for non-sensitized and sensitized groups.
Figure 3.

The figure shows a graph with the x-axis representing different treatment groups: No pretreatment, Antihistamine treatment, Dexamethasone treatment, and Combination treatment. The y-axis represents Rectal Temperature AUC with values ranging from -1500 to 0. The graph includes data points for various substances: Sensitized, Tripolidine, Cimetidine, Single dose Dex, Multi dose Dex, Oral Dex Start Day, and CV-6209.
Figure 4.
Figure 5.
Figure 6.

- No pretreatment
- Antihistamine treatment
- Dexamethasone treatment
- Combination treatment

<table>
<thead>
<tr>
<th>mMCP-1 (ng/mL)</th>
<th>Sensitized</th>
<th>Triprolidine</th>
<th>Cimetidine</th>
<th>Single dose Dex</th>
<th>Multi dose Dex</th>
<th>Oral Dex Start Day</th>
<th>CV-6209</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>0</td>
<td>-7</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>+</td>
</tr>
</tbody>
</table>
Figure 8.