Comparative Medicine
Copyright 2007
by the American Association for Laboratory Animal Science

Vol 57, No 4
August 2007
Pages 349–354

Carbamoylcholine Chloride Induces a Rapid Increase in IL6 in the Nasal Cavity of C57BL/6 Mice

Xiaoying Lu,[2] Lindsey C Pingel,[2] Kindra K Burnell,[2] Joseph E Cavanaugh,[3] and Kim A Brogden[1,2,*]

Mice are widely used as models to study the roles of chemokines and cytokines in immune and inflammatory responses. In our work to determine the basal levels of cytokines in saliva, nasal wash fluid (NWF), bronchoalveolar lavage fluid (BALF), and serum of mice, we found that injection of carbamoylcholine chloride, used to stimulate saliva production, induced variations in the interleukin (IL) 6 levels of NWF and BALF supernatants. To characterize this response, C57BL/6 mice were given 10 μg carbamoylcholine chloride intraperitoneally and euthanized at 0, 1, 3, 6, 12, 24, 48, 72, and 96 h after injection. IL6 was increased in NWF supernatants by 2 to 3 h, remained elevated for 24 h, and declined by 48 h after injection. To determine whether carbamoylcholine chloride increased Th1 cytokine (IL2, IL12[p70], and interferon γ), Th2 cytokine (IL4, IL5, and IL10), granulocyte–macrophage colony-stimulating factor (GM-CSF), or proinflammatory cytokine (IL1β, tumor necrosis factor α, and IL6 in saliva and serum) levels, mice were given 10 μg carbamoylcholine chloride and euthanized. In 47 mice, all cytokine levels in saliva supernatants, NWF supernatants, BALF supernatants, and serum were within normal reported levels (range, 1 to 364 pg/ml); in the serum of the remaining 3 mice, GM-CSF, IL1β, and IL2 levels were increased. In summary, carbamoylcholine chloride induces a rapid, elevated IL6 response in the nasal cavity and respiratory tract of mice but does not alter the levels of other Th1, Th2, or proinflammatory cytokines.

Abbreviations: BALF, bronchoalveolar lavage fluid; GM-CSF, granulocyte–macrophage colony-stimulating factor; IL, interleukin; IP, intraperitoneal; NWF, nasal wash fluid; PBS, phosphate buffered saline; TNF, tumor necrosis factor

Mice are widely used as models to study the roles of chemokines and proinflammatory cytokines in innate and acquired immunity and acute and chronic inflammatory responses.[8] Despite extensive information on the concentrations of cytokines in murine serum, little is known about the identity and concentration of cytokines in the supernatants of saliva, nasal wash fluid (NWF), and bronchoalveolar lavage fluid (BALF). Such information is pertinent to studies assessing innate and adaptive immune responses in the oronasal cavity.[2] In determining the levels of chemokines and cytokines in the serum and supernatants of saliva, NWF, and BALF of C57BL/6 mice, we found unexplained variations in the concentrations of IL6 in the NWF supernatants. Closer examination showed that carbamoylcholine chloride, a parasympathic stimulator of saliva production, was involved. In light of these observations, we tested the hypothesis that intraperitoneal (IP) injection of carbamoylcholine chloride induces a rapid, increased IL6 response in the nasal cavity of C57BL/6 mice.

Materials and Methods

Reagents. We prepared 0.01 M sodium phosphate buffer (pH 7.2) containing 0.14 M sodium chloride (PBS) by using pyrogen-free reagent water (Lonza Walkersville, Walkersville, MD). Representative samples of this preparation contained 0.0017 ± 0.0003 (mean ± standard error of the mean [SEM]; n = 3) endotoxin units per milliliter (QCL-1000 Chromogenic Limulus Amebocyte Lysate Assay, Lonza Walkersville). A 100-μg/ml solution of carbamoylcholine chloride (Sigma Chemical, St Louis, MO) was prepared by using 0.1 M PBS (pH 7.2) and contained 0.032 ± 0.007 endotoxin units per milliliter (n = 6); 0.1 ml of this solution was used to stimulate saliva secretion. The endotoxin concentrations of these solutions are below the United States Pharmacopeia limits for common injectables of 0.25 EU/ml.[4]

Mice. We used 115 adult female C57BL/6 mice (*Mus musculus domesticus*; age, 5 to 7 mo; Charles River Breeding Laboratories, Willingham, MA) throughout the study.

Husbandry and health status. Mice were housed in The University of Iowa Medical Laboratory rodent facility. The facility has been accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, International, since November 1994; is a registered research facility with the United States Department of Agriculture (no. 42-R-0004); and has a Public Health Services Animal Welfare Assurance (A3021-01) on file. All mice were handled humanely according to The University of Iowa Animal Care and Use protocol number 0404050.

All mice were housed in ventilated microisolation caging (Thoren Caging Systems, Hazelton, PA) in groups of 5 and fed a conventional rodent diet. Sterilized water (0.2-μm filter) was provided by an automatic watering system (Edstrom Industries, Waterford, WI).

C3H/HEJ sentinel mice are used in the rodent facility health monitoring program which uses 1 cage of sentinel mice per 60 to 70 cages in a dirty-bedding system; sentinels are not exposed directly to monitored colonies. Overall health status is assessed quarterly in November (clinical testing), February (comprehensive testing), May (clinical testing), and August (comprehensive serology with inhouse parasitology exam). The clinical testing program monitors the serologic status of mice for murine hepa-

Received: 8 Jan 2007. Revision requested: 1 Feb 2007. Accepted: 11 Apr 2007.
[1]Department of Periodontics and [2]Dows Institute for Dental Research, College of Dentistry, and [3]Department of Biostatistics, College of Public Health, The University of Iowa, Iowa City, IA.
[*]Corresponding author. Email: kim-brogden@uiowa.edu

titis virus, Sendai virus, Theiler murine encephalomyelitis virus, reovirus 3, mouse parvovirus, minute virus of mice, and mouse rotavirus. The comprehensive testing program monitors the serologic status of mice for mouse hepatitis virus, Sendai virus, pneumonia virus of mice, reovirus 3, Theiler murine encephalomyelitis virus, *Ectromelia*, mouse adenovirus types 1 and 2, polyoma virus, *Mycoplasma pulmonis*, mouse parvovirus, minute virus of mice, mouse rotavirus, and lymphocytic choriomeningitis virus; uses PCR for detection of *M. pulmonis*; assays for internal and external parasites by examination of pelage and tape tests and microscopic examination of all cecal contents; and cultures for microbiologic pathogens in the nasopharynx and cecum. Serologic results from sentinel mice are posted on the website of the University of Iowa Office of Animal Resources.

Helicobacter spp. has been detected in this facility, but routine testing for these bacteria is no longer performed. Mouse parvoviruses (minute virus of mice, mouse parvovirus) are endemic in this facility (screened quarterly). *Pasteurella pneumotropica* has been detected in this facility (screened yearly), and norovirus was detected in several rooms in this facility in May 2006, the first time this facility was checked for this pathogen. Results from sentinel mice are posted on the website of the University of Iowa Office of Animal Resources.

Collection of samples. We injected 88 mice IP with 0.1 ml of 0.1 M PBS (pH 7.2) containing 100 µg/ml carbamoylcholine chloride, and 27 mice were given an IP injection of 0.1 ml of 0.1 M PBS (pH 7.2) only. Saliva was collected with a pipetter fitted with a 200-µl plastic tip and centrifuged in a microfuge at 2300 × *g* for 10 min to pellet cells and debris. The saliva supernatant was removed and stored at –80 °C without inhibitors.

All 115 mice then were euthanized by carbon dioxide overdose. After euthanasia, blood was collected from the subclavian vein and centrifuged in a microfuge at 2300 × *g* for 10 min.[10,12] Serum was removed and stored at –80 °C without inhibitors.

The nasal cavity was washed with 200 µl saline, and the NWF was collected with a pipetter fitted with a 20 µl plastic tip and centrifuged in a microfuge at 2300 × *g* for 10 min to pellet cells and debris. The NWF supernatant was removed and stored at –80 °C without inhibitors.

The trachea was exposed, and the respiratory tract was washed 2 times with 200 µl saline; the BALF aliquots were combined and centrifuged in a microfuge at 2300 × *g* for 10 min to pellet cells and debris. The BALF supernatant was removed and stored at –80 °C without inhibitors.

Cytokine production. We determined the concentrations (pg/ml) of 10 cytokines in all saliva supernatant, serum, NWF supernatant, and BALF supernatant samples of all 115 mice. Concentrations of Th1 cytokines (IL2, IL12[p70], and IFNγ), Th2 cytokines (IL4, IL5, and IL10), GM-CSF, and proinflammatory cytokines (IL1β, IL6, TNFα) in saliva supernatants, serum, NWF supernatants, and BALF supernatants were determined by use of a multiplex fluorescent bead-based immunoassay (Kit 48-004, Upstate Biotechnology, Lake Placid, NY). Samples were incubated with antimouse multicytokine beads at 4 °C for 18 h. Unbound material was removed by filtration. Antimouse multicytokine biotin reporter was added, and reactions were incubated at room temperature for 1.5 h in the dark. Streptavidin–phycoerythrin then was added, and the plates were incubated at room temperature for 30 min. Stop solution was added, and the plates were read in the plate reader (model 100 IS, Luminex, Austin, TX). Concentrations of cytokines in each sample were extrapolated from those of standards by use of Beadview software (Upstate Biotechnology).

Experimental design. To test the hypothesis that concentrations of IL6 in NWF supernatants are correlated with time after IP injection of carbamoylcholine chloride, 11 mice were euthanized at 1, 2, 3, or 4 h after injection. To further characterize the phenomena and to determine the duration of the IL6 response in NWF and BALF supernatants, 54 mice were divided into 2 groups; 27 mice were injected IP with 0.01 M PBS (pH 7.2), whereas the remaining 27 were injected IP with carbamylcholine chloride in 0.01 M PBS (pH 7.2). At 0, 1, 3, 6, 12, 24, 48, 72, and 96 h after injection, 3 mice from each group were euthanized for collection of serum, NWF supernatants, and BALF supernatants. To determine whether carbamoylcholine chloride alters the concentrations of IL6 and 9 other cytokines, 50 C57BL/6 mice each received 10 µg carbamoylcholine chloride by IP injection and were euthanized immediately thereafter for collection of saliva, NWF, BALF, and serum for determination of cytokine levels (GM-CSF, IFNγ, IL1β, IL2, IL4, IL5, IL6, IL10, IL12[p70], and TNFα). These results were compared with cytokine concentrations previously reported in the literature.

Statistical analysis. The trend between IL6 response (y_t) and time after IP injection of carbamoylcholine chloride (t) was tested by calculating the Spearman correlation coefficient (r_s) for the data pairs (t,y_t) and determining whether the correlation coefficient was significantly different from 0. A logistic growth model was fit to the data pairs (t,y_t) to characterize the relationship between IL6 response (y_t) and elapsed time after IP injection (t). The model was fit so that the estimated mean response at the initial time, $t = 0$ h, and at the maximal time, $t = 4$ h, would correspond to the sample mean response at these 2 times. The Gauss–Newton algorithm was used.

The distribution of the concentrations of cytokines in saliva supernatants, NWF supernatants, BALF supernatants, and serum was summarized by using the first quartile (Q_1, the 25th percentile), median (M, the 50th percentile), third quartile (Q_3, the 75th percentile), and interquartile range ($IQR = Q_3 - Q_1$). These statistics were used to construct box plots (or box-and-whisker plots) of the samples. Box plots are a convenient graphic tool for illustrating and comparing skewed data distributions. In a box plot, the top and bottom edges of a split box represent the third quartile and first quartile of the sample, respectively. The split in the box represents the median. The height of the box corresponds to the interquartile range. Line segments ('whiskers') extend from the top edge of the box to the maximal value and from the bottom edge to the minimal value. However, if either line segment exceeds 1.5 × *IQR* in length, it is trimmed so that it only extends to the most extreme value contained within the interval

$$Q_1 - (1.5 \times IQR) \text{ to } Q_3 + (1.5 \times IQR).$$

In this case, data values falling outside this interval are plotted with special symbols and regarded as outliers.

All analyses were conducted using SAS (version 9.1, SAS Institute, Cary, NC). PROC NLIN was used to fit the logistic growth model.

Results

Carbamoylcholine chloride is used routinely to increase the volume and flow of saliva for collection.[7,10,12] In our preliminary work, we assessed the IL6 response in NWF supernatants of 5 mice per cage daily for 5 d (n = 25 mice). We observed an unexplained variation in the concentrations of IL6 in the NWF su-

Table 1. IL6 concentrations in 50 C57BL/6 mice given 10 µg carbamoylcholine chloride by IP injection

		IL6 concentration (pg/ml)			
Mouse No.	Day	Saliva	Serum	NWF	BALF
Replication 1					
1, 2, 3, 4, 5	1	36 (12)	71 (47)	4414 (2198)	237 (189)
6, 7, 8, 9, 10	2	50 (35)	22 (12)	140 (81)	61 (6)
11, 12, 13, 14, 15	3	32 (4)	53 (26)	3902 (1035)	426 (363)
16, 17, 18, 19, 20	4	64 (31)	31 (14)	250 (318)	58 (20)
21, 22, 23, 24, 25	5	35 (5)	27 (9)	123 (66)	55 (10)
Replication 2					
30, 31, 33, 38, 45	1	10 (10)	64 (30)	51 (23)	17 (12)
42, 51, 52, 56, 59	2	9 (4)	53(40)	46 (28)	33 (35)
27, 37, 47, 54, 57	3	8 (5)	72 (33)	39 (20)	18 (4)
26, 36, 39, 40, 60	4	7 (5)	48 (26)	1797 (2006)	118 (126)
29, 32, 43, 49, 55	5	10 (12)	53 (27)	3313 (1861)	215 (239)

Day-to-day variations were seen in the concentrations of IL6 in the NWF supernatants and BALF supernatants on days 1 and 3 (replication 1) and days 4 and 5 (replication 2).
Data are given as: mean IL6 concentration of 5 mice (standard deviation).

pernatants of 10 mice from 2 cages on days 1 and 3 (Table 1). We then assessed the IL6 response in NWF supernatants of 5 mice randomly selected from 5 cages for 5 d (n = 25 mice). Again, we observed an unexplained variation in the concentrations of IL6 in the NWF supernatants of 10 mice selected on days 4 and 5 (Table 1). Close examination of the procedures used during these days suggested that a 2- to 3-h delay occurred between the IP injection of carbamoylcholine chloride for the collection of saliva and the euthanasia of mice for the collection of serum, NWF, and BALF and might be related to the elevated IL6 levels.

To test the hypothesis that increased concentrations of IL6 in NWF supernatants are correlated with an increase in time after IP injection of carbamoylcholine chloride, 11 C57BL/6 mice each received 10 µg carbamoylcholine chloride by IP injection and euthanized at 0, 1, 2, 3 and 4 h after injection. IL6 concentrations in the NWF supernatants significantly increased with time after IP injection of carbamoylcholine chloride. Based on the Spearman correlation coefficient, the trend was statistically significant (r_s = 0.7733, P = 0.0052).

A logistic growth model that characterizes this rapid increase is shown in Figure 1. The functional form of the fitted model is given by

$$\hat{y}_t = \frac{3967}{1 + 310.2 \exp(-3.317t)},$$

where $\hat{y}_t$= represents the estimated mean IL6 response based on an elapsed time of t h after IP injection.

To determine the duration of the IL6 response in NWF supernatants and BALF supernatants, mice were divided into 2 groups, given either an IP injection of 0.01 M PBS (pH 7.2) or carbamylcholine chloride in PBS, and euthanized at 0, 1, 3, 6, 12, 24, 48, 72, and 96 h after injection for collection of serum, NWF supernatants, and BALF supernatants. At 3 h, IL6 concentrations in NWF supernatants of mice that received carbamylcholine chloride started to increase and stayed elevated for 24 h above the IL6 concentrations in the NWF supernatants of control mice receiving PBS (Figure 2). IL6 concentrations in the NWF supernatants of mice receiving carbamylcholine chloride returned to the baseline concentrations in the NWF supernatants of control mice at 48, 72, and 96 h. At 3 h, IL6 concentrations in BALF supernatants of mice receiving carbamylcholine chloride also started to increase and stayed elevated above the IL6 concentrations in the BALF supernatants of control mice receiving PBS (Figure 3). IL6 was not induced by 0.01 M PBS (pH 7.2) at any time. None of 9 other cytokines (for example, GM-CSF, IFNγ, IL1β, IL2, IL4, IL5, IL10, IL12[p70], and TNFα) were elevated in mice receiving an IP injection of either 0.01 M PBS (pH 7.2) or carbamylcholine chloride in PBS (data not shown), suggesting that the induction of IL6 was unique. Median concentrations of these 9 cytokines in NWF supernatants ranged from 2 to 32.0 pg/ml (mean concentrations ranged from 2 to 33 pg/ml).

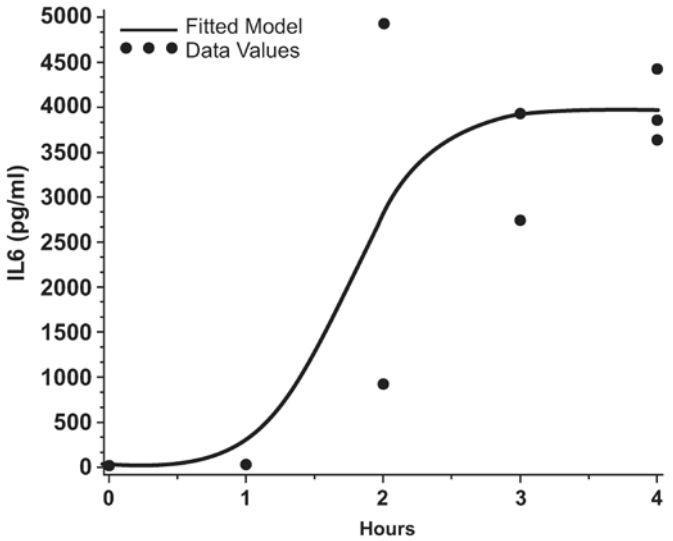

Figure 1. A model showing the increase in IL6 concentrations in the nasal wash fluid supernatants of mice. Eleven C57BL/6 mice each received 10 µg carbamoylcholine chloride by IP injection and were euthanized at 0, 1, 2, 3, and 4 h.

To determine whether carbamoylcholine chloride alters the concentrations of 9 other cytokines (and IL6 in saliva and serum), 50 C57BL/6 mice each received 10 µg carbamoylcholine chloride

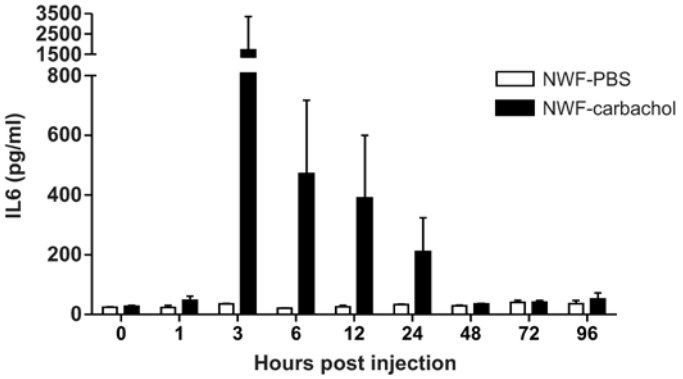

Figure 2. A comparison of IL6 responses induced in the nasal wash fluid supernatants of mice given carbamoylcholine chloride or 0.01 M PBS (pH 7.2) by IP injection. Twenty-seven C57BL/6 mice each received PBS, and 27 mice each received 10 µg carbamoylcholine chloride in PBS. At 0, 1, 3, 6, 12, 24, 48, 72, and 96 h after injection, 3 mice from each group were euthanized for collection of nasal wash fluid.

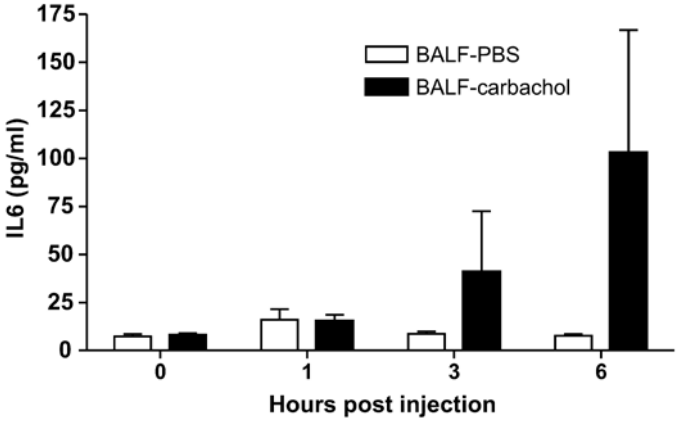

Figure 3. A comparison of IL6 responses induced in the bronchoalveolar lavage fluid supernatants of mice given carbamoylcholine chloride or 0.01 M PBS (pH 7.2) by IP injection. Twenty-seven C57BL/6 mice each received PBS, and 27 mice each received 10 µg carbamoylcholine chloride in PBS. At 0, 1, 3, and 6 h after injection, 3 mice from each group were euthanized for collection of bronchoalveolar lavage fluid.

by IP injection and immediately were euthanized for collection of saliva, NWF, BALF, and serum for determination of cytokines. The basal levels of these cytokines are shown in Figures 4 through 7. Median concentrations of these 9 cytokines in saliva supernatants ranged from 2.3 to 36.0 pg/ml (mean concentrations ranged from 6.1 to 31.3 pg/ml; Figure 4). Median concentrations of these 9 other cytokines in serum ranged from 4.6 to 55.0 pg/ml (mean concentrations ranged from 7.4 to 231.3 pg/ml; Figure 5). Three mice had elevated levels of GM-CSF (2280, 5000, and 328 pg/ml), IL1β (2040, 52, and 348 pg/ml), and IL2 (2020, 4820, and 218 pg/ml), likely skewing these results slightly. Median concentrations of these 9 cytokines in NWF supernatants ranged from 2.3 to 45.1 pg/ml (mean concentrations ranged from 4.3 to 57.0 pg/ml; Figure 6). Median concentrations of these 9 cytokines in BALF supernatants ranged from 2.5 to 34.9 pg/ml (mean concentrations ranged from 8.0 to 32.5 pg/ml; Figure 7). Variations in IL6 were seen in the NWF supernatants (14 to 8240 pg/ml) and BALF supernatants (5 to 918 pg/ml) of these 50 mice (Table 1).

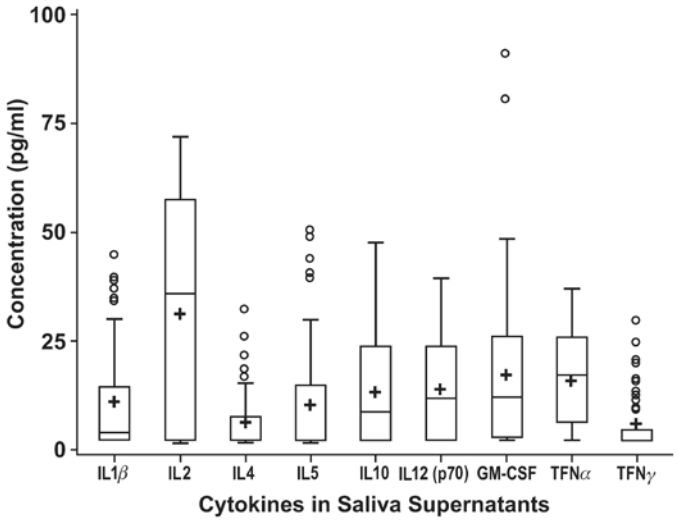

Figure 4. Box plots showing the concentrations of cytokines in murine saliva supernatants from mice. Fifty C57BL/6 mice each received 10 µg carbamoylcholine chloride by IP injection for collection of saliva. Median cytokine concentrations in saliva supernatants ranged from 2.3 to 36.0 pg/ml. Mean cytokine concentrations are indicated with +.

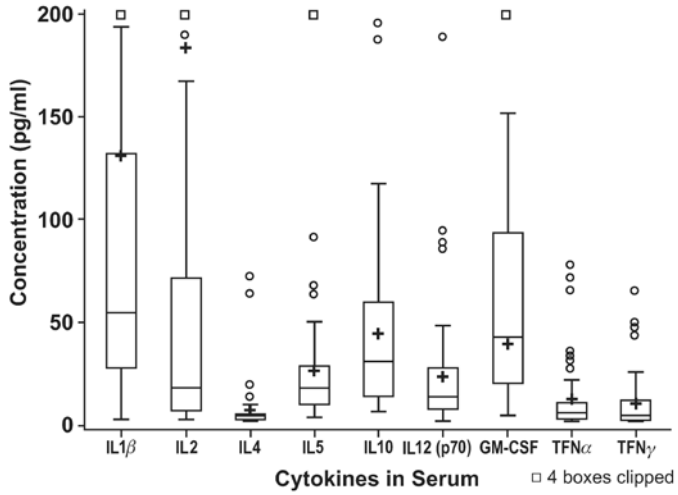

Figure 5. Box plots showing the concentrations of cytokines in murine serum from mice. Fifty C57BL/6 mice each received 10 µg carbamoylcholine chloride by IP injection and immediately were euthanized for collection of blood. Median cytokine concentrations in serum ranged from 4.6 to 55 pg/ml. Mean cytokine concentrations are indicated with +. Note that the plots for IL1β, IL2, IL5, and GM-CSF are clipped at the top, due to extremely large values in these samples.

Discussion

We wanted to establish baseline concentrations of cytokines in the saliva supernatants, serum, NWF supernatants, and BALF supernatants of normal C57BL/6 mice for extended work on the ability of defensins to induce innate and adaptive immune responses.[2] However, during the course of these studies, we observed a unique phenomenon: IP injection of carbamoylcholine chloride used to increase the flow and volume of saliva induced a rapid and elevated IL6 response in the nasal cavity of C57BL/6 mice. More importantly, the rapid production of high concen-

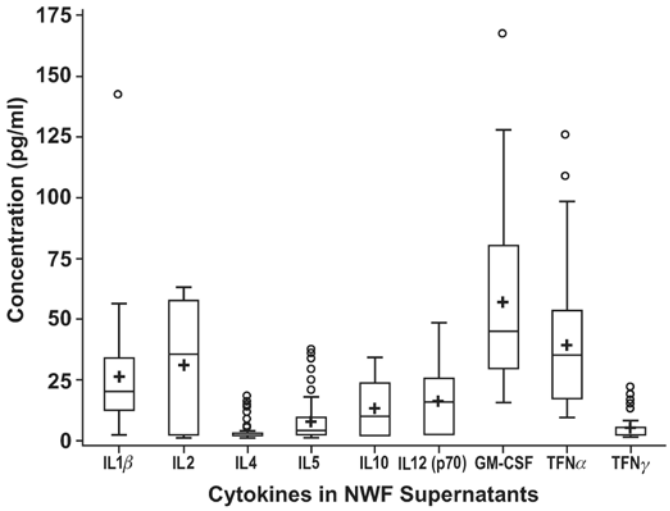

Figure 6. Box plots showing the concentrations of cytokines in murine nasal wash fluid supernatants from mice. Fifty C57BL/6 mice each received 10 µg carbamoylcholine chloride by IP injection and immediately were euthanized for collection of nasal wash fluids. Median cytokine concentrations in nasal wash fluid supernatants ranged from 2.3 to 45.1 pg/ml. Mean cytokine concentrations are indicated with +.

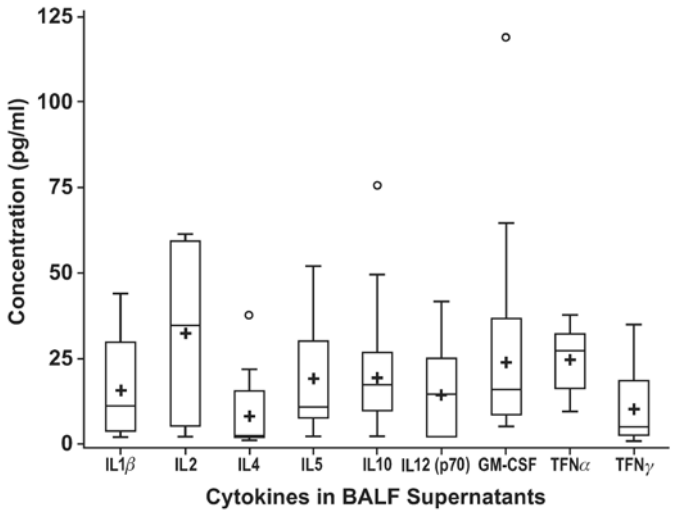

Figure 7. Box plots showing the concentrations of cytokines in murine bronchoalveolar lavage fluid supernatants from mice. Fifty C57BL/6 mice each received 10 µg carbamoylcholine chloride by IP injection and immediately were euthanized for collection of bronchoalveolar lavage fluid. Median cytokine concentrations in bronchoalveolar lavage fluid supernatants ranged from 2.5 to 53.5 pg/ml. Mean cytokine concentrations are indicated with +.

Table 2. Reported basal levels of cytokines in serum and the bronchoalveolar lavage fluid (BALF) of mice.

Cytokine	Fluid	Strain of mice	Gender	Concentration (pg/ml)	Reference
IL1β	Serum	C57BL/6	Female	98.7 (9.2)	9
	Serum	CD1	Male	113.8 (4.2)	8
IL2	Serum	C57BL/6	Female	25.2 (1.0)	9
	Serum	CD1	Male	9.4 (1.5)	8
IL4	Serum	CD1	Male	0.1 (0.0)	8
IL5	Serum	C57BL/6	Female	2.7 (2.7)	9
	Serum	CD1	Male	4.8 (0.6)	8
IL6	Serum	C57BL/6	Female	48.2 (9.4)	9
	Serum	CD1	Male	10.2 (2.8)	8
	BALF	C3HeB/FeJ	Unknown	1.3 (1.3)	11
IL10	Serum	C57BL/6	Female	15.0 (12.6)	9
	Serum	CD1	Male	16.0 (4.0)	8
IL12[p70]	Serum	CD1	Male	76.9 (10.8)	8
	BALF	C3HeB/FeJ	Unknown	14.3 (2.7)	11
TNFα	Serum	C57BL/6	Female	75.4 (20.4)	9
	Serum	CD1	Male	224.8 (27.0)	8, 9
	BALF	C3HeB/FeJ	Unknown	10.7 (6.0)	11
IFNγ	Serum	C57BL/6	Female	13.0 (0.5)	9
	Serum	CD1	Male	388.7 (29.3)	8
	BALF	C3HeB/FeJ	Unknown	20.0 (8.5)	11
GM-CSF	Serum	CD1	Male	30.8 (2.4)	8
	BALF	C3HeB/FeJ	Unknown	5.7 (2.5)	11

Data are given as: mean concentration (standard deviation).
Female mice were in diestrous stage.

trations of IL6 was induced primarily in the nasal cavity, and in the lungs to a lesser extent, but not in the saliva supernatants or serum. The reason for this compartmentalized response is not yet known. Carbamoylcholine chloride is a parasympathomimetic drug that mimics the action of acetylcholine.[3] Acetylcholine stimulates 'muscarinic' receptors, which are found in many organs in the body.[11] Stimulation of muscarinic receptors in the nose leads to hypersecretion and vasodilation. However, the mechanism of

increased IL6 in NWF fluid induced by carbamoylcholine chloride needs further study.

This study also extensively characterized the basal profiles of 9 cytokines in saliva supernatants, NWF supernatants, and BALF supernatants of mice by using multiplex immunoassays. Of the 50 mice tested, 47 had varied levels of IL1β, IL2, IL4, IL5, IL10, IL12(p70), GM-CSF, IFNγ, and TNFα in their saliva supernatants, serum, NWF supernatants, and BALF supernatants (Figures 4 to 7). Three mice had elevated levels GM-CSF, IL1β, and IL2 in their serum, and 20 mice had elevated concentrations of IL6 in their NWF supernatants (Table 1). Whether these differences are due to a preexisting condition or administration of the carbamoylcholine chloride is not known. There was no indication that these mice showed any clinical signs of being sick during this study, nor did they appear lethargic, have ruffled fur, or huddle together with littermates.

Cytokine levels reported in other studies are very similar to those seen in our study (Table 2). For example, similar ranges of murine cytokines have been seen in seminal fluid;[6,9] serum;[1,6,9] and BALF.[5]

In summary, these results demonstrate that carbamylcholine chloride can induce a rapid, elevated IL6 response in the nasal cavity of C57BL/6 mice as early as 1 h after IP injection. NWF should be collected within 1 h if carbamylcholine chloride is used to collect saliva in mice for studies assessing the concentrations of proinflammatory cytokines including IL6.

Acknowledgments

This work was supported by funds from grant number R01 DE014390 from the National Institute of Dental and Craniofacial Research, National Institutes of Health. We are grateful to James D Herd for preparation of the figures and to Elizabeth A Schmitt (Cairn Communications, Mahtomedi, MN) for critically reading the manuscript.

References

1. **Bobrowski WF, McDuffie JE, Sobocinski G, Chupka J, Olle E, Bowman A, Albassam M.** 2005. Comparative methods for multiplex analysis of cytokine protein expression in plasma of lipopolysaccharide-treated mice. Cytokine **32:**194–198.
2. **Brogden KA, Heidari M, Sacco RE, Palmquist D, Guthmiller JM, Johnson GK, Jia HP, Tack BF, McCray PB.** 2003. Defensin-induced adaptive immunity in mice and its potential in preventing periodontal disease. Oral Microbiol Immunol **18:**95–99.
3. **Budavari S, O'Neil MJ, Smith A, Heckelman PE, Kinneary JF.** 1996. Merck research laboratories division of Merck and company. Whitehouse Station (NJ): The Merck Publishing Group.
4. **Food and Drug Administration, Department of Health, Education, and Welfare.** 1985. ITG subject: bacterial endotoxins/pyrogens. inspector's technical guide, vol 40. Washington (DC): Department of Health and Human Services, Public Health Service.
5. **George CL, White ML, Kulhankova K, Mahajan A, Thorne PS, Snyder JM, Kline JN.** 2006. Early exposure to a nonhygienic environment alters pulmonary immunity and allergic responses. Am J Physiol Lung Cell Mol Physiol **291:**L512–L522.
6. **Gopichandran N, Ekbote UV, Walker JJ, Brooke D, Orsi NM.** 2006. Multiplex determination of murine seminal fluid cytokine profiles. Reproduction **131:**613–621.
7. **Katz J, Ward DC, Michalek SM.** 1996. Effect of host responses on the pathogenicity of strains of *Porphyromonas gingivalis*. Oral Microbiol Immunol **11:**309–318.
8. **Legrand N, Weijer K, Spits H.** 2006. Experimental models to study development and function of the human immune system in vivo. J Immunol **176:**2053–2058.
9. **Orsi NM, Gopichandran N, Ekbote UV, Walker JJ.** 2006. Murine serum cytokines throughout the estrous cycle, pregnancy, and postpartum period. Anim Reprod Sci **96:**54–65.
10. **Russell MW, Wu HY.** 1991. Distribution, persistence, and recall of serum and salivary antibody responses to peroral immunization with protein antigen I/II of *Streptococcus mutans* coupled to the cholera toxin B subunit. Infect Immun **59:**4061–4070.
11. **White MV.** 1995. Muscarinic receptors in human airways. Allergy Clin Immunol **95:**1065–1068.
12. **Wu HY, Russell MW.** 1993. Induction of mucosal immunity by intranasal application of a streptococcal surface protein antigen with the cholera toxin B subunit. Infect Immun **61:**314–322.