Letter to the Editor

Occupational endotoxin exposure and a novel luminol-enhanced chemiluminescence assay of nasal lavage neutrophil activation

To the Editor:

Methods to assess environmental endotoxin exposures might not accurately reflect personal exposure, and personal biomarkers of endotoxin would be valuable in determining inhaled endotoxin exposures. A method to detect endotoxin in human blood has been reported based on neutrophil-dependent chemiluminescence,[1] and a modification to use the assay with nasal neutrophils from subjects who have been exposed to endotoxin demonstrated increased activation in response to exogenous LPS *in vitro*.[2] The current study (which was approved by our research ethics board) evaluated the assay as a biomarker of *in vivo* nasal exposure to endotoxin and dusts in 33 animal care workers (ACWs; 70% women; mean age, 32.5 ± 9 years; 1 current smoker) compared with 20 nonexposed control subjects (85% women; mean age, 36.4 ± 9 years; 1 current smoker). ACW exposures included mice (100% of workers), rats (52%), guinea pigs (24%) and rabbits (20%). Atopy (as determined based on a positive skin prick test response to ≥1 of 13 common aeroallergen extracts) was not significantly different between groups (14 [54%] of 26 ACWs tested [4 with positive test results to a laboratory animal extract] and 25% of control subjects, $P = .07$). Histories of asthma (ie, cough, shortness of breath, and chest tightness) and allergy were slightly but not significantly more common in the exposed group than in the control subjects (21% vs 10% with asthma and 58% vs 45% with a self-reported history of allergy). Daily nasal symptoms were reported in 52% of ACWs and 30% of control subjects ($P = .16$). Seventy percent of ACWs had worked in this job for less than 3 years, but almost all (93%) reported cleaning at least 50 animal cages per day. Twenty-one percent of ACWs reported worsened nasal or chest symptoms at work.

Nasal lavage (NAL) in ACWs was performed (1) at baseline on a Monday morning before workplace contact with animals; (2) before exposure but later during the week just before the animal cages were changed; and (3) immediately at the end of the same cage-changing day (after exposure). The NAL procedure was modified from that described previously.[3] Briefly, 5 mL of warmed normal saline solution (0.9%) was instilled into each nostril with a syringe while the volunteer tilted his or her head backward and did not breathe or swallow. The solution was retained in the nasopharyngeal region by means of palatal pressure for 10 seconds, after which the volunteer expelled the nasal lavage fluid (NALF) into a sterile 50-mL plastic centrifuge tube, and the sample was immediately transferred to a 15-mL plastic centrifuge tube for centrifugation (1100*g* for 15 minutes at 15°C; Eppendorf 5810R refrigerated centrifuge [Eppendorf, Hamburg, Germany]). The supernatant was isolated to measure endotoxin concentration by using the Pyrochrome LAL assay (Associates of Cape Cod, Inc, East Falmouth, Mass), according to the manufacturer's protocol. The white cells and aliquots of the remaining supernatant were frozen at −80°C. Endotoxin was measured in NALF supernatants with the Pyrochrome LAL assay. Total protein was measured in NAL samples with the Bradford assay.[4,5] All samples were collected in a clean environment away from the animal facility to avoid contamination of the samples with endotoxin. Venous blood samples were collected at the time of the ACWs' first and third NAL samplings. Control subjects had nasal and blood samples collected on 2 occasions, on the morning and afternoon of a single day, at times similar to the collections from the ACWs.

The blood samples were drawn in K_2EDTA Vacutainer collection tubes (BD, Oakville, Ontario, Canada) and then transported to the laboratory at ambient temperature for immediate Endotoxin Activity assay.[6] Cell composition in the collected NALF and blood samples was determined by using a Cell-Dyn Model 3500 (Abbott Diagnostics, Abbott Park, Ill). Neutrophil activation was measured in both NALF and blood samples by using the luminol-enhanced chemiluminescence assay (Fig 1). Measurement of cytokines (IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, GM-CSF, IFN-γ, and TNF-α) in the NALF samples was performed with a multiplex bead-based cytokine panel (Bio-Plex Cytokine Assay; Bio-Rad Laboratories, Hercules, Calif) on a Luminex 100TM system (Invitrogen, Toronto, Ontario, Canada).

Air sampling was performed by using both area and personal sampling of ACWs during cage-changing activities. Sampling filters were stored at −80°C, extracted according to previously described methods,[7,8] and analyzed as a batch by using the Pyrochrome LAL assay.

Statistical analysis consisted of Pearson χ^2 tests or Fisher exact tests (where required) for categorical variables and *t* tests or Wilcoxon tests for continuous variables. All statistical analyses were conducted with SAS (version 9.2; SAS Institute, Inc, Cary, NC)

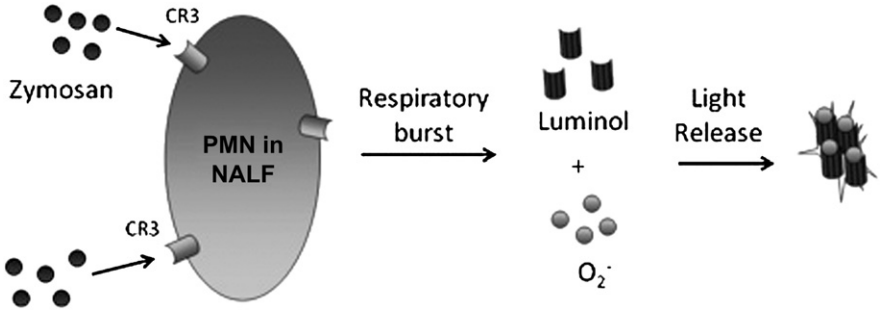

FIG 1. Chemiluminescence in nasal lavage fluid *(NALF)* samples. Zymosan binds activated CR3 receptors on a polymorphonuclear neutrophil *(PMN)*, resulting in a respiratory burst releasing reactive oxygen species. Oxygen radicals then combine with luminol, causing light release, which is measured with a chemiluminometer.

TABLE I. Blood and nasal lavage results

	ACWs (n = 33)		Control subjects (n = 20)		*P* value
	No.*	Median (IQR)	No.	Median (IQR)	ACWs vs control subjects
Blood neutrophil count (10^9/L)					
Baseline	32	3.1 (2.4-4.8)	—	—	
Before exposure	20	2.8 (2.4-3.9)	20	3.2 (2.8-3.6)	.62
After exposure	30	3.7 (2.8-5.3)	20	3.9 (3.6-5.2)	.48
Blood endotoxin (Endotoxin Activity assay)					
Baseline	30	0.40 (0.31-0.47)	—	—	
Before exposure	21	0.39 (0.32-0.45)	20	0.42 (0.23-0.49)	.76
After exposure	31	0.37 (0.29-0.52)	20	0.41 (0.28-0.59)	.42
NAL neutrophil count (10^9/L)					
Baseline	31	0.005 (0.003-0.030)	—	—	
Before exposure	29	0.011 (0.004-0.030)	20	0.050 (0.016-0.085)	.005
After exposure	31	0.003 (0.002-0.017)	20	0.050 (0.020-0.070)	.0003
NALF endotoxin (EU/mL)					
Baseline	31	0.23 (0.12-0.63)	—	—	
Before exposure	32	0.37 (0.12-0.76)	20	0.61 (0.44-0.96)	.04
After exposure	32	0.29 (0.12-0.71)	20	0.61 (0.38-1.16)	.03
NAL neutrophil activation (RLU/neutrophil)					
Baseline	30	19.8 (8.9-106.5)	—	—	
Before exposure	28	27.3 (11.7-65.1)	19	7.3 (1.9-36.9)	.006
After exposure	31	77.8 (14.3-120.0)	20	5.4 (2.4-31.5)	.0002
NALF IL-8 (pg/mL^3)					
Baseline	29	74.0 (27.3-134.5)	—	—	
Before exposure	30	82.9 (52.6-184.3)	18	74.5 (46.2-201.7)	.73
After exposure	31	54.9 (32.7-90.0)	19	83.9 (39.3-142.3)	.38
NALF total protein (mg/mL)					
Baseline	28	0.021 (0.012-0.050)			
Before exposure	30	0.016 (0.010-0.31)	18	0.011 (0.007-0.031)	.54
After exposure	31	0.020 (0.011-0.041)	19	0.012 (0.009-0.030)	.16

Blood and NAL results are expressed as medians (interquartile ranges). There were no significant statistical differences from baseline (ACWs only) or preshift to postshift value within groups. Among ACWs, for the baseline test, the time since the last work exposure or days free of occupational exposure was 2 days in 27 workers, 3 days in 3 workers, 4 days in 2 workers, and more than 5 days in 1 worker. Among ACWs, for the baseline test, the time since any animal exposure was 0 to 3 hours in 13 workers, 4 to 16 hours in 3 workers, and more than 16 hours in 17 workers.

*Number of samples that could be analyzed: not all samples could provide all results.

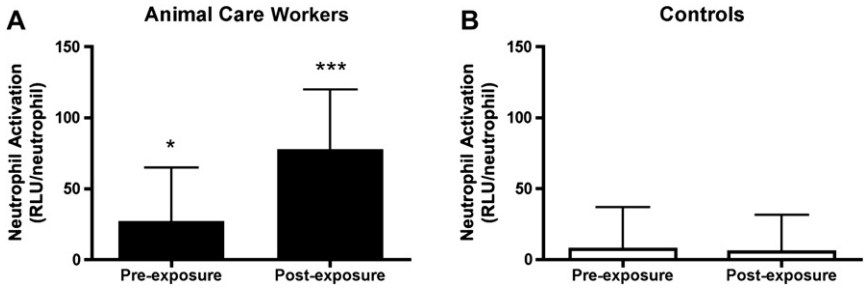

FIG 2. Median NAL neutrophil activation in control subjects **(A)** and ACWs **(B)** obtained before and after exposures. NAL neutrophil activation did not change between the pre-exposure and postexposure periods, but results were significantly different between control subjects and ACWs. *$P = .006$ and ***$P = .0002$ versus control subjects at the same time point.

and GraphPad Prism 4 (GraphPad Software, Inc, San Diego, Calif) software.

Laboratory results are shown in Table I. There was a significant increase in both preshift and postshift neutrophil activation in NAL samples from ACWs versus that seen in control subjects ($P = .006$ and $P = .0002$, respectively; Fig 2). The increased levels within the ACWs did not differ significantly throughout the week, despite a 2-day weekend off work, and did not differ significantly among subgroups with atopy or rhinitis. Interestingly, NAL neutrophil counts were lower before and after shifts in ACWs compared with those seen in control subjects at similar times ($P = .005$ and $P = .0003$), as were NALF endotoxin levels indicated by means of LAL assay ($P = .04$ and $P = .03$). NALF total protein concentrations were not significantly different in the ACWs from those seen in the control subjects, and although higher levels might have been expected in the ACWs,

differences in dilution did not clearly appear to entirely explain the lower cell counts and LAL results in this group. Other possible reasons for a lower NAL neutrophil count in the ACWs, which we could not explore further in this study, might include neutrophil margination, apoptosis, or both associated with endotoxin exposure or other components of the dust, or both. The greater proportion of atopic subjects among the ACWs than among the control subjects potentially might have resulted in more eosinophils and some reduction in neutrophil counts in NALF, but the total white cell counts were too low to provide an accurate NALF eosinophil count, and differential white cell counts were not obtained in NALF. Other measures did not differ significantly between or within groups.

Air-sampling results for endotoxin varied among the 3 facilities for area and personal sampling results: facility 1, area levels of 81 to 118 pg/m^3 and personal levels of 73 to 207 pg/m^3; facility 2, area levels of 38 to 1,060 pg/m^3 and personal levels of 260 to 1,360 pg/m^3; and facility 3, area levels of 260 to 1,226 pg/m^3 and personal levels of 1,670 to 2,660 pg/m^3. However, no significant differences in nasal neutrophil activation were found between facilities, perhaps reflecting a threshold effect with nonsignificant further increases or perhaps reflecting additional influencing exposures.

Our findings suggest that luminol-enhanced chemiluminescence in NALF might be a noninvasive personal biomarker of local endotoxin or other components of the dust in the exposure of ACWs. It is not affected by NALF dilution, unlike NAL cell counts and fluid assays, which are less useful at reflecting exposures. Environmental endotoxin exposures might not always accurately reflect personal exposure because workers generally wear respiratory protection, and there was no increase in this study in levels of the other personal biomarkers that might be expected to reflect endotoxin and other dust exposures. Similarly, although previous studies have investigated personal biomarkers of endotoxin exposure,[9] none have been able to successfully quantify inhaled endotoxin exposures in human NALF.[10,11] Our findings indicate that further investigation of the luminol-enhanced chemiluminescence assay of NAL neutrophil activation is warranted to determine whether this response is explained by endotoxin or by other dust components in this and other settings in which endotoxin exposure can occur.

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