Lipopolysaccharide augments IgG and IgE responses of mice to the latex allergen Hev b 5

Jay E. Slater, MD,[a] Elizabeth J. Paupore, BS,[a] Michael R. Elwell, DVM, PhD,[b] and Wava Truscott, PhD[c] *Washington, DC, Herndon, Va, and San Diego, Calif*

Background: LPS is a common contaminant in the health care environment and in latex examination gloves.
Objective: We sought to investigate the role of LPS in enhancing the immune responses of mice to inhaled latex allergen.
Methods: As our model allergen, we used a fusion protein containing the potent latex allergen Hev b 5. BALB/c mice were lightly anesthetized and given repeated intranasal doses of saline, LPS, and/or Hev b 5. The doses were given in 2 courses separated by a 6-week period, with the first course consisting of 6 doses and the second consisting of 3 doses.
Results: After the first set of immunizations, mice given Hev b 5 alone had no detectable IgG1 or IgE responses to Hev b 5, whereas mice given the antigen along with LPS had significant responses (IgG1, 0.73 U ± 0.05; IgE, 0.88 U ± 0.2). No enhancement of specific IgG2a was observed. A stimulatory effect of LPS on all 3 immunoglobulin types was apparent after the second course. Lymphocytes from mice immunized with LPS and Hev b 5 had increased proliferation to Hev b 5 and its fusion partner.
Conclusions: LPS may be an important immunoadjuvant for the development of allergic reactions to latex protein allergens. (J Allergy Clin Immunol 199;102:977-83.)

Key words: *Latex, endotoxin, lipopolysaccharide, mouse, IgE, IgG1, T_{H1}, T_{H2}*

Latex allergy is a major medical problem for health care providers, children with spina bifida, and children undergoing repeated surgical procedures. Latex allergens are present in many medical and consumer devices, but high-allergen powdered latex gloves have been implicated repeatedly as the most important source of exposure, both for the initial sensitization and subsequent clinical reactions. Although the degree and route of exposure have been suggested as possible risk factors for latex protein sensitization, this has not been experimentally or clinically verified, and other factors have not been systematically excluded.

Abbreviations used
HPF: High-power field
MBP: Maltose-binding protein
TBS-T: Tris-buffered saline with Tween

Recently, Williams and Halsey[1] have reported that bacterial LPS levels are elevated, sometimes markedly so, in latex gloves. This is important because LPS has emerged as a key signal in many of the responses of the mammalian immune system to gram negative infection, ranging from neutralizing antibodies to septic shock. In early studies, LPS was identified as the ubiquitous "pyrogen" in contaminated injectables that elicited febrile responses in recipients. Subsequent investigations indicated that LPS induces fever by causing the release of IL-1, or "endogenous pyrogen," from circulating monocytes and macrophages. Most recent investigations have confirmed that IL-1β is required for the inflammatory effects of LPS in mice.[2,3]

In addition, LPS is a B-cell mitogen and strong in vivo adjuvant. In particular, LPS was repeatedly observed to potentiate IgG1 and IgE responses to coinjected antigens.[4,5] The adjuvant properties of LPS are not a consequence of B-cell mitogenesis; athymic mice, which have normal B-cell responses to LPS, are unresponsive to the adjuvant effects of LPS.[4,6] LPS is a cosignal in the induction of immunoglobulin class switching to the IgE[7,8] and IgA[9] isotypes, and the high concentration of LPS in the gastrointestinal tract may be an essential in vivo signal to B cells within Peyer's patches.

In murine macrophages LPS appears to elicit the transcription of TNF-α, IL-1α, IL-1β, IL-6, IL-10, IL-12, and IFN-γ, as well as the release of TNF-α, IL-6, and IL-12 protein.[10,11] LPS acts in part by interacting with the membrane receptor CD14 and through the activation of NFκB, which can occur by CD14-dependent and independent mechanisms.[12] The production of IL-12 by murine macrophages stimulated with LPS occurs independent of T-cell interactions and is potentiated by IFN-γ.[13] IL-12, in turn, is a potent signal that favors the development of T_{H1}-type immune responses.

From [a]the Center for Molecular Mechanisms of Disease Research, Children's Research Institute, Children's National Medical Center, Washington; [b]Experimental Pathology Laboratories, Inc, Herndon; and [c]Safeskin Corporation, San Diego.
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Reprint requests: Jay E. Slater, MD, Laboratory of Immunobiochemistry, US Food and Drug Administration, Center for Biologics Evaluation and Research, 1401 Rockville Rd, Rockville, MD 20852-1448.

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However, in vivo studies suggest that LPS may elicit a broad range of immune responses, with evidence of both T_{H1} and T_{H2} activation. Industrial exposure to endotoxin has been associated with flu-like illnesses and asthma.[14] Among patients with asthma, the endotoxin content of the house dust is strongly correlated with the severity of their asthma.[15] In challenge studies asthmatic subjects experience bronchospasm when exposed to 20 μg of LPS by inhalation, whereas normal subjects experience no spirometric changes in spite of significant systemic neutrophilia and elevation in C reactive protein levels.[16-18] Bronchoalveolar lavage studies have confirmed that LPS inhalation leads to rapid recruitment of neutrophils into the lungs associated with increased recovery of TNF-α, IL-1β, and IL-8.[19]

Thus previous work has identified LPS as an important and ubiquitous proinflammatory agent. Although some in vitro studies indicate that LPS increases the generation of T_{H1}-type cytokines, in vivo work suggests that LPS may potentiate T_{H2}-type responses. Recent work suggests that latex gloves are frequently contaminated with significant amounts of LPS. In this study we examined the hypothesis that LPS could affect the immune responses of mice to a recombinant *Hevea* latex allergen, Hev b 5. In particular, we administered Hev b 5 and LPS to mice by the respiratory route and measured the specific Ig and lymphocyte proliferative responses.

METHODS

Hev b 5

The sequence cDNA Hev b 5 (839 bp) was cloned and expressed from a *Hevea brasiliensis* latex cDNA library as previously described (GenBank accession number U42640[20]). The sequence was expressed in the pMAL/c-2 plasmid (New England Biolabs) as part of a maltose-binding protein (MBP) fusion protein (rHev b 5/MBP). rHev b 5/MBP and MBP were biotinylated with N-hydroxysuccinimidobiotin as previously described.[21]

LPS

LPS from *Escherichia coli* serotype 0111:B4 was used in all experiments (Sigma).

Immunization

Mice were sensitized to Hev b 5/MBP in the presence or absence of LPS by nasal installation. Female BALB/c mice (6 to 8 weeks old) were anesthetized with methoxyflurane, and 25 μL of sterile saline, containing either Hev b 5/MBP (10 μg), LPS (10 μg), or both, was applied to the right or left naris and was rapidly aspirated by the mouse. Application was repeated every other day for 6 doses. After a 6-week rest period, 3 more doses were applied on alternate days. Mice were bled every 2 weeks from the tail vein or the retroorbital plexus. All procedures were approved by the institutional Animal Research Committee in accordance with American Association for Accreditation of Laboratory Animal Care guidelines.

We attempted to reduce the LPS content of the recombinant Hev b 5/MBP by using polymyxin B agarose chromatography (Sigma) and by extensive washing of immobilized Hev b 5/MBP with endotoxin-free buffer, without measurable success. The endotoxin content of the recombinant protein remained between 5 and 50 EU/μg protein (LAL ELISA, Bio Whittaker). Therefore we controlled for any effect of the contaminating endotoxin by adding an equivalent amount of LPS to all negative control cultures. The LPS preparation that we used had endotoxin activity equivalent to 25 EU/pg by limulus amebocyte lysate assay. Therefore we added 2 pg of LPS as a control for each microgram of Hev b 5/MBP added to the splenocyte cultures.

Antigen-specific IgG subclass ELISA

Polyvinyl chloride microtiter plates (Falcon Microtest III) were coated with rHev b 5/MBP (10 ng/well) overnight in 0.1 mol/L sodium bicarbonate and blocked with 1% BSA in 50 mmol/L Tris and 150 mmol/L NaCl (pH 7.4) containing 0.02% Tween 20 (dilution buffer). Serum was added in dilution buffer. After overnight incubation, wells were washed and incubated with, in series, biotinylated anti-mouse IgG1 or biotinylated anti-mouse IgG2a (1:1000 vol/vol, Binding Site) and peroxidase-labeled streptavidin (1:1000 vol/vol, Kirkegaard and Perry). After further washes, the wells were developed with TMB peroxidase substrate (Kirkegaard and Perry) and read at 450 nm. With each assay, a standard curve was generated by using serial dilutions of a polyclonal ascites fluid generated from BALB/c mice immunized with rHev b 5/MBP in Freund's adjuvants. The normalized titer was determined by comparison of the sample absorbance to the standard curve. One unit represents the absorbance extrapolated to undiluted standard serum.

To control for any contaminating LPS, parallel experiments were conducted in which the wells were coated with 2 fg or 0.05 EU/well LPS in bicarbonate. These control experiments were not performed to measure anti-LPS antibody (see below) but to control for the effect of contaminating LPS on the measurement of the anti-Hev b 5 response.

Antigen-specific IgE ELISA

IgE specific for rHev b 5 and for MBP were determined in assays identical to the total IgE ELISA except that biotinylated rHev b 5/MBP or biotinylated MBP (100 ng/well) were added instead of biotinylated anti-IgE. With each assay, a standard curve was generated by using serial dilutions of a polyclonal ascites fluid generated from BALB/c mice immunized with rHev b 5/MBP in alum. The normalized titer was determined by comparison of the sample absorbance to the standard curve. One unit represents the absorbance extrapolated to undiluted standard serum.

Total IgG1 ELISA

Polystyrene (Immulon 4) microtiter plates were coated with goat anti-mouse IgG1 (1:10,000 vol/vol; Sigma) overnight in 0.1 mol/L sodium bicarbonate and blocked with dilution buffer. Serum was added in dilution buffer. After 2 hours, wells were washed and incubated with, in series, biotinylated anti-mouse IgG1 (1:1000 vol/vol, Binding Site) and peroxidase-labeled streptavidin (1:1000 vol/vol, Kirkegaard and Perry). After further washes, the wells were developed with TMB peroxidase substrate (Kirkegaard and Perry) and read at 450 nm. A standard curve was generated by using serial dilutions of a polyclonal ascites fluid containing the monoclonal IgG1-κ MOPC21 (Sigma). The normalized titer was determined by comparison of the sample absorbance with the standard curve.

Total IgE ELISA

Polystyrene (Immulon 4) microtiter plates were coated with rat monoclonal anti-mouse IgE (Pharmingen; 100 ng/well) overnight in 50 μL of 0.1 mol/L sodium bicarbonate and blocked with 1% BSA.

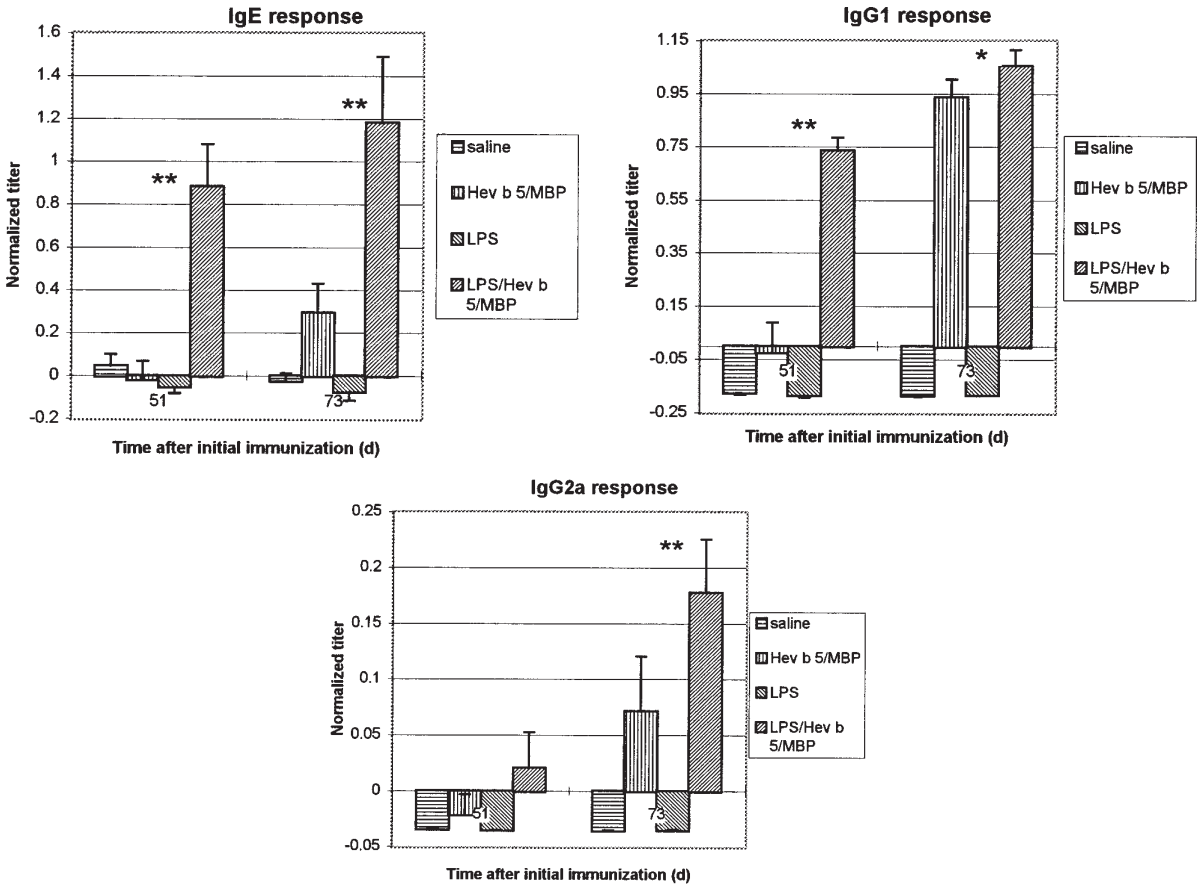

FIG 1. Specific antibody responses to Hev b 5/MBP after the first and second immunization courses. Mice were administered repeated intranasal doses of saline, LPS, and/or Hev b 5/MBP. The priming course consisted of 6 doses on days 1 to 12, and the boosting course was 3 doses on days 54 to 58. Specific antibody to Hev b 5/MBP was determined by ELISA as described. A standard curve was generated with each assay by using sera in aliquots from hyperimmunized mice, and the normalized titer was determined by comparison to the standard curve. A normalized titer of 1.0 would be observed if the test serum had the same specific antibody titer as the identically diluted standard serum. Before immunization, no antibody to Hev b 5 was detected (not shown). After the priming course, specific IgG1 and IgE responses were significantly higher in the mice administered Hev b 5/MBP together with LPS compared with mice given Hev b 5/MBP alone. No significant effect was seen in the specific IgG2a level. After the boosting immunization, the specific IgE responses of the mice receiving both LPS and Hev b 5/MBP remained significantly elevated over the responses in mice receiving Hev b 5/MBP alone. The IgG1 responses were comparable in the 2 groups. IgG2a levels were higher in the Hev b 5 + LPS group compared with the Hev b 5 group. Each data point represents the average of sera from 3 mice ± SD. $*P < .05$; $**P < .005$.

Serum was added in dilution buffer. After overnight incubation, wells were washed, and biotinylated anti-mouse IgE (1:1000 vol/vol; Binding Site) was added in dilution buffer. After 2 hours of incubation, wells were washed, and peroxidase-labeled streptavidin (Kirkegaard and Perry; 100 ng/well) was added in dilution buffer. The wells were incubated for 1 hour more, washed, developed with TMB, and read at 450 nm. The results were compared with a standard curve generated with monoclonal mouse IgE (Pharmingen).

Anti-LPS IgM and IgG ELISA

Anti-LPS IgM and IgG ELISA was performed by a modification of the method described by Freudenberg et al.[22] LPS was dissolved in endotoxin-free water at 10 mg/mL and then diluted to 10 μg/mL in ethanol-chloroform (9:1 vol/vol). The stock solution was applied to polystyrene microtiter plates (0.1 mL/well; Immulon 4) and allowed to evaporate at room temperature. The wells were then washed in 50 mmol/L Tris and 150 mmol/L NaCl (pH 7.4) containing 0.02% Tween 20 (TBS-T) and blocked with 0.1% BSA in TBS-T. Sera were added in TBS-T and, after overnight incubation at 4°C, IgG and IgM were detected with the appropriate horseradish peroxidase conjugate (Kirkegaard and Perry) and TMB substrate.

Lung pathology

Mice were sacrificed by cervical dislocation 2 weeks after the first and second immunizations. For 1 mouse in each group, lungs were excised, fixed in buffered formalin overnight, and transferred to 70% ethanol. The tissues were then trimmed, processed, embedded in paraffin, sectioned, and stained with hematoxylin and eosin or with Alcian blue and Periodic Acid Schiff. The inflammatory

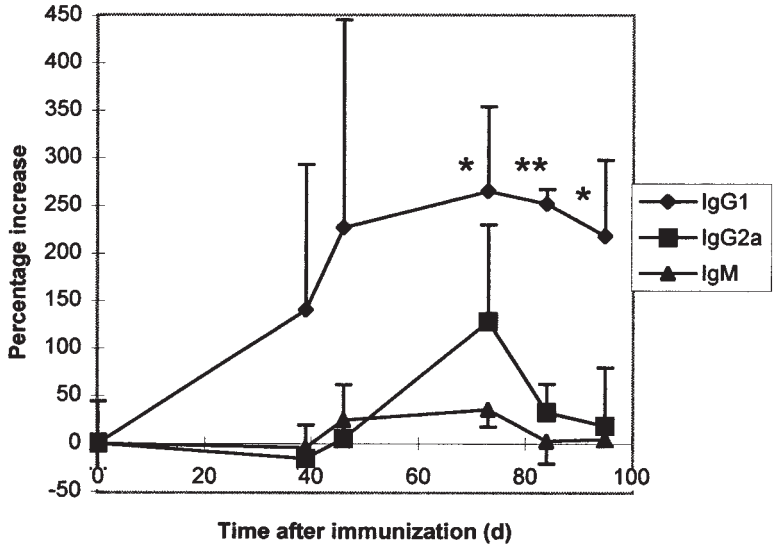

FIG 2. Specific antibody responses to LPS. Microtiter wells were coated with 1 μg of LPS dissolved in ethanol-chloroform, and LPS-specific IgG and IgM was determined as described in Methods. Preimmune mice had no measurable anti-LPS response above baseline. Because no hyperimmune sera were available for standard curves, percentage increase in the absorbance at 590 nm was determined and plotted on the ordinate. Each data point represents the average of sera from 4 mice ± SD. **P* < .05; ***P* < .005.

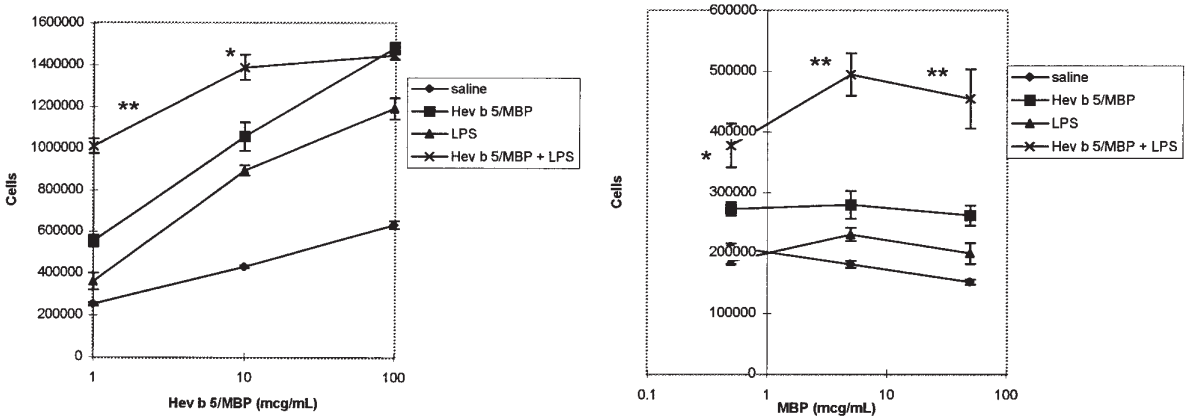

FIG 3. Proliferation of splenocytes from mice immunized with Hev b 5/MBP, LPS, or both. Mice were killed 16 days after the second immunization series. Splenocytes were prepared and erythrocytes were removed by hypotonic lysis. Cells (10^5) were incubated with Hev b 5/MBP, MBP, and LPS in serum-free culture medium. Cell proliferation was estimated at 2 days with a colorimetric assay. LPS elicited no proliferative responses in the range of concentrations used (not shown; see Methods). When cells were incubated in the presence of MBP alone, only the splenocytes from the mice immunized with Hev b 5/MBP and LPS together had increased proliferation compared with cells from unimmunized mice. However, when cells were incubated in the presence of Hev b 5/MBP, increased proliferation was observed in cells from animals sensitized with Hev b 5/MBP and LPS alone, as well as from animals sensitized with both compounds. The studies were done in triplicate, and each data point represents the average ± SD. **P* < .05; ***P* < .005 for comparison of splenocytes from mice immunized with Hev b 5/MBP and LPS together with those from mice immunized with Hev b 5/MBP alone.

cells surrounding 5 to 10 bronchi per section (a minimum of 2000 cells per slide) were counted at a magnification of 1000× and averaged.

Lymphocyte preparation and culture

Mice were killed by cervical dislocation 16 days after the second immunization. Lungs were excised and fixed in buffered formalin. Splenocytes were prepared, and erythrocytes were removed by hypotonic lysis. Cells (10^5) were incubated for 2 to 7 days with Hev b 5/MBP (1 to 100 μg/mL), MBP (0.5 to 50 μg/mL), and LPS (0.2 to 20 pg/mL or 5 to 500 EU/mL) in serum-free culture medium (Aim V, Life Technologies) supplemented with 50 μmol/L β-mercaptoethanol.

Lymphocyte proliferation

Cell proliferation was estimated at 2 and 7 days by using a colorimetric assay. MTT tetrazolium salt (Sigma) was added to the culture wells to a final concentration of 0.5 mg/mL. After incubation for 4 hours at 37°C, cells were lysed by the addition of 0.5 to 1 volumes of 10% SDS in 0.01 mol/L HCl.

Statistics

Changes in lymphocyte numbers, lymphocyte proliferation, and antibody production were compared by a 2-tailed Student's *t* test.

RESULTS

Antibody responses

Hev b 5–specific IgG1, IgG2a, and IgE were all elevated after the 2 immunization courses in the mice receiving Hev b 5/MBP. When LPS was added to the regimen, the specific IgE and IgG1 responses were measurably greater after the first course, and specific IgE levels remained higher after the second course. In particular, specific IgE (0.88 U ± 0.2) and IgG1 (0.73 U ± 0.05) responses were clearly detectable after the first series of nasal immunizations (days 1 through 12) only in the mice that were immunized with both the Hev b 5 and the LPS. After the second series of immunizations (days 54 through 58), IgE (0.29 U ± 0.1) and IgG1 (0.94 U ± 0.07) responses appeared in the mice receiving Hev b 5/MBP alone, although the specific IgE level in the mice receiving Hev b 5/MBP in the presence of LPS was significantly higher (1.18 U ± 0.31) (Fig 1). In contrast, LPS had no significant effect on total IgG1 and IgE levels in Hev b 5/MBP-treated or untreated animals (data not shown).

Specific IgG2a levels were not observed in any of the immunization groups after the priming course but appeared after the second course in the animals immunized with Hev b 5/MBP and Hev b 5/MBP + LPS. Here too the titers from the Hev b 5/MBP + LPS mice were significantly higher than the titers in the mice immunized with Hev b 5/MBP alone (0.18 U ± 0.05 compared with 0.07 U ± 0.05), although the enrichment of the specific IgG2a titer after the second course was only 157% compared with a 307% increase in specific IgE (Fig 1).

As expected, the mice receiving LPS also mounted antibody responses to the LPS itself (Fig 2). Because these sera contained anti-LPS antibodies and the Hev b 5/MBP preparation contained contaminating LPS, we examined the degree to which contaminating LPS in the coating protein might account for antibody binding in the IgG1 and IgG2a assays. In these assays the microtiter wells are coated with 10 ng of rHev b 5/MBP, and therefore we coated the control wells with 2 fg of LPS under identical conditions. No binding of antibody to the corresponding amount of LPS was detected under these conditions (data not shown).

Lymphocyte proliferation

Splenocytes from mice treated with Hev b 5/MBP proliferated when incubated with Hev b 5/MBP and, to a lesser degree, with MBP alone. Splenocytes from mice treated with both Hev b 5/MBP and LPS proliferated more when incubated with both antigens than did the cells from the mice primed with Hev b 5/MBP alone. Surprisingly, cells from the mice treated with LPS alone also proliferated in a dose-response manner when incubated with Hev b 5/MBP. This effect was not seen in the cells incubated with MBP alone (Fig 3).

Because LPS is a known B-cell mitogen, we examined the degree to which contaminating LPS might account for the lymphocyte proliferation and cytokine release observed with rHev b 5/MBP. LPS was added to control wells in amounts corresponding to 2 pg/μg rHev b 5/MBP. In multiple experiments (not shown) there was no proliferation to the small amount of LPS added.

The lung sections from the mice were evaluated for the presence of perivascular or peribronchial aggregates of lymphocytes after the first and the second rounds of immunizations. Minimal differences were noted after the first round, but all of the lung specimens from the mice immunized with 2 rounds of Hev b 5/MBP, LPS, or both had significant peribronchial, perivascular, and subpleural lymphoid cell aggregates (Fig 4). Peribronchial inflammation was assessed by counting the inflammatory cells per high-power field (HPF) surrounding the bronchi. The lung from the mouse immunized with saline alone had 203 ± 30 cells/HPF, compared with 452 ± 46 cells/HPF, 336 ± 40 cells/HPF, and 253 ± 40 cells/HPF for the lungs from the mice immunized with Hev b 5/MBP alone, LPS alone, and Hev b 5/MBP + LPS, respectively ($P < .05$ for each group compared with the control). There was no evidence of increased mucus secretion by PAS/Alcian blue staining in any of the lung specimens (not shown).

DISCUSSION

LPS may be a common contaminant in the hospital environment. Williams and Halsey[1] have demonstrated that latex gloves may contain significant amounts of LPS, ranging from 0.09 to 2837 ng of LPS per gram of glove material, and that the LPS is readily eluted from the surface of the gloves. The results described in this study are consistent with the hypothesis that LPS accentuates antibody and cellular responses to at least 1 of the latex protein antigens, Hev b 5. Thus the presence of LPS that is released concurrently with latex proteins may significantly increase the risk of allergic reactions to latex proteins.

The mechanism of this action by LPS is uncertain. LPS is a strong adjuvant. Human studies have suggested that endotoxin exposure can lead to the appearance of T_{H2}-type cytokines in the blood.[23] But in vitro studies also suggest that LPS-stimulated macrophages release IL-12, which in turn increases the release of IFN-γ. The likelihood of T_{H1} or T_{H2} responses may depend on the specific antigen and the phenotype of the immunized animal.

Although we have shown, as expected, that mice exposed to LPS make anti-LPS antibodies (Fig 2), this was not our focus in the current investigations. However, we were surprised to observe that splenocytes from mice exposed to LPS alone had a modest proliferative

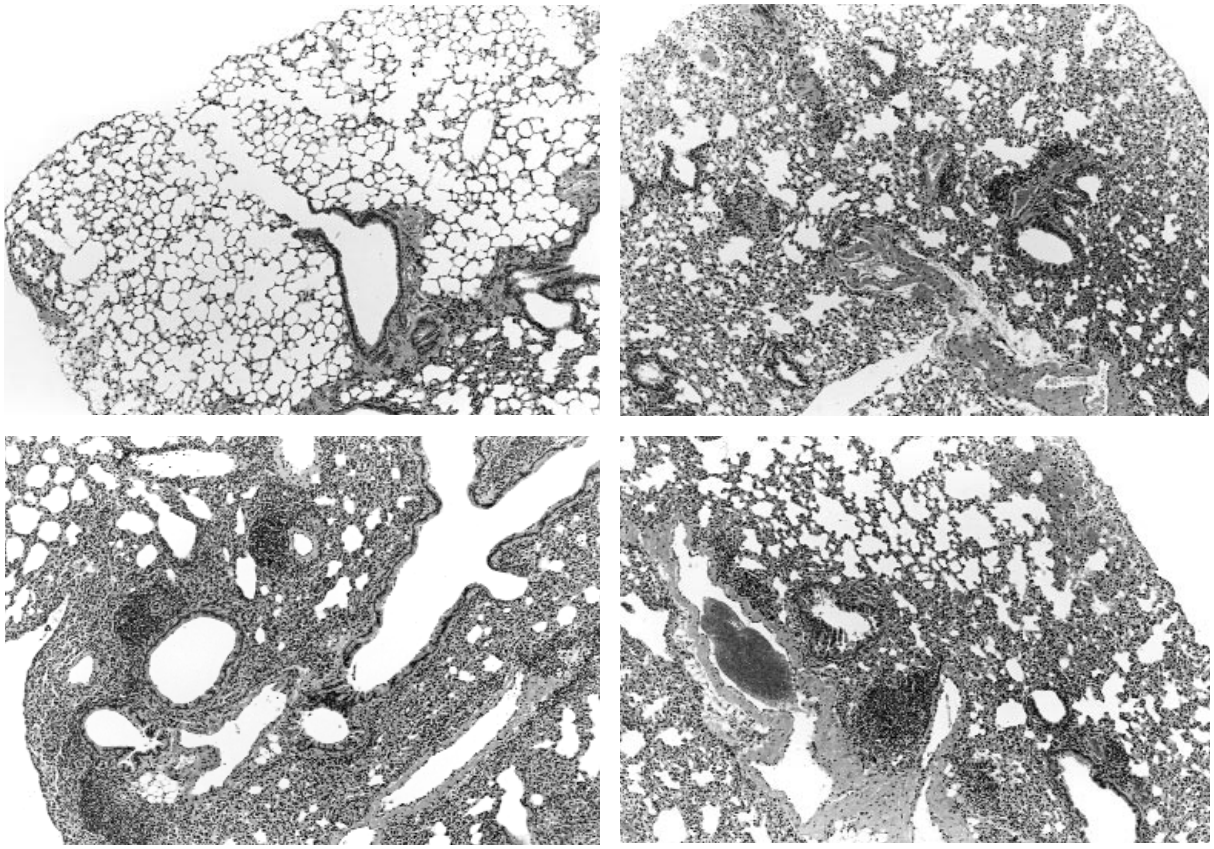

FIG 4. Histopathology of the lungs of immunized mice. Mice from each of the 4 immunization groups were killed after the first and second immunization courses. Lungs were excised, fixed in buffered formalin overnight, and transferred to 70% ethanol. The tissues were then trimmed, processed, embedded in paraffin, sectioned, and stained with hematoxylin and eosin or with Alcian blue and PAS. No differences among the immunization groups were seen in the Alcian blue/PAS–stained sections (not shown). After the first series of immunizations, only minimal lymphocytic infiltrates were seen in the lungs of the animals receiving Hev b 5/MBP, LPS, or both (not shown). However, after the second series of nasal immunizations, significant peribronchial, perivascular, and subpleural lymphoid cell aggregates were seen in the immunized animals. *Upper left,* saline; *upper right,* Hev b 5/MBP; *lower left,* LPS; *lower right,* Hev b 5/MBP + LPS. Magnification: 20×.

response to Hev b 5/MBP but not MBP alone (Fig 3). The most likely explanation for this is that the mice were exposed to latex proteins, including native Hev b 5, in the course of handling and routine care. Although nonlatex gloves are used predominantly in our Animal Research Facility, other investigators occasionally bring their own gloves into the unit, with likely low-level exposure to our animals as a result. Such exposure is not sufficient to elicit measurable responses in naive mice or antibody responses in LPS-primed mice, but the LPS-primed mice may have enough exposure to antigen to have a primed set of Hev b 5–sensitive lymphocytes. These lymphocytes would not be expected to recognize MBP, which is not found in native Hev b 5.

In summary, these studies demonstrate that microgram amounts of LPS can significantly augment IgG and IgE antibody responses of BALB/c mice to the cloned *Hevea* latex allergen Hev b 5. The mechanism of this adjuvant effect remains to be identified, but this observation is consistent with several previous studies in humans and animals. The degree of LPS exposure in this experiment is similar to what might be expected with inhalational exposure to some latex gloves and other sources of endotoxin in the hospital environment, and these observations should raise concern about the possibility that environmental endotoxin increases the likelihood of sensitization to latex antigens in susceptible individuals.

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