Endotoxin contamination of ovalbumin suppresses murine immunologic responses and development of airway hyper-reactivity

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Running title: Endotoxin contamination of ovalbumin induces tolerance
Abstract

The reversible airway hyper-reactivity (AHR) of asthma is modeled by sensitizing and challenging mice with aerosolized ovalbumin. However the C57BL/6 murine strain does not display the large increase in circulating IgG and IgE antibodies found in human atopy and asthma. We found that commercial ovalbumin was contaminated with LPS in amounts sufficient to fully activate endothelial cells in an in vitro assay of the first step of inflammation. Desensitization of TLR4 by LPS pretreatment suppressed the inflammatory effect of ovalbumin. The presence of LPS was occult because it does not require serum presentation and, like the LPS of Salmonella minnesota, was not suppressed by polymyxin B. Purified ovalbumin did not activate endothelial cells in vitro; however endotoxin-free ovalbumin was far more effective than commercial material in stimulating IgE production and respiratory dysfunction in a C57/BL6 murine model of AHR. Moreover, endotoxin-free ovalbumin induced lung inflammation with alveolar enlargement and destruction in a histologic pattern that differed from the changes caused by commercial, endotoxin-contaminated ovalbumin. Reconstitution of purified ovalbumin with Salmonella minnesota LPS decreased lung inflammation, decreased changes in lung function, and suppressed anti-ovalbumin antibody concentrations. We conclude endotoxin contaminates ovalbumin preparations, and that endotoxin co-administration with the ovalbumin antigen creates a state of tolerance in a murine model of AHR. Co-exposure to endotoxin and antigen occurs in humans through organic dusts, so murine models of AHR may reflect the clinical situation, but models based on commercial ovalbumin do not accurately reflect the effect of protein antigen alone on animal physiology.
**Introduction**

Asthma is a chronic lung disease characterized by airway hyper-responsiveness (AHR) to allergens, airway edema, and increased mucus secretion. Increased levels of circulating IgE and IgG1 antibody and a propensity to allergic responses, atopy, are associated with the development of asthma. The natural history of the early phases of asthma remain ill-defined with a multitude of genes underlying predisposition towards this disease, variation in environmental exposure to triggers, access to medical care and a lengthy interval before the development of symptoms all contribution to the complexity of the natural history of this disease. Animal models of AHR, where control of the timing of exposure to the initiating antigen, the use of a defined allergen trigger, and genetic manipulation are possible, are defining the roles of cytokines and inflammatory and immune cells (1-3).

The role of acute and chronic inflammation in altering in airway structure and function in animal models suggests that pro-inflammatory agents could contribute to the development of AHR (4). A premier candidate in this category is LPS (LPS) of gram-negative bacteria. LPS is a potent and pleiotropic inflammatory agent that is a component of tobacco smoke (5), house dust (6) and even extracts used in allergen testing (7). Co-exposure to LPS or β (1 → 3) glucans along with particulate antigens is common in certain agricultural and industrial settings that produce organic dusts (8). Segmental installation of LPS into human lung results in an intense local inflammatory response characterized by a rapid neutrophilic influx (9,10) followed by a monocytic and eosinophilic response a day or two later (10). This progression of cellular influx is mirrored, we find (11), in a murine model of AHR using ovalbumin sensitization and
aerosol challenge. Why exposure to ovalbumin would mirror changes wrought by endotoxin is unknown.

Ovalbumin has long been employed as a model immunogen as a single antigen to reduce the complexity of modeling AHR. Ovalbumin induces IgE accumulation in the serum of mice, although there is a significant variation in the amount and antibody class among strains (12,13), and at least in BALB/c mice anti-ovalbumin IgE injection alone induces AHR (14). We find, however, that commercial preparations of ovalbumin are highly contaminated with an inflammatory endotoxin that was not sequestered by polymyxin B treatment. We purified large amounts of ovalbumin to an endotoxin-free state and then examined how co-administration of an inflammatory and immunologic trigger, a likely scenario outside the laboratory setting, affects the development of AHR in a murine model of this disease. We report that co-administration of endotoxin with the ovalbumin immunogen suppresses changes in lung function, alters lung pathology, and immunoglobin production.
Materials and Methods

Reagents and chemicals—Reagents and their sources were as follows: HBSS, BioWhittaker (Walkersville, MD); human serum albumin, Baxter Healthcare (Glendale, CA); CD14 neutralizing monoclonal antibody MY4, Coulter Corp. (Miami, FL); TNFα and IL-1β were the products of R&D Systems (Minneapolis, MN); ovalbumin, S. minnesota LPS (R595), E. coli LPS (0111:B4), polymyxin B sulfate, and all other chemicals unless otherwise noted were from Sigma (St Louis, MO). We examined four lots of ovalbumin with similar results; the data was collected using any of several batches of ovalbumin. N-palmitoyl-S-2,3-bis(palmitoyloxy)-2(RS)-propyl]-(-R)-cysteinyl-seryl-lysyl-lysyl-lysyl-lysine (Pam3CSK4) was conjugated with N-palmitoyl-S-2,3-bis(palmitoyloxy)-2(RS)-propyl]-(-R)-cysteine by the Chemical Synthesis core facility (University of Utah).

Endothelial cell culture, neutrophil isolation, and adhesion assays—Primary cultures of human umbilical vein endothelial cells (HUVEC) and [111In]oxine labeled neutrophils used in adhesion assays were prepared as previously described (15) and use of these materials was approved the University of Utah’s IRB. Values of 111In-neutrophils tightly bound to endothelial cells during a 5 min co-incubation represent the average and range of two samples for each condition from at least two similar experiments.

Endotoxic activity in ovalbumin—Endotoxin-like contaminants in ovalbumin were visualized by SDS-PAGE followed by silver staining with periodic acid activation as described previously (16). Endotoxin was quantitated by a Limulus amebocyte lysate assay (QCL-1000) purchased from BioWhittaker (Walkersville, MD). Commercial and purified ovalbumin were tested for contamination with E. coli and S. minnesota LPS as
standards. Commercial ovalbumin was diluted until the endotoxic activity entered the linear range of the standard, whereas purified ovalbumin was assayed in an undiluted form. We attempted to remove endotoxin from commercial ovalbumin (grade V) with endotoxin-affinity resin (END-X®) (Associates of Cape Cod, Inc.; Falmouth, MA), polymyxin B beads (Sigma), Extracti-Gel® D detergent removing gel (Pierce; Rockford, IL) after incubating ovalbumin in 1% nonidet P40 (Roche; Indianapolis, IN). Commercial ovalbumin was also precipitated with 60% (NH₄)₂SO₄, as described previously (17). None of these procedures successfully removed the endotoxic material from the large amounts of ovalbumin needed for animal studies.

Ovalbumin purification from chicken egg white—Ovalbumin was prepared from chicken egg white as described (18, 19), except that all steps were performed under sterile conditions, all solutions used in the purification were filtered through a 0.22 μm sterile filter unit (Nalgen, Rochester, NY), and a few modifications were made to accommodate the large amounts (>100 g) of ovalbumin being purified. Chicken eggs were washed with antibacterial soap, aseptically punctured, and their white transferred into autoclaved glassware with a sterile syringe and needle in a laminar flow hood. The white from 6 eggs was made to 500 ml with 50 mM Tris-HCl (pH 9.0) containing 10 mM β-mercaptoethanol and incubated overnight at 4°. The precipitate was removed by centrifugation at 48,000 x g for 5 min and 150 ml portions of the egg white solution were loaded onto a XK 50/60 Q Sepharose Fast Flow FPLC Column (Amersham Pharmacia Biotech, Piscataway, NJ) at flow rate of 7.5 ml/min. The column was washed with 50 mM Tris-HCl (pH 9.0) (Buffer A) until OD₂₈₀ returned to the baseline; the non-bound material was defined as fraction 1. The column was developed with a linear gradient of
100% buffer A to 50% buffer A and 50% buffer B [0.3 M NaCl in 50 mM Tris-HCl (pH 9.0)], and held at this mixture until ovalbumin finished eluting. This was fraction 2. The column was then stripped of remaining lipids and proteins by washing with 1M NaOH, 70% EtOH and 2M NaCl, which was collected as fraction 3. The collected material was concentrated with an Amicon ultrafiltration membrane (MW cutoff of 30,000) (Millipore, Bedford, MA), the buffer was then substituted with endotoxin-free phosphate buffered saline (Sigma), and then reconcentrated until the pH stabilized between pH 7.2 and 7.4 (a minimum of three exchanges were required for this result). Protein concentration was determined by BCA assay (Pierce, Rockford, IL), adjusted to 20 mg/ml, and stored at −70°C until use.

Ovalbumin immunization, airway sensitization, histology, and plethysmography—Pathogen-free 3-week-old female C57BL/6 mice were purchased from Simenson (Gilroy, CA). Upon delivery, the mice were kept in a pathogen-free animal facility and were given food and water *ad libitum*. The Institutional Animal Care and Use Committee at the University of Utah approved these animal experiments. Ovalbumin sensitization by injection and aerosolization, and collection of histologic specimens, was described previously (11). Penh estimation of lung function of sensitized mice was analyzed by whole-body barometric plethysmography (Buxco Electronics, Inc., Sharon, CT) after methacholine challenge as described previously (11). Breathing patterns were assessed immediately after methacholine challenge using the variable Penh. Penh, or enhanced pause, is a unit-less calculated number that increases with bronchoconstriction (20), although we find it does not accurately reflect lung compliance (11).
Anti-ovalbumin IgE ELISA—Mice were euthanized after plethysmography, their blood was collected by cardiac puncture, their serum collected after centrifugation at 500 x g for 5 min, and then stored at -70°C. Specific IgE that bound ovalbumin antigen was quantitated by ELISA. High capacity EIA plates (Corning, Corning, NY) were coated with the cognate ovalbumin sample (50 μg/ml) overnight at 4°C. The wells were washed thrice with PBS containing 0.5% Tween-20, the plates were blocked with 2% HSA for 2 h at 37°C, washed thrice with PBS/Tween-20 and probed for 2 h at 37°C with biotin-conjugated goat anti-mouse (BD Biosciences Pharmingen; Franklin Lakes, NJ) and then HRP-conjugated strepavidin (Biosource International; Camarillo, CA).
Results

Commercial ovalbumin is inflammatory—Inflammation is initiated and localized by activation of endothelium (21). LPS stimulates endothelial cells via TLR4 receptors, and induces expression of TLR2 receptors that respond to other endotoxins including bacterial lipoproteins (22). We used human umbilical vein endothelial cells to probe for pro-inflammatory material by treating these cells for 4 h with buffer containing either human serum albumin or chicken ovalbumin before their state of activation was assessed by overlaying the monolayer with quiescent human PMN (23). Pyrogen-free human serum albumin did not induce endothelial cell-dependent PMN adhesion (Figure 1), but even low concentrations of ovalbumin proved to be equivalent to phorbol myristate acetate (PMA) or \textit{E. coli} LPS in stimulating endothelial cells. The response of endothelial cells to \textit{E. coli} LPS is enhanced by the LPS-binding protein and soluble CD14 of serum, and serum similarly potentiated the response to low concentrations of ovalbumin. Higher concentrations of ovalbumin, however, did not require these accessory proteins.

\textit{LPS-like material is present in commercial ovalbumin, but cannot easily be suppressed by polymyxin B}—Resolution of commercial ovalbumin by SDS-PAGE and staining with a periodate-Schiff reaction showed the presence of material with the characteristics of LPS (not shown), so we assessed the endotoxic content of this ovalbumin with a \textit{Limulus} amebocyte lysate assay. We found that as little as 3 \textmu g/ml of ovalbumin contained about 0.5 EU/ml of endotoxic activity (Figure 2A). This is a considerable amount of endotoxin, but since we do not know its identity we cannot calculate its mass in the absence of an appropriate standard. However extrapolating from the LPS of \textit{E. coli}, we estimate that 1 mg of this batch of ovalbumin could be contaminated with as much as 10 \textmu g of LPS.
We tested whether the inflammatory material in commercial ovalbumin was the endotoxic contaminant we quantified in the Limulus assay. For this we pretreated, or not, each of the agonists with polymyxin B, an antibiotic that sequesters LPS. We found (Figure 2B) that polymyxin B abolished the stimulation of endothelial cells by our positive control, E. coli LPS, whereas it was only partially effective at this concentration when mixed with even low concentrations of ovalbumin. In this experiment we used a submaximal amount of ovalbumin (3 µg/ml) to be sure that the ratio of polymyxin B to endotoxin was high, but even so polymyxin B was ineffective in neutralizing the endothelial cell agonist(s).

*LPS from Salmonella minnesota is resistant to polymyxin B sequestration and does not require CD14 presentation*—Chicken eggs can be contaminated with Salmonella and the TLR4 receptor responds to the endotoxin of this bacteria as it does to that of E. coli (24), so we determined whether Salmonella LPS differed from E. coli LPS as a target for polymyxin B sequestration. To test this, LPS from the two bacteria was pretreated with polymyxin B and endothelial cell activation was assayed in serum-containing medium in the continued presence of polymyxin B. E. coli LPS became inflammatory in this assay at 100 ng/ml, and even at ten times this concentration its inflammatory effect was completely suppressed by polymyxin B (Figure 3A). In contrast, Salmonella LPS (Figure 3B) was considerably more potent than the LPS from E. coli in activating endothelial cells, and it was completely resistant to the effects of polymyxin B. Accordingly, we found (not shown) that columns containing immobilized polymyxin B were unable to remove the biologic activity from commercial ovalbumin.
We determined whether the effect of *Salmonella* LPS on endothelial cells—like that of *E. coli*—was enhanced by the presence of serum and the soluble CD14 (sCD14) it contains (25). We preincubated endothelial cells in the presence or absence of serum, or with the blocking anti-CD14 monoclonal antibody MY4. The serum-supplied sCD14 was important at low concentrations of *E. coli* LPS because MY4 completely inhibited endothelial cell activation at LPS concentrations of 100 µg/ml and below (Figure 3C). Above this concentration, the response to *E. coli* LPS became both serum- and CD-14 independent. In contrast, activation of endothelial cells by *Salmonella* LPS was only partially dependent on serum and sCD14, and only at low LPS concentrations. The LPS of *Salmonella* (Figure 3D) displays several of the characteristics of the biologically active material in purchased ovalbumin in that it is potent, largely independent of serum, and minimally affected by polymyxin B.

*LPS, but not an endotoxic lipopeptide, desensitized endothelial cells to commercial ovalbumin*—We explored the nature of the inflammatory agent in commercial ovalbumin by distinguishing LPS from non-LPS endotoxins because both prokaryotic proteins displaying a lipid-modified N-terminal cysteinylation residue and LPS are a potent endothelial cell agonists (26,27). We selectively desensitized endothelial cells to either LPS or endotoxic proteins by a 24 h pre-exposure to *Salmonella* LPS or a synthetic lipid-modified endotoxic peptide Pam3CSK4. These agents activated the endothelial cell monolayers (not shown), but after 24 h most of the response had faded (note the slightly higher background in media/LPS and media/ Pam3CSK4 compared to the open bar depicting media pre-treated/media challenged monolayers in Figure 4A). Challenge of these buffer- or endotoxin-pretreated cells with TNFα or IL-1β showed they still
mounted an inflammatory response to unrelated inflammatory agents (Figure 4A). These cells were, however, selectively unable to respond to a subsequent exposure to the desensitizing agonist. Thus cells pre-exposed to *Salmonella* LPS did not respond to a subsequent exposure to this LPS, but did respond to Pam$_3$CSK$_4$. The converse also was true—Pam$_3$CSK$_4$ desensitized only to a second exposure to Pam$_3$CSK$_4$ and not to a subsequent LPS challenge.

With the controls validating this approach established, we desensitized endothelial cells for 24 h with purified ovalbumin as a stringent control, or with *Salmonella* LPS or Pam$_3$CSK$_4$ and then challenged these cells with purified or commercial ovalbumin for 4 h. Cells pre-incubated with purified ovalbumin and then challenged with purified ovalbumin were not activated (open bars), while those pre-incubated with *Salmonella* LPS or Pam$_3$CSK$_4$ showed a low level of residual activation as before (left bars of Fig. 4B). Cells pre-incubated with purified ovalbumin and then challenged with commercial ovalbumin still displayed a strong inflammatory response—compare the open bars in Figure 4B. In contrast, cells desensitized to *Salmonella* LPS (the dark bar) showed a complete lack of a response to commercial ovalbumin. Conversely, endothelial cells desensitized to Pam$_3$CSK$_4$ fully responded to a subsequent challenge with commercial ovalbumin. Commercial ovalbumin—then—must contain an endotoxin that is recognized in the same way as *Salmonella* LPS.

*Contamination of commercial ovalbumin accounts for its inflammatory effects*—We purified large amounts of ovalbumin from fresh eggs to find that the purified ovalbumin—in marked contrast to the starting material—was without effect on endothelial cell monolayers (Figure 5). The presence of serum did not increase the
sensitivity of the cells to purified ovalbumin, which it did for low —and not high— amounts of commercial ovalbumin. We added *Salmonella* LPS to the purified ovalbumin and found that this combination behaved as higher concentrations of commercial ovalbumin: there was high basal activity that was insensitive to the presence of serum, polymyxin B or anti-CD14 MY4 antibody. We noted that ovalbumin enhanced the effect of *Salmonella* LPS over free LPS (compare the unfilled bars of the rightmost two groups of columns), but we have no direct evidence that ovalbumin aids in the presentation of LPS to endothelial cells.

*Ovalbumin contamination affects development of murine AHR*—Commercial ovalbumin has been widely used as an antigen in murine models of airway hyper-responsiveness (AHR). We determined whether purified ovalbumin, in the absence of a concomitant inflammatory signal from contaminating endotoxin, induced AHR, and whether the addition of *Salmonella* LPS to this endotoxin-free material recapitulated the results obtained with commercial ovalbumin. Mice were sensitized on day 0 with either endotoxin-free PBS, purified ovalbumin, ovalbumin reconstituted with *Salmonella* LPS, commercial ovalbumin or *Salmonella* LPS alone. These mice were then exposed to these same agents in an aerosolized form on day 8 and then every day for 9 days starting on day 14 as described (11,28). To detect AHR, the breathing patterns of these mice were analyzed on days 15, 18, 22 of the protocol in awake unrestrained animals by plethysmography—to calculate the Penh variable—just after each mouse was challenged with methacholine.

Mice challenged with commercial ovalbumin developed small changes—about two fold—in their airway reactivity (Figure 6) by day 22 when challenged with
minimally effective dose of methacholine (15 mg/ml). We tested purified, endotoxin-free ovalbumin in this model, but unexpectedly found that this proved to be significantly better at sensitizing the mice than the commercial material. By example, as early as day 18 mice sensitized with the endotoxin-free ovalbumin responded to the methacholine challenge—and this response was greatly increased by day 22 of the model. *Salmonella* LPS alone, when used at a concentration giving a response equivalent to commercial ovalbumin in the *Limulus* amebocyte assay, did not differ in its effects from the vehicle control. We then combined this amount of *Salmonella* LPS with purified ovalbumin to find that this combination suppressed the effect of the purified protein in sensitizing the mice to the methacholine challenge. The combination of *Salmonella* LPS and endotoxin-free ovalbumin therefore behaved much as the purchased protein preparation. We conclude that endotoxin can be a powerful negative modulator in the development of AHR in this model.

*Lung inflammation and structural changes induced by purified and commercial ovalbumin*—We examined lung sections from mice subjected to the AHR model to find that the sections from PBS-exposed control mice were unremarkable (Figure 7A). In contrast, sections from mice sensitized and challenged with either purified or commercial ovalbumin showed distinct—and non-equivalent—changes over time. Lung consolidation was evident in animals receiving purified ovalbumin (note the overall increase in tissue staining), but this was not a prominent feature of mice receiving commercial ovalbumin. Enlargement (Figure 7B) of the areas outlined in Figure 7A confirm the impression of consolidation and cellularity. Among the notable changes were perivascular infiltrates, thickened septa around airways and thickened alveolar walls, and the presence of alveolar
edema—as shown by the pink staining material—in the lung sections of mice receiving purified ovalbumin. In contrast to the histology of animals challenged with purified ovalbumin, the alveoli of mice receiving commercial ovalbumin were clearly dilated. The alveolar destruction and dilatation caused by commercial ovalbumin is similar to the emphysematous-like lesions found after long term intratracheal exposure of mice to LPS (29). Supplementation of purified ovalbumin with *Salmonella* LPS suppressed—but not completely—formation of perivascular infiltrates and alveolar thickening. The addition of *Salmonella* LPS to the purified ovalbumin also caused some alveolar dilatation, but—at least at this concentration—did not completely recapitulate the changes caused by exposure to commercial ovalbumin.

*Circulating IgE levels differ after immunization with commercial or purified ovalbumin*—We determined whether LPS contamination suppressed the immune response to ovalbumin, or just the development of AHR, and so quantified the levels of IgE in serum from each group of mice. We found that mice sensitized to commercial ovalbumin displayed a minor increase in circulating total (Figure 8A) or ovalbumin-specific (Figure 8B) IgE levels, but that mice treated with purified ovalbumin demonstrated a large increase in IgE over time. We also found that addition of *Salmonella* LPS to the purified material suppressed the animal’s immunologic response to the antigen and inhibited IgE accumulation.
Discussion

Murine models of AHR and other immunologic disorders—where genetic manipulation is possible—promise to be invaluable in understanding complicated disease. The use of a single immunogen and a defined time of sensitization in the murine model eliminate two key variables in human disease. Here we show that the most commonly employed immunogen in models of murine AHR is sufficiently contaminated with an endotoxin to affect the inflammatory system. We anticipated that removing endotoxin from the immunogen used in the initial intraperitoneal injection and in the subsequent aerosol exposures would suppress development of AHR because we would have removed an adjuvant from the immunogen, and because chronic LPS exposure causes persistent lung inflammation (29). Instead, we saw that the effect of this endotoxic material was to suppress the development of AHR, lung consolidation, and IgE accumulation.

Adhesion of leukocytes to endothelial cells is an initial step in the inflammatory response to endotoxin, and so we used these cells to probe for bioactivity. We found endothelial cells to be fully activated by a few micrograms of commercial ovalbumin, where we estimate—based on the Limulus amebocyte assay—that as much as 1% of the mass may be endotoxin. The response of the LAL assay varies with the nature of the LPS being analyzed, so we lack an appropriate standard to accurately define its mass in ovalbumin. A recent publication (30) also detected endotoxic activity in ovalbumin solutions, although at levels less than we measured, that did not affect the development of lung disease in BALB/c mice. Also in BALB/c mice, addition of low levels of LPS induces an IgE-promoting Th2-type response while higher amounts of LPS induce a Th1
response (31). This study, unlike most murine models of AHR, did not use alum as an adjuvant so this may account for the difference we find in a model using C57/Bl6 mice, but there also are large differences in the response to ovalbumin among inbred strains of mice (32). Among these differences is the very low production of ovalbumin-specific IgE antibody by C57/BL6 mice relative to BALB/c mice (32), although we show here that the presence of contaminating endotoxin in ovalbumin significantly suppresses the production of these antibodies in C57/BL6 mice.

Endotoxin in commercial ovalbumin did not behave like *E. coli* LPS in that it was resistant to sequestration by polymyxin B and insensitive to the presence of soluble CD14 and LPS-binding protein in serum. The LPS of the gram negative bacterium *Salmonella* also proved to be resistant to polymyxin B inhibition (33) (*vide supra*) and we find to also not require presentation by CD14 or LPS-binding protein. The principle in commercial ovalbumin responsible for endothelial cell activation seemed to act through the same receptor—likely TLR4 (34)—as *Salmonella* LPS because selective desensitization to this LPS prevented cellular activation by commercial ovalbumin. We conclude an endotoxin contaminates commercial ovalbumin, and its nature makes it less susceptible to suppression by methods that are effective in blocking the effects of *E. coli* LPS. This property makes standard approaches to detecting or suppressing endotoxic activity by chromatography over immobilized polymyxin B ineffective.

A recent editorial “Eat dirt—the hygiene hypothesis and allergic diseases” (35) encapsulates the idea that the gradual increase in the prevalence of autoimmune disease stems from a gradual decrease in early childhood infections obtained through better public health measures. A study relating children’s exposure to endotoxin with hay
fever, atopic asthma, and atopic sensitization supports the conclusion that a subject's environmental exposure to endotoxin might have a crucial role in developing tolerance to ubiquitous natural allergens (36). Endotoxins contaminate organic dusts (8) and subchronic inhalation of LPS in a murine model causes persistent airway disease in endotoxin-responsive mice (37). On the other hand, other approaches have suggested that early exposure to endotoxin independently increases the risk of developing wheeze (38) and suggest that chronic inhalation of endotoxin may contribute to the development of asthma—although the timing of endotoxin exposure and of course genotype affect how endotoxins affect the response to allergens (39). Asthma is a particularly complex autoimmune disorder, and unraveling its natural history in animal models with mixed antigen and adjuvant is problematic.

Egg white proteins physically bind pathogenic bacteria to preventing upper urinary tract infections (40) during egg formation, and indeed we found that even fresh eggs steriley prepared contained sufficient endotoxic material to fully activate endothelial cells. Purification of ovalbumin was the only effective means we found to circumvent the effect of endotoxic contamination: Extracti-Gel® D beads, precipitation by SDS (41), sequestration with immobilized polymyxin B, and phenol extraction of LPS (42) were ineffective or did not provide soluble antigen (not shown). We conclude that Salmonella-like material in commercial ovalbumin is a potent, serum-independent, and polymyxin B-resistant inflammatory agent(s) that caused immune tolerance in C57/Bl6 mice. Such contaminants complicate lessons derived from murine models of airway hyperreactivity that use unpurified ovalbumin as the immunogen.
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References


Footnotes

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Figure Legends

Figure 1. Commercial ovalbumin induces an endotoxic response from human endothelial cells. Confluent monolayers of primary human umbilical vein endothelial cells were incubated for 4 h with buffer (HBSS/A) containing either no additions or 1 μM PMA, 1 μg/ml \textit{O111:B4 E. coli} LPS [LPS(E)], or commercial ovalbumin (cOVA) at the stated concentrations in the presence or absence of 5% pooled human serum. After this incubation, we assessed endothelial cell activation by quantifying their ability to bind quiescent human neutrophils. For this, the monolayers were washed with HBSS/A and \textsuperscript{111}In-labeled PMN were added for 5 min before unattached and loosely attached PMN were removed with two washes. The fraction of bound and adherent PMN was determined by gamma counting and is expressed as a fraction of the total recovered cells. The values represent the mean ± range of two samples for each condition.

Figure 2. Endotoxic activity is present in commercial ovalbumin, and is resistant to sequestration by polymyxin B. Panel A. Endotoxic activity was determined with a \textit{Limulus} amebocyte lysate assay as describe in “Methods.” The amount of activity in endotoxin free PBS, \textit{O111:B4 E. coli} LPS [LPS(E); 100 ng/ml] or commercial ovalbumin (cOVA; 3 μg/ml) is shown. B. Polymyxin B (PMB) does not effectively block the activity of commercial ovalbumin. The designated agonists in HBSS/A containing 5% pooled human serum were treated with polymyxin B (10 μg/ml) for 5 min, or not, before addition to monolayers of endothelial cells at final concentrations of: 1μM PMA; 100 ng/ml of \textit{O111:B4 E. coli} LPS [LPS(E)]; 3 μg/ml commercial ovalbumin (cOVA). After
incubation for 4 h, the level of endothelial cell activation was assessed with quiescent
\(^{111}\)In-PMN as in Figure 1.

**Figure 3. Polymyxin B blocks the endotoxic activity of *E. coli*, but not *Salmonella minnesota* LPS, and *S. minnesota* LPS does not require presentation by soluble CD14.** *E. coli O111:B4* LPS [LPS(E)] (Panel A), or *Salmonella minnesota* LPS [LPS(S)] (Panel B), was incubated in 5% pooled human serum with 10 \(\mu\)g/ml polymyxin B, or not, for 5 min and then added to endothelial cell monolayers for a 4 h incubation at the designated concentration. After this incubation, \(^{111}\)In-labeled PMN were used to judge the state of activation of the monolayer as before. Panel C and D. Endothelial cells were incubated for 10 min in the presence or absence of 5% pooled human serum or in pooled serum containing CD14 neutralizing antibody (MY4). The monolayers were then incubated for 4 h with *O111:B4 E. coli* (Panel C) or *Salmonella minnesota* LPS (Panel D) at the stated final concentrations. After this incubation, the fraction of \(^{111}\)In-labeled PMN bound to the monolayers was determined as before.

**Figure 4. Selective desensitization of LPS signaling blocks endothelial cell activation by commercial ovalbumin.** Panel A. Selectivity controls. Confluent monolayers of primary human umbilical vein endothelial cells were incubated for 24 h with serum-free growth media alone, or media containing 1 \(\mu\)g/ml *Salmonella minnesota* LPS [LPS(S)] or 5 \(\mu\)g/ml Pam\(_3\)CSK\(_4\) [Pam3Cys]. After this pre-incubation to desensitize the cells to LPS or the lipid-modified peptide Pam\(_3\)CSK\(_4\), we challenged the monolayers of human endothelial cells for 4 h with 5 units TNF\(\alpha\), 1 \(\mu\)g/ml IL-1\(\beta\), 1 \(\mu\)g/ml *S. minnesota* LPS...
[LPS(S)] or 5 µg/ml Pam3CSK4. Endothelial cell activation was assessed by quantifying the adhesion of initially quiescent 111In-PMN—as in Figure 1. Note that the activation state of endotoxin-treated cells does not return to the baseline after 24h of agonist stimulation (that is, the two shaded bars on the left depicting PMN adhesion to endothelial cells stimulated for 24h are well above the background of cells never exposed to an agonist in the open bar). However, these cells still respond to a second round of stimulation—unless the stimulus was the same as the desensitizing agonist. Open bars, no desensitizing agent; dark bars, LPS(S)-pre-treated monolayers; and, light bars, Pam3CSK4-treated monolayers. Panel B. Desensitization of endothelial cell receptor(s) for commercial ovalbumin. Endothelial cell monolayers were desensitized as in the above panel with media containing purified ovalbumin (open bars), S. minnesota LPS (dark bars) or Pam3CSK4 (light bars) and then challenged for 4 h with 1 mg/ml purified or commercial ovalbumin. The fraction of 111In-PMN that adhere in 5 min to endothelial cells treated in this way was quantified and presented as in Figure 1. Again note that the state of endothelial cell activation had not returned to baseline after 24 h exposure to either of the two endotoxins (since the two shaded bars are higher than the open bar when the endothelial cells were quiescent for the 24h desensitization period). The pre-incubated endothelial cells were still activated by a 4 h exposure to commercial ovalbumin—but only if they had not first been desensitized to LPS.

**Figure 5.** Purified ovalbumin is not inflammatory, but enhances the effect of *Salmonella minnesota* LPS. Agonists were pre-treated for 10 min with either media, or media containing 5% pooled human serum without or with polymyxin B (10 µg/ml) or
MY4 (10 μg/ml) as stated. Endothelial cell monolayers were then incubated with commercial ovalbumin treated as before at a low concentration [cOVA(L); 200 μg/ml] or higher concentration [cOVA(H); 2 mg/ml], or the cells were exposed to purified ovalbumin at the higher 2 mg/ml concentration (pOVA), Salmonella minnesota LPS [LPS(S); 1 μg/ml] or the combination of purified ovalbumin and Salmonella LPS [pOVA + LPS(S)] at these concentrations. Incubation with the agents proceeded for 4 h before their ability to bind 111In-PMN was determined as before.

**Figure 6. Purified ovalbumin induces large changes in Penh, which are suppressed by LPS addition.** Mice were sensitized with aluminum hydroxide mixed with either endotoxin free PBS, commercial ovalbumin (cOVA), purified ovalbumin (pOVA), OVA plus Salmonella LPS (pOVA/LPS) or Salmonella LPS alone [LPS(S)]. These mice were then exposed to an aerosol containing the same antigen under the protocol described in “Material and Methods”. Changes in breathing patterns were analyzed using the Penh variable obtained during plethysmography following challenge with methacholine on the stated day after sensitization. Open circles present the individual fold change in Penh, while the closed circle shows the mean of each set.

**Figure 7. Lung histopathology reveals structural changes caused by ovalbumin.** Panel A. Survey micrographs of lung tissue sections to illustrate gross histopathology. Lung sections from mice sensitized with endotoxin-free PBS, commercial ovalbumin, purified ovalbumin or purified ovalbumin supplemented with Salmonella minnesota LPS as in Figure 6. Lungs were removed on days 15, 18 and 22 of the model, sectioned and
stained with hematoxylin and eosin as described in “Methods.” Caudal lobes (top three rows) and the cranial lobe of the lung of the same animal (lower row) are shown at 40-fold magnification. The inset boxes are the regions selected for the lower panel. Panel B. Higher magnification (200x) of the boxed regions of Panel A.

**Figure 8. Commercial ovalbumin contains an unidentified immunosuppressant affecting IgE accumulation.** Serum from the individual mice used for plethysmography in the foregoing figure was analyzed for circulating (A) total or (B) ovalbumin-specific IgE content by ELISA as described in “Methods.” The values represent the mean ± S.D. of at least three sera from each group of mice.
Figure 1
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Figure 2
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Figure 4
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Figure 5

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Figure 6
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Figure 8
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Endotoxin contamination of ovalbumin suppresses murine immunologic responses and
development of airway hyper-reactivity
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