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Immunogenicity of antigens in boiled alginate microspheres

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Abstract—Vaccine efficacy can be enhanced by delivery of antigens in synthetic microspheres. The process of antigen incorporation into microspheres can expose fragile antigens to damaging conditions, such as high temperatures, and to bacterial contamination. Maintenance of immunogenicity of several antigens and reduction of bacterial load in alginate microspheres following boiling was evaluated. Mice were immunized subcutaneously, initially and again 21 days later, with either non-boiled or boiled microspheres containing ovalbumin (OVA), a culture supernatant vaccine of *Pasteurella haemolytica* (PHV), or a potassium thiocyanate extract of *P. multocida* (PTE). Serum samples were obtained prior to immunization and at the time of euthanasia 28 days later. Culture of microspheres showed that boiling completely eliminated aerobic bacterial growth for OVA-containing microspheres, and reduced growth by a factor of 10^4 for PTE microspheres. More bacteria were cultured after boiling than before for PHV microspheres. ELISA performed on serum and intestinal lamina propria explant supernatants showed that immunogenicity of PHV microspheres was not altered by boiling. Boiled OVA microspheres were still able to stimulate a significant serum IgG anti-OVA titer in mice, but boiled PTE microspheres completely lacked immunogenicity. Elispot assays of spleens showed that only PHV microspheres were able to retain immunogenicity after boiling. Results indicate that boiling is not an effective means for reducing the bacterial load of alginate microspheres and that the process is associated with a diminution of vaccine immunogenicity.

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INTRODUCTION

Vaccine efficacy is improved by delivery in ways that protect critical antigenic epitopes from rapid degradation. This is particularly true in the case of orally administered preparations which encounter the risk of enzymatic degradation in the digestive tract. Microencapsulation is a unique way to protect orally-delivered antigens and stimulate their uptake by Peyer's patches, sites of lymphoid tissue in the gastrointestinal tract [1]. Incorporation of antigen into microspheres has been shown to be an effective means of inducing serum and secretory antibody responses following oral immunization [2]. One of the most common materials used to encapsulate antigens is poly (D,L-lactide-*co*-glycolide) (DL-PLG) [3]. Although DL-PLG particles are readily taken up by Peyer's patches, where the antigen is then processed by the immune system, their production requires organic solvents which could threaten the integrity of fragile antigens.

Sodium alginate is a natural polysaccharide formed by sea weed and which cross-links into a solid material when mixed with divalent cations. Alginate has been used for incorporation of beta-islet cells for the experimental treatment of diabetes [4]. Alginate microspheres have also been used for immunization of rabbits against *P. multocida*, an important bacterial pathogen [5].

It is important that compounds administered as vaccines maintain immunogenicity in the face of harsh environmental conditions and remain free of contaminant bacteria so that the process of vaccination does not expose the animal to potentially infectious pathogens. In this regard, methods of disinfection, including use of sterile technique during production, must be considered so that vaccine preparations are acceptable. The study described here examined maintenance of antigen immunogenicity and reduction of bacterial load in alginate microspheres following exposure to high temperatures (boiling).

METHODS

Animals

Ten to twelve week-old, female Balb/c mice obtained from the Purdue University Biological Sciences Breeding Colony were used in these studies. Mice were serologically free of major viral and bacterial pathogens. The rooms in which the mice were housed were maintained at 70–72°F and were illuminated on a 12/12-h light/dark cycle. The mice were allowed *ad libitum* access to feed (Laboratory Rodent Chow, Purina Mills, Inc., Richmond, IN, USA) and tap water supplied through a water bottle and sipper tube. Use of the animals in this study was approved by the Purdue University Institutional Animal Care and Use Committee.

Immunogens

Potassium thiocyanate extract (PTE) of *P. multocida* (serotype 3,12,15 : D) was prepared as previously described [6]. Briefly, *P. multocida* was grown to confluence on tryptic soy agar containing 5% sheep blood and harvested in 6 ml of equal parts saline and 1.0 M KSCN. Following incubation at 37°C for 6 h, whole cells were removed by centrifugation at 8000 *g* for 10 min, and the supernatant was dialyzed extensively against 0.01 M Tris-hydrochloride–0.32 M NaCl–0.01% NaN_3 buffer (pH 8.0). The extract was then concentrated with a Centriprep-10 concentrator (Amicon, Inc., Beverly, MA, USA) and sterilized by passage through a 0.22-μm filter.

Ovalbumin (OVA) (Grade V, Sigma Chemical Co., St. Louis, MO, USA) and a culture supernatant vaccine (PHV) of *P. haemolytica* (1-Shot^{fi}, Pfizer Animal Health, Exton, PA, USA) were both purchased from commercial suppliers.

Alginate microsphere preparation

Alginate microsphere containing 100 ug ml^{-1} of either PTE or PHV or 10 mg ml^{-1} of OVA were prepared as previously described [5, 7]. Briefly, sodium alginate, medium viscosity (Kelco, Inc., Chicago, IL, USA) was dissolved in distilled water at a 1.5% w/v concentration with constant stirring. Immunogens were added to the alginate solution to create a final alginate concentration of 1.2% w/v. The mixture was placed in a syringe pump (Harvard Instruments, Inc., South Natick, MA, USA) and infused into an atomizer (Turbotak, Inc., Ontario, Canada). The alginate/immunogen mixtures were then sprayed into a 1.5% w/v $CaCl_2$ solution placed 40 cm from the tip of the atomizer. Particles were separated from the $CaCl_2$ by low-speed centrifugation and placed in a 0.05% solution of poly-L-lysine (M_w 100 000; Sigma Chemical Co., St. Louis, MO, USA) with stirring for 30 min at room temperature. The size of the alginate spheres ranged from 1 to larger than 50 μm in diameter, with 70% of the spheres less than 10 μm in diameter as measured by a Microtrak Series 9200 particle analyzer (Leeds and Northrup Co., Northwales, PA, USA).

Portions of each batch of microspheres were boiled in an attempt to sterilize the material. In this regard, microspheres were brought to a rolling boil by placing a glass tube containing the microspheres in a beaker containing boiling water for a period of 10 min. After cooling, microspheres were cultured and then used to immunize the mice.

Enumeration of microsphere bacterial load was performed by decanting fluid once the beads had settled to the bottom of a 15-ml comical centrifuge tube. Fifty microliters of beads were added to 950 μl of PBS with 0.1 M EDTA. The suspension was thoroughly mixed on a high speed vortex for 3 min, and the beads allowed to settle to the bottom of the tube over 30 min. One hundred microliters of the supernatant was then plated for lawn growth on tryptic soy agar with 5% blood. After 24 h incubation at 37°C, bacterial colony forming units were counted on each plate.

Electron microscopy of microspheres

Microspheres were prepared for electron microscopy using previously described methods [8]. Briefly, 100 μl of microspheres were allowed to settle to the bottom of a 1.5 ml microcentrifuge tube. The supernatant $CaCl_2$ was removed and replaced with 1% of OsO_4 in distilled water. The microspheres were gently dispersed in the OsO_4 by shaking every 10 min and held at 20°C for 1 h. The microspheres were then allowed to settle by gravity followed by centrifugation at 200 *g* for 1 min. Supernatant was removed and the spheres were washed twice with distilled water. For preparation for scanning electron microscopy, spheres were dehydrated through a 30–100% ethanol gradient, washed twice in freon-113, and then pipetted onto a nucleopore filter for mounting on a specimen stub. The spheres were sputter coated with gold and viewed on a scanning electron microscope (Model ISI-100A, Topcon Technologies, Inc., Paramus, NJ, USA). Electron micrographs were taken of all typical fields.

Immunization of mice and sample collection

Groups of five mice each were immunized subcutaneously with 0.5 ml of either microspheres containing 10 mg ml^{-1} of OVA, microspheres containing 100 μg ml^{-1} of PTE, or microspheres containing 100 μg ml^{-1} of PHV. All mice were immunized initially and boosted 21 days later.

Serum was harvested by retroorbital venipuncture prior to and 28 days following initial immunization. Blood collection was performed on mice which were anesthetized by inhalation of methoxyflurane (Metofane^{fi}, Pitman-Moore, Inc., Mundelein, IL, USA). Mice were euthanized 28 days after initial immunization and the spleen and intestinal lamina propria were harvested from each using sterile technique.

Lamina propria tissue explants were placed in sterile polystyrene wells containing RPMI 1640 media with 5% fetal calf serum (Sigma Chemical Co., St. Louis, MO, USA). Tissues were incubated at 37°C under a 5% CO_2 atmosphere. After 72 h of incubation, explant supernatants were harvested, centrifuged at 8000 *g* to remove cellular debris, and frozen at −20°C until assayed for specific antibody activity by ELISA.

Enzyme-linked immunosorbent assay (ELISA)

Serum samples were assayed for anti-PTE, PHV, or OVA IgG_1, IgG_2, IgM, and IgA activity as previously described [6]. Briefly, polystyrene microtiter wells (Immulon 2, Dynatech Laboratories, Chantilly, VA, USA) were each coated with 0.5 μg per well of immunogen (OVA, PTE, or PHV) diluted in phosphate buffered saline (PBS, pH 7.4). Immediately before addition of samples, immunogen was removed, and the wells were washed with PBS containing 0.05% (v/v) Tween 20 followed by a 1-h incubation at room temperature with 0.1% gelatin (Sigma Chemical Co.,

St. Louis, MO, USA) to block non-specific binding. Samples were also added to uncoated negative control wells and processed the same as samples added to coated wells. Serial dilution of serum samples from 1 : 25 to 1 : 25 600 and undiluted for all other fluids were added at 50 μl per well, in triplicate. The plates with sample were incubated at room temperature for 3 h, washed extensively with PBS–Tween 20 and 50 μl of peroxidase conjugated goat antibodies to mouse IgA, IgM, IgG_1, and IgG_2 (Bethyl Laboratories, Montgomery, TX, USA) were added to each well. Following incubation and washing, substrate (*o*-phenylaminediamine, Sigma Chemical Co., St. Louis, MO, USA) was diluted in 0.05 M phosphate-citrate buffer (pH 5.0) was added to each well. After 30 min of incubation at room temperature, the optical density of wells was measured at 490 nm using a V_{max} microplate reader (Molecular Devices, Inc., Menlo Park, CA, USA). Specific immunoglobulin activity is expressed as the greatest dilution of sample with detectable activity at least three standard deviations greater than the negative control wells.

ELISPot assay

At the time of necropsy, the spleens were placed in Iscove's medium containing 10% fetal clone I (Hyclone Laboratories, Inc., Logan, UT, USA) and 1% penicillin-streptomycin and Amphotericin B (Sigma) added. The tissue was then macerated with sterile forceps. The medium was centrifuged at 200 *g* for 10 min, and the cell pellet was washed twice with medium and resuspended. A sample of the cell suspension was stained with trypan blue exclusion dye, and the number of live cells estimated with a hemocytometer.

Enzyme immunoassay–radioimmunoassay flat-bottom high-binding 96-well plates (Costar Corp., Cambridge, MA, USA) were coated with 2.5 μg of either PTE, PHV, or OVA per ml in PBS (100 μl per well) and incubated overnight at 4 °C. On the following day, plates were washed three times with PBS–0.05% Tween 20 (v/v) and blocked with 100 μl of PBS–0.01% bovine serum albumin (Sigma) per well at 37 °C for 1 h. Plates were then washed twice with PBS–Tween and once with PBS, and 100 μl containing 10^5 cells from each tissue was added per well. Plates were placed at 37 °C for 4 h and washed three times with PBS–Tween. Goat anti-mouse IgA, IgM, IgG_1, and IgG_2 (Zymed Laboratories, Inc., South San Francisco, CA, USA) were diluted to optimal working concentration (1 : 20 000) with PBS–Tween and absorbed with a 1 : 1000 dilution of either PTE, PHV, or OVA. Following overnight incubation at 4 °C, the plates were washed three times with PBS–Tween, and rabbit anti-goat IgG (heavy and light chains) conjugated to alkaline phosphatase (Southern Biotechnology Associates, Birmingham, AL, USA) was diluted to 1 : 1000 with PBS–Tween and absorbed with the appropriate immunogen. Seventy-five microliters of conjugated antibody was added to each well. Plates were incubated for 3 h at room temperature and washed three times with PBS–Tween. Substrate was prepared by diluting 1 part 3% (w/v) agarose (ultrapure, high melting temperature; Gibco BRL, Grand Island, NY, USA) with 4 parts 5-bromo-4-chloro-3-indolylphosphate (5-BCIP) (Sigma). The agarose was first melted in a boiling

water bath and then mixed with 5-BCIP. The mixture was kept at 40°C by placing the tube containing the mixture in a beaker containing hot water. One hundred microliters of the warm mixture were added to each well. Plates were incubated for 10 min at room temperature and placed in a humid chamber overnight at 4°C. Spots were counted with a Photo-Zoom inverted microscope (Cambridge Instruments), and the number of spots per six wells was averaged and recorded as the mean number of antibody-secreting cells (ASC) per 10^6 cells.

Statistical evaluation of data

Means and standard errors of ELISA and ELIspot values were compared using the Wilcoxon rank sum test [9]. Statistical significance was reached when $p \leqslant 0.05$.

RESULTS

Electron microscopy of microspheres

Most unboiled microspheres, containing any of the three antigens, had generally smooth surfaces with minimal aggregation of the spheres (Fig. 1). In contrast, boiled microspheres demonstrated uneven surfaces with moderate pitting and aggregation (Fig. 2). The electron microscopic appearances of microspheres containing each of the three antigens were similar.

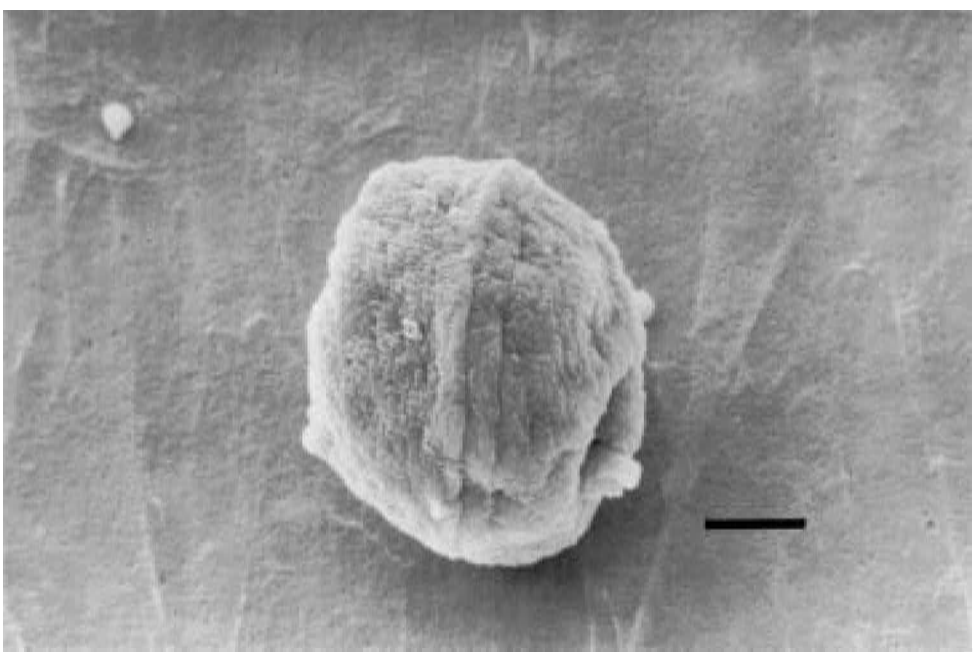

Figure 1. Scanning electron micrograph of non-boiled alginate microsphere containing ovalbumin (1500 × magnification, bar = 1 μm). The surface is smooth with minimal pitting and aggregation between microspheres.

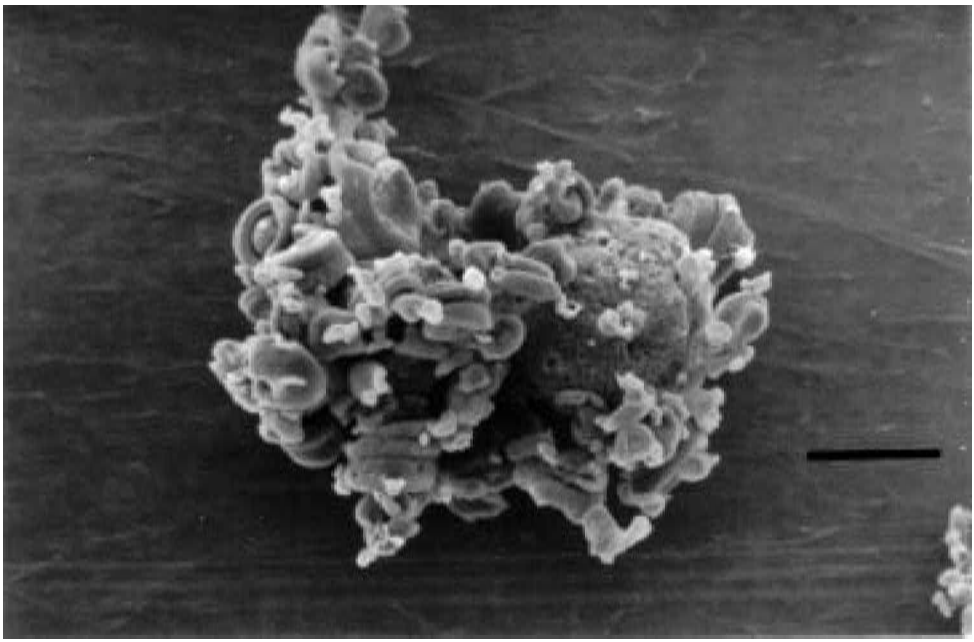

Figure 2. Scanning electron micrograph of boiled alginate microsphere containing ovalbumin (1000× magnification, bar = 2 μm). The surface is rough with substantial pitting and aggregation between microspheres.

Table 1.
Mean bacterial colony forming units (CFUs) cultured from microspheres[a]

Type of microsphere	Number of CFUs before boiling	Number of CFUs after boiling
OVA	3×10^7	NG
PHV	2×10^5	2×10^6
PTE	2×10^7	2×10^3

[a] Numbers of colony forming units (CFUs) of bacteria shown are means from cultures of three samples for each group. Samples were plated for lawn growth on TSA with 5% sheep blood and incubated for 24 h at 37°C before CFUs were counted. NG = no growth; since samples were diluted, the one that demonstrated no growth could have contained bacteria at levels below detection with these methods. OVA = ovalbumin-containing microspheres, PHV = *P. haemolytica* vaccine-containing microspheres, and PTE = *P. multocida* thiocyanate extract-containing microspheres.

Bacterial load of microspheres

Results from bacterial culture of microspheres are shown in Table 1. Boiling of the microspheres completely eliminated all aerobic vegetative bacteria from microspheres containing OVA. Bacterial load was reduced by a factor of 10^4 in microspheres containing PTE. A greater number of colony forming units (CFUs)

were cultured from microspheres containing PHV after boiling, in contrast to the other groups. Contaminating bacteria were of multiple species, based upon colony morphology.

Enzyme-linked immunosorbent assay (ELISA)

The serum antibody response to immunization with microspheres containing ovalbumin is shown in Fig. 3. Mice immunized with either boiled or nonboiled microspheres containing OVA demonstrated strong serum IgG responses to OVA, with no significant difference between these groups. Significant increases in anti-OVA IgM titers were also detected in these mice. Although anti-OVA IgA increased in mice immunized with boiled microspheres containing OVA, this increase was not significant.

Mice immunized with PHV in either boiled or nonboiled microspheres developed strong serum anti-PHV IgG responses that were not significantly different between the two groups (Fig. 4). A statistically significant increase in anti-PHV IgM was detected in mice immunized with boiled microspheres containing PHV. None of the groups examined demonstrated significant IgA anti-PHV activity.

A significant increase in anti-PTE IgG activity was observed only in mice immunized with nonboiled microspheres containing PTE (Fig. 5). Significant increases in anti-PTE IgM nor IgA were not detected in either of the treatment groups.

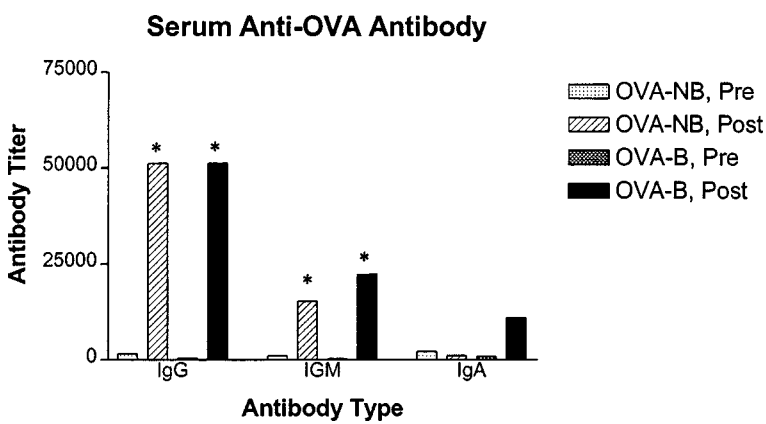

Figure 3. Titers of serum anti-OVA antibody both before and 28 days after immunization. Mice immunized with either boiled or non-boiled microspheres containing OVA developed significant IgG anti-OVA activity. Error bars are not evident due to consistency of results between individuals within groups. Significant increase compared to control is indicated by the $*$ symbol. OVA-NB, Pre = samples from mice before immunization with non-boiled microspheres containing OVA; OVA-NB, Post = samples from mice after immunization with non-boiled microspheres containing OVA; OVA-B, Pre = samples from mice before immunization with boiled microspheres containing OVA; OVA-B, Post = samples from mice after immunization with boiled microspheres containing OVA.

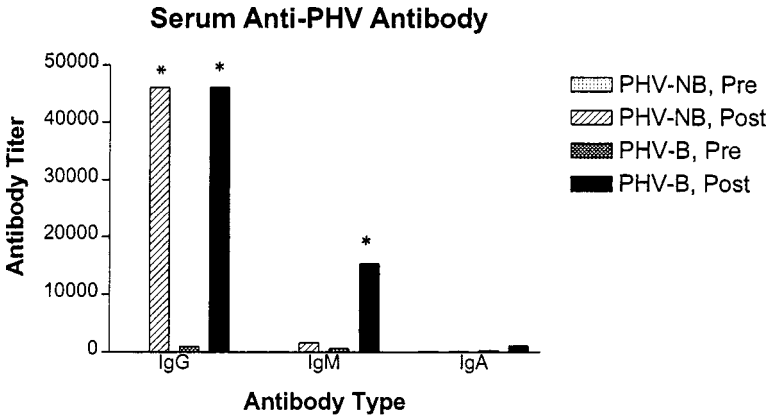

Figure 4. Titers of serum anti-PHV antibody both before and 28 days after immunization. Mice immunized with either boiled or non-boiled microspheres containing PHV developed significant anti-PHV IgG activity. Error bars are not evident due to consistency of results between individuals within groups. Significant increase compared to control is indicated by the * symbol. PHV-NB, Pre = samples from mice before immunization with non-boiled microspheres containing PHV; PHV-NB, Post = samples from mice after immunization with non-boiled microspheres containing PHV; PHV-B, Pre = samples from mice before immunization with boiled microspheres containing PHV; PHV-B, Post = samples from mice after immunization with boiled microspheres containing PHV.

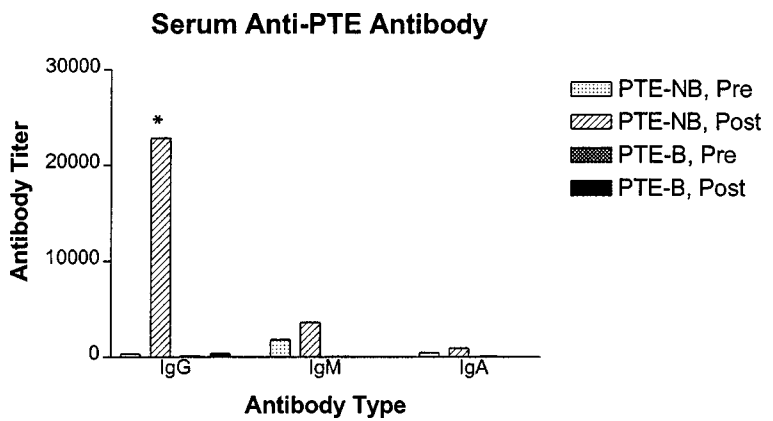

Figure 5. Titers of serum anti-PTE antibody both before and 28 days after immunization. Mice immunized with non-boiled microspheres developed a significant serum IgG anti-PTE response. Error bars are not evident due to consistency between individuals within groups. Significant increase compared to control is indicated by the * symbol. PTE-NB, Pre = samples from mice before immunization with non-boiled microspheres containing PTE; PTE-NB, Post = samples from mice after immunization with non-boiled microspheres containing PTE; PTE-B, Pre = samples from mice before immunization with microspheres containing PTE; PTE-B, Post = samples from mice after immunization with microspheres containing PTE.

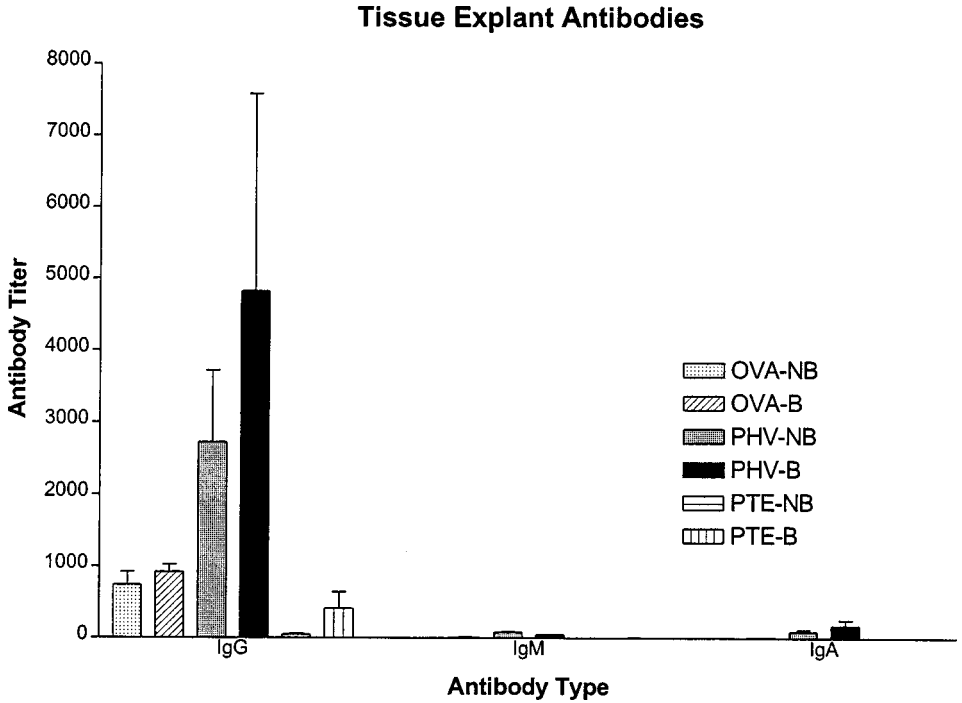

Figure 6. Titers of tissue explant supernatant antibody after immunization. Mice immunized with either boiled or non-boiled microspheres containing PHV developed marked, but not significant ($p \geqslant 0.05$), anti-PHV IgG titers. OVA-NB = non-boiled microspheres containing OVA; OVA-B = boiled microspheres containing OVA; PHV-NB = non-boiled microspheres containing PHV; PHV-B = boiled microspheres containing PHV; PTE-NB = non-boiled microspheres containing PTE; PTE-B = boiled microspheres containing PTE; bars represent standard error of the mean.

ELISA of tissue explant supernatants demonstrated marked IgG antigen-specific titers in mice immunized with either boiled or nonboiled PHV, with no significant difference between groups (Fig. 6). No appreciable IgM nor IgA activity was detected in explant supernatants for any of the treatment groups.

ELIspot assay

Results for the ELIspot assay are shown in Fig. 7. Increases in numbers of detectable IgG-positive spots were noted in tissues from animals dosed with either boiled or non-boiled microspheres containing ovalbumin and for boiled microspheres containing PHV, however these increases were not significant compared to controls.

Significant increases in numbers of IgM-positive spots were noted in tissues from mice dosed with either boiled or non-boiled microspheres containing PHV, with no significant difference between these two groups. No significant increases in IgM-positive spots were noted for any of the other treatment groups.

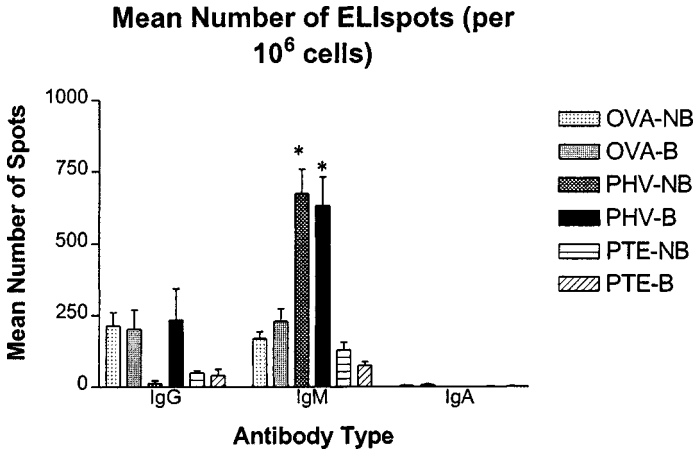

Figure 7. Mean number of ELIspots per 1.0×10^6 spleen cells. Only samples from mice immunized with non-boiled and boiled microspheres containing PHV demonstrated significant ($p \leqslant 0.05$) increases in mean numbers of IgM-positive ELIspots (indicated by the $*$ symbol). OVA-NB = non-boiled microspheres containing OVA; OVA-B = boiled microspheres containing OVA; PHV-NB = non-boiled; microspheres containing PHV; PHV-B = boiled microspheres containing PHV; bars represent standard error of the mean.

There was no marked numbers of IgA-positive spots detected in tissues from mice in any of the treatment groups.

DISCUSSION

Many infectious pathogens, including *P. haemolytica* and *P. multocida*, initially invade the body at mucosal surfaces such as those which line the gastrointestinal, respiratory, and genitourinary tracts [10, 11], thus effective immunization might involve methods to stimulate the local mucosal immune response at these sites. Because the mucosal immune system is linked, exposure to an antigen at one mucosal surface often results in an immune response to that same antigen at multiple mucosal sites [12].

Although non-replicating antigens can be used to successfully stimulate protective immunity [13–15], their efficacy is limited by both dilution and degradation in harsh environments such as the gastrointestinal tract, where low pH and enzymes may interact to render antigens nonimmunogenic. For these reasons, methods to protect antigens for delivery to immunoreactive tissues would be particularly valuable.

Microspheres of various polymers have been shown to be highly effective in delivering antigens to sites such as intestinal Peyer's patches, resulting in strong immunity in mice, monkeys, and rabbits [2, 16–18]. Previously, we found that rabbits could be immunized against the respiratory pathogen *P. multocida* by incorporating PTE into alginate microspheres which were orally administered in the drinking water. In contrast to microspheres made from many other materials,

production of alginate microspheres does not involve the use of organic solvents that could destroy fragile antigens.

Maintenance of antigen immunogenicity in the face of harsh conditions, such as high temperatures, and reduction of microbial content are important factors in the production of useful vaccines. Exposure to harmful conditions such as organic solvents or high temperatures could destroy fragile antigens, and thereby render the vaccine ineffective. In addition, the need for sterility of alginate microencapsulated materials which are administered through the gastrointestinal tract is less critical than for parenterally-administered compounds; however, the possibility of delivery of compounds, including vaccines, by parenteral routes requires that methods for disinfection of alginate microspheres be explored. In the present study, the effect of exposure to boiling on the immunogenicity of antigen encapsulated in alginate microspheres, and the utility of boiling as a simple means of reducing the bacterial load of alginate microspheres were studied.

Results from bacterial culture of boiled and non-boiled microspheres provided conflicting results. Boiling completely eliminated bacterial growth from microspheres containing ovalbumin, and reduced the number of bacteria cultured from microspheres containing PTE by a factor of 10^4. In contrast, ten times more bacteria were cultured from microspheres containing PHV after boiling. It is unclear why reduction of bacterial load in the microspheres by boiling is affected in a seeming antigen-dependent fashion. Conceivably, microsphere conformation could be altered by the type of incorporated antigen in such a way as to provide harborage for microbial organisms, although scanning electron microscopy did not demonstrate any detectable differences between types of microspheres either before or after boiling. The PHV antigen used is a live bacteria/toxoid preparation; thus, it may be that while boiling did not entirely eliminate bacteria, the process did expose antigenic bacteria that had been encapsulated within the alginate. Exposure of a live, replicating antigen such as this would also explain the marked serum and tissue explant antibody and ELIspot responses to PHV. The origin of the contaminating bacteria is uncertain, but likely resulted from the use of non-sterile, unfiltered alginate and water in preparation of the microspheres. The same materials were used to prepare all microspheres.

Immunogenicity was diminished by boiling for microspheres containing either PTE or OVA. In the case of PTE, boiling completely destroyed immunogenicity as evaluated by ELISA of serum and tissue explant supernatants, and by ELIspot. Immunogenicity of microspheres containing PHV was significant both before and after boiling as measured by all three immunologic assays. That bacterial load was increased after boiling suggests that the boiling procedure may have been misperformed for microspheres containing this particular antigen. Immunogenicity of microspheres containing OVA was reduced by boiling as indicated by diminished responses in the ELIspot assay and ELISA of tissue explant supernatants. However, OVA-containing microspheres still maintained substantial immunogenicity as evidenced by significant ELISA titers both before and after boiling. It is worth noting

again that boiling also completely eliminated detectable bacteria from microspheres in this group.

The difficulty with sterilization of polymerized material, specifically alginate, has been investigated by others [19]. In that study, chlorine was investigated as a means of reducing the number of bacteria entrapped within alginate gels. Although the purpose of the study was to use alginate as a model to determine the ability of chlorine to penetrate bacterial biofilms, it did show that alginate could not be well penetrated by chlorine to kill bacteria. The process of boiling apparently destroys the immunogenicity of some, but not all, microsphere-incorporated antigens. The temperature increase associated with boiling altered the appearance of alginate microspheres, causing them to aggregate and become pitted. Nonetheless, microspheres were not destroyed and thus apparently retained their polymerized state. An alternative approach to encapsulating antigens without bacterial contamination might be to sterilize all production equipment and to handle materials in a controlled aseptic environment.

In summary, boiling of antigen-containing alginate microspheres is a means which can reduce the bacterial load of alginate microspheres, albeit inconsistently. The method is limited, however, by significant diminution of the immunogenicity of incorporated antigens.

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REFERENCES

1. D. T. O'Hagan, D. Rahman, J. P. McGhee, H. Jeffery, M. C. Davis, P. Williams and S. S. Davis, *Immunology* **73**, 239 (1991).
2. D. T. O'Hagan, J. P. McGhee, J. Holmgren, A. Mowat, A. M. Donachie, K. H. G. Mills, W. Gaisford, D. Rahman and S. J. Challacombe, *Vaccine* **11**, 149 (1993).
3. J. Eldridge, C. Hammond, J. Meulbroek, J. Staas, R. Gilley and T. Tice, *J. Control. Rel.* **11**, 205 (1990).
4. F. Lim and A. M. Sun, *Science* **210**, 908 (1980).
5. M. A. Suckow, T. L. Bowersock, H. Park and K. Park, *J. Biomater. Sci. Polymer Edn* **8**, 131 (1996).
6. D. H. Ringler, G. K. Peter, C. E. Chrisp and D. F. Keren, *Infect. Immunol.* **49**, 498 (1985).
7. K. K. Kwok, M. J. Groves and D. J. Burgess, *Pharmaceut. Res.* **8**, 341 (1991).
8. O. Smidsrod, *Faraday Disc.* **57**, 263 (1974).
9. D. A. Fraser, *Nonparametric Methods in Statistics*. John Wiley, New York (1957).
10. J. C. Glorioso, G. W. Jones, H. G. Rush, L. J. Pentler, C. A. Darif and J. E. Coward, *Infect. Immunol.* **35**, 1103 (1982).
11. S. J. Czinn and J. G. Nedrud, *Infect. Immunol.* **59**, 2359 (1991).
12. S. Fossum, in: *Histophysiology of The Immune System*, B. Rolstad (Ed.), p. 599. Plenum Press, New York (1988).

13. M. A. Suckow, T. L. Bowersock, K. Nielsen, C. E. Chrisp, P. L. Frandsen and E. B. Janovitz, *Lab. Anim. Sci.* **45**, 526 (1995).
14. D. Bessen and V. A. Fischetti, *Infect. Immunol.* **56**, 2666 (1988).
15. D. Kaul and P. L. Ogra, *Dev. Biol. Stand.* **95**, 141 (1998).
16. J. Eldridge, J. K. Staas, J. A. Meulbroek, J. R. McGhee, T. R. Tice and R. M. Gilley, *Mol. Immunol.* **28**, 287 (1991).
17. C. E. McQueen, E. C. Boedeker, R. Reid, D. Jarboe, M. Wolf, M. Le and W. R. Brown, *Vaccine* **11**, 201 (1993).
18. D. T. O'Hagan, J. P. McGee, R. Boyle, D. Gumaer, X. M. Li, B. Potts, C. Y. Wang and W. C. Koff, *J. Control. Rel.* **36**, 75 (1995).
19. X. Xu and P. S. Stewart, *Biotech. Bioengineer.* **49**, 93 (1996).