Systemic Immunization With Hsp60 Alters the Development of Chlamydial Ocular Disease

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Purpose. To determine whether immunization with recombinant Hsp60 would exacerbate ocular pathology on challenge with viable chlamydial elementary bodies.

Methods. Guinea pigs were immunized either subcutaneously with recombinant Hsp60 or both subcutaneously with recombinant Hsp60 and ocularly with attenuated Salmonella typhimurium expressing the guinea pig inclusion conjunctivitis (GPIC) Hsp60 antigen. All animals were challenged in the conjunctiva with the agent of GPIC, and the degree of gross ocular pathology was determined. Immunoglobulin G (IgG) and immunoglobulin A (IgA) antibody titers to Hsp60 were measured in ocular secretions as a measure of the degree of immunization.

Results. In primary and challenge GPIC infection, the degree of gross ocular pathology was lower in the immunized group. The presence of high IgA and IgG antibody titers to Hsp60 in tears suggested that the response may have been modified by the presence of blocking antibodies that either may have removed the antigen quickly or prevented interaction with sensitized T cells. In contrast to subcutaneous immunization, the combined immunization regimen, consisting of subcutaneous recombinant Hsp60 followed by ocular inoculation of the attenuated Salmonella, resulted in no difference in gross pathology after reinfection of guinea pigs with GPIC.

Conclusions. These data indicated that the immunization with Hsp60 did not produce exacerbated disease on challenge with viable organisms; however, the data suggested that the route of administration, form of antigen, or both may be critical in the disease process. Invest Ophthalmol Vis Sci. 1995;36:1344-1351.

It has been well documented in the human and in the primate that trachoma results primarily from an immunopathologic response to repeated or chronic infection with Chlamydia trachomatis. Although the use of a whole elementary body vaccine for trachoma is not endorsed, it is of interest to determine those chlamydial components responsible for eliciting the pathologic response. By definition, these may not be the optimal components of a vaccine and should in fact be deleted from a potential vaccine.

Eliciting such a response is the 57-kd heat shock protein (Hsp60) of Chlamydia. Watkins et al originally reported that a Triton-X 100 extract of elementary bodies (EB) from the C. psittaci agent of guinea pig inclusion conjunctivitis (GPIC) could elicit a pathologic response in the eyes of previously infected guinea pigs that resembled the reaction to a primary GPIC infection. The guinea pig–GPIC model was found to be an excellent model for trachoma because repeated infection can produce a disease similar to that appearing in humans. Morrison et al later cloned and identified the active component of this extract as the chlamydial homologue of Escherichia coli GroEL. The homologous protein isolated from C. trachomatis was found also to be capable of inducing a delayed-type hypersensitivity (DTH) response in the eyes of previously infected monkeys. Although it is not clear if there are other chlamydial antigens that can elicit a DTH response, it seems obvious from these experiments that any potential vaccine against Chlamydia should not include the Hsp60 protein.

However, one caveat of the above studies was that...
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the response was always elicited with purified protein in a buffer containing Triton X-100, an artificial situation. No experiments have been reported in which animals sensitized to Hsp60 by prior immunization had more severe reactions when challenged with live EB. The purpose of this study was, thus, to determine whether immunization with Hsp60 would actually elicit an exacerbated pathologic response when animals were challenged in the eye with viable GPIC.

METHODS

Experimental Animals

Female Hartley strain guinea pigs, each weighing 450 to 500 g, were obtained from Sasco Laboratories (Omaha, NE). All animals were housed individually in cages covered with fiberglass filter tops and given food and water ad libitum. Unless stated otherwise, each experimental group routinely consisted of five animals. Experiments were repeated at least once. All animal experiments adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Culture of Chlamydiae and Assessment of Infection

Purification of GPIC for antigen and for infection purposes was performed according to standard practices previously described. Stocks for infection were prepared in McCoy cells while antigen was prepared in HeLa cells. The infection was assessed by the collection of conjunctival scrapings using a dental plastic instrument and then staining with Giemsa. The inclusion score was determined as the percentage of epithelial cells containing chlamydial inclusions.

Ultraviolet (UV) light-inactivated GPIC was prepared by irradiating a suspension of GPIC approximately 10 cm from a Germidical lamp (Westinghouse Sterilamp #63615L) for 2 hours with constant stirring. This procedure has been found to inactivate 100% of the organisms present.

Infection of Guinea Pigs

Guinea pigs were infected in the conjunctiva by instilling 20 µl of GPIC suspended in sucrose–phosphate buffer (2-SP) directly into the conjunctival sac. In some cases, only one eye was inoculated. In the assessment of infection in these animals, both eyes were observed for the development of disease, but in no case was contamination of the uninoculated eye detected. Infection doses were generally approximately 10³ inclusion-forming units of GPIC. This dose, though perhaps unrealistically high in comparison to humans, was used to ensure a larger amount of antigen to provide the maximum chance to elicit the pathologic response.

Purification of rHsp60

Recombinant HSP60 (rHsp60) expressed by JM109 (pGP57) was purified by sucrose density gradient centrifugation as described by McMullin and Hallberg with some modifications. An overnight culture (4 ml) of JM109 (pGP57) was used to inoculate 1 liter of Luria–Bertani medium plus ampicillin (50 µg/ml) and grown to OD₆₀₀ = 1.0.

The culture was placed on ice for 15 minutes and then centrifuged for 15 minutes at 6000 RPM in a JA-10 rotor (Beckman Instruments) at 4°C. The cells were resuspended in 40 ml of lysozyme solution (1 mg/ml lysozyme, 30 mM Tris-HCl, pH 8.0, 1 mM ethylenediaminetetraacetic acid, 20% sucrose (wt/vol) and were kept on ice for 30 minutes. The resultant spheroplasts were then centrifuged for 10 minutes at 2800g at 4°C. The cells were resuspended in 8 ml of buffer TM (50 mM Tris-HCl, pH 7.0, 10 mM MgCl₂) on ice and sonicated for 5 to 10 seconds. The lysate was then centrifuged at 26,000g for 15 minutes, and the supernatant was collected. Two milliliters (approximately 25 mg total protein) of the supernatant was layered onto a 15% to 30% continuous sucrose gradient made with buffer TM in 25 X 89 mm Beckman Ultra Clear tubes (Palo Alto, CA). An SW28 rotor was run at 25,000 RPM and 4°C for 18 hours. Fractions (2 ml) were collected and analyzed by SDS–PAGE and Coomassie blue staining. Peak fractions containing rHsp60 were used for subsequent experiments (Fig. 1).

Generation of Salmonella typhimurium Expressing Chlamydial rHsp60

S. typhimurium strain SL3261 was prepared for electrophoresis in water according to BioRad (Richmond, CA). Gene Pulser instructions. Electrocompetent SL3261 cells (4 X 10⁹ colony-forming units in 40 µl) were mixed with pGP57 plasmid DNA (10 µg in 10 µl water) in a 0.2 cm gene pulser cuvette and electrophoresed using a BioRad Gene Pulser (25 µFD and 1.5 kV) equipped with Pulse Controller (1000 OHMS). Ampicillin-resistant colonies were grown to OD₆₀₀ = 0.7 and frozen at –70°C in Luria–Bertani medium supplemented with 10% glycerol. Isolates were assessed by SDS–PAGE for expression of rHsp60. A positive isolate designated SL3261 (pGP57) expressed high levels of rHsp60 (approximately 5% to 10% of bacterial dry weight) and was used for subsequent inoculations of animals (Fig. 2).

Immunization and Assessment of Immune Response

Guinea pigs were immunized subcutaneously (SC) with 50 µg of rHsp60 suspended in phosphate-buff-
FIGURE 1. Purification of GroEL. A Coomassie blue stained gel of each fraction from the sucrose gradient purification procedure (10 μl per lane). Lane 1 represents the bottom of the gradient, and lane 19 represents the top. Molecular weights (kd) are indicated at the right. The top arrow indicates the position of rHsp60; the bottom arrow indicates the position of rGsp10 (GroES homologue).

Serum and ocular secretions were obtained as described previously. Antibodies to Hsp60 were measured using a standard enzyme-linked immunosorbent assay with either GPIC elementary bodies or rHsp60 as antigens. For each antigen, 0.5 μg were used per well in a 96-well microtiter plate. Immunoglobulin G (IgG) antibodies to either antigen were measured using peroxidase-labeled rabbit anti-guinea pig IgG (ICN, Costa Mesa, CA), and immunoglobulin A (IgA) antibodies were measured using rabbit anti-guinea pig α-chain (ICN) followed by peroxidase-labeled goat anti-rabbit IgG (ICN).

Assessment of Pathologic Changes
Pathologic changes were assessed using a modification of the 0 to 4+ scale described by Watkins et al. Briefly, palpebral and bulbar conjunctiva are evaluated for erythema, edema, and exudation. The scores are defined as follows: slight erythema or edema of either the palpebral or bulbar conjunctiva, 1+; definite erythema or edema of either the palpebral or bulbar conjunctiva, 2+; definite erythema or edema of both the palpebral or bulbar conjunctiva, 3+; definite erythema or edema of both the palpebral or bulbar conjunctiva and the presence of exudate, 4+. In most experiments, two individuals evaluated the animals although it was not possible to blind the individuals as to the identity of the animals. In the majority of the cases, the scores were reported to be the same by each grader. If there was disagreement, the mean of the scores was taken.

RESULTS
Subcutaneous Immunization With rHsp60
To determine whether immunization with rHsp60 could exacerbate an ocular challenge with viable GPIC, five guinea pigs were injected subcutaneously with 50 μg of purified rHsp60 in Freund’s incomplete adjuvant on three separate occasions at 2-week inter-
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A B

FIGURE 2. Salmonella typhimurium SL3261 expressing chlamydial Hsp60. Strains: lane 1, SL3261; lane 2, SL3261(PGP57). Molecular weights (kd) are indicated at the left. The arrow indicates the position of rHsp60.

vals. Two weeks after the last immunization, the immunized animals and four control unimmunized animals were inoculated in the left eye only with 10^6 inclusion-forming units of GPIC, and the development of gross pathology was monitored daily. Conjunctival inclusion scrapings were collected every 3 days for the determination of inclusion scores. Interestingly, the animals immunized with rHsp60 did not have enhanced pathology but actually had a small but significantly (P < 0.0001) lower level of pathologic changes than did the control unimmunized animals, according to a two-way analysis of variance with repeated measures on two factors (treatment and days) (Fig. 3). Furthermore, it was interesting to note that reactions were not seen by 24 to 48 hours after inoculation, which would have been the time a response was expected if there were a strong classic DTH reaction. In contrast, there was no difference in the course of the infection between the two groups as assessed by inclusion scores (Fig. 4).

This experiment was repeated, and a group of animals immunized with UV light-inactivated EB was included to determine if a similar reduced response was observed. Once again, the response of the rHsp60-immunized animals was reduced when compared to the controls, as was the response of guinea pigs immunized with UV-GPIC EB (Fig. 5). As observed in the first experiment, immunization with rHsp60 had no

FIGURE 3. Effect of immunization with rHsp60 by the subcutaneous route on the development of pathologic changes in the eye in a primary guinea pig inclusion conjunctivitis infection.

FIGURE 4. Effect of immunization with Hsp60 by the subcutaneous route on the course of ocular infection with guinea pig inclusion conjunctivitis.
FIGURE 5. Effect of subcutaneous immunization with rHsp60 on a primary and a challenge infection with guinea pig inclusion conjunctivitis (GPIC) in comparison to immunization with ultraviolet light-inactivated GPIC elementary bodies. Animals were given a primary infection only in the left eye. The challenge data in this Figure represent the response in the right eye when reinfected 30 days after infection.

Effect on the course of the primary ocular infection when assessed by the percentage of conjunctival cells bearing inclusions on a Giemsa-stained conjunctival scraping, whereas immunization with UV–GPIC did reduce the intensity of infection, as seen before.\(^{13}\) The animals were then reinfected with viable GPIC in both eyes either 30 or 75 days after the primary infection to determine if the rHsp60 immunization followed by infection in one eye would accentuate the course of a second infection. In actual clinical disease in humans, it is the repeated antigenic challenge that apparently results in the production of immunopathology, so this experiment was more realistic in this regard. Reinfection with live GPIC in the previously infected left eye did not result in any obvious pathologic changes (data not shown). In contrast to the left eye, pathologic changes were noted in the previously uninfected right eye after the infection on day 30 (Fig. 5). However, the pathologic response was significantly lower \((P < 0.001)\) in the immunized group when compared to UV–GPIC-immunized and unimmunized controls.

When separate immunized groups, also given a primary infection in the left eye, were reinfected in both eyes 75 days after the primary infection, pathologic changes were seen in both eyes; in both scenarios, the pathologic response was lower in the UV–GPIC-immunized and the rHsp60-immunized groups than in the control group (Fig. 6). These data were provocative because they were contrary to what was expected. Rather than producing an exacerbated response by immunizing with rHsp60 before ocular challenge with viable chlamydiae, immunization actually seemed to provide a statistically significant measure of protection against pathologic changes, albeit not great in magnitude.

The IgG and IgA antibody responses to Hsp60 in ocular secretions before reinfection at 30 and 75 days after the primary infection was determined to assess the degree of reactivity to each antigen. At each time, the response to Hsp60 was significantly greater in the rHsp60-immunized guinea pigs when compared to controls, although a strong response was also noted in the group immunized with UV–GPIC EB (Table 1).

Immunization With Attenuated \textit{Salmonella typhimurium} Expressing rHsp60

One explanation for these results was that SC immunization was unable to elicit T cells with the appropriate receptors necessary to home to the mucosal site. Thus, it may be essential to stimulate a local mucosal T cell response for a local DTH response to be elicited by GPIC challenge. To test this hypothesis, guinea pigs were immunized with attenuated \textit{S. typhimurium} expressing rHsp60. One group of guinea pigs was immunized SC with 50 \(\mu\)g of purified rHsp60 in Freund’s incomplete adjuvant, followed 2 weeks later with ocular inoculation of recombinant bacteria, and 2 weeks
after that with a combined ocular bacterial inoculation and SC purified rHsp60 inoculation. A second group received three SC inoculations with rHsp60 in Freund’s incomplete adjuvant at 2-week intervals. A third group was not immunized. Both immunization regimens resulted in the production of specific serum and secretion IgG (Table 2) when assessed just before the primary infection, indicating that the system was indeed primed. The IgG response in the SC immunized group was much greater than the response elicited by the combined ocular and SC immunization regimen.

Two weeks after the last immunization, all guinea pigs were given a primary infection with 10^7 inclusion-forming units of GPIC in both eyes. As before, the pathologic response resulting from the primary infection was lower in the SC immunized group than in the control group (data not shown). The combined SC–ocular immunized group had an equally low response. When the course of the infection was compared between the groups, no differences in the level or length of infection were noted, and the infection was, in fact, similar to the infection seen in Figure 4.

However, on reinfection 30 days after the primary infection, only the SC group demonstrated a decreased pathologic reaction. The SC–ocular combined immunization group had the same level of response as did the control animals (Fig. 7A). Once again, responses were not seen at 24 to 48 hours after inoculation. Similar data were obtained when the experiment was repeated (Fig. 7B). When the IgG antibody response to Hsp60 was determined in ocular secretions before the day 30 challenge, SC immunization was once again found to elicit a higher response than the SC–ocular immunization group (3.38 ± 0.22 versus 2.28 ± 0.27, respectively; P < 0.0001).

**DISCUSSION**

In this study, we attempted to determine the effect of immunization of guinea pigs with purified rHsp60 on subsequent ocular infection with GPIC. Because Hsp60 has been shown to elicit a DTH response in

### TABLE 1. Antibody Titers to Hsp60 in Ocular Secretions Before Reinfection at Either 30 or 75 Days After a Primary Ocular Infection

<table>
<thead>
<tr>
<th>Immunization</th>
<th>Day 30</th>
<th>Day 75</th>
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<tbody>
<tr>
<td></td>
<td>Immunoglobulin G</td>
<td>Immunoglobulin A</td>
</tr>
<tr>
<td>Hsp60</td>
<td>3.16 ± 0.26</td>
<td>1.72 ± 0.37</td>
</tr>
<tr>
<td>UV–GPIC</td>
<td>2.26 ± 0.49</td>
<td>1.90 ± 0.37</td>
</tr>
<tr>
<td>None</td>
<td>1.72 ± 0.46</td>
<td>1.90 ± 0.37</td>
</tr>
</tbody>
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* Log_{10} ± SD.

### TABLE 2. Antibody Titers to Hsp60 in Serum and Ocular Secretions Before the Primary Infection

<table>
<thead>
<tr>
<th>Immunization</th>
<th>Serum IgG</th>
<th>Ocular Secretions IgG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subcutaneous ocular alone</td>
<td>3.82 ± 0.22</td>
<td>1.96 ± 0.64</td>
</tr>
<tr>
<td>Subcutaneous ocular alone</td>
<td>3.70 ± 0.32</td>
<td>0.70 ± 0.41</td>
</tr>
</tbody>
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* Log_{10} ± SD.

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**FIGURE 7.** Comparison of subcutaneous immunization with rHsp60 and combined subcutaneous immunization with rHsp60 and ocular immunization with viable Salmonella typhimurium expressing guinea pig inclusion conjunctivitis rHsp60. Panels A and B represent two separate experiments.
Although it is also possible that the DTH reaction was not primed by immunization through the SC route, pre-

vious immunization regimen elicited high titers of IgG and IgA antibodies to Hsp60. It is conceivable that the 

antibody could "block" the Hsp60 or effect its removal so that a DTH response is not initiated or is diminished.15 Hsp60 as a local site.15 Immunization with whole GPIC elementary bodies did not reduce the pathologic response to 

reinfection.

The mechanism of this protective response remains to be defined, but it could be related to a strong antibody response to Hsp60 in ocular secretions. The subcutaneous immunization regimen elicited high titers of IgG and IgA antibodies to Hsp60. It is conceivable that the 

antibody could "block" the Hsp60 or effect its removal so that a DTH response is not initiated or is diminished. 

Although it is also possible that the DTH response was not primed by immunization through the SC route, previous studies have demonstrated that immunization of guinea pigs with purified Hsp60 by the SC route does 

clicit strong lymphocyte proliferative responses when peripheral blood lymphocytes are incubated with Hsp60 

antigen in vitro (Kincy and Rank, unpublished data, 1992). We have noted in a murine model of reactive 

arthritis that in the absence of antibody, chlamydial antigen remains at the local site longer, and the pathologic 

reaction is markedly enhanced. Thus, the arthritis model indicates that antibody can downregulate chlamydial disease.

In contrast, when animals were immunized with a regimen that combined subcutaneous immunization with 

rHsp60 and immunization locally in the conjunctiva with avirulent S. typhimurium expressing GPIC 

rHsp60, no protective response was noted. In fact, the intensity of the pathologic response was similar to that of the control unimmunized animals. These data suggest that the route of exposure to Hsp60 may be critical in the development of a pathologic response, although further experiments with immunization by other mucosal routes will be necessary to confirm these observations. In natural exposure of individuals to C. trachomatis in trachoma-endemic areas, people are exposed by the ocular route, and a DTH response occurs even in situations in which apparently few organ- 

isms can be isolated.9 Similarly, in the guinea pig, immunization by the ocular route with rHsp60, expres-

sed by a viable organism that can gain access to the intracellular environment, also elicits a response comparable to the natural infection. However, if ani-

mals are previously immunized by the same rHsp60 through a parenteral route, the pathologic response on challenge with viable organisms is diminished. Thus, in the development of a vaccine for trachoma or for genital tract disease, immunization through the mucosal route may actually be less desirable because of the potential for the induction of a pathologic event, especially because it is not clear if Hsp60 is the only antigen capable of eliciting an immunopatho-

logic response.

An explanation for this phenomenon might be that previous local immunization followed by GPIC infection 

may elicit a larger population of Hsp60-specific T cells that could home to the local site, thereby shifting the 

balance away from any blocking function of local antibody. This explanation is more likely for the results of the secondary infection because that response is pre-

dominantly a DTH response.3 An alternative explanation is that the immunization dose associated with the 

S. typhimurium recombinant may have been insufficient to elicit the "protective" response in comparison to im-

munization with purified RHsp60 administered subcutaneously. However, we have noted that ocular immuniza-

tion with the S. typhimurium recombinant is sufficient to elicit a local mucosal antibody response to Salmonella 

antigens (data not shown).

In contrast to reinfection, the primary GPIC infection in the guinea pig results in a vigorous acute inflam-

matory reaction that can mask a DTH response. It is not clear how immunization with rHsp60 could alter this response because acute inflammation arises from mechanisms not necessarily related to the ac-

quired immune response.

Key Words

Chlamydia, heat shock protein, trachoma, guinea pig, immu-

nization

Acknowledgments

The authors thank Dr. Richard Morrison for his generous contribution of his clone expressing the GPIC Hsp60 and Aleisha Shurley for her excellent technical assistance.
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