

Heat Shock Proteins HSP27 and HSP70 Are Present in the Skin and Are Important Mediators of Allergic Contact Hypersensitivity¹

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Proteomic analysis of murine skin has shown that a variety of heat shock proteins (HSPs) are constitutively expressed in the skin. Using murine allergic contact hypersensitivity as a model, we investigated the role of two heat shock proteins, HSP27 and HSP70, in the induction of cutaneous cell-mediated immune responses. Immunohistochemical examination of skin specimens showed that HSP27 was present in the epidermis and HSP70 was present in both the epidermis and dermis. Inhibition of HSP27 and HSP70 produced a reduction in the 1-fluoro-2,4-dinitrobenzene contact hypersensitivity response and resulted in the induction of Ag-specific unresponsiveness. Treatment of dendritic cell cultures with recombinant HSP27 caused in the up-regulation of IL-1 β , TNF- α , IL-6, IL-12p70, and IL-12p40 but not IL-23p19, which was inhibited when Abs to HSP27 were added. The 1-fluoro-2,4-dinitrobenzene-conjugated dendritic cells that had been treated with HSP27 had an increased capacity to initiate contact hypersensitivity responses compared with control dendritic cells. This augmented capacity required TLR4 signaling because neither cytokine production by dendritic cells nor the increased induction of contact hypersensitivity responses occurred in TLR4-deficient C3H/HeJ mice. Our findings indicate that a cascade of events occurs following initial interaction of hapten with the skin that includes increased activity of HSPs, their interaction with TLR4, and, in turn, increased production of cytokines that are known to enhance Ag presentation by T cells. The results suggest that HSPs form a link between adaptive and innate immunity during the early stages of contact hypersensitivity. *The Journal of Immunology*, 2009, 182: 675–683.

Heat shock proteins (HSPs)³ are a highly conserved family of intracellular proteins that are constitutively expressed in virtually all nucleated cells. Their synthesis is enhanced by exposure to a variety of stressful conditions, including heat, toxins, oxidative stress, and glucose deprivation (1). HSPs have been categorized into various families (HSP110, HSP90, HSP70, HSP60, HSP27, and other small HSPs) depending on their molecular weights and functional attributes (2, 3).

Increasing evidence suggests that HSPs may play important roles in both innate and adaptive immunity by acting as chaperones for immunologically important molecules such as MHC class I, Igs, TCRs, and TLRs. HSPs can also deliver chaperoned haptens and Ags from non-APCs to MHC molecules of APCs. As a result, HSPs have been found to be remarkably potent in their ability to elicit T cell-mediated immune responses to tu-

mors, viruses, and minor histocompatibility Ags (4). HSPs that make their way to the extracellular milieu also serve as cytokines to activate innate functions of APCs. Depending upon the mode of tissue damage, the release of HSPs may also play an immunoregulatory role in vivo (5).

As in any other organ system, several HSPs are constitutively expressed in the skin and can be up-regulated as a result of exposure to stresses such as heat, cold shock, PGs, arsenite, and oxidative stress (2, 3). Using proteomic mapping, we have shown that six molecular chaperones, HSP27, HSP60, HSP70, HSP84, ER60, and GRP78, are constitutively expressed in the skin of C3H/HeN and BALB/c mice. Of the multiple protein molecular chaperones identified in the skin proteome, only HSP27 was found at predominantly higher levels in the epidermis (6). The 27-kDa HSP (HSP27, small HSP was originally named as HSP25 in mice; in this manuscript, the molecule will be termed HSP27) form large oligomers that can act as molecular chaperones and can protect cells from heat shock and oxidative stress in vitro. Also, expression of HSP27 is reduced or absent in basal cell carcinoma, squamous cell carcinoma, and other malignancies arising from keratinocytes, suggesting that the lack of HSP27 might be a marker of epidermal malignancy (7, 8). Another relevant HSP family is the 70-kDa family. The 70-kDa family is among the most abundant and most thoroughly investigated. HSP70 family members are expressed constitutively within keratinocytes and are elevated in both epidermis and dermis after skin samples are heat shocked in vivo and in vitro (9, 10). Stressors such as UV light and 815-nm diode laser treatment of skin may also elevate their levels in keratinocytes (11, 12).

Despite the fact that the skin is proficient at initiating and eliciting immune responses and that a variety of HSPs have important immunological activities, the role of HSPs in the

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³ Abbreviations used in this paper: HSP, heat shock protein; CHS, contact hypersensitivity; DNFB, 1-fluoro-2,4-dinitrobenzene; DNBS, 2,4-dinitrobenzene sulfonic acid; DC, dendritic cell; BMDC, bone marrow-derived DC.

immunopathology of the skin has not been investigated. Using murine allergic contact hypersensitivity (CHS) as a model, the studies presented here were designed to examine the role of HSP27 and HSP70, two HSPs that are expressed in the skin, in the induction of cutaneous cell-mediated immune responses. We found that inhibition of HSP27 and HSP70 retarded the development of CHS to 1-fluoro-2,2-dinitrobenzene (DNFB) and led to the induction of tolerance to that hapten. We also observed that HSP27 treatment of dendritic cells (DCs) led to an increase in the secretion of several cytokines that are known to be involved in Ag presentation to T cells and an augmented capacity to initiate CHS responses. Finally, we found that the effect of HSPs on DCs did not occur in TLR4-deficient mice, indicating that components of the innate immune system were required for HSP27 effects.

Materials and Methods

Animals and reagents

Adult female, 6- to 8-wk-old C3H/HeN mice were obtained from Charles River Laboratories and adult female, 6- to 8-wk-old C3H/HeJ mice were purchased from The Jackson Laboratory. Animals were maintained in accordance with institutional guidelines.

Normal goat IgG and goat polyclonal anti-HSP27 and anti-HSP70 IgG were purchased from Santa Cruz Biotechnology. Alexa Fluor 488-conjugated donkey anti-goat IgG, Alexa Fluor 594-conjugated goat anti-rabbit IgG, and Texas Red X-phalloidin and sheep anti-rat IgG Dynabeads were from Invitrogen. Hybridoma lines GK1.5 (anti-CD4), Lyt-2 (anti-CD8), and HB-32 (anti-Ia^b) were acquired from American Type Culture Collection. CD3e, CD45R/B220, and recombinant GM-CSF were obtained from BD Pharmingen. Recombinant HSP25 (The 27-kDa HSP was originally named as HSP25 in mice; in this manuscript, the molecule will be termed HSP27) was purchased from Calbiochem. DNFB, 2,4-dinitrobenzene sulfonic acid (DNBS), LPS, polymyxin B, and rIL-4 and were purchased from Sigma-Aldrich.

Immunofluorescent staining

Staining for HSP27 and HSP70 was done as described in our previous studies (6). Briefly, fixed skin sections were incubated with anti-HSP27/anti-HSP70 primary Ab and were then treated with Alexa Fluor 488-conjugated donkey anti-goat IgG; tissues incubated with Texas Red X-phalloidin were subsequently treated with Alexa Fluor 594 goat anti-rabbit IgG. Following incubation with secondary Ab, all tissues were counterstained with 20 μ g/ml Hoechst 33258 and mounted in polyvinyl alcohol mounting medium. Fluorescent emissions were detected with a Zeiss Axiovert 100 microscope with epifluorescent optics and analyzed with LSM 3.8 software (Zeiss). The dilution factor for anti-HSP27 and anti-HSP70 Abs was 1/100.

Ab application on skin

To assess the contribution of HSP27 and 70 in CHS, the abdominal skin of mice was prepared by removing hair with an electric trimmer in conjunction with gentle brushing of the skin with a soft bristle toothbrush for 30 strokes. This was followed by epicutaneous application of 2 μ g of control Ab, anti-HSP27, anti-HSP70 Ab, or a combination of anti-HSP27 and anti-HSP70 Ab in PBS for 2 h under occlusion with a bioocclusive dressing (Tegaderm; 3M). The site was examined for erythema and edema using a modified Draize scoring system of 0–3 (13). All animals used for experiments had a Draize score of <1. Studies were also performed which showed that application of Ab to the shaved abdominal skin allowed Ab to penetrate into the epidermis and dermis (see Fig. 2).

CHS to DNFB

CHS to DNFB was performed as described previously (14). Briefly, the shaved abdominal skin of C3H/HeN mice was sensitized on day 0 with 25 μ l of a 0.5% solution of DNFB in a 4:1 mixture of olive oil:acetone. Five days later, a challenge dose of 20 μ l of 0.2% DNFB was painted on the ear after measuring baseline ear thickness. The increase in ear swelling was measured at 24-h intervals to quantitate the magnitude of the CHS response as described previously (14, 15).

Generation of bone marrow-derived DC (BMDC)

BMDC were prepared from C3H/HeN and C3H/HeJ mice as described, with some modifications (16). Briefly, bone marrow cells from femurs and

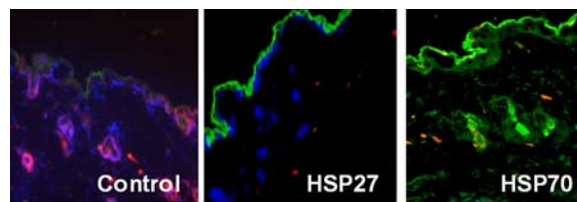


FIGURE 1. HSP27 is expressed in the epidermis while HSP70 is expressed in both the epidermis and dermis in C3H/HeN mice (A). HSP27 and HSP70 expression in naive skin samples were collected from naive mice and stained with HSP27 or HSP70 Ab. *Left panel* is naive skin incubated with control Abs; *middle panel* is naive skin treated with HSP27 Abs; and the *right panel* is naive skin treated with HSP70 Abs.

tibiae were incubated in RPMI 1640 medium with a mixture of Abs against Ia^k, CD45R/B220, Lyt-2, and GK1.5 (2 μ g/10⁶ cells) on ice for 1 h and washed once with HBSS after lysis of RBCs. Different cellular populations were removed from the cell suspension by Ab-mediated depletion using sheep anti-rat IgG Dynabeads according to the manufacturer's instructions. Cells were washed once with HBSS and cultured in 10% FCS/RPMI 1640 medium supplemented with recombinant mouse GM-CSF (10 ng/ml) and IL-4 (10 ng/ml) in 6-well plates (5 $\times$ 10⁵ cells/well). On day 5, one-half of the medium was replaced with fresh medium and cells were stimulated on the following day for the experiments.

Stimulation of DNFB-primed lymph node cells with hapten-conjugated BMDC for cytokine production

To assess Ag-specific cytokine production, BMDC were used for in vitro stimulation of primed lymph node cells as described elsewhere (14). Briefly, mice were pretreated with anti-HSP27/70 or species-matched control Ab as described earlier and were then sensitized with DNFB in a 4:1 mixture of olive oil:acetone on day 0. On day +5, mice were sacrificed and lymph node cell suspensions were prepared by gentle pressure through a wire mesh screen. Ten million BMDC were suspended in 1 ml of DNBS solution (5 mM in PBS) for 15 min. The cells were then washed three times with RPMI 1640 containing 5% FCS and resuspended in the culture medium. DNFB-primed lymph node cells (2 $\times$ 10⁶/ml) were stimulated with DNBS-labeled BMDC (2 $\times$ 10⁵/ml). Cytokine concentrations in culture supernatants were measured 48 h after cultures by cytokine-specific ELISA.

Assessment of tolerance

Tolerance to DNFB was assessed by a modification from the protocol of Schwarz et al. (17). On day –14, the abdominal skin of mice was treated as described above for Ab application on the skin. Two micrograms of control Ab, anti-HSP27, anti-HSP70, or a combination of anti-HSP27 and anti-HSP70 Abs in PBS was applied to the skin for 2 h under occlusion with a bioocclusive dressing. Immediately thereafter, 25 μ l of 0.5% DNFB was applied topically to the Ab-treated site. On day 0, mice were resensitized on the dorsal skin, a site that had not been treated with Ab, with 25 μ l of 0.5% DNFB. Five days later, mice were challenged with 20 μ l of 0.2% DNFB on the ear. The increase in ear swelling was measured at 24-h intervals as described above.

To evaluate antigenic specificity of tolerance, mice were treated in the same manner as above. One group of anti-HSP27- and anti-HSP70 Ab-treated mice was sensitized with 25 μ l of 0.5% DNFB on day –14, but on day 0 they were sensitized with 50 μ l of 3% oxazolone on their shaved dorsal side and 5 μ l on each footpad. On day +5, hapten-sensitized and control mice were challenged with 20 μ l of 0.2% DNFB or 1% oxazolone on the ear. The increase in ear swelling was measured at 24-h intervals as described above.

Adoptive transfer of regulatory cells

Mice were treated epicutaneously with 2 μ g of control or anti-HSP27 and anti-HSP70 Ab on their shaved abdomen for 2 h as described above in *Ab application on the skin*. Subsequently, 25 μ l of 0.5% DNFB was applied topically to the abdomen on the Ab-treated site. On day +5, donor mice from the two groups were sacrificed and single-cell lymphocyte suspensions were prepared from draining lymph nodes. Lymph node cells, 50 $\times$ 10⁶ in 200 μ l of PBS, were injected i.v. into the tail vein of naive recipient mice. The recipients were contact sensitized immediately after adoptive transfer of the lymph node cells with 25 μ l of 0.5% DNFB on the shaved

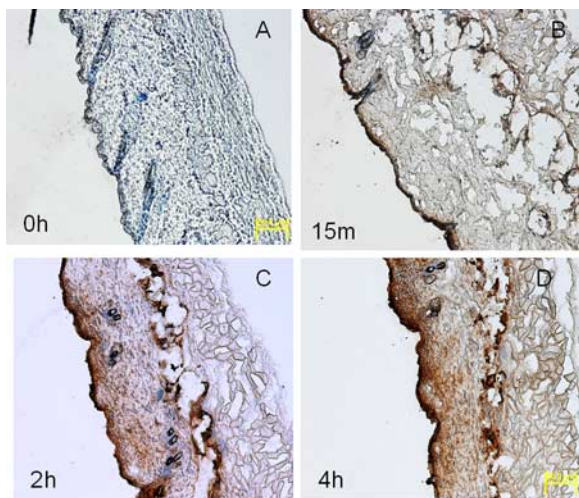


FIGURE 2. Ab penetration into the skin. Goat IgG was applied on the shaved back and animals were sacrificed at the indicated times. Frozen sections were cut as 7- μ m-thick sections and fixed in methanol for 10 min and then rehydrated with PBS. After blocking with 3% H_2O_2 in rat serum, the sections were incubated with biotin-labeled anti-goat IgG for 1 h. The sections were incubated with streptavidin-HRP and then after washing with PBS were incubated with diaminobenzidine for 10 min and then counterstained with hematoxylin and visualized under a light microscope. Goat IgG was applied on the skin and sections were cut after various time intervals. Peroxidase staining with diaminobenzidine was done as indicated in *Materials and Methods*. A, Control skin; B, Ab-applied skin after 15 min; C, Ab-applied skin after 2 h; and D, Ab-applied skin after 4 h. There were two mice per group and each experiment was repeated twice with the same results.

abdominal skin. On day +5, they were challenged with 20 μ l of 0.2% DNFB on the ear. The increase in ear swelling was measured at 24-h intervals as described above.

In vitro stimulation of BMDC with HSP27

BMDC were stimulated with 1 μ g/ml recombinant HSP27. LPS was used as a positive control for inducing DC maturation. Polymyxin B (10 μ g/ml) was added to the samples before stimulation to prevent LPS contamination. After 24 h of culture at 37°C in the presence of 5% CO_2 , culture supernatants were collected and stored at -20°C until cytokine measurements were performed.

In vitro measurement of cytokine production

Cytokine concentrations in culture supernatants from stimulated BMDC were measured using cytokine-specific ELISA kits from Invitrogen according to the manufacturer's instructions.

Assessment of CHS response after injection of hapten-labeled BMDC

BMDC (naive and HSP27 stimulated) from C3H/HeN and C3H/HeJ mice were labeled with DNBS according to previously established protocols, with some modifications (14). Ten million cells were suspended in 1 ml of DNBS solution (5 mM in PBS) for 15 min. The cells were then washed three times with RPMI 1640 containing 5% FCS and resuspended in the culture medium. One million cells were injected s.c. on the right and left ventral side of each group of mice. Later, the mice were ear challenged with 20 μ l of 0.2% DNFB. The positive control groups were sensitized with 25 μ l of 0.5% DNFB and on day +5 they were ear challenged with 20 μ l of 0.2% DNFB. The increase in ear swelling was measured at 24-h intervals as described above.

Statistical analysis

Data were analyzed by one-tailed Student's *t* test, and the *p* values are indicated in the text and figure legends. The group difference was compared using the ANOVA test followed by Tukey's post hoc test for multiple comparison adjustment in one experiment. Differences were considered significant at *p* < 0.05.

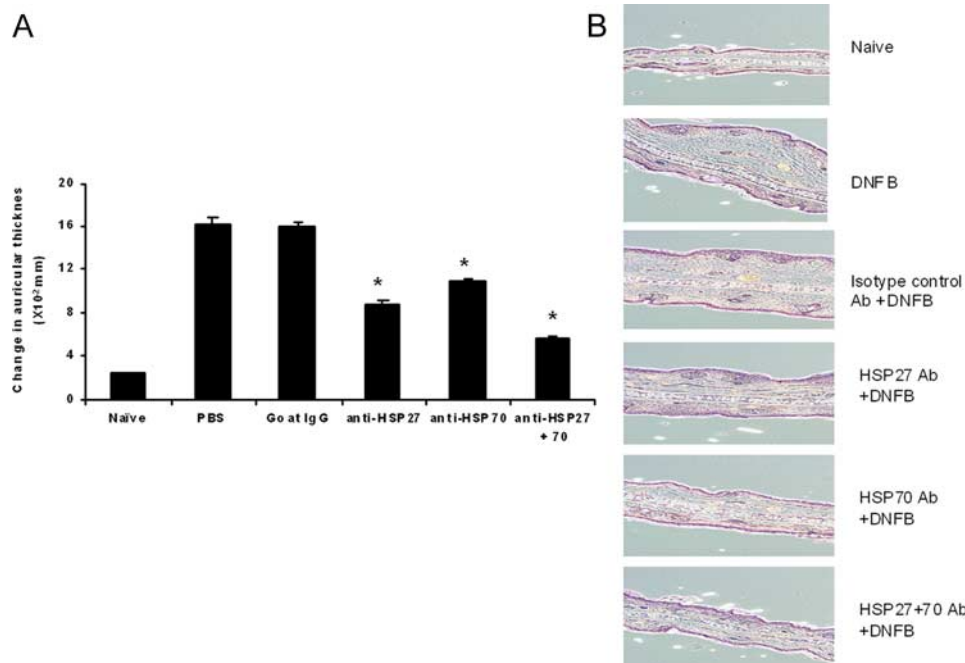


FIGURE 3. CHS to DNFB is inhibited by pretreatment of skin with Abs to HSPs 27 (HSP27) and 70 (HSP70). A, C3H/HeN mice were treated with anti-HSP Abs (2 μ g/mouse in 100 μ l of PBS). After 2 h, the mice were sensitized with DNFB on the Ab-treated site. The mice were ear challenged 5 days later and auricular thickness was measured at 24 h. The application of anti-HSP Abs significantly inhibited the CHS response (*, *p* < 0.05). Results were expressed as change in auricular thickness $\pm$ SEM. There were four mice per group and each experiment was repeated twice with the same results. The results are from one representative experiment. The group difference was compared using the ANOVA test followed by Tukey's post hoc test for multiple comparison adjustment. B, Histologic sections were taken from representative mice at 24 h after DNFB challenge, stained with H&E, and photomicrographs were taken (original magnification, $\times 10$ for all sections).

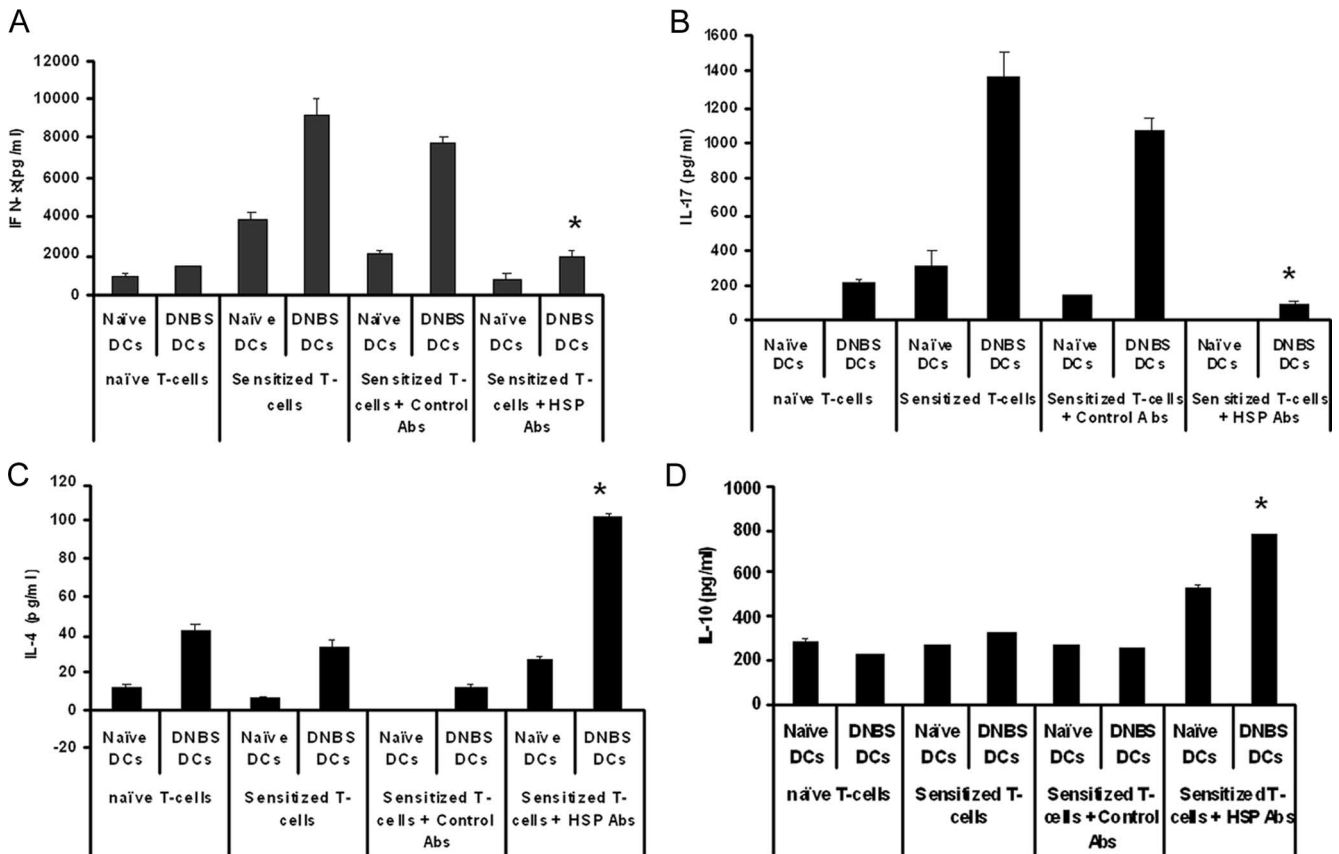


FIGURE 4. Effect of HSP27 and -70 on cytokines by draining lymph node cells after DNFB sensitization in C3H/HeN mice. C3H/HeN mice were pretreated with anti-HSP27 and anti-HSP70 or species-matched control Abs and sensitized with 25 μ l of 0.5% DNFB. Primed lymph node cells were isolated after 5 days from the draining lymph nodes of DNFB-sensitized mice and placed in cultures with DNBS-labeled BMDC. Treatment of BMDC with anti-HSP27/70 Ab-treated lymphocytes significantly inhibited the production of IL-17 and IFN- γ compared with untreated BMDC (*, $p < 0.05$). There was a corresponding increase in IL-4 and IL-10 production under the same conditions. The results are the mean $\pm$ SD with three mice per group and each experiment was repeated twice with identical results. The data shown are from one representative experiment.

Results

Localization of HSP27 and HSP70 in the murine skin

In previous studies, proteomic analysis of the skin of C3H/HeN and BALB/c mice revealed that there was constitutive expression of the heat shock proteins HSP27 and HSP70 (6). To identify where in the skin these molecules were expressed, sections of skin were subjected to immunohistochemical analysis. HSP27 was only detected in the superficial layers of epidermis, whereas HSP70 was present throughout the entire epidermis and also to some extent in the dermis (Fig. 1).

It has been demonstrated that under certain conditions, topically applied proteins and DNA can reach deeper levels of the skin (18–21). To determine whether the procedures we had used allowed Ab to penetrate, Ab was applied to the skin under a bioocclusive dressing as described in *Materials and Methods*. By immunohistochemical analysis, the Ab could only be detected in the most superficial portion of the epidermis after 15 min. But with greater lengths of time up to 4 h, the Ab had penetrated to the dermis and s.c. tissue (Fig. 2).

Heat shock proteins HSP27 and HSP70 play a role in the CHS response to DNFB

Having determined that Abs could penetrate into the epidermis and dermis, experiments were performed to assess the functional relevance of HSP27 and HSP70 in cutaneous cell-mediated immune responses. To do this, C3H/HeN mice were contact sensitized to

DNFB after anti-HSP27 and anti-HSP70 Abs had been applied topically to abdominal skin that had been prepared in the manner described above. Inhibition of the induction of DNFB CHS could be achieved by pretreatment with both anti-HSP27 and anti-HSP70 Abs (Fig. 3). When both anti-HSP27 and anti-HSP70 Abs were applied simultaneously, there was an additive effect leading to greater inhibition of CHS than with either alone (Fig. 3). When goat IgG was used as a species-matched control, there was no inhibition, indicating that the effect of topical Ab treatment was specific for the anti-HSP Abs used (Fig. 3). Inhibition of the induction of DNFB CHS with anti-HSP27 was dose dependent with maximum inhibition occurring at 2 μ g/mouse. Similar results were obtained with anti-HSP70 (data not shown).

Studies on the contact hypersensitivity response to DNFB have demonstrated that this response is mediated by IFN- γ and IL-17 in mice (14, 22). Experiments were therefore also performed in which hapten-primed lymph node cells were isolated from the draining lymph nodes of DNFB-sensitized mice and placed in culture with DNBS-labeled BMDC. This served as an alternative method of assessing the immune response to topically applied hapten. Hapten-labeled BMDC stimulated the production of IL-17 and IFN- γ by primed lymph node cells, whereas they were not able to stimulate naïve lymph node cells (Fig. 4). Pretreatment of mice with anti-HSP27 and anti-HSP70 Ab significantly inhibited the production of IL-17 and IFN- γ compared with untreated BMDC (Fig. 4, $p < 0.05$). We also observed a corresponding increase in

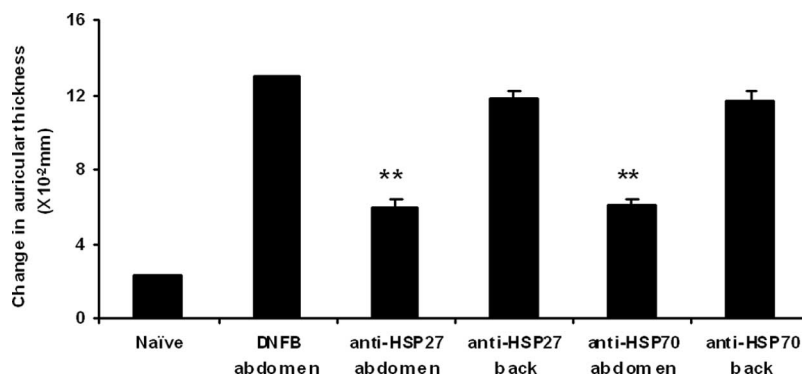


FIGURE 5. Application of anti-HSP Abs to the skin inhibits CHS to DNFB through a local effect. C3H/HeN mice were treated with anti-HSP27 or anti-HSP70 Ab on the abdomen or back as indicated. All mice, with the exception of the negative control, were then sensitized with DNFB on the abdomen. Application of hapten to the Ab-treated site produced significant inhibition of the CHS response (**, $p < 0.001$). In contrast, administration of anti-HSP27 or HSP70 Abs to the back but with hapten sensitization to the abdomen resulted in an ear swelling response that was not significantly different ($p > 0.05$) from the PBS-treated control. Results are expressed as change in auricular thickness $\pm$ SEM. There were four mice per group and each experiment was repeated twice with identical results. The data shown are from one representative experiment.

IL-4 and IL-10 production under the same conditions. It is possible that treatment of anti-HSP27 and anti-HSP70 Ab shifts the response from a Th1/Th17 to a Th2 phenotype at least in vitro.

Inhibition of CHS to DNFB by anti-HSP Abs is a local effect of the Abs

To assess whether the effect of topically applied anti-HSP Abs was a local or systemic one, separate panels of mice were treated with anti-HSP27 or anti-HSP70 on the abdomen or the back, and attempts were then made to sensitize both panels of mice on the abdomen. Mice that had been treated with anti-HSP27 or anti-HSP70 on the abdomen, showed significant inhibition of the DNFB CHS response (Fig. 5), confirming the results shown in Fig. 3. In contrast, mice treated with anti-HSP Ab on the back, but having received a sensitizing dose of DNFB on the abdomen, had no inhibition of CHS ($p > 0.05$). These results show that the inhibitory effect of topical application of this dose of anti-HSP Abs is a local occurrence, restricted to the site of Ab treatment.

Induction of hapten-specific tolerance by HSP-27 and -70 Abs

Experiments were then conducted to determine whether epicutaneous application of anti-HSP Ab followed by DNFB application

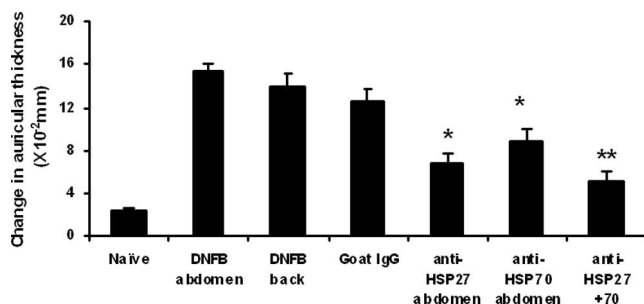


FIGURE 6. Application of DNFB to sites of HSP treatment renders mice tolerant to that hapten. C3H/HeN mice were treated with HSP Ab on the shaved abdomen. DNFB was applied to the Ab site as described elsewhere. Fourteen days later, mice were sensitized with DNFB on the shaved back and were subsequently ear challenged after 5 days. Ear swelling responses indicate that the inhibition of CHS response produced by the HSP Ab persisted and was significant (*, $p < 0.05$ and **, $p < 0.001$) in these mice. Results are expressed as change in auricular thickness $\pm$ SEM. There were four mice per group and each experiment was repeated twice with identical results. The data shown are from one representative experiment.

had an effect on subsequent attempts to sensitize mice to that hapten. Animals that had been sensitized with DNFB on anti-HSP Ab-treated skin were resensitized with 0.5% DNFB after a resting period of 14 days and were then ear challenged with DNFB 5 days after that. The ear swelling response of mice pretreated with anti-HSP Ab was suppressed compared with positive controls despite the fact that the second attempt to sensitize mice was through normal skin (Fig. 6). This indicated that mice treated with anti-HSP Ab followed by hapten application had become tolerant to DNFB.

To determine whether suppression of the induction of CHS was hapten specific and to exclude the possibility that the mice treated with a combination of anti-HSP27 and anti-HSP70 Abs and hapten were also nonresponsive to other haptens given subsequently, the following experiment was performed. Panels of mice were treated with a combination of anti-HSP27 and anti-HSP70 Abs followed immediately thereafter by application of DNFB to the Ab-treated site. Two weeks later, animals were treated with either a sensitizing dose of DNFB or a different hapten (oxazolone). Five days

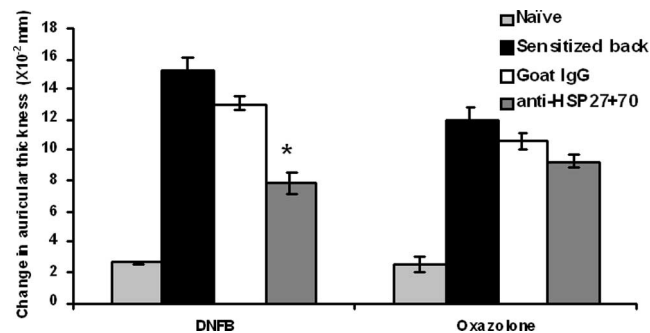


FIGURE 7. Tolerance to DNFB following HSP Ab treatment is Ag specific. C3H/HeN mice were treated with HSP Ab on the abdomen, after which 0.5% DNFB was applied to the same site. After 14 days, the mice were resensitized on the shaved back with either 0.5% DNFB or 3% oxazolone. Mice were ear challenged 5 days later with the same hapten that had been applied to the back. Ear swelling responses indicate that the inhibition of the CHS response produced by the HSP Ab was present in the DNFB back-sensitized group (*, $p < 0.05$), but was not present in the oxazolone back-sensitized mice ($p > 0.05$). Results are expressed as change in auricular thickness $\pm$ SEM. There were four mice per group and each experiment was repeated twice with identical results. The data shown are from a representative experiment.

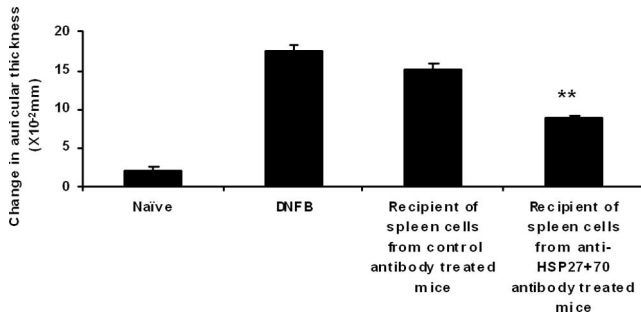


FIGURE 8. Inhibition of DNFB CHS by anti-HSP Abs can be adoptively transferred by splenocytes. C3H/HeN mice were treated with either a combination of anti-HSP27 and anti-HSP70 or control Ab and the site of Ab application was then treated with 0.5% DNFB. Five days later, 50×10^6 splenocytes from these mice were transferred into naive syngeneic recipients. The recipients were then sensitized and ear challenged with DNFB. The adoptive transfer of splenocytes from HSP-treated donor mice led to significant inhibition (**, $p < 0.001$) of CHS in the recipient naive mice compared with the control recipient mice. Results are expressed as change in auricular thickness $\pm$ SEM. There were four mice per group and each experiment was repeated twice with identical results. The data shown are from one representative experiment.

after that, they were ear challenged with the same hapten that had been applied 5 days earlier. Although mice treated with DNFB plus anti-HSP Abs and then resensitized with DNFB had a significantly suppressed response to DNFB, mice treated with DNFB plus anti-HSP Ab and then sensitized with oxazolone behaved like normal mice in their ear swelling response to oxazolone (Fig. 7). The results indicate that these animals are able to render a full immune response to other haptens and that the combination of anti-HSP Abs followed by hapten sensitization induces hapten-specific tolerance.

Tolerance to DNFB by HSP27 and HSP70 can be adoptively transferred

In other systems, tolerance to DNFB in mice is frequently accompanied by the development of regulatory T cells which inhibit the response on subsequent encounter with hapten (15, 17). To examine whether the unresponsiveness to DNFB which occurred following HSP-Ab treatment was also associated with the develop-

ment of these cells, adoptive transfer studies were performed. Mice, skin painted with a sensitizing dose of DNFB following treatment with anti-HSP-Ab or species-matched control Ab, served as donors of spleen cells that were transferred to naive syngeneic recipients. These recipient animals were then sensitized and ear challenged with DNFB. Mice that received cells from animals that were unresponsive to DNFB by pretreatment with HSP-Abs developed a significantly suppressed ear swelling response ($p < 0.001$) compared with positive controls that had not received cells, but that had been immunized and ear challenged to DNFB and to negative controls that had not received cells nor had been sensitized but had been ear challenged to DNFB (Fig. 8). Furthermore, suppression could not be adoptively transferred ($p > 0.05$) with spleen cells from donors treated with species-matched control Ab before sensitization to DNFB. These results indicate that unresponsiveness to DNFB could be adoptively transferred by spleen cells.

HSP27 augments cytokine production and the induction of CHS by DCs

There is evidence to indicate that HSPs augment the presentation of Ag to T cells (23–26). Experiments were therefore conducted to determine whether the modulation of CHS response by HSPs occurred by influencing the function of DCs. Initial studies in this regard revealed that topical administration of anti-HSP27 and anti-HSP70 Abs had no significant effect on densities of epidermal Langerhans cells and there was no difference on the migration of DCs to regional lymph nodes after hapten application (data not shown). Studies were next performed in which BMDC were stimulated with HSP27 protein for 24 h and then evaluated for the production of IL-1 β , IL-12p35, IL-23p19, and IL-12p40, all of which are involved in the presentation of hapten to T cells.

To exclude the possibility that any changes in cytokine production were caused by contamination by LPS, polymyxin B was added to cultures. The addition of HSP27 to cultures augmented the production of IL-1 β , IL-12p35, IL-12p40, IL-6, and TNF- α but not IL-23p19 in C3H/HeN mice (Fig. 9).

Several reports have shown that recombinant HSPs are frequently contaminated with TLR ligands (27, 28). To determine whether this was the case in our system, cultures were treated with both recombinant HSP27 protein and Abs to HSP27 at the same time. The cultures were then examined for production of IL-12p35,

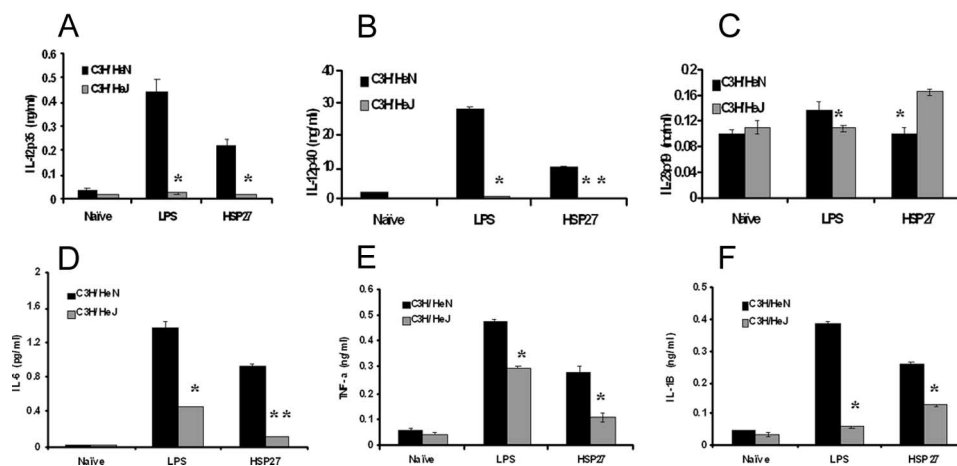
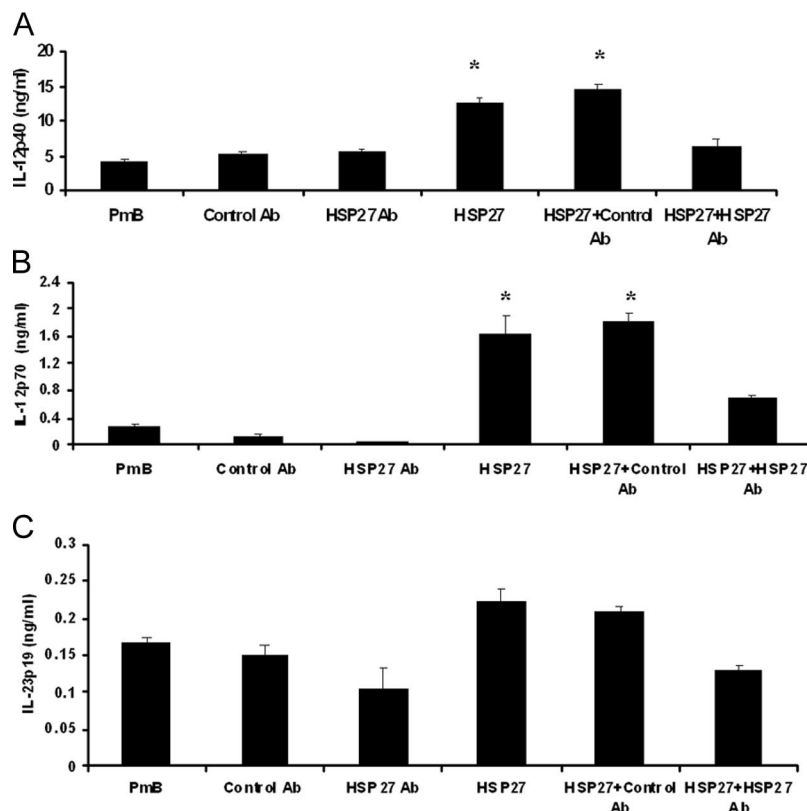


FIGURE 9. Production of proinflammatory cytokines by HSP27-stimulated BMDC is dependent on TLR4. BMDC from C3H/HeN and C3H/HeJ mice were treated with recombinant mouse HSP27. Polymyxin B (10 μ g/ml) was added to the culture to rule out any LPS contamination. There was a significant increase in IL-12p35 (A), TNF- α (E), and IL-1 β (F) (*, $p < 0.05$) and IL-12p40 (B) and IL-6 (D; **, $p < 0.001$) in C3H/HeN mice compared with C3H/HeJ mice. C3H/HeJ mice, on the other hand, showed an increase in IL-23p19 (C; *, $p < 0.05$) on stimulation with HSP27. There were two mice per group and each experiment was repeated twice with identical results. The data shown are from one representative experiment.

FIGURE 10. Production of proinflammatory cytokines by HSP27-stimulated BMDC from C3H/HeN mice can be blocked by anti-HSP27 Abs. Cultures were treated with HSP27 in a manner identical to that of Fig. 9. Abs to HSP27 were added to separate cultures. There was a significant increase in IL-12p35 (*, $p < 0.05$) and IL-12p40 production and, upon addition of HSP27 Ab (5 $\mu\text{g/ml}$), there was significantly less production of these cytokines. There were two mice per group and each experiment was repeated twice with identical results. The data shown are from one representative experiment.



IL-12p40, and IL-23p19. Treatment of cultures increased production of those cytokines, whereas addition of Abs to HSP27 abrogated that response, indicating that it was HSP27 that stimulated the BMDC to produce the cytokines rather than a contaminant in the HSP27 preparation (Fig. 10).

Requirement of functional TLR4 on DCs for HSP27-induced cytokine production and augmentation of the induction of CHS

Studies were also conducted to evaluate the contribution of TLR4 to the augmented cytokine response in DCs elicited by HSP27. To accomplish this, BMDC from C3H/HeJ mice, which have a functional deficiency in TLR4 signaling due to a mutation in the *TLR4* gene, were compared with BMDC from C3H/HeN mice, which have normal TLR4 signaling (Fig. 9). In contrast to BMDC from C3H/HeN mice, there was no augmentation of IL-12p35 or IL-12p40 and only a modest increase in IL-1 β , TNF- α , and IL-6 in BMDC from C3H/HeJ mice. Even for IL-1 β , TNF- α and IL-6,

HSP27-induced production was significantly less than in BMDC from C3H/HeN mice. IL-23p19, however, was significantly increased in BMDC from C3H/HeJ following treatment with HSP27, whereas levels of this cytokine were unaffected in BMDC from C3H/HeN mice.

Treatment of hapten-conjugated BMDC with HSP27 augments their contact-sensitizing capacity

We used DNBS-conjugated HSP27-treated BMDC from C3H/HeN and C3H/HeJ mice to immunize naive C3H/HeN and C3H/HeJ recipients. HSP27-stimulated DNBS-labeled DCs from C3H/HeN mice were much more proficient at immunizing both C3H/HeN and C3H/HeJ mice to DNFB than control DCs labeled with DNBS but not treated with HSP27 (Fig. 11). In contrast, HSP27-stimulated DNBS-labeled DCs from C3H/HeJ mice were comparable to control DCs labeled with DNBS (Fig.

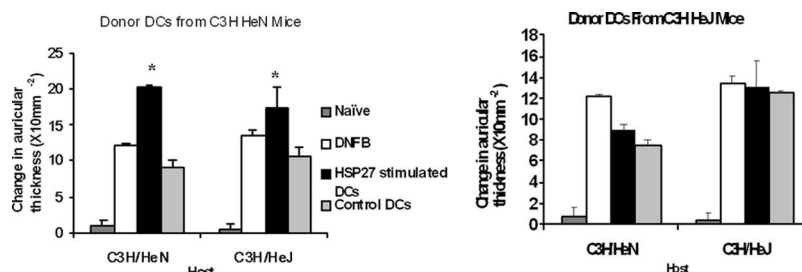


FIGURE 11. Immunization with HSP27-stimulated DNBS-labeled BMDC from C3H/HeN mice augments the induction of CHS. Naive and HSP27-stimulated BMDC from C3H/HeN and C3H/HeJ mice were labeled with DNBS and injected s.c. into naive C3H/HeN mice or C3H/HeJ mice. CHS was then assessed according to the protocol as described in *Materials and Methods*. There was a significant increase in the CHS response (*, $p < 0.05$) on injection of DNBS-labeled and HSP27-stimulated BMDC as compared with DNBS-labeled naive BMDC from C3H/HeN mice when they were transferred to C3H/HeN or C3H/HeJ mice. In contrast, HSP27-stimulated DNBS-labeled BMDC from C3H/HeJ mice were not effective in eliciting an enhanced CHS response when transferred to either C3H/HeN or C3H/HeJ mice on challenge. There were three mice per group and each experiment was repeated twice with identical results. The data are from a representative experiment.

11). These results indicated that HSP27 alters the immunizing capacity of DCs through a TLR4-dependent process in vivo.

Discussion

Our results suggest that the initial steps that occur during the induction of CHS responses are more complex than previously conceptualized. Traditionally, it was thought that haptens were taken up by Langerhans cells and other cutaneous DCs, proinflammatory cytokines were produced, and following migration of DCs to regional lymph nodes, presentation of Ag to subpopulations of T lymphocytes occurred (29, 30). It now seems likely that HSPs and elements of the innate immune system play a much greater role in the initial stages of this process than previously thought. Using an in vivo model of allergic CHS, we found that HSP27 and HSP70 are key participants in the induction of immune responses to topically applied haptens. When Abs to HSP27 and/or HSP70 were applied to the skin and then the hapten DNFB was applied to the same site, the induction of CHS was suppressed and Ag-specific immunological unresponsiveness developed. Further analysis showed that incubation of DCs with recombinant HSP27 augmented the production of IL-1 β , IL-12p35, and IL-12p40 and those cells were much more proficient at immunizing animals to DNFB. Moreover, the effect of HSP27 was mediated through the TLR4 pathway, since increased production of IL-1 β , IL-12p35, and IL-12p40 augmented the capacity of DCs from C3H/HeN mice to initiate CHS responses, but this augmentation did not occur in DCs of C3H/HeJ mice, which have a mutation in the TLR4 gene. These findings are consistent with the hypothesis that following topical application of hapten, the induction of CHS involves HSPs, which in turn activate TLR4 in DCs. TLR4 activation then serves as a stimulus for production of IL-1 β , IL-12p35, and IL-12p40 by DCs, which increases their effectiveness at initiating T cell-mediated immune responses and controls the types of T cells that elicit and regulate the CHS response.

We became interested in investigating the in vivo contribution of HSPs 27 and 70 to CHS responses when we found that large amounts of these molecules were constitutively expressed in the skin of mice (6). In vitro studies have implicated the role of HSPs in adaptive and innate immune responses (5, 31). These evolutionarily conserved proteins chaperone antigenic moieties into the appropriate intracellular channels in DCs for presentation of Ag to T cells. They also promote DC maturation and they stimulate DC production of IL-1 β , IL-12p35, and IL-12p40, all of which are known to promote Th1 and Tc1 cell-mediated immune responses. The immunomodulatory properties of HSPs have been exploited to stimulate immune responses by incorporating them into vectors used for vaccination against viral and tumor Ags (32–34). Moreover, HSP70 peptide complexes isolated from the brains of experimental autoimmune encephalomyelitis mice have been used to promote NK-induced tolerance to the disease in healthy mice (35). HSP70 has previously been shown to function in association with MHC class I and II molecules as part of the endogenous pathway of Ag presentation. HSP70 is capable of binding to antigenic peptides and markedly increases the efficiency of MHC class I and II presentation (36). HSPs are expressed in all organisms and in different subcellular compartments (37). The ability of HSPs to bind antigenic peptides and deliver them to APCs forms the basis for their potential role in the generation of peptide-specific T lymphocyte responses. New biochemical and structural studies have recently emerged to support the role of HSPs in some, but not all, models of Ag processing and presentation. Ag recognition by TLRs results in activation of the innate immune system and leads to the secretion of many inflammatory mediators such as TNF- α , IL-6, and several chemokines. Therefore, innate immunity is likely

to play an important role both in the initiation and perpetuation of the inflammatory processes. It has been demonstrated that two members of the small HSP family, A crystallin and HSPB8, were able to activate DCs by inducing maturation and cytokine production in a rheumatoid arthritis model (38). *Toxoplasma gondii*-derived HSP70 (*T. gondii* HSP70) has also been shown to stimulate murine DC maturation via TLR4 through the MyD88-independent signal transduction cascade (39).

The contribution of HSPs to in vivo models of autoimmune and inflammatory disease has received little attention. These proteins are overexpressed in the synovial tissue of rheumatoid arthritis patients and in involved tissues of animals with experimental autoimmune encephalitis and collagen-induced arthritis (35, 38, 40). Our experiments indicate that a deficiency of HSPs in vivo will alter the T cell repertoire that develops in response to hapten. Greater numbers of immunoregulatory T cells and fewer effector T cells developed under conditions in which HSP27 was deficient and this was associated with a shift in the balance of IL-12 and IL-23 that were produced in response to hapten.

The role of TLR4 in the biologic activity of HSPs is not without precedent. Asea et al. (41) have previously shown that TLR2 and TLR4 are required for cytokine production by purified human monocytes and the THP-1 monocyte cell line in response to HSP70. HSP70-induced IL-12 production by DCs also has been shown to require TLRs (41). In addition, Flohé et al. (42) have shown that HSP60 treatment of BMDC results in their maturation and release of TNF- α , IL-12, and IL-1 β . However, DCs from C3H/HeJ mice with a mutation at the TLR4 locus failed to respond to HSP60. In this study, we were able to demonstrate that TLRs are required for production of these cytokines in DCs and that this has consequences in vivo.

Our findings highlight the complex interplay between the adaptive and innate immune system during the early stages of the development of CHS involving HSPs, TLR4, and production of cytokines known to augment presentation of Ag to T cells. In support of this concept are the findings of Di Nardo et al. (43), who have shown that cathelicidins, antimicrobial peptide components of the innate immune system, inhibit the development of DNFB CHS. Cathelicidins are antimicrobial peptides that have been shown to have both pro- and anti-inflammatory activities; with respect to CHS, they interfere with DNFB-induced activation of TLR4. These findings are consistent with our results and provide additional evidence that the cellular composition of the inflammatory response with respect to regulatory T cells, effector T cells, and the cytokines that they produce is determined at least in part by the different types and concentrations of molecules of the innate immune system that are present following the skin's initial encounter with Ag.

Many aspects of the immunological consequences of a localized deficiency of HSP27 and HSP70 in the skin are reminiscent of the effect of low doses of UV radiation on cutaneous immune responses. Like UV exposure, reducing the activity of HSP27 and HSP70 inhibited the induction of CHS. In both cases, this was due to a reduction in the Ag-presenting capacity in a confined area of skin. Also in both systems, application of hapten to the UV or HSP Ab-treated site resulted in Ag-specific unresponsiveness (2). These findings raise the possibility that alterations in the expression of HSP27 and HSP70 occur after UV exposure and that such alterations contribute to UV-induced immune suppression.

Our findings indicate that treatment of DCs with HSP27 resulted in the production of several cytokines that are involved in the Ag presentation process. Although it is tempting to attribute the defect in Ag presentation that we observed in HSP-deficient skin solely to an inability to synthesize these cytokines, we are mindful of the

fact that HSPs have additional effects on APCs, including the channeling of Ags into the appropriate pathways for Ag processing and display on MHC proteins, the maturation of DCs, and the production of other cytokines.

Significant progress has been made in elucidating the cellular and molecular mechanisms involved in the immune response to haptens applied topically to the skin. The finding that HSPs participate in the induction of CHS means that they may be attractive targets with which to manipulate the immune response to cutaneous Ags. Addition of HSPs may increase immune responses and therefore may be useful as adjuvants for vaccination, whereas inhibition of their activity may be beneficial in the prevention of autoimmune and immunological hypersensitivity diseases of the skin such as allergic CHS.

Disclosures

The authors have no financial conflict of interest.

References

1. Srivastava, P. 2002. Roles of heat-shock proteins in innate and adaptive immunity. *Nat. Rev. Immunol.* 2: 185–194.
2. Trautinger, F. 2001. Heat shock proteins in the photobiology of human skin. *J. Photochem. Photobiol. B* 63: 70–77.
3. Morris, S. D. 2002. Heat shock proteins and the skin. *Clin. Exp. Dermatol.* 27: 220–224.
4. Srivastava, P. K., A. Menoret, S. Basu, R. J. Binder, and K. L. McQuade. 1998. Heat shock proteins come of age: primitive functions acquire new roles in an adaptive world. *Immunity* 8: 657–665.
5. Hauet-Broere, F., L. Wieten, T. Guichelaar, S. Berlo, R. van der Zee, and W. Van Eden. 2006. Heat shock proteins induce T cell regulation of chronic inflammation. *Ann. Rheum. Dis.* 65: 65–68.
6. Huang, C. M., K. W. Foster, T. DeSilva, J. Zhang, Z. Shi, N. Yusuf, K. R. Van Kampen, C. A. Elmetts, and D. C. Tang. 2003. Comparative proteomic profiling of murine skin. *J. Invest. Dermatol.* 121: 51–64.
7. Gandour-Edwards, R., M. McClaren, and R. R. Isseroff. 1994. Immunolocalization of low-molecular-weight stress protein HSP 27 in normal skin and common cutaneous lesions. *Am. J. Dermatopathol.* 16: 504–509.
8. Kiriya, M. T., M. Oka, M. Takehana, and S. Kobayashi. 2001. Expression of a small heat shock protein 27 (HSP27) in mouse skin tumors induced by UVB-irradiation. *Biol. Pharm. Bull.* 24: 197–200.
9. Muramatsu, T., H. Tada, N. Kobayashi, M. Yamaji, T. Shirai, and T. Ohnishi. 1992. Induction of the 72-kD heat shock protein in organ-cultured normal human skin. *J. Invest. Dermatol.* 98: 786–790.
10. Trautinger, F., I. Trautinger, I. Kindas-Mugge, D. Metz, and T. A. Luger. 1993. Human keratinocytes in vivo and in vitro constitutively express the 72-kDa heat shock protein. *J. Invest. Dermatol.* 101: 334–338.
11. Zhou, X., V. A. Tron, G. Li, and M. J. Trotter. 1988. Heat shock transcription factor-1 regulates heat shock protein-72 expression in human keratinocytes exposed to ultraviolet B light. *J. Invest. Dermatol.* 111: 194–198.
12. Souil, E., A. Capon, S. Mordon, A. T. Dinh-Xuan, B. S. Polla, and M. Bachelet. 2001. Treatment with 815-nm diode laser induces long-lasting expression of 72-kDa heat shock protein in normal rat skin. *Br. J. Dermatol.* 144: 260–266.
13. Van Kampen, K. R., Z. Shi, P. Gao, J. Zhang, K. W. Foster, D. T. Chen, D. Marks, C. A. Elmetts, and D. C. Tang. 2005. Safety and immunogenicity of adenovirus-vectored nasal and epicutaneous influenza vaccines in humans. *Vaccine* 23: 1029–1036.
14. Xu, H., N. DiIulio, and R. Fairchild. 1996. T cell populations primed by hapten sensitization in contact sensitivity are distinguished by polarized patterns of cytokine production: interferon γ -producing (Th1) effector CD8⁺ T cells and interleukin (IL) 4/IL-10-producing (Th2) negative regulatory CD4⁺ T cells. *J. Exp. Med.* 183: 1001–1012.
15. Elmetts, C. A., P. R. Bergstresser, R. E. Tigelaar, P. J. Wood, and J. W. Streilein. 1983. Analysis of the mechanism of unresponsiveness produced by haptens painted on skin exposed to low dose ultraviolet radiation. *J. Exp. Med.* 158: 781–794.
16. Inaba, K., M. Inaba, N. Romani, H. Aya, M. Deguchi, S. Ikehara, S. Muramatsu, and R. M. Steinman. 1992. Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor. *J. Exp. Med.* 176: 1693–1702.
17. Schwarz, A., A. Maeda, K. Kernebeck, H. Van Steeg, S. Beissert, and T. Schwarz. 2005. Prevention of UV radiation-induced immunosuppression by IL-12 is dependent on DNA repair. *J. Exp. Med.* 201: 173–179.
18. Herrick, C. A., H. MacLeod, E. Glusac, R. E. Tigelaar, and K. Bottomly. 2000. Th2 responses induced by epicutaneous or inhalational protein exposure are differentially dependent on IL-4. *J. Clin. Invest.* 105: 765–775.
19. Choi, M. J., J. H. Kim, and H. I. Maibach. 2006. Topical DNA vaccination with DNA/lipid based complex. *Curr. Drug Deliv.* 3: 37–45.
20. Jensen, J. M., R. Fölster-Holst, A. Baranowsky, M. Schunck, S. Winoto-Morbach, C. Neumann, S. Schütze, and E. Proksch. 2004. Impaired sphingomyelinase activity and epidermal differentiation in atopic dermatitis. *J. Invest. Dermatol.* 122: 1423–1431.
21. Yarosh, D. B. Liposomes in investigative dermatology. 2001. *Photodermatol. Photoimmunol. Photomed.* 17: 203–212.
22. He, D., L. Wu, H. K. Kim, H. Li, C. A. Elmetts, and H. Xu. 2006. CD8⁺ IL-17-producing T cells are important in effector functions for the elicitation of contact hypersensitivity responses. *J. Immunol.* 177: 6852–6858.
23. Javid, B., P. A. MacAry, and P. J. Lehner. 2007. Structure and function: heat shock proteins and adaptive immunity. *J. Immunol.* 179: 35–40.
24. Hauet-Broere, F., L. Wieten, T. Guichelaar, S. Berlo, R. van der Zee, and W. Van Eden. 2006. Heat shock proteins induce T cell regulation of chronic inflammation. *Ann. Rheum. Dis.* 65: 65–68.
25. Van Eden, W., R. Van der Zee, and B. Prakken. 2005. Heat-shock proteins induce T-cell regulation of chronic inflammation. *Nat. Rev. Immunol.* 5: 318–330.
26. Callahan, M. K., M. Garg, and P. K. Srivastava. 2008. Heat-shock protein 90 associates with N-terminal extended peptides and is required for direct and indirect antigen presentation. *Proc. Natl. Acad. Sci. USA* 105: 1662–1667.
27. Floto, R. A., P. A. MacAry, J. M. Boname, T. S. Mien, B. Kampmann, J. R. Hair, J. R. O. S. Huey, E. N. Houben, J. Pieters, C. Day, et al. 2006. Dendritic cell stimulation by mycobacterial Hsp70 is mediated through CCR5. *Science* 314: 454–458.
28. Ye, Z., and Y. H. Gan. 2007. Flagellin contamination of recombinant heat shock protein 70 is responsible for its activity on T cells. *J. Biol. Chem.* 282: 4479–4484.
29. Xu, H., B. Bjarnason, and C. A. Elmetts. 2000. Sensitization versus elicitation in allergic contact dermatitis: potential differences at cellular and molecular levels. *Am. J. Contact Dermatol.* 11: 228–234.
30. Cruz, P. D., Jr., and P. R. Bergstresser. 1990. Antigen processing and presentation by epidermal Langerhans cells: induction of immunity or unresponsiveness. *Dermatol. Clin.* 8: 633–647.
31. Pockley, A. G. 2003. Heat shock proteins as regulators of the immune response. *Lancet* 362: 469–476.
32. Heikema, A., E. Agsteribbe, J. Wilschut, and A. Huckriede. 1997. Generation of heat shock protein-based vaccines by intracellular loading of gp96 with antigenic peptides. *Immunol. Lett.* 57: 69–74.
33. Pavlenko, M., A. K. Roos, C. Leder, L. O. Hansson, R. Kiessling, E. Levitskaya, and P. P. P. 2004. Comparison of PSA-specific CD8⁺ CTL responses and anti-tumor immunity generated by plasmid DNA vaccines encoding PSA-HSP chimeric proteins. *Cancer Immunol. Immunother.* 53: 1085–1092.
34. Multhoff, G. 2006. Heat shock proteins in immunity. *Handb. Exp. Pharmacol.* 172: 279–304.
35. Galazka, G., M. Stasiulek, A. Walczak, A. Jurewicz, A. Zylicz, C. F. Brosnan, C. S. Raine, and K. W. Selmaj. 2006. Brain-derived heat shock protein 70-peptide complexes induce NK cell-dependent tolerance to experimental autoimmune encephalomyelitis. *J. Immunol.* 176: 1588–1599.
36. Binder, R. J., N. E. Blachere, and P. K. Srivastava. 2001. Heat shock protein-chaperoned peptides but not free peptides introduced into the cytosol are presented efficiently by major histocompatibility complex I molecules. *J. Biol. Chem.* 276: 17163–17171.
37. Gallucci, S., and P. Matzinger. 2001. Danger signals: SOS to the immune system. *Curr. Opin. Immunol.* 13: 114–119.
38. Roelofs, M. F., W. C. Boelens, L. A. Joosten, S. Abdollahi-Roodsaz, J. Geurts, L. U. Wunderink, B. W. Schreurs, W. B. van den Berg, and T. R. Radstake. 2006. Identification of small heat shock protein B8 (HSP22) as a novel TLR4 ligand and potential involvement in the pathogenesis of rheumatoid arthritis. *J. Immunol.* 176: 7021–7027.
39. Aasai, F., M. S. Rodriguez Pena, H. S. Mun, H. Fang, T. Mitsunaga, K. Norose, H. K. Kang, Y. S. Bae, and A. Yano. 2006. *Toxoplasma gondii*-derived heat shock protein 70 stimulates maturation of murine bone marrow-derived dendritic cells via Toll-like receptor 4. *Cell Stress Chaperones* 11: 13–22.
40. Haug, M., C. P. Schepp, H. Kalbacher, G. E. Dannecker, and U. Holzer. 2007. 70-kDa heat shock proteins: specific interactions with HLA-DR molecules and their peptide fragments. *Eur. J. Immunol.* 37: 1053–1063.
41. Asea, A., M. Rehli, E. Kabingu, J. A. Bochs, O. Bare, P. E. Auron, M. A. Stevenson, and S. K. Calderwood. 2002. Novel signal transduction pathway utilized by extracellular HSP70: role of Toll-like receptor (TLR) 2 and TLR4. *J. Biol. Chem.* 277: 15028–15034.
42. Flohé, S. B., J. Bruggemann, S. Lendemann, M. Nikulina, G. Meierhoff, S. Flohe, and H. Kolb. 2003. Human heat shock protein 60 induces maturation of dendritic cells versus a Th1-promoting phenotype. *J. Immunol.* 170: 2340–2348.
43. Di Nardo, A., M. H. Braff, K. R. Taylor, C. Na, R. D. Granstein, J. E. McInturff, S. Krutzik, R. L. Modlin, and R. L. Gallo. 2007. Cathelicidin antimicrobial peptides block dendritic cell TLR4 activation and allergic contact sensitization. *J. Immunol.* 178: 1829–1834.