

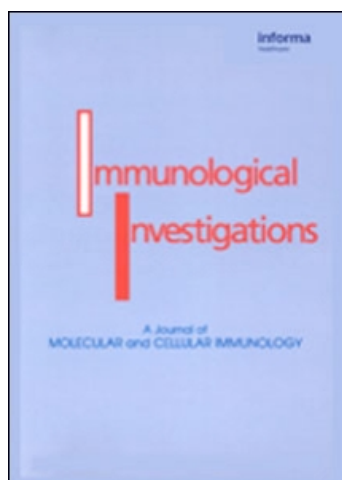
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Autologous Hsp70 Induces Antigen Specific Th1 Immune Responses in a Murine T-Cell Lymphoma

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Heat Shock protein-70 derived from tumor cells is highly immunogenic and induces specific anti-tumor immune response by directly activating cytotoxic CD8⁺ T cells. Additionally, Hsp70 is known to be a strong activator of antigen presenting cells and therefore, up regulates the production of pro-inflammatory cytokines and chemokines. In this study, we have shown the effect of tumor-derived Hsp70 on the induction of delayed type hypersensitivity reaction in a T cell lymphoma bearing mice. The autologous Hsp70 augments contact hypersensitivity and delayed type hypersensitivity responses in mice challenged with allergen in vehicle and antigens respectively. The adoptive transfer of splenocytes derived from Hsp70 immunized mice is able to enhance delayed type hypersensitivity response in antigen challenged normal and DL-bearing host. Furthermore, adoptive transfer of macrophages incubated with autologous Hsp70 also enhances DTH reactivity in mice. The pro-inflammatory cytokines and C-C chemokines are found to be elevated in the DTH footpad extract of antigen challenged normal and DL-bearing mice. Increased production of IFN- γ and MIP-1 α suggest that autologous Hsp70 augments the recruitment of antigen specific Th1 cells, which further secretes pro-inflammatory cytokines and C-C chemokines mediating the hypersensitivity reaction upon challenge with antigens.

Keywords Hsp70, DTH, Pro-inflammatory cytokines, Chemokines, Dalton's lymphoma.

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INTRODUCTION

Heat shock proteins (HSPs) are a highly conserved group of proteins found in all organisms (Lindquist et al., 1988). In response to many environmental stresses, synthesis of these proteins is enhanced (Collins et al., 1982; Ferris et al. 1988; Karl et al., 1986; Kurtz et al., 1986). HSPs are constitutively expressed at lower levels and play vital roles in maintaining cell functions (Pellham et al., 1989). HSPs have been grouped into six different families of related proteins on the basis of their size, sequence homologies and antigenic cross-reactivities (Deshaies et al., 1988). Members of Hsp70 family plays major role in the folding, refolding and translocation of polypeptides (Cheng et al., 1989; Hemmingsen et al., 1988; Ostermann et al., 1989; Reading et al., 1989; William et al., 1988).

Hsp70 over expression leads to an increased chaperoning of antigenic peptides into a particular subset of macrophages or other antigen presenting cells, leading to their efficient presentation via MHC class I and MHC class II pathways (Zihai, 1997). It activates the resting antigen presenting cells to take up and process the tumor antigens and up-regulates the expression of costimulatory molecules necessary for T cell activation (Suto et al., 1995).

Therefore, Hsp70 chaperone an entire array of antigenic peptides generated by a tumor, instead of one or few selected antigenic peptides and helps in their processing and presentation through MHC class I pathways. *In vivo* administration of autologous Hsp70 leads to the generation of profound and effective anti-tumor immunity (Wang et al., 2000).

In addition to peptide binding and delivery, Hsp70 can serve as a carrier of tumor peptides that are effective immunogenic for B cells, CD4⁺ and CD8⁺ T cells without requiring an adjuvant (Lehner et al., 2000; Roman et al., 1996; Suzue et al., 1996), but its role in eliciting delayed type hypersensitivity, which is the typical *in vivo* manifestation of the cell-mediated immunity characterized by activation and recruitment predominantly of T cells and macrophages and by resultant swelling at 24 to 48 hrs at the site of antigen entry in previously sensitized host, is not well studied. It is well known that DTH is mediated by T helper 1 (Th1) cells secreting predominantly IFN- γ and IL-2.

IFN- γ was previously demonstrated to be a major mediator of lymphocyte recruitment at the DTH reaction site (Issekutz et al., 1988) by stimulating the production of chemokines such as IL-8 (Larsen et al., 1995), monocyte chemoattractant protein 1 (MCP-1) (Rand et al., 1996), macrophage inflammatory protein 1 α (MIP-1 α) (Doyle et al., 1997) and macrophage migration inhibitory factor (MIF) (Bernhagen et al., 1996) in the macrophages. Moreover, the expression of interferon-inducible protein-10 (IP-10) (Gautam et al., 1994) and RANTES (Devergne et al., 1994) (regulated upon activation, normal T cell expressed and secreted) was reported in contact sensitivity and in DTH granulomas, respectively.

Several studies have shown that mycobacterial HSP can induce inflammatory cytokines (Friedland et al., 1993; Ohashi et al., 2000) and C-C chemokines (Lehner et al., 2000) by stimulating monocytes or dendritic cells (DCs). Therefore, in this study, we sought to investigate the effect of extracellular autologous Hsp70 administration on the elicitation of delayed type hypersensitivity reaction in mice bearing a transplantable T cell lymphoma of spontaneous origin, designated as Dalton's lymphoma and the production of pro-inflammatory cytokines and C-C chemokines in the DTH footpad of DL-bearing host. Dalton's lymphoma mimics human malignancies and progress as solid tumors or as ascites in the peritoneum of tumor-bearing host. Its progression is very rapid in syngenic BALB/c ($H2^d$) mice. It is highly immunosuppressive and invasive, causing death of the host in a relatively short time with little sign of host's control.

MATERIALS AND METHODS

Mice and Tumor System. Inbred populations of BALB/c ($H2^d$) strain of mice of either sex were used at 8 to 12 weeks of age. The mice were given food and water *ad libitum* under pathogen free condition and were treated with utmost care in an approved animal room facility of Department of Zoology, Banaras Hindu University. For tumor system, healthy mice of either sex at 8 to 12 weeks of age were injected intraperitoneally with 1.5×10^6 DL cells (in 0.5 ml PBS). The DL cells for transplantation were obtained from ascitic fluid of DL-bearing mice, where the yield of the cells is higher and maintained in ascitic form *in vivo* by serial transplantation.

Isolation and Purification of Hsp70 from DL Cells. The Hsp70 were isolated and purified as previously described (Peng et al., 1997) with minor modifications. A total of 10ml of packed all pellet of DL cells, cultured *in vitro* in thermal stress condition, was homogenized in 40 ml of hypertonic buffer A without detergent (10 mM NaHCO_3 , 0.5 mM PMSF, pH 7.1) and centrifuged at $1,000,000 \times g$. The pellet was dissolved in buffer B (20 mM NaCl, 15 mM 2-ME, 3 mM MgCl_2 , 0.5 mM PMSF, pH 7.5) loaded onto Sephadex column (G-75 from Sigma). Further, elute from Sephadex column was loaded on ADP-agarose column (Sigma) equilibrated with buffer B. The column was washed extensively with buffer B until protein was undetectable in the elute by absorbance at 280 nm. The buffer of the elute from ADP-agarose column was changed for buffer C (20 mM Na_2PO_4 , 20 mM NaCl, pH 7.0) and the elute was loaded onto a DEAE-Sephacel column (Sigma) in buffer D (130 mM NaCl in buffer A). The protein content in the different fractions was measured by the Lowry method with BSA as standard.

Preparation of Whole Cell Extract of Tumor. Tumor extract (TE) was prepared from Dalton's Lymphoma (DL) cells by a method as described previously (An et al., 1998). Briefly, DL-cells were isolated from peritoneum of

DL-bearing host mice in chilled serum-free culture medium RPMI-1640 supplemented with 10% FCS (v/v), 100 units/ml penicillin, 100 µg/ml streptomycin and 100 µg/ml gentamicin. Cells were maintained in a 5% CO₂ atmosphere at 37°C (Sheldon, OR, USA) in plastic petridishes. Non-adherent cells were isolated and washed with sterile PBS, pH 7.3 twice by centrifugation at 100 × g for 10 minutes at 4°C and homogenized in a lysis buffer (50 mM Tris-HCl, pH 8.0, 5 mM EDTA, 150 mM NaCl, 0.5% SDS, 0.5 mM phenylmethylsulfonyl fluoride, and 0.5 mM dithiothreitol) for 30 min at 4°C. After that, the lysates were centrifuged and the supernatants were collected as whole cell extracts.

Macrophage Isolation and Culture. Peritoneal macrophages were harvested from normal healthy mice of either sex by a standard method. Briefly, macrophages were harvested by peritoneal lavage using chilled serum-free culture medium RPMI 1640 after killing by pain free cervical dislocation. Macrophage monolayer was prepared by incubating for 2 hrs in round plastic petridishes (Tarson, Kolkata, India) at 37°C in CO₂ incubator. The culture was washed three times with lukewarm serum-free medium with gentle flushing to ensure that all DL cells and other non-adhering cells were removed. More than 95% of the adhering cell population was that of macrophages as determined by morphology and non-specific esterase staining.

Antigen Pulsing of Peritoneal Exudate Cells and Immunization Protocol. Cultured peritoneal exudate cells (PEC) were washed twice with RPMI-1640 culture medium supplemented with 2% FCS (v/v) and once with sterile PBS. 1×10^7 PEC were incubated for 30 min in PBS alone or PBS containing OVA, TE, OVA + TE at 37°C and 5% CO₂ environment and then washed as above. Viable PECs were enumerated and 1×10^4 viable Ag-pulsed PECs were injected s.c. into the flanks of each mouse. Viability before injections was always greater than 70% as confirmed by trypan blue staining.

Assessment of the Contact Hypersensitivity. Mice were shaved on their dorsum and 24 hrs later mice were sensitized with 0.5% DNFB (1-Fluoro-2,4-dinitrobenzene) in vehicle (4:1 acetone and olive oil). Mice were resensitized 2nd consecutive day and were challenged by 0.25% DNFB in vehicle (4:1 acetone and olive oil) 5 days later on their ears. The change in ear thickness was measured 24 hrs post-challenge, previously described as the time of maximal response using a micrometer (Back et al., 1982). Control mice received DNFB on their ears (were challenged) without prior sensitization.

Assessment of the Delayed-Type Hypersensitivity Response. DTH response to OVA, BSA or TE was estimated by footpad swelling. Mice were sensitized to OVA, BSA or TE by an intradermal injection of 50 µl of 1mg/ml emulsified with complete Freund's adjuvant at 2 sites on the abdomen. Five days after the immunization, the mice were challenged by an injection of 25 µl of 2 mg/ml in PBS into left rear footpad, while the other rear footpad received a complete volume of PBS as a control. Footpad swelling was measured using engineer's micrometer at a given time after the challenge. The magnitude of the DTH

response was determined as the difference in footpad thickness between sensitized mice and PBS injected mice.

Measurement of Cytokine and Chemokines. Cytokine and chemokines in DTH footpad was determined by sandwich ELISA. Briefly, footpad of antigen-challenged mice were cut off, minced, homogenized in disruption buffer (7.5 mM NaHPO₄, 10 mM EDTA, 1% SDS) supplemented with the protease inhibitors leupeptin (1 mg/ml), pepstatin (1 mg/ml) and phenylmethylsulfonyl fluoride (100 mg/ml) and centrifuged at 100 × g. 96 well ELISA plates (Tarson, Kolkata, India) were coated overnight at room temperature with 0.5 µg/ml monoclonal anti-mice antibody for IFN- γ and MIP-1 α (R&D Systems, Abingdon, United Kingdom). After washing with PBS/0.05% Tween, nonspecific binding sites were blocked with PBS/0.05% Tween/2% BSA for 2 hrs at 37°C. Subsequently, wells were washed and incubated with 100 µl of footpad extract or 100 µl of standard for 1 hr at 37°C. Recombinant murine IFN- γ and MIP-1 α was used as standard (range, 3 to 3000 pg/ml). Samples and standard were diluted in PBS/0.05% Tween/0.2% BSA. Blank values were obtained from wells incubated with medium alone. After washing with PBS/0.05% Tween, wells were incubated with 0.5 µg/ml rabbit anti-mice IFN- γ and MIP-1 α for 1 hr at 37°C. After washing, bound antibody was detected using alkaline phosphatase conjugated goat anti-rabbit IgG antibody (Banglore Genie, Bangalore, India) diluted in PBS/0.05% Tween/0.2% BSA at a dilution of 1:5000 for 60 minutes followed by addition of 50 µl of p-nitro phenyl phosphate (NPP) 1mg/ml in enzyme substrate buffer), and the absorbance was read at 405 nm after 10 min using a ELISA Plate Reader (Biorad, Hercules, CA, USA).

Tumor Regression Assay. After immunotherapy, one mouse of each group was sacrificed for counting DL cells or for histological examinations of the tumor, and remaining animals were observed for tumor growth. DL cells were simply counted on hemocytometer and calculated as number of DL cells per ml of ascitic fluid. Tumor derived Hsp70 immunized mice were subjected to survival analysis.

Statistical Analysis. The unpaired and paired student's *t*-test was used to test the significance of data obtained in experimental settings for IFN- γ and MIP-1 α . The survival of mice was analyzed by log-rank test and plotted on a Kaplan–Meier plot. The data were taken as significant at *p* < 0.05. All statistical analyses of data were performed on Sigma plot Version 8.0 (Systat Software, San Jose, CA, USA).

RESULTS

Effect of Hsp70 on the Induction of Contact Hypersensitivity. To test the effect of Hsp70 on the level of CHS response, groups of BALB/c mice were injected intraperitoneally with 50 µg/ml of Hsp70 before sensitization with DNFB on days –4 and –2. Control group of mice is administered with sterile

PBS on same days. Both groups of mice were then sensitized with DNFB on their shaved dorsum on days 0 and 1. Five days after first sensitization, the left ear of mice was challenged with DNFB and increase in ear thickness was noted at different time periods of 24, 48 and 72 hrs. Hsp70 administration significantly enhances the CHS response in both normal healthy and DL-bearing mice. DNFB sensitization stimulates CHS response in contralateral ear also, which were abolished by the administration of heat inactivated Hsp70 preparation (data not shown). The tumor-bearing host develops significantly less CHS response in comparison to normal mice (Fig. 1). The *in vivo* injection of Hsp70 significantly enhances the CHS responses in DL-bearing host (Fig. 2), which indicates that exogenous Hsp70 application modulates the induction of CHS in tumor-bearing host.

Effect of Hsp70 on the Induction of Delayed Type Hypersensitivity. To examine, whether exogenous Hsp70 has an implication in the development of DTH response, we injected 50 µg/ml tumor derived Hsp70 intraperitoneally in a group of mice before sensitization on day -4 and -2. After that, mice were sensitized with 50 µl of OVA or BSA in the left footpad with leur tip Hamilton syringe on days 0 and 1. Five days later, mice were challenged with 25 µl of OVA or BSA into left footpad and swelling of footpad were measured. The Hsp70 treatment induces the development of DTH response up to 50% increment in footpad swelling. These results suggest that the Hsp70 is capable of modulating the DTH response in terms of footpad swelling. Further, we tested whether induction of DTH at one site has a contralateral effect at another site, and we injected groups of sensitized mice in the left rear footpads with 50 µg/ml of Hsp70.

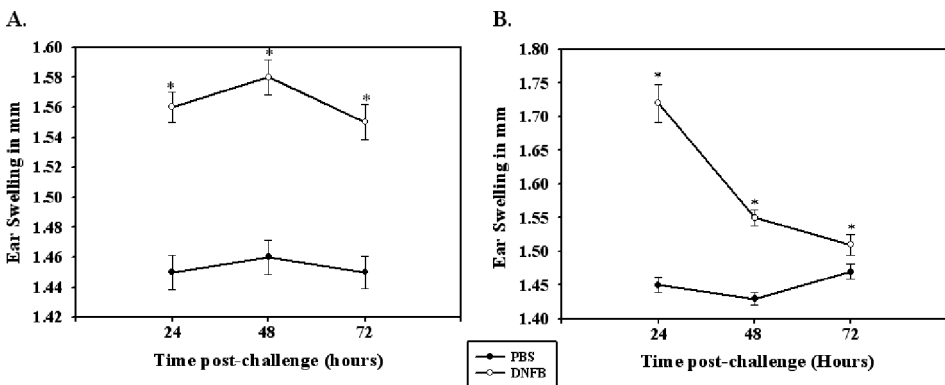


Figure 1: Effect of Hsp70 on hapten sensitized CHS response in normal healthy mice. Mice were immunized with autologous Hsp70, sensitized with 0.5% DNFB in vehicle and 5 days after, challenged with 0.25% DNFB in vehicle on left ear. After challenge, the increase in ear thickness was measured in control mice injected with only sterile PBS (A), and Hsp70 immunized normal healthy mice (B) at different time intervals of 24, 48 and 72 hrs. The data are presented as mean of difference in ear thickness $\pm$ SE_M. The results shown are representative of three independent experiments.

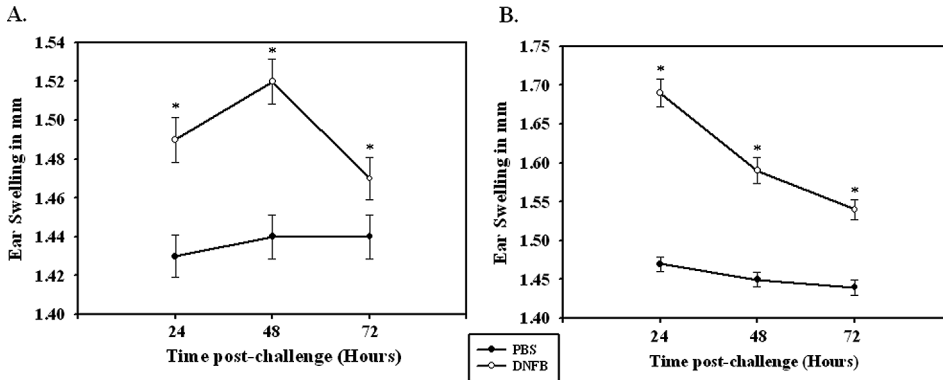


Figure 2: Effect of Hsp70 on hapten sensitized CHS response in DL-bearing mice. Mice were immunized with autologous Hsp70, sensitized with 0.5% DNFB in vehicle and 5 days after, challenged with 0.25% DNFB in vehicle on left ear. After challenge, the increase in ear thickness was measured in control DL-bearing mice injected with only sterile PBS (A), and Hsp70 immunized DL-bearing mice (B) at different time intervals of 24, 48 and 72 hrs. The data are presented as mean of difference in ear thickness $\pm$ SE_M. The results shown are representative of three independent experiments.

Contralateral paws received equal volume of sterile PBS. After 24 hrs, we challenged both footpads with 25 μ g of OVA or BSA. DTH responses were induced in Hsp70-injected paws in both normal healthy (Fig. 3) and DL-bearing host (Fig. 4), and significant swelling in the contralateral paw was also observed. The observation revealed that this effect was dependent on the timing of antigenic challenge (Jacysyn et al., 2001). The treatment of heat inactivated tumor derived Hsp70 abrogated the induction of DTH response in both normal healthy (Fig. 3C) and DL-bearing mice (Fig. 4C). Induction of DTH responses was maximal if challenge in the footpads occurred at least 24 hrs after injection of Hsp70.

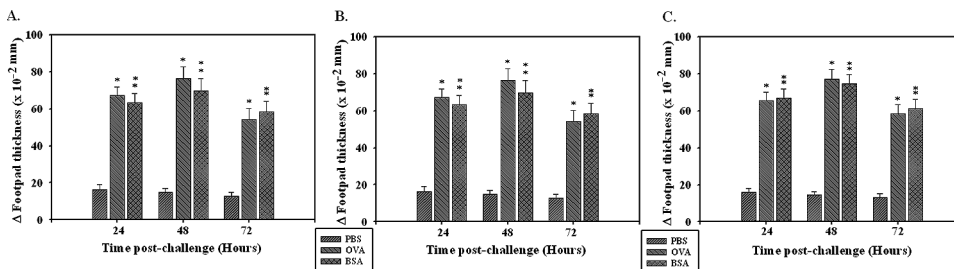


Figure 3: Effect of autologous Hsp70 on delayed type hypersensitivity reaction in normal healthy mice. Both control and Hsp70 immunized normal healthy mice were sensitized with OVA or BSA in PBS into left rear footpad by leur tip Hamilton syringe and 5 days later, challenged with OVA or BSA in PBS. The increase in thickness of the rear left footpad of control (A), Hsp70 immunized normal healthy mice (B), and normal healthy mice immunized with heat inactivated Hsp70 (C) were measured at different time intervals of 24, 48 and 72 hrs. Data are presented as mean of difference of footpad thickness $\times 10^{-2} \pm$ SE_M. The results shown are representative of three independent experiments.

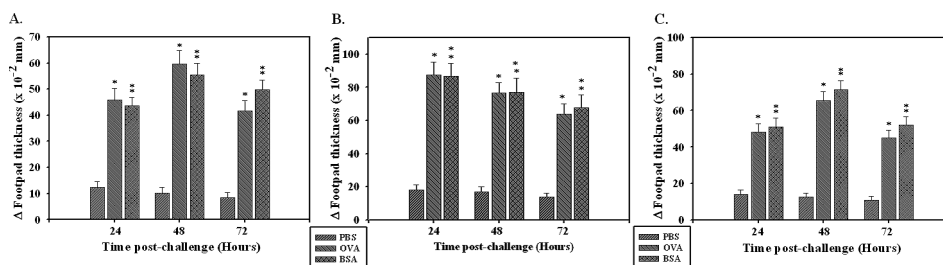


Figure 4: Effect of autologous Hsp70 on delayed type hypersensitivity reaction in DL-bearing mice. Both control and Hsp70 immunized DL-bearing mice were sensitized with OVA or BSA in PBS into left rear footpad by leur tip Hamilton syringe and 5 days later, challenged with OVA or BSA in PBS. The increase in thickness of the rear left footpad of control (A), Hsp70 immunized DL-bearing mice (B), and DL-bearing mice immunized with heat inactivated Hsp70 (C) were measured at different time intervals of 24, 48 and 72 hrs. Data are presented as mean of difference of footpad thickness $\times 10^{-2} \pm \text{SE}_{\text{M}}$. The results shown are representative of 3 independent experiments.

Effect of Adoptive Transfer of Splenocytes from Hsp70 Injected Mice on DTH. Tumor-derived Hsp70 treatment before antigen sensitization enhances the ability of adoptively transferred T cells and antigen to induce footpad swelling in recipient. In contrast, control T cells obtained from mice injected with only sterile PBS were also capable of inducing footpad swelling in antigen challenged recipient normal healthy (Fig. 5A) and DL-bearing mice (Fig. 5B). Splenocytes from mice injected with tumor derived Hsp70 significantly enhances the footpad swelling and generates strong DTH reactivity in both normal healthy (Fig. 5C) and DL-bearing (Fig. 5D) mice, indicating the exogenous Hsp70 application stimulates Th1 polarization at the site of antigen delivery.

Further, we mixed splenocytes derived from sensitized mice able to transfer DTH responses with an equal number of splenocytes derived from DL-bearing mice that were not capable to transfer DTH responses, and injected the combined splenocyte populations with antigen into naïve mice. There was no suppression of the resulting DTH response (data not shown).

Effect of Adoptive Transfer of Macrophages from Hsp70 Injected Mice on DTH. We used naïve mice as donor. Peritoneal exudate cells were incubated with or without Hsp70 with OVA, tumor cell extract or OVA + tumor cell extract overnight. The incubated peritoneal exudate cells enriched with macrophages (1×10^6 cells) injected locally to recipient mice that had been sensitized to OVA, TE or OVA + TE and tested DTH response after 24 hrs. Macrophages harvested from Hsp70-treated mice were able to induce DTH responses in sensitized normal healthy (Fig. 6C) and DL-bearing (Fig. 6D) mice. Macrophages incubated with tumor cell extract were also induces DTH responses almost in similar magnitude but significantly lower than the mice injected intraperitoneally with tumor derived Hsp70. This observation

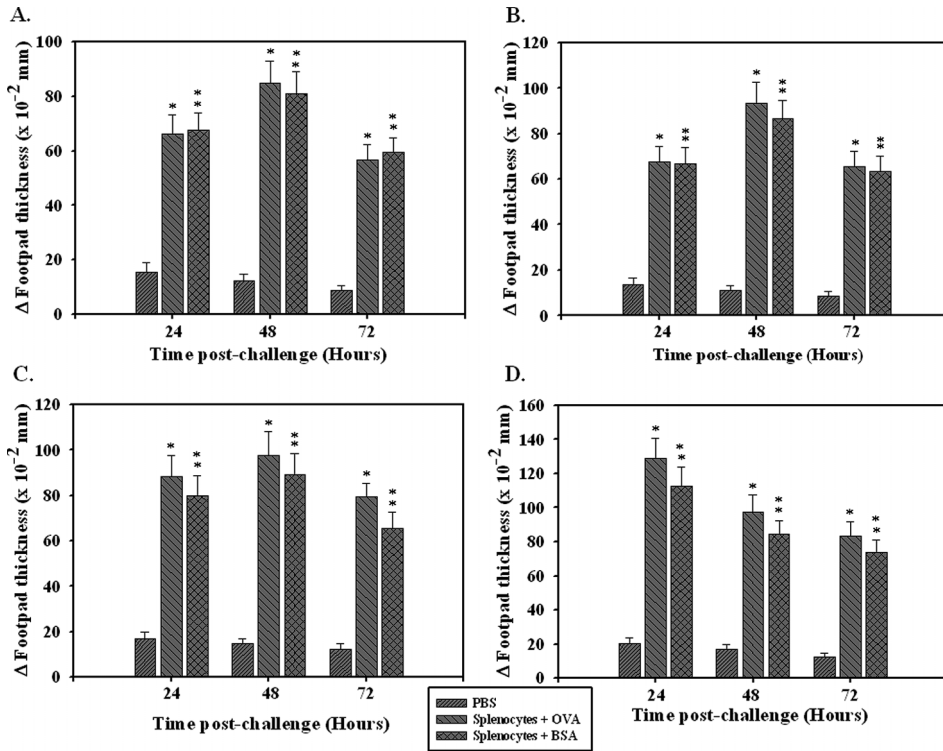


Figure 5: Effect of transfer of splenocytes from Hsp70 immunized donor. 2×10^7 Splenocytes from PBS, PBS + OVA or PBS + BSA treated normal healthy mice were injected into normal healthy mice (A), control DL-bearing mice (B), autologous Hsp70 immunized normal healthy mice (C), and autologous Hsp70 immunized DL-bearing (D), and increase in footpad thickness was measured after 24, 48 and 72 hrs. Data are presented as mean of difference in footpad thickness $\times 10^{-2} \pm \text{SE}_M$. The results shown are representative of three independent experiments. p-values for adoptive transfer of splenocytes from PBS injected mice versus OVA immunized mice is < 0.05 and BSA immunized mice is < 0.05 .

indicates that Hsp70 potentiates immune responses to specific antigens by enhancing the antigen presentation machinery. Further, we incubated macrophages with Hsp70 in culture for 1hr and then incubated overnight with OVA, or TE or OVA + TE. Macrophages treated with Hsp70 potentially increased the DTH responses in these mice, whereas macrophages incubated with heat-inactivated Hsp70 showed significantly lower DTH response similar to the splenocytes harvested from heat-inactivated Hsp70 injected mice (Fig. 7).

Proinflammatory Cytokine and C-C Chemokine Production of DTH Footpad. Proinflammatory cytokines and chemokines are important mediators for elicitation of DTH responses. Therefore, to assess the production of these mediators IFN (Interferon)- γ and MIP-1 α (Macrophage Inflammatory Protein-1 α), the DTH footpads of different groups of mice such as mice

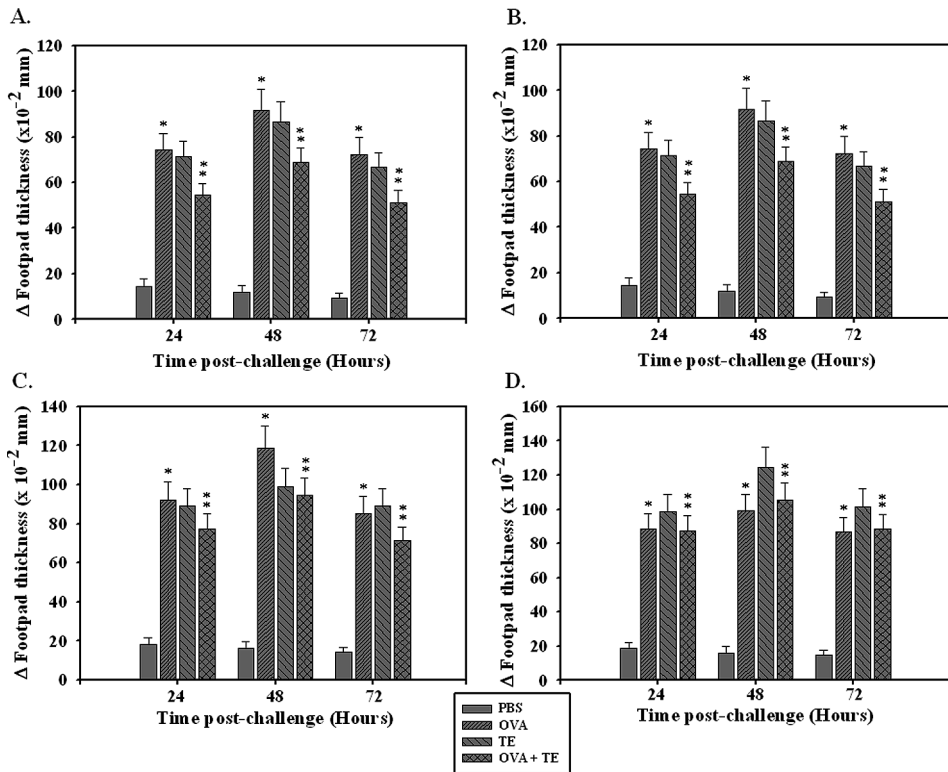


Figure 6: Effect of antigen pulsed Hsp70 treated macrophage transfer on DTH response. Macrophages were harvested from normal healthy mice and incubated for 24 hrs with culture medium alone or culture medium containing OVA, TE or OVA + TE for 24 hrs and 1×10^7 macrophages were transferred to normal healthy mice (A), control DL-bearing mice (B), autologous Hsp70 immunized normal healthy mice (C), and autologous Hsp70 immunized DL-bearing mice (D). The increase in footpad thickness was measured after 24, 48 and 72 hrs. Data are presented as mean of difference in footpad thickness $\times 10^{-2} \pm \text{SE}_M$. The results shown are representative of three independent experiments. p-values for adoptive transfer of macrophages incubated with PBS versus macrophages incubated with OVA in the medium < 0.05 and macrophages incubated with TE + OVA in the medium is < 0.05.

sensitized with OVA or TE pre-treated with tumor derived Hsp70 and challenged with OVA, were cutoff, minced and soluble materials were extracted as described in Materials and Methods. Supernatants collected after centrifugation was assayed for $\text{IFN-}\gamma$ and $\text{MIP-1}\alpha$ by Sandwich ELISA. We observed significantly enhanced production of $\text{IFN-}\gamma$ in the DTH footpad extracts but almost not in the control footpad extract (Fig. 8A).

Similarly, the $\text{MIP-1}\alpha$ production were induced in Hsp70 pretreated DTH footpads of PBS, OVA and TE sensitized mice (Fig. 8B). Treatment with heat-inactivated tumor derived Hsp70 almost abrogated the production of $\text{IFN-}\gamma$ and $\text{MIP-1}\alpha$ in DTH footpad extract (Right panel of Fig. 8 A,B), which indicates that Hsp70 derived from DL cells enhances the Th1 cytokine and C-C

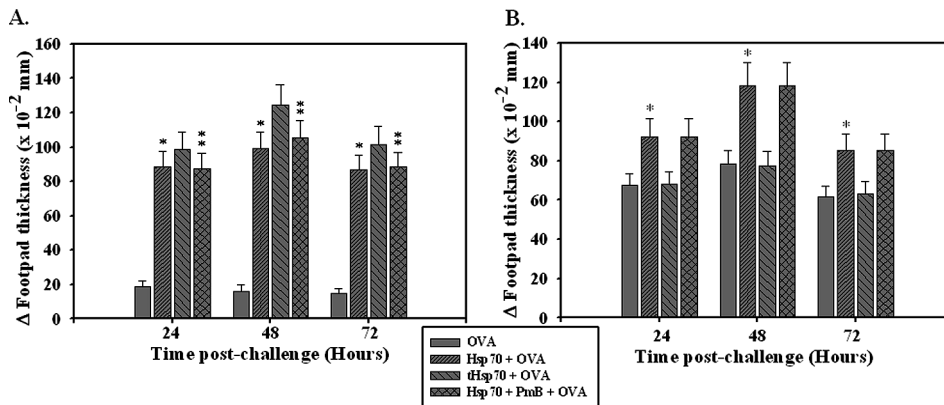


Figure 7: Effect of heat inactivation of Hsp70 on DTH response upon splenocytes and macrophages transfer. 2×10^7 Splenocytes from OVA injected normal healthy mice immunized with autologous Hsp70, autologous Hsp70 + PmB and heat inactivated Hsp70 were injected into normal healthy mice (A) and macrophages harvested from normal healthy mice and incubated for 24 hrs with culture medium alone or culture medium containing OVA for 24 hrs and 1×10^7 macrophages were transferred to normal healthy mice (B), and increase in footpad thickness was measured after 24, 48 and 72 hrs. Data are presented as mean of difference in footpad thickness $\times 10^{-2} \pm \text{SE}_M$. The results shown are representative of three independent experiments.

chemokine production at the site of antigen delivery that results into enhanced DTH reactivity in antigen challenged normal healthy and DL-bearing mice.

Induction of DL-Cell Rejection by Autologous Hsp70 and Increase in Longevity of Mice Next, we examined the effects of tumor derived Hsp70 on the protective immunity and DL cell progression in the host mice. All the mice that received autologous Hsp70 survived for more than 45 days. In contrast, those mice received only sterile PBS or Hsp70 derived from normal liver did not show any longevity and died within 32 days of DL cell transplantation (Fig. 9A). Further to test the efficacy of Hsp70, the mice were injected with Hsp70 derived from S-180 cells did not show any improvement in survival time and all DL-bearing mice were died within 32 days of life span. These data suggested that repeated injection of autologous Hsp70 preparations derived from DL cells to DL-bearing mice resulted in both the rejection of DL cells and a survival enhancement of DL-bearing host.

Treatment with autologous Hsp70 resulted in a reduced growth rate of DL cells as indicated by the reduction in numbers of DL cell count following Hsp70 immunization (Fig. 9B). In contrast, when the mice were injected with sterile PBS or Hsp70 derived from liver of normal healthy mice, they did not show any reduction in growth rate of DL cells and did not show any survival benefit or longevity enhancement in tumor-bearing host.

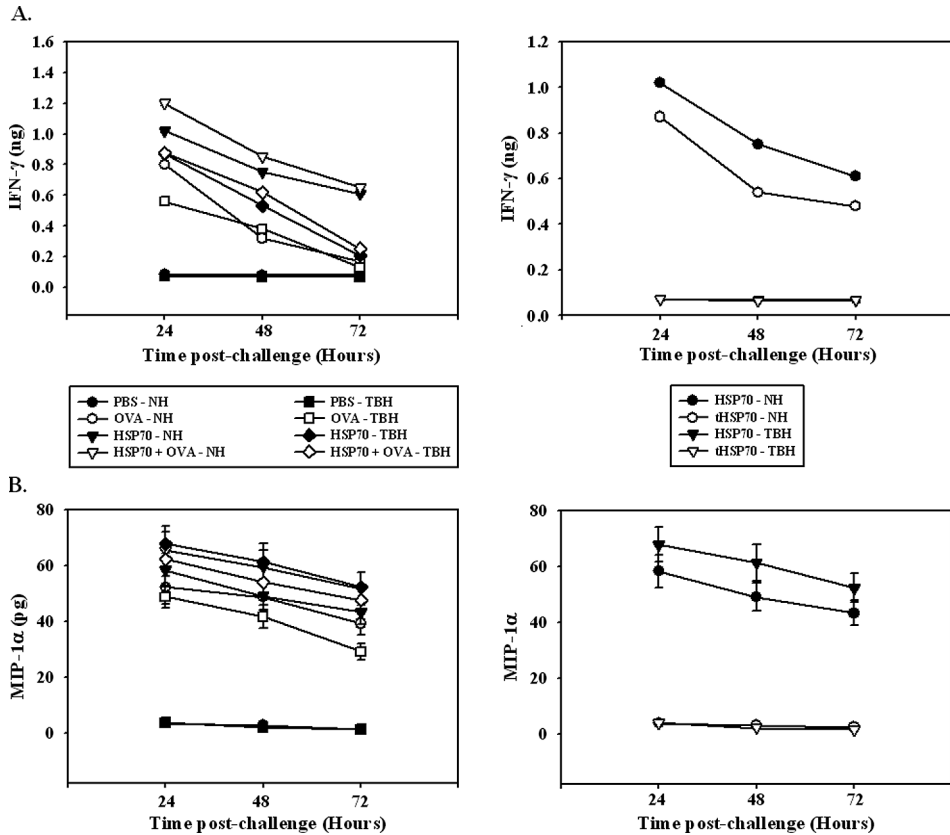


Figure 8: Effect of autologous Hsp70 on pro-inflammatory cytokine and C-C chemokine production in DTH footpad. Control group of mice and autologous Hsp70 immunized normal healthy mice and DL-bearing mice were sensitized with OVA and challenged. The left footpad of challenged mice was cut off at different time intervals of 24, 48 and 72 hrs, and IFN- γ (A) and MIP-1 α (B) level were measured as described in Materials and methods. Data are presented as mean $\pm$ SE_M. P-value for Hsp70 immunized normal healthy mice versus Hsp70 immunized DL-bearing host, $p < 0.05$. The right panel shows the effect of immunization of heat inactivated Hsp70 on IFN- γ and MIP-1 α production in DTH footpad. The results shown are representative of three independent experiments.

DISCUSSION

Previously, we reported on the capacity of autologous Hsp70 to induce Th1 polarization by activating polarized type II macrophages in the tumor-bearing host (Kumar et al., 2007). Now, we present the evidence that autologous Hsp70, if injected into tumor-bearing host, can trigger strong DTH responses in antigen challenged tumor-bearing mice. The DTH reaction is mainly elicited by Th1 type of cells. Thus, autologous Hsp70 can act as potent immunogens (Udono et al., 1993) as well as elicitors of Th1 type CD4⁺ T cell responses. To our knowledge, this is the first report of augmentation of DTH by autologous

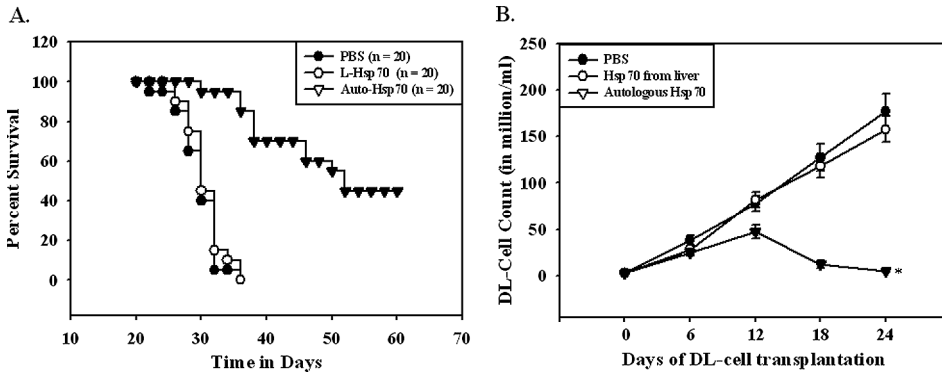


Figure 9: Effect of Hsp70 on survival of DL-bearing mice and DL-cell number. 3×10^6 DL-cells in 0.5 ml of PBS were injected i.p. and at 2nd and 9th day of post-DL transplantation, only sterile PBS as a control, Hsp70 from liver of normal healthy mice and autologous Hsp70 were injected intraperitoneally. Survival of DL-bearing host were monitored and analyzed by Kaplan Meier Plot (A). Further, DL-cells were harvested at indicated time intervals and counted. The results shown are representative of individual three experiments.

Hsp70 in tumor-bearing host, and is consistent with a recent study on mice, in which DTH was enhanced by the administration of synthetic peptides complexed with *Mycobacterium tuberculosis* Hsp70 (Román et al., 1997). The response due to Hsp70 appears to be in part via direct effects upon antigen presenting cells. Intra-peritoneal administration of autologous Hsp70 led to the stimulation of primary peritoneal exudate cells to produce proinflammatory cytokines and chemokines.

Heat treatment to Hsp70 derived from DL cells, conversely, inhibited the DTH response in antigen challenged both normal healthy and DL-bearing host, which indicates that the augmentation of DTH in Hsp70 injected mice upon antigen challenge is solely due to the effect of tumor derived Hsp70. However, the mechanism(s) that account for this increase in DTH reactivity induced by autologous Hsp70 are largely unknown. The treatment of macrophage culture with bacterial Hsp70, has been reported to increase the steady state levels of cytokine mRNA for a number of inflammatory cytokines, including interleukin-1 α , interleukin-1 β and interleukin-6, tumor necrosis factor- α and granulocyte-macrophage colony stimulating factor (Retzlaff et al., 1994).

As macrophages are thought to play a central role in DTH response (Gajewski et al., 1991), the increase in DTH response upon adoptive transfer of macrophages incubated with Hsp70 might suggest that the DTH inducing capacity of autologous Hsp70 could be due to its ability to upregulate production of pro-inflammatory cytokines by macrophages. Splenocytes from Hsp70 injected mice were able to enhance DTH reactions and produce a contralateral effect in antigen challenged mice. Similar to this observation, peritoneal exudate cells incubated with Hsp70 were able to enhance DTH reactions and produce a contralateral effect when pulsed with the antigen used to trigger the DTH

reactions. It is possible that presentation of antigen by APCs to CD4⁺ T lymphocytes in the presence of Hsp70-peptide complex may generate responsive T cells that augment DTH reactivity in recipient mice. Trafficking of these cells, or the APCs themselves, may explain the contralateral effect in the antigen challenged mice (Ghivizzani et al., 1998).

The results from the TE-pulsed PECs experiments suggest that viable cells within the epidermis can induce protective anti-tumor immunity against the DL cells. The slow progression and rapid loss of DL cell number indicate that incubation with tumor-derived Hsp70 enhances cross-presentation of tumor antigen by PECs in culture. However, the adoptive transfer of TE-pulsed PECs induces strong DTH reactivity but significantly lower than OVA peptide in Hsp70 immunized both normal healthy and DL-bearing mice.

Further, to validate the possible explanation of mechanism(s) by which Hsp70 augments the DTH reactivity in tumor-bearing host, we investigated the level of Th1 cytokines and C-C chemokine in the DTH footpad. The presence of higher level of IFN- γ and MIP-1 α in DTH footpad extract of antigen challenged mice supports the assumption that Hsp70 induces Th1 polarization at the DTH site. Additionally, it also indicates that administration of autologous Hsp70 stimulates the local APCs and the expansion of Th1 cell clones at the site of antigen delivery. The outcome of DTH response is determined *in vivo* by the interaction of Th1 and Th2 cell clones (Rizzo et al., 1995). In our study, the augmentation of DTH response in DL-bearing mice on passive transfer of splenocytes from immunized mice indicates that immunization increases the population of Th1 cell clone in tumor-bearing host, that resulted into enhanced DTH reactivity, probably by modulating the effector stage of DTH reactivity through the release of Th1 cytokines and down regulating the release of Th2 cytokines.

DL-bearing host, normally, experiences highly suppressed state of immunity as previously reported in other form of implantable tumor models (Loeffler et al., 1992; Mizoguchi et al., 1992). The induction of delayed type hypersensitivity on Hsp70 administration indicates that state of immune suppression or immune dysfunction in DL-bearing host can be modulated, at least in early stage of DL cell progression, to allow enhancement of specific antitumor immune responses. This rejection of tumor may be due to sustained expansion of CD8⁺ T cells (Shrikant et al., 1999) or by the recruitment and activation of macrophages and NK cells (Bartholomae et al., 2004) at the tumor site. Collectively, this study suggests that autologous Hsp70 may assist development of Th1 cell-mediated immunity in the tumor-bearing host that further extends its use in chaperone-based immunotherapy to cancers.

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