60-kDa heat shock protein of *Chlamydia pneumoniae* is a target of T-cell immune response

C.M. AUSIELLO [1], R. PALAZZO [1], F. SPENSIERI [1], G. FEDELE [1], R. LANDE [1], A. CIERVO [1], G. FIORONI [2], A. CASSONE [1]

[1] Department of Infectious, Parasitic, and Immune-Mediated Diseases, Istituto Superiore di Sanità, Rome, Italy
[2] Department of Internal Medicine, Catholic University, Rome, Italy

ABSTRACT: Inflammatory processes contribute to the pathogenesis and complications of atherosclerosis and coronary heart disease (CHD). Several findings indicate that chlamydial heat shock proteins (HSP) may represent a particularly strong antigenic stimulus, able to induce specific humoral (Ab) and T-cell-mediated immune responses (CMI) linking infection by *Chlamydia pneumoniae* (CP) to immuno-pathological sequelae such as atherosclerosis and CHD. We have here evaluated the ability of chlamydial recombinant (r) HSP60 and rHSP10 to induce specific immune responses in human peripheral blood lymphocytes and in murine models. rHSP60, but not rHSP10, was shown to induce proliferation and Interferon-gamma secretion in lymphocytes of randomly selected blood donors, as well as to generate and detect delayed-type hypersensitivity response in HSP60-vaccinated mice. Overall, the present study provides new hints to evaluate a previous exposition to CP using rHSP60 in humans. Thus the evaluation of specific HSP60 CMI response in healthy subject could be useful to monitor the reactivity to *Chlamydia pneumoniae* possibly providing a link to CHD pathologies. (J Biol Regul Homeost Agents 2005; 19:)

KEY WORDS: Chlamydia pneumoniae*, rHSP60, rHSP10, Lymphocyte proliferation*

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INTRODUCTION

It is now well established that inflammatory processes contribute to the pathogenesis and complications of atherosclerosis and coronary heart disease (CHD) (1-3). Hypercholesterolemia, modified lipoproteins and infection by several organisms have been implicated in the triggering of inflammation (4, 5). Recently, there has been renewed interest in the possibility that one or more CHD pathologies might be associated with chronic infection by *Chlamydia pneumoniae* (CP), a common respiratory, intracellular pathogen (6, 7).

Several findings indicate that chlamydial heat shock proteins (HSP), in particular the 60-kDa HSP (HSP60), may represent a particularly strong antigenic stimulus, linking specific humoral (Ab) and T-cell-mediated immune responses (CMI) to immuno-pathological sequelae (8-11).

Recently the co-localization of CP and HSP60 in the atheromatous plaque (12-14) and plaque infiltration with human and chlamydial specific T-cell clones of Th1 and Th0 cytokine profile (9, 15) have been reported. In addition, it is well documented that protective immunity against infection by *Chlamydia* requires induction of a Th1-oriented immune response (16, 17).

The aim of this study was to evaluate the capacity of CP-HSP60 and -HSP10 to induce specific T-cell immune responses in healthy human individuals and in immunized mice. Thus, CP-recombinant (r) HSP60 and -rHSP10 were generated under non-denaturing conditions (18) and used to assess proliferation and Interferon-gamma (IFN-γ) in human peripheral blood mononuclear cells (PBMC). Moreover, the induction of a classical T memory response was evaluated *in vivo* by measuring the delayed-type hypersensitivity (DTH) reaction in immunized mice.

MATERIALS AND METHODS

Recombinant (r) HSP antigens, CP Elementary Bodies (EB) and other reagents

rHSP60 and rHSP10 were obtained as described in detail by Ciervo et al (18). The rHSPs were incubated with 10 µg/mL polymyxin B-4% beaded agarose (Sigma Chemical co, St. Louis, USA) to remove *E. coli* LPS. Preparations were tested for the presence of endotoxin by end-point chromogenic *Limulus* amebocyte lysate methods (LAL Endochrome, Charles River Endosafe, Charleston, SC). Endotoxin levels were below 0.2 endotoxin units (EU)/mg of

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proteins (below 0.02 ng/mg protein).

Elementary bodies (EB) from *C. pneumoniae* were obtained from strain Parola (kindly provided by Dr. P. Saikku, KTL, Oulu, FI), propagated in Hep-2 cells (American Type Culture Collection, Rockville MD). Hep-2 cells were grown in Eagle's MEM (Gibco Life Technologies, Grand Island, NY) supplemented with fetal calf serum (FCS, Gibco Life Technologies) as described (18). Heat-inactivated (HI) EB of CP (96 °C, 1 h) were used as antigen.

Staphylococcus enterotoxin B (SEB) was obtained from Sigma (Sigma Chemicals Co) and purified protein derivative (PPD) from *M. tuberculosis* was obtained from Serum Staten Institute (Copenhagen, Denmark). Anti HLA-DR (AA65) monoclonal antibody (mAb) was kindly donated by F. Malavasi (Università di Torino, Italy).

Human peripheral blood mononuclear cells (PBMC) isolation, culture, proliferation and cytokine assays

PBMC from 26 healthy donors (courtesy of Dr Girelli, "Centro Trasfusionale dell'Università", Rome, Italy) were examined for T-cell proliferation induced by CP preparations. PBMC were isolated from venous heparinized blood samples by centrifugation on Ficoll gradient (Lympholyte-H, Cedarlane, Hornby, Ontario, Canada), washed twice and suspended in RPMI medium (ICN-Flow, Aurora, OH) supplemented with 5% pooled AB serum and antibiotics (Penicillin 100 IU/mL, Streptomycin 0.1 µg/mL; Hyclone Laboratories, Logan, UT) (19).

PBMC proliferation was measured by using $2x10^5$ cells/well in 0.2 mL complete medium, in triplicate, in 96 flat bottom microwell trays (Falcon, Becton Dickinson, Lincoln Park, New Jersey, USA) in the presence of the predetermined optimal doses of stimulants (rHSP60, rHSP10 at 10 mg/mL and HI-EB at 10^5/mL). The cultures were harvested after 7 days stimulation. DNA synthesis was evaluated by using ^{3}H-thymidine (Amersham Biosciences, UK) at 0.5 µCi and incorporation measured (19). Data are reported as Stimulation Index (SI), i.e. the ratio between the antigen-stimulated PBMC culture and the unstimulated one. The cpm (±SE) mean value of unstimulated culture of PBMC was 0.7 ± 0.1. A CMI proliferative response was considered positive (CMI^+) when the SI was greater or equal to 4 (20).

Cytokine production was measured in cultures of $2x10^6$ PBMC/mL in 0.5 mL complete medium obtained from six randomly selected donors in the presence of CP antigen preparations at the concentration used for cell proliferation. Culture supernatants were collected at 48 h and used to measure IFN-γ by ELISA (Quantikine, R&D systems, Inc., Minneapolis, MN, USA) (19).

Mouse immunization and DTH assay

Purified recombinant proteins were mixed with complete Freund's adjuvant (Sigma Chemical Co.) and used to immunize CD2F1 mice (9 animals, 10 µg of protein each, sc). At weekly intervals, three additional antigen boosts (10 mg) in incomplete Freund's adjuvant (Sigma Chemical Co.) were given to each animal. DTH response was tested 10 days after the last booster (21). Five mg of each antigen in 20 µL of saline were injected into the footpad of mice and the DTH reaction was recorded 24 h later by measuring the footpad swelling with a caliper. The results were expressed as the increase in footpad swelling (right hind) over that of the saline-injected (left hind) counterpart. Data, expressed in mm x 10^{-2}, are the means ± SE of nine mice per group.

Statistical analyses

Statistical descriptive analyses were carried out using the SPSS Inc (Chicago, IL) statistical package. Differences between mean values were assessed by two-tailed Student's t test and were statistically significant for p values < 0.05.

Results

T-cell response to CP HSPs in human PBMC

To evaluate if CP HSPs were the target of specific immune responses in normal human subjects, the ability of these proteins to induce a proliferative response in PBMC from randomly selected blood donors was studied in comparison with the proliferation induced by heat-inactivated CP elementary bodies (HI-EB), used as control CP antigen. In preliminary experiments, PBMC were cultured in the presence of different doses of each CP preparation and it was found that the optimal dose of HI-EB in responsive subjects was 10^5 HI-EB/mL, whereas the optimal dose of the rHSPs was 10 µg/mL. Figure 1A shows that the PBMC of more than half the subjects examined had a positive proliferative response to HI-EB (greater than SI cut-off, i.e., 4) and that PBMC of about half of subjects studied did also proliferate in response to stimulation with rHSP60. In contrast, only 1 subject responded with a consistent lymphocyte proliferation to rHSP10.

rHSP60 could be endowed with mitogenic or superantigenic properties. To clarify this point, a boiled preparation of rHSP60 was used in several donors and shown that T-cell proliferation response was not affected. Yet, a small mitogenic component was probably present in the preparation since cord blood lymphocytes showed a low-level proliferation when stimulated by rHSP60, approaching to the cut-off limit (data not shown). Since, however, a similar, low-level proliferation of naïve T-cells was also induced by the rHSP10 preparation, it is possible that the mitogenic contaminant could be a component of the bacterial expression system.

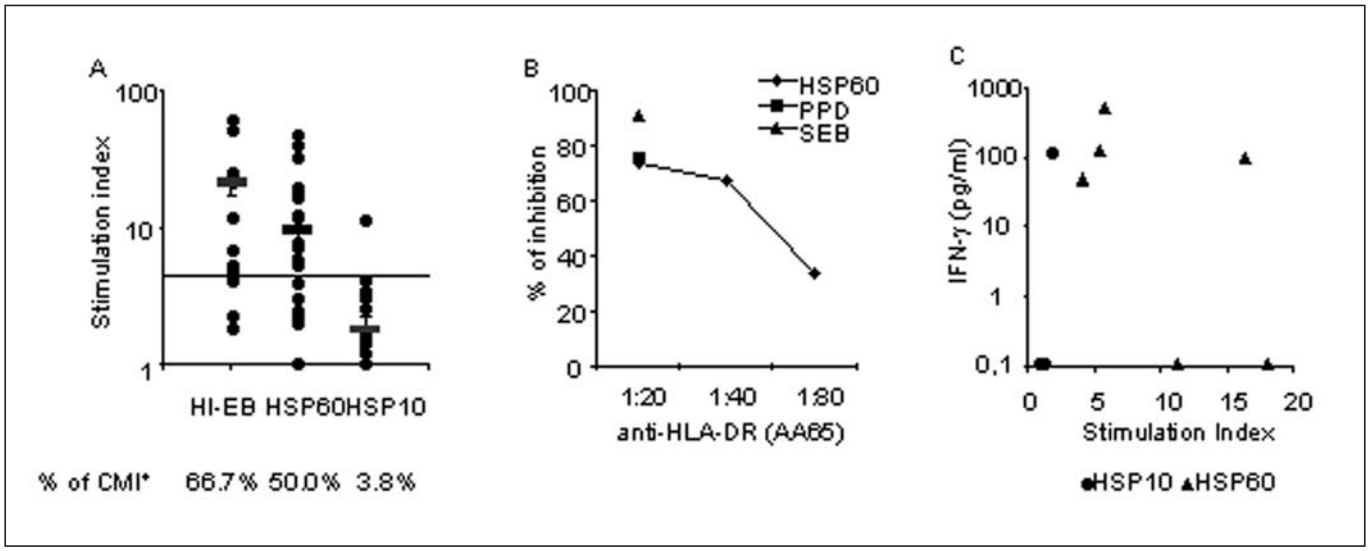

Fig. 1 - rHSP60 induce proliferation (A) of antigenic type (B) and specific IFN-γ (C) in human PBMC.
(A) PBMC ($2x10^5$ cells/well in 0.2 mL) were cultured in the presence of indicated stimuli. Proliferation was measured after 7 days by ^{3}H-Thymidine incorporation. Data are reported as dispersion of stimulation index values of all PBMC donors tested. Percentage of positive proliferative responses to the stimuli is also indicated. For the definition of SI, CMI positivity and technical details, see text.
(B) rHSP60-, PPD- or SEB-induced proliferation are inhibited by the addition to the cultures of blocking anti HLA-DR mAb (AA65). Proliferation is measured as in A. Data are expressed as percentage of inhibition of proliferation induced in absence of the anti HLA-DR mAb (stimulation index obtained in activated PBMC were: 18, 49 and 385 for rHSP60, PPD and SEB respectively). For technical details, see text.
(C) MC ($2x10^5$ cells/well in 0.2 mL) were cultured in the presence of indicated stimuli. IFN-γ (pg/mL) and proliferation (stimulation index) obtained in six PBMC preparations are shown. IFN-γ and proliferations were measured after 2 and 7 days, respectively. For further technical details, see text.

Figure 1B shows that the proliferation induced by rHSP60 is substantially and dose-dependently inhibited by the addition to the cultures of a blocking anti HLA-DR mAb (AA65). Overall, the data above constitute indirect evidence that the proliferation induced by the recombinant protein is of antigenic rather than mitogenic or superantigenic type.

To further evaluate the immunological properties of rHSP60, the levels of IFN-γ released upon PBMC activation was studied in six rHSP60-responsive donors. The results obtained indicate that rHSP60 was able to induce high levels of IFN-γ in 5 out of 6 PBMC cultures. Interestingly, there was no clear match between IFN-γ production levels and proliferation, as indicated by high cytokine levels in some PBMC cultures associated with low levels of proliferation, and *viceversa* (Fig. 1C).

All together, the data shown above clearly indicate that a major proportion of normal healthy adults have memory T-cell responses to chlamydial antigens, in particular to rHSP60, consistent with previous exposure to CP, and in keeping with epidemiological observations (22, 23).

T-cell response to CP HSP in CD2F1 mice

We next examined whether rHSPs were also able to induce B and T-cell responses in naïve animals. To this aim, mice were immunized with either rHSP60 or rHSP10, and both humoral (antibody) and cellular (DTH) responses were assessed. Elevated IgG levels with the expected specificity were found in serum of all CD2F1 mice inoculated with rHSP60, while those inoculated with rHSP10 did not show a consistent antibody response (Ciervo et al, 2004, submitted). As shown in Figure 2, rHSP60-immunized mice manifested a consistent DTH response with a footpad thickness reaction to rHSP60 significantly higher than that obtained in non-immunized or rHSP10-immunized animals ($P = 0.01$). These data indicate that rHSP60 protein was able to elicit a T-cell recall immune response in immunized mice whereas rHSP10 was unable to induce a memory response. Shamed-immunized mice challenged with each recombinant antigen did not express any DTH response.

Discussion

In this study, the role played by HSPs of CP in their ability to drive specific T-cell response was addressed. First of all, we noticed that a relatively high proportion of randomly selected healthy blood donors expressed a positive proliferative response to CP antigenic preparations. CMI responses were similarly prevalent in PBMC stimulated by the EB or the rHSP60, but not rHSP10, of CP.

Accordingly, the rHSP60 antigen was strongly immunogenic and able to generate/detect a strong

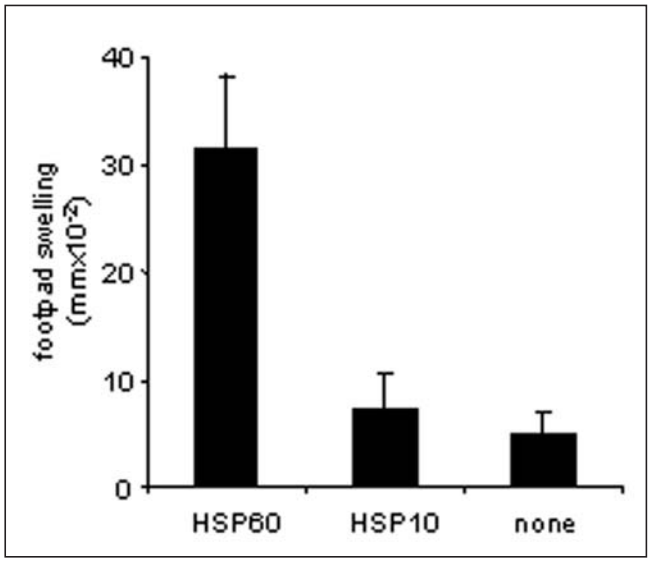

Fig. 2 - rHSP60 induce specific DTH response in rHSP60 vaccinated mice. rHSP60, rHSP10 or saline were injected into the footpad of CD2F1 mice and DTH reaction was recorded 24h later by measuring the footpad swelling. Results were expressed as the increase in footpad swelling (right hind) over that of the saline-injected (left hind) counterpart. Data, expressed in mm x 10^{-2}, are the means ± SE of 9 mice per group.

DTH reaction in mice, confirming that rHSP60 protein was able to elicit a T recall immune response in immunized mice whereas rHSP10 was unable to induce a memory response.

More than 50% of subjects studied showed a positive response to CP preparations. In particular, about 50% specifically proliferates and secretes IFN-γ to rHSP60. The T-cell response is specific and indicate a true T-memory response, as enlighten by the inhibition of proliferation, in a dose-dependet manner, by anti-HLA class II mAb.

All this indicates a previous exposure to CP, a finding which is in accordance with the high prevalence of antibody response to CP found in many previous studies in healthy adults (22). In this respect, a recent study performed in Finland, in a prospective study of detection of *Chlamydia pneumoniae* antibodies in children between 7 months and 8 years of age, indicates that 10% of the children had a clearly positive IgG and IgA antibody value, suggesting that CP infection, often asymptomatic, occurs commonly already at an early age (23).

Our data also confirms that HSP60 is a major, immunodominant antigen expressed during infection by this microorganism (8, 11, 18, 24).

An aspect of interest of our study is also the unresponsiveness to HSP10 preparation of human PBMC and vaccinated mice, which is in contrast with the results obtained with the homologue protein of *Chlamydia trachomatis*. In fact sero-reactivity to *Chlamydia trachomatis* HSP10 correlates with severity of human genital tract disease. In addition while HSP60 and HSP10 were both recognized by infertile women, reactivity to HSP10 specifically discriminates the infertile women with tubal factor infertility factors (25, 26).

It has been suggested that HSPs play a role in the pathogenesis of chlamydial infections and in some immuno-inflammatory diseases which appear to be associated with chlamydial infection (27, 28). Amino acid sequence homologies between chlamydial HSP60 and bacterial and human counterparts have been detected, and antigenic epitopes were shown to cross-react extensively (7, 29, 30). On this basis, it has been assumed that chronic infection with CP and other bacteria might represent a persistent antigenic stimulus capable of intensifying a generalized response to the HSP protein family, and possibly, autoimmunity (9, 15, 31). Circulating HSP60 antigen is elevated in CP infection and in subjects with atherosclerosis (24, 31), and both HSP60 and CP have been detected in the atheromatous plaque (13). Acellular components of CP are a potent stimulus for cytokine production, and this mechanism may have an important role in the inflammatory aspects of atherogenesis (32).

Thus, induction of specific immune responses to HSPs together with the production of pro- or anti-inflammatory cytokines, and infiltration of cytotoxic, HSP60-specific T-cells within the plaque have been advocated to link infection to atherosclerosis and some of its acute sequelae (9, 29). Consistent with this, elevated anti-HSP60 IgG levels are thought to be a marker of coronary artery disease (8, 10).

Overall, the present study provides new hints to evaluate a previous exposition to CP using rHSP60 to induce specific immune responses in healthy human individuals. The critical balance between protective and detrimental immune responses induced by potent immunomodulators such as HSPs in the setting of infection by CP may play a role in chronic inflammatory sequelae of atherosclerosis.

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Reprint requests to:
C.M. Ausiello, MD
Unit of Anti-Infectious Immunity
Department of Infectious, Parasitic,
and Immuno-Mediated Diseases
Istituto Superiore di Sanità,
Viale Regina Elena, 299
00161 Rome, Italy
ausiello@iss.it

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