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CD4 and CD8 T cell response to the rHSP60 from *Klebsiella pneumoniae* in peripheral blood mononuclear cells from patients with ankylosing spondylitis

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ABSTRACT

Objective. To determine the processing pathways used by peripheral blood mononuclear cells (PBMC) and present the rHSP60Kp, and the T cell subpopulations involved in the response, in patients with ankylosing spondylitis (AS) **Methods.** The lymphoproliferative response to the rHSP60Kp in PBMC from 14 HLA-B27$^+$ AS patients and 15 B27$^-$ healthy controls was assessed by ^{3}H-TdR incorporation. The processing pathways for the rHSP60Kp were analyzed by ^{3}H-TdR incorporation in fresh PBMC from patients using homologous PBMC preincubated with the antigen and specific inhibitors: chloroquine, N-acetyl-L-leucil-L-leucil-L-nor-leucinal (LLnL) or brefeldin A (BFA), fixed with p-formaldehyde (fixed APC). The CD4$^+$/CD8$^+$ T cell subpopulation activated with the antigen was determined by three colours flow cytometry in PBMC from patients. **Results.** Eight out of fourteen patients showed positive lymphoproliferative responses to the rHSP60Kp while none of the healthy controls responded ($p < 0.012$). In five patients S.I. was above 4.0. In these patients lymphoproliferation was lower when chloroquine and LLnL was used and it became negative with BFA, indicating that both pathways are used. CD4+ and CD8+ T cells populations expressed CD69 when activated by the rHSP60Kp. **Conclusions.** Our results suggest that CD4 and CD8 T cells participate in the response to the rHSP60Kp in B27+ AS patients.

Key words. Ankylosing spondylitis. HLA-B27. Antigen processing. HSP60. *Klebsiella pneumoniae.*

Respuesta de linfocitos T CD4 y CD8 contra la rHSP60 de* Klebsiella pneumoniae *en células mononucleares de sangre periférica de pacientes con espondilitis anquilosante

RESUMEN

Objetivo. *Determinar las vías utilizadas por las células mononucleares de sangre periférica (CMSP) de pacientes con espondilitis anquilosante para procesar a la rHSP60 de* Klebsiella pneumoniae *(rHSP60Kp) y las subpoblaciones de linfocitos T involucrados en la activación.* ***Métodos.*** *Se determinó la respuesta linfoproliferativa, por incorporación de ^{3}H-TdR en CMSP, en presencia de la rHSP60Kp, en 14 pacientes con EA HLA-B27+ y en 15 sujetos sanos HLA-B27-. La ruta de procesamiento y presentación de la rHSP60Kp se determinó por incorporación de ^{3}H-TdR en las CMSP de los pacientes utilizando como células presentadoras a las CMSP homólogas, preincubadas con el antígeno y los inhibidores específicos: cloroquina, brefeldina A y N-acetil-L-leucil-L-leucil-L-nor-leucinal (LLnL), y fijadas con p-formaldehído. Se evaluaron las subpoblaciones de linfocitos T CD4+ y CD8+ que expresaron CD69, frente al antígeno, por citometría de flujo.* ***Resultados.*** *Ocho de los 14 pacientes y ninguno de los sujetos sanos, tuvo respuesta linfoproliferativa positiva (IE > 3.0) contra la rHSP60Kp ($p < 0.012$). En cinco de los pacientes el I.E. fue superior a 4.0. En estos pacientes la linfoproliferación disminuyó cuando se utilizó cloroquina y LLnL, y se hizo negativa cuando se utilizó BFA, lo que indica que ambas vías son empleadas. Las subpoblaciones de linfocitos T (CD4+ y CD8+) expresaron CD69 frente al antígeno.* ***Conclusiones.*** *Nuestros resultados sugieren que ambas poblaciones de linfocitos T: CD4+ y CD8+ participan en la respuesta a la rHSP60Kp.*

Palabras clave. *Espondilitis anquilosante. HLA-B27. Procesamiento de antígeno. HSP.* Klebsiella pneumoniae.

INTRODUCTION

Ankylosing spondylitis (AS) is a chronic inflammatory disease of unknown aetiology in which immunogenetic and environmental factors are involved.[1] Immunogenetic factors are clearly represented by AS strong association to HLA-B27 (90% of AS patients carry the HLA-B27 gene) and the environment by the possible role of *Klebsiella sp.* and some other bacteria in AS pathogenesis.[2-9] We have previously reported both lymphoproliferative and IgG responses to electroeluted HSP60 from *Klebsiella pneumoniae* in HLA-B27 positive AS patients.[10-14] We also reported in a theoretical study that there is a nonapeptide in the HSP60Kp protein (residues 117-125) with high affinity for the B27 molecule.[12] Therefore, CD8 T cells could participate in the cellular response to this antigen and in AS pathogenesis.[15]

HLA-B27 physiologically binds antigenic peptides and thus presents them to CD8+ T cells. Single HLA-B27 restricted cytotoxic T lymphocytes have been described in AS patients with specificity for bacterial and viral peptides that show cross-reactivity with self antigens. Additionally, CD8 T cells have been reported to maintain inflammatory process even after the eradication of the microbial pathogen.[15,16]

In principle, antigens may be processed for presentation to T cells through two distinct pathways. Endogenous proteins are processed through the cytosolic pathway, while exogenous antigens, such as HSP60Kp, follow the endocytic pathway and induce MHC class II restricted responses.[17,18] However, the classical view of cytosolic and endosomic pathways for antigen presentation are not absolute. There are several extracellular antigens that are presented in class I molecules,[19-25] and in this case, it has been reported that the antigen processing occurs in the cytosol.[26] Then, if the HSP60Kp is processed by the cytosolic pathway this could help us to better understand the relationship of this antigen with AS, and the possible involvement of CD4 and CD8 T cells in the response to this protein.

However, to support this idea it is important to know if the rHSP60Kp is able to activate $CD4^+$ and/or $CD8^+$ T cells, and therefore it is necessary to demonstrate if this molecule could be processed by both the endocytic and cytosolic pathways. The aim of this work was to explore the processing pathways for the rHSP60Kp in AS patients and to determine the T cell subpopulations activated by the antigen.

MATERIAL AND METHODS

Patients and controls

Fourteen B27+ patients with AS, independently of the time of onset, diagnosed according to current diagnostic criteria[27] at the "Servicio de Reumatología" from "Hospital General de Mexico", were included. All of them were receiving non-steroidal anti-inflammatory drugs. Fifteen healthy subjects, none of them B27+, were included as control group. After written consent was given by each patient or control subject, they were bled by venipuncture and PBMC were obtained by using Lymphoprep (Nycomed, Oslo, Norway).

Purification of rHSP60Kp

Recombinant HSP60Kp (rHSP60Kp) was obtained from *E. coli* transformed with the plasmid containing the corresponding gene, as we have previously reported,[12] and purified in the Ni-NTA column system as described by the supplier (QIAGEN Inc. Valencia CA, USA). Purity was analyzed by gel electrophoresis under reducing conditions (12% SDS-PAGE) and concentration was determined by the Bradford method.[28]

Lymphoproliferative response to rHSP60Kp

In order to select subjects responding to the rHSP60Kp, PBMC from patients and healthy subjects were obtained using Lymphoprep (Nycomed, Oslo, Norway). Viability was determined by trypan blue (Sigma Chemical Company, St. Louis, MO, USA) exclusion. 10^5 viable PBMC, resuspended in 200 µL of AIM-V medium (Gibco, Life Technologies. Rockville, MD. USA), were plated in each well of a 96 well round bottom polystyrene microplate (Nunc, Roskilde, Denmark); 2 µg of rHSP60Kp or Concanavalin A (Con A, Sigma Chemical Co., St. Louis, MO, USA) as activation control, were added. Assays were done in triplicate. Plates were incubated for 5 days under 5% CO_2 atmosphere. After incubation, the wells were pulsed with ^{3}H-TdR at 1 µCi/well concentration (Amersham International, Bucks, UK) for the last 7 h. After harvesting onto glass filters, ^{3}H-TdR incorporation was measured by liquid scintillation spectroscopy.[13] Conditions were selected from a dose response curve previously determined.

Results were expressed as stimulation index (SI). SI ≥ 3.0 was considered positive[13] (Stimulation Index = cpm of cells with antigen/cpm of cells without antigen).

Determination of the processing pathway for rHSP60Kp

First, in order to validate the experimental design we used varidase (Wyeth, S.A. de C.V., México), an antigen reported to be processed only by the endocytic pathway, in three intradermally positive healthy subjects, that in PBMC had a positive lymphoproliferative response to this antigen.[29-31]

To elucidate the routes by which the rHSP60Kp is processed, 10^6 PBMC from each one of the five high responding AS patients were incubated in a final volume of 500 µL of AIM-V medium with 6 µg of rHSP60Kp and one of the specific inhibitors at the following final concentration selected from a dose response curve: 10 µg/mL brefeldin A (BFA), 10 µg/mL chloroquine (clathrin mediated transport and endosomic inhibitors, respectively) (34) or 1.0 µg/mL of N-acetyl-L-leucil-L-leucil-L-nor-leucinal (LLnL, a proteasome inhibitor)[32] all of them purchased from Sigma Chemical Co., St. Louis, MO, USA. PBMC were incubated 5 hours at 37°C in 5% CO_2, then centrifuged to remove the supernatant and fixed with 0.5% p-formaldehyde (Sigma Chemical Co., St. Louis, MO, USA) in PBS for 15 minutes at room temperature (RT). Para-formaldehyde excess was blocked by additional 20 minutes incubation with 0.1 M glycine (Sigma Chemical Co., St. Louis, MO, USA). Cells were washed twice with MEM (Sigma Chemical Co., St. Louis, MO, USA) and resuspended in AIM-V medium to be used as fixed antigen presenting cells (Fixed APC). PBMC incubated with antigen but without inhibitors and PBMC incubated with neither antigen nor inhibitors, but fixed as described above, were used as positive and negative controls, respectively.

To elucidate the processing pathways used by PBMC from AS patients for the rHSP60Kp, lymphoproliferation assays were done, briefly: 10^5 Fixed APC were added to an equal number of homologous Fresh PBMC, in 96 well round bottom polystyrene microplates and incubated 6 days at 37 °C in 5% CO_2. As a control for toxicity of each one of the Fixed APC, the mixture of fresh PBMC and Fixed APC was stimulated with Con A. Proliferation was determined by ^{3}H-TdR, as described above. Results of Fresh PBMC stimulated by Fixed APC with inhibitors were expressed in stimulation index and compared with the positive control.

CD4/CD8 subpopulations response to rHSP60Kp

To confirm which subpopulation of T cells were activated by the rHSP60Kp in the AS patients, the responses were evaluated by three colour flow cytometry, using CD69 as an early activation marker.[33,34] Briefly, 10^6 PBMC from lymphoproliferative responding AS patients were incubated with either 2 µg of rHSP60Kp or Con A for 12 hours at 37°C in 5% CO_2. The conditions used were selected from a dose response curve. Afterwards, following manufacturer instructions, cells were stained for 20 minutes at room temperature with the following monoclonal antibodies obtained from Becton Dickinson (San José, CA, USA): a) γ1-FITC/ γ1-PE/CD3-PeRCP b) CD4-FITC/CD69-PE/CD3-PeRCP and c) CD8-FITC/ CD69-PE/CD3-PeRCP washed with FACS-FLOW (Becton Dickinson) and fixed with 0.5% p-formaldehyde. Samples were run on a FACSCalibur (Becton-Dickinson), and data was collected on 5000 T lymphocytes (gated by CD3-PeRCP and side scatter properties), and analyzed using the Cellquest software (Becton-Dickinson Biosciences).

Statistical analysis

The non-parametric Fisher exact test was used to compare the lymphoproliferative response to the rHSP60Kp between PBMC from AS patients and from healthy subjects. The Mann-Whitney U test was used to compare the response to Con A between PBMC from AS patients and healthy subjects, as well as, to compare the lymphoproliferative response to Con A between fresh PBMC from AS patients stimulated with Fixed APC and fresh PBMC from AS patients stimulated by Con A. In all cases the Sigma stats v2.03 software was used.

RESULTS

Proliferative response to rHSP60Kp

Eight out of the 14 patients studied had a positive lymphoproliferative response to the rHSP60Kp (S.I. ≥ 3.0) and none of the healthy subjects responded ($p = 0.012$). In the group of patients five had a S.I. high above 4.0 so that they were chosen for these inhibition studies. All patients and healthy subjects responded to Con A as expected (Table 1).

rHSP60Kp Processing Pathway Determination

Varidase, an antigen processed by the endocytic pathway, was used to validate our experimental design in which BFA, chloroquine and LLnL were used as specific inhibitors. Our results showed that lymphoprolife-

Table 1. Lymphoproliferative response to rHSP60Kp and Con A in Fresh PBMC of AS patients and healthy subjects.

	As patients		Healthy subjects	
	Con A	rHSP60Kp	Con A	rHSP60Kp
1	284	8.7*	406	0.7
2	32.9	1.1	460.9	0.9
3	5.3	5.3*	78.6	0.7
4	129	2.3	113.6	1.8
5	198.15	5.7*	130.9	0.1
6	100.46	2.1	26.8	0.8
7	208	13.3*	230.33	1
8	87.3	15.1*	69.2	2
9	12.9	1.4	280.25	1
10	69.7	4.2	231.8	0.4
11	9.17	0.5	48.2	0.2
12	8.6	0.93	190	0.5
13	51.11	3.18	6.9	1.1
14	580	3	532	1.2
15	-	-	215	0.8
Frequency of positive response S.I. = 3.0	14/14	8/14	15/15	0/15

No statistical differences in the response to Con A between groups was observed (p = 0.994), but the response to rHSP60Kp was statistically different (p = 0.012). * Patients selected to analyze the antigen processing pathways.

ration became negative (S.I. < 3.0) with chloroquine and BFA (10 µg/mL), but not with the LLnL (1.0 µg/mL) in the three studied subjects (Figure 1).

In the five selected patients, the lymphoproliferative response of Fresh PBMC stimulated by Fixed APC incubated with rHSP60Kp was above 4.0. The S.I. with chloroquine and LLnL were lower than S.I. without inhibitors (Figures 2A and 2B). When BFA was used, lymphoproliferation became negative (SI < 3.0) (Figure 2C).

No differences between the responses to Con A of Fresh PBMC from AS patients incubated in the presence or absence of different homologous Fixed APC was observed, p = 0.383.

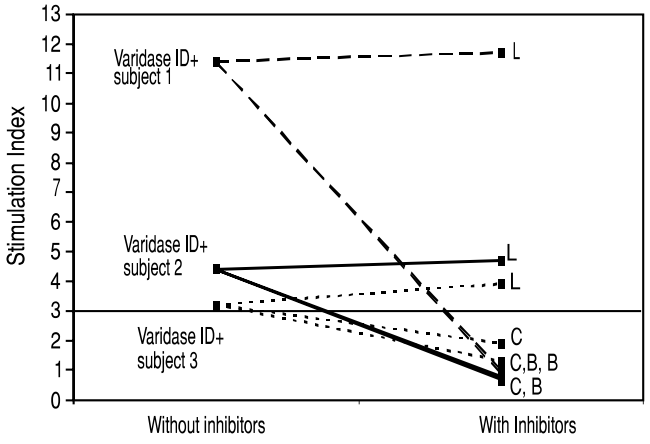

Figure 1. *Inhibition of the lymphoproliferative response to varidase in three intradermally positive healthy subjects. Fresh PBMC were stimulated with homologous Fixed APC incubated with varidase in the presence or absence of specific inhibitors. The response became negative when chloroquine (C) and BFA (B) were used but with LLnL (L) no change was observed. The horizontal line indicates the cut point (S.I. ≥ 3.0) for positive response.*

T cell subpopulations activated with the rHSP60Kp

In order to confirm the results suggested by the processing pathway determinations, T cell subpopulations activated with the rHSP60Kp or with Con A as a control, were determined by measuring the CD69 expression by three colours flow cytometry in four of the five patients studied. In figure 3, a representative activation of PBMC from one AS patient expressing CD69 by both CD4 and CD8 T cell subpopulations in the presence of rHSP60Kp, and Con A are shown. Averages of percentage of CD4 and CD8 T cells activated with Con A or with rHSP60Kp in all patients analyzed are graphed in Figure 4a and 4b, respectively.

DISCUSSION

We have previously reported the association between the HSP60Kp and AS by cellular and humoral immune response.[10-14] However, it became impor-

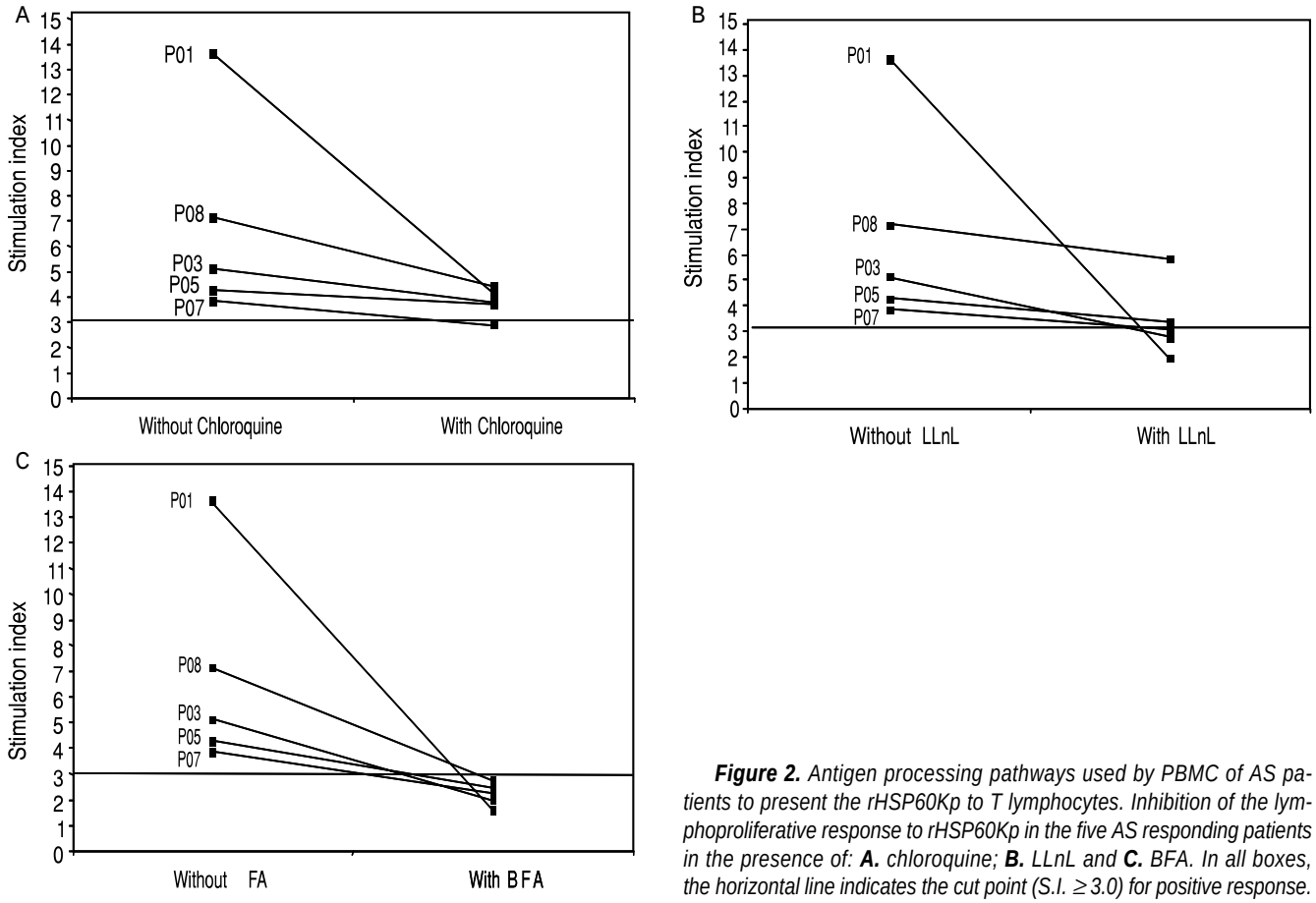

Figure 2. *Antigen processing pathways used by PBMC of AS patients to present the rHSP60Kp to T lymphocytes. Inhibition of the lymphoproliferative response to rHSP60Kp in the five AS responding patients in the presence of:* ***A.*** *chloroquine;* ***B.*** *LLnL and* ***C.*** *BFA. In all boxes, the horizontal line indicates the cut point (S.I. ≥ 3.0) for positive response.*

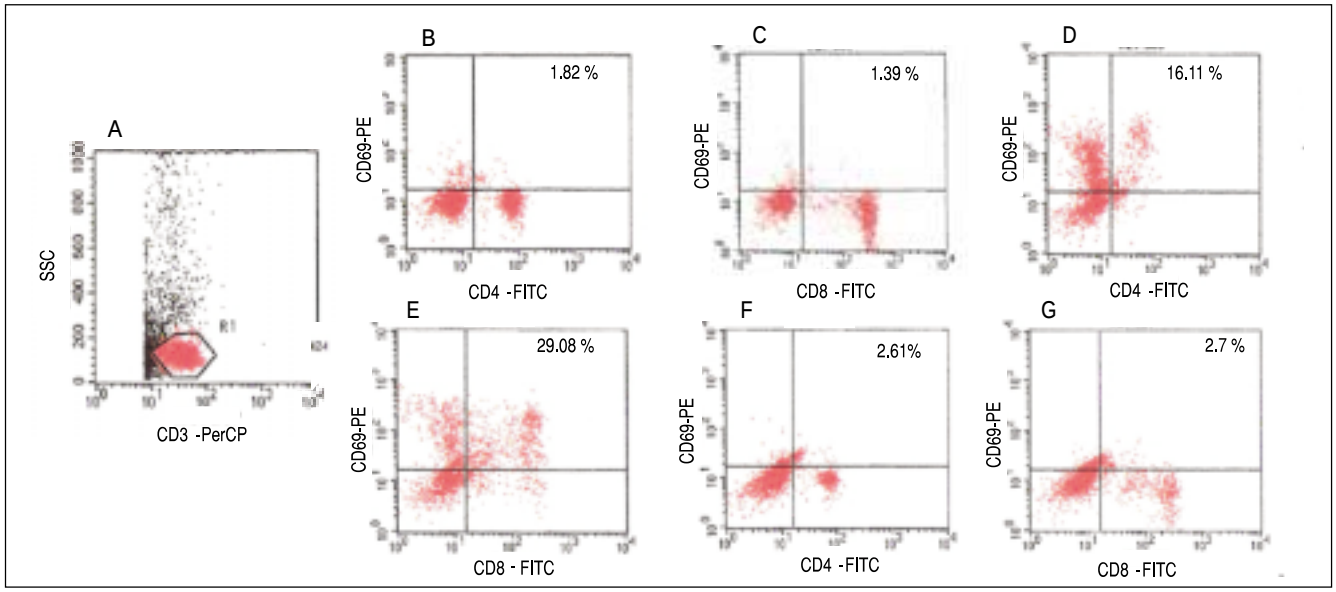

Figure 3. *Representative dot plots showing CD69 expression on CD4 and CD8 T cells from PBMC of AS patients. T cells were stained with anti-CD4 or anti-CD8 FITC and anti-CD69-PE. CD3+ T cells stained with PerCP were selected from the gated area in Panel* ***A****. Cells shown in Panels* ***B*** *and* ***C****: control without Con A or antigen, Panels* ***D*** *and* ***E*** *activated with Con A, and Panels* ***F*** *and* ***G****: activated with the rHSP60Kp. In the right corner of each panel the percentage of activated cells calculated by the software are shown. In panels* ***D****,* ***E****,* ***F*** *and* ***G*** *the percentage values shown are the result of the subtraction of the respective controls (b and c).*

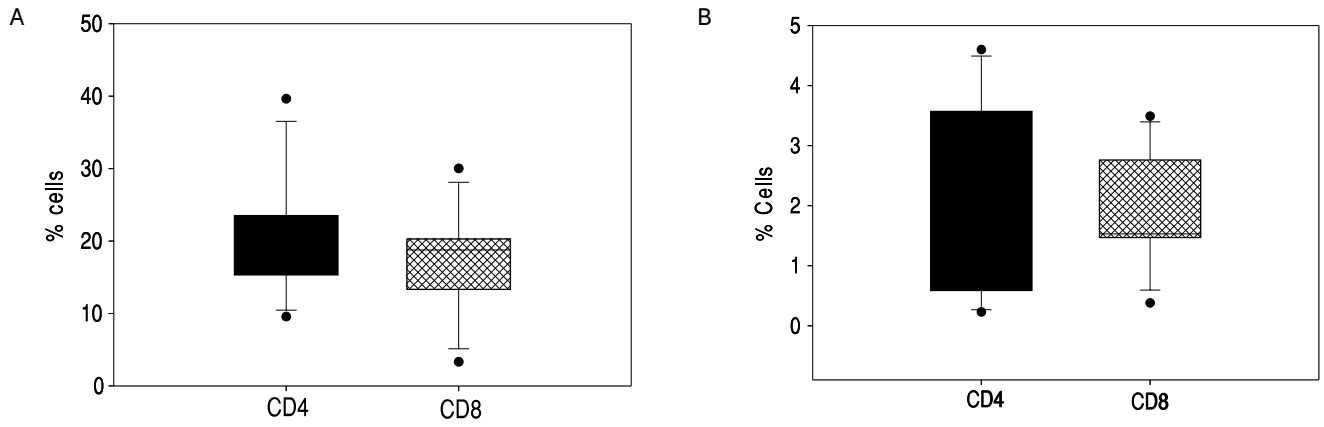

Figure 4. *Percentage of activated CD4 and CD8 T cells, measured by CD69 expression in a three-colour flow cytometer. PBMC from lymphoproliferative responder AS patients were incubated with:* ***A.*** *Con A or* ***B.*** *rHSP60Kp. Median, 25 and 75 percentile are shown. In all cases, values for control without Con A or antigen were subtracted, respectively. No statistical differences were found in CD4 or CD8 T cell activation for Con A or for antigen, as determined by the non-parametric Mann-Whitney test (p = 0.05).*

tant to know the T cell subpopulation involved in these responses to support its association with the AS pathogenesis.

The aim of this work was to determine the processing pathway for the rHSP60Kp used by PBMC from AS patients and then propose the participation of one or both of the T cells subpopulations in the response. First of all, we confirmed our previous results[13] that PBMC from the majority of HLA-B27+ AS patients' lymphoproliferate in the presence of the recombinant protein in contrast to healthy subjects which do not recognize it (Table 1). It would have been important to analyze HLA B27+ healthy subjects as well as HLA-B27-patients but in the duration of the study (almost two years) it was not possible to get them. It is important to point out that the frequency of B27 in the Mexican population is between 3-5% and that in the group of patients at the Hospital General not more than 8% are B27- (personal communication). Throughout the experiments Con A was used as a control of viability and functionality of the cells. As expected, the mitogenic response, as well as to antigen, was very variable as described by others. That is why, as a consensus, positive results are always reported as S.I. ³ 3.0, independently of the absolute values.[13,35-37]

To determine the processing pathways for the rHSP60Kp, we validated our experimental design by using varidase as antigen. PBMC from three intradermally positive healthy subjects were incubated with antigen with or without inhibitors and then fixed to be used as APC for homologous Fresh PBMC. As expected, varidase is processed only by the endocytic pathway.[29,30] As shown in figure 1, lymphoproliferation was positive (S.I. > 3.0) when Fresh PBMC were stimulated by homologous Fixed APC incubated with antigen only, while the response was negative (S.I. < 3.0) when Fresh PBMC were stimulated by homologous Fixed APC incubated with antigen and either chloroquine or BFA. Chloroquine increases the pH within the acidic vacuolar compartments and inhibits the maturation of class II molecules by preventing the hydrolysis of the li chain. BFA inhibits the exocytosis of newly synthesized proteins from the endoplasmic reticulum to the Golgi apparatus; therefore, it inhibits the endocytic and cytosolic pathways.[32] In addition, Fresh PBMC stimulated with homologous Fixed APC incubated with antigen and LLnL did not diminish the lymphoproliferative response (Figure 1). LLnL is an inhibitor of calpain and cathepsins and blocks the proteolytic sites of proteasome, thus decreasing the supply of degraded peptides transported into the endoplasmic reticulum lumen to assembly with nascent MHC class I molecules.[32,38] These results led us to use the same experimental design to analyze the processing pathways for rHSP60Kp in PBMC from AS patients.

Despite the fact that eight out of fourteen AS patients had a positive lymphoproliferative response to the rHSP60Kp, in only five of them the processing pathways used by PBMC to process the rHSP60Kp could be analyzed, because they had a SI above 4.0 when Fixed APC incubated with antigen in the absence of inhibitors were used to stimulate homologous Fresh PBMC. When Fresh PBMC were stimulated by Fixed APC incubated in the presence of antigen and either chloroquine or LLnL, the S.I. were lowered, suggesting that both pathways are

used to process the rHSP60Kp (Figure 2a and 2b). It has been reported that heat shock proteins could be presented by cross-presentation to CD8 T cells[20] and that in healthy subjects, this cross presentation could be involved in tolerance induction of CD8 T cells, but in the case of patients with autoimmune pathology, it could be involved in the induction of the disease.[20] As expected, when Fixed APC were incubated in the presence of antigen and BFA, lymphoproliferation became negative in all patients analyzed (Figure 2c).

One important control in our experimental design was to demonstrate that fixed APC were not toxic for Fresh PBMC. In order to test this, Fresh PBMC were stimulated by Con A in the presence of each one of the Fixed APC obtained by incubation with antigen, with or without inhibitors. Fresh PBMC were able to proliferate in response to Con A in the presence of the different Fixed APC. Therefore, the system was not toxic and negative S.I. could be taken as specific inhibition.

Unfortunately, all healthy subjects analyzed did not show lymphoproliferative response to the rHSP60Kp, however, this fact could support the association of this antigen with the disease.

We found that both CD4 and CD8 T cells are activated by the rHSP60Kp in PBMC from lymphoproliferative responding AS patients, as measured by the expression of CD69[33,34] after exposure to the rHSP60Kp protein (Figure 4). Although the expression of CD69 as an early activation marker does not necessarily correlate with proliferation, and it has been demonstrated that it is less sensitive than thymidine uptake, it has been used by many authors to identify T cell subpopulations involved in mitogen or antigen responses.[37,39] The percentages of CD69 rHSP60Kp positive cells were low but in the range accepted to be considered positive by others.[40] Responses to Con A were very variable, as expected from the results obtained in the proliferation assays. Our results suggest that both T cell subpopulations are involved in the response to rHSP60Kp which is important considering that in AS pathogenesis CD8 T cells could be involved.[2,15,25]

The results obtained by flow cytometry reinforce those obtained in the processing pathways determinations. That is, CD8 T cells recognize antigenic peptides from the rHSP60Kp. The lymphoproliferation observed could be explained by the activation of both CD4 and CD8 T cells. The antibody responses reported before, by our group, could also be explained in terms of the specific activation and cooperation of CD4 cells.[10,11,14]

These results can help us understand the role of the HSP60Kp in AS pathogenesis, because of the association of this protein with the disease and with the response by CD4 and CD8 T cells.

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