J. Physiol. Biochem., **59** (1), 1-10, 2003

"2-6-11" motif in heat shock protein 60 and central nervous system antigens: a preliminary study in multiple sclerosis patients

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(Received on May 20, 2002)

E. RUIZ-VÁZQUEZ and P. DE CASTRO. *"2-6-11" motif in heat shock protein 60 and central nervous system antigens: a preliminary study in multiple sclerosis patiens.* J. Physiol. Biochem., **59** (1), 1-10, 2003.

Multiple sclerosis (MS) is a chronic inflammatory demyelinating disease of the central nervous system (CNS) whith unknown etiology and pathogenesis. A local autoimmune process involving activation of autoreactive T cells against CNS protein components is likely crucial in the development of MS lesions. Myelin-reactive T cells are believed to be primed in the periphery during infections by antigens of bacterial or viral origin via molecular mimicry, a postulated mechanism that might account for the trigger of an autoimmune response on the basis of sequence homology between foreign and self determinants. Immune responses to heat shock proteins (hsp) have been implicated in the initiation or progression of a number of autoimmune diseases. Hsp may function as immunodominant targets during the immune response evoked by pathogens, and theoretically a cross-reactive response to sequences shared by these immunogens and autoantigens in the CNS may contribute to the pathogenesis of MS. We examined the immune response of peripheral blood mononuclear cells (PBMNc) from MS patients and healthy subjects elicited by peptides derived from hsp60 containing a common structural motif ("2-6-11" motif) already described, which is also present in CNS putative antigens. This structural pattern consists of an apolar residue or Lys at position 2, Pro always at position 6, and Glu, Asp or Lys at residue 11. Results reported here are indicative of maturation of peripheral blood monocytes towards a differentiated $CD14^+CD16^+DR^+$ cell and release of pro-inflammatory cytokines consistent with a Th1-like pattern. These are typical features exhibited by immune cells implicated in autoimmune responses.

Key words: Cytokines, Heat shock protein 60, Molecular mimicry, Monocyte phenotype, "2-6-11" motif, Multiple sclerosis.

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Multiple sclerosis (MS) is a demyelinating disease of the central nervous system (CNS) characterized by a chronic inflammatory reaction dominated by lymphocytes and macrophages, plaque-like demyelination, and astroglial sclerosis. Demyelinated lesions, considered to be the result of an autoimmune process (19), are often symmetrical and can be found throughout the CNS. The course of the disease can be categorized as either relapsing-remitting, chronic, or progressive. Relapses often follow infections, most commonly of the upper respiratory system or gastrointestinal tract.

The animal model of MS, experimental autoimmune/allergic encephalomyelitis (EAE), has demonstrated that $CD4^+$ T cells specific for various myelin components are potentially encephalitogenic (29, 32). $CD4^+$ T helper cells can be broadly categorized into one of several subsets based on the cytokines they produce upon activation. Th1 cells secrete pro-inflammatory cytokines such as IL-2, interferon γ (IFNγ), and tumor necrosis factor (TNF) α and β; Th2 cells secrete cytokines such as IL-4, IL-5, IL-6, IL-10 and IL-13 which aid in antibody class-switching; and Th0 express both patterns (23). EAE is induced by immunization with myelin proteins and exhibits the sequential expression of Th1- and Th2-type cytokines, which correlate with disease and recovery, respectively (14). Extensive data in humans indicate that myelin-reactive cells are part of the normal T cell repertoire both in MS patients and healthy subjects (24). T cells specific for peptides of myelin basic protein (MBP) and proteolipid protein (PLP), the two major components of myelin, occur at a relatively higher precursor frequency in patients with MS and are found to be in a state of *in vivo* activation and clonal expansion as opposed to healthy individuals (31). The minor components of myelin have also been studied regarding their possible role as auto-antigens in MS. Myelin oligodendrocyte glycoprotein (MOG) can induce pathogenic, demyelinating auto-antibodies, and this antibody response is higher in MS patients than in controls or patients affected with other neurological diseases. T cell response directed to MOG is more prevalent in MS than in controls (13). Myelin associated protein (MAG), 2'-3'-cyclic nucleotide 3'-phosphodiesterase (CNPase) and transaldolase exhibited lower level T cell reactivity. Some non-myelin CNS antigens have also been implicated in the pathogenesis of MS, such as the families of stress proteins belonging to the heat shock protein 60 (hsp60) and 70 (hsp70), which are expressed constitutively in normal CNS tissue. Inflammation in the CNS can be associated with altered expression of hsp, that may function as targets in the development of chronic disease. The small hsp αB crystallin detected in white matter from MS lesions is not present in unaffected myelin, and elicits strong proliferative responses of peripheral blood T cells from patients with MS and healthy controls (30). T cell responses to hsp60 and 70 have been found in cerebrospinal fluid (CSF) and peripheral blood (PB) of patients with MS (27), but it is still uncertain whether these responses are specific or secondary events from the inflammatory process.

Hsp60 amino acidic sequence analysis revealed a number of peptides containing the "2-6-11" motif ("2-6-11" peptides), a common structural pattern consisting basically in the presence of certain amino acid (aa) residues at fixed positions with respect to a Pro (position 6), an apolar residue or a Lys at position 2, and a Glu, Asp or Lys at position 11 (16, 17). In pre-

vious works it has been shown that PBMNc from patients with autoimmune conditions such as Graves' disease, Hashimoto's disease and primary biliary cirrhosis exhibited activation features after culture with "2-6-11" peptides derived from putative autoantigens. Further, monocyte subsets from healthy subjects redistributed as freshly isolated circulating monocytes from autoimmune conditions. Immune activation was manifested by expression of activation markers, release of pro-inflammatory cytokines such as IL-1α, IL-1β, TNFα, IL-2, IFNγ, and induction of nitric oxide synthase (10, 15, 16). In MS, T cells contribute to myelin damage via secretion of different cytokines and inflammatory mediators, such as nitric oxide (NO) and other reactive nitrogen species. The work reported here was aimed at studying the immune response elicited by "2-6-11" peptides derived from human hsp60 in lymphomononuclear cells from MS patients and controls, since hsp60 has been suggested to be a cross-reacting agent in autoimmune responses (11), and similar "2-6-11" sequences are also present in putative CNS antigens in the context of MS. The results indicate maturation of peripheral blood monocytes induced by "2-6-11" peptides and release of pro-inflammatory cytokines consistent with a Th1-like cytokine pattern.

Materials and Methods

Subjects.– Peripheral venous blood was obtained from 10 healthy subjects, 5 patients with the relapsing-remitting form of Multiple Sclerosis and 5 patients with the secondary progressive MS. All patients were in remission at the time of blood sampling, and none of the patients or controls were on corticorteroids or immunosuppressive therapy at the time of blood drawing. Informed consent was obtained from all individuals.

Peptides.– Peptides 15 amino acids in length were synthesized by the solid phase method as described (21) with the Fmoc modifications (2). Two peptides from previous works (16) were used as positive controls. Pa1: NVLGAPKKLNESQAV, not present in natural proteins, and Pa2: QVLASPGSCLDEFRV, from α1 collagen. From human hsp60, two peptides, P50: KKQSKPVTTPEEIA, P51: VVTEIPKEEKDPGM containing the "2-6-11" motif were synthesized. As a negative control we used Pc5: MLRLPTVFRQMRPV derived from hsp60 and lacking this motif. In all these peptides an extra valine residue at the C-end was added for convenience of the synthesis.

Cell culture.– Lymphomononuclear cells (PBMNc) were obtained from peripheral blood of healthy donors and MS patients by centrifugation over Ficoll-Hypaque as reported in the literature (3). Cells were incubated at $1x10^6$ per milliliter in RPMI 1640 medium supplemented with 2.5% autologous serum, 2mM L-glutamine, penicillin (100U/ml), and streptomycin (100 μg/ml) in a 5% CO2 humified atmosphere. Isolation of monocytes was performed taking advantage of their adherence to the culture plates. After 4-6h at 37 °C, nonadherent cells were removed with the supernatant and the plates were washed three times with phosphate buffered saline (PBS). Adherent cells $CD14^+$ were carefully detached from the surface of the culture plates by gentle friction and resuspended in a small volume of PBS. Peptides were added to PBMNc and monocyte cultures to give a

ratio of 30 μg/10^6 cells. The incubation time was 24h except for IL-2. In order to avoid the binding of IL-2 to its soluble receptor, which is present in the culture medium, a specific antibody (anti-h-IL2 Ab BT563, Biotest Pharma, Germany) was added at the beginning of the culture (2.5 μg/10^6 cells). This modification increased the incubation time necessary to measure IL-2 release by up to 7 days (10, 16, 22).

Cytokine determination.– Cytokine production was determined by using commercial ELISA kits (Genzyme, Cambridge, MA). For the determination of the different cytokines IL-1β, IL-2, IL-4, TNFα, and IFNγ, PBMNc were placed on 16 mm diameter wells (2x10^6 cell/ml in each well). After the incubation period an aliquot of 100 μl of the supernatant of each well was harvested and determination of cytokines was performed by duplicate. Sensitivity of these assays was 50 pg/ml.

Flow cytometry analysis.– Adherent cells, consisting mainly of monocytes, were suspended in PBS containing 0.1% sodium azide at a density of 10^7 per ml. Aliquots of cell suspensions (100 μl) were incubated with antibodies labeled with fluorescein isothiocyanate (FICT) or phycoerythrin (PE). Monoclonal antibodies used were: anti-CD14 (Leu-M3)-FITC, anti-CD16 (Leu-11a)-PE, anti-HLA-DR-PE and isotype control antibodies labeled with either FITC or PE. Monoclonal antibodies were from Becton Dickinson. Incubations were performed at 4 °C for 30 minutes in the dark. Cells were washed twice with cold PBS 0.1% sodium azide and fixed with 1% paraformaldehyde and stored in the dark at 4 °C until analysis. Fixed cells (5x10^4) were analyzed with the EPICS-Profile II (Coulter Electronics, Hialea, FL). Monocytes were gated according to their light-scattering properties. Isotype antibodies of irrelevant specificity were used as negative control. The expression of HLA-DR irrelevant specificity were used as negative control. The expression of HLA-DR and CD14+ were classified as high or low, according to their fluorescence intensity related to antigen expression.

Statistical analysis.– Data were analyzed by ANOVA and Fisher PlsD tests; a p value less than 0.05 was considered to be significant.

Results

"2-6-11" motif in CNS antigens.– The majority of the proteins reported to be significant in MS etiopathogenesis contained the "2-6-11" structural motif in their sequences (Table I): myelin associated glycoprotein (MAG) amino acids (aa) (187-178, 409-418, 506-497), myelin oligodendrocyte glycoprotein (MOG) aa (96-87), myelin proteolipid protein (PLP) aa (153-144), 2'-3-cyclic nucleotide 3í-phosphodiesterase (CNPase) aa (191-182, 307-316), αB-crystallin aa (159-150), hsp60 aa (68-85, 157-166, 546-555), hsp70 aa (95-86, 112-121, 122-131, 143-152). The more abundant 17.2- and 18.5 kD isoforms of myelin basic protein (MBP) in adult CNS contain this motif at residues (68-59). The exon 2-containing isoforms 20.2- and 21.5 kD expressed during developmental myelin formation and during remyelination (5) maintained the same peptide but at residues (94-85).

Cytokine production.– Cytokines released to the medium were determined after incubating PBMNc in presence of

Table I. *"2-6-11" motif in CNS antigens.*

Antigen	Sequence	Position
Myelin associated glycoprotein	**G**LVA**P**EGLG**E**	187-178
	VEFA**P**VLLL**E**	409-418
	AGGF**P**LELS**K**	506-497
Myelin Basic Protein 17.2 kD/18.5kD	**A**TRA**P**HHSD**K**	68-59
Myelin Basic Protein 20.2kD/21.5kD	**A**TRA**P**HHSD**K**	94-85
Myelin oligodendrocyte glycoprotein	**G**RYE**P**AQDG**D**	96-87
Myelin proteolipid protein	**V**FKD**P**HGLW**K**	153-144
2'-3'-cyclic nucleotide	**G**FYL**P**LFDK**E**	191-182
3'-phosphodiesterase (CNPase)	LQLW**P**SDVD**K**	307-316
αB-crystallin	**I**TRE**P**GSVQ**K**	159-150
hps60	**G**WGS**P**KVTK**D**	68-85
	KQSK**P**VTTP**E**	157-166
	VTE**I**PKEEK**D**	546-555
hsp70	**V**VMF**P**WHKM**D**	95-86
	IKFL**P**FKV**V**E	112-121
	KKTK**P**YIQV**D**	122-131
	VVTV**P**AYFN**D**	143-152

synthetic peptides corresponding to fragments of hsp60 spanning the "2-6-11" motif ("2-6-11" peptides). Pa1 and Pa2 were used as positive controls and Pc5, lacking this motif, as negative control. Figure 1 shows the release of pro-inflammatory cytokines when PBMNc from healthy subjects and MS patients were incubated with peptides. Spontaneous release of cytokines was higher in patients with MS, reflecting perhaps an *in vivo* activated state already reported by others (33). The amount of cytokine released to the incubation medium in presence of "2-6-11" peptides was also higher in MS compared to healthy controls. It has been suggested that in MS, circulating mononuclear cells have an enhanced capacity to recognize antigens. Thus, MS T cells cultured with various autoantigens more often expressed IL-2 receptor and this recognition is sustained by IL-2 production (20). No IL-4 release was detected with any treatment (data not shown).

Phenotypic analysis of monocytes from MS patients and healthy donors.– Two-color immunofluorescence analysis of the monocyte population coexpressing CD14 and HLA-DR or CD14 and CD16 was performed (Table II). As in previous works (10, 15), $CD14^{++}/DR^{high}$ and $CD14^{+}CD16DR^{high}$ monocytes subsets were larger in the autoimmune condition than in healthy subjects. After short term culture (7 days) with "2-6-11" peptides derived from hsp60 and the active peptide Pa1, an increase in the subsets $CD14^{++}CD16^{+}DR^{high}$ was observed, as well as a decrease in the proportion of

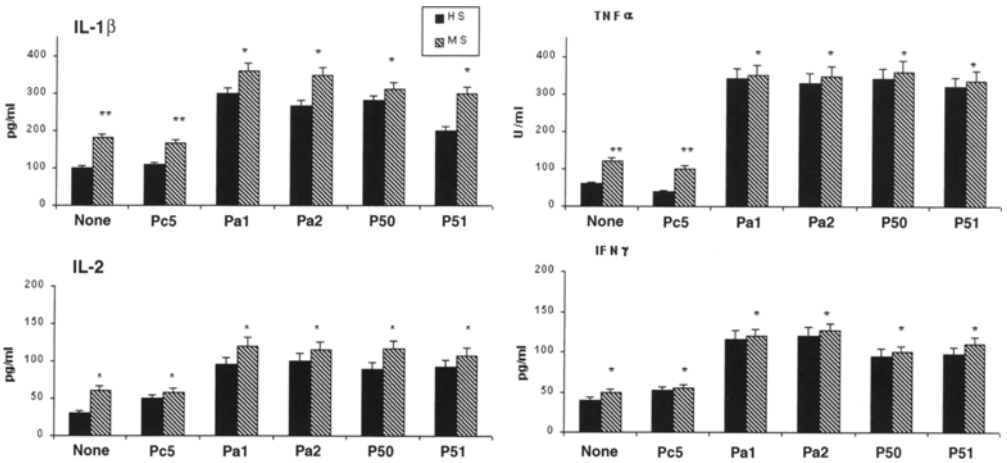

Fig. 1. *Cytokines released by PBMNc obtained from healthy subjects (HS) and multiple sclerosis (MS) patients treated with active peptides.*
Values represent the mean + SD of duplicates of 10 independent experiments. Spontaneous release of cytokines between cells obtained from healthy subjects and multiple sclerosis patients was statistically significant (** $p < 0.001$). Differences between cells treated with active peptides and negative control peptides were statistically significant (* $p < 0.05$).

Table II. *Phenotypic characterization of monocytes from 10 healthy subjects (HS) and 10 patients with MS treated with active peptides.*
Values represent the mean + SD of independent determinations. RPMI, non treated. * p<0.05 compaired with its corresponding values from healthy subjects.

	HS				MS			
	RPMI	Pa1	P50	P51	RPMI	Pa1	P50	P51
$CD14^{++}DR^{low}$	65 ± 3	38 ± 2	37 ± 7	32 ± 5	30 ± 1*	28 ± 9	27 ± 8	25 ± 6
$CD14^{++}DR^{hig}$	25 ± 5	40 ± 3	18 ± 4	15 ± 3	38 ± 2*	44 ± 6	42 ± 9*	42 ± 4*
$CD14^{++}CD16^{+}DR^{high}$	12 ± 6	15 ± 8	18 ± 1	15 ± 9	36 ± 7*	46 ± 3*	43 ± 2*	43 ± 3*

$CD14^{++}$ monocytes. This was also true for monocytes from healthy subjects. "2-6-11"peptides induced activation of monocytes, triggering their differentiation towards a cell type described as specialized in antigen presentation and migration into tissues (34). It is remarkable that after culture with active peptides subsets of monocytes from controls resemble freshly isolated monocytes from MS patients, as has been reported by others (15).

Discussion

In this study, we have analyzed the *in vitro* immune responsiveness of lymphomononuclear cells from MS patients and healthy subjects to peptides derived from hsp60 sharing a common structural motif ("2-6-11" motif). Hsp60 has been implicated in the pathogenesis of MS, but the exact pathogenic role of hsp-reactive T cells in the development of MS lesion remains controversial. Anti-human hsp60

and anti-mycobacterial hsp65 and hsp70 T cell clones have been isolated from synovial fluid of patients with rheumatoid arthritis (RA) and from CSF of patients with MS but with no evidence of mycobacterial disease (9, 27), suggesting a role of these cells in the inflammation characteristic of an autoimmune process. The T cell population that preferentially recognizes hsp, γδ T cells, have been demonstrated at areas of demyelination, where hsp are upregulated (28). Hsp are among the most phylogenetically conserved proteins, and may act as dominant immunogens in the immune response evoked by pathogens (4). Responses to bacterial hsp may lead to immune responses to host hsp or auto-antigens sharing sequence homology via molecular mimicry (26). Homology may be necessary at only a few of the amino acids comprising a T cell epitope, but the cross-reactive response has to involve self determinants pathogenic for the disease. This mechanism helps to explain the connection between infections in MS patients and disease exacerbations.

Pathogens mimic host epitopes to avoid immune response, since there is little, anergic, or suppressed autoreactive T cell repertoire. Elimination of self-reactive cells by mechanisms of T cell regulation (peripheral tolerance) from the immune repertoire would restrict hsp-response to non-conserved, microbe-specific areas of the molecules. However, the host immune response detects pathogen hsp, and this response includes reactivity to mammalian hsp (self hsp) (1). Pair-wise sequence of human hsp60 (accession number: I53042) and its mycobacterial homologous (accession number: P42384), showed that "2-6-11" peptides are not located in conserved regions of the molecule. Peptide (68-85) (Table I) has a bacterial homologous at residues (41-51). The microbial counterpart to the human (157-166) replaces Pro at position 6 of the motif, which abolishes peptide functionality (17). Peptide (546-555) is entirely absent in microbial hsp since the C-terminal portion is unique. To our knowledge, no "2-6-11" peptide from mycobacterium or human hsp60 has been specifically reported to be implicated in autoimmune responses. However, our results are indicative of immune activation when mononuclear cells are cultured in presence of these peptides. Cells from healthy donors also underwent phenotypic changes towards populations found in peripheral blood of patients with autoimmune diseases (10, 15). These results are encouraging in the search of a specific component within the heterogeneous populations of $CD4^+$ T cells sensitized to myelin-related antigens found in MS CNS. It has been postulated that individuals with weak pathogen-specific T cell repertoire can be susceptible to develop MS after encounters with pathogens, because of their failure to properly fight infections (12). Weak pathogen-specific T cell repertoire may become autoreactive by expanding autoreactive T cells able to recognize cross-reactive, non-immunodominant epitopes of the pathogens. Perhaps a "2-6-11"- directed T cell repertoire would tune in this setting.

In EAE, expansion of autoreactive T cells with bacterial or viral peptides was shown to contribute to worsening, but not to the initiation of the disease. Activation of autoreactive T cells with the autoantigen itself is necessary to trigger disease (5). CORREALE *et al.* (7) studied the variations in the cytokine secretion pattern predominant either during exacerbations or in clinical remissions in MS using a panel of T cell clones specific for PLP epi-

tope (142-153), containing the "2-6-11" motif. That work demonstrated that acute attacks in MS occur with preferential activation of Th1-like T cell clones; on the contrary, remissions are characterized by Th0, Th1 and Th2-like subpopulations, with increase of transforming growth factor (TGF)-β production, a cytokine that acts as an inhibitor of lymphocyte proliferation and also interferes in monocyte and NK cell function. Recognition of PLP (142-153) occurred in the context of multiple HLA-DR alleles and T cell receptor (TCR) V beta usage for this epitope showed a marked diversity in both MS patients and normal subjects (8). This heterogeneity in antigen recognition in MS also may represent the epitope spreading process manifested as disease progression. Epitope spreading is presumably the result of endogenous priming to new self-determinants released during the inflammatory or autoimmune process. Works on intramolecular spreading have been focused on MBP-specific repertoire (25). Intermolecular spreading has been already demonstrated in the detection of a subsequent response to PLP in mice initially sensitized with MBP (18). We have found interesting the fact that most of the putative CNS antigens in the context of MS shared "2-6-11" peptides, may be released in the CNS as a consequence of myelin breakdown during the genesis of the MS lesion. Whether this hypothetical "2-6-11"-rich inflammatory ensemble is actually present in the MS brain and to what aspects contributes to the progression of the disease remains unknown. Further studies aimed at studying "2-6-11" peptides from CNS antigens will enable us to asses their role as targets for the autoimmune response that might be taking place during the course of MS.

Acknowledgements

E.R.V. was the recipient of a fellowship from Fundación Esclerosis Múltiple, Spain.

E. RUIZ-VÁZQUEZ y P. DE CASTRO. *Motivo "2-6-11" en proteína de estrés 60 y antígenos del sistema nervioso central: resultados preliminares en pacientes con esclerosis múltiple.* J. Physiol. Biochem., **59** (1), 1-10, 2003.

La esclerosis múltiple (EM) es una enfermedad desmielinizante crónica e inflamatoria del sistema nervioso central (SNC) de etiología y patogénesis desconocidas. Un proceso inmunológico local que implique la activación de células T autorreactivas hacia proteínas del SNC es probablemente crucial para el desarrollo de la EM. Se cree que las células T que reaccionan ante la mielina son predispuestas en la periferia durante infecciones por antígenos de origen viral o bacteriano mediante mimetismo molecular, lo que podría influir en el desarrollo de una respuesta autoinmune, debido a la homología de secuencia entre determinantes propios y foráneos. Las respuestas inmunológicas a proteínas de estrés (hsp) se han implicado en la iniciación o progresión de varias enfermedades autoinmunes. Las hsp pueden funcionar como antígenos dominantes durante la respuesta inmunológica provocada por patógenos, y –teóricamente– una respuesta cruzada hacia secuencias compartidas por estos inmunógenos y antígenos propios en el SNC podría contribuir a la patogénesis de la EM. En este trabajo se examina la respuesta de células linfomononucleares de sangre periférica de pacientes con esclerosis múltiple y sujetos control, producida por péptidos derivados de hsp60 que contienen un motivo estructural común ya descrito (motivo "2-6-11"), que también está presente en proteínas del SNC. Este modelo estructural consiste en un residuo apolar o Lys en la posición 2, Pro siempre en la posición 6 y el residuo 11 es Glu, Asp o Lys. Los resultados aquí descritos son indicativos de la maduración de monocitos de sangre periférica a un tipo de célula diferenciada $CD14^+CD16^+DR^+$, y de la

liberación de citoquinas pro-inflamatorias que concuerdan con un perfil de activación de células Th-1. Estas son características típicas de las células inmunológicas implicadas en respuestas autoinmunitarias.

Palabras clave: Antígenos del SNC, Citoquinas, Esclerosis múltiple, Mimetismo molecular, Motivo estructural "2-6-11", Proteína de estrés 60.

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