The expression of αB-crystallin in multiple sclerosis brains: where, when, how and why?

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The expression of αB-crystallin in multiple sclerosis brains: where, when, how and why?

De expressie van αB-crystalline in multipele sclerose hersenen: waar, wanneer, hoe en waarom?

PROEFSCHRIFT

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(H. Jekkers)

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Abbreviations

AC	Astrocyte
APC	Antigen-presenting cell
APL	Altered peptide ligand
AS	Ankylosing spondylitis
BBB	Blood Brain Barrier
CD	Cluster of differentiation
cDNA	Copy DNA
cNOS	Constitutive NO-synthase
CNPase	2',3'-Cyclic nucleotide 3'-phosphohydrolase
CNS	Central nervous system
CPE	Cytopathological effect
CSF	Cerebrospinal fluid
DC	Dendritic cell
DNA	Deoxyribonucleic acid
EAE	Experimental autoimmune/allergic encephalomyelitis
EAL	Early active lesion
EBV	Epstein Barr virus
ELISA	Enzyme-linked immunosorbent assay
ERK	Extracellular-responsive kinase
FCS	Fetal calf serum
GFAP	Glial fibriallary acidic protein
GM-CSF	Granulocyte migrating-colony stimulating factor
HHV-6	Humam herpes virus-6
HAM/TSP	HLTV-associated myelopathy/ tropical spastic paraparesis
HLA	Human leukocyte antigen
HNK	Human natural killer cell marker
rp-HPLC	Reversed-phase high-performance liquid chromatography
HSP	Heat shock protein
HTLV	Human T lymphotrophic virus
IDDM	Insulin-dependent diabetes mellitus
IFA	Immunofluorescent assay
IFN	Interferon
I-CAM	Intercellular adhesion molecule
Ig	Immunoglobulin
IL	Inactive lesion
IL	Interleukine
iNOS	Inducible NO-synthase
IP-10	IFN-γ-inducible protein 10
JNK	c-Jun N-terminal kinase
LAL	Late active lesion
LFA	Lymphocyte function-associated antigen
LSM	Lymphocyte separation medium
MΦ	Macrophage
MAG	Myelin-associated glycoprotein
MAPK	Mitogen-activated protein kinase
MAPKAPK-2	MAPK-activated protein kinase 2
MBP	Myelin basic protein
MCP	Macrophage chemoattractant protein
MHC	Major histocompatibility complex
MIP	Macrophage inflammatory protein
MMP	Matrix metalloproteinase
MOG	Myelin oligodendrocyte glycoprotein
mRNA	Messenger RNA
MRI	Magnetic resonance imaging
MS	Multiple Sclerosis
NAWM	Normal appearing white matter
NF-κB	Nuclear factor-κB
NHS	Normal human serum
NO	Nitric oxide
OBP	Oligoclonal banding pattern
ODC	Oligodendrocyte
OND	Other neurological diseases
ORO	Oil red O
PAGE	Polyacrylamide gelelectrophoresis
PBMC	Peripheral blood mononuclear cells
PLP	Proteolipid protein
PM	Post mortem
PML	Progressive leukoencephalopathy
pp-MS	Primary progressive MS
RA	Rheumathoid Arthrititis
RANTES	Regulated on activation, normal T cells expressed and secreted
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rr-MS	Relapsing-remitting MS
RT-PCR	Reverse transcriptase polymerase chain reaction
SAPK	Stress-activated protein kinase
SDS	Sodium dodecyl sulfate
sp-MS	Secondary progressive MS
SSPE	Subacute sclerosing panencephalitis
STAT	Signal transducer and activator of transcription
TCID	Tissue culture infectious dose
TCR	T cell receptor
TGF	Transforming growth factor
TNF	Tumor necrosis factor
UV	Ultraviolet
V-CAM	Vascular cell adhesion molecule
VLA	Very late antigen

1.

General introduction

1. The Theme....

Multiple Sclerosis (MS) is the most common neurological disease of young adults in the Western world, affecting about 1 per 1,000. Hallmark of the disease is the local occurence of inflammatory reactions in the central nervous system (CNS) white matter, leading to focal areas of demyelination (lesions or plaques). The insulating white matter that is wrapped around axons (the myelin sheath) is degraded and axons and oligodendrocytes are often lost. As a result, signal conduction is impaired giving rise to many different neurological symptoms. Since local inflammatory reactions occur repetitively, MS generally is chronic and progressive. The many (Multiple) lesions eventually evolve into dysfunctional scars (Sclerosis) that are visible macroscopically in the CNS of patients upon obduction, hence the disease's name (1, 2).

1.1. Clinical features of multiple sclerosis

The start of MS generally manifests itself in people between ages 20 and 40 years, and twice as many women as compared to men are affected. Because almost any system of the CNS may be damaged, a spectrum of clinical signs and symptoms including impaired vision, extreme fatigue, spasms and paralysis of a variety of muscle systems is the result (3, 4). The clinical course of MS is highly variable. After onset, the disease most frequently follows a relapsing-remitting course (RR-MS) with transient neurological deficits that usually tend to resolve completely at the beginning of the disease. In this phase, symptom-free periods are alternated with exacerbations. Sometimes exacerbations are preceded by special occasions like physical exercise, mental tension or a bacterial or viral infection, but more often they appear without any traceable direct cause. With time RR-MS may change into a more progressive phase; the relapsing-progressive or secondary-progressive (SP) course with incomplete recovery or steady progression of disease. A subset of patients is characterized by a more insidious onset with steady detoriation of neurological function and an overall poorer prognosis. This form of MS, characterized by the absence of the relapsing-remitting period, is called chronic-progressive or primary-progressive (PP).
The diversity of symptoms of MS patients makes it difficult to unambiguously diagnose a patient on basis of symptoms only. A diagnosis of clinically definite MS requires a demyelinating disorder of the CNS, with onset at young age, that affects at least two distinct sites in the CNS with at least two temporally separate exacerbations and without alternative explanation. In order to support the diagnosis electrophysiological tests, analysis of the cerebrospinal fluid (CSF) for the presence of oligoclonal immunoglobulins, and magnetic resonance imaging (MRI) of the brain and spinal cord are being used (5, 6).

1.2. Etiology

Despite intensive scientific and clinical research on MS, the etiology of this disease remains largely unknown. It is, however, generally accepted that both genetic and environmental factors contribute to the immune-mediated pathogenesis of MS.

1.2.1. Genetic factors

There are clear genetic influences on susceptibility to MS. Studies of familial MS reveal that in 26% of monozygotic twin pairs both twins are affected, as compared with 4% of dizygotic twins and 1.9% of non-twin siblings with MS (7). It is believed that the genetic influence on the suspectibility to attract MS is not modulated by a single gene but rather by several genes that contribute jointly. Most of the candidate genes that have been identified over the last decade are immunologically relevant. Certain human leukocyte antigen (HLA)-class I genes are weakly associated with MS (HLA-A3 and HLA-B7), as well as, to a stronger extent, certain HLA-class II genes. In particular, HLA-DR4 (Italian and Arab populations), HLA-DR6 (Mexican and Japanese populations), HLA-DR15 (caucasian populations) and several different HLA-DQ genes have been reported (8). In addition, studies have revealed weak associations of other genes of the HLA gene cluster on chromosome 6 with MS (9). Most notable are the MS-associated polymorphisms in interleukin-1 (IL-1) receptor antagonist genes (10) and transporter genes that are involved in antigen processing (11). Although there are also studies that describe a linkage between genes of the T cell receptor (TCR) complex and MS (12), others using the same experimental strategies have failed to obtain similar results.

1.2.2. The environment

The prevalence rate for MS increases with latitude and is particularly high in northern Europe and north America. Geographic differences in prevalence may be explained by differences in genetic make-up of different populations but also by environmental factors such as nutrition (13), UV-radiation (14) or exposure to infectious agents. Involvement of the latter in the pathogenesis of MS is supported by epidemiological studies, clinical experience and parallels of MS to infectious demyelinating disorders. Epidemiological studies on the prevalence of MS include analyses of "MS-epidemics", for example those on the Faroer islands (15, 16), and indicate an involvement of an unknown pathogen(s). Other studies amongst migrants moving from high-prevalence areas to low-prevalence areas, show that these migrants attain the lower risk if they move before puberty (17, 18). However, the decrease in risk is not conferred with migration after puberty. This indicates that a cofactor mediating the risk to acquire MS at a later age has been contracted already by the age of fifteen and that young children, even in high risk areas, are not affected (yet) by this predetermining cofactor. It also shows that there is a prolonged period between acquisition of this factor and the clinical onset of symptoms suggesting either a infectious agent with a very long latency period

or a multifactorial etiology. It could also be that the absence of a primary infection early in life (and the following delayed primary infection) rather than the presence of it plays a role (17). The data on infection with Epstein-Barr virus (EBV) in this context are particularly intriguing in that a history of infectious mononucleosis (the clinical manifestation of a delayed primary EBV infection) is associated with an increased risk of developing MS later on (19), the latter thus being dependent on the age of primary infection.
Clinically, viral or bacterial infections may trigger exacerbations once MS has manifested itself. Most notable are infections of the upper respiratory tract such as by influenza virus (20, 21). The still poorly understood beneficial effect of interferon-β (IFN-β), an antiviral agent, on the clinical course of MS could also be interpreted as suggestive for viral involvement.
Finally, parallels of MS to infectious demyelinating diseases support the idea that transmissable agents might play a role in MS. Several virus-induced chronic demyelinating CNS diseases have been described (e.g. progressive multifocal leukoencephalopathy (PML) (22), HTLV-I associated myelopathy/tropical spastic paraparesis (HAM/TSP) (23) as well as more acute demyelinating CNS diseases following infection with measles (subacute sclerosing panencephalitis (SSPE)), varicella and vaccinia, illustrative of the potential of viruses to cause demyelinating disorders (24).

1.2.3. Autoimmunity
Normally the immune system does not react against self components. Several mechanisms are used to maintain tolerance to self- or autoantigens. Among others, possible autoreactive T cells are removed by a process called negative selection in the thymus (25). However, this deletion is far from complete, especially for autoantigens that are not expressed in the thymus. Thus, many potentially autoreactive T cells escape the process of negative selection and become part of the peripheral T cell repertoire. Non-reactivity of those autoreactive T cells is in part maintained by ignorance. Proteins that are sequestered in immune privileged sites, such as the testes and the brain, are usually not seen by the immune system (26). Another mechanism by which tolerance is maintained is that, in order to become activated, resting or naive T cells require appropriate costimulatory signals in addition to recognition of the major histocompatibility complex (MHC)-peptide complex. The distribution of costimulatory signals is restricted to professional antigen presenting cells (APC). When naive T cells encounter (self) antigen on non-professional APC in the absence of costimulation this generally leads to the induction of (peripheral) tolerance (Fig. 1.1) (27). Autoreactive T cells may be peripherally deleted, anergized, actively supressed, or deviated towards a non-agressive phenotype (28-30).

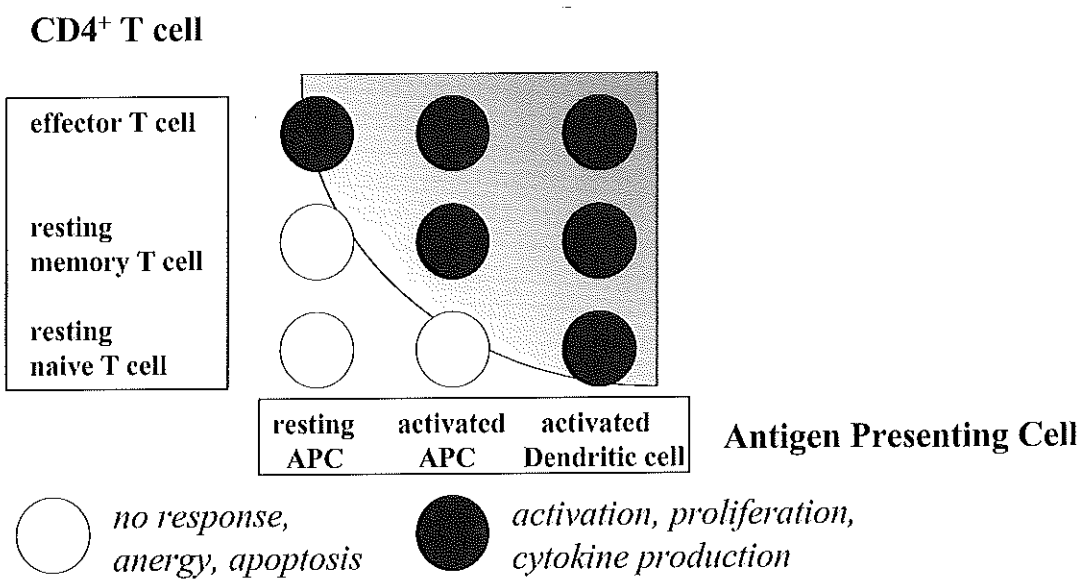

Figure 1.1. The requirements for T cell activation (adapted from (31)).

Despite all these precautions, autoimmunity exists. Both B cell-mediated autoimmune diseases as well as T cell-mediated autoimmune diseases are known. Examples of presumably T cell-mediated autoimmune diseases in humans are rheumatoid arthritis (RA), ankylosing spondylitys (AS), insulin-dependent diabetes mellitus (IDDM) and MS. Currently, the most accepted hypothesis is that MS is due to a $CD4^+$ T cell-mediated autoimmune response directed against myelin antigen(s).
Several findings support the idea that immunological factors are involved in the pathogenesis of MS. The genes that determine disease susceptibility are all encoding proteins that are part of the immune system (see 1.2.1.). Furthermore, the beneficial therapeutic effects of certain immune-suppressive agents like corticosteroids, and the disease-aggrevating effects of immunostimulators like IFN-γ are suggestive of immunological participation in the pathogenesis of MS. Also, it has been known since the 1950's that persistent high levels of immunoglobulins (Igs) are present in the CSF but not in serum of most patients with MS (32). Antibodies in the CSF are secreted by a restricted number of plasma cells leading to the oligoclonal banding pattern (OBP) when CSF is separated by isoelectric focusing. OBP is found in over 90% of MS patients and is used frequently as a laboratory test to support the diagnosis of MS (33, 34). The cause nor target of the persistent intrathecal Ig production is known, although antibodies against measles and rubella virus have been demonstrated repeatedly (35).

Histopathology of MS-affected brain tissue shows perivascular infiltrates consisting of T cells, B cells and macrophages (36). In addition, increased expression of HLA class II on macrophages, microglia and astrocytes and of costimulatory molecules like CD80 and CD40 on macrophages has been demonstrated in immunologically active lesions (Table 1.1). Thus, the key components for the tri-molecular interaction required for T cell (re)activation are present in MS lesions, i.e. TCR, HLA class II and (auto)antigen. Given the observation that activated helper T cells (see 1.3) can readily be detected within active MS lesions but not in the periphery of MS patients it appears reasonable to assume that these T cells have encountered their antigen locally. Since many MS lesions show no traces of a pathogen, the alternative source of antigens that could be responsible for activating these T cells is the target tissue itself and in particular the structural unit formed by oligodendrocytes and myelin. Additional circumstantial evidence for an autoimmunological etiology of MS stems from the analogy of the disease with an experimental animal model for disease, experimental autoimmune encephalomyelitis (EAE). Most frequently the model is induced in rodents, although also non-human primates are used. EAE is an experimentally induced demyelinating disease of the CNS and can be induced either by active immunization or by the passive transfer of encephalitogenic T cells. For active immunization, whole spinal cord preparations, myelin proteins or peptides derived thereof are mixed with (in)complete Freund's adjuvant and injected subcutaneously. Activated T cells isolated from the spleen and lymph nodes of actively immunized animals can be used to transfer the disease to naive recipients (passive transfer). Many different EAE models have been developed with clinical features that may be acute monophasic, chronic progressive, or relapsing remitting (37, 38). These features are dependent on the animal strain, on the induction protocol and on the antigen that is used. Although EAE is not a spontaneous disease in laboratory animals and does not resemble MS completely, it has provided considerable insight in the immunopathology of demyelinating autoimmune diseases. It must be kept in mind, however, that the immunization with CNS-antigens that results in EAE may not be relevant to pathological events in MS or that other, non-myelin antigens are (also) involved.
Where the involvement of the immune system in the pathogenesis of MS is undisputed, the autoimmune nature of the response is still poorly documented. In humans, T cells specific for several myelin (self) antigens can be cultured from peripheral blood of healthy controls as easily as from peripheral blood of MS patients (38-41). Accumulated data gradually begin to raise the question whether or not the peripheral T cell response to myelin antigens is any different between MS patients and healthy controls. To date, there is no definitive answer to the question how myelin-specific lymphocytes may initiate MS in humans.

Table 1.1. Markers of immune activation as observed in MS lesions.

Activation markers	Molecules	Cellular source	Ref.
Cytokines	IL-1β	Astrocytes, Mϕ/μglia	(42-44)
	IL-2	T cells	(42, 44, 45)
	IL-4	T cells	(44, 45)
	IL-6	Astrocytes, Mϕ/μglia	(43, 45-47)
	IL-10	Astrocytes, T cells	(44-46)
	IL-12p40	Mϕ/μglia	(48)
	IFN-α	Mϕ/μglia	(49)
	IFN-β	Astrocytes, Mϕ/μglia	(49)
	IFN-γ	Astrocytes, T cells	(44, 45, 49)
	TNF-α	Astrocytes, Mϕ/μglia, T cells	(44, 45, 50, 51)
	TNF-β	Astrocytes, Mϕ/μglia, T cells	(46, 51)
	TGF-β	Astrocytes, Mϕ/μglia	(44, 52, 53)
Chemokines	GM-CSF	Astrocytes	(54)
	IL-8	Astrocytes, Mϕ/μglia	(55, 56)
	IP-10	Astrocytes	(57)
	MCP-1, -2, -3	Astrocytes	(58, 59)
	MIP-1α	Astrocytes, Mϕ/μglia	(60)
	MIP-1β	Mϕ/μglia	(60)
	RANTES	Astrocytes, T cells	(60, 61)
Costimulation and adhesion molecules	CD40	Mϕ/μglia	(62)
	CD40 ligand (gp39)	T cells	(62)
	CD80	Mϕ/μglia	(48)
	CD86	Mϕ/μglia	(48)
	VCAM-1	Endothelium	(44)
	ICAM-1	Endothelium	(44)
	E-Selectin	Endothelium	(63)
	VLA-4	T cells	(44)
	LFA-1	T cells	(44)
Antibodies	α-MOG	B cells	(64, 65)
	α-MBP	B cells	(64-66)
Complement	CD88 (C5aR)	Astrocytes, Mϕ/μglia	(67)

1.3. Pathogenesis

Most of what is known about the pathogenesis of MS has originated from detailed histopathological studies. MS lesions are characterized most importantly by demyelination, axonal damage (68-70) and the occurence of reactive astrogliosis. Immunologically active lesions are characterized by the presence of infiltrates consisting predominantly of lymphocytes and macrophages (36). Detectability of myelin degradation products within these macrophages in combination with the presence of activation markers on these macrophages can be used to further establish lesional age (71-73). Whereas the pathology of the MS lesion leaves little in doubt that immunological mechanisms are involved (Table 1.1), the exact order of events is not yet completely understood. For all that, the pathogenesis of MS can be divided classically in three stages, i.e. activation of (myelin-specific) T cells, entry of these T cells into the CNS followed by antigen recognition and impairment of the blood-brain barrier (BBB), and finally injury of the myelin/oligodendrocyte (ODC) complex (Fig. 1.2).

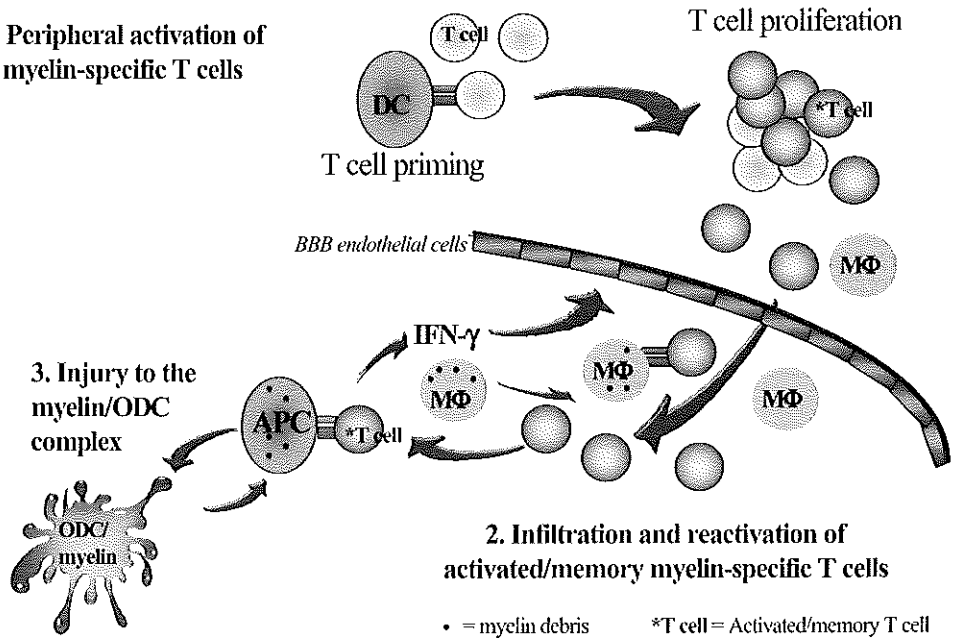

Figure 1.2. Schematic overview of the processes involved in the pathogenesis of MS.

1.3.1. Activation

The CNS has been recognized as an immunologically privileged site. The brain and spinal cord exist behind a functional barrier (the BBB) which prevents the diffusion of immunoglobulins and complement, and inhibits the traffic of cellular elements of the immune system. The endothelium in the brain differs from that in other tissues in having continuous tight junctions and specialized transport molecules. Together with its associated astrocyte foot processes the endothelium forms a barrier that restricts the passage of cells and large molecules into the brain. In addition to this lack of conventional immune surveillance there are low levels of constitutive HLA class I and II expression when compared to other parenchymal organs and there is an absence of dendritic cells or other cells capable of activating resting T cells in the brain (74, 75). This renders it highly unlikely that resting (myelin-specific) T cells can be activated in the brain, although this possibility can not be ruled out formally. One can hypothesize that a local event in the brain (e.g. a viral infection) changes the immunological environment in such a way that the organ itself actively recruits T cells and gets equiped for succesful T cell priming. Alternatively, myelin-specific T cells might be activated in the periphery. Several hypotheses have been put forward that include T cell activation by the release of self antigen from the (infected) brain (76, 77), activation of myelin-specific T cells in an antigen non-specific manner by superantigenic stimulation (78, 79) and activation of myelin-specific T cells by activation against (viral) antigens that crossreact at the peptide level with myelin antigens (viral mimicry (80, 81)) or against antigens that are present in the periphery as well as in the brain (31). These latter need not necessarilly be myelin antigens and could include viral antigens and antigens that are not constitutively present in myelin.

Activation of T cells in the periphery might have occurred years before they eventually encounter their antigen in the CNS. Memory T cells, specific for myelin antigens, might even be activated during early childhood. In order to (re)-activate these memory cells two mechanisms could be envisioned. First, encounter of their antigen in the periphery might lead to re-activation and subsequent entry of these activated T cells into the CNS. Secondly, the CNS itself might actively recruit memory T cells. This might occur when the BBB is impaired or expresses unusual amounts of adhesion molecules. In this way, peripheral activation of T cells need not preceed the demyelinating process directly. The T cell-recruiting capacity of the CNS itself may be sufficient to initiate lesional formation.

1.3.2. CNS entry, antigen recognition and BBB impairment

Once activated, there is evidence that T cells, regardless of their specificity, are able to gain access to the CNS by crossing the BBB (82-85). If these activated T cells are virus-specific $CD8^+$ T cells, they might recognize their putative antigen on all infected glia cells that are able to express HLA class I. However, at this moment, the most accepted

hypothesis is that $CD4^+$ T cells of the T helper 1 type are most important in the induction of the following inflammatory proces. The nature of the cells that can function as (non-professional) APC to these $CD4^+$ T cells in this initial phase of lesional formation is still unclear, but microglia, perivascular macrophages, endothelial cells and even astrocytes have been shown able to express HLA class II molecules. Activated T cells that have crossed the BBB and encountered their antigen locally will start to produce cytokines (mainly IL-2 and IFN-γ), thereby activating resident CNS cells to express elevated levels of HLA molecules as well as influencing the properties of the BBB. Endothelial cells that make up the BBB will start to express elevated levels of adhesion molecules such as E-selectin, vascular cell adhesion molecule-1 (VCAM-1), and intercellular adhesion molecule-1 (ICAM-1) that facilitate the arrest and extravasation of blood-borne T cells and macrophages (86, 87). This process will eventually result in serious disruption of the BBB leading to the facilitated influx of even more lymphocytes.

1.3.3. Myelin destruction

When the inflammatory lesion in the brain is initiated several immunological mechanisms including soluble factors, humoral factors, complement, and infiltrating macrophages contribute to the destruction of myelin. Although oligodendrocytes do not seem to be direct targets for $CD4^+$ T cells, since there is no evidence for HLA class II expression or antigen presenting capacity, they are extremely sensitive for soluble factors such as interferon gamma (IFN-γ) and tumor necrosis factor α (TNF-α), that are produced by infiltrating T cells and macrophages (88-90). Another mechanism that may lead to demyelination is the specific recognition and opsonization of myelin sheaths by antibodies that are present on the extracellular surface of myelin and oligodendrocytes. Up to now, however, there are only few myelin antigens known to be exposed at the extracellular surface. Myelin oligodendrocyte glycoprotein (MOG) appears to be an important target antigen for antibody-mediated demyelination as it had been extensively shown that MOG-specific antibodies possess demyelinating activity in EAE-models (91-93). Furthermore, MOG- and MBP-specific antibodies have recently been shown to be bound to disintegrating myelin around axons in acute MS lesions and in lesions as they occur in marmosets with EAE (64, 65). Destruction of antibody-opsonized myelin might then be accomplished directly by complement or alternatively by receptor-mediated phagocytosis by macrophages (94-96). Electron microscopical examination of early plaques revealed that this process is characterized by the interaction of coated pits and vesicles on macrophages with myelin sheaths, suggesting that macrophages react with opsonized myelin through upregulated Fc- and complement receptors. Uptake of myelin by macrophages could lead to myelin-derived antigen presentation to $CD4^+$ T cells and thereby contribute to the phenomenon of epitope spreading and to aggravation of the disease. In addition to this, macrophages

produce nitric oxide (NO), oxygen radicals and proteolytic enzymes that are probably involved in the direct destruction of myelin (97, 98).

1.3.4. Heterogeneity of MS lesions

Recent evidence suggests that the inflammatory demyelinating lesions as found in the CNS of MS patients might be induced by a variety of different pathological mechanisms (99). Especially with regard to the survival of oligodendocytes during the inflammatory proces, there are considerable differences between different MS cases. Some cases e.g. show relative sparing of oligodendrocytes whereas others show almost complete loss of oligodendrocytes within lesions (100, 101). It is possible to distinguish about five types of lesions, all with different pathologies (102). In some types of lesions there is evidence for the involvement of antibodies in the demyelination proces, whereas in others there seems to be more evidence for a primary oligodendrocyte defect (e.g. viral infection). In addition to different effector mechanisms of demyelination, another source of variability in the pathogenesis of MS lesions resides in the possible target antigen of (auto)immunity. Multiple myelin and non-myelin antigens can be the target for an encephalitogenic T cell reaction possibly generating different types of lesions (103). Within a single MS case, however, the pattern of demyelination appears to be the same regardless of the localization or stage of the lesions.

1.4. Therapies

Most therapies for MS are based on drugs that suppress the immune response in a non-antigen specific manner. Apart from corticosteroids as prednisone and methylprednisolone that exert strong short-term anti-inflammatory and immunosuppressive actions, several immunosuppressants including azathioprine and cyclophosphamid may have some beneficial effects in terms of reducing exacerbation rate and clinical progression. These drugs have, however, serious side-effects when given for a longer period of time. The most promising new drug at this moment is IFN-β that may act through immunomodulatory rather than through immunosuppressive effects. Its action is probably largely due to its antagonistic effect on IFN-γ although it is tempting to speculate on its effects on viral infections (104). Treatment with IFN-β results in a decrease of the exacerbation rate, a significant reduction in disease burden as assessed by magnetic resonance imaging (MRI) and a significant reduction in disease progression(105-106). Similar effects have been documented for treatment with copolymer-1 (107). In addition, a number of treatment attempts that try to block the different pathogenic mechanisms thought to be involved in MS are currently underway or will be started. These include blockade of adhesion and costimulatory molecules with antibodies against VCAM and CD40, administration of immunomodulatory

cytokines as IL-10 and transforming growth factor β (TGF-β) and vaccination with TCR peptides. Finally, several antigen-specific therapies are under investigation, most of them based on MBP, including studies that make use of altered peptide ligand (APL) and oral tolerization strategies (108). Detailed evaluations of these studies have not been published yet.

2. The Players....

In this section I will introduce the topics that need introduction to fully appreciate the research described in this thesis. By definition this focus will lead to an incomprehensive list of topics, since many more factors are involved in the pathogenesis of MS.
First, a brief description of glia cells is provided. Secondly, there is an introduction in the (biochemical) nature of myelin, the target of the autoimmune response. Thirdly, heat shock proteins (hsp) are introduced, and in particular the small hsp αB-crystallin. Finally, possible mediators for the enhanced expression of heat shock proteins in MS lesions are introduced.

2.1. Glia cells

The CNS is one of the most complicated systems present in mammals. It contains two distinct classes of cells: neuronal cells and glia cells, of which glia cells are far better equiped to play an active role in immunological processes. Opportunities for studying the neuroimmunology of human demyelinating diseases have altered over the last decades with the increasing possibilities to culture glia cells *in vitro*.
Glia cells can be classified as microglia or macroglia cells. Microglia are the resident macrophages of the brain and they are thought to play a major role in immunologically mediated events in the CNS. They are able to secrete a variety of cytokines and express HLA class II and costimulatory molecules upon activation (109, 110). In addition, microglia have been shown to possess antigen presenting capacity *in vitro* and to be able to reactivate T cells in such a way that these exert their effector functions (75). Activation of microglia as seen in MS lesions is probably most often caused by interactions between microglia and infiltrating cells (111). Immunohistochemical data, however, suggest the presence of patches in normal appearing white matter (NAWM) that contain HLA class II-expressing, activated microglia in the absence of any sign of infiltration (112, 113). To date it is unclear whether these abnormalities reflect endogenous neurodegenerative processes in MS patients that could contribute to disease or that they only develop following the formation of MS lesions elsewhere in the tissue.
Macroglia cells can be subdivided into astrocytes and oligodendrocytes. Astrocytes are the largest glia cells and can be characterized immunohistochemically by the presence of glial fibrillary acidic protein (GFAP). They support neurons and oligodendrocytes both structurally and functionally, e.g. by secreting growth factors. In MS lesions astrocytes have been shown to secrete a variety of cytokines (most notably IL-6 and TGF-β) that have the potential to modulate immune reactions. There is no consensus yet

as to what extent astrocytes express HLA class II in MS lesions and whether they play a role in antigen presentation (114, 115). Following the initial immune-modulated events, astrogliosis is a well known phenomenon in MS lesions. This proliferation of astrocytes results in the formation of scar-like tissue. Whether this process is beneficial (it might help counteracting the immunological response) or rather aggrevates disease by precluding remyelination remains controversial (116).
Oligodendrocytes synthesize, assemble and maintain myelin by producing large membrane sheaths that are wrapped around axons several times, thus forming an insulating layer, the myelin sheath. Unlike Schwann cells, that myelinate single axons in the peripheral nervous system, oligodendrocytes ensheathe short segments of up to 50 neighboring axons. Immunohistochemically, oligodendrocytes can be characterized by the presence of myelin proteins such as MOG, myelin basic protein (MBP), proteolipid protein (PLP) and 2',3'-cyclic nucleotide 3'-phosphohydrolase (CNPase). Since differentiated oligodendrocytes are not themselves thought to be capable of proliferation or migration, the assumption has to be made that repair is effected by progenitor cells. The inability to proliferate combined with the sensitivity of oligodendrocytes to cytokines as TNF-α (88, 89) makes them vulnerable and, once lost, hard to replace. The fate of oligodendrocytes in MS lesions is diverse; in some lesion types there is a relative sparing of oligodendrocytes whereas in others there is extensive loss (see above). There is a controversy on the manner in which oligodendrocytes are lost in MS lesions (117-120) but most recent literature points to non-programmed cell death rather than apoptosis. From an immunological point of view oligodendrocytes are most likely to play a passive role in the inflammatory proces. Oligodendrocytes express cytokine receptors and FAS by which they are able to react to their environment but they are not able to express HLA class II molecules or to secrete immunomodulatory molecules as cytokines.

2.2. Myelin

CNS myelin consists of a dynamic complexity of potential autoantigens. Myelin sheaths are composed of 70-75% lipids and 25-30% proteins. The two most abundant proteins in myelin are the hydrophobic transmembrane PLP that accounts for about half the total protein mass in myelin and the strongly hydrophilic MBP that represents 10 to 15% of all protein. In addition, myriad other proteins are found, each of which usually do not represent more than 1% of the total protein mass. These minor myelin proteins include myelin-associated glycoprotein (MAG), MOG, CNPase, transaldolase-H and several kinases, acyltransferases and methylases. Many of these proteins can exist as different isoforms, generated by differential splicing of primary transcripts (121). Also, it is quite conceivable that (subtle) differences exist in the composition of MS-affected myelin and that of control cases. Such differences have for example already been documented for

molecular chaperones including αB-crystallin, hsp27 and hsp70 that are present at higher levels in MS-affected myelin (see below) (122, 123). Another factor that contributes to the structural complexity of CNS myelin is posttranslational modification. As a rule, CNS myelin proteins are covalently modified following their biosynthesis (124). Glycosylation, acylation, acetylation, methylation, deamidation, and phosphorylation are among the common modifications that may influence the recognition of peptides derived from these proteins by T cells and may even generate altered self determinants.

2.3. Heat shock proteins

Heat shock proteins (hsp) or stress proteins can be produced by both prokaryotic and eukaryotic cells in response to a wide variety of stressful insults such as heat shock, hypoxia, ischaemia, metabolic disruption and various inflammatory mediators. They play a role in protection against irreversible cell damage and they promote recovery of the cells experiencing stress. Most hsp are also expressed constitutively in unstressed cells at lower levels. Hsp act as molecular chaperones that regulate transport of proteins across membranes and they prevent inappropriate protein-protein interaction and aggregation of damaged or misfolded proteins. They are categorized into several families on the basis of their molecular weight (125,126).
The role of hsp in autoimmune diseases has been studied extensively and there is evidence for a possible involvement in several diseases (127). Hsp60 is suggested to be involved as an autoantigen in atherosclerosis (128), type I diabetes (129), MS (130, 131) and most notably rheumatoid arthritis (RA) (132), and it has been shown to regulate immune responses in animal models for diabetes (133), MS (134, 135) and RA (136). The small stress proteins hsp27 and αB-crystallin are known to be upregulated in glia cells in neurological diseases such as Alexander's disease, Alzheimer's disease, MS and Parkinson (122, 137, 138).
In MS lesions hsp are thought to be recognized by γδ T cells, a distinct subpopulation of T cells mainly concentrated in mucosal membranes. These γδ T cells are thought to be activated against microorganisms that have invaded the host and to crossreact with self hsp. In some active MS lesions γδ T cells were found to be enriched possibly due to clonal activation (131). Furthermore, it has been shown that γδ T cells can lyse oligodendrocytes with a certain degree of specificity and it has been suggested that the antigen recognized by these cells belong to the family of hsp such as hsp65 and hsp70 (139, 140). In this thesis, an alternative mechanism for the involvement of hsp, more specific the small hsp αB-crystallin, in the pathogenesis of MS will be proposed incriminating $CD4^+$ αβ T cells rather than γδ T cells (see below).

2.3.1. Hsp60, hsp27 and αB-crystallin

Of all existing hsp, only the three that have been part of the studies as presented here will be introduced briefly. Hsp60 is synthesized constitutively in most cells and can be induced to high levels following several forms of stress. It is present in all species analyzed so far and exhibits a remarkable sequence homology among pro- and eukaryotes. The ubiquitious occurence and the evolutionary conservation suggest that hsp60 may be playing an essential role in the cell. Hsp60 is localized in the mitochondrial matrix in eukaryotes and is known to interact with multiple proteins during translocation and/or folding (141).

Hsp27 is, like αB-crystallin, a member of the family of small hsp. Apart from considerable sequence similarity they share the ability to form 17S cytoplasmic particles and to relocalize from the cytoplasm into or around the nucleus during and after heat shock. The hsp27 gene is estrogen-responsive and has been found to be expressed in several estrogen-sensitive human tissues and breast tumors (142, 143).

α-Crystallin is one of the major structural proteins in the vertebrate eye lens. It occurs as large aggregates composed of two gene products, αA- and αB-crystallin. In humans, the αB-crystallin gene encodes a 175 residue polypeptide with a molecular mass of about 20 kDa. αB-Crystallin is expressed constitutively in the cardiac and skeletal muscle, lung and brain albeit at much lower levels than in the eye lens where it functions as a molecular chaperone (144, 145). The protein can be post-translationally modified in a variety of ways. It can be glycosylated, deamidated, truncated, the C-terminal lysine can serve as a substrate for transglutaminase and serine phosphorylation can occur at three sites (146). Enhanced expression levels of αB-crystallin have been reported in response to heat shock, arsenite exposure, ceramide, hypertonic shock, dexamethasone and TNF-α (147-149). Phosphorylation of αB-crystallin can be recognized directly by T cell receptors (150). Since phosphorylated residues are not always present in the protein but appear and disappear as the result of physiological stress (in particular oxidative stress), it is conceivable that different forms of αB-crystallin might result in different forms of immune responses (151).

2.4. Inflammatory mediators

The major research questions of this thesis concentrate on the cause(s) and the context of enhanced hsp (in particular that of αB-crystallin) expression in glia cells in MS lesions. Since numerous inflammatory mediators are present in the inflamed brain these are the most obvious candidates to influence the expression of hsp in glia cells. Cytokines, chemokines, matrix metalloproteinases (MMP), nitric oxide and reactive oxygen species are produced by both infiltrating as well as by resident glia cells (see Table 1.1). Again, only these mediators will be introduced that are used in the studies described in this thesis.

2.4.1. Cytokines

The inflammatory reaction in the CNS as it occurs in MS is accompanied by the expression of cytokines, both by infiltrating leukocytes as well as by glia cells. Although $CD4^+$ T-helper 1 (Th1) cells are thought to be the most important T cell type in mediating the immune response in MS lesions, no clear dichotomy is observed locally in the expression of pro- or anti-inflammatory cytokines. T-helper cells can be divided depending on the type of cytokines they produce into Th1 and Th2 subtypes. This division has been studied most extensively in the murine system, in which it was found that the subsets are correlated with different functional abilities. With respect to cytokine production, Th1 cells predominantly produce IL-2 and IFN-γ, whereas Th2 cells are known to stimulate B cell proliferation and differentiation (humoral immunity) by secreting IL-4, IL-5, IL-6 and IL-10. Both types of cytokines seem to operate simultaneously in MS lesions. In addition, IL-1β and TNF-α (probably produced by macrophages/microglia) can be detected in lesions as well as TGF-β, a cytokine thought to down-regulate cell- mediated immune responses. This complex mixture of cytokines is likely to exert its effect on cells of the immune system as well as on resident glia cells that express the right receptors. These glia cells can react by producing cytokines themselves, by altering their immunomodulatory capacities (e.g. by enhancing the expression of HLA class II molecules at their surface), by undergoing programmed cell death and by the enhanced expression of hsp.

2.4.2. ROS and NO

Upon activation blood-borne macrophages and microglia can produce, among other mediators (see above), reactive oxygen species (ROS) and nitric oxide (NO). ROS (e.g. superoxide, hydrogen peroxide and hydroxyl) normally play an important role in the antimicrobial defense of an organism but they have also been shown to play a pathogenic role in several diseases. ROS are highly reactive molecules that can react rapidly with other molecules. When phagocytotic cells are activated by appropriate stimuli, they show a marked increase in oxygen consumption, which is often referred to as the respiratory burst. The activation of the NAPDH oxidase complex, associated with the plasma membrane, results in the formation of several ROS. Especially the CNS is vulnerable to damage mediated by ROS since it is relatively short of antioxidants. In addition, the membrane lipids in the CNS are very rich in polyunsaturated fatty acids, that become easily peroxidated. Oligodendrocytes degenerate upon exposure to ROS and myelin is easily damaged by ROS (152, 153).

NO is a free radical that is produced by neurons, endothelial cells and phagocytes. In macrophages, NO is synthesized from L-arginine in a reaction catalyzed by inducible NO synthase (iNOS). iNOS, in turn, can be activated by several cytokines, including TNF-α and IFN-γ. *In vitro* studies have shown that microglia cells exert their cytotoxicity towards oligodendrocytes via NO (154). However, apart from cytostatic

and cytotoxic effects on several cell types and microbes, NO can also exert immune suppressive effects by interfering with the production of ROS by macrophages and by inhibiting the proliferation of T cells (155). Since cell membranes are permeable to both ROS and NO and thus no specific receptors are needed, they can directly exert an effect on all cell types present in the direct vicinity.
Both ROS and NO are thought to be implicated in the pathogenesis of MS (156-158) and iNOS has been demonstrated in perivascular cells of the macrophage lineage in MS lesions (159, 160).

2.5. Viruses

Many viruses have been proposed as the causative agent in MS including measles, rubella, herpes viruses and retroviruses (161-164). Also many mechanisms have been proposed by which a viral infection could lead to an autoimmune response against myelin as is supposed to play a role in MS. First, host cellular components may be released together with or even incorporated in newly formed virions upon cytolysis of virus-infected glia cells (76). In this way, glia cell components (e.g. myelin) may become available for presentation to the immune system. Secondly, killing of virally-infected (glia) cells as part of a normal immune response and the release of a variety of cytotoxic products may lead to bystander damage to other, non-infected cells. This, in turn, could lead to activation of autoreactive T cells (77).
Thirdly, viruses may influence or directly infect the endothelial cells that make up the BBB, causing BBB impairment. This could result in increased visibility of CNS antigens to the cellular immune system (165, 166). Alternatively, viral infection does not necessarily need to take place in the CNS. Molecular mimicry (structural similarity between pathogen-specific determinants and CNS myelin determinants) could cause pathogen-activated T cells to cross-react against myelin (80, 81). In addition, myelin-reactive T cells could get activated by superantigens produced by bacteria and viruses (78, 79). Superantigens activate T cells on the basis of their TCR Vβ-usage, regardless of their antigen specificity. Finally, viral infection of the CNS by itself could inititate a virus-specific immune response resulting in demyelination without the involvement of an autoimmune response. In spite of all this, there has been no convincing evidence of a single virus causing MS.
In this thesis a working hypothesis is formulated in which Epstein Barr Virus (EBV) plays an important role (see section 3) and experiments are described with human herpes virus-6 (HHV-6); reason to introduce them both.

2.5.1. EBV

EBV, also known as human herpes 4, is a γ-herpes virus and has been associated with MS for a long time. Infection with EBV during infancy is usually asymptomatic. Following infection, EBV persists for life in a latent state, mainly in peripheral B cells. With regards to MS, all MS patients are EBV seropositive, whereas 90-95% of healthy controls are seropositive (167, 168). Furthermore, serum and CSF titers of antibodies directed against EBV are significantly increased in MS patients (169). A possible explanation is that EBV-infection is a necessary co-factor in the development of clinical MS. In addition, delayed primary infection of EBV, characterized clinically as IM, leads to a more than 2-fold higher risk for the development of MS (19).

As far as the mechanisms by which EBV could be involved in MS is concerned, three hypotheses have been put forward (167). Firstly, EBV could directly cause MS by latent persistent infection, although there are no data on EBV infection of the CNS. Secondly, EBV could modulate an autoimmune response against myelin. Viral mimicry (see above) has been suggested since MBP-specific T cell clones, isolated from MS patients, have been shown to cross-react with seven viral peptides, including EBV-derived peptides (80). Thirdly, transactivation of latent (retro)viruses upon co-infection could play a role (170-172). We recently postulated a fourth mechanism by which EBV could be involved in the pathogenesis of MS (see section 3).

2.5.2. HHV-6

Recently, HHV-6 has been added to the list of MS-associated viruses (173, 174). HHV-6 is a β-herpesvirus that was discovered in 1986 as a human B-lymphotropic virus (175) and it is the causal agent of exanthem subitum in children. In later studies it was found that the virus predominantly infects $CD4^+$ T cells and monocytes/macrophages, and that it also has the capability to infect astrocytes, microglia and oligodendrocytes *in vitro* (176, 177). HHV-6 is a common virus and thought to be latently present in approximately 90% of the adult population. After primary infection the virus is able to establish a persistent infection throughout life. It is interesting to note the pleiotrophic nature of HHV-6, which infects both cells of the immune system and cells of the CNS. HHV-6 might activate the immune system against a virus in the periphery and as a result, virus-specific activated T cells might enter the CNS and recognize glia cells that are infected with the same virus.

Several studies add evidence to the possible involvement of HHV-6 in the pathogenesis of MS. Increased IgM serum antibody responses to HHV-6 early antigen were measured in patients with RR-MS as compared to healthy controls. Furthermore, active HHV-6 infection as measured by the presence of HHV-6 DNA in serum could be demonstrated in 30% of MS patients and not in any of the controls (174). In another study (173) an indirect immunofluorescence assay was used to demonstrate that cerebrospinal fluid (CSF) from MS patients had a higher frequency (39.4 %) of, in this

case, IgG antibody to HHV-6 late antigens compared with CSF from other neurological diseases (OND; 7.4%). In addition, 16.7% of these CSF samples contained HHV-6 DNA (none of the controls did) and serum samples showed an increase in both IgM and IgG responses to HHV-6 early proteins in MS patients as compared to OND. These observations would be consistent with the notion that a subpopulation of MS patients have active, ongoing HHV-6 infections. However, it should be noted that not all studies on the possible association between HHV-6 and MS have produced positive data. Secondly, HHV-6 antigens were detected by immunohistochemistry in oligodendrocytes of MS patients only (178). Furthermore, HHV-6 was most frequently present around the lesions suggesting that activation of HHV-6 in oligodendrocytes might precede the inflammatory response leading to lesional development. However, this argument could also be turned around in that HHV-6 (re)-activation in glia cells and maybe even infection of glia cells, would be the result of the inflammatory reaction rather than the cause.
Thirdly, the potential of HHV-6 to play a role in the demyelination proces is suggested by several case-studies. In some acquired immune deficiency syndrom (AIDS) patients there is evidence for active HHV-6 infection in the CNS and HHV-6-infected cells were present only in areas of demyelination (179). In addition, fulminant demyelinating encephalomyelitis associated with HHV-6 infection has been reported in a 21-year old woman with normal immune parameters (180), HHV6-activation in conjunction with JC virus infection is associated with the demyelinative lesions of progressive multifocal leukoencephalopathy (181), and chronic myelopathy and fatal encephalomyelitis-associated HHV-6 infection has been reported repeatedly (182, 183). Although circumstantial, together these reports are indicative of involvement of HHV-6 in the pathogenesis of MS, without claiming a role as the causative agent for MS (184).

3. The Scenario....

Over the last few years a hypothesis has been put forward by our group describing a mechanism by which T cell recognition of the small hsp αB-crystallin could lead to inflammatory reactions in the brain and demyelination as observed in MS (31). The initial observation that αB-crystallin is the immunodominant human T cell antigen in MS-affected myelin and not in myelin from healthy controls (as described in chapter 2) inspired us to study this antigen in greater detail. Despite the fact that this hypothesis was formulated during the research period in which this thesis was prepared I will depict the general mechanism in the introduction already (Fig. 1.3), so that it can be supplemented, adapted (if necessary) and discussed in the general discussion.
MS is depicted as a two stage process in which peripheral activation of T cells to αB-crystallin and enhanced expression of αB-crystallin in the CNS play a central role. Stage 1 comprises the activation of αB-crystallin-specific T cells in the periphery, and could take place years (or even decades) before the development of clinical disease. Recently, we showed that infection of peripheral blood mononuclear cells (PBMC) with EBV leads to HLA-DR-restricted presentation of αB-crystallin to T cells (185). As a result, a pool of αB-crystallin-specific memory T cells will be generated that may be maintained by periodical reactivation of latent (EBV) infection. This pool of memory T cells specific for αB-crystallin plays a central role in the hypothesis, and recently we found direct evidence for the existence of such a memory pool (Plomp, unpublished results).
Stage 2 involves local stress in the CNS, leading to enhanced expression of αB-crystallin in oligodendrocytes and/or astrocytes and maybe even tissue damage. In addition to the enhanced expression of αB-crystallin, this stressor might locally induce microglia to express immunomodulatory molecules such as HLA class II and even costimulatory molecules. Patches of activated microglia in NAWM, suggesting local activation, in MS patients have recently been described (112, 113). Local stress might also result in the enhanced expression of adhesion molecules on endothelial cells of the BBB or maybe even cause direct BBB impairment, both leading to an influx of lymphocytes. These lymphocytes enter "stressed"tissue, where the costimulatory potential of (local) APC has been stimulated (the danger signal (27)). The accumulation of αB-crystallin in astrocytes and the oligodendrocyte/myelin complex, the infiltration of (αB-crystallin-specific memory) T cells, and the enhanced costimulatory potential of the stressed target tissue, may provide the ingredients for the development of a pathogenic local inflammatory response. Alternatively, reactivation of latent viral infections in the periphery might lead to (re)-activation of αB-crystallin-specific T cells, that should be able to cross an intact BBB. Upon encounter of locally presented αB-crystallin (even without co-stimulation) these T cells could then exert their effector functions.

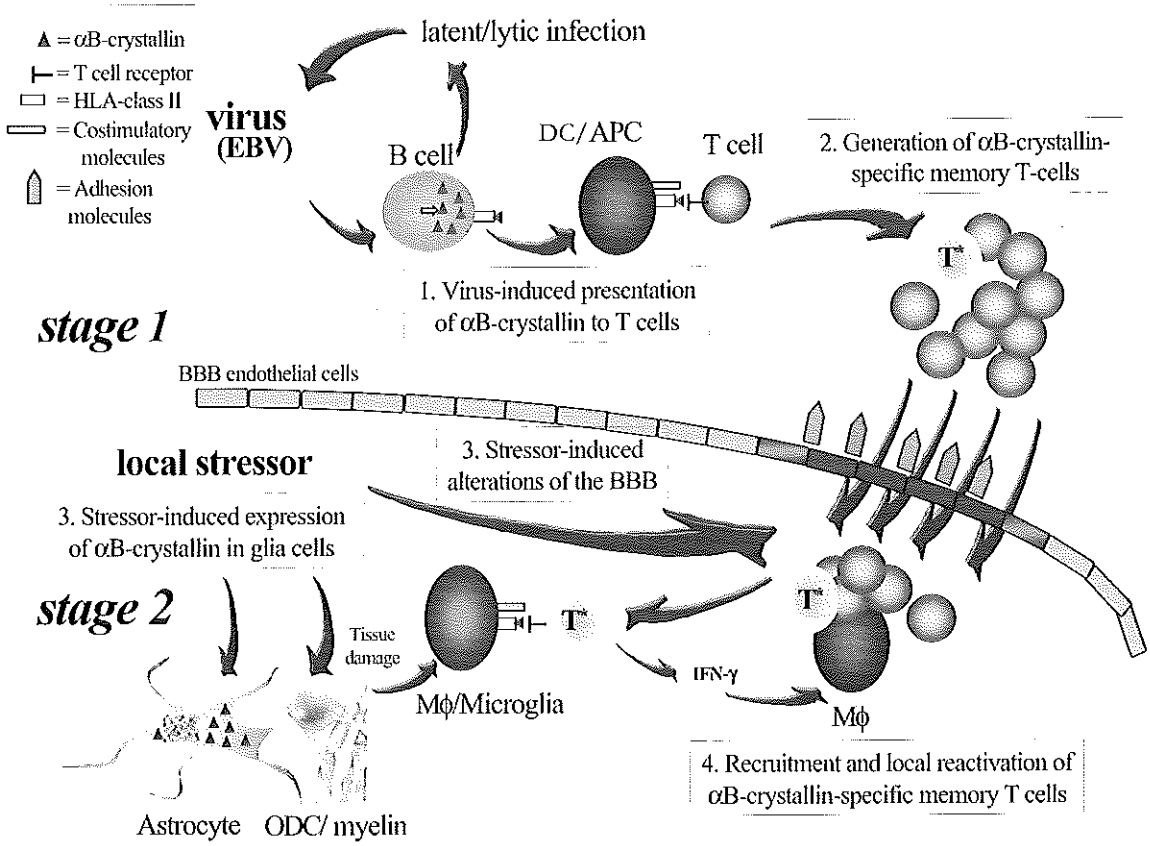

Figure 1.3. Tentative model depicting αB-crystallin as a key autoantigen in the development of inflammatory lesions in MS.

In this thesis the focus will be on stage 2, in which the CNS is involved. Several assumptions had to be made in formulating a hypothesis in which αB-crystallin plays a key role in the initiation of the demyelinating process, and some of them were (in part) clarified by studies presented in this thesis. First, local presentation of αB-crystallin to T cells should take place in the CNS, preferably in the presence of co-stimulation. Second, the cellular source of αB-crystallin that might be presented to T cells in the CNS is not clear. αB-Crystallin might be derived from damaged astrocytes, or alternatively from damaged oligodendrocytes or might even be a part of the myelin complex. If the latter is the case, then it remains unclear whether phagocytosis of myelin membranes would

result in functional presentation to T cells of αB-crystallin, that is present only in minute amounts in myelin. Third, the stressor that is responsible for the accumulation of αB-crystallin in glia cells is unknown. Adding to the complexity is the fact that the nature of this stressor should not necessarily be limited to one specific type of stressor; several different stressors might exert the same result.

4. Outline of this thesis.

Following the general introduction, **chapter 2** describes the initial experiments that led to the extensive research over the past few years on αB-crystallin. HPLC-fractionated CNS myelin proteins of MS patients were used as an antigen source to measure proliferative responses of peripheral blood mononuclear cells (PBMC) towards the individual protein fractions. It was found that fraction 8 contained a highly immunogenic protein, αB-crystallin. T cell responses towards this protein were indistinguishable between MS patients and healthy controls when MS-myelin was used as an antigen source. However, when control myelin was used as an antigen source, no proliferative responses, neither from MS patients nor from control donors, against the fraction corresponding to fraction 8 was observed. This indicated that the cause for MS might not be found in aberrations in the peripheral immune system, but rather in the CNS itself. In the same study we applied immunohistochemistry to demonstrate enhanced expression of αB-crystallin in MS lesions as compared to normal appearing white matter (NAWM) and we identified the cell types in which αB-crystallin was expressed, viz. astrocytes and oligodendrocytes. These observations instigated further research of our group on several aspects of αB-crystallin. One line of research is focussed on the characterization of the human immune response towards αB-crystallin. Another line of research examined the role of αB-crystallin in animal models of demyelinating diseases (mouse and rat EAE-models) and focussed on the immunological implications of phosphorylation of αB-crystallin (van Stipdonk, thesis). The aim of the studies described in this thesis was to gain insight in the regulation of αB-crystallin in astrocytes and oligodendrocytes.
Chapter 3 describes the expression pattern of αB-crystallin in glia cells at different stages of MS lesional development. It was shown that already in the earliest stages of lesional development αB-crystallin was present in both oligodendrocytes and astrocytes. Also, the expression pattern of αB-crystallin in oligodendrocytes was markedly different from that in astrocytes, both with regard to kinetics as with regard to the distribution pattern within the lesion. Finally, comparison of staining patterns for αB-crystallin and hsp60 suggested different regulatory pathways for both hsp in glia cells.
In **chapter 4** it was investigated whether αB-crystallin is presented to T cells in MS lesions, using a combined *in situ-in vitro* approach. The *in situ* data revealed that αB-crystallin could be detected in a subset of myelin-phagocytosing macrophages, in the close vicinity of infiltrating T cells. Comparison of several known markers for myelin-degradation products and *in vitro* data strongly suggested that detection of αB-crystallin within myelin-phagocytosing macrophages is limited to a very short period of time, and could therefore be used as a marker for very recent myelin-phagocytosis. Additional

evidence for functional presentation of myelin-derived αB-crystallin to T cells by macrophages is given by *in vitro* experiments in which αB-crystallin-primed T cells were shown to proliferate in response to macrophages that were fed whole myelin membranes.
The next three chapters are devoted on studies of hsp expression in glia cells at both the mRNA and the protein level, to answer the question why αB-crystallin is present at enhanced levels in MS lesions. Furthermore, it was investigated whether different hsp would respond in the same way to stressful stimuli. **Chapter 5** describes in detail the validation of a competitive rt-PCR assay that was developed in order to be able to quantify mRNA levels of three different hsp, viz. αB-crystallin, hsp27 and hsp60. In **chapter 6** this assay was applied, combined with an immunohistochemical approach, to study the regulation of these hsp in post mortem human astrocytes in response to a series of different cytokines, known to be abundantly present in MS lesions. Markedly different patterns of expression in response to both type 1 as well as type 2 cytokines were found for the three hsp studied. For αB-crystallin it was found that only TNF-α was able to enhance its expression levels. However, the subcellular localization of αB-crystallin (nuclear) was not consistent with the localization as observed in MS lesions (cytosolic).
The subcellular localization of αB-crystallin in post mortem astrocytes was investigated in more detail in **chapter 7**. In this chapter it is demonstrated that oxidative stress results in p38-MAPK-mediated phosphorylation of αB-crystallin at serine residue 59. In addition it is shown that this phosphorylation plays a role in the redistribution of αB-crystallin from the nucleus to the cytosol that occurs following exposure to oxidative stress.
In **chapter 8** the same approach as in chapter 6 was applied on post mortem oligodendrocytes. The idea that oligodendrocytes use other regulatory pathways than astrocytes with regard to αB-crystallin expression, as put forward in chapter 3, could not be confirmed in this study. In reponse to several pro-inflammatory cytokines, most notably TNF-α, oligodendrocytes show enhanced levels of αB-crystallin mRNA. In response to a combination of these pro-inflammatory cytokines however, oligodendrocytes did not enhance levels of αB-crystallin mRNA levels, thereby resembling astrocytes. Attempts to clarify the mechanism(s) responsible for this phenomenon are described also.
Recently, it has been shown that αB-crystallin expression in B cells can be enhanced by EBV infection leading to subsequent presentation of αB-crystallin to T cells. Inspired by this observation we studied the effects of infection of post mortem astrocytes with another herpes virus, HHV-6. These studies are described in **chapter 9**. Interesting to note is that post mortem astrocytes were easily infected by HHV-6 but that the astrocytoma celline U373 was not susceptible for infection. In addition, we described a new method to quantitate the tissue culture infectious dose ($TCID_{50}$) based on

immunofluorescence and demonstrated that the expression of αB-crystallin was not directly affected by HHV-6 infection.
In **chapter 10** results are summarized and discussed. Furthermore the hypothesis as put forward in section 3 is discussed and supplemented with results from this thesis. Finally, suggestions for future research are given.

5. References.

1. Amor, S., D. Baker, L. Layward, K. McCormack, and J.M. van Noort. 1997. Multiple sclerosis: variations on a theme. *Immunol Today 18*:368.

2. Ffrench-Constant, C. 1994. Pathogenesis of multiple sclerosis. *Lancet 343*:271.

3. Weinshenker, B.G. 1998. The natural history of multiple sclerosis: update 1998. *Semin Neurol 18*:301.

4. Waxman, S.G. 1988. Clinical course and electrophysiology of multiple sclerosis. *Adv Neurol 47*:157.

5. Poser, C.M., D.W. Paty, L. Scheinberg, W.I. McDonald, F.A. Davis, G.C. Ebers, K.P. Johnson, W.A. Sibley, D.H. Silberberg, and W.W. Tourtellotte. 1983. New diagnostic criteria for multiple sclerosis: guidelines for research protocols. *Ann Neurol 13*:227.

6. Fazekas, F., F. Barkhof, M. Filippi, R.I. Grossman, D.K. Li, W.I. McDonald, H.F. McFarland, D.W. Paty, J.H. Simon, J.S. Wolinsky, and D.H. Miller. 1999. The contribution of magnetic resonance imaging to the diagnosis of multiple sclerosis. *Neurology 53*:448.

7. Ebers, G.C., K. Kukay, D.E. Bulman, A.D. Sadovnick, G. Rice, C. Anderson, H. Armstrong, K. Cousin, R.B. Bell, W. Hader, D.W. Paty, S. Hashimoto, J. Oger, P. Duquette, S. Warren, T. Gray, P. O'Connor, A. Nath, A. Auty, L. Metz, G. Francis, J.E. Paulseth, T.J. Murray, W. Pryse-Phillips, and N. Risch. 1996. A full genome search in multiple sclerosis. *Nat Genet 13*:472.

8. Ebers, G.C., A.D. Sadovnick, and N.J. Risch. 1995. A genetic basis for familial aggregation in multiple sclerosis. Canadian Collaborative Study Group. *Nature 377*:150.

9. Haines, J.L., M. Ter-Minassian, A. Bazyk, J.F. Gusella, D.J. Kim, H. Terwedow, M.A. Pericak-Vance, J.B. Rimmler, C.S. Haynes, A.D. Roses, A. Lee, B. Shaner, M. Menold, E. Seboun, R.P. Fitoussi, C. Gartioux, C. Reyes, F. Ribierre, G. Gyapay, J. Weissenbach, S.L. Hauser, D.E. Goodkin, R. Lincoln, K. Usuku, and J.R. Oksenberg. 1996. A complete genomic screen for multiple sclerosis underscores a role for the major histocompatability complex. The Multiple Sclerosis Genetics Group. *Nat Genet 13*:469.

10. Crusius, J.B., A.S. Pena, B.W. Van Oosten, G. Bioque, A. Garcia, C.D. Dijkstra, and C.H. Polman. 1995. Interleukin-1 receptor antagonist gene polymorphism and multiple sclerosis [letter]. *Lancet 346*:979.

11. Middleton, D., G. Megaw, C. Cullen, S. Hawkins, C. Darke, and D.A. Savage. 1994. TAP1 and TAP2 polymorphism in multiple sclerosis patients. *Hum Immunol 40*:131.

12. Seboun, E., M.A. Robinson, T.H. Doolittle, T.A. Ciulla, T.J. Kindt, and S.L. Hauser. 1989. A susceptibility locus for multiple sclerosis is linked to the T cell receptor beta chain complex. *Cell 57*:1095.

13. **Esparza, M.L., S. Sasaki, and H. Kesteloot.** 1995. Nutrition, latitude, and multiple sclerosis mortality: an ecologic study. *Am J Epidemiol 142*:733.

14. **Hutter, C.D. and P. Laing.** 1996. Multiple sclerosis: sunlight, diet, immunology and aetiology. *Med Hypotheses 46*:67.

15. **Kurtzke, J.F.** 1993. Epidemiologic evidence for multiple sclerosis as an infection. *Clin Microbiol Rev 6*:382.

16. **Kurtzke, J.F., K. Hyllested, A. Heltberg, and A. Olsen.** 1993. Multiple sclerosis in the Faroe Islands. 5. The occurrence of the fourth epidemic as validation of transmission. *Acta Neurol Scand 88*:161.

17. **Alter, M., Z.X. Zhang, Z. Davanipour, E. Sobel, J. Zibulewski, G. Schwartz, and G. Friday.** 1986. Multiple sclerosis and childhood infections. *Neurology 36*:1386.

18. **Gale, C.R. and C.N. Martyn.** 1995. Migrant studies in multiple sclerosis. *Prog Neurobiol 47*:425.

19. **Haahr, S., N. Koch-Henriksen, A. Moller-Larsen, L.S. Eriksen, and H.M. Andersen.** 1995. Increased risk of multiple sclerosis after late Epstein-Barr virus infection: a historical prospective study. *Mult Scler 1*:73.

20. **Panitch, H.S.** 1994. Influence of infection on exacerbations of multiple sclerosis. *Ann Neurol 36 Suppl*: S25.

21. **Edwards, S., M. Zvartau, H. Clarke, W. Irving, and L.D. Blumhardt.** 1998. Clinical relapses and disease activity on magnetic resonance imaging associated with viral upper respiratory tract infections in multiple sclerosis. *J Neurol Neurosurg Psychiatry 64*:736.

22. **Stoner, G.L.** 1993. Polyomavirus models of brain infection and the pathogenesis of multiple sclerosis. *Brain Pathol 3*:213.

23. **Greenberg, S.J.** 1995. Human retroviruses and demyelinating diseases. *Neurol Clin 13*:75.

24. **Rice, G.P.** 1992. Virus-induced demyelination in man: models for multiple sclerosis. *Curr Opin Neurol Neurosurg 5*:188.

25. **Kappler, J.W., N. Roehm, and P. Marrack.** 1987. T cell tolerance by clonal elimination in the thymus. *Cell 49*:273.

26. **Ohashi, P.S., S. Oehen, K. Buerki, H. Pircher, C.T. Ohashi, B. Odermatt, B. Malissen, R.M. Zinkernagel, and H. Hengartner.** 1991. Ablation of "tolerance" and induction of diabetes by virus infection in viral antigen transgenic mice. *Cell 65*:305.

27. **Matzinger, P.** 1994. Tolerance, danger, and the extended family. *Annu Rev Immunol 12*:991.

28. **Van Parijs, L. and A.K. Abbas.** 1998. Homeostasis and self-tolerance in the immune system: turning lymphocytes off. *Science 280*:243.

29. **Kruisbeek, A.M. and D. Amsen.** 1996. Mechanisms underlying T-cell tolerance. *Curr Opin Immunol 8*:233.

30. **Schwartz, R.H.** 1996. Models of T cell anergy: is there a common molecular mechanism? *J Exp Med 184*:1.

31. **van Noort, J.M., A.C. van Sechel, M.J. van Stipdonk, and J.J. Bajramovic.** 1998. The small heat shock protein alpha B-crystallin as key autoantigen in multiple sclerosis. *Prog Brain Res 117*:435.

32. **Laterre, E.C., A. Callewaert, J.F. Heremans, and Z. Sfaello.** 1970. Electrophoretic morphology of gamma globulins in cerebrospinal fluid of multiple sclerosis and other diseases of the nervous system. *Neurology 20*:982.

33. **Kamp, H.H., P.R. Bar, E.H. van den Doel, and A. Elderson.** 1985. Albumin and immunoglobulin-G in the cerebrospinal fluid and the diagnosis of multiple sclerosis. *Clin Neurol Neurosurg 87*:3.

34. **Bollengier, F., P. Delmotte, and A. Lowenthal.** 1976. Biochemical findings in multiple sclerosis. III. Immunoglobulins of restricted heterogeneity and light chain distribution in cerebrospinal fluid of patients with multiple sclerosis. *J Neurol 212*:151.

35. **Rostrom, B., H. Link, M.A. Laurenzi, S. Kam-Hansen, E. Norrby, and B. Wahren.** 1981. Viral antibody activity of oligoclonal and polyclonal immunoglobulins synthesized within the central nervous system in multiple sclerosis. *Ann Neurol 9*:569.

36. **Powell, H.C. and P.W. Lampert.** 1983. Pathology of multiple sclerosis. *Neurol Clin 1*:631.

37. **Miller, S.D. and E.M. Shevach.** 1998. Immunoregulation of experimental autoimmune encephalomyelitis: editorial overview. *Res Immunol 149*:753.

38. **Martin, R. and H.F. McFarland.** 1995. Immunological aspects of experimental allergic encephalomyelitis and multiple sclerosis. *Crit.Rev Clin Lab Sci 32*:121 .

39. **Burns, J., A. Rosenzweig, B. Zweiman, and R.P. Lisak.** 1983. Isolation of myelin basic protein-reactive T-cell lines from normal human blood. *Cell Immunol 81*:435.

40. **Jingwu, Z., R. Medaer, G.A. Hashim, Y. Chin, E. van den Berg-Loonen, and J.C. Raus.** 1992. Myelin basic protein-specific T lymphocytes in multiple sclerosis and controls: precursor frequency, fine specificity, and cytotoxicity. *Ann Neurol 32*:330.

41. **Pette, M., K. Fujita, B. Kitze, J.N. Whitaker, E. Albert, L. Kappos, and H. Wekerle.** 1990. Myelin basic protein-specific T lymphocyte lines from MS patients and healthy individuals. *Neurology 40*:1770.

42. **Hofman, F.M., R.I. von Hanwehr, C.A. Dinarello, S.B. Mizel, D. Hinton, and J.E. Merrill.** 1986. Immunoregulatory molecules and IL 2 receptors identified in multiple sclerosis brain. *J Immunol 136*:3239.

43. **Merrill, J.E.** 1992. Proinflammatory and antiinflammatory cytokines in multiple sclerosis and central nervous system acquired immunodeficiency syndrome. *J Immunother 12*:167.

44. **Cannella, B. and C.S. Raine.** 1995. The adhesion molecule and cytokine profile of multiple sclerosis lesions]. *Ann Neurol 37*:424.

45. **Woodroofe, M.N. and M.L. Cuzner.** 1993. Cytokine mRNA expression in inflammatory multiple sclerosis lesions: detection by non-radioactive in situ hybridization. *Cytokine. 5*:583.

46. **Link, H.** 1998. The cytokine storm in multiple sclerosis. *Mult Scler 4*:12.

47. **Maimone, D., G.C. Guazzi, and P. Annunziata.** 1997. IL-6 detection in multiple sclerosis brain. *J Neurol Sci 146*:59.

48. **Windhagen, A., J. Newcombe, F. Dangond, C. Strand, M.N. Woodroofe, M.L. Cuzner, and D.A. Hafler.** 1995. Expression of costimulatory molecules B7-1 (CD80), B7-2 (CD86), and interleukin 12 cytokine in multiple sclerosis lesions. *J Exp Med 182*:1985.

49. **Traugott, U. and P. Lebon.** 1988. Multiple sclerosis: involvement of interferons in lesion pathogenesis. *Ann Neurol 24*:243.

50. **Hofman, F.M., D.R. Hinton, K. Johnson, and J.E. Merrill.** 1989. Tumor necrosis factor identified in multiple sclerosis brain. *J Exp Med 170*:607.

51. **Selmaj, K., C.S. Raine, B. Cannella, and C.F. Brosnan.** 1991. Identification of lymphotoxin and tumor necrosis factor in multiple sclerosis lesions. *J Clin Invest 87*:949.

52. **De Groot, C.J., L. Montagne, A.D. Barten, P. Sminia, and D. Van, V.** 1999. Expression of transforming growth factor (TGF)-beta1, -beta2, and - beta3 isoforms and TGF-beta type I and type II receptors in multiple sclerosis lesions and human adult astrocyte cultures. *J Neuropathol Exp Neurol 58*:174.

53. **Peress, N.S., E. Perillo, and R.J. Seidman.** 1996. Glial transforming growth factor (TGF)-beta isotypes in multiple sclerosis: differential glial expression of TGF-beta 1, 2 and 3 isotypes in multiple sclerosis. *J Neuroimmunol 71*:115.

54. **Battistini, L., F.R. Fischer, C.S. Raine, and C.F. Brosnan.** 1996. CD1b is expressed in multiple sclerosis lesions. *J Neuroimmunol 67*:145.

55. **Xia, M.Q. and B.T. Hyman.** 1999. Chemokines/chemokine receptors in the central nervous system and Alzheimer's disease. *J Neurovirol 5*:32.

56. **Hesselgesser, J. and R. Horuk.** 1999. Chemokine and chemokine receptor expression in the central nervous system. *J Neurovirol 5*:13.

57. **Balashov, K.E., J.B. Rottman, H.L. Weiner, and W.W. Hancock.** 1999. CCR5(+) and CXCR3(+) T cells are increased in multiple sclerosis and their ligands MIP-1alpha and IP-10 are expressed in demyelinating brain lesions. *Proc Natl Acad Sci U S A 96*:6873.

58. **McManus, C., J.W. Berman, F.M. Brett, H. Staunton, M. Farrell, and C.F. Brosnan.** 1998. MCP-1, MCP-2 and MCP-3 expression in multiple sclerosis lesions: an immunohistochemical and in situ hybridization study. *J Neuroimmunol 86*:20.

59. **Van, D., V, J. Tekstra, R.H. Beelen, C.P. Tensen, and C.J. De Groot.** 1999. Expression of MCP-1 by reactive astrocytes in demyelinating multiple sclerosis lesions. *Am J Pathol 154* :45.

60. **Simpson, J.E., J. Newcombe, M.L. Cuzner, and M.N. Woodroofe.** 1998. Expression of monocyte chemoattractant protein-1 and other beta- chemokines by resident glia and inflammatory cells in multiple sclerosis lesions. *J Neuroimmunol 84*:238.

61. **Hvas, J., C. McLean, J. Justesen, G. Kannourakis, L. Steinman, J.R. Oksenberg, and C.C. Bernard.** 1997. Perivascular T cells express the pro-inflammatory chemokine RANTES mRNA in multiple sclerosis lesions. *Scand J Immunol 46*:195.

62. **Gerritse, K., J.D. Laman, R.J. Noelle, A. Aruffo, J.A. Ledbetter, W.J. Boersma, and E. Claassen.** 1996. CD40-CD40 ligand interactions in experimental allergic encephalomyelitis and multiple sclerosis. *Proc Natl Acad Sci U S A 93*:2499.

63. **Washington, R., J. Burton, R.F. Todd, W. Newman, L. Dragovic, and P. Dore-Duffy.** 1994. Expression of immunologically relevant endothelial cell activation antigens on isolated central nervous system microvessels from patients with multiple sclerosis. *Ann Neurol 35*:89.

64. **Genain, C.P., B. Cannella, S.L. Hauser, and C.S. Raine.** 1999. Identification of autoantibodies associated with myelin damage in multiple sclerosis. *Nat Med 5*:170.

65. **Raine, C.S., B. Cannella, S.L. Hauser, and C.P. Genain.** 1999. Demyelination in primate autoimmune encephalomyelitis and acute multiple sclerosis lesions: a case for antigen-specific antibody mediation. *Ann Neurol 46*:144.

66. **Gerritse, K., C. Deen, M. Fasbender, R. Ravid, W. Boersma, and E. Claassen.** 1994. The involvement of specific anti myelin basic protein antibody-forming cells in multiple sclerosis immunopathology. *J Neuroimmunol 49*:153.

67. **Gasque, P., S.K. Singhrao, J.W. Neal, O. Gotze, and B.P. Morgan.** 1997. Expression of the receptor for complement C5a (CD88) is up-regulated on reactive astrocytes, microglia, and endothelial cells in the inflamed human central nervous system. *Am J Pathol 150*:31.

68. Narayanan, S., L. Fu, E. Pioro, N. De Stefano, D.L. Collins, G.S. Francis, J.P. Antel, P.M. Matthews, and D.L. Arnold. 1997. Imaging of axonal damage in multiple sclerosis: spatial distribution of magnetic resonance imaging lesions. *Ann Neurol 41*:385.

69. De Stefano, N., P.M. Matthews, L. Fu, S. Narayanan, J. Stanley, G.S. Francis, J.P. Antel, and D.L. Arnold. 1998. Axonal damage correlates with disability in patients with relapsing- remitting multiple sclerosis. Results of a longitudinal magnetic resonance spectroscopy study. *Brain 121*:1469.

70. Ferguson, B., M.K. Matyszak, M.M. Esiri, and V.H. Perry. 1997. Axonal damage in acute multiple sclerosis lesions. *Brain 120*:393.

71. Bruck, W., N. Sommermeier, M. Bergmann, U. Zettl, H.H. Goebel, H.A. Kretzschmar, and H. Lassmann. 1996. Macrophages in multiple sclerosis. *Immunobiology 195*:588.

72. Bruck, W., P. Porada, S. Poser, P. Rieckmann, F. Hanefeld, H.A. Kretzschmar, and H. Lassmann. 1995. Monocyte/macrophage differentiation in early multiple sclerosis lesions. *Ann Neurol 38*:788.

73. Lassmann, H. and H.M. Wisniewski. 1979. Chronic relapsing experimental allergic encephalomyelitis: morphological sequence of myelin degradation. *Brain Res 169*:357.

74. Carson, M.J., C.R. Reilly, J.G. Sutcliffe, and D. Lo. 1998. Mature microglia resemble immature antigen-presenting cells. *Glia 22*:72.

75. Ford, A.L., E. Foulcher, F.A. Lemckert, and J.D. Sedgwick. 1996. Microglia induce CD4 T lymphocyte final effector function and death. *J Exp Med 184*:1737.

76. Dalgleish, A.G., J.K. Fazakerley, and H.E. Webb. 1987. Do human T-lymphotrophic viruses (HTLVs) and other enveloped viruses induce autoimmunity in multiple sclerosis? *Neuropathol Appl Neurobiol 13*:241.

77. Miller, S.D., B.L. McRae, C.L. Vanderlugt, K.M. Nikcevich, J.G. Pope, L. Pope, and W.J. Karpus. 1995. Evolution of the T-cell repertoire during the course of experimental immune-mediated demyelinating diseases. *Immunol Rev 144*:225.

78. Kotb, M. 1995. Infection and autoimmunity: a story of the host, the pathogen, and the copathogen. *Clin Immunol Immunopathol 74*:10.

79. Racke, M.K., L. Quigley, B. Cannella, C.S. Raine, D.E. McFarlin, and D.E. Scott. 1994. Superantigen modulation of experimental allergic encephalomyelitis: activation of anergy determines outcome. *J Immunol 152*:2051.

80. Wucherpfennig, K.W. and J.L. Strominger. 1995. Molecular mimicry in T cell-mediated autoimmunity: viral peptides activate human T cell clones specific for myelin basic protein. *Cell 80*:695.

81. **Fujinami, R.S. and M.B. Oldstone.** 1985. Amino acid homology between the encephalitogenic site of myelin basic protein and virus: mechanism for autoimmunity. *Science 230*:1043.

82. **Lassmann, H.** 1997. Basic mechanisms of brain inflammation. *J Neural Transm Suppl 50*:183.

83. **Wekerle, H.** 1993. T-cell autoimmunity in the central nervous system. *Intervirology 35*:95.

84. **Westland, K.W., J.D. Pollard, S. Sander, J.G. Bonner, C. Linington, and J.G. McLeod.** 1999. Activated non-neural specific T cells open the blood-brain barrier to circulating antibodies. *Brain 122*:1283.

85. **Hickey, W.F.** 1991. Migration of hematogenous cells through the blood-brain barrier and the initiation of CNS inflammation. *Brain Pathol 1*:97.

86. **Rossler, K., C. Neuchrist, K. Kitz, O. Scheiner, D. Kraft, and H. Lassmann.** 1992. Expression of leucocyte adhesion molecules at the human blood-brain barrier (BBB). *J Neurosci Res 31*:365.

87. **Lee, S.J. and E.N. Benveniste.** 1999. Adhesion molecule expression and regulation on cells of the central nervous system. *J Neuroimmunol 98*:77.

88. **Vartanian, T., Y. Li, M. Zhao, and K. Stefansson.** 1995. Interferon-gamma-induced oligodendrocyte cell death: implications for the pathogenesis of multiple sclerosis. *Mol Med 1*:732.

89. **Selmaj, K., C.S. Raine, M. Farooq, W.T. Norton, and C.F. Brosnan.** 1991. Cytokine cytotoxicity against oligodendrocytes. Apoptosis induced by lymphotoxin. *J Immunol 147*:1522.

90. **Andrews, T., P. Zhang, and N.R. Bhat.** 1998. TNFalpha potentiates IFNgamma-induced cell death in oligodendrocyte progenitors. *J Neurosci Res 54*:574.

91. **Bernard, C.C., T.G. Johns, A. Slavin, M. Ichikawa, C. Ewing, J. Liu, and J. Bettadapura.** 1997. Myelin oligodendrocyte glycoprotein: a novel candidate autoantigen in multiple sclerosis. *J Mol Med 75*:77.

92. **Genain, C.P., M.H. Nguyen, N.L. Letvin, R. Pearl, R.L. Davis, M. Adelman, M.B. Lees, C. Linington, and S.L. Hauser.** 1995. Antibody facilitation of multiple sclerosis-like lesions in a nonhuman primate. *J Clin Invest 96*:2966.

93. **Linington, C., M. Bradl, H. Lassmann, C. Brunner, and K. Vass.** 1988. Augmentation of demyelination in rat acute allergic encephalomyelitis by circulating mouse monoclonal antibodies directed against a myelin/oligodendro-cyte glycoprotein. *Am J Pathol 130*:443.

94. **Mosley, K. and M.L. Cuzner.** 1996. Receptor-mediated phagocytosis of myelin by macrophages and microglia: effect of opsonization and receptor blocking agents. *Neurochem Res 21*:481.

95. **DeJong, B.A. and M.E. Smith.** 1997. A role for complement in phagocytosis of myelin. *Neurochem Res 22*:491.

96. **Smith, M.E.** 1999. Phagocytosis of myelin in demyelinative disease: a review. *Neurochem Res* 24:261.

97. **van der Goes, A., J. Brouwer, K. Hoekstra, D. Roos, T.K. van den Berg, and C.D. Dijkstra.** 1998. Reactive oxygen species are required for the phagocytosis of myelin by macrophages. *J Neuroimmunol* 92:67.

98. **Ruuls, S.R., J. Bauer, K. Sontrop, I. Huitinga, H.B. 't, and C.D. Dijkstra.** 1995. Reactive oxygen species are involved in the pathogenesis of experimental allergic encephalomyelitis in Lewis rats. *J Neuroimmunol* 56:207.

99. **Lucchinetti, C.F., W. Brueck, M. Rodriguez, and H. Lassmann.** 1998. Multiple sclerosis: lessons from neuropathology. *Semin Neurol* 18:337.

100. **Ozawa, K., G. Suchanek, H. Breitschopf, W. Bruck, H. Budka, K. Jellinger, and H. Lassmann.** 1994. Patterns of oligodendroglia pathology in multiple sclerosis. *Brain* 117:1311.

101. **Bruck, W., M. Schmied, G. Suchanek, Y. Bruck, H. Breitschopf, S. Poser, S. Piddlesden, and H. Lassmann.** 1994. Oligodendrocytes in the early course of multiple sclerosis. *Ann Neurol* 35:65.

102. **Lassmann, H.** 1998. Neuropathology in multiple sclerosis: new concepts. *Mult Scler* 4:93.

103. **Storch, M. and H. Lassmann.** 1997. Pathology and pathogenesis of demyelinating diseases. *Curr Opin Neurol* 10:186.

104. **Yong, V.W., S. Chabot, O. Stuve, and G. Williams.** 1998. Interferon beta in the treatment of multiple sclerosis: mechanisms of action. *Neurology* 51:682.

105. **Rudick, R.A., D.E. Goodkin, L.D. Jacobs, D.L. Cookfair, R.M. Herndon, J.R. Richert, A.M. Salazar, J.S. Fischer, C.V. Granger, J.H. Simon, J.J. Alam, N.A. Simonian, M.K. Campion, D.M. Bartoszak, D.N. Bourdette, J. Braiman, C.M. Brownscheidle, M.E. Coats, S.L. Cohan, D.S. Dougherty, R.P. Kinkel, M.K. Mass, F.E. Munschauer, R.L. Priore, and R.H. Whitham.** 1997. Impact of interferon beta-1a on neurologic disability in relapsing multiple sclerosis. The Multiple Sclerosis Collaborative Research Group (MSCRG). *Neurology* 49: 358.

106. **Paty, D.W. and D.K. Li.** 1993. Interferon beta-1b is effective in relapsing-remitting multiple sclerosis. II. MRI analysis results of a multicenter, randomized, double-blind, placebo-controlled trial. UBC MS/MRI Study Group and the IFNB Multiple Sclerosis Study Group. *Neurology* 43:662.

107. **Comi, G. and L. Moiola.** 1997. Copolymer-1. *Baillieres Clin Neurol* 6:495.

108. **van Noort, J.M.** 1998. Antigen-specific therapies in multiple sclerosis. *Biotherapy* 10:237.

109. **Gonzalez-Scarano, F. and G. Baltuch.** 1999. Microglia as mediators of inflammatory and degenerative diseases. *Annu Rev Neurosci* 22:219.

110. **Benveniste, E.N.** 1997. Role of macrophages/microglia in multiple sclerosis and experimental allergic encephalomyelitis. *J Mol Med 75*:165.

111. **Sedgwick, J.D., A.L. Ford, E. Foulcher, and R. Airriess.** 1998. Central nervous system microglial cell activation and proliferation follows direct interaction with tissue-infiltrating T cell blasts. *J Immunol 160*:5320.

112. **Li, H., M.L. Cuzner, and J. Newcombe.** 1996. Microglia-derived macrophages in early multiple sclerosis plaques. *Neuropathol Appl Neurobiol 22*:207.

113. **Hayes, G.M., M.N. Woodroofe, and M.L. Cuzner.** 1987. Microglia are the major cell type expressing MHC class II in human white matter. *J Neurol Sci 80*:25.

114. **Aloisi, F., F. Ria, G. Penna, and L. Adorini.** 1998. Microglia are more efficient than astrocytes in antigen processing and in Th1 but not Th2 cell activation. *J Immunol 160*:4671.

115. **Tan, L., K.B. Gordon, J.P. Mueller, L.A. Matis, and S.D. Miller.** 1998. Presentation of proteolipid protein epitopes and B7-1-dependent activation of encephalitogenic T cells by IFN-gamma-activated SJL/J astrocytes. *J Immunol 160*:4271.

116. **Fawcett, J.W. and R.A. Asher.** 1999. The glial scar and central nervous system repair. *Brain Res Bull 49*:377.

117. **Bonetti, B. and C.S. Raine.** 1997. Multiple sclerosis: oligodendrocytes display cell death-related molecules in situ but do not undergo apoptosis. *Ann Neurol 42*:74.

118. **Bonetti, B., C. Stegagno, B. Cannella, N. Rizzuto, G. Moretto, and C.S. Raine.** 1999. Activation of NF-kappaB and c-jun Transcription Factors in Multiple Sclerosis Lesions : Implications for Oligodendrocyte Pathology. *Am J Pathol 155*:1433.

119. **Bauer, J., H. Wekerle, and H. Lassmann.** 1995. Apoptosis in brain-specific autoimmune disease. *Curr Opin Immunol 7*:839.

120. **Dowling, P., W. Husar, J. Menonna, H. Donnenfeld, S. Cook, and M. Sidhu.** 1997. Cell death and birth in multiple sclerosis brain. *J Neurol Sci 149*:1.

121. **Campagnoni, A.T.** 1988. Molecular biology of myelin proteins from the central nervous system. *J Neurochem 51*:1.

122. **Aquino, D.A., E. Capello, J. Weisstein, V. Sanders, C. Lopez, W.W. Tourtellotte, C.F. Brosnan, C.S. Raine, and W.T. Norton.** 1997. Multiple sclerosis: altered expression of 70- and 27-kDa heat shock proteins in lesions and myelin. *J Neuropathol Exp Neurol 56*:664.

123. **van Noort, J.M., A.C. van Sechel, J.J. Bajramovic, M. el Ouagmiri, C.H. Polman, H. Lassmann, and R. Ravid.** 1995. The small heat-shock protein alpha B-crystallin as candidate autoantigen in multiple sclerosis. *Nature 375*:798.

124. **Williams, K.A. and C.M. Deber.** 1993. The structure and function of central nervous system myelin. *Crit Rev Clin Lab Sci 30*:29.

125. **Morimoto, R.I., M.P. Kline, D.N. Bimston, and J.J. Cotto.** 1997. The heat-shock response: regulation and function of heat-shock proteins and molecular chaperones. *Essays Biochem 32*:17.

126. **Hightower, L.E.** 1991. Heat shock, stress proteins, chaperones, and proteotoxicity. *Cell 66*:191.

127. **Kaufmann, S.H.** 1990. Heat shock proteins and the immune response. *Immunol Today 11*:129.

128. **Wick, G., G. Schett, A. Amberger, R. Kleindienst, and Q. Xu.** 1995. Is atherosclerosis an immunologically mediated disease? *Immunol Today 16*:27.

129. **Kiessling, R., A. Gronberg, J. Ivanyi, K. Soderstrom, M. Ferm, S. Kleinau, E. Nilsson, and L. Klareskog.** 1991. Role of hsp60 during autoimmune and bacterial inflammation. *Immunol Rev 121*:91.

130. **Selmaj, K., C.F. Brosnan, and C.S. Raine.** 1992. Expression of heat shock protein-65 by oligodendrocytes in vivo and in vitro: implications for multiple sclerosis. *Neurology 42*:795.

131. **Selmaj, K., C.F. Brosnan, and C.S. Raine.** 1991. Colocalization of lymphocytes bearing gamma delta T-cell receptor and heat shock protein hsp65+ oligodendrocytes in multiple sclerosis. *Proc Natl Acad Sci U.S A 88*:6452.

132. **De Graeff-Meeder, E.R., R. van der Zee, G.T. Rijkers, H.J. Schuurman, W. Kuis, J.W. Bijlsma, B.J. Zegers, and W. van Eden.** 1991. Recognition of human 60 kD heat shock protein by mononuclear cells from patients with juvenile chronic arthritis. *Lancet 337*:1368.

133. **Elias, D., D. Markovits, T. Reshef, R. van der Zee, and I.R. Cohen.** 1990. Induction and therapy of autoimmune diabetes in the non-obese diabetic (NOD/Lt) mouse by a 65-kDa heat shock protein. *Proc Natl Acad Sci U S A 87*:1576.

134. **Gao, Y.L., C.F. Brosnan, and C.S. Raine.** 1995. Experimental autoimmune encephalomyelitis. Qualitative and semiquantitative differences in heat shock protein 60 expression in the central nervous system. *J Immunol 154*:3548.

135. **Birnbaum, G., L. Kotilinek, S.D. Miller, C.S. Raine, Y.L. Gao, P.V. Lehmann, and R.S. Gupta.** 1998. Heat shock proteins and experimental autoimmune encephalomyelitis. II: environmental infection and extra-neuraxial inflammation alter the course of chronic relapsing encephalomyelitis. *J Neuroimmunol 90*:149.

136. **van Eden, W., J.E. Thole, R. van der Zee, A. Noordzij, J.D. van Embden, E.J. Hensen, and I.R. Cohen.** 1988. Cloning of the mycobacterial epitope recognized by T lymphocytes in adjuvant arthritis. *Nature 331*:171.

137. **Bajramovic, J.J., H. Lassmann, and J.M. van Noort.** 1997. Expression of alphaB-crystallin in glia cells during lesional development in multiple sclerosis. *J Neuroimmunol 78*:143.

138. **Iwaki, T., T. Wisniewski, A. Iwaki, E. Corbin, N. Tomokane, J. Tateishi, and J.E. Goldman.** 1992. Accumulation of alpha B-crystallin in central nervous system glia and neurons in pathologic conditions. *Am J Pathol 140*:345.

139. **Freedman, M.S., R. Bitar, and J.P. Antel.** 1997. gamma delta T-cell-human glial cell interactions. II. Relationship between heat shock protein expression and susceptibility to cytolysis. *J Neuroimmunol 74*:143.

140. **Freedman, M.S., S. D'Souza, and J.P. Antel.** 1997. gamma delta T-cell-human glial cell interactions. I. In vitro induction of gammadelta T-cell expansion by human glial cells. *J Neuroimmunol 74*:135.

141. **Bukau, B. and A.L. Horwich.** 1998. The Hsp70 and Hsp60 chaperone machines. *Cell 92*:351.

142. **Welsh, M.J. and M. Gaestel.** 1998. Small heat-shock protein family: function in health and disease. *Ann N Y Acad Sci 851*:28.

143. **Ciocca, D.R., S. Oesterreich, G.C. Chamness, W.L. McGuire, and S.A. Fuqua.** 1993. Biological and clinical implications of heat shock protein 27,000 (Hsp27): a review. *J Natl Cancer Inst. 85*:1558.

144. **Dubin, R.A., E.F. Wawrousek, and J. Piatigorsky.** 1989. Expression of the murine alpha B-crystallin gene is not restricted to the lens. *Mol Cell Biol 9*:1083.

145. **Klemenz, R., E. Frohli, R.H. Steiger, R. Schafer, and A. Aoyama.** 1991. Alpha B-crystallin is a small heat shock protein. *Proc Natl Acad Sci U S A 88*:3652.

146. **Groenen, P.J., K.B. Merck, W.W. de Jong, and H. Bloemendal.** 1994. Structure and modifications of the junior chaperone alpha-crystallin. From lens transparency to molecular pathology. *Eur J Biochem 225*:1.

147. **Head, M.W., E. Corbin, and J.E. Goldman.** 1994. Coordinate and independent regulation of alpha B-crystallin and hsp27 expression in response to physiological stress. *J Cell Physiol 159*:41.

148. **Scheier, B., A. Foletti, G. Stark, A. Aoyama, U. Dobbeling, S. Rusconi, and R. Klemenz.** 1996. Glucocorticoids regulate the expression of the stressprotein alpha B- crystallin. *Mol Cell Endocrinol 123*:187.

149. **Chang, Y., A. Abe, and J.A. Shayman.** 1995. Ceramide formation during heat shock: a potential mediator of alpha B- crystallin transcription. *Proc Natl Acad Sci U S A 92*:12275.

150. **van Stipdonk, M.J., A.A. Willems, S. Amor, C. Persoon-Deen, P.J. Travers, C.J. Boog, and J.M. van Noort.** 1998. T cells discriminate between differentially phosphorylated forms of alphaB-crystallin, a major central nervous system myelin antigen. *Int Immunol 10*:943.

151. **Ito, H., K. Okamoto, H. Nakayama, T. Isobe, and K. Kato.** 1997. Phosphorylation of alphaB-crystallin in response to various types of stress. *J Biol Chem 272*:29934.

152. **Kim, Y.S. and S.U. Kim.** 1991. Oligodendroglial cell death induced by oxygen radicals and its protection by catalase. *J Neurosci Res 29*:100.

153. **Konat, G.W. and R.C. Wiggins.** 1985. Effect of reactive oxygen species on myelin membrane proteins. *J Neurochem 45*:1113.

154. **Merrill, J.E., L.J. Ignarro, M.P. Sherman, J. Melinek, and T.E. Lane.** 1993. Microglial cell cytotoxicity of oligodendrocytes is mediated through nitric oxide. *J Immunol 151*:2132.

155. **MacMicking, J., Q.W. Xie, and C. Nathan.** 1997. Nitric oxide and macrophage function. *Annu Rev Immunol 15*:323.

156. **Smith, K.J., R. Kapoor, and P.A. Felts.** 1999. Demyelination: the role of reactive oxygen and nitrogen species. *Brain Pathol 9*:69.

157. **Parkinson, J.F., B. Mitrovic, and J.E. Merrill.** 1997. The role of nitric oxide in multiple sclerosis. *J Mol Med 75*:174.

158. **Cross, A.H., P.T. Manning, R.M. Keeling, R.E. Schmidt, and T.P. Misko.** 1998. Peroxynitrite formation within the central nervous system in active multiple sclerosis. *J Neuroimmunol 88*:45.

159. **Oleszak, E.L., E. Zaczynska, M. Bhattacharjee, C. Butunoi, A. Legido, and C.D. Katsetos.** 1998. Inducible nitric oxide synthase and nitrotyrosine are found in monocytes/macrophages and/or astrocytes in acute, but not in chronic, multiple sclerosis. *Clin Diagn Lab Immunol 5*:438.

160. **De Groot, C.J., S.R. Ruuls, J.W. Theeuwes, C.D. Dijkstra, and D. Van, V.** 1997. Immunocytochemical characterization of the expression of inducible and constitutive isoforms of nitric oxide synthase in demyelinating multiple sclerosis lesions. *J Neuropathol Exp Neurol 56*:10.

161. **Brahic, M. and J. Jewell.** 1989. Multiple sclerosis as a viral disease. *Res Immunol 140*:176.

162. **Monteyne, P., J.F. Bureau, and M. Brahic.** 1998. Viruses and multiple sclerosis. *Curr Opin Neurol 11*:287.

163. **Rasmussen, H.B., H. Perron, and J. Clausen.** 1993. Do endogenous retroviruses have etiological implications in inflammatory and degenerative nervous system diseases? *Acta Neurol Scand 88*:190.

164. **Dalgleish, A.G.** 1997. Viruses and multiple sclerosis. *Acta Neurol Scand Suppl 169*:8.

165. **Hurwitz, A.A., J.W. Berman, and W.D. Lyman.** 1994. The role of the blood-brain barrier in HIV infection of the central nervous system. *Adv Neuroimmunol 4*:249.

166. **Allen, I. and B. Brankin.** 1993. Pathogenesis of multiple sclerosis--the immune diathesis and the role of viruses. *J Neuropathol Exp Neurol 52*:95.

167. **Munch, M., J. Hvas, T. Christensen, A. Moller-Larsen, and S. Haahr.** 1997. The implications of Epstein-Barr virus in multiple sclerosis--a review. *Acta Neurol Scand Suppl 169*:59.

168. **Munch, M., K. Riisom, T. Christensen, A. Moller-Larsen, and S. Haahr.** 1998. The significance of Epstein-Barr virus seropositivity in multiple sclerosis patients? *Acta Neurol Scand 97*:171.

169. **Sumaya, C.V., L.W. Myers, G.W. Ellison, and Y. Ench.** 1985. Increased prevalence and titer of Epstein-Barr virus antibodies in patients with multiple sclerosis. *Ann Neurol 17*:371.

170. **Diaz, J.J., M.D. Dodon, N. Schaerer-Uthurralt, D. Simonin, K. Kindbeiter, L. Gazzolo, and J.J. Madjar.** 1996. Post-transcriptional transactivation of human retroviral envelope glycoprotein expression by herpes simplex virus Us11 protein. *Nature 379*:273.

171. **Flamand, L., I. Stefanescu, D.V. Ablashi, and J. Menezes.** 1993. Activation of the Epstein-Barr virus replicative cycle by human herpesvirus 6. *J Virol 67*:6768.

172. **Geng, Y.Q., B. Chandran, S.F. Josephs, and C. Wood.** 1992. Identification and characterization of a human herpesvirus 6 gene segment that trans activates the human immunodeficiency virus type 1 promoter. *J Virol 66*:1564.

173. **Ablashi, D.V., W. Lapps, M. Kaplan, J.E. Whitman, J.R. Richert, and G.R. Pearson.** 1998. Human Herpesvirus-6 (HHV-6) infection in multiple sclerosis: a preliminary report. *Mult Scler 4*:490.

174. **Soldan, S.S., R. Berti, N. Salem, P. Secchiero, L. Flamand, P.A. Calabresi, M.B. Brennan, H.W. Maloni, H.F. McFarland, H.C. Lin, M. Patnaik, and S. Jacobson.** 1997. Association of human herpes virus 6 (HHV-6) with multiple sclerosis: increased IgM response to HHV-6 early antigen and detection of serum HHV-6 DNA. *Nat Med 3*:1394.

175. **Salahuddin, S.Z., D.V. Ablashi, P.D. Markham, S.F. Josephs, S. Sturzenegger, M. Kaplan, G. Halligan, P. Biberfeld, F. Wong-Staal, and B. Kramarsky.** 1986. Isolation of a new virus, HBLV, in patients with lymphoproliferative disorders. *Science 234*:596.

176. **He, J., M. McCarthy, Y. Zhou, B. Chandran, and C. Wood.** 1996. Infection of primary human fetal astrocytes by human herpesvirus 6. *J Virol 70*:1296.

177. **Albright, A.V., E. Lavi, J.B. Black, S. Goldberg, M.J. O'Connor, and F. Gonzalez-Scarano.** 1998. The effect of human herpesvirus-6 (HHV-6) on cultured human neural cells: oligodendrocytes and microglia. *J Neurovirol 4*:486.

178. **Challoner, P.B., K.T. Smith, J.D. Parker, D.L. MacLeod, S.N. Coulter, T.M. Rose, E.R. Schultz, J.L. Bennett, R.L. Garber, and M. Chang.** 1995. Plaque-associated expression of human herpesvirus 6 in multiple sclerosis. *Proc Natl Acad Sci U S A 92*:7440.

179. **Knox, K.K. and D.R. Carrigan.** 1995. Active human herpesvirus (HHV-6) infection of the central nervous system in patients with AIDS. *J Acquir Immune Defic Syndr Hum Retrovirol 9*:69.

180. **Novoa, L.J., R.M. Nagra, T. Nakawatase, T. Edwards-Lee, W.W. Tourtellotte, and M.E. Cornford.** 1997. Fulminant demyelinating encephalomyelitis associated with productive HHV-6 infection in an immunocompetent adult. *J Med Virol* 52:301.

181. **Mock, D.J., J.M. Powers, A.D. Goodman, S.R. Blumenthal, N. Ergin, J.V. Baker, D.H. Mattson, J.G. Assouline, E.J. Bergey, B. Chen, L.G. Epstein, and B.M. Blumberg.** 1999. Association of human herpesvirus 6 with the demyelinative lesions of progressive multifocal leukoencephalopathy. *J Neurovirol* 5:363.

182. **Carrigan, D.R., D. Harrington, and K.K. Knox.** 1996. Subacute leukoencephalitis caused by CNS infection with human herpesvirus-6 manifesting as acute multiple sclerosis. *Neurology* 47:145.

183. **Kamei, A., S. Ichinohe, R. Onuma, S. Hiraga, and T. Fujiwara.** 1997. Acute disseminated demyelination due to primary human herpesvirus-6 infection. *Eur J Pediatr 156*:709.

184. **Jacobson, S.** 1998. Association of human herpesvirus-6 and multiple sclerosis: here we go again? *J Neurovirol* 4:471.

185. **van Sechel, A.C., J.J. Bajramovic, M.J. van Stipdonk, C. Persoon-Deen, S.B. Geutskens, and J.M. van Noort.** 1999. EBV-induced expression and HLA-DR-restricted presentation by human B cells of alphaB-crystallin, a candidate autoantigen in multiple sclerosis. *J Immunol 162*:129.

2.

The small heat-shock protein αB-crystallin as candidate autoantigen in multiple sclerosis.

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The identification of key antigens in human autoimmune diseases is a crucial step towards the development of specific intervention. The autoantigen(s) relevant to multiple sclerosis (MS) probably reside in myelin of the central nervous system, the target of the disease[1]. Here we examine proliferative responses of human peripheral blood T cells to the complete collection of myelin proteins fractionated by reversed-phase high-performance liquid chromatography. Myelin isolated from MS-affected brain contained a single protein fraction to which T cells from MS patients and from healthy controls showed dominant responses. This highly immunogenic protein was identified as αB-crystallin, a small heat-shock protein. Immunohistochemical examination of MS lesions revealed the presence of oligodendrocytes and astrocytes with raised αB-crystallin expression, which were not found in unaffected myelin. Our findings indicate that αB-crystallin serves as immunodominant myelin antigen to human T cells when expressed at the elevated levels found in active MS lesions.
To examine responses of human T cells to the complete collection of myelin proteins from the central nervous system (CNS), short-term cultures were raised *in vitro* by priming peripheral blood mononuclear cells (PBMC) from 24 HLA-typed donors with total myelin protein. Proteins used for this purpose were prepared from either healthy control myelin or from myelin purified from MS-affected white matter. Five PBMC donors were clinically defined MS patients and the other nineteen were healthy control subjects. After one cycle of restimulation with total myelin protein, T cells were collected and tested for proliferative responses to each of the forty reversed-phase high-performance liquid chromatography (RP-HPLC) fractions prepared from the same protein preparation as that used for priming. Figure 2.1*a* shows the RP-HPLC profile of fractionated total myelin protein from MS-affected brain, which is virtually identical to that from control material. Data from the proliferation assays were essentially the same in all cases, irrespective of HLA-type or clinical status of the donor. A representative set of data from five donors is shown in Fig. 2.1*b*. The data in Fig. 2.1 illustrate two points. First, bulk T-cell responses after priming with all CNS myelin proteins were always directed primarily at the various minor myelin proteins contained in HPLC fractions 7-20. In contrast, responses to the major components proteolipid protein or myelin basic protein were barely significant. These findings extend our previous observations that the relative immunogenicity of individual CNS myelin proteins to human T cells bears no apparent relationship to their relative abundance in myelin[2]. Second, the data in Fig. 2.1 reveal a distinct difference between responses to proteins derived from either healthy myelin or MS-affected tissue. In the latter case, a consistently predominant response was detected against the contents of RP-HPLC fraction 8. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) showed that this fraction contained only one detectable protein, with an apparent relative molecular mass (M_r) of about 23K (Fig. 2.2). The same protein was detected as the single component in the corresponding fraction derived from the control preparation but in smaller amounts.

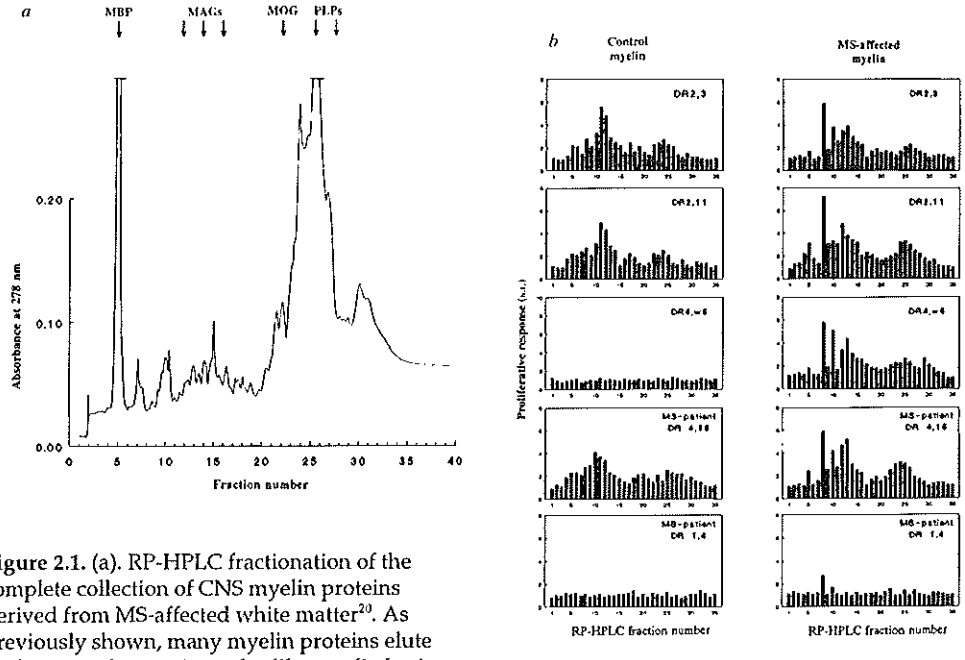

Figure 2.1. (a). RP-HPLC fractionation of the complete collection of CNS myelin proteins derived from MS-affected white matter[20]. As previously shown, many myelin proteins elute as sharp single protein peaks, like myelin basic protein (MBP) (fractions 5/6) and myelin/ oligodendrocyte glycoprotein (MOG; fraction 22). Others, such as proteolipid protein (PLP) or myelin-associated glycoprotein (MAG), exist as differentially modified species with varying hydrophobicities and elute over a series of adjacent fractions. In this way, MAG is recovered in fractions 10-15 and PLP in fractions 22-32.
(b). Proliferative responses of myelin-primed T-cell cultures to HPLC-fractionated CNS myelin proteins from either control myelin (left panels) or MS-affected myelin (right panels). s.i., Stimulation index, defined as (response with antigen)/(response without antigen).

To identify the 23K protein in fraction 8, the protein was purified from MS-affected white matter by RP-HPLC. Direct amino-acid sequencing of the purified preparation was unproductive, indicative of an N-terminal modification. Thus, the 23K protein was trypsinized and a selection of the resulting fragments was purified by RP-HPLC and sequenced. As a result, four internal sequences were identified that were identical to sequences of human αB-crystallin. The amino-acid composition of the purified 23K protein was consistent with the full sequence of human αB-crystallin. In line with this the behaviour of purified human 23K protein and bovine αB-crystallin was identical in RP-HPLC and in SDS-PAGE. Furthermore, polyclonal rabbit antibodies raised against the purified 23K human protein were crossreactive with bovine αB-crystallin upon western blotting (Fig. 2.2).

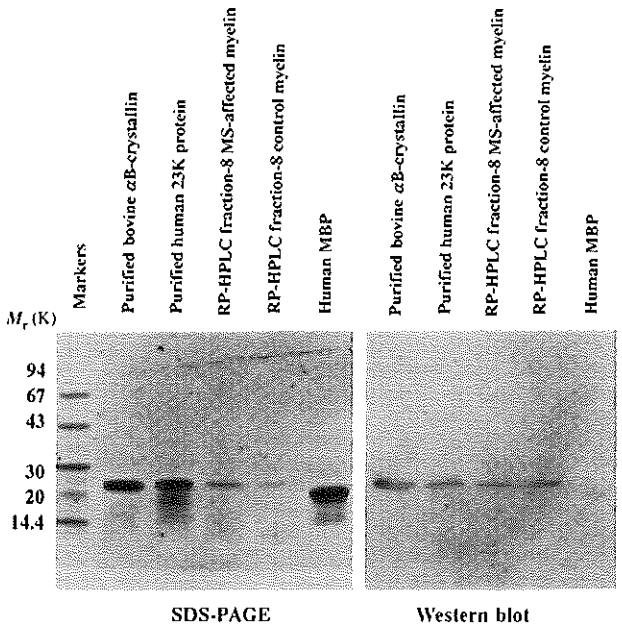

Figure 2.2. SDS-PAGE and western blot analysis of the contents of HPLC fractions 8, compared to bovine αB-crystallin and the 23K protein contained in HPLC fractions 8. Western blotting reveals crossreactivity of a polyclonal rabbit serum raised against purified 23K protein towards bovine αB-crystallin. Sequence analysis of tryptic peptides from the 23K protein yielded the following sequences: LFDQFR (human αB-crystallin residues 23-28), YLR (redidues 48-50), APSWFDTGLSEMR (residues 57-69) and IPADVDP(L) (residues 124-131).

To verify that αB-crystallin is indeed the immunogenic component in HPLC fraction 8, responses of myelin-primed T cells to fraction 8 were compared with those against αB-crystallin purified from bovine eye lens, which is identical to the human homologue at all but four of the 175 amino-acid positions. Figure 2.3 illustrates that responses of myelin- primed T cells to HPLC fraction 8 and purified αB-crystallin were comparable in proliferation as well as in their release of the pro-inflammatory cytokines interferon-γ and interleukin-2. Conversely, bulk T-cell cultures primed with purified bovine αB-crystallin were strongly crossreactive to total myelin protein as well as to the contents of HPLC fraction 8. Earlier reports identifying αB-crystallin as a small heat-shock protein [3,4] prompted us to test whether quantitative differences in the local expression of αB-crystallin could explain our findings (Fig. 2.1*b*). Expression of αB-crystallin was examined by immunohistochemistry of 34 MS plaques corresponding to different developmental stages from 28 MS patients and five control white-matter samples with no neuropathological condition. In all but two inactive plaques, αB-crystallin-expressing glial cells were found grouped within or at the edge of the lesional area. No, or very few, glial cells with αB-crystallin expression could be detected in control white matter or unaffected white matter from MS brains (data not shown). To identify the cellular origin of αB-crystallin in MS lesions, double staining was performed with myelin/oligoden-

drocyte glycoprotein and myelin-associated glycoprotein as a marker for oligodendrocytes, and glial fibrillary acidic protein as a marker for astrocytes (Fig. 2.4) [5,6]. αB-crystallin colocalized with oligodendroglial as well as with astroglial markers in both acute and chronic MS plaques. Intense staining of oligodendrocytes for αB-crystallin was frequent in early lesions whereas astrocytic staining predominated in older lesions. Some staining of myelin itself was also distinguishable, but was close to

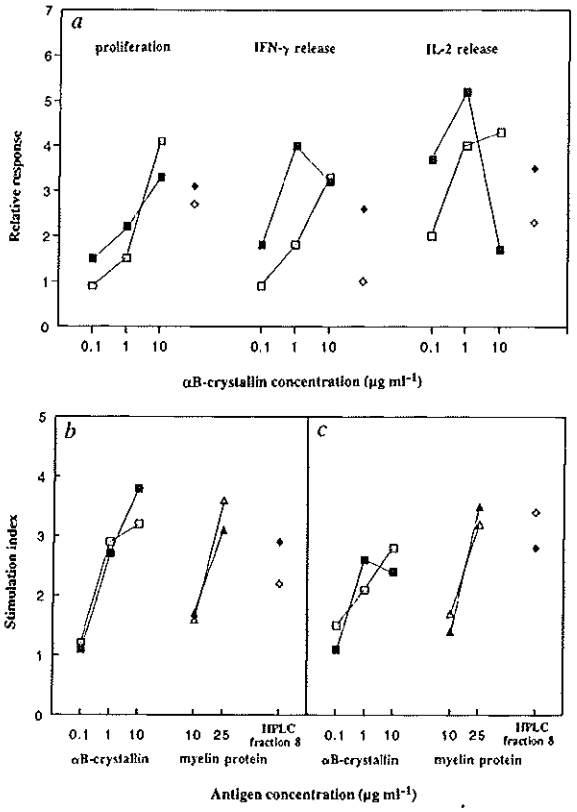

Figure 2.3. Human T cells show full crossreactivity between total myelin protein, the contents of HPLC fraction 8, and αB-crystallin purified from bovine eye lens, identifying αB-crystallin as the immunogenic component in HPLC fraction 8.
(a). T cells primed with total myelin protein respond to purified αB-crystallin equally as well as they do to the contents of HPLC fraction 8. T cells from two donors (open symbols: DR2,4; closed symbols: MS patient DR4,15) were primed against total myelin protein. After 2 weeks, bulk responses against the contents of HPLC fraction 8 derived from MS-affected myelin (diamonds) were compared to those against varying amounts of purified αB-crystallin (squares). Proliferative responses as well as release of the pro-inflammatory cytokines IFN-γ and IL-2 are expressed relative to the levels found in the absence of any antigen.
(b). T cells primed against αB-crystallin crossreact with total myelin protein and the contents of HPLC fraction 8.
(c). T cells primed with the contents of HPLC fraction 8 crossreact with both αB-crystallin and total myelin protein, For b and c, PBMC were used from two other donors (open symbols: MS patients DR2,4; closed symbols: control donor DR 11,14). PBMC were primed with purified αB-crystallin at 1 μg ml^{-1} or with the contents of HPLC fraction 8. After two weeks of culture, T cells were collected and assayed for proliferative responses to the antigens indicated. At the dosage of fraction 8 used in all experiments, the concentration of αB-crystallin as a component of this fraction was estimated by SDS-PAGE and western blotting at ~1 μg ml^{-1}.

background. There was no colocalization of αB-crystallin with markers specific for infiltrated T-cells, B cells or macrophages. As previously reported[7-10], enhanced αB-crystallin expression in some glial cells was also evident in white matter samples from donors affected by other neuropathological diseases such as Alzheimer's, Parkinson's, Huntington's, Pick's and Lewy body diseases (data not shown), but staining of glial cells was generally less intense than in MS lesions and was lost more rapidly at higher serum dilutions. Other heat-shock proteins, including cognates of the Hsp60, Hsp70 and Hsp90 families, have been detected in glial cells in MS lesions[11-13], but the factors inducing these larger heat-shock proteins are probably different from those that trigger expression of the small Hsp αB-crystallin[14, 15]. Also, rather than triggering responses in T cells with γδ T-cell receptors, Hsp60 and Hsp70 may help mount responses by γδ-bearing T cells[11, 13]. Flow cytometry of short-term T-cell cultures raised against αB-crystallin provided no evidence for recruitment of γδ T cells by αB-crystallin *in vitro* (data not shown).
Our key finding is that αB-crystallin acts as immunodominant myelin antigen to human T cells when expressed at the increased levels found within and immediately around active MS lesions in the human brain. Stress-induced expression of αB-crystallin in human disease has so far been found primarily in CNS glial cells and much less - if at all - in other types of cells or tissues[7, 10, 16]. Neurodegenerative diseases, neurotropic infection and oncogene expression are among the factors found so far to be associated with elevated αB-crystallin expression in the CNS. Constitutive expression occurs in several other tissues, including eye lens, cardiac muscle and kidney epithelial cells[7,17,18]. Together, these data indicate that the expression of αB-crystallin is unlikely on its own to trigger demyelinating autoimmunity in MS, but may contribute to the amplification of local inflammatory responses in parallel with disturbance of the blood-brain barrier, cytokine production and expression of major histocompatibility antigens, and of co-stimulatory and adhesion molecules. Our data indicate that peripheral blood T cells from MS patients and healthy controls respond similarly to αB-crystallin, as they do to other self antigens[1,19], which suggests that local antigen-presenting features of the target tissue itself, rather than any aberrance of the peripheral T-cell repertoire, are crucial to the development of autoimmunity in MS. The fact that the immunodominant myelin antigen identified here is expressed at locally regulated levels is in line with this view. MS lesions may develop if pro-inflammatory factors - including the autoantigen itself - accumulate locally beyond a threshold of control as a result of stress-producing events such as local immune responses to viral antigens. We propose that αB-crystallin in this context serves as key myelin antigen in the development of MS.

Methods

rp-HPLC and T cell proliferation assays

CNS myelin was purified by density-gradient centrifugation[21] from human white matter and collected as two distinct pools of samples. One pool contained myelin from eleven clinically definite MS patients; all samples include MS plaques, as confirmed by magnetic resonance imaging and histopathological examination. Control myelin was prepared from white matter from 21 control brains. Total myelin protein was extracted and delipidated as described[20]. Before using the total protein extract as antigen, lyophilized protein was dissolved into 2-chloroethanol/0.1% triofluoroacetic acid and dialysed extensively against water. Protein concentrations were determined by amino-aced analysis and equal amounts of proteins derived from either myelin preparation were used throughout all experiments. Peripheral blood mononuclear cells (PBMC) from each donor were cultured in RPMI1640 (Dutch modification; supplied with 100 U ml^{-1} penicillin, 0.1 mg ml^{-1} strptomycin, 2 mM l-glutamine, 1 mM sodium pyruvate, 10 mM HEPES pH 7.4 and 10% pooled human serum) at 2 x 10^5 cells per 200 µl at 37°C in a humidified stove containing 5% CO_2. Total myelin protein was added as priming antigen at 25 µg ml^{-1}. After 7 days, growing T cells were restimulated with 10^5 irradiated (30Gy) autologous PBMC per 200 µl and fresh antigen (25 µg ml^{-1}). After another 4 days, recombinant human IL-2 was added to a final concentration of 50 U ml^{-1}. At day 14, T cells were collected, pooled and tested for proliferative responses to various antigens by seeding 5 x 10^4 T cells together with 5 x 10^4 irradiated autologous PBMC and antigen in 200 µl of fresh culture medium. After 3 days, [^{3}H]thymidine was added (20KBq per well) and after another 24 h of culture, thymidine incorporation was determined using a ß-plate counter. For use as a test antigen, HPLC-fractionated proteins were lyophilized, redissolved in 2-chloroethanol/01.% trifluoroacetic acid and dialysed against water. Protein concentrations were determined by amino-acid analysis. A fixed proportion of the contents of each fraction was added as antigen to give a final protein concentration of ~50 µg ml^{-1} for the fractions containing the largest amounts of protein (fractions 5/6 and 24-27). Varying the dose of antigen yielded data consistent with the altered dose, indicating that the low responses to MBP or PLP were not due to inappropriate dosage (data not shown). Release of IFN-γ and IL-2 were determined using culture supernatants drawn following a 2-day culture with the antigen indicated. Concentrations of IFN-γ were determined by ELISA (Central Laboratory of the Blood Transfusion Service, Amsterdam) and of IL-2 using the CTLL-L assay. Briefly, 5 x 10^3 CTLL-L cells were exposed to culture supernatant for 24 h before being pulsed with 20 kBq [^{3}H]thymidine.

SDS-PAGE and Western Blotting

Bovine αB-crystallin was purified by RP-HPLC from a commercial preparation of αB-crystallin (Sigma). Standard SDS-PAGE analysis was done using an 8-25% gradient

polyacrylaminde gel (Pharmacia LKB). Equal samples of the contents of HPLC fraction 8 derived from either control myelin or MS-affected myelin were used for comparison. For western blotting a 1:25 dilution of rabbit serum raised against purified 23K protein from MS-affected myelin was used.

Immunohistochemistry
Formalin-fixed paraffin-embedded human white-matter sections (5 μm) were deparaffinized in xylene and hydrated in ethanol. Following blocking of endogenous peroxidase activity and a 10-min incubation 10% FCS, sections were stained for the different markers. Staining for αB-crystallin was done with a 1:1,500 dilution in 10% (vol/vol) FCS of polyclonal rabbit antibodies raised against αB-crystallin purified from MS-affected human myelin. After rinsing, binding was visualized using biotinylated donkey anti-rabbit antibodies and, subsequently, avidin-labelled alkaline phosphatase and fast red (Sigma). Specificity of the αB-crystallin staining was confirmed by the observation that preincubating polyclonal rabbit antiserum with RP-HPLC-purifies bovine αB-crystallin from eye lens at 5 μg ml^{-1} completely blocked the staining observed. No blocking was observed when the serum was preincubated with a 5 μg ml^{-1} purified bovine αB-crystallin, which shares about 60% sequence identity with αB-crystallin. MOG staining was performed with a murine anti-MOG monoclonal antibody, followed by peroxidase-conjugated goat anti-mouse antibody (Dianova Jackson) and 3'-diaminobenzidine. GFAP was visualized using a murine monoclonal antibody against GFAP (Boehringer Mannheim); secondary steps were the same as for anti-MOG staining except for the use of choronaphthol as a chromogen. Counterstaining was performed using haematoxilin.

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References

1. Martin, R., McFarland, H.F. & MacFarlin, D.E.A. Rev. Immun. **10**, 153-187 (1992).
2. Van Noort, J.M. et al J. Neuroimmun. **46**, 67-72 (1993).
3. Ingolia, T.D. & Craig, E.A. Proc. natn. Acad. Sci. U.S.A. **79**, 2360-2364 (1982)
4. Klemenz, R., Frohli, E., Steiger, R.H., Schafer, R. & Aoyama, A. Proc. natn. Acad. Sci. U.S.A. **88**, 3652-3656 (1991).
5. Ozawa, K. et al. Brain **117**, 1311-1322 (1994).
6. Bruck, W. et al. Ann. Neurol. **35**, 65,-73 (1994).
7. Iwaki, T., Kume-Iwaki, A., Liem, R.K.H. & Goldman, J.E. Cell **57**, 71-78 (1989).
8. Head, M.W., Corbin, E. & Goldman, J.E. J. Cell. Physiol. **159**, 41-50 (1994).
9. Iwaki T. et al Am. J. Path. **140**, 345-356 (1992).
10. Lowe, J. et al. J. Path. **166**, 61-68 (1992).
11. Selmaj, K., Brosnan, C.F. & Raine, C.S. Proc. natn. Acad. Sci. U.S.A. **88**, 6452-6456 (1991).
12. Wucherpfennig, K.W. et al Proc.natn. Acad. Sci. U.S.A. **89**, 4588-4592 (1992).
13. Salvetti, M. et al. J. Autoimmun. **5**, 691-702 (1992).
14. Karlik, C.C., Coutu, M.D. & Fryberg, E.A. Cell **38**, 711-719 (1984).
15. Hiromi, Y. & Hotta, Y. EMBO J. **4**, 1681-1687 (1985).
16. De Jong, W.W., Lubsen, N.H. & Kraft, H.J. Prog. Ret. Eye Res **13**, 391-442 (1994).
17. Kato, K. et al. J. biol. Chem. **167**, 7718-7725 (1992).
18. Zantema, A., Verlaan-De Vries, M., Maasdam, D., Bol, S. & van der Eb, A. J. biol. Chem. **267**, 12936-12941 (1992).
19. Pette, M. et al. Neurology **40**, 1770-1778 (1990).
20. Van Noort J.M., El Ouagmiri, M., Boon, J. & Van Sechel, A.C. J. Chromat. **653**, 155-161 (1994).
21. Norton, W.T. & Poduslo, S.E. J. Neurochem. **31**, 749-757 (1973).

3.

Expression of αB-crystallin in glia cells during lesional development in multiple sclerosis

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Abstract

The small heat shock protein αB-crystallin was recently identified as a dominant human T-cell antigen in myelin derived from Multiple Sclerosis (MS) patients. Using immunohistochemical techniques, oligodendrocytes as well as astrocytes in MS lesions were shown to express αB-crystallin. In the present study we examined the expression of αB-crystallin, human natural killer cell marker (HNK-1; as a marker for immature oligodendrocytes) and heat shock protein 60 (hsp60) in glia cells at different stages of MS lesion development, i.e. in early active lesions, late active lesions, and inactive lesions.

The results demonstrate that already at the earliest stages of lesional development a subpopulation of oligodendrocytes express detectable levels of αB-crystallin. In active lesions about 5-10% of all oligodendrocytes were found to express αB-crystallin, whereas in inactive lesions the relative number of αB-crystallin-expressing oligodendrocytes was approximately tenfold less. For astrocytes the relative number of αB-crystallin-expressing cells was 40-50% for all three types of lesions. Also, αB-crystallin-expressing oligodendrocytes and astrocytes displayed different patterns of distribution in lesional areas. These data suggest different regulatory pathways for αB-crystallin expression in either type of glia cell.

No correlation was found between expression patterns of HNK-1 and αB-crystallin indicating that the subpopulation of αB-crystallin-expressing oligodendrocytes consisted of both mature and immature oligodendrocytes. In addition, no correlation was found between expression of hsp60 and αB-crystallin in MS lesions suggesting different regulatory pathways for either hsp.

Keywords: Multiple Sclerosis; αB-crystallin; glia cells; heat shock proteins

1. Introduction

Multiple Sclerosis is a chronic disease characterized by localized myelin destruction within the central nervous system (CNS). Although the exact etiology and pathogenesis of MS are unknown, it is generally accepted that autoimmunity is involved. The autoantigen(s) relevant to MS probably reside in CNS myelin, the target of the disease. In a recent study, αB-crystallin was found to be a dominant human T-cell antigen in myelin derived from MS patients (van Noort et al., 1995).
αB-Crystallin is abundant in the vertebrate eye lens, where it is part of the structural α-crystallin protein complex. Expression can also be found in several other organs (Bhat and Nagineni, 1989; Iwaki et al., 1989) including skeletal muscle, kidney epithelial cells and glia cells (Iwaki et al., 1990) where it is known to act as a heat shock protein (Ingolia and Craig, 1982; Klemenz et al., 1991; de Jong et al., 1993).
The group of proteins known as stress proteins (or heat shock proteins) are induced in a wide variety of cells in response to physiological insults. They play a role in preventing the aggregation of immature or damaged proteins. Based upon their molecular weight, heat shock proteins (hsp) are categorized into several families i.e. hsp90, hsp70, hsp60 and the small hsp (Morimoto et al., 1990; Hightower, 1991). Members of the same family usually share highly conserved structures. Likewise, αB-crystallin shares significant homologies with the other small mammalian heat shock protein hsp27 (Merck et al., 1993).
As regards the expression of αB-crystallin in the CNS standard immunohistochemical techniques fail to detect the protein in healthy white matter. Induced expression can be seen in several neurodegenerative diseases such as Alzheimer's disease, Alexander's disease and Pick's disease and recently also in MS (Iwaki et al., 1992; Head et al., 1993; Iwaki et al., 1993; Renkawek et al., 1994) where within lesional areas oligodendrocytes as well as astrocytes were shown to express αB-crystallin (van Noort et al., 1995). No data are available, however, with regard to αB-crystallin expression during disease development nor with regard to possible differences between αB-crystallin-expressing oligodendrocytes and αB-crystallin-expressing astrocytes. To clarify the possible role of αB-crystallin as an autoantigen in the development of MS, defining its cellular localization in MS lesions at different stages is an important step.
To examine the expression of αB-crystallin in astrocytes as well as in oligodendrocytes sections of previously characterized MS lesions were used (Ozawa et al., 1994). Using immunohistochemical techniques we studied αB-crystallin expression in glia cells and classified these cells as oligodendrocytes or astrocytes based on morphological features. Serial sections were stained for astrocyte or oligodendrocyte-specific markers, glial fibrillary acidic protein (GFAP) and myelin oligodendrocyte glycoprotein (MOG), respectively, in order to determine total numbers of either type of glia cell in the tissue samples. Furthermore, we examined possible colocalization of αB-crystallin with the HNK-1 antigen, a marker for immature oligodendrocytes, Prineas et al., 1989) as well as with hsp60. The results indicate different regulatory pathways of αB-crystallin in

oligodendrocytes as compared to astrocytes. In addition, the patterns of αB-crystallin expression differ from those of HNK-1 and hsp60.

2. Materials and methods

2.1. Neuropathology

Paraffin-embedded autopsy tissue from 14 patients with clinically definite multiple sclerosis was studied (Table 3.1). In the present study lesion samples were used that had already been categorized into the following three categories according to previously established criteria (Brück et al., 1994).
Early Active Lesions (EAL) are characterized by massive infiltration of the brain tissue by leukocytes. Macrophages contain early myelin degradation products: minor myelin proteins, such as myelin oligodendrocyte glycoprotein (MOG) and 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNPase) are still detectable inside vesicles by immunohistochemistry. These lesions also contain a high number of macrophages carrying the MRP 14 epitope. Previous data show that MRP 14 is expressed on macrophages only during a short period of time concurrent with extravasation into inflammatory lesions (Bhardway et al., 1992). Late Active Lesions (LAL) are characterized by more advanced myelin degradation. Macrophages contain myelin degradation products that can be stained with antibodies against major myelin proteins, such as proteolipid protein (PLP) and myelin basic protein (MBP) but not with antibodies against the aforementioned minor myelin proteins. Inactive Lesions (IL) are characterized by little or no leukocyte infiltration and by complete demyelination. If present, macrophages contain degradation products without detectable myelin proteins and they show little expression of the MRP 14 marker. Macrophages still express the late macrophage antigen detected by the 25F9 antibody (Zwadlo et al., 1986).

2.2. Immunocytochemistry

Immunocytochemistry was performed on formalin-fixed paraffin-embedded brain sections (5 μm) from 14 patients with clinically definite MS. Sections were deparaffinized in xylene and hydrated using ethanol. Following blocking of endogenous peroxidase activity and a 10-min incubation in 10% (vol/vol) FCS, sections were stained overnight for the following different markers at 4°C. To identify astrocytes a murine monoclonal anti-glial fibrillary acidic protein (GFAP; Boehringer Mannheim, Germany) antibody was used. For identification and characterization of oligodendrocytes murine monoclonal anti-myelin oligodendrocyte glycoprotein (MOG; 8-18C5, Piddlesden et al., 1993), murine monoclonal anti-human myelin basic protein (MBP; Boehringer Mannheim, Germany) and murine monoclonal anti-human natural killer cells (HNK-1; Leu-7, Becton and Dickinson, Mountain View, CA) antibodies were

used. For detection of the two heat shock proteins we used rabbit polyclonal anti-αB-crystallin (van Noort et al., 1995) and murine monoclonal anti-human heat shock protein 60 (hsp60; StressGen, Victoria, Canada) antibodies. Nine sections were selected for HNK-1 and hsp 60 stainings; three from each lesion type.
The secondary antibodies used were horseradish peroxidase-conjugated swine anti-rabbit antibody, horseradish peroxidase-conjugated goat anti-mouse antibody (DAKO, Glostrup, Denmark), biotinylated donkey anti-rabbit antibodies and avidin-labelled alkaline phosphatase (Sigma). Following a 1-h incubation at room temperature, binding was visualized using 3-amino-9-ethylcarbazole (AEC), 3'-diaminobenzidine (DAB) or Fast Red (Sigma) as a chromogen. Counterstaining was performed using haematoxylin. Negative controls included serum of a non-immunized rabbit as a first antibody and a primary antibody omission control.

2.3. Quantitative evaluation and statistical analysis

The number of cells stained by immunocytochemistry per square unit was determined by counting the cells on serial sections. Counting of αB-crystallin-expressing cells was performed twice with a variability of <10%. Appropriate areas of the sections were selected according to patterns of demyelination seen in the sections. αB-Crystallin-positive glia cells were classified as oligodendrocytes or astrocytes based on morphological criteria.
Relative numbers of αB-crystallin-expressing oligodendrocytes and astrocytes were calculated by comparing serial sections stained for αB-crystallin, MOG or GFAP respectively. For each lesional stage, accumulated numbers of αB-crystallin-positive oligodendrocytes or astrocytes were compared to the total number of either type of glia cell (Table 3.1).
For statistical analysis of the data, early active lesions and late active lesions were considered as one group (active lesions) and compared to the group of inactive lesions. A non-parametric group test (Wilcoxon-test) was employed on these two groups (Siegel, 1956).

3. Results

3.1. αB-Crystallin expression in MS lesions

Already at the earliest stages of lesional development, oligodendrocytes were found to express detectable levels of αB-crystallin. No αB-crystallin expression could be detected in healthy white matter (in close proximity of the lesions) in the same samples. In EAL and LAL about 5-10% of all oligodendrocytes were found to express αB-crystallin, whereas in IL, the percentage of αB-crystallin-expressing oligodendrocytes was approximately tenfold less, a statistically significant decrease (Table 3.1). In addition, the staining intensity of the oligodendrocytes in IL was markedly less than that in AL. It is striking that only a subpopulation of the cells produced detectable amounts of αB-crystallin (Fig. 3.1). The size of this subpopulation of oligodendrocytes varied considerably between different lesions, even between those of similar developmental stage. While generally only 5-10% of the oligodendrocytes in active lesions showed detectable expression of αB-crystallin, some lesions contained up to 64% of positive oligodendrocytes. For astrocytes the relative number of αB-crystallin-expressing cells was 40-50% for all three types of lesions (Fig. 3.2). The small decrease in the relative numbers of αB-crystallin-expressing astrocytes in LAL can be attributed to an increase in total numbers of astrocytes in LAL, presumably due to astrogliosis. In general, the distribution pattern of αB-crystallin-expressing astrocytes was patch-like; in some areas nearly all astrocytes were found to express αB-crystallin, while in another area of the same lesion no αB-crystallin-expressing astrocytes could be detected at all. This expression pattern in astrocytes differed markedly from that in oligodendrocytes since in the latter type expression in individual cells was found more randomly throughout the lesions (Fig. 3.2 shows the more general expression pattern in astrocytes in IL as opposed to the more individual expression in oligodendrocytes as shown in Fig. 3.1).

3.2. No correlation between expression of the HNK-1 marker and αB-crystallin

In order to examine the possibility that αB-crystallin-expressing oligodendrocytes are primarily immature cells, their localization was compared to expression patterns of the HNK-1 marker. We selected nine lesions and compared the staining patterns of αB-crystallin and HNK-1, using serial sections. Oligodendrocytes were classified on the basis of the distribution of the HNK-1 antigen. Cytoplasmic granular staining at one pole of the nucleus was considered to be the staining pattern for mature oligodendrocytes whereas intense surface and cytoplasmic HNK-1 reactivity was taken to identify immature oligodendrocytes (Prineas et al., 1989). Noteworthy is that not all mature oligodendrocytes were positive for HNK-1 and that only few immature oligodendrocytes were detected in MS lesions. The appearance of immature oligodendrocytes was restricted to active lesions. No correlation was observed between expression of αB-crystallin and the maturation state of oligodendrocytes (Fig. 3.3a-c).

Table 3.1. Expression of αB-crystallin in glia cells within MS lesions of different developmental stages.

	αB-crystallin expression in oligodendrocytes				αB-crystallin expression in astrocytes			
Lesion #	αBc⁺ oligo's	MOG⁺ cells	sample size (mm^3)	relative expression (%)	αBc⁺ astro's	GFAP⁺ cells	sample size (mm^3)	relative expression (%)
Early active lesions (EAL)								
J24/81-1	273	729	27	37.4	31	517	27	6
J46/89-3a	42	1273	16	3.8	0	6	16	0
95-84-5	126	663	39	19	499	675	39	73.9
J172/81-2	171	7590	23	2.3	308	491	23	62.7
Total	612	10255	105	**5.9**	838	1689	105	**49.6**
Late active lesions (LAL)								
436/76a	15	1027	6.5	1.5	251	292	6.5	86
J39/80/8	94	147	3.5	63.9	82	123	3.5	66.7
J22/81/3a	201	378	9	53	526	756	9	69.2
J46/89-3b	56	594	33	9.4	32	2195	33	1.5
J366/80a	40	660	2.5	6	18	27	2.5	66.7
142/87	51	1311	28.5	3.9	919	1528	28.5	60.7
Total	457	4117	94	**11.2**	1828	4921	94	**37.1**
Inactive lesions (IL)								
436/76b	6	1634	9.5	0.4	151	342	9.5	44.1
245/79	0	88	3.5	0	59	197	3.5	29.9
J22/81/3b	0	377	14.5	0	514	914	14.5	56.2
217/74b	31	541	23.5	5.7	313	773	23.5	40.5
92-79/5	0	1059	6.5	0	16	76	6.5	21.1
J361/82	0	35	11.5	0	0	150	11.5	0
J366/80b	6	128	8.5	4.7	41	77	8.5	53.2
J229/84-3	0	333	11.5	0	8	123	11.5	71.5
Total	37	4195	89	**0.8**	1182	2652	89	**44.6**

Total cell numbers for oligodendrocytes and astrocytes per lesion were determined by counting the number of MOG-positive cells and the number of GFAP-positive cells in serial sections of the area of interest. The relative expression for each lesional stage is calculated by dividing the total number of αB-crystallin-expressing oligodendrocytes or astrocytes by the total number of either cell type in the areas examined. αBc = αB-crystallin

3.3. Comparison between the expression of two heat shock proteins: αB-crystallin and hsp60

To examine whether αB-crystallin expression correlates with upregulation of other hsp we studied hsp60 expression in the same nine samples as were selected for the study of HNK-1 expression. Astrocytes, oligodendrocytes as well as endothelial cells could be stained for hsp60. Subcellular distribution of hsp60, detected in the mitochondrial matrix as described before (Jindal et al., 1989) was clearly different from that of αB-crystallin, which was localized more diffusely in the cytosol. In contrast to αB-crystallin, hsp60 could also be detected in unaffected white matter. Yet, the frequency of cells expressing hsp60 as well as the number of positive granules per cell was strongly increased in lesional areas as compared to non-lesional areas. Furthermore, hsp60 expression was more pronounced in EAL and LAL as compared to IL. Comparing the serial sections of the lesions showed that although in the majority of areas both hsp are expressed, the overall pattern of expression of αB-crystallin is different from that of hsp60 (Fig. 3.4a-c). In general there were more cells expressing hsp60 than αB-crystallin, implying that induced expression of αB-crystallin is a more selective event in MS lesions than induced expression of hsp60.

4. Discussion

In a recent study we showed that αB-crystallin may act as an immunodominant CNS myelin antigen to human T cells and that its expression can be detected in oligodendrocytes as well as in astrocytes in the lesional areas of MS brains (van Noort et al., 1995). The present study was undertaken to examine expression of αB-crystallin in either type of glia cell at different stages of lesional development in MS. We also examined to what extent expression of αB-crystallin correlates with the state of maturity of oligodendrocytes as well as with expression of another heat shock protein i.e. hsp60. Data obtained in this study show that already at the earliest stages of lesional development, a subpopulation of oligodendrocytes are expressing detectable levels of αB-crystallin. This would be consistent with a role of aB-crystallin as a T-cell antigen even at the earliest stages of lesional development. Generally, only 5-10% of all oligodendrocytes express αB-crystallin and positive cells can be found scattered among negative ones. A similar heterogeneity with regard to expression of hsp70 has been reported also for murine oligodendrocytes (Satoh et al., 1991). In order to acquire more information about the subpopulation of αB-crystallin-expressing oligodendrocytes, we compared the distribution of these cells with the distribution pattern of the HNK-1 antigen, a marker for immature oligodendrocytes. Since no correlation was found, it appears unlikely that αB-crystallin is an antigen expressed exclusively by immature oligodendrocytes.
Furthermore, our data indicate that expression of αB-crystallin is regulated differently in oligodendrocytes as compared to astrocytes, as has been described earlier for other hsp (Satoh et al., 1991; Freedman et al., 1992; Satoh and Kim, 1995). First, the distribution

pattern of αB-crystallin-expressing oligodendrocytes differs from that of αB-crystallin-expressing astrocytes. Such astrocytes are distributed in a patch-like manner, contrasting the random distribution of positive oligodendrocytes throughout the lesion. Secondly, in inactive lesions, a tenfold reduction of αB-crystallin-expressing oligodendrocytes occurs relative to active lesions, whereas αB-crystallin-expression in astrocytes does not change. This reduction in numbers of αB-crystallin-expressing oligodendrocytes in IL cannot be ascribed to mere oligodendrocyte loss in developing lesions (Table 3.1); sufficient numbers of oligodendrocytes survive that would have allowed detection of αB-crystallin in these cells. In addition, a comparison between expression patterns of αB-crystallin and another heat shock protein, hsp60 brings up several interesting points. Whereas expression of αB-crystallin is restricted to cells in the lesion area, hsp60 is also constitutively expressed in non-stressed glia cells and endothelial cells. In addittion it is shown that although in the majority of the lesional areas both hsp are expressed, there are regions where either αB-crystallin or hsp60 is expressed. Secondly, the localization of hsp60 is mitochondrial (Jindal et al., 1989) in contrast to the cytosolic localization of αB-crystallin. Relocalization of hsp60 from the mitochondria into the cytosol of stressed cells, as was reported earlier for lesions in EAE experiments (Gao et al., 1995) was not observed in our material, nor did we observe any surface expression of hsp60 on lymphocytes or oligodendrocytes, contrary to what has been suggested by other studies (Selmaj et al., 1991; Freedman et al., 1992; Gao et al., 1995).
The differences in distribution and localization between hsp60 and αB-crystallin suggest markedly different regulatory pathways. It is known that not all stress proteins are upregulated in response to the same stimuli, and our *in situ* observations match well with previously reported data (Hightower, 1991; Kato et al., 1992; Wiegant et al., 1994). Even stress proteins that belong to the same family do not always react to the same stimuli (Chang et al., 1995); αB-crystallin in astrocytomas, for example, is known to be upregulated in response to TNF-α, whereas hsp27 levels remain low (Head et al., 1994). The apparent differences in the regulatory pathways for different hsp might open ways of selective modulation of distinct hsp (Lee et al., 1995).

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References

Bhardway, R.S., Zotz, C., Zwadlo-Klarwasser, G., Roth, J., Goebeler, M. and Mahnke, K.1992. The calcium-binding proteins MRP8 and MRP14 form a membrane-associated heterodimer in a subset of monocytes/macrophages present in acute but absent in chronic inflammatory lesions. Eur. J. Immunol. 22, 1891-1897.

Bhat, S.P. and Nagineni, C.N. 1989. αB subunit of lens-specific protein α-crystallin ispresent in other ocular and non-lenticular tissues. Biochem. Biophys. Res. Commun. 158, 319-325.

Brück, W., Schmied, M., Suchanek, G., Brück, Y., Breitschopf, H., Poser, S., Piddlesden, S. and Lassmann, H. 1994. Oligodendrocytes in the early course of multiple sclerosis. Ann. Neurol. 35, 65-73.

Chang, Y., Abe, A. and Shayman, J.A. 1995. Ceramide formation during heat shock: A potential mediator of αB-crystallin transcription. Proc. Natl. Acad. Sci. USA 92, 12275-12279.

De Jong, W.W., Leunissen, J.A.M. and Voorter, C.E.M. 1993. Evolution of the α-crystallin/small heat-shock family. Mol. Biol. Evolut. 10, 103-126.

Freedman, M.S., Buu, N.N., Ruijs, T.C.J., Williams, K. and Antel, J.P. 1992. Differential expression of heat shock proteins by human glial cells. J. Neuroimmunol. 41, 231-238.

Gao, Y.L., Brosnan, C.F. and Raine, C.S. 1995. Experimental Autoimmune Encephalomyelitis; Qualitative and semiquantatative differences in heat shock protein 60 expression in the central nervous system. J. Immunol. 154, 3548-3556.

Head, M.W., Corbin, E. and Goldman J.E. 1993. Overexpression and abnormal modification of the stress proteins αB-crystallin and hsp27 in Alexander disease. Am. J. Pathol. 143, 1743-1752.

Head, M.W., Corbin, E. and Goldman, J.E. 1994. Coordinate and independent regulation of αB-crystallin and hsp27 expression in response to physiological stress. J. Cell. Physiol. 159, 41-50.

Hightower, L.E. 1991. Heat shock, stress proteins, chaperones and proteotoxicity. Cell 66, 191-197.

Ingolia, T.D. and Craig, E.A. 1982. Four small heat shock proteins are related to each other and to mammalian α-crystallin. Proc. Natl. Acad. Sci. USA 79, 2360-2364.

Iwaki, T., Kume-Iwaki, A., Liem, R.K.H. and Goldman, J.E. 1989. αB-crystallin is expressed in non-lenticular tissues and accumulates in Alexander's disease brain. Cell 57, 71-78.

Iwaki, T., Kume-Iwaki, A. and Goldman J.E. 1990. Cellular distribution of αB-crystallin in non-lenticular tissues. J. Histochem. Cytochem. 38, 31-39.

Iwaki, T., Wisniewski, T., Iwaki, A., Corbin, E., Tomokane, N., Tateishi and Goldman, J.E. 1992. Accumulation of αB-crystallin in central nervous system glia and neurons in pathologic conditions. Am. J. Pathol. 140, 345-356.

Iwaki, T., Iwaki, A., Tateishi, J., Sakaki, Y. and Goldman, J.E. 1993. αB-crystallin and 27-kd heat shock protein are regulated by stress conditions in the central nervous system and accumulate in Rosenthal fibers. Am. J. Pathol. 143, 487-495.

Jindal, S., Dudani, A.K., Singh, B., Harley, C.B. and Gupta, R.S. 1989. Primary structure of a human mitochondrial protein homologous to bacterial and plant chaperonins and to 65 kD mycobacterial antigen. Mol. Cell Biol. 9, 2279-2283.

Kato, S., Hirano, T., Umahara, M., Kato, F., Herz, F. and Ohama, E. 1992. Comparative immunohistochemical study on the expression of αB-crystallin, ubiquitin and stress-response protein 27 in ballooned neurons in various disorders. Neuropath. Appl. Neurobiol. 18, 335-340.

Klemenz, R., Frohli, E., Steiger, R.H., Schafer, R. and Aoyama A. 1991. αB-crystallin is a small heat shock protein. Proc. Natl. Acad. Sci. USA 88, 3652-3656.

Lee, Y.J., Berns, C.M., Galoforo, S., Erdos, G., Cho, J.M. and Corry, P.M. 1995. Differential effect of 1-(5-isoquinolinesulfonyl)-2-methylpiperazine (H-7) on αB-crystallin and hsp70 gene expression in murine cell lines. Biochem. Pharmacol. 50(8), 1149-1155.

Merck, K.B., Horwitz, J., Kersten, M., Overkamp, P., Gaestel, M., Bloemendal, H. and de Jong, W.W. 1993. Comparison of the homologous carboxy-terminal domain and tail of α-crystallin and small heat shock protein. Mol. Biol. Rep. 18, 209-215.

Morimoto, R.I., Tissieres, A. and Georgopoulos, C. 1990. In: Morimoto, R.I., Tissieres, A. and Georgopoulos, C. (Eds.), Stress proteins in Biology and Medicine. Cold Spring Harbor Labaratory Press, Cold Spring Harbor, NY, 1-36.

Ozawa, K., Suchanek, G., Breitschopf, H., Brück, W., Budka, H., Jellinger, K., and Lassmann H. 1994. Patterns of oligodendroglia pathology in multiple sclerosis. Brain 117, 1311-1322.

Piddlesden, S.J., Lassmann, H., Zimprich, F., Morgan, B.P. and Linington, C. 1993. The demyelinating potential of antibodies to myelin oligodendrocyte glycoprotein is related to their ability to fix complement. Am. J. Pathol. 143, 555-564.

Prineas, J.W., Kwon, E.E., Goldenberg, P.Z., Ilyas, A.A., Quarles, R.H., Benjamins, J.A. and Sprinkle T.J. 1989. Multiple Sclerosis: oligodendrocyte proliferation and differentiation in fresh lesions. Lab. Invest. 61, 489-503.

Renkawek, K., Voorter, C.E.M., Bosman, G.J., van Workum, F.P. and de Jong W. 1994. Expression of αB-crystallin in Alzheimers disease. Acta Neuropathol. 87, 155-160.

Satoh, J-I., Yamamura, T., Kunishita, T. and Tabira, T. 1991. Heat-inducible expression of 72-kilodalton heat shock protein (hsp72) on cultured mouse oligodendrocytes and astrocytes. J. Neuroimmunol. Suppl. 1, 98.

Satoh, J-I. and Kim, S.U. 1995. Differential expression of heat shock protein hsp27 in human neurons and glial cells in culture. J. Neurosci. Res. 41 (6), 805-818.

Selmaj, K., Brosnan, C., Raine, C.S. 1991. Colocalization of lymphocytes bearing γδ T-cell receptor and heat shock protein hsp65$^+$ oligodendrocytes in multiple sclerosis. Proc. Natl. Acad. Sci. USA. 88, 6452-6456.

Siegel, S. 1956. Non parametric statistics (for the behavourial sciences). Mc Graw-Hill Book Company, New York, pp. 75-83.

Van Noort, J.M., Van Sechel, A.C., Bajramovic, J.J., El Ouagmiri, M., Polman, C.H., Lassmann, H., Ravid, R. 1995. The small heat shock protein αB-crystallin as candidate autoantigen in multiple sclerosis. Nature 375, 798-801.

Wiegant, F.A., Souren, J.E., Van Rijn, J. and Van Wijk, R. 1994. Stressor-specific induction of heat shock proteins in rat hepatoma cells. Toxicol. 94, 143-159.

Zwadlo G., Schlegel, R. and Sorg, C.A. 1986. A monoclonal antibody to a subset of human monocytes found only in the peripheral blood and inflammatory tissues. J. Immunol. 137, 512-518.

4.

Presentation of αB-crystallin to T cells in active Multiple Sclerosis lesions: an early event following inflammatory demyelination[1]

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Abstract

In the development of multiple sclerosis (MS), (re)activation of infiltrating T cells by myelin-derived antigens is considered to be a crucial step. Previously, αB-crystallin has been shown to be an important myelin antigen to human T cells. Since αB-crystallin is an intracellular heat shock protein the question arises at what stage, if any, during lesional development in MS this antigen becomes available for $CD4^+$ T cells.
In 3 out of 10 active MS lesions αB-crystallin could be detected inside phagocytic vesicles of perivascular macrophages, colocalizing with myelin basic protein (MBP) and myelin oligodendrocyte glycoprotein (MOG). Although the detectability of MOG in phagosomes is considered as a marker for very recent demyelination, MOG was detected in more macrophages and in more lesions than αB-crystallin. The disappearance of αB-crystallin from macrophages even before MOG was confirmed by *in vitro* studies; within 6 h after myelin-uptake αB-crystallin disappears from the phagosomes.
αB-Crystallin-containing macrophages colocalized with infiltrating T cells and they were characterized by expression of MHC-II, CD40 and CD80. In order to examine functional presentation of myelin antigens to T cells, purified macrophages were pulsed *in vitro* with whole myelin membranes. These macrophages activated both myelin-primed and αB-crystallin-primed T cells in terms of proliferation and IFN-γ secretion. In addition, αB-crystallin-pulsed macrophages activated myelin-primed T cells to the same extent as myelin-pulsed macrophages, whereas MBP-pulsed macrophages triggered no response at all.
These data indicate that, in active MS lesions, αB-crystallin is available for functional presentation to T cells early during inflammatory demyelination.

Keywords: heat-shock protein, autoimmunity, antigen presentation, myelin proteins, EAE/MS

Abbreviations used in this paper: CNPase, 2',3'-cyclic nucleotide 3'-phosphohydrolase; CNS, central nervous system; EAE, experimental allergic encephalomyelitis; GFAP, glial fibrillary acidic protein; MAG, myelin associated glycoprotein; MBP, myelin basic protein; MOG, myelin oligodendrocyte glycoprotein; MS, multiple sclerosis; ORO, Oil red O; PLP, proteolipid protein

Introduction

Multiple sclerosis (MS) is a chronic demyelinating disease of the central nervous system (CNS) characterized by focal areas of demyelination (lesions or plaques). Although the exact etiology of MS is unknown, it is generally accepted that autoimmunity is involved and that the autoantigen(s) probably reside in CNS myelin, the target of the immune response (1, 2). Myelin consists of numerous candidate autoantigenic proteins amongst which are major myelin constituents like myelin basic protein (MBP) and proteolipid protein (PLP), and minor myelin constituents like myelin oligodendrocyte glycoprotein (MOG), myelin associated glycoprotein (MAG) and 2',3'-cyclic nucleotide 3'-phosphohydrolase (CNPase). In the complete collection of proteins extracted from MS-affected myelin the dominant human antigen for $CD4^+$ T cells appears to be αB-crystallin (3), a small heat shock protein (4, 5).
αB-Crystallin is present at enhanced levels in the cytosol of oligodendrocytes and astrocytes in MS lesions, where it is upregulated already at the earliest stages of lesional formation (6). Elevated expression of αB-crystallin in the oligodendrocyte/myelin complex correlates strongly with the immunological activity of the lesion. In order to locally (re)activate T cells, a potential autoantigen must be presented to the cellular immune system. In immunologically active MS lesions, characterized by ongoing or recent demyelination and the presence of inflammatory infiltrates, several myelin proteins can be detected in macrophages (7), suggesting processing and presentation of these proteins to the cellular immune system.
Detectability of such myelin proteins in macrophages, together with activation markers of macrophages, can be used to assess lesional age (8). Detection of minor myelin constituents like MOG and/or CNPase in phagocytic vesicles inside macrophages indicate recent demyelination. Macrophages containing vesicles that show immunoreactivity for the major myelin constituents MBP and/or PLP, but not for MOG or CNPase, are indicative of more advanced myelin degradation. Inactive lesions may show infiltration of T cells and of macrophages containing either empty vacuoles or myelin-protein-negative vesicles, whereas the most frequently found lesions in MS patients are even older and contain less, if any, immunological activity.
In addition to immunohistochemical analyses, that do not provide information with respect to the functional relevance of (auto)antigen presentation, there has been extensive research on autoantigen-specific T cell responses in MS (9). Most of these *in vitro* studies have been performed with purified myelin proteins, assuming that during demyelination these proteins will be liberated from the fatty myelin membrane and will subsequentially be processed and presented by antigen presenting cells (APC) to T cells. The role of αB-crystallin as a putative autoantigen has been studied with protein extracts of myelin *in vitro* (3), and αB-crystallin is localized in the cytosol of oligodendrocytes and astrocytes (6) in MS lesions. So far it has thus remained unclear whether αB-crystallin actually becomes presented to MHC class II-restricted T cells in MS lesions.

We therefore investigated whether presentation of αB-crystallin to the cellular immune sytem occurs in MS lesions, using a combined *in situ-in vitro* approach. In this report, we show that in demyelinating MS lesions αB-crystallin can be detected inside vesicles belonging to the endosomal/lysosomal pathway in a subset of myelin-phagocytosing macrophages. These perivascular macrophages express MHC class II molecules as well as CD80 and CD40 and they are found in close proximity of infiltrating T cells, suggesting presentation of myelin-derived αB-crystallin to these T cells. Functional presentation of myelin-derived αB-crystallin to T-cells was demonstrated *in vitro* by proliferative responses and IFN-γ production of αB-crystallin primed T-cells in response to macrophages fed with whole myelin membranes. The response of myelin-primed T-cells to macrophages fed with whole myelin was similar to their response to macrophages fed with purified αB-crystallin alone, showing that much of the human T cell response to APC fed with whole myelin is accounted for by the recognition of myelin-derived αB-crystallin. The present study demonstrates that as an early event after myelin phagocytosis in MS lesions αB-crystallin becomes available to T cells, suggesting an important role of this autoantigen in the pathogenesis of MS.

Materials and Methods

Immunohistochemistry.
Immunohistochemistry was performed on snap-frozen brain sections (10 μm) from 6 patients with clinically definite MS and on 2 control cases (obtained from MS Tissue Bank, Institute of Neurology, London). Ten MS lesions were selected for the presence of active demyelination using Oil red O (ORO)-positive macrophage infiltration as a marker for myelin uptake. These active lesions were analysed for cellular infiltrates and myelin-derived protein detectability in macrophages. ORO staining was performed as described previously. Sections were fixed in ice-cold acetone with 0.03% H_2O_2 (to block endogenous peroxidase) for 10 min. Following air-drying, the sections were incubated overnight at 4°C with primary antibodies in 0.1% BSA and rinsed extensively with PBS+0.05% Tween-20. Primary antibodies used for glial cell and myelin protein detection were rabbit polyclonal anti-glial fibrillary acidic protein (α-GFAP, Zymed), murine monoclonal anti-myelin oligodendrocyte glycoprotein (α-MOG, clone 8-18C5; provided by Dr. Sarah Piddlesden, University of Melbourne, Melbourne, Australia) and murine monoclonal anti-human myelin basic protein (α-MBP; Boehringer Mannheim). To study markers of immunological relevance the following primary antibodies were used, murine monoclonal anti-MHC class II (α-HLA-D, Novocastra), murine monoclonal anti-CD40 (provided by Dr. Mark de Boer, Tanox Pharma B.V., Amsterdam, The Netherlands), murine monoclonal anti-CD68 (DAKO), murine monoclonal anti-CD80 (provided by Dr. Carine Dello, Innogenetics, Ghent, Belgium) and rabbit polyclonal anti-CD3 (DAKO). For detection of αB-crystallin we used rabbit polyclonal

anti-αB-crystallin w3/13 (3) and rabbit polyclonal anti-lap70, directed against the C-terminal 14 amino acids of αB-crystallin provided by Dr. Gerard Stege (University of Nijmegen, Nijmegen, The Netherlands). The secondary antibodies used were biotinylated horse-anti-mouse (Vector) and biotinylated donkey-anti-rabbit (Life Sciences). They were incubated in 1% BSA+1% normal human serum (NHS) for 45 min at room temperature. After rinsing with PBS+0.05% Tween-20 peroxidase labeled streptavidin (Gibco BRL) in 1% BSA+1% NHS was added for 45 min at room temperature.
Binding of the antibodies was visualized using 3'-diaminobenzidine (DAB) as a chromogen. Counterstaining was performed using haematoxylin. Negative controls included serum of non-immunized mice or rabbits as a first antibody and primary antibody ommision controls.
As an additional specificity assay for the αB-crystallin staining, antibodies were supplied with partly digested αB-crystallin (incubation time = 120 min; see below) at a 10 µg/ml concentration and incubated for 1 h at room temperature before use in the abovementioned immunohistochemical procedure.

In vitro myelin uptake assay.
24-well plates were incubated for 2 h at room temperature with 0.5 ml (2mg/ml) human fibronectin (CLB, Amsterdam) per well. Peripheral blood mononuclear cells (PBMC) at a concentration of 2.10^6/ml in RPMI + 10% FCS + streptomycin (50mg/ml), penicillin (100 IU/ml) and 1 mM glutamine was added per well and incubated for 48 h at 37°C and 5% CO_2. The supernatant was discarded and the cells were washed twice with RPMI. The adherent monocytes were incubated for 90 min with 0.5 ml RPMI+4% NHS and fed with 20 µg MS patient-derived myelin. The supernatant was discarded and cells were washed twice with RPMI. At various timepoints cells were harvested using 0.5 ml RPMI+5 mM EDTA per well. Cells were left on ice for 15-30 min before they were harvested and centrifuged for 5 min at 1500 rpm. Cells were resuspended in PBS+0.1% BSA and used for cytospins at 500 rpm. Cytospins were analysed for macrophage purity using the anti-CD68 monoclonal antibody and found to contain > 85% monocytes. Immunohistochemistry was performed as described above.

Western Blotting and in vitro degradation of αB-crystallin.
Myelin samples were lyophilized and dissolved in sample buffer (60 mM Tris-HCl (pH 6,8), 2% SDS, 10% glycerol, 5% 2-mercapto-ethanol, 0.01% bromophenol blue). They were subjected to standard SDS-PAGE analysis using a 8-25% gradient polyacrylamide gel (Pharmacia LKB). Western blots were analyzed for the presence of αB-crystallin using rabbit polyclonal anti-lap70 and alkaline phosphatase labeled swine-anti-rabbit antibodies (DAKO). Degradation of αB-crystallin was performed *in vitro* by the addition of 50 µg/ml cathepsin B (28 U/mg) and 50 µg/ml cathepsin D (8.5 U/mg) to a 2 mg/ml solution of αB-crystallin in 50 mM NaAc (pH 5.0) at 37°C. Cathepsin B and D were pre-

incubated in NaAc/HAc buffer supplied with 0.14 M 2-mercaptoethanol. Samples were taken at various timepoints and diluted 10-fold in 0.5 M Tris (pH 9.0) buffer to terminate proteolyitc activity. Western blot analysis of these samples using the polyclonal antibodies w3/13 and lap70 was performed as described above.

Antigens.
Whole myelin derived from an MS patient was isolated by density-gradient centrifugation, as described (10). Human myelin basic protein (MBP) was isolated from human myelin, as described previously (11). Human recombinant αB-crystallin was prepared as follows. αB-crystallin encoding mRNA derived from the astrocytoma cell line U373 was amplified by RT-PCR using primers, to which SacI and KpnI restriction sites were added at the 5' end of the upstream primer and HindIII and PstI restriction sites at the 5' end of the downstream primer. Primersequences are:
5'-GAGCTCGGTACCATGGACATCGCCATC and 5'-GGAAGCTTCTGCAGCTATTTCTTGGGGGCTGC.
The PCR product was digested with KpnI/HindIII and subcloned into the KpnI/HindIII site of vector pQE30 (Qiagen). The sequence of the construct was confirmed by sequence analysis using the ABI Prism^{tm} automatic sequencing system (Perkin Elmer). Histidine-tagged recombinant protein was isolated using a Ni-NTA column, followed by rp-HPLC purification. Identity of the protein was confirmed by Western blot analysis using αB-crystallin-specific mAb (3).

T cell proliferation assay.
PBMC from healthy control subjects were cultured in RPMI1640 medium (Dutch modification) supplemented with 100 U/ml penicilin, 0.1 mg/ml streptomycin, 2 mM L-glutamine, 1 mM sodium pyruvate, 10 mM HEPES (pH 7.4), and 5% NHS at 2 x 10^5 cells per 100 µl in round-bottom wells at 37°C and 5% CO_2 in the presence of 10 µg/ml human recombinant αB-crystallin or 25 µg/ml MS-affected myelin. On day 5 and 8, 10% Lymphocult-T (Biotest Seralc) in culture medium was added as source of growth-promoting cytokines including IL-2. At day 10 the T cells were harvested and antigen specificity was determined in a standard proliferation assay using purified monocytes/macrophages as antigen presenting cells.
In order to isolate monocytes/macrophages, 6-well plates were incubated overnight at room temperature with 250 µl (1 mg/ml) human plasma fibronectin (Life technologies). PBMC were seeded at a concentration of 2 x 10^6 /ml per well in culture medium and incubated for 2 h at 37°C and 5% CO_2. Non-adherent cells were removed by washing the wells twice with RPMI. After an additional 72 h incubation in culture medium at 37°C and 5% CO_2, the wells were washed with RPMI and adherent monocytes/macrophages were trypsinized using 0.25% w/v porcine trypsin (Sigma) in 0.12 M NaCl; 50 mM KCl; 10 mM $NaHCO_3$, 0.7 mM EDTA; 20 mM HEPES; 0.1 % w/v glucose. Purity of the monocyte/macrophage population was determined by direct immunofluorescence surface staining using mAbs directed against CD14, CD3 and

CD19 conjugated with FITC or phycoerytrin (Becton Dickinson) and analysed on a FACScalibur flow cytofluorometer using Cell Quest software (Becton Dickinson). The population contained > 85 % monocytes/macrophages. Proliferation assays were performed by seeding 5 x 10^4 T cells with varying doses of irradiated (30 Gray) monocytes/macrophages in the presence of 25 µg/ml human recombinant αB-crystallin or MS-affected myelin in 200 µl culture medium in flat-bottom wells. After 72 h incubation 20 kBq [^{3}H]-thymidine (Amersham Life Sciences) was added per well. After 16 h, [^{3}H]-thymidine incorporation was determined by using a beta plate counter (Canberra Packard).

IFN-γ assay.
To determine interferon-γ (IFN-γ) release in response to the antigens examined in the proliferation assays, 100 µl of culture supernatant was harvested prior to the addition of [^{3}H]-thymidine. IFN-γ production was determined by ELISA using a commercially available detection kit (Biosource).

Results

αB-Crystallin is present in phagocytic vesicles in a subset of myelin-phagocytosing macrophages in active MS lesions.
Lesions were selected on the basis of presence of inflammatory infiltrates and active demyelination. Myelin uptake, as revealed by ORO-positive lipids and MBP-immunostaining inside macrophages, indicated that ORO and MBP were detectable in numerous macrophages distributed throughout these lesions. In correspondence with our previous findings (6), such lesions contain oligodendrocytes and astrocytes with clearly elevated levels of αB-crystallin (Fig. 4.1 A). In three of these lesions αB-crystallin could also be detected in phagocytic vesicles of perivascular macrophages.
Colocalization of ORO, MBP, MOG and αB-crystallin in macrophages, as demonstrated by the use of serial sections (Fig. 4.1 B-E), demonstrated that all αB-crystallin containing macrophages were actively phagocytosing myelin. Moreover, αB-crystallin and MOG-containing macrophages were far less abundant than MBP or ORO-positive macrophages. They were restricted to the perivascular spaces and represented a small subset of MBP-positive macrophages only. In addition, MBP-containing macrophages were detected in more lesions than MOG- and αB-crystallin-containing macrophages and MOG was detected inside phagocytic vesicles in two lesions in which αB-crystallin was undetectable in macrophages (Table 4.1). The pattern of antigen-laden macrophages therefore suggested a temporal relationship in the disappearance of individual myelin antigens from phagocytic vesicles after myelin uptake in the following order: αB-crystallin, MOG, MBP.

αB-Crystallin-laden macrophages reflect very recent myelin phagocytosis.
In vitro experiments were performed to gain more insight into the detectability of individual myelin proteins within intracellular vesicles in macrophages. MS-affected myelin was fed to monocytes/macrophages freshly isolated from PBMC. This myelin contained small amounts of αB-crystallin as confirmed by Western blotting (data not shown). By immunohistochemical criteria, macrophages pulsed *in vitro* with whole myelin were indistinguishable from myelin-laden macrophages in MS lesions (Fig. 4.2 A-C). Within 6 h following myelin uptake αB-crystallin disappeared from phagocytic vesicles, whereas MBP and MOG remained detectable well after this period (Fig. 4.2 D-F). The order in which individual myelin antigens disappear from macrophages following myelin-uptake is a function of the sensitivity and specificity of the antibodies used to detect the antigens, the relative concentration of each antigen in myelin at the time of phagocytosis and the relative rate of degradation of each antigen in the endosomal pathway of macrophages. For the *in vitro* experiment of Fig. 4.2, these parameters are probably comparable to those that apply to Fig. 4.1. The antibodies used to stain the macrophages were the same as those used to stain the tissue sections of Fig. 4.1, the myelin membranes used as an antigen source in Fig. 4.2 were extracted from MS brains, and in both cases phagocytozed myelin appeared to undergo degradation in the endolysosomal compartment. These data therefore confirm the suggested order of disappearance of individual myelin antigens from phagocytic vesicles after myelin uptake in MS lesions (Table 4.1) and support the notion that the detectability of αB-crystallin in phagocytosing macrophages reflects very recent myelin uptake.

αB-Crystallin enters a proteolytic pathway inside macrophages after myelin phagocytosis.
In order to verify that the vesicles in which αB-crystallin was visible belong to the endosomal/lysosomal pathway, two antisera were used that discriminate between intact and cathepsin-degraded αB-crystallin. Recognition of αB-crystallin by the lap70 polyclonal antiserum is dependent on the integrity of the C-terminal amino acid sequence. This sequence is highly susceptible to cleavage by both cathepsin B and D, two major endosomal/lysosomal proteases. *In vitro* degradation followed by amino acid analyses revealed that cathepsin B recognizes two major scissile bonds in the C-terminus of αB-crystallin, i.e. T_{170}-A_{171} and K_{174}-P_{175} (data not shown). As shown by Western blotting, only the w3/13 antiserum recognizes αB-crystallin degradation products generated by cathepsin B and D digestion, albeit for a limited period of time (Fig. 4.3 A-C). The lap70 antiserum almost exclusively recognizes intact αB-crystallin, indicating that the integrity of the C-terminus of αB-crystallin is indeed rapidly lost upon proteolytic degradation.
Only w3/13 was able to detect αB-crystallin in phagocytic vesicles inside macrophages, whereas both antibodies proved able to recognize αB-crystallin expressed in the cytosol of astrocytes and oligodendrocytes. This confirms that αB-crystallin-positive vesicles indeed belong to the endosomal/lysosomal pathway. The pattern of recognition also

Table 4.1. Immunohistochemical analysis of myelin degradation products in actively demyelinating lesions.

Sample No.	Diagnosis	Age (Y)	DD (Y)	Cause of death	Sample Type	Presence of ORO$^+$ mϕ	Presence of MBP$^+$ mϕ	Presence of MOG$^+$ mϕ	Presence of αB-crystallin$^+$ mϕ
488-95	MS	47	20	Bronchopneumonia	Brain	++++	++++	+++	+++
488-96	MS	47	20	Bronchopneumonia	Brain	++++	++++	++	++
444-67	MS	29	8	Bronchopneumonia	Brain	++++	+++	++	+
488-94	MS	47	20	Bronchopneumonia	Brain	++++	++++	++	-
410-26	MS	64	14	Septacaemia	Brain	++	++	+	-
360-44	MS	60	28	Bronchopneumonia	Brain	++++	++++	-	-
444-67	MS	29	8	Bronchopneumonia	Brain	++++	++	-	-
413-75	MS	59	20	Bronchopneumonia	Brain	+	+	-	-
444-23	MS	29	8	Bronchopneumonia	Spinal Cord	++++	-	-	-
396-13	MS	37	10	Bronchopneumonia	Spinal Cord	+++	-	-	-
505-10	Control	62	-	Myocardial Infarct	Brain	-	-	-	-
501-10	Control	65	-	Myocardial Infarct	Brain	-	-	-	-

DD = Disease duration (in years).
+ ≤ 5 positive perivascular infiltrates
++ ≤ 10 positive perivascular infiltrates
+++ > 10 positive perivascular infiltrates
++++ widespread presence of positive macrophages, not restricted to perivascular spaces

renders it highly unlikely that the transient detection of αB-crystallin in macrophages reflects endogenous production of αB-crystallin rather than myelin-uptake.

Colocalization of T-cells, costimulatory molecules and macrophages that contain minor myelin proteins in perivascular infiltrates.
In order to examine where antigen presentation is most likely to take place, we analyzed the MS lesions for the presence of T cells, MHC class II molecules and the costimulatory molecules CD80 and CD40. MHC class II molecules were distributed widely on macrophages, microglia and astrocytes throughout the lesional area. In contrast, expression of the costimulatory molecules CD80 and CD40 on T cells and macrophages was found to be more restricted to the perivascular regions often localized

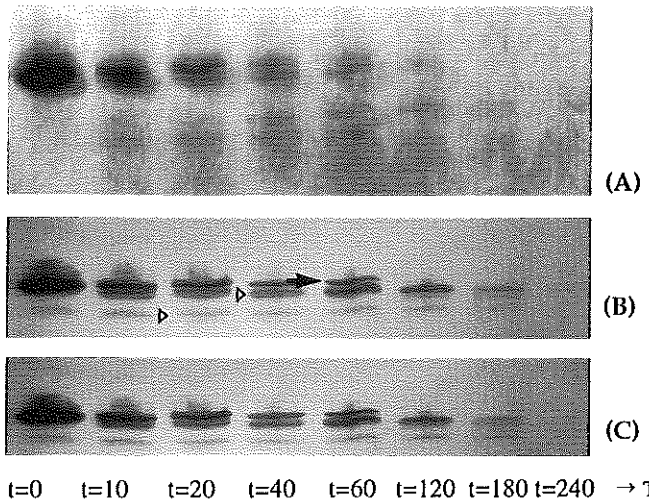

Figure 4.3. Recognition of αB-crystallin degradation products by the w3/13 antibody but not by the lap70 antibody. Purified αB-crystallin was digested *in vitro* with a mixture of cathepsin B and cathepsin D and samples taken at various points in time were analysed by SDS-gelelectrophoresis (A) and Western blotting. Western blot analysis revealed that polyclonal w3/13 (B) is able to recognize intact αB-crystallin (arrow) as well as several smaller degradation products (open arrowheads). This in contrast to the lap70 polyclonal (C), directed against the C-terminal 14 amino acids of αB-crystallin, that reacts almost exclusively with the intact αB-crystallin. The faint band below intact αB-crystallin presumably reflects N-terminal degradation.

at the lesion edge. Analysis of serial sections revealed that recent phagocytic activity, as shown by the presence of MOG in phagocytic vesicles of perivascular macrophages, colocalized with the presence of MHC class II and costimulatory molecules on macrophages as well as with the presence of T cells in the direct vicinity (Fig. 4.1 F-I). Antigen presentation in these lesions is thus most likely to take place in the perivascular regions, colocalizing with recent myelin phagocytosis.

In vitro uptake of whole myelin by macrophages leads to presentation of αB-crystallin to T cells. Next we investigated whether uptake of total myelin by monocytes/macrophages not only leads to protein degradation but also to presentation of αB-crystallin to T cells. Macrophages were fed *in vitro* with myelin membranes derived from MS-affected brain. As a read-out for antigen presentation, proliferative responses and IFN-γ production were monitored of αB-crystallin- and myelin-primed T-cells derived from the same donor as the macrophages. Proliferative responses of αB-crystallin-primed T cells were found against both αB-crystallin and myelin-fed macrophages (Fig. 4.4 A). In addition, these T cells produce readily detectable amounts of IFN-γ, indicative of antigen-specific

activation (Fig. 4.4 C). Similarly, myelin-primed T cells also proliferated and produced IFN-γ in response to either myelin or αB-crystallin (Fig. 4.4 B and 4.4 D). It is remarkable to note that the strength of the proliferative responses as well as the amount of IFN-γ produced by these T cells in response to αB-crystallin-fed macrophages are comparable with the responses measured against macrophages pulsed with total myelin. These results are in marked contrast to the lack of proliferation and IFN-γ production shown in response to macrophages fed with the major myelin constituent MBP.

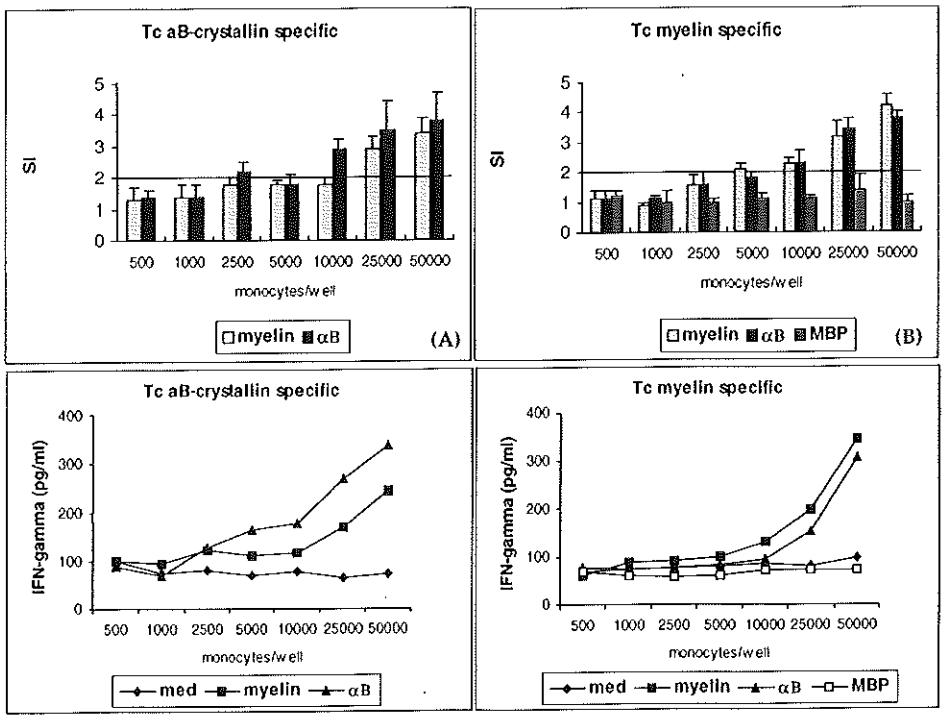

Figure 4.4. *In vitro* uptake of whole myelin by macrophages leads to presentation of αB-crystallin to T cells. PBMC were cultured for 10 days in the presence of either αB-crystallin (A and C) or whole myelin (B and D). T cells were harvested and antigen-specific proliferation was determined using increasing doses of myelin-, αB-crystallin-, or MBP-fed macrophages. The use of a standard proliferation assay (A and B) shows that αB-crystallin-primed T cells recognize αB-crystallin as well as myelin. MS myelin-primed T cells proliferate in reponse to myelin as well as to αB-crystallin-pulsed APC. In contrast, no proliferation was found in response to macrophages pulsed with the major myelin-constituent MBP. Both T-cell populations produce IFN-γ in response to either myelin or αB-crystallin (C and D), indicative of antigen-specific activation. med = medium.

All T cell responses were antigen specific since tetanus toxoid (TT)-primed T cells did neither proliferate nor produce IFN-γ in response to MBP-, αB-crystallin- or myelin-fed APC, while showing marked responses to APC fed with TT (data not shown).

Discussion

We have examined whether αB-crystallin, a candidate autoantigen in MS, is presented to the cellular immune system in demyelinating MS lesions. Therefore, we performed a detailed immunohistochemical analysis of 10, difficult to obtain, active demyelinating MS lesions.
The immunohistochemical analysis confirmed the previously reported enhanced expression of αB-crystallin in the cytosol of oligodendrocytes and astrocytes already during early phases of lesion formation. In this study, we show that intracellular vesicles in a subset of myelin-digesting macrophages contain αB-crystallin in addition to other myelin proteins. The use of two different antisera that discriminate intact from partially degraded αB-crystallin showed that inside these macrophages αB-crystallin enters a proteolytic pathway, most likely resulting in MHC class II-restricted presentation of αB-crystallin-derived peptides to T cells.
The detectability of myelin proteins in phagocytosing macrophages in MS lesions using immunohistochemistry has been described in great detail and is used to estimate lesional age (8). Major myelin constituents like MBP remain detectable in phagocytic vesicles inside macrophages for a longer period of time than minor myelin constituents like CNPase or MOG. Lesions or lesional areas containing macrophages positive for minor myelin proteins reflect recent demyelination and are therefore classified as early active lesions (7, 8). Since αB-crystallin is a minor myelin constituent one would expect the detectability of this protein inside macrophages to be limited. The pattern of αB-crystallin-positive macrophages in actively demyelinating lesions was consistent with this expectation. A small number of MBP-positive macrophages and a larger number of MOG-positive macrophages were also found to be positive for αB-crystallin. Detectability of αB-crystallin in macrophages is thus an even more selective feature than the detectability of MOG. *In vitro* assays in which macrophages were fed MS brain-derived total myelin confirmed that the detectability of αB-crystallin in phagocytosing macrophages is indeed limited to a very short period of time, i.e. less than 6 h, compared to MBP and MOG which were still present after 6 h (A. van der Goes et al., in preparation). Thus, detectability of αB-crystallin inside macrophages can be used as a marker for very recent myelin uptake.
Macrophages that contained endosomal/lysosomal vesicles positive for MBP, MOG and αB-crystallin, expressed MHC class II as well as co-stimulatory molecules CD80 and CD40, and they were found in the close proximity of infiltrating T cells. This indicates that all factors required for productive (re)activation of T cells accumulate in the areas

where MOG- and αB-laden macrophages were observed. The strict perivascular localization of APC that are fully equiped to present antigen and deliver the necessary co-stimulatory signals is in concordance with earlier reports describing expression patterns of CD80 and CD40 in MS lesions (12-15). Experimental allergic encephalomyelitis (EAE) studies using mannosylated liposome-encapsulated dichloromethylene diphosphonate to deplete peripheral monocytes (16-18) have shown an important role for hematogenous, perivascular macrophages in the induction of EAE. Also, bone marrow chimera studies showed that the inflammatory response in EAE can start, proceed and end virtually in the absence of resident microglia that may also be able to present antigen in the right MHC class II context (19). Recently, hematogenous macrophages were shown to modulate local activation of T cells in the brain and subsequent migration of macrophages into the brain parenchyma in adoptively transferred EAE (20). Functional interaction between monocytes/macrophages and T cells is likely to play a key role in the early phases of MS lesion development (21, 22). The perivascular localization of the phagocytosing CD68-positive cells strongly suggests that these cells are infiltrating hematogenous macrophages and not resident microglia. Recently, there have been reports that local activation of resident microglia in normal appearing white matter in MS brains is followed by myelin phagocytosis as an initiating or very early event in MS pathogenesis, followed by the recruitment of large numbers of hematogenous macrophages (23). In our studies minor myelin protein-positive microglia in unaffected white matter were not observed. The most recent myelin breakdown in our material, as assessed by the detectability of minor myelin proteins in macrophages, was found to occur in the perivascular spaces. Clustering of such bloodvessels was often found at the edges of the lesion.

The absence of dendritic cells in the CNS renders it highly unlikely that the interactions between APC and T cells in the perivascular spaces lead to activation of naive T cells against myelin-derived antigens. Therefore, we would strongly favour the idea that, if these T cells are to play a role in the pathogenesis of MS, they must already have been primed against myelin antigens in the periphery. Several mechanisms have been decribed for such a peripheral priming against CNS-specific autoantigens, many of which hypothesize the involvement of infectious agents (8, 24-27). Recently, we described a novel mechanism for the activation of peripheral T cells to αB-crystallin, involving virus-induced presentation of this antigen by EBV-infected B lymphocytes to peripheral T cells (28).

The presence of αB-crystallin inside phagocytic vesicles in macrophages in the vicinity of infiltrating T cells strongly suggests functional antigen presentation to T cells. In order to confirm functional presentation of myelin-derived αB-crystallin to T cells we used peripheral monocytes/macrophages fed with total myelin membranes. Total myelin membranes contain 75% lipids, whereas of the remaining 25% protein fraction MBP makes up about 12% and αB-crystallin only makes up 0.5%. Despite this low levels

of expression in whole myelin we showed that much of the total T cell response to total myelin is accounted for by the responses to αB-crystallin. This is consistent with the previously reported vigorous T cell responses to αB-crystallin (3). Although we took great care in purifing monocytes from peripheral blood we can not rule out that small numbers of dendritic cells or B cells might have had a role in antigen presentation in addition to macrophages in these *in vitro* assays. Even if this were the case, however, our data still show that with MS-affected myelin as starting material, αB-crystallin becomes available for T cell recognition and acts as a dominant antigen.
In summary, data presented here show that the presence of αB-crystallin in myelin-phagocytosing macrophages can be used as a marker for very recent myelin uptake. In these macrophages αB-crystallin enters the endosomal/lysosomal compartment which generally leads to MHC class II-restricted antigen presentation. Functional presentation of total myelin-derived αB-crystallin was demonstrated using hematogenous macrophages as APC in an *in vitro* approach. The localization and immunological properties of αB-crystallin containing macrophages strongly suggests that functional presentation of αB-crystallin to T cells takes place in the perivascular spaces of active MS lesions. As an early event following myelin phagocytosis αB-crystallin becomes available to the cellular immune system. The vigorous response of T-cells to this protein is likely to initiate (29) or to enhance the ongoing immune response via chemokine/cytokine-induced attraction of macrophages and modulation of the properties of the blood brain barrier.

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References

1. Amor, S., D. Baker, L. Layward, K. McCormack, and J. M. van Noort. 1997. Multiple sclerosis: variations on a theme. *Immunol. Today 8:368.*

2. ffrench-Constant, C. 1994. Pathogenesis of multiple sclerosis. *Lancet 343:271.*

3. Van Noort, J. M., A. C. van Sechel, J. J. Bajramović, M. El Ouagmiri, C. H. Polman, H. Lassmann, and R. Ravid. 1995. The small heat shock protein αB-crystallin as candidate autoantigen in multiple sclerosis. *Nature 375:798.*

4. Ingolia, T. D. and E. A. Craig. 1982. Four small heat shock proteins are related to each other and to mammalian α-crystallin. *Proc. Natl. Acad. Sci. USA 79:2360.*

5. Klemenz, R., E. Frohll, R. H. Steiger, R. Schafer, and A. Aoyama. 1991. αB-crystallin is a small heat shock protein. *Proc. Natl. Acad. Sci. USA 88:3652.*

6. Bajramović, J. J., H. Lassmann, and J. M. van Noort. 1997. Expression of αB-crystallin in glia cells during lesional development in multiple sclerosis. *J. Neuroimmunol. 78:143.*

7. Brück, W., M. Schmied, G. Suchanek, Y. Brück, H. Breitschopf, S. Poser, S. Piddlesden, and H. Lassmann. 1994. Oligodendrocytes in the early course of multiple sclerosis. *Ann. Neurol. 35:65.*

8. Bruck, W., P. Porada, S. Poser, P. Rieckmann, F. Hanefeld, H. A. Kretzschmar, and H. Lassmann. 1995. Monocyte/macrophage differentiation in early multiple sclerosis lesions. *Ann Neurol. 38:788.*

9. Martin, R., and H. F. McFarland. 1995. Immunological aspects of experimental allergic encephalomyelitis and multiple sclerosis. *Crit. Rev. Clin. Lab. Sci. 32:121.*

10. Norton, W. T., and S. E. Poduslo. 1973. Myelination in rat brain: method of myelin isolation. *J. Neurochem. 21:749.*

11. van Noort, J. M., M. el Ouagmiri, J. Boon, and A. C. van Sechel. 1994. Fractionation of central nervous system myelin proteins by reversed-phase high-performance liquid chromatography. *J. Chromatogr. B. Biomed. Appl. 653:155.*

12. De Simone, R., A. Giampaolo, B. Giometto, P. Gallo, G. Levi, C. Peschle, and F. Aloisi. 1995. The costimulatory molecule B7 is expressed on human microglia in culture and in multiple sclerosis acute lesions. *J. Neuropathol. Exp. Neurol. 54:175.*

13. Williams, K., E. Ulvestad, and J. P. Antel. 1994. B7/BB-1 antigen expression on adult human microglia studied in vitro and in situ. *Eur. J. Immunol. 24:3031.*

14. Windhagen, A., J. Newcombe, F. Dangond, C. Strand, M. N. Woodroofe, M. L. Cuzner, and D. A. Hafler.1995. Expression of costimulatory molecules B7-1 (CD80), B7-2 (CD86), and interleukin 12 cytokine in multiple sclerosis lesions. *J Exp Med. 182:1985.*

15. Gerritse, K., J. D. Laman, R. J. Noelle, A. Aruffo, J. A. Ledbetter, W. J. Boersma, and E. Claassen.1996. CD40-CD40 ligand interactions in experimental allergic encephalomyelitis and multiple sclerosis. *Proc. Natl. Acad. Sci. USA. 93:2499.*

16. Bauer, J., I. Huitinga, W. Zhao, H. Lassmann, W. F. Hickey, and C. D. Dijkstra. 1995. The role of macrophages, perivascular cells, and microglial cells in the pathogenesis of experimental autoimmune encephalomyelitis. *Glia. 15:437.*

17. Brosnan, C. F., M. B. Bornstein, and B. R. Bloom. 1981. The effects of macrophage depletion on the clinical and pathologic expression of experimental allergic encephalomyelitis. *J. Immunol. 126:614.*

18. Huitinga, I., N. van Rooijen, C. J. de Groot, B. M. Uitdehaag, and C. D. Dijkstra. 1990. Suppression of experimental allergic encephalomyelitis in Lewis rats after elimination of macrophages. *J. Exp. Med. 172:1025.*

19. Hickey, W. F., and H. Kimura. 1988. Perivascular microglial cells of the CNS are bone marrow-derived and present antigen in vivo. *Science. 239:290.*

20. Tran, E. H., K. Hoekstra, N. van Rooijen, C. D. Dijkstra, and T. Owens. 1998. Immune invasion of the central nervous system parenchyma and experimental allergic encephalomyelitis, but not leukocyte extravasation from blood, are prevented in macrophage-depleted mice. *J. Immunol. 161:3767.*

21. Benveniste, E. N. 1997. Role of macrophages/microglia in multiple sclerosis and experimental allergic encephalomyelitis. *J. Mol. Med. 75:165.*

22. Bauer, J., S. R. Ruuls, I. Huitinga, and C. D. Dijkstra. 1996. The role of macrophage subpopulations in autoimmune disease of the central nervous system. *Histochem. J. 28:83.*

23. Li, H., M. L. Cuzner, and J. Newcombe. 1996. Microglia-derived macrophages in early multiple sclerosis plaques. *Neuropathol. Appl. Neurobiol. 22:207.*

24. Van Noort, J. M and S. Amor. 1998. Cell biology of autoimmune diseases. *Int. Rev. Cytol. 178:127.*

25. Dalgleish, A. G., J. K. Fazakerley, and H. E. Webb. 1987. Do human T-lymphotrophic viruses (HTLVs) and other enveloped viruses induce autoimmunity in multiple sclerosis? *Neuropathol. Appl. Neurobiol. 13:241.*

26. Racke, M. K., L. Quigley, B. Cannella, C. S. Raine, D. E. McFarlin, and D. E. Scott. 1994. Superantigen modulation of experimental allergic encephalomyelitis: activation of anergy determines outcome. *J. Immunol. 152:2051.*

27. Wucherpfennig, K. W. and J. L. Strominger. 1995. Molecular mimicry in T cell-mediated autoimmunity: viral peptides activate human T cell clones specific for myelin basic protein. *Cell. 80:695.*

28. van Sechel, A. C., J. J. Bajramović, M. J. van Stipdonk, C. Persoon-Deen, S. B. Geutskens, and J. M. van Noort. 1999. EBV-induced expression and HLA-DR-restricted presentation by human B cells of alpha B-crystallin, a candidate autoantigen in multiple sclerosis. *J Immunol. 162:129.*

29. Van Noort, J. M., A. C. van Sechel, M. J. B. van Stipdonk, and J. J. Bajramović. 1998. The small heat-shock protein αB-crystallin as key autoantigen in multiple sclerosis. *In Progress in Brain Research,* Vol. 117. F.W. van Leeuwen, A. Salehi, R.J. Giger, A.J.G.D. Holtmaat, J. Verhaagen, editors. Elsevier Science B.V. p. 435.

5.

The stress-kit: a new method based on competitive reverse transcriptase-polymerase chain reaction to quantify the expression of human αB-crystallin, hsp27, and hsp60

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Abstract

We describe a reverse transcriptase-polymerase chain reaction method for the semi-quantitative detection of mRNAs encoding the human heat shock proteins αB-crystallin, hsp27 and hsp60. The method involves the co-amplification of cellular mRNA-derived cDNA with a dilution series of a competitor fragment (internal standard), using one primer pair common to both templates. Internal standards were based on cellular-derived cDNA engineered to be slightly smaller in order to differentiate between the target and the standard upon electrophoretic separation. Initial cDNA quantitations can be corrected for possible variations during cDNA-synthesis by standardizing to the levels of β-actin-encoding cDNA.
We show that the co-amplified templates accumulate in a parallel manner with the cellular-derived cDNA throughout both the exponential and the non-exponential phase of amplification. Furthermore, we illustrate the utility of this technique by quantifying increased expression of αB-crystallin, hsp27 and hsp60 mRNA in astroglioma cells upon heat shock.

Introduction

Several lines of evidence indicate that the cellular mechanisms by which hsp expression is regulated in various types of cells e.g. in the human central nervous system (CNS) are different (Freedman et al 1992; Satoh and Kim 1995; Bajramović et al 1997). In addition, there is also evidence for differential expression patterns of different hsp in the same cell type in response to the same stimulus (Wiegant et al 1994; Lee et al 1995; Head et al 1996). Primary rat astrocytes for example show an increase in mRNA expression levels of αB-crystallin but not of hsp27 in response to TNF-α stimulation (Head et al 1994). Such observations indicate that hsp expression, in response to stressors other than heat shock, are regulated by differential control mechanisms rather than by uniform mechanisms. In many cases the promoter sequences that are involved are unknown or still await characterization. Consequently, predictions on possible hsp induction by specific stressors is prevented. In order to allow studies on the regulation of hsp, reliable methods to quantitate hsp-encoding mRNAs are an important prerequisite. In this paper we describe a sensitive competitive reverse transcriptase-ploymerase chain reaction (rt-PCR) method to quantify the mRNA levels of three human hsp viz. αB-crystallin, hsp27 and hsp60. These three hsp were selected on basis of their presumed involvement in several diseases. αB-Crystallin has been described as a putative autoantigen in multiple sclerosis (MS; van Noort et al 1995), hsp27 is associated with enhanced tumorgenicity of a variety of cell types (Garrido et al 1998; Lemieux et al 1997) and hsp60 is thought to be involved in the pathogenesis of atherosclerosis (Roma and Catapano 1996), type I diabetes (Abulafia et al 1999), MS (Selmaj et al 1992), and rheumatoid arthritis (De Graeff-Meeder et al 1991). Quantitation by PCR is difficult because the efficiency of PCR amplification may vary among samples mainly due to differences in the quality of mRNA/cDNA preparations and to variations occurring during the PCR reaction. Therefore we chose to design a competitive PCR in which a competitor control fragment (internal standard) is co-amplified along with sample cDNA using one set of primers within a single PCR-mixture (Wang et al 1989; Siebert and Carrick 1992). The internal standard was engineered to be slightly smaller than the target in order to allow separation of the respective amplicons using agarose gel electrophoresis. PCR reaction tubes containing the target samples were spiked with a dilution series of internal standard. When the molar ratio of the PCR products generated from target and internal standard is equal to one, the amount of target cDNA is equal to that of the internal standard. Since the amount of internal standard is known, the amount of target can thus be determined. Initial cDNA quantitations were corrected for possible variations during cDNA-synthesis by standardizing to cDNA levels of the endogenous housekeeping gene β-actin. In this report we describe the optimization and validation of this assay.

Material and Methods

In vitro culture of the U373 MG astroglioma cell line

U373 MG (Number HTB-17; American Type Culture Collection, Manassas, VA) cells were cultured in 1:1 v/v Dulbecco's Modified Eagle's Medium (high glucose; Life Technologies, Breda, The Netherlands) /HAMF10 (with L-glutamine; Life Technologies) + 10% v/v FCS (Gibco; Life Technologies) and antibiotic supplement (penicillin 100U/ml and streptomycin 0.1 mg/ml) at 37°C in a humidified atmosphere containing 5% CO_2. Cells (2.5×10^5) were seeded into poly-L-lysine (PLL, 15 µg/ml; Sigma, St. Louis, MO) coated 25 cm^2 flasks. To passage cells they were rinsed with PBS, incubated for 5-10 min with 0.25% w/v porcine trypsin (Sigma) at 37°C, washed once with culture medium containing 10% v/v FCS and replated in PLL-coated flasks. Confluent cell cultures were exposed to heat shock at 43°C by the addition of an equal volume of prewarmed medium at 49°C. Cells were left for 30 min at 43°C before they were allowed to recover at 37°C.

Competitive rt-PCR

Total RNA was isolated directly from cells in the culture flasks using RNAzolB according to the manufacturer's protocol (Campro Scientific, Veenendaal, The Netherlands) and precipitated with isopropanol. Using 2.5 µg of RNA as a template, 50 µl of copy DNA (cDNA) was produced using the Reverse Transcription System (Promega, Madison, WI). For amplification, sample cDNA (1 µl), internal standard cDNA (1 µl) and the appropriate primer pair (1 µl for each primer of a 20 µM solution) were added to 1 µl 10 mM dNTP mix, 5 µl 10x PCR-buffer (0.1 M Tris/HCl (pH 8.4), 0.5 M KCl and 0.6 mg BSA/ml), 1 µl Taq-mix (PCR-buffer containing 1 U *Taq* polymerase (Life Technologies, Amsterdam, The Netherlands)) and 39 µl Depc-H_2O. For β-actin and hsp60 22.5 mM $MgCl_2$, for αB-crystallin 30.0 mM $MgCl_2$ and for hsp27 15.0 mM $MgCl_2$ was added to the 10x PCR buffer. Competitive rt-PCR was performed at 30 cycles for β-actin, αB-crystallin and hsp60 and at 27 cycles for hsp27. One cycle consisted of 30 s melting at 94°C, 30 s annealing at 57°C and 30 s elongation at 72°C. Primers used and amplified fragment sizes are given in Table 5.1. Sample cDNA was amplified together with increasing amounts of the appropriate internal standard (see below). The 50% equivalence point was calculated following agarose gel (2%) electrophoresis and densitometrical analysis (software: Bioprofil V6.0, Vilber Lourmat, France) of the ethidium bromide-stained amplicons. Linear regression was applied on five 2-fold dilution analyses centered around the 50% equivalence point. Initial cDNA quantitations were corrected for possible variations that might have occurred during cDNA-synthesis by standardizing to the levels of β-actin-encoding cDNA and thus

converted into relative content values. When absolute cDNA values for β-actin were below 0.1 ng/ml, cDNA synthesis was repeated using more mRNA.

Development of the internal standards

Internal standards were developed using RNA of unstimulated U373 MG cells. The mRNA was transcribed into cDNA and used for rt-PCR as described above. After agarose gel electrophoresis the PCR-product was isolated from gel and purified using the Geneclean kit (Bio 101 Inc., Vista, CA). Purified PCR-products were then cloned into a pGEM-T vector (Promega) and competent *E. coli* cells (strain JM109) were transformed with these vectors. Positive (white) clones were selected using a conventional blue-white screening method. Isolated plasmids (Qiagen, Leusden, The Netherlands) were subjected to restriction analysis in order to verify the length of the insert. Correct plasmids were subjected to digestion with specificially chosen restriction enzymes resulting in amplified fragments that were approximately 50 bp shorter than the original fragments. For β-actin Bal I and Bsu36 I (Promega) were used, for αB-crystallin BamH I (Promega) and Dra II (Roche, Almere, The Netherlands), for hsp27 Bsu36 I and Nhe I (Promega) and for hsp60 Hpa I (Promega) and Dra II (Roche). Non-homologous sticky ends generated by the different restriction enzymes were blunt-ended by treatment with 1 U/μl Mung Bean nuclease (Promega) for 30 min at 30°C. After subsequent ligation and transformation of the plasmids to *E. coli*, clones were analyzed by restriction analysis. Plasmids displaying the expected restriction pattern were selected and sequenced using the T7 sequencing kit (Pharmacia, Roosendaal, The Netherlands) to confirm the correct sequence of the complete internal standards.

Parallellism assays

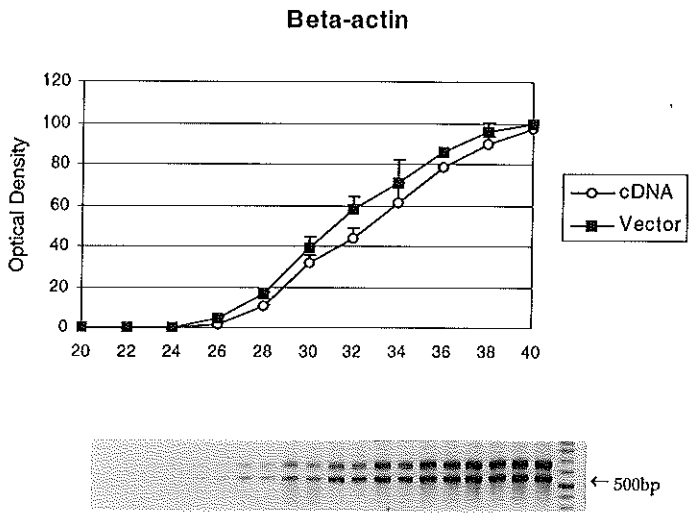
Parallellism assays were performed using approximately the same amounts of (predetermined) sample cDNA and internal standard. Rt-PCR was performed as described above. From cycle 20 until cycle 40, PCR reactions of two samples were terminated every two cycli. These samples were then subjected to gel electrophoresis to compare amplification efficiencies of the sample cDNA and the internal standard by densitometrical analysis.

Results and Discussion

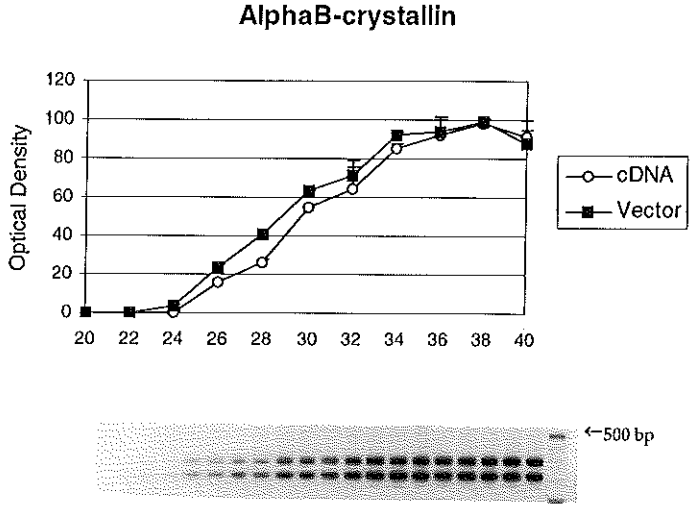
A competitive rt-PCR assay was developed in order to allow quantitation of mRNA levels of three human heat shock proteins. Table 5.1 shows the sequences of the primers selected and the amplicon lengths that were generated. All amplified fragments were analyzed by sequence-analysis (data not shown). Internal standards were synthesized and used to quantify mRNA-derived cDNA levels of hsp and β-actin (as described). cDNA levels of the housekeeping gene β-actin were used to correct for possible variations during cDNA synthesis from different samples.
To assess whether amplification efficiencies of sample cDNA and of the (smaller) internal standards were comparable, parallellism assays were performed (Becker-André and Hahlbrock 1989; Gilliland et al 1990). Results show that the amplification of the internal standards and the samples are indeed parallel up to the plateau phase (figure 5.1). Since the amplification efficiencies were identical for targets and internal standards throughout the amplification process, the amount of hsp-cDNA in the total cDNA can theoretically be calculated at any cycle number in the range examined (Bouaboula et al, 1992). However, it should be noted that during the competitive PCR for hsp27 at higher cycle numbers (≥cycle 32) heteroduplex formation, composed of single stranded (ss) internal standard cDNA and ss sample cDNA, was observed (figure 5.1c). This heteroduplex formation, visible on the gel as a faint third band that migrated slower than the sample cDNA, can interfere with precise quantification. This phenomenon, also reported by others (Henley et al 1996; Boer and Ramamoorthy 1997), was observed only when a 1:1 ratio of sample and internal standard was used. In our system, heteroduplex formation can be prevented either by using fewer PCR cycles for quantitation of hsp27 cDNAs, or by performing agarose gel electrophoresis under denaturing conditions. As fewer amplification cycles (25-30) still generate a sufficient amount of amplicons for quantitation this solution for prevention of heteroduplexes is preferable.

Table 5.1. Primers used to amplify internal standards and sample cDNA and the amplified fragment sizes.

mRNA	Upstream primer 5'----------3'	Downstream primer 5'----------3'	*Sample cDNA (bp)*	*Internal standard (bp)*
β-Actin	AAGATGACGCAGATCATGTTTGAG	AGGAGGAGCAATGATCTTGATCTT	649	557
αB-Crystallin	AGCTGGTTTGACACTGGACT	GCAATTCAAGAAAGGGCATC	372	315
Hsp27	TCCCTGGATGTCAACCACTT	CAAAAGAACACACAGGTGGC	399	356
Hsp60	TTCGATGCATTCCAGCCTTG	TTGGGCTTCCTGTCACAGTT	440	388

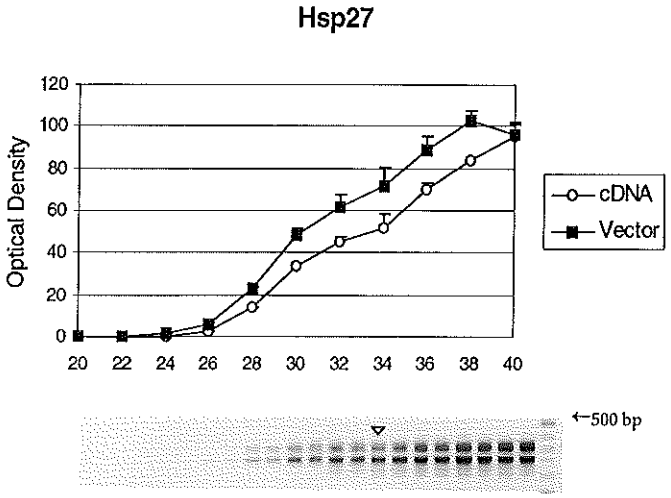


(A) β-actin parallellism assay with 1.25 ng internal standard/ml.

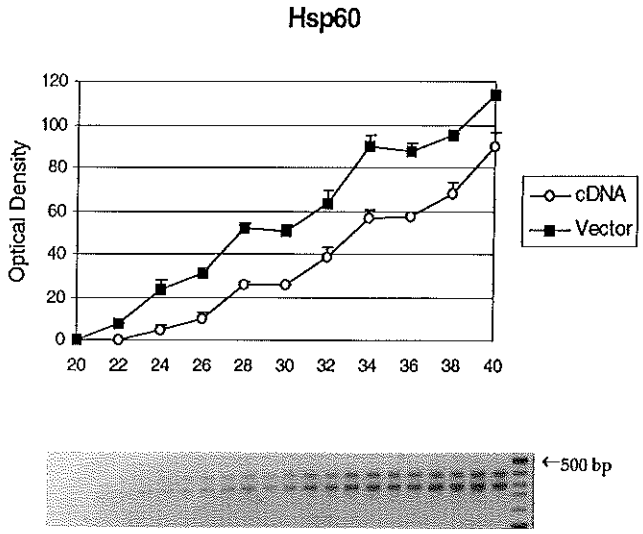


(B) αB-Crystallin parallellism assay with 0.5 ng internal standard/ml.

Figure 5.1 A-B. Parallellism assays for β-actin and αB-crystallin. Parallellism assays were performed using approximately the same amounts of internal standard and sample cDNA. Starting at cycle 20 and ending at cycle 40 amplicons were sampled in duplo every second cycle. Amplicon samples were subjected to gelelectrophoresis (lower figures) and densitometrical analysis (upper graphs; each point is the mean of 2 analyses).



(C) Hsp27 parallellism assay with 0.4 ng internal standard/ml. The extra band appearing as a result of heteroduplex formation (visible ≥ cycle 32) is indicated by an open arrowhead at cycle 34.



(D) Hsp60 parallelism assay with 2 ng internal standard/ml.

Figure 5.1 C-D. Parallellism assays for hsp27 and hsp60. Parallellism assays were performed using approximately the same amounts of internal standard and sample cDNA. Starting at cycle 20 and ending at cycle 40 amplicons were sampled in duplo every second cycle. Amplicon samples were subjected to gelelectrophoresis (lower figures) and densitometrical analysis (upper graphs; each point is the mean of 2 analyses).

The applicability of the technique was tested by subjecting U373 MG astroglioma cells to heat shock and analyzing hsp mRNA levels at three subsequent points in time. cDNA values for all proteins, as well as both their standardization to β-actin cDNA levels and final standardization to the levels of unstimulated cells, are shown in Table 5.2.

Table 5.2. β-Actin, αB-crystallin, hsp27 and hsp60 mRNA-levels in U373 cells at different points in time following heat shock as determined by competitive rt-PCR[a].

Time (h)	β-Actin	αB-Crystallin	Hsp27	Hsp60
cDNA (ng/ml)				
0	0.695	0.006	0.103	0.244
1	0.724	0.005	0.121	0.248
2	0.624	0.008	0.444	0.756
4	0.17	0.007	0.141	0.485
Relative contents (corrected per time for β-actin)				
0	1	0.008	0.149	0.351
1	1	0.007	0.168	0.342
2	1	0.012	0.712	1.212
4	1	0.039	0.827	2.853
Relative values (relative to unstimulated values at t = 0)				
1	1	0.88	1.128	0.975
2	1	1.515	4.795	3.456
4	1	4.861	5.567	8.14

[a]Sample cDNA was amplified together with a dilution series of internal standards and the 50% equivalence point was calculated following gel electrophoresis and densitometrical analysis of the amplicons. Relative contents were calculated by dividing the hsp values at a given time point by the β-actin value for that given time point. Subsequently relative values as compared to the unstimulated U373 cells (t = 0 h) were calculated.

Quantitation of mRNA-derived cDNA was highly reproducible, even when separately synthesized cDNA batches of the same mRNAs were used (data not shown). Figure 5.2 shows that heat shock indeed resulted in an upregulation of the mRNAs of all three hsp tested. This was confirmed at the protein level by immunohistochemistry (data not shown).

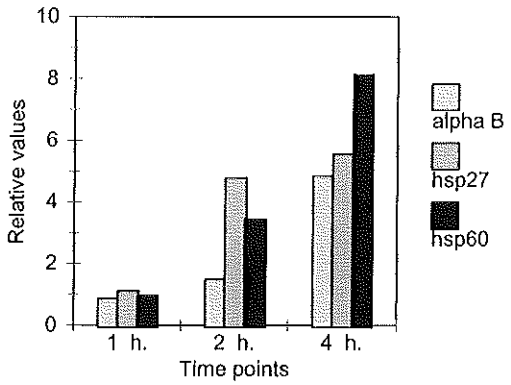


Figure 5.2. Upregulation of αB-crystallin, hsp27 and hsp60 following heat shock of U373 cells.
Relative values of αB-crystallin, hsp27 and hsp60 mRNA levels are shown as calculated in Table 5.2.

Interestingly, the kinetics of the three hsp studied differed. Where both hsp27 and hsp60 mRNA levels were upregulated within two hours, αB-crystallin showed markedly slower kinetics. The sensitivity of the quantitative PCR-assay was within the 10-100 picogram hsp cDNA/ml range. mRNA isolated from 5,000-10,000 U373 cells still allowed the production of sufficient amounts of cDNA to perform reproducible quantitative PCR analyses. However, sensitivity should be assessed for each cell type separately since constitutive hsp mRNA levels vary considerably between different types of cells.
The differential expression patterns of hsp in the same cell type in response to the same stimulus (Wiegant et al 1994; Lee et al 1995; Head et al 1996; Bajramović et al 1999) indicate that hsp expression in response to stressors other than heat shock is regulated by differential control mechanisms rather than by uniform mechanisms. The assay described here provides a powerful tool to analyze such control mechanisms for αB-crystallin, hsp27 and hsp60 in response to various stimuli.

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References

Abulafia-Lapid, R Elias, D Raz, I Keren-Zur, Y Atlan, H Cohen, IR (1999) T-cell proliferative responses of type I diabetes patients and healthy individuals to human hsp60 and its peptides. J. Autoimmun. **12(2)**,121-129.

Bajramović, JJ Lassmann, H van Noort, JM (1997) Expression of αB-crystallin in glia cells during lesional development in multiple sclerosis. J. Neuroimmunol. **78**, 143-151.

Bajramović, JJ Bsibsi, M Geutskens, SB Hassankhan, R Verhulst, KC Stege, GJJ de Groot, CJA van Noort, JM (1999) Differential expression of stress proteins in human adult astrocytes in response to cytokines. *Submitted.*

Becker-André, M and Hahlbrock, K (1989) Absolute mRNA quantification using the polymerase chain reaction (PCR). A novel approach by a PCR aided transcript titration assay (PATTY). Nucleic Acids Res. **17(22)**, 9437-9446.

Boer, PH and Ramamoorthy, J (1997) How to correct for errors in mRNA quantitation by competitive PCR due to heteroduplex formation of amplification products. Cell. Mol. Biol. **43(6)**, 841-850.

Bouaboula, M Legoux, P Pességué, B Delpech, B Dumont, X Piechaczyk, M Casellas, P Shire, D (1992) Standardization of mRNA titration using a polymerase chain reaction method involving co-amplification with a multispecific internal control. J. Biol. Chem. **267 (30)**, 21830-21838.

De Graeff-Meeder, ER van der Zee, R Rijkers, GT Schuurman, HJ, Kuis, W, Bijlsma, JWJ Zegers, BJM van Eden, W (1991) Recognition of human 60 kD heat shock protein by mononuclear cells from patients with juvenile chronic arthritis. Lancet **337**, 1368-1372.

Freedman, MS Buu, NN Ruijs, TCJ Williams, K Antel, JP (1992) Differential expression of heat shock proteins by human glial cells. J. Neuroimmunol. **41**, 231-238.

Garrido, C Fromentin, A Bonnotte, B Favre, N Moutet, M Arrigo, AP Mehlen, P Solary, E (1998) Heat shock protein 27 enhances the tumorigenicity of immunogenic rat colon carcinoma cell clones. Cancer Res. **58(23)**, 5495-5499.

Gilliland, G Perrin, S Blanchard, K Bunn, HF (1990) Analysis of cytokine mRNA and DNA: detection and quantitation by competitive polymerase chain reaction. Proc. Natl. Acad. Sci. USA **87(7)**, 2725-2729.

Head, MW Corbin, E Goldman, JE (1994) Coordinate and independent regulation of αB-crystallin and hsp27 expression in response to physiological stress. J. Cell. Physiol. **159**, 41-50.

Head, MW Hurwitz, L Goldman, JE (1996) Transcription regulation of alpha B-crystallin in astrocytes: analysis of HSF and AP1 activation by different types of physiological stress. J. Cell. Sci. **109 (Pt 5)**, 1029-1039.

Henley, WN Shuebel, KE and Nielsen, DA (1996) Limitations imposed by heteroduplex formation on quantitative RT-PCR. Biochem. Biophys. Res. Commun. **226(1)**, 113-117.

Lee, YJ Berns, CM Galoforo, S Erdos, G Cho, JM Corry, PM (1995) Differential effect of 1-(5-isoquinolinesulfonyl)-2-methylpiperazine (H-7) on αB-crystallin and hsp70 gene expression in murine cell lines. Biochem. Pharmacol. **50(8),** 1149-1155.

Lemieux, P Oesterreich, S Lawrence, JA Steeg, PS Hilsenbeck, SG Harvey, JM Fuqua, SA (1997) The small heat shock protein hsp27 increases invasiveness but decreases motility of breast cancer cells. Invasion Metastasis **17(3),** 113-123.

Roma, P and Catapano, AL (1996) Stress proteins and atherosclerosis. Atherosclerosis **127(2),** 147-154.

Satoh, JI and Kim, SU (1995) Differential expression of heat shock protein hsp27 in human neurons and glial cells in culture. J. Neurosci. Res. **41 (6),** 805-818.

Selmaj, K Brosnan, CF Raine, CS (1992) Expression of heat shock protein-65 by oligodendrocytes in vivo and in vitro: implications for multiple sclerosis. Neurology **42(4),** 795-800.

Siebert, PD and Carrick JW (1992) Competitive PCR. Nature. **359,** 557-558.

Van Noort, JM van Sechel, AC Bajramović, JJ El Ouagmiri, M Polman, CH Lassmann, H Ravid, R (1995) The small heat shock protein αB-crystallin as candidate autoantigen in multiple sclerosis. Nature **375,** 798-801.

Wang, AM Doyle, MV and Mark DV (1989) Quantitation of mRNA by the polymerase chain reaction. Proc. Natl. Acad. Sci. USA. **86(24),** 9717-21.

Wiegant, FA Souren, JE van Rijn, J van Wijk R (1994) Stressor-specific induction of heat shock proteins in rat hepatoma cells. Toxicol. 94, 143-159.

6.

Differential expression of stress proteins in human adult astrocytes in response to cytokines

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Abstract

Various lines of evidence suggest a close relationship between heat shock proteins (hsp) and several autoimmune diseases such as arthritis, diabetes and multiple sclerosis. While enhanced expression of hsp in autoimmune diseases is often regarded as a non-specific bystander effect of the inflammatory process, surprisingly little is known on hsp regulation by inflammatory mediators such as cytokines. In this study cytokine-induced expression of hsp60, hsp27 and αB-crystallin was studied in cultures of primary human adult astrocytes at the mRNA as well as at the protein level. We show differential hsp expression patterns in response to pro-inflammatory and immunoregulatory cytokines. Hsp60 expression was found to be enhanced in response to cytokines as diverse as IL-1β, TNF-α, IL-4, IL-6 and IL-10. Upregulation of hsp27, however, was primarily induced by immunoregulatory cytokines like IL-4, IL-6 and TGF-β whereas αB-crystallin expression was found to be enhanced by the pro-inflammatory cytokine TNF-α only. None of the cytokines studied was able to enhance expression of all three hsp simultaneously. These results show that in human astrocytes induced expression of hsp27 and αB-crystallin is dependent on the presence of a defined set of stimuli, while induced expression of hsp60 is a much less selective event. This highly differential pattern of hsp expression in response to inflammatory mediators known to play an important role in the pathogenesis of autoimmune diseases indicates that hsp responses are specific rather than non-specific bystander responses.

Keywords: Human; Astrocytes; Multiple sclerosis; Heat shock proteins; Cytokines

1. Introduction

Heat shock proteins (hsp) or stress proteins are produced by both prokaryotic and eukaryotic cells in response to a wide variety of stressful insults such as heat shock, hypoxia, ischaemia, metabolic disruption and various inflammatory mediators. They play a role in protection against irreversible cell damage and they promote recovery of the cells experiencing stress. Several stress proteins are also expressed constitutively in unstressed cells at lower levels. Hsp act as molecular chaperones that regulate transport of proteins across membranes and they prevent inappropriate protein-protein interaction and aggregation of damaged or misfolded proteins. They are categorized into several families on basis of their molecular weight (Morimoto et al., 1990; Hightower, 1991).

The role of hsp in autoimmune diseases has been studied extensively and there is evidence for possible involvement in several diseases (Kaufmann, 1990). Hsp60 is suggested to be involved as an autoantigen in atherosclerosis (Wick et al., 1995) type I diabetes (Kiessling et al., 1991), multiple sclerosis (MS) (Selmaj et al., 1991 and 1992) and rheumatoid arthritis (RA) (De Graef-Meeder et al., 1991), and it has been shown to regulate immune responses in animal models for diabetes (Elias et al., 1990), multiple sclerosis (Gao et al., 1995; Birnbaum et al., 1998) and RA (Van Eden et al., 1988). The small stress protein αB-crystallin (Klemenz et al., 1991) has been identified as the immunodominant human T cell antigen in MS-affected myelin (Van Noort et al., 1995), and enhanced expression has been reported in the cytosol of oligodendrocytes (the myelin forming cells) and astrocytes already at the earliest stages of lesional development (Bajramovic et al., 1997). In other neurological diseases such as Alexander's disease, Alzheimer's disease and Parkinson's disease upregulation of the small heat shock proteins αB-crystallin as well as hsp27 has been found in glia cells (Aquino et al., 1997; Head et al., 1993; Iwaki et al., 1989, 1992 and 1993; Renkawek et al., 1994).

There are indications that the cellular mechanisms by which hsp expression is regulated among various cell types of the human central nervous system (CNS) are different (Bajramovic et al., 1997; D'Souza et al., 1994; Freedman et al., 1992; Satoh and Kim, 1995). The potential effects that the type of cell can have on the regulation of hsp-expression seriously hampers expression studies when ill-defined cell cultures or tumour cell lines are used. In addition, there is also some evidence for differential expression patterns of different hsp in the same cell type in response to the same stimulus (Bajramovic et al., 1997, Head et al., 1996; Hitotsumatsu et al., 1996). Primary rat astrocytes, for example, show enhanced αB-crystallin and hsp27 mRNA expression in response to heat shock, whereas only αB-crystallin mRNA expression is enhanced in response to TNF-α stimulation (Head et al., 1994). These observations suggest that hsp expression may be regulated by differential control mechanisms rather than by uniform mechanisms.

The presence of numerous inflammatory mediators produced by both glia cells and infiltrating cells in the inflamed brain (Benveniste, 1998; Cannella and Raine, 1995; Woodroofe and Cuzner, 1993) could influence the expression of hsp in glia cells. Since glial cells of the CNS such as astrocytes, oligodendrocytes and microglia have been reported to express a wide variety of cytokine receptors (D' Souza et al., 1994; Otero and Merrill, 1994) it could well be that the enhanced expression of αB-crystallin, hsp27 and hsp60 in astrocytes in MS lesions might be caused by the presence of these cytokines.
In this study we addressed the questions whether elevated expression of hsp in astrocytes in MS lesions is the inevitable result of the presence of cytokines and whether all hsp react in the same way to these mediators. We used a semi quantitative competitive reverse transcriptase (rt)-PCR assay to measure mRNA levels and immunocytochemistry for protein detection and localization. Human adult post-mortem astrocytes were used rather than astrocytoma cell lines in order to avoid artefacts caused by the tumorigenic transformation of those lines.
We demonstrate here remarkably differential hsp expression patterns in response to pro-inflammatory and immunoregulatory cytokines. Hsp60 expression was found to be enhanced in response to a variety of cytokines. In contrast, upregulation of hsp27 was primarily induced by immunoregulatory cytokines like IL-4, IL-6 and TGF-β whereas αB-crystallin expression was found to be enhanced by the pro-inflammatory cytokine TNF-α only. None of the cytokines studied was able to enhance expression of all three hsp simultaneously. In human astrocytes the enhanced expression of the small heat proteins hsp27 and αB-crystallin in response to cytokines is a much more selective event than that of hsp60. The highly differential pattern of hsp expression in response to inflammatory mediators known to play an important role in the pathogenesis of autoimmune diseases indicates that hsp responses are specific rather than non-specific bystander responses. This could have implications for the type of immune response that is being raised against these proteins.

2. Materials and methods

2.1. Donors

For all experiments post mortem material derived from two healthy controls was used; characteristics of the donors are stated below. Individual PCR and immunohistochemical experiments were performed with astrocytes derived from one donor in one experiment. All experiments were repeated at least once with astrocytes from a different donor.

Donor#	Sex	Age	PM delay	Cause of death
S97-145	F	55	5h 35m	Intracerebral rupture
S98-142	M	85	4h 35m	Respiratory insufficiency

2.2. In vitro culture of human adult astrocytes

Human adult post-mortem astrocytes were obtained as previously described (De Groot et al., 1997). Briefly, tissue samples from subcortical white matter or corpus callosum were collected and meninges and visible blood vessels were removed before mincing the tissue into cubes of ≤2mm^3. The tissue fragments were incubated at 37°C for 20 minutes in a Hank's balanced salt solution containing 0.25% w/v porcine trypsin (Sigma, St. Louis, MO), 0.2 mg/ml EDTA, 1 mg/ml glucose and 0.1 mg/ml bovine pancreatic DNase I. After digestion, cell suspensions were gently triturated, washed and taken into culture. Cells were grown in 1:1 v/v Dulbecco's Modified Eagle's Medium (high glucose; Life Technologies, Breda, The Netherlands) /HAMF10 (with L-glutamine; Life Technologies) + 10% v/v FCS (Gibco; Life Technologies) and antibiotic supplement (penicillin 100U/ml and streptomycin 0.1 mg/ml) at 37°C in a humidified atmosphere containing 5% CO_2. To avoid contamination of the astrocyte cultures with meningeal and blood monocyte-derived macrophages, cell suspensions were grown overnight in uncoated tissue culture flasks allowing monocytes/macrophages to adhere. Subsequently, the supernatant was transferred to poly-L-lysine (15 µg/ml; Sigma)-coated 80 cm^2 flasks and after 48 h medium was changed to remove unattached cells and myelin debris. To passage the adherent cells they were rinsed with PBS, incubated for 5-10 min with 0.25% w/v porcine trypsin at 37°C, washed once with culture medium containing 10% v/v FCS and replated in 25 cm^2 flasks or chamberslides (Nunc, Roskilde, Denmark). Cells were used at early passage (not after passage 8) and stimulation assays were performed with post-confluent cultures. Recombinant human cytokines and chemokines were used at the following concentrations: IL-1β (500 U/ml; Peprotech, Rocky Hill, NJ), IL-4 (250 U/ml; Peprotech), IL-6 (200 U/ml; Peprotech), IL-10 (10 U/ml; Peprotech), IL-12 (500 U/ml; Peprotech), IFN-γ (500 U/ml; Peprotech), TNF-α (500 U/ml; Peprotech), TGF-β1 (200 U/ml; Calbiochem, La Jolla, CA) and RANTES (100 ng/ml; Peprotech). Heat shock controls were generated by performing heat shock at 43°C. An equal volume of prewarmed medium at 49°C was added to the cultures and left for 30 min at 43°C before cells were allowed to recover.

2.3. Semi quantitative competitive RT-PCR

RNA was isolated directly from the cells in the culture flasks (0.5-1 X 10^6 cells/time-point) using RNAzolB (Campro Scientific, Veenendaal, The Netherlands) and isopropanol precipitation. Using 2.5 µg of mRNA as a template, copy DNA was produced using the Reverse Transcription System (Promega, Madison, WI). For amplification, cDNA (1 µl) was added to 1 µl 10 mM dNTP mix, 5 µl 10 X Taq-polymerase (Life Technologies, Amsterdam, The Netherlands) and 43 µl PCR-buffer (0.01 M Tris/HCl (pH 8.4), 0.05 M KCl and 0.06 mg BSA/ml). Competitive rt-PCR was performed at 30 cycles for β-actin, αB-crystallin and hsp60 and at 27 cycles for hsp27. One cycle consisted of 30 s melting at 94°C, 30 s annealing at 57°C and 30 s elongation at 72°C. For β-actin and hsp60 2.25 mM $MgCl_2$, for αB-crystallin 3.0 mM $MgCl_2$ and for hsp 27 1.5 mM $MgCl_2$ was added to the PCR buffer. Primers used were (from 5' to 3') for

β-actin *sense*: AAG ATG ACG CAG ATC ATG TTT GAG, *antisense*: AGG AGG AGC AAT GAT CTT GAT CTT at 15 pmol/µl (Perkin-Elmer, Norwalk, CT), for αB-crystallin *sense*: AGC TGG TTT GAC ACT GGA CT, *antisense*: GCA ATT CAA GAA AGG GCA TC at 20 pmol/µl (Perkin-Elmer), for hsp27 *sense*: TCC CTG GAT GTC AAC CAC TT, *antisense*: CAA AAG AAC ACA CAG GTG GC at 20 pmol/µl (Perkin-Elmer) and for hsp60 *sense*: TTC GAT GCA TTC CAG CCT TG, *antisense*: TTG GGC TTC CTG TCA CAG TT at 20 pmol/µl (Perkin-Elmer). For internal standards, plasmids were used that carried target cDNA sequences modified to be approximately 50 bp shorter than the sample cDNA but that still contained the same primer binding sites. Sequence analysis was performed to confirm the correct sequence of the plasmids. Parallellism assays were performed to verify equal amplification efficiencies of sample and plasmid cDNA (data not shown). Sample cDNA was amplified together with internal standards at known concentrations and the 50% equivalence point was calculated following gel electrophoresis and densitometrical analysis of the amplified products. Relative cDNA values were calculated by dividing the hsp values at a given time point by the β-actin value for that given time point.
Variabilities in relative cDNA levels for unstimulated astrocytes were:
For donor S97-145; Hsp60 [0.002-0.004], Hsp27 [0.06-0.1], αB-crystallin [0.01-0.02]
For donor S98-142; Hsp60 [0.006-0.01], Hsp27 [0.01-0.04], αB-crystallin [0.008-0.05]

2.4. Immunocytochemistry
Immunocytochemistry was performed on chamberslides with 8 chambers per slide. After stimulation, samples were fixed in acetone + 0.03% v/v H_2O_2 (to block endogenous peroxidase) for 10 minutes. Following air-drying, the slides were incubated overnight at 4°C with primary antibodies in PBS + 0.1% w/v BSA and rinsed extensively with PBS + 0.05% w/v Tween-20. To establish purity of the cell cultures rabbit polyclonal anti-glial fibrillary acidic protein (α-GFAP, Zymed, So. San Francisco, CA) was used; as a rule cell cultures were >98% GFAP positive. For detection of hsp, we used affinity-purified rabbit polyclonal anti-lap70 (Katholieke Universiteit Nijmegen, the Netherlands) directed against the C-terminal 14 amino acids of αB-crystallin, murine monoclonal anti-hsp27 (Stressgen, Victoria, Canada) and murine monoclonal anti-hsp60 (Stressgen).
The secondary antibodies used were biotinylated horse-anti-mouse (Vector, Burlingame, CA) and biotinylated donkey-anti-rabbit (Amersham Life Science, Roosendaal, The Netherlands) in PBS + 1% w/v BSA + 1% v/v Normal Human Serum (NHS) for 45 min at room temperature. After rinsing with PBS + 0.05% w/v Tween-20 peroxidase-labelled streptavidin (Gibco; Life Technologies) in PBS + 1% w/v BSA + 1% v/v NHS was added for 45 min at room temperature.
Binding of the antibodies was visualized using 3'-diaminobenzidine (DAB) as a chromogen. Counterstaining was performed using haematoxylin. Negative controls included serum of non-immunized mice or rabbits as a first antibody and primary antibody omission controls. In order to compare expression levels of a certain hsp, each

chamberslide was stained for one hsp at different timepoints in response to the same stimulus and contained a negative staining control as well as unstimulated astrocytes. Protein expression was measured by the eye, but objectified by the comparison of photomicroscopical pictures taken with the same shutterspeed of unstimulated and stimulated astrocytes that were processed on the same chamberslide. Analysis of the slides was done blinded to the stimulation used.

3. Results

3.1. Differential expression of hsp mRNA in human adult astrocytes in response to cytokines. Hsp and β-actin mRNA-derived cDNA levels were measured following stimulation of human post-mortem astrocytes with a panel of cytokines. Stimulations were performed for either short periods (0-24 h) or prolonged periods (48-96 h) of time and amounts of hsp cDNA relative to β-actin were calculated for each timepoint. Subsequently, stimulation indices were calculated by dividing the relative amounts of hsp cDNA by values determined for unstimulated astrocytes. After an initial screening for all timepoints the experiments presented in Table 6.1 focus on the timeframes where changes in hsp mRNA-derived cDNA values were measured in these initial experiments. If no changes in hsp cDNA levels were measured in response to a specific cytokine a set of four representative timepoints is presented. Per individual stimulus a dataset of timepoints was obtained using cells from one donor; for all experiments astrocytes of two control donors were used. Stimulation indices (SI) ≥3 were considered relevant. Heat shock was used as a positive control for enhanced mRNA expression levels of hsp. Indeed, heat shock resulted in measurable upregulation of all three hsp studied (Table 6.1). Figure 6.1 shows the maximum SI for each hsp after stimulation with a particular cytokine. Despite the fact that some inter-donor variability with regards to the kinetics and absolute values of the measured hsp upregulations occurred, observed phenomena were reproducible. The variability was mainly due to variability in relative cDNA values of unstimulated astrocytes that differed somewhat between the different donors (see also Materials and Methods). The data (Fig. 6.1) reveal that stimulation with different cytokines leads to markedly different patterns of induced hsp cDNA expression. Even the small hsp do not respond to the same stimuli in the same way despite their high degree of structural homology (Ingolia and Craig; 1982).

Table 6.1. Hsp mRNA-derived cDNA stimulation indices in response to different stimuli.

Stimulus	Hsp60	Hsp27	αB-C.	Stimulus	Hsp60	Hsp27	αB-C.
IL-1β	**Donor:**	***S97-145***		**IL-4**		***S98-142***	
30'	1	1	1	4h	0.8	0.8	1.1
60'	1	0.8	0.7	8h	1.6	1.9	1.9
4h	**7.2**	1.9	1.2	24h	1.2	0.8	0.8
24h	1.6	0.9	0.7	48h	**3.6**	2.4	2.9
IFN-γ		***S98-142***		**IL-6**		***S97-145***	
30'	1	0.3	1.3	30'	0.6	0.7	0.6
60'	2.1	0.6	1	60'	0.9	1.4	0.9
4h	2.8	1.1	1	4h	**8.4**	**6.8**	0.8
24h	**3.7**	2.3	1.2	24h	**4.5**	**8.1**	1.3
48h	2.9	2.7	1	48h	2.5	0.4	2.1
72h	2.8	2.4	1				
TNF-α		***S97-145***		**IL-10**		***S98-142***	
4h	2	0.4	0.9	2h	1.2	0.9	0.8
24h	**6.3**	0.6	**4**	4h	**4.3**	0.8	1.1
48h	1.4	0.4	**9.8**	6h	**5.1**	1.3	1.2
72h	0.6	1.8	1.2	24h	**5.3**	1.7	1.4
IL-1β, IFN-γ, TNF-α		***S98-142***		**TGF-β**		***S98-142***	
30'	2.9	2.4	2.1	4h	0.5	1.3	1.4
60'	**3.3**	**6.6**	**3.8**	24h	0.9	**4.2**	1.5
4h	2.4	**3.8**	2.4	48h	0.5	**5.5**	2.1
24h	1.9	**4.6**	2.3	72h	0.3	**4.6**	1.7
48h	1.1	2.7	1.3	96h	0.4	**7.3**	1.6
IL-12		***S98-142***					
60'	1.3	0.8	0.7				
4h	1.7	0.6	0.8				
8h	1.6	0.6	0.4				
24h	2.4	0.6	0.2	**HS, 30' at 43°C**		***S98-142***	
RANTES		***S98-142***		30'	1.5	1.4	1.3
60'	1.6	1.8	1.9	60'	1.9	2.2	1.9
4h	0.8	1.1	1.2	2h	**8.6**	2.1	**3.9**
8h	0.9	0.8	0.6	4h	**5.4**	**4.8**	**5.3**
24h	1.6	1.3	0.8	24h	1.7	1.6	**3.5**

For each time point hsp cDNA levels were first divided by the cDNA level of β-actin (a housekeeping gene) at that given time point to acquire relative cDNA levels. These were then divided by the relative cDNA levels of unstimulated astrocytes (taken as a mean of two separate analyses) yielding the stimulation indices (SI). αB-C.= αB-crystallin, HS = Heat shock. Marked in bold are SI ≥3.

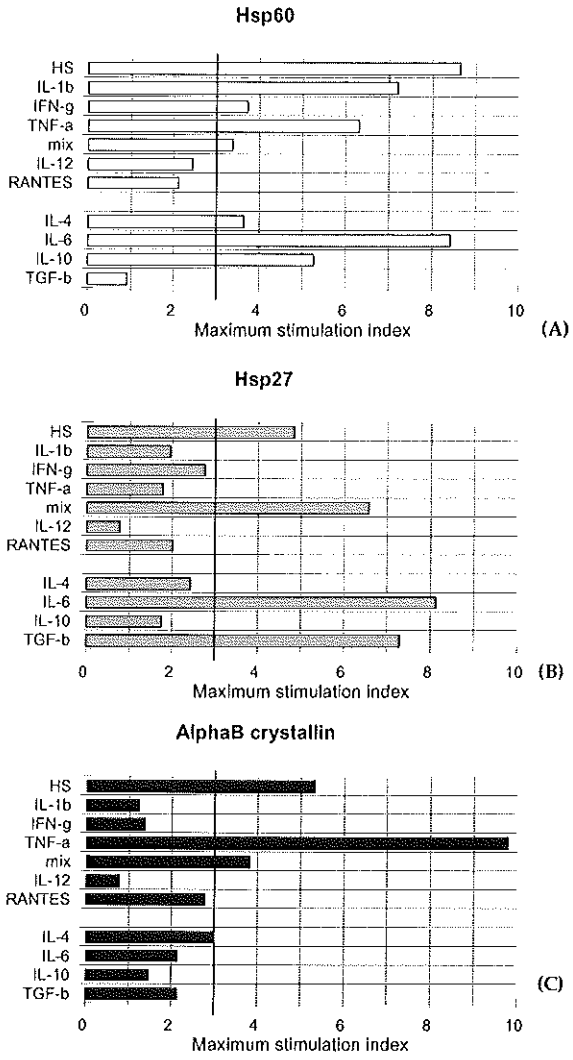

Figure 6.1. Maximum stimulation indices (SI) of relative cDNA levels for each hsp after stimulation with particular cytokines. In Fig. 6.1a the maximum SI of hsp60 in response to specific cytokines are shown, in Fig. 6.1b those of hsp27 and in Fig. 6.1c those of αB-crystallin. The bold line indicates SI=3, SI ≥3 were considered relevant. Mix is a mixture of the pro-inflammatory cytokines IL-1β, IFN-γ and TNF-α. Hsp60 is shown to respond to cytokines as diverse as IL-1β, IFN-γ, TNF-α, IL-4, IL-6 and IL-10, whereas primarily immunoregulatory cytokines like IL-6 and TGF-β induced hsp27 upregulation. αB-crystallin is responsive to the pro-inflammatory cytokine TNF-α only.

3.2. Hsp60 mRNA levels are enhanced in response to both pro-inflammatory and immunoregulatory cytokines.
Hsp60 mRNA levels were strongly enhanced by stimulation with IL-1β, TNF-α, IL-6 and IL-10 (Fig. 6.1a). In addition IFN-γ and IL-4 were also able to enhance hsp60 mRNA levels, albeit to a lesser extent (Fig. 6.1a). IL-12, RANTES and TGF-β did not affect hsp60 mRNA levels. The mix of proinflammatory cytokines (IL-1β, IFN-γ and TNF-α), that was added to the cells in order examine a potential synergistic effect (Merrill, 1991) of the individual cytokines in this mix, was also able to enhance hsp60 mRNA levels. To our surprise, however, the expected synergistic effect was not observed. In contrast, a lower level of enhancement of hsp60 mRNA was observed with the mix than with TNF-α or IL-1β alone. In general the response of hsp60 in astrocytes to cytokines was acute (occurring within 24 h) with a slow response to IL-4 as the only exception (Table 6.1). These data show that the expression of hsp60 mRNA can be enhanced by a variety of cytokines, both pro-inflammatory and immunoregulatory, and that enhanced expression is not the result of a certain well-defined subset of cytokines.

3.3. Hsp27 mRNA levels are enhanced mainly in response to immunoregulatory cytokines
Hsp27 mRNA levels were strongly enhanced by stimulation with the immunoregulatory cytokines IL-6 and TGF-β (Figure 6.1b). The responsiveness to IL-6 is no unique feature of hsp27, since it is shared with hsp60 also with regards to the kinetics (Table 6.1). The effect of TGF-β on hsp27 mRNA transcription however, is more selective and has not been described before. This response was cumulative in that prolonged stimulation resulted in continuously enhanced hsp27 mRNA levels (Table 6.1). In contrast to the moderate enhancement of hsp60 mRNA expression levels to the mix of pro-inflammatory cytokines, hsp27 mRNA levels were increased rapidly when astrocytes were stimulated with the same cytokine mix indicating a powerful synergistic effect of the combination on the expression levels of hsp27 (Table 6.1).

3.4. Upregulation of αB-crystallin mRNA levels is more selective than that of hsp27 or hsp60.
TNF-α was the only individual cytokine tested capable of inducing upregulation of αB-crystallin mRNA levels. The effect of TNF-α on αB-crystallin mRNA levels started after 24 h of stimulation (Table 6.1). The pro-inflammatory cytokine mix also caused enhanced αB-crystallin mRNA levels, with more rapid kinetics than TNF-α alone. However, this upregulation of mRNA did not attain the same maximum level as that determined with TNF-α alone (Fig. 6.1c).

3.5. Hsp protein upregulation correlates with enhanced mRNA levels.
We also studied the effect of cytokines on protein levels and on the subcellular localization of hsp using immunocytochemistry. Post-mortem astrocytes were cultured on chamberslides, stimulated for different timeperiods with cytokines and subsequently analysed by immunocytochemistry. Unstimulated and stimulated astrocytes were cultured on the same chamberslide in order to enable simultaneous processing resulting

in identical immunocytochemical conditions for all chambers on one slide. This allowed semi-quantitative analysis of protein levels. In general, we found that enhanced protein levels occurred either simultaneously with or shortly after mRNA upregulation was observed, consistent with what would be expected.

Table 6.2. Induced heat shock protein expression patterns in cultures of primary human adult astrocytes in response to different stimuli.

Stimulus	Hsp60	Hsp27	αB-C.
Heat Shock	++	+	+
IL-1β	++	-	-
IFN-γ	-	-	-
TNF-α	+	-	++
IL-1β+IFN-γ+TNF-α	+	+	-
IL-12	-	-	-
RANTES	-	-	-
IL-4	+	+	-
IL-6	++	++	-
IL-10	+	-	-
TGF-β	-	++	-

Human adult astrocytes were cultured on chamberslides, stimulated for different times with cytokines and analysed by immunocytochemistry. Enhanced protein levels occurred at the same time point or shortly (1-2 h) after observed mRNA upregulation. αB-C.= αB=crystallin.
- = no enhanced expression + = enhanced expression ++ = strongly enhanced expression

Enhanced expression of hsp60 was found in response to the same cytokines that caused enhanced mRNA expression with the exception of IFN-γ where no increased protein levels were observed (Table 6.2). The punctate, cytosolic localisation of hsp60 was compatible with mitochondrial staining (Jindal et al, 1989). The localisation of the protein did not change in response to any of the stimuli used (Fig. 6.2a). Enhanced expression of hsp27 also correlated with the observed pattern of mRNA upregulation. However, although staining for hsp27 demonstrated enhanced expression following stimulation with IL-4, no enhanced hsp27 mRNA synthesis was observed under the same stimulation conditions (Table 6.2). This indicates that IL-4 may stabilize existing mRNA or lead to the dissociation of aggregated hsp27 resulting in increased reactivity to the antibody used. Hsp27 in unstimulated astrocytes was mainly found in the cytoplasm and to a lesser extent in the nucleus, while upregulation did increase protein levels in both subcellular sites (Fig. 6.2b). The only cytokine capable of enhancing αB-crystallin protein levels in astrocytes was TNF-α (Table 6.2). The mix of IL-1β, IFN-γ and TNF-α did not result in enhanced expression of αB-crystallin protein. αB-Crystallin was localised mainly in the nucleus although minute amounts could be found in the cytosol of unstimulated astrocytes as well. This distribution pattern did not change after stimulation with TNF-α (Fig. 6.2c).

4. Discussion

The major finding of this study, the first one on cytokine-induced hsp expression in human adult astrocytes, is that the three hsp studied respond in a strikingly different way to various cytokines. The data reveal differential hsp expression patterns in response to pro-inflammatory and immunoregulatory cytokines. Hsp60 expression was found to be enhanced in response to cytokines as diverse as IL-1β, TNF-α, IL-4, IL-6 and IL-10. Upregulation of hsp27, however, was primarily induced by immunoregulatory cytokines such as IL-4, IL-6 and TGF-β whereas αB-crystallin expression was found to be enhanced by the pro-inflammatory cytokine TNF-α only. None of the cytokines studied was able to enhance expression of all three hsp simultaneously. The absence of enhanced mRNA expression of any of the hsp studied in response to the pro-inflammatory cytokine IL-12 and the chemokine RANTES might be the result of the absence of appropriate receptors on astrocytes for those mediators. However, the absence of enhanced hsp protein expression in response to IFN-γ cannot be attributed to such a lack of receptors since astrocytes did respond to IFN-γ stimulation by upregulating their MHC class II molecules (data not shown). The highly differential pattern of hsp expression in response to cytokines adds to our previously described observations on differential hsp expression in MS lesions (Bajramovic et al., 1997) and emphasises that stress responses are specific rather than more general bystander responses.

Cytokine-induced initiation of hsp60 mRNA transcription is a less selective event than induction of the two smaller hsp. Since the promoter sequence of hsp60 has not been analysed the transcription factors involved in its regulation remain to be established. Our results suggest that the hsp60 promoter would include consensus sequences for a variety of transcription factors including STAT-3 (activated by IL-6 and IL-10 (Kishimoto et al., 1995)), STAT-6 (activated by IL-4 (Lu et al., 1997)) and NF-κB (activated by IL-1β and TNF-α (O'Neill, 1995)). The sensitivity of the promoter of hsp60 to transcription factors other than heat shock factor is in concordance with the recently described transcriptional activation of hsp90 in human PBMC by IL-6 via STAT-3 (Stephanou et al., 1997) and in human T cells by IL-4 via STAT-6 (Metz et al., 1996). The relative abundance of hsp60 in MS lesions correlates very well with the promiscuous responsiveness of the hsp60 promoter to both pro-inflammatory and anti-inflammatory mediators that are known to be present in considerable amounts in MS lesions.
Our data indicate that the stress protein hsp27 is responsive to immunoregulatory rather than to pro-inflammatory cytokines. Again, the promoter of hsp27 has not been analysed, so consensus binding motifs that could be involved still await identification. Our results suggest that the hsp27 promoter may include responsive elements for STAT-3 or NF-IL-6 and for TGF-β- induced smad3-smad4 and AP-1 (Wong et al., 1999). It is interesting to note the fact that the two most potent upregulators of hsp27, TGF-β and IL-6, are cytokines that can be produced by glia cells in the brain itself. Both cytokines have clear proliferative effects on post-mortem astrocytes (data not shown) and enhanced expression of hsp27 may therefore be specifically related to astrocyte growth. In support of this idea is the enhanced hsp27 expression observed in rapidly proliferating astrocytomas (Hitotsumatsu et al., 1996). The expression of hsp27 in astrocytes in MS lesions could thus perhaps also be explained by the astrogliosis as observed in MS lesions.
The selective inducibility of αB-crystallin by TNF-α only is remarkable when compared to the expression patterns of the two other hsp. Our results are in full concordance with a report on the inducibility of αB-crystallin (and not hsp27) in rat astrocytes by TNF-α (Head et al., 1994). The sensitivity of αB-crystallin to TNF-α can be explained by the presence of both an NF-κB and an AP-1 consensus site in the promoter sequence of αB-crystallin (Quax-Jeuken et al., 1985; Srinivasan, 1994). TNF-α-induced expression of αB-crystallin leads to the accumulation of this hsp in the nucleus and not in the cytosol (see Fig. 6.2c). This pattern of expression is clearly different from what is observed in MS lesions where enhanced expression of αB-crystallin is restricted to the cytosol of astrocytes (Bajramovic et al., 1997). The difference in intracellular localisation of αB-crystallin in post-mortem astrocytes as compared to that in astrocytes in MS lesions is unlikely to be the result of an *in vitro* artefact since stimulation of post-mortem astrocytes with 25 μM H_2O_2 did result in enhanced expression and cytosolic localisation of αB-crystallin comparable to what is seen in MS lesions (data not shown). Together, our data indicate that elevated expression of αB-crystallin in astrocytes in MS lesions is unlikely to be the result of any of the individual cytokines known to be present in MS

lesions, including those secreted by activated pro-inflammatory T cells. We therefore favour the idea that the upregulation of αB-crystallin in astrocytes in MS lesions, an early event in their development, is mediated by endogenous signals in the CNS rather than by an ongoing T-cell response. Such endogenous signals could involve oxidative stress, apoptosis or viral infection, all known to occur frequently in MS-affected brains. The selective upregulation pattern of αB-crystallin in response to cytokines as compared to the relative promiscuous pattern found for hsp60 upregulation probably results in different frequencies with which the immune system encounters these hsp. In addittion, it is likely that the immunological context in which these antigens are being seen by the immune system might differ. Together this might have consequences for the type of immune response that is being raised towards these different hsp. It has been reported that T-cell responses to hsp60 are of the immunoregulatory rather than of the Th1 type (Van Roon et al., 1997; Van Eden et al., 1998). This in marked contrast to the observed type 1 T-cell responses directed against the more selectively expressed αB-crystallin (Van Noort et al., 1998; Van Sechel et al., 1999).

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References

Aquino, D.A., Capello, E., Weisstein, J., Sanders, V., Lopez, C., Tourtellotte, W.W., Brosnan, C.F., Raine, C.S., Norton, W.T. 1997. Multiple sclerosis: altered expression of 70- and 27 kDa heat shock proteins in lesions and myelin. J. Neuropathol. Exp. Neurol. 56(6), 664-672.

Bajramović, J.J., Lassmann, H., van Noort, J.M. 1997. Expression of αB-crystallin in glia cells during lesional development in multiple sclerosis. J. Neuroimmunol. 78, 143-151.

Benveniste, E.N. 1998. Cytokine actions in the central nervous system.Cytokine Growth Factor Rev. 9, 259-75.

Birnbaum, G, Kotilinek, L., Miller, S.D., Raine, C.S., Gao, Y.L., Lehmann, P.V., Gupta, R.S. 1998. Heat shock proteins and experimental autoimmune encephalomyelitis. II: environmental infection and extra-neuraxial inflammation alter the course of chronic relapsing encephalomyelitis. J. Neuroimmunol. 90(2), 149-161.

Cannella, B., Raine, C.S. 1995. The adhesion molecule and cytokine profile of multiple sclerosis lesions. Ann. Neurol. 37, 424-435.

D'Souza, S.D., Antel, J.P., Freedman, M.S. 1994. Cytokine induction of heat shock protein expression in human oligodendrocytes: An interleukin-1-mediated mechanism. J. Neuroimmunol. 50, 17-24.

De Graeff-Meeder, E.R., van der Zee, R., Rijkers, G.T., Schuurman, H.J., Kuis, W., Bijlsma, J.W.J., Zegers, B.J.M., van Eden, W. 1991. Recognition of human 60 kD heat shock protein by mononuclear cells from patients with juvenile chronic arthritis. Lancet 337, 1368-1372.

De Groot, C.J.A., Langeveld, C.H., Jongenelen, C.A.M., Montagne, L., van der Valk, P., Dijkstra, C.D. 1997. Establishment of human adult astrocyte cultures derived from postmortem multiple sclerosis and control brain and spinal cord regions: Immunophenotypical and functional characterization. J. Neurosci. Res. 49, 342-354.

Elias, D., Markovits, D., Reshef, T., van der Zee, R., Cohen, I.R. 1990. Induction of autoimmune diabetes in the non-obese diabetic (NOD/Lt) mouse by a 65 kDA heat shock protein. Proc. Natl. Acad. Sci. USA 87, 1576-1580.

Freedman, M.S., Buu, N.N., Ruijs, T.C.J., Williams, K., Antel, J.P. 1992. Differential expression of heat shock proteins by human glial cells. J. Neuroimmunol. 41, 231-238.

Gao, Y.L., Brosnan, C.F., Raine, C.S. 1995. Experimental Autoimmune Encephalomyelitis; Qualitative and semiquantatative differences in heat shock protein 60 expression in the central nervous system. J. Immunol. 154, 3548-3556.

Head, M.W., Corbin, E., Goldman, J.E. 1993. Overexpression and abnormal modification of the stress proteins αB-crystallin and hsp27 in Alexander disease. Am. J. Pathol. 143, 1743-1752.

Head, M.W., Corbin, E., Goldman, J.E. 1994. Coordinate and independent regulation of αB-crystallin and hsp27 expression in response to physiological stress. J. Cell. Physiol. 159, 41-50.

Head, M.W., Hurwitz, L., Goldman, J.E. 1996. Transcription regulation of alpha B-crystallin in astrocytes: analysis of HSF and AP1 activation by different types of physiological stress. J. Cell. Sci. 109 (Pt 5), 1029-1039.

Hightower, L.E. 1991. Heat shock, stress proteins, chaperones and proteotoxicity. Cell 66, 191-197.

Hitotsumatsu, T., Iwaki, T., Fukui, M., Tateishi, J. 1996. Distinctive immunohistochemical profiles of small heat shock proteins (heat shock protein 27 and αB-crystallin) in human brain tumors. Cancer 77 (2), 352-361.

Ingolia, T.D., Craig, E.A. 1982. Four small heat shock proteins are related to each other and to mammalian α-crystallin. Proc. Natl. Acad. Sci. USA 79, 2360-2364.

Iwaki, T., Kume-Iwaki, A., Liem, R.K.H., Goldman, J.E. 1989. αB-crystallin is expressed in non-lenticular tissues and accumulates in Alexander's disease brain. Cell 57, 71-78.

Iwaki, T., Wisniewski, T., Iwaki, A., Corbin, E., Tomokane, N., Tateishi J., Goldman, J.E. 1992. Accumulation of αB-crystallin in central nervous system glia and neurons in pathologic conditions. Am. J. Pathol. 140, 345-356.

Iwaki, T., Iwaki, A., Tateishi, J., Sakaki, Y., Goldman, J.E. 1993. αB-crystallin and 27-kd heat shock protein are regulated by stress conditions in the central nervous system and accumulate in Rosenthal fibers. Am. J. Pathol. 143, 487-495.

Jindal, S., Dudani, A.K., Singh, B., Harley, C.B., Gupta, R.S. 1989. Primary structure of a human mitochondrial protein homologous to bacterial and plant chaperonins and to 65 kD mycobacterial antigen. Mol. Cell Biol. 9, 2279-2283.

Kaufmann, S.H, 1990. Heat shock proteins and the immune response. Immunol. Today. 11,129-136.

Kiessling, R., Grönberg, A., Ivanyi, J., Söderström, K., Ferm, M., Kleinau, S., Nilsson, E., Klaresklog, L. 1991. Role of hsp60 during autoimmune and bacterial inflammation. Immunol. Rev. 121, 91-111.

Kishimoto, T., Akira, S., Narazaki, M., Taga, T. 1995. Interleukin-6 family of cytokines and gp130. Blood 86(4), 1243-1254.

Klemenz, R., Frohll, E., Steiger, R.H., Schafer, R., Aoyama, A. 1991. αB-crystallin is a small heat shock protein. Proc. Natl. Acad. Sci. USA 88, 3652-3656.

Lu, B., Reichel, M., Fisher, D.A., Smith, J.F., Rothman, P. 1997. Identification of a STAT6 domain required for IL-4 induced activation of transciption. J. Immunol. 159(3), 1255-1264.

Merrill, J.E. 1991. Effects of Interleukin-1 and Tumor Necrosis Factor-α on astrocytes, microglia, oligodendrocytes, and glia precursors in vitro. Dev. Neurosci. 13, 130-137.

Metz, K., Ezernieks, J., Sebald, W., Duschl, A. 1996. Interleukin-4 upregulates the heat shock protein Hsp90α and enhances transcription of a reporter gene coupled to a single heat shock element. FEBS Lett. 385 (1-2), 25-28.

Morimoto, R.I., Tissieres, A., Georgopoulos C. 1990. In: Morimoto, R.I., Tissieres, A., Georgopoulos, C.(Eds.), Stress proteins in Biology and Medicine, Cold Spring Harbor Labaratory Press, Cold Spring Harbor, NY, 1990, pp. 1-36.

O'Neill, L.A. 1995. Interleukin-1 signal transduction. Int. J. Clin. Lab. Res. 25(4), 169-177.

Otero, G.C., Merill, J.E. 1994. Cytokine receptors on glial cells. Glia 11(2), 117-128.

Quax-Jeuken, Y., Quax, W., van Rens, G., Khan, M., Bloemendal, H. 1985. Complete structure of the αB-crystallin gene: Conservation of the exon-intron distribution in the two non-linked α-crystallin genes. Proc. Natl. Acad. Sci. USA 82, 5819-5823.

Renkawek, K., Voorter, C.E.M., Bosman, G.J., van Workum, F.P., de Jong, W. 1994. Expression of αB-crystallin in Alzheimers disease. Acta Neuropathol. 87, 155-160.

Satoh, J-I., Kim, S.U. 1995. Differential expression of heat shock protein hsp27 in human neurons and glial cells in culture. J. Neurosci. Res. 41 (6), 805-818.

Selmaj, K., Brosnan, C., Raine, C.S, 1991. Colocalization of lymphocytes bearing γδ T-cell receptor and heat shock protein hsp65$^+$ oligodendrocytes in multiple sclerosis. Proc. Natl. Acad. Sci. USA. 88, 6452-6456.

Selmaj, K., Brosnan, C.F., Raine, C.S. 1992. Expression of heat shock protein-65 by oligodendrocytes in vivo and in vitro: implications for multiple sclerosis. Neurology 42(4), 795-800.

Srinivasan, A., Bhat, S.P. 1994. Complete structure and expression of the rat αB-crystallin gene. DNA and Cell Biol. 13 (6), 651-661.

Stephanou, A, Amin, V., Isènberg, D.A., Akira, S., Kishimoto, T., Latchman, D.S. 1997. Interleukin 6 activates heat-shock protein 90 beta gene expression. Biochem. J. 321 (Pt 1), 103-106.

Van Eden, W., Thole, J.E., van der Zee, R., Noordzij, A., van Embden, J.D., Hensen, E.J., Cohen, I.R. 1988. Cloning of the mycobacterial epitope recognized by T lymphocytes in adjuvant arthritis. Nature 311, 171-173.

Van Eden, W., van der Zee, R., Taams, L.S., Prakken, A.B., van Roon, J., Wauben, M.H. 1998. Heat-shock protein T-cell epitopes trigger a spreading regulatory control in a diversified arthritogenic T-cell response. Immunol. Rev. 164, 169-174.

Van Noort, J.M., van Sechel, A.C., Bajramović, J.J., El Ouagmiri, M., Polman, C.H., Lassmann, H., Ravid, R. 1995. The small heat shock protein αB-crystallin as candidate autoantigen in multiple sclerosis. Nature 375, 798-801.

Van Noort, J.M., van Sechel, A.C., van Stipdonk, M.J.B., Bajramović, J.J. 1998. The small heat-shock protein αB-crystallin as key autoantigen in multiple sclerosis. In: Van Leeuwen, F.W., Salehi, A., Giger, R.J., Holtmaat, A.J.G.D., Verhaagen, J. (Eds.), Progress in Brain Research, Vol. 117, Elsevier Science B.V. 1998, pp. 435-452.

Van Roon, J.A., van Eden, W., van Roy, J.L., Lafeber, F.L., Bijlsma, J.W. 1997. Stimulation of suppressive T cell responses by human but not bacterial 60-kD heat-shock protein in synovial fluid of patients with rheumatoid arthritis. J. Clin. Invest. 100(2), 459-463.

Van Sechel-Plomp, A.C., Bajramović, J.J., van Stipdonk, M.J.B., Geutskens, S.B., van Noort, J.M. 1999. Activation of autoreactive helper T cells by virus-induced presentation of a self antigen. J. Immunol. 162(1), 129-135.

Wick, G., Schett, G., Amberger, A., Kleindienst, R., Xu Q. 1995. Is atherosclerosis an immunologically mediated disease? Immunol. Today 16 (1), 27-33.

Wong, C., Rougier-Chapman, E.M., Frederick, J.P., Datto, M.B., Liberati, N.T., Li, J.M., Wang, X.F. 1999. Smad3-smad4 and AP-1 complexes synergize in transcriptional activation of the c-Jun promoter by transforming growth factor beta. Mol. Cell. Biol. 19(3), 1821-1830.

Woodroofe, M.N., Cuzner, M.L. 1993. Cytokine mRNA expression in inflammatory multiple sclerosis lesions: detection by non-radioactive in situ hybridization. Cytokine 5(6), 583-588.

7.

Phosphorylation of the small heat shock protein αB-crystallin at serine-59 causes a major shift in intracellular distribution of the protein in human astrocytes.*

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Abstract

In multiple sclerosis the small heat shock protein αB-crystallin has been shown to accumulate in the cytosol of astrocytes. The molecular triggers for this accumulation are unknown. In this study we examined the effects of reactive oxygen species (ROS) on the expression of αB-crystallin in adult human astrocytes.
αB-Crystallin phosphorylated at either serine-19 or serine-45 was mainly found in the nuclei of astrocytes, and remained unaffected by oxidative stress. Also, levels of αB-crystallin-encoding-mRNA did not change upon oxidative stress. However, within minutes following oxidative stress, high levels of αB-crystallin phosphorylated at serine-59 (αB-59^P) appeared in the cytosol. Filamentous staining patterns suggested a close association of this form of αB-crystallin with cytoskeletal elements. This filamentous expression pattern of αB-59^P preceded diffuse cytosolic accumulation of αB-crystallin. Inhibition of H_2O_2-induced activation of p38-MAPK by the synthetic inhibitor SB202190 selectively abrogated the phosphorylation of αB-crystallin at serine-59. More importantly, it also reduced the concomitant cytosolic accumulation of αB-crystallin implicating an important role for phosphorylation of αB-crystallin at serine-59 in determining the intracellular distribution of the protein.
The presence of αB-59^P in a subset of αB-crystallin-expressing astrocytes in MS lesions demonstrates that phosphorylation of αB-crystallin at serine-59 occurs *in vivo*. Together, our data indicate that the apparent cytosolic accumulation of αB-crystallin in astrocytes under conditions of oxidative stress primarily results from p38-MAPK-mediated phosphorylation at serine-59.

[1] *The abbreviations used are: ERK, extracellularly responsive kinase; hsp, heat shock protein; JNK, c-Jun N-terminal kinase; MAPK, mitogen activated protein kinase; MAPKAPK2, MAPK-activated protein kinase 2; MS, multiple sclerosis; NO, nitric oxide; SAPK, stress-activated protein kinase; ROS, reactive oxygen species; TNF-α, tumor necrosis factor-α*

Introduction

In the pathogenesis of the autoimmune disease multiple sclerosis (MS) the small heat shock protein (hsp) αB-crystallin is thought to play an important role. αB-Crystallin is the immunodominant T-cell antigen in MS-affected myelin (1), the target of the disease. In MS-affected brain tissue αB-crystallin is present at enhanced levels in the cytosol of astrocytes and oligodendrocytes (2) and it becomes locally presented to T cells (3). As the increased or altered expression of αB-crystallin in glia cells may help trigger the local inflammatory process in MS, identification of the factors that mediate this expression is the goal of our studies.

The distribution pattern of αB-crystallin-expressing astrocytes in MS lesions strongly suggests the involvement of a soluble mediator in the altered expression of αB-crystallin in this cell type (2). Many inflammatory mediators have been demonstrated in MS lesions that might influence the expression of αB-crystallin (4). *In vitro* studies on cytokine-induced expression of hsp-mRNA in human astrocytes indeed revealed upregulation of αB-crystallin mRNA levels in response to TNF-α . However, αB-crystallin expression in these astrocytes was restricted almost exclusively to the nucleus (5, 6) and the cytosolic localization of the protein as observed in MS lesions could not be reproduced *in vitro* by any of the cytokines used.

Reactive oxygen species (ROS) such as nitric oxide (NO) and H_2O_2 in MS lesions (7, 8, 9, 9) represent alternative triggers for cytosolic expression of αB-crystallin in astrocytes. ROS have been described to activate all three subfamilies of the mitogen-activated protein kinase (MAPK) superfamily. These are the extracellularly responsive kinases (ERKs), the c-Jun N-terminal kinases (JNKs) that are also known as the stress-activated protein kinases (SAPKs) and the p38-MAPKs. Several reports have demonstrated that ROS-induced phosphorylation of hsp27, another small hsp, is associated with a decrease in its aggregation state (10, 11, 12, 13), thereby affecting its intracellular distribution.

The phosphorylation of αB-crystallin has been studied in some detail in the U373 astroglioma cell line (14). αB-Crystallin contains three serine residues that are susceptible to phosphorylation (15) and two MAPKs have been described to be involved. p44-MAPK, a member of the ERKs, phosphorylates αB-crystallin at serine residue 45, whereas p38-MAPK (supposedly by activating MAPKAPK2) seems to be responsible for the phosphorylation of serine residue 59 (16). No biological function has been ascribed to the phosphorylation(s) of αB-crystallin yet.

In this study we investigated whether ROS influence mRNA levels, intracellular distribution and phosphorylation of αB-crystallin in adult human astrocytes from post mortem brains . In addition, we studied the effects of ROS on the activation of the three members of the MAPK superfamily, and their relationship to the phosphorylation of αB-crystallin. Finally, we analyzed the phosphorylation profile of αB-crystallin in MS lesions.

Experimental procedures

Donors

For all experiments post mortem material derived from donors without a history of neurological disease was used; characteristics of the donors are given below. Post mortem (PM) delay times were < 5h. Individual PCR and immunohistochemical experiments were performed with astrocytes derived from one donor in one experiment. All experiments were repeated at least once with astrocytes from a different donor.

Donor#	Sex	Age	PM delay	Clinical history	Cause of death
S95-064	F	51	4h	ALS	Aspiration pneumonia
S98-142	M	85	4h 35m	Healthy	Respiratory insufficiency

In vitro culture of human adult astrocytes

Human adult post-mortem astrocytes were obtained from control donors as previously described (17). Briefly, tissue samples from subcortical white matter or corpus callosum were collected and meninges and visible blood vessels were removed before mincing the tissue into cubes of $\leq 2mm^3$. The tissue fragments were incubated at 37°C for 20 minutes in a Hank's balanced salt solution containing 0.25% w/v porcine trypsin (Sigma, St. Louis, MO), 0.2 mg/ml EDTA, 1 mg/ml glucose and 0.1 mg/ml bovine pancreatic DNase I. After digestion, cell suspensions were gently triturated, washed and taken into culture. Cells were grown in 1:1 v/v Dulbecco's Modified Eagle's Medium (high glucose; Life Technologies, Breda, The Netherlands) /HAMF10 (with L-glutamine; Life Technologies) + 10% v/v FCS (Gibco; Life Technologies) and antibiotic supplement (penicillin 100U/ml and streptomycin 0.1 mg/ml) at 37°C in a humidified atmosphere containing 5% CO_2. To avoid contamination of the astrocyte cultures with meningeal and blood monocyte-derived macrophages, cell suspensions were grown overnight in uncoated tissue culture flasks allowing monocytes/macrophages to adhere. Subsequently, the supernatant was transferred to poly-L-lysine (15 µg/ml; Sigma)-coated 80 cm^2 flasks (Nunc, Roskilde, Denmark) and after 48 h medium was changed to remove unattached cells and myelin debris. To passage the adherent cells they were rinsed with PBS, incubated for 5-10 min with 0.25% w/v porcine trypsin at 37°C, washed once with culture medium containing 10% v/v FCS and replated in 25 cm^2 flasks or chamberslides (Nunc). Assays were performed with post confluent cultures.

Reagents
10 M (30%) H_2O_2 (Perhydrol) was purchased from Merck (Darmstadt, Germany). S-Nitroso-N-acetylpenicillamine (SNAP) and the protein kinase inhibitor 4-(4-Fluorophenyl)-2-(4-hydroxyphenyl)-5-(4-pyridyl)1 H-imidazole.HCl (SB202190.HCl) were purchased from Calbiochem (La Jolla, CA). SNAP and SB202190.HCl were dissolved in milliQ water and used at concentrations of 5 mM and 10 µM respectively.

Semi-quantitative competitive rt-PCR
RNA was isolated directly from the cells in the culture flasks using RNAzolB™ (Campro Scientific, Veenendaal, The Netherlands) and isopropanol precipitation. Using 2.5 µg of mRNA as a template, copy DNA was produced using the Reverse Transcription System (Promega, Madison, WI). For amplification, cDNA (1 µl) was added to 1 µl 10 mM dNTP mix, 5 µl 10x Taq-polymerase (Life Technologies, Amsterdam, The Netherlands) and 43 µl PCR-buffer (0.01 M Tris/HCl (pH 8.4), 0.05 M KCl and 0.06 mg BSA/ml). Competitive rt-PCR for β-actin, αB-crystallin, hsp27 and hsp60 were performed as described previously (18). Briefly, as internal standards plasmids were used that carried target cDNA sequences modified to be approximately 50 bp shorter than the sample cDNA but that still contained the same primer binding sites. Sample cDNA was amplified together with internal standards at known concentrations and the 50% equivalence point was calculated following gel electrophoresis and densitometrical analysis of the amplified products. Relative cDNA values were calculated by dividing the hsp values at a given time point by the β-actin value for that given time point.

Immunofluorescence and immunohistochemistry
Immunofluorescence was performed on chamberslides with 8 chambers per slide. After stimulation, samples were fixed in acetone + 0.03% v/v H_2O_2 for 10 minutes. Following air-drying, the slides were incubated overnight at 4°C with primary antibodies in PBS + 0.1% w/v BSA and rinsed extensively with PBS + 0.05% w/v Tween-20. To establish purity of the cell cultures rabbit polyclonal anti-glial fibrillary acidic protein (α-GFAP, Zymed, So. San Francisco, CA) was used; as a rule cell cultures were >98% GFAP positive. For detection of αB-crystallin we used affinity-purified rabbit polyclonal anti-lap70 (Katholieke Universiteit Nijmegen, the Netherlands) directed against the C-terminal 14 amino acids of αB-crystallin and phospho-specific rabbit polyclonal antibodies against αB-crystallin. Specificity of these antibodies was confirmed by blocking studies. Primary antibodies were incubated overnight at 4°C together with 10 µg bovine eye lens-derived αB-crystallin/ml. In order to visualize activation of members of the MAPK superfamily we used the "PhosphoMAPK-sampler" kit (New England Biolabs, Beverly, MA) containing phospho-specific polyclonal antibodies against phosphorylated p38-MAPK, p44-MAPK and SAPK/JNK.

The secondary antibodies used were biotinylated horse-anti-mouse (Vector, Burlingame, CA) and biotinylated donkey-anti-rabbit (Amersham Life Science, Roosendaal, The Netherlands) in PBS + 1% w/v BSA + 1% v/v Normal Human Serum (NHS) for 45 min at room temperature. After rinsing with PBS + 0.05% w/v Tween-20, FITC-labelled streptavidin (DAKO, Glostrup, Denmark) in PBS + 1% w/v BSA + 1% v/v NHS was added for 45 min at room temperature. Negative controls included serum of non-immunized mice or rabbits as a first antibody and primary antibody omission controls.
Immunohistochemistry was performed on 5 µm frozen tissue sections of 3 MS-patients. Sections were fixed in acetone + 0.03% v/v H_2O_2 for 10 minutes and stained overnight for the following different markers at 4°C. To verify demyelination murine monoclonal anti-myelin oligodendrocyte glycoprotein (MOG; 8-18C5, (19)) was used. For the detection of αB-crystallin affinity-purified rabbit polyclonal anti-lap70 and the phospho-specific rabbit polyclonal antibodies were used. The secondary antibodies and the protocol used were the same as described for immunofluorescence. As a conjugate, streptavidin-labelled peroxidase (DAKO) was used. Following a 1 h incubation at room temperature, binding was visualized using 3'-diaminobenzidine (DAB; Sigma) as a chromogen. Counterstaining was performed using haematoxylin.

Results

Oxidative stress affects the localization of αB-crystallin in astrocytes but does not affect transcription levels

For the detection of αB-crystallin in human glia cells, affinity-purified polyclonal rabbit antibodies (lap70) directed against the C-terminal sequence of the protein was used. Lap70 is suitable for use in immunohistochemistry, immunocytochemistry and Western blotting. When lap70 was used to stain unstimulated cultured astrocytes the expression of αB-crystallin appears almost exclusively restricted to the nucleus (Fig. 1A). Upon stimulation with H_2O_2 at concentrations up to 500µM, the intracellular distribution of αB-crystallin in human post mortem astrocytes dramatically changed (Fig 1B). While nuclear expression levels appeared to decrease, cytosolic localization of αB-crystallin was strongly increased (Fig. 1C). After 180 min of stimulation astrocytes could be detected with an inverse intracellular distribution of αB-crystallin as compared to unstimulated astrocytes, i.e. an exclusive cytosolic distribution (Fig. 1D). Stimulation with 5 mM of the NO-donor S-nitroso-N-acetylpenicillamine (SNAP) produced the same effect (data not shown).
In order to examine whether the apparent accumulation of αB-crystallin in the cytosol was the result of increased mRNA synthesis, levels of mRNA encoding αB-crystallin, hsp27 and hsp60 were quantified using competitive rt-PCR. Hsp and β-actin mRNA levels were measured following stimulation of astrocytes with different concentrations of H_2O_2. As a positive control, hsp mRNA levels were determined after exposure of the

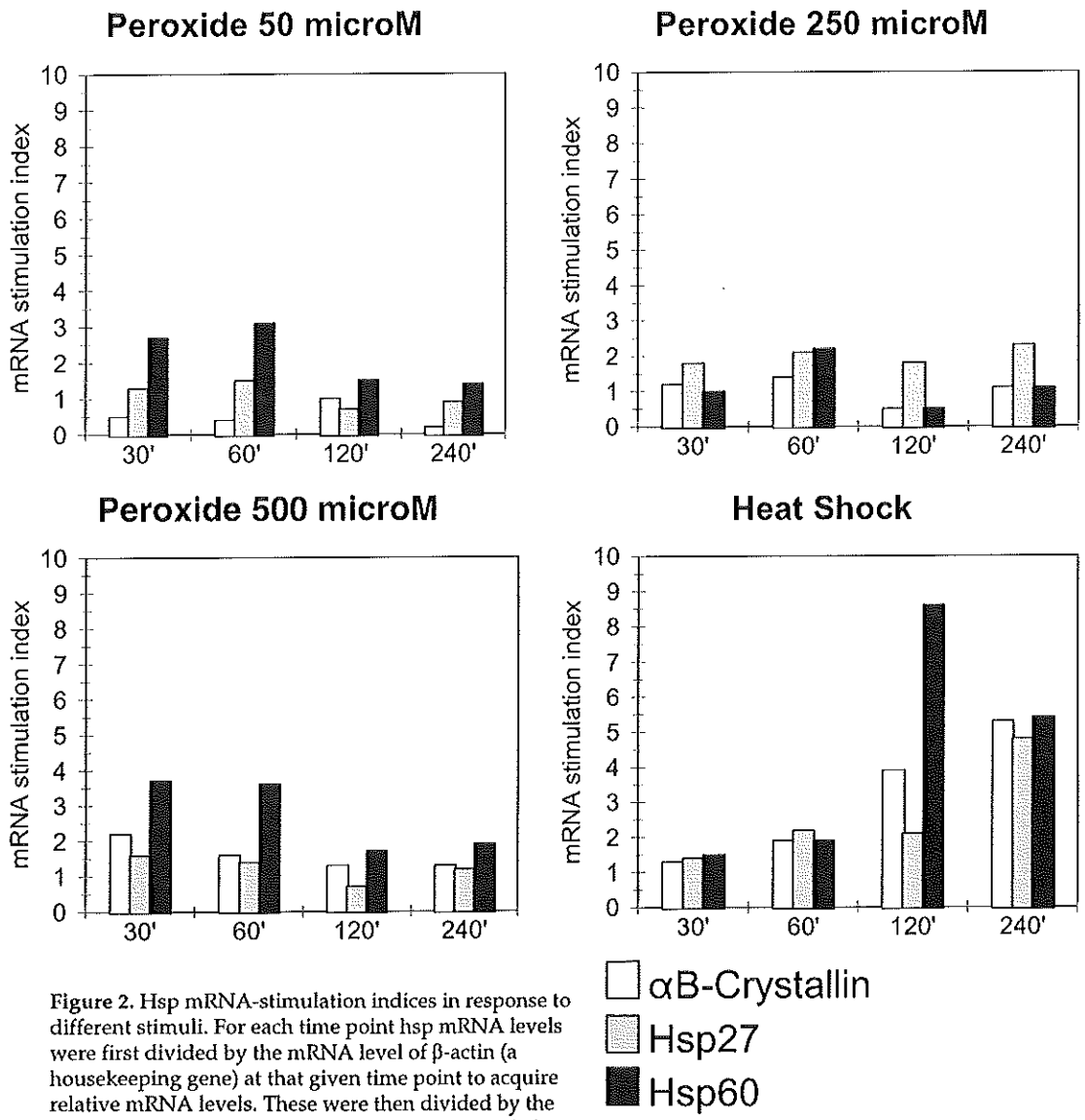

Figure 2. Hsp mRNA-stimulation indices in response to different stimuli. For each time point hsp mRNA levels were first divided by the mRNA level of β-actin (a housekeeping gene) at that given time point to acquire relative mRNA levels. These were then divided by the relative mRNA levels of unstimulated astrocytes (taken as a mean of two separate analyses) yielding the stimulation indices.

cells to heat shock at 43 °C. Amounts of hsp-encoding mRNA relative to that of β-actin-encoding mRNA were calculated for each timepoint. Subsequently, stimulation indices were calculated by dividing the relative amounts of hsp-mRNA by the values as determined for unstimulated astrocytes.
The data reveal that for neither of the two related small hsp, αB-crystallin and hsp27, mRNA levels were increased in response to H_2O_2 over a period of 4 h. By contrast, hsp60-mRNA levels were moderately increased after stimulation with H_2O_2, albeit with high concentrations of H_2O_2. Exposure of astrocytes to heat shock did result in increased mRNA levels of all three hsp studied thereby confirming the ability of the assay to monitor physiologically relevant changes in hsp-mRNA levels (Fig. 2).

Oxidative stress causes rapid phosphorylation of αB-crystallin on serine residue 59
For the detection of various phosphorylated forms of αB-crystallin in response to stimulation with 500μM H_2O_2 antibodies directed against αB-crystallin phosphorylated either at serine residue 19, 45 or 59 were used. In unstimulated astrocytes, αB-crystallin phosphorylated at serine-19 (designated αB-19^P) and serine-45 (αB-45^P) were localized exclusively in the nucleus, although αB-45^P could also be detected in the cytosol of mitotic astrocytes. This latter observation is in line with previous reports (16). αB-59^P was present at low to undetecable levels in the cytosol (Fig 3). Upon H_2O_2 stimulation, levels or localization of neither αB-19^P (Fig. 3A) nor αB-45^P (Fig. 3B) markedly changed, although a slight increase of αB-19^P levels in the cytosol of stimulated astrocytes could be detected after prolonged stimulation (120-180 min). By contrast, already after 1-2 min of H_2O_2 stimulation a dramatic increase in the levels of αB-crystallin phosphorylated at serine-59 (αB-59^P) was detected in the cytosol (Fig. 3C). Staining for αB-59^P produced a cytoplasmic filamentous staining pattern that gradually changed (starting at approximately 60 min) to a more diffuse cytosolic staining pattern. αB-59^P levels peaked at 30-60 min after H_2O_2 stimulation, after which they slowly decreased (Fig. 3C).
In unstimulated astrocytes, expression of αB-crystallin as detected by lap70, was mainly restricted to the nucleus. Starting at 60 min of H_2O_2 stimulation, increasing amounts of αB-crystallin became detectable in the cytosol and continued to increase until 180 min (Fig. 3D). This lagged behind the increased cytoplasmic detectability of αB-59^P by around 60 min and coincided with the change from a filamentous to a more diffuse staining pattern for αB-59^P. Specificity of all stainings was confirmed by blocking studies of the primary antibodies using bovine eye lens-derived αB-crystallin.

Oxidative stress activates both p38-MAPK and p44-MAPK but not SAPK/JNK
In order to examine which kinases could possibly be responsible for the rapid phosphorylation of αB-crystallin at serine-59 astrocytes were stained with antibodies against the activated (phosphorylated) forms of p38-MAPK, p44-MAPK and SAPK/JNK. In unstimulated post-mortem astrocytes activated p44-MAPK (Fig. 4C) and SAPK/JNK were detectable but almost no activated p38-MAPK (Fig. 4A). Within 5 min

following H_2O_2 stimulation, levels of phosphorylated p38-MAPK and p44-MAPK markedly increased whereas no changes were observed in the levels of phosphorylated SAPK/JNK (data not shown). Activated p38-MAPK was mainly localized in the cytosol (Fig. 4B) and levels peaked at 15 min after H_2O_2 stimulation. By contrast, activated p44-MAPK was mainly localized in the nucleus (Fig. 4D).

Inhibition of p38-MAPK abrogates the phosphorylation of αB-crystallin at serine59 and reduces cytosolic accumulation of αB-crystallin

Selective inhibition of p38-MAPK by preincubation of astrocytes with the synthetic inhibitor SB202190 for 60 min prior to 15 min stimulation with 500μM H_2O_2 produced two marked effects on the expression of αB-crystallin. Firstly, the H_2O_2-induced increase in cytosolic αB-59^P (Fig. 5A-D) was abrogated, whereas levels of αB-19^P and αB-45^P were unaffected (data not shown). This indicates that, in human astrocytes, H_2O_2-induced phosphorylation of αB-crystallin at serine-59 is mediated by p38-MAPK.
Secondly, inhibition of p38-MAPK with SB202190 prior to stimulation with H_2O_2, reduced the H_2O_2-induced cytosolic accumulation of αB-crystallin as recognized by the lap70 antibody. Whereas the cytosolic accumulation of αB-crystallin was markedly reduced, the appearance of empty nuclei was hardly affected (Table 1). Thus, phosphorylation of αB-crystallin at serine 59 by p38-MAPK plays a significant role in the cytosolic accumulation of αB-crystallin.

Table 1. Inhibition of p38-MAPK reduces the 500 μM H_2O_2-induced relocalization of αB-crystallin.

	Intracellular distribution of αB-crystallin			
Stimulus	Nucleus	Nucleus/Cytosol	Cytosol	Total #
None	621 (65%)	330 (35%)	0 (0%)	951
500 μM H_2O_2	77 (6%)	1004 (76%)	237 (18%)	1318
SB202190 + 500 μM H_2O_2	768 (31%)	1455 (59%)	243 (10%)	2466

Cells were either unstimulated, stimulated for 180 min using 500 μM H_2O_2 or pre-incubated for 60 min with SB202190 prior to stimulation for 180 min with 500 μM H_2O_2. Cells were categorized in three categories on basis of the intracellular distribution of αB-crystallin. Categories contained cells with either exclusive nuclear expression (nucleus), cells with both nuclear and cytosolic expression (nucleus/cytosol) or exclusive cytosolic expression (cytosol) of αB-crystallin. Analysis of the cells was performed blinded to the conditions used.

In MS lesions phosphorylation of αB-crystallin on serine residue 59 is detected in a subset of αB-crystallin-expressing astrocytes

To investigate whether phosphorylation of αB-crystallin also occurs in developing MS lesions, three MS lesions were analyzed for the presence of the three different phosphorylated forms of αB-crystallin. Serial sections were analyzed using immunohistochemistry. Whereas in all three MS lesions astrocytes and oligodendrocytes contained considerable amounts of αB-crystallin, as determined by staining with the lap70 antibody, phosphorylated forms of αB-crystallin were far less abundantly present (Fig. 6). Two lesions contained detectable amounts of αB-crystallin phosphorylated at serine 59, whereas no other forms of phosphorylated αB-crystallin were detected. Expression of αB-59^P was restricted to astrocytes. Those astrocytes were localized at the lesional edges representing a subset of astrocytes that were stained with the lap70 antibody.

Discussion

In this study we demonstrate that although ROS do not increase mRNA transcription levels of αB-crystallin, they do affect the intracellular distribution of αB-crystallin via p38-MAPK-dependent phosphorylation of αB-crystallin on serine residue 59. In addition, the presence of αB-59^P in astrocytes in MS lesions demonstrates the physiological relevance of phosphorylation on this residue.

Whereas in cultured unstimulated astrocytes the expression of αB-crystallin was mainly restricted to the nucleus, in astrocytes that were stimulated with H_2O_2 or nitric oxide increasing levels of αB-crystallin could be detected in the cytosol with increasing stimulation times. Since H_2O_2 stimulation did not result in an increase of αB-crystallin mRNA levels, the accumulation of cytosolic protein is not likely to be the result of *de novo* synthesis of αB-crystallin. Rather, cytosolic αB-crystallin might represent αB-crystallin that has been relocalized from another subcellular compartment. In support of this idea are the data on phosphorylation patterns of αB-crystallin in response to oxidative stress. The rapid detectability of phosphorylated αB-crystallin on serine residue 59 demonstrates that, although undetectable by the lap70 antibody, considerable amounts of αB-crystallin were already present in the cytoplasm of astrocytes. The decrease in αB-59^P staining after 180 min of H_2O_2 stimulation when compared to 30 min of H_2O_2 stimulation is probably caused by dephosphorylation of αB-crystallin. This illustrates the transient nature of phosphorylation at serine-59.

The initial filamentous staining pattern of αB-59^P in response to oxidative stress can be explained by interaction of αB-crystallin with intermediate filaments (20, 21, 22, 23). In this state, the C-terminal epitope that is recognized by lap70 appearantly is inaccessible for the antibody. The increase in cytosolic expression of αB-crystallin, as detected by lap70, coincides with a change in the filamentous staining pattern of αB-59^P to a more diffuse pattern. This could well be the result of disaggregation of αB-crystallin resulting

in increased visibility to the lap70 antibody. Alternatively, the accumulation of cytosolic αB-crystallin might be the result of relocalization of nuclear αB-crystallin. This could explain the increasing number of cultured astrocytes with an exclusively cytosolic distribution upon prolonged H_2O_2 stimulation times.
In order to examine possible interactions between the H_2O_2-induced altered intracellular distribution of αB-crystallin and its phosphorylation at serine-59, p38-MAPK was selectively inhibited with the synthetic inhibitor SB202190. This abrogated H_2O_2-induced phosphorylation of αB-crystallin at serine-59 (as reported earlier for the astroglioma celline U373 (14)), demonstrating that in human astrocytes H_2O_2-induced phosphorylation of αB-crystallin at serine-59 is mediated by p38-MAPK. More importantly, inhibition of p38-MAPK also reduced the H_2O_2-induced cytosolic accumulation of αB-crystallin, indicating that p38-MAPK-induced phosphorylation of αB-crystallin at serine-59 plays a role in determining the intracellular distribution of αB-crystallin. The acute increase of αB-59^P levels in the cytoplasm (already at 1 min after H_2O_2 stimulation) combined with the predominant cytoplasmic localization of activated p38-MAPK in astrocytes, suggests that phosphorylation of αB-crystallin that was already present in the cytoplasm preceded the shift in intracellular distribution of the protein.
The above is consistent with the idea that phosphorylation of αB-crystallin on intermediate filaments results in cytosolic accumulation of αB-crystallin, and renders it highly unlikely that relocalization from the nucleus would be phosphorylation-dependent.
The detection of αB-59^P in astrocytes in MS lesions demonstrates that phosphorylation at serine-59 also occurs *in vivo*. The distribution pattern of αB-59^P-positive astrocytes reveals several interesting points. Firstly, only a small subpopulation of αB-crystallin-expressing astrocytes were found to contain αB-59^P probably reflecting the transient nature of phosphorylation at serine-59. Secondly, αB-59^P-positive astrocytes were localized at the lesional edges where the (auto)immunological activity is concentrated. Consequently it is likely that the concentration of inflammatory mediators like cytokines, chemokines and reactive oxygen species (ROS) is high in these areas. Therefore, detection of αB-59^P in astrocytes in MS lesions might reflect recent stimulation of these astrocytes with a stressor capable of activating p38-MAPK.

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References

1. van Noort, J.M., van Sechel, A.C., Bajramovic, J.J., el Ouagmiri, M., Polman, C.H., Lassmann, H., and Ravid, R. (1995) *Nature* **375**, 798-801
2. Bajramovic, J.J., Lassmann, H., and van Noort, J.M. (1997) *J Neuroimmunol* **78**, 143-151
3. Bajramovic, J.J., Plomp, A.C., van der Goes, A., Koevoets, C., Newcombe, J., Cuzner, M.L., and van Noort, J.M. *Submitted for publication*
4. Cannella, B. and Raine, C.S. (1995) *Ann Neurol* **37**, 424-435
5. Head, M.W., Corbin, E., and Goldman, J.E. (1994) *J Cell Physiol.* **159**, 41-50
6. Bajramovic, J.J., Bsibsi, M., Geutskens, S.B., Hassankhan, R., Verhulst, K.C., Stege, .J., De Groot, C.J., and van Noort, J.M. (2000) *J Neuroimmunol* (in press)
7. Cross, A.H., Manning, P.T., Keeling, R.M., Schmidt, R.E., and Misko, T.P. (1998) *J Neuroimmunol* **88**, 45-56
8. Smith, K.J., Kapoor, R., and Felts, P.A. (1999) *Brain Pathol* **9**, 69-92
9. Parkinson, J.F., Mitrovic, B., and Merrill, J.E. (1997) *J Mol Med* **75**, 174-186
10. Clerk, A., Michael, A., and Sugden, P.H. (1998) *Biochem J* **333**, 581-589
11. Mehlen, P., Hickey, E., Weber, L.A., and Arrigo, A.P. (1997) *Biochem Biophys Res Commun* **241**, 187-192
12. Mehlen, P., Kretz-Remy, C., Briolay, J., Fostan, P., Mirault, M.E., and Arrigo, A.P. (1995) *Biochem J* **312**, 367-375
13. Mehlen, P., Mehlen, A., Guillet, D., Preville, X., and Arrigo, A.P. (1995) *J Cell Biochem* **58**, 248-259
14. Ito, H., Okamoto, K., Nakayama, H., Isobe, T., and Kato, K. (1997) *J Biol Chem* **272**, 29934-29941
15. Smith, J.B., Sun, Y., Smith, D.L., and Green, B. (1992) *Protein Sci* **1**, 601-608
16. Kato, K., Ito, H., Kamei, K., Inaguma, Y., Iwamoto, I., and Saga, S. (1998) *J Biol Chem* **273**, 28346-28354
17. De Groot, C.J., Langeveld, C.H., Jongenelen, C.A., Montagne, L., Van, D., V, and Dijkstra, C.D. (1997) *J Neurosci Res* **49**, 342-354
18. Bajramovic, J.J., Geutskens, S.B., Bsibsi, M., Boot, M., Hassankhan, R., Verhulst, K.C., and van Noort, J.M. (2000) *Cell Stress Chaperones.* (in press)
19. Piddlesden, S.J., Lassmann, H., Zimprich, F., Morgan, B.P., and Linington, C. (1993) *Am J Pathol* **143**, 555-564
20. Perng, M.D., Muchowski, P.J., van, d., I, Wu, G.J., Hutcheson, A.M., Clark, J.I., and Quinlan, R.A. (1999) *J Biol Chem* **274**, 33235-33243
21. Perng, M.D., Cairns, L., van, d., I, Prescott, A., Hutcheson, A.M., and Quinlan, R.A. (1999) *J Cell Sci* **112**, 2099-2112
22. Muchowski, P.J., Valdez, M.M., and Clark, J.I. (1999) *Invest Ophthalmol Vis.Sci* **40**, 951-958
23. Djabali, K., de Nechaud, B., Landon, F., and Portier, M.M. (1997) *J Cell Sci* **110**, 2759-2769

8.

Cytokine-induced expression of stress proteins in human adult oligodendrocytes

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Abstract

The immunogenic nature of heat shock proteins (hsp) combined with the enhanced expression of hsp in the oligodendrocyte/myelin complex in immunologically active multiple sclerosis (MS) brain lesions suggest an important role for hsp in the pathogenesis of this disease. While enhanced expression of hsp in autoimmune diseases is often regarded as a non-specific bystander effect of the inflammatory process, surprisingly little is known on their regulation by inflammatory mediators such as cytokines. In MS lesions both pro- and anti-inflammatory cytokines are present and might mediate expression levels of different hsp in oligodendrocytes. In this study cytokine-induced expression of two hsp, *viz.* hsp60 and αB-crystallin was studied in cultures of primary human adult oligodendrocytes at the mRNA level and compared to cytokine-induced hsp expression patterns as found in astrocytes.
We show that αB-crystallin mRNA levels are upregulated in both oligodendrocytes and astrocytes in response to TNF-α. In addition, αB-crystallin mRNA levels in oligodendrocytes were also enhanced by stimulation with IFN-γ. The selective upregulation of hsp60 mRNA in oligodendrocytes in response to IL-6 and TNF-α is in marked contrast with the data on cytokine-induced hsp60 mRNA levels in astrocytes. Many more cytokines were found able to induce hsp60 mRNA levels in this cell type. Surprisingly, comparison of stimulation with a combination of IL-1β, IFN-γ and TNF-α and stimulation with either of these cytokines alone showed antagonistic rather than synergistic effects on cytokine-induced hsp-mRNA expression levels. This effect was seen in both oligodendrocytes and astrocytes. In astrocytes, the combination of IL-1β, IFN-γ and TNF-α strongly enhanced expression of TNF-α receptor II (TNF-α RII), while none of the individual cytokines did. Upregulation of TNF-α RII will result in competition between TNF-α RI and TNF-α RII for the available TNF-α, leading to a different use of signal transduction pathways. The absence of a death domain from TNF-α RII, whereas such a domain is present on TNF-α RI, may explain why TNF-α in combination with IL-1β and IFN-γ has much less effect on the expression levels of hsp-mRNA than TNF-α alone.

Keywords: Multiple Sclerosis, Heat Shock Proteins, Oligodendrocytes, Cytokines

1. Introduction

Heat shock proteins (hsp) or stress proteins are produced by both prokaryotic and eukaryotic cells in response to a wide variety of stressful insults such as heat shock, hypoxia, ischaemia, metabolic disruption and various inflammatory mediators. Hsp act as molecular chaperones that regulate transport of proteins across membranes and they prevent inappropriate protein-protein interaction and aggregation of damaged or misfolded proteins (Hightower, 1991; Morimoto et al., 1997). Based upon their molecular weight hsp are categorized into several families i.e. hsp90, hsp70, hsp60 and the small hsp; hsp27 and αB-crystallin (Welsh and Gaestel, 1998; van den IJssel et al., 1999).

There is evidence for the possible involvement of hsp in a variety of (autoimmune) diseases amongst which MS (Georgopoulos and McFarland, 1993; Birnbaum and Kotilinek, 1997). Expression of hsp65/60 has been found on the surface of immature oligodendrocytes in MS lesions, colocalizing with T cells bearing the γδ T-cell receptor (Selmaj et al., 1991). Since γδ T cells are thought to function in a cytotoxic fashion in association with hsp this colocalization might imply a functional relationship (Freedman et al., 1997a; Freedman et al., 1997b). In addition there is evidence for the involvement of hsp60 in the pathogenesis of the animal model for MS, experimental autoimmune encephalomyelitis (EAE) (Gao et al., 1995; Birnbaum et al., 1996). The evidence for an involvement of αB-crystallin, one of the small hsp, in the pathogenesis of MS is even more provocative. Of all the proteins present in MS-affected myelin, αB-crystallin has been identified as the immunodominant human T cell antigen (van Noort et al., 1995). In MS lesions, αB-crystallin was demonstrated to be expressed at enhanced levels in the cytosol of oligodendrocytes and astrocytes already at the earliest stages of lesional development (Bajramovic et al., 1997), and in immunologically active MS lesions αB-crystallin was demonstrated in the endolysosomal compartment of myelin-phagocytosing macrophages colocalizing with infiltrating T lymphocytes. Furthermore, *in vitro* functional MHC-restricted presentation of αB-crystallin to T cells by macrophages fed with total (MS-derived) myelin membranes was demonstrated.

The enhanced expression of hsp in glia cells as observed in MS lesions must be the result of a preceding stressfull event and is often regarded as a non-specific bystander effect of the ongoing inflammatory process. However, even in the presence of a mixture of potential initiators of hsp expression as is the case in MS lesions, the expression patterns of certain hsp are remarkably different (Aquino et al., 1997; Bajramovic et al., 1997). In addition, recent studies demonstrate highly differential expression patterns for hsp in astrocytes in response to a variety of stimuli (Head et al., 1994; Head et al., 1996, Bajramovic et al., in press *a*). There are also indications that the cellular mechanisms by which hsp expression is regulated among various cell types of the human central nervous system (CNS) are different (Freedman et al., 1992; Satoh et al., 1992; Satoh and Kim, 1995). These observations suggest that hsp expression may be regulated by

differential control mechanisms rather than by uniform mechanisms.
The causes of enhanced expression of αB-crystallin and hsp60 in oligodendrocytes in MS lesions are not known, but it could well be that they are responsive to different stimuli present in MS-affected tissue. Glial cells of the CNS such as astrocytes, oligodendrocytes and microglia are able to produce and to respond to the variety of inflammatory mediators that are present in the inflamed brain and cytokine receptors for IL-1 (Blasi et al., 1999), IL-2 (Otero and Merrill, 1997), IL-6 (Watanabe et al., 1996), IFN-γ (Torres et al., 1995) and TNF-α (Dopp et al., 1997) have been demonstrated on oligodendrocytes, both *in vitro* as *in situ* (Otero and Merrill, 1994). Pro-inflammatory cytokines have been shown to mediate the expression of hsp72 in human oligodendrocytes, and an IL-1-mediated common pathway has been suggested for this phenomenon (D'Souza et al., 1994). However, nothing is known about the effect of IL-1 or other cytokines on the expression levels of other hsp than hsp72 in human oligodendrocytes.
In this study we adress the questions whether elevated expression of αB-crystallin and of hsp60 in oligodendrocytes in MS lesions is the inevitable result of the presence of pro-inflammatory mediators and whether these hsp all react in the same way to these mediators. Based on the cytokine receptor profile of oligodendrocytes we selected a panel of (pro-inflammatory) cytokines known to be present in MS lesions and studied their effects on the expression of αB-crystallin and hsp60 in cultures of primary human adult oligodendrocytes. In addition, we compared hsp expression patterns with those obtained for human adult astrocytes. Finally, we describe experiments designed to gain insight in the unexpected expression patterns of hsp that were obtained in response to stimulation with a combination of pro-inflammatory cytokines in both glia celltypes.

2. Materials and methods

2.1. Donors

For all astrocyte experiments post mortem brain white matter derived from two healthy controls was used (for characteristics see chapter 6). For oligodendrocyte-experiments post mortem brain white matter derived from the following donors was used: S98-290 (Alzheimer's disease), S98-286 (Alzheimer's disease), S99-86 (Alzheimer's disease), S99-95 (healthy control).

2.1. In vitro culture of post mortem oligodendrocytes and astrocytes

Adult human post mortem oligodendrocytes were obtained from control and Alzheimer's disease donors. Briefly, tissue samples from brain or spinal cord were collected and meninges and visible blood vessels were removed before mincing the tissue into cubes of $\leq 2mm^3$. The tissue fragments were incubated at 37°C for 20 min in a Hank's balanced salt solution (± 10 ml/g tissue) containing 0.25% w/v porcine trypsin (Sigma, St. Louis, MO), 0.2 mg/ml EDTA, 1 mg/ml glucose and 0.1 mg/ml bovine pancreatic DNase I (Sigma). The supernatant was discarded (no centrifugation) and the pellet was resuspended in 1:1 v/v Dulbecco's Modified Eagle's Medium (high glucose; Life Technologies, Breda, The Netherlands)/HAMF10 (with L-glutamine; Life Technologies) + 5% v/v FCS (Gibco; Life Technologies) + antibiotic supplement (penicillin 100 U/ml and streptomycin 0.1 mg/ml), washed once and passed through a nylon cellstrainer (100 µm; Becton Dickinson Labware Europe, Meylan Cedex, France). Following centrifugation at 1500 rpm for 7 min, the pellet was resuspended in 40 ml Percoll solution (40 ml myelin gradient buffer (see below) + 11.7 ml Percoll + 1.3 ml 1.5 M NaCl; density = 1.03 g/ml). On top of this solution 6 ml of myelin gradient buffer (base: 20 mM $Na_2HPO_4 \cdot H_2O$; acid: 6 mM $NaH_2PO_4 \cdot H_2O$; base was freshly mixed with acid (1:4; pH=7.4), subsequently 140 mM NaCl, 5 mM KCl, 2 g glucose/l and 0.2% (w/v) BSA were added) was carefully added and the solution was centrifuged at 2590 rpm for 30 min (no brakes!). The myelin-containing upper band was discarded and the pellet was washed, incubated in ery-shock buffer (0.2 M NH_4Cl, 13 mM $KHCO_3$; pH= 7.4) for 20 min on ice, washed again and transferred into 25 or 75 cm^2 flasks (Corning Costar Europe, Badhoevedorp, The Netherlands). After 48 h of incubation at 37°C in a humidified atmosphere containing 5% CO_2, unattached cells and myelin debris were collected, washed, if necessary centrifuged using an LSM gradient (ICN Biomedicals, Aurora, OH) to remove dead cells and debris and replated in 6-well plates (Nunc, Roskilde, Denmark). Cells were continuously replated until attachment of cells to the plate in 24 h of less then 5 cells/well was achieved. Subsequently, cells were stimulated and used for PCR or immunohistochemical analysis.

Adult post mortem astrocytes were obtained and cultured as previously described (De Groot et al., 1997; chapter 6). Cells were used at early passage (not after passage 8) and stimulation assays were performed with post confluent cultures.

2.2. *Reagents*
For oligodendrocytes recombinant human cytokines were used at the following concentrations: IL-1β (200 U/ml; Peprotech, Rocky Hill, NJ), IL-2 (100 U/ml; Peprotech), IL-6 (200 U/ml; Peprotech), IFN-γ (250 U/ml; Peprotech), TNF-α (250 U/ml; Peprotech) and the pro-inflammatory mix of IL-1β (200 U/ml), IFN-γ (250 U/ml) and TNF-α (250 U/ml). For astrocytes recombinant human cytokines were used at the following concentrations: IL-1β (500 U/ml), IL-6 (200 U/ml), IFN-γ (500 U/ml), TNF-α (500 U/ml) and the pro-inflammatory mix of IL-1β (500 U/ml), IFN-γ (500 U/ml) and TNF-α (500 U/ml).
Heat shock controls were generated by performing heat shock at 43°C. An equal volume of prewarmed medium of 49°C was added to the cultures and left for 30 min at 43°C before cells were allowed to recover.

2.3. *RT-PCR*
RNA was isolated from pelleted cells using RNAzolB (Campro Scientific, Veenendaal, The Netherlands) and isopropanol precipitation. Using 2.5 of mRNA (or the total precipitate if the total mRNA yield was less then 2.5 μg) as a template, copy DNA was produced using the Reverse Transcription System (Promega, Madison, WI). For amplification, cDNA (1 μl) was added to 1 μl 10 mM dNTP mix, 5 μl 10x Taq-polymerase (Life Technologies, Amsterdam, The Netherlands) and 43 μl PCR-buffer (0.01 M Tris/HCl (pH 8.4), 0.05 M KCl and 0.06 mg BSA/ml). Competitive rt-PCR for β-actin, hsp60 and αB-crystallin was performed as decribed in detail (Bajramovic et al; in press *b*). The PCR was tested for applicability with the low mRNA yields derived from oligodendrocytes and proved to be highly reproducible when used. Briefly, plasmids were used as internal standards that carried target cDNA sequences modified to be approximately 50 bp shorter than the sample cDNA but that still contained the same primer binding sites. Sequence analysis was performed to confirm the correct sequence of the plasmids and parallellism assays were performed to verify equal amplification efficiencies of sample and plasmid cDNA. Sample cDNA was amplified together with internal standards at known concentrations and the 50% equivalence point was calculated following gelelectrophoresis and densitometrical analysis of the amplified products. Relative mRNA values were calculated by dividing the hsp values at a given time point by the β-actin value for that given time point. In order to be able to quantitate even very low cDNA amounts, SYBR-Green (Molecular Probes, Leiden, The Netherlands) staining was sometimes performed instead of the routinely used ethidium bromide for densitometrical analysis.
For the semi-quantitative analysis of cytokine receptor mRNA levels, PCR conditions were as follows: for both IFN-γ receptors 30 cycli were used (2.25 mM $MgCl_2$ concentration was added to the PCR reaction), for IL-1 receptors 1 and 2 and for both TNF-α receptors 40 cycli were used (1.5 mM $MgCl_2$ concentration was added to the PCR reaction). Primers used are given in table 8.1. The β-actin cDNA values that were

obtained by competitive rt-PCR were used to equalize the samples for the amounts of cDNA. The standardized samples were then used for the cytokine receptor PCRs.

Table 8.1. Cytokine-receptor specific primers used.

mRNA	Upstream primer 5'----------3'	Downstream primer 5'----------3'	Sample cDNA (bp)
IL-1 RI	GAAACTACCCGTTGCAGGAGACGG	GCTGGGCTCACAATCACAGGCC	304
IL-1 RII	GGAGGACTCTGGCACCTACGTC	CCAGGGCCACATCGTGTACGAG	297
IFN-γ RI	CCAGATCATGCCACAGGTCC	TCGACTTCCTGCTCGTCTCC	328
IFN-γ RII	CGCTCCTCAGCACCCGAAGA	CCAGCTCAGCTCGAAGGCGT	284
TNF-α RI	CCGTGCCCAGTTCCACCTTC	TTCCTTCCAGCGCAACGGGG	285
TNF-α RII	CACCTGCTGATCACAGCGCC	GGCTTGGGAGGAGCACTGTG	250

IL-1 RI and IL-1 RII primers are specific for IL-1 receptor I and IL-1 receptor II respectively. IFN-γ RI primers amplify mRNA of the ligand binding chain-α of the IFN-γ receptor whereas IFN-γ RII primers are specific for the non-ligand chain-β of the IFN-γ receptor. TNF-α RI primers are specific for the TNF-α receptor I (containing the "death domain") whereas TNF-α RII primers amplify mRNA of the TNF-α receptor II.

2.4. Immunofluorescence

Immunofluorescence was performed on cytospins of stimulated cells. After stimulation, cells were cytofuged at 700 rpm for 8 min and fixed in acetone + 0.03% v/v H_2O_2 (to block endogenous peroxidase) for 10 minutes. Following air-drying of the slides they were incubated overnight at 4°C with primary antibodies in 0.1% w/v BSA and rinsed extensively with PBS + 0.05% w/v Tween-20. To establish purity of the cell cultures rabbit polyclonal anti-glial fibrillary acidic protein (α-GFAP, Zymed, So. San Francisco, CA) as an astrocyte marker, murine monoclonal anti-CD68 (DAKO, Glostrup, Denmark) as a microglia marker, murine monoclonal anti-2',3'-cyclic nucleotide 3'-phosphodiesterase (CNPase; Boehringer Mannheim, Mannheim, Germany) and rabbit polyclonal anti-αB-crystallin (α-lap70, Katholieke Universiteit Nijmegen, the Netherlands) as oligodendrocyte markers were used; as a rule cell cultures were >95% CNPase positive and contained less than 1% CD68- or GFAP-positive cells. For detection of heat shock proteins rabbit polyclonal anti-αB-crystallin (Katholieke Universiteit Nijmegen, the Netherlands) directed against the C-terminal 14 amino acids of αB-crystallin and murine monoclonal anti-hsp60 (Stressgen) were used. Rabbit polyclonal anti-TNF-α receptor I (Sanvertech, Heerhugowaard, The Netherlands) and anti-TNF-α receptor II (Sanvertech) were used to detect TNF-α receptors.
The secondary antibodies (biotinylated horse-anti-mouse (Vector, Burlingame, CA) and biotinylated donkey-anti-rabbit (Amersham Life Science, Roosendaal, The Netherlands)) were incubated in 1% w/v BSA + 1% v/v Normal Human Serum for 45

min at room temperature. After rinsing with PBS + 0.05% w/v Tween-20 samples were incubated with FITC-labelled streptavidin (DAKO) in 1% w/v BSA + 1% v/v Normal Human Serum for 45 min at room temperature. Processed cytospins were embedded using Vectashield (Vector Laboratories, Burlingame, CA).
Negative controls included serum of non-immunized mice or rabbits as a first antibody and primary antibody omission controls. In order to compare expression levels of hsp, cytospins that were stained for the same hsp were photographed using the same shutterspeed. In all cases unstimulated oligodendrocytes were used as the reference.

3. Results and discussion

3.1. Cytokine-induced upregulation of hsp60-encoding mRNA in human oligodendrocytes is a more selective event than in astrocytes

Hsp and β-actin mRNA-derived cDNA levels were measured following stimulation of human post-mortem astrocytes and oligodendrocytes with a panel of cytokines. Amounts of hsp-cDNA relative to β-actin were calculated for each timepoint. Subsequently, stimulation indices were calculated by dividing the relative amounts of hsp cDNA by values determined for unstimulated astrocytes. Stimulation indices ≥ 3 were considered relevant. Heat shock was used as a positive control for enhanced mRNA expression levels of hsp. In astrocytes, heat shock indeed resulted in measurable upregulation of both hsp. In oligodendrocytes, only αB-crystallin-encoding mRNA levels were markedly increased, whereas hsp60-encoding mRNA levels were relatively unaffected (Table 8.2).
Relative cDNA levels of both hsp to β-actin in unstimulated astrocytes varied between 0.002-0.01 for hsp60 and between 0.008-0.05 for αB-crystallin. For unstimulated oligodendrocytes relative cDNA values were markedly higher. They varied between 0.1-0.6 for hsp60 and between 2-6 for αB-crystallin. The relatively high levels of constitutively expressed cytosolic αB-crystallin in unstimulated oligodendrocytes (Fig. 8.1) could even be used as an additional marker to distinguish oligodendrocytes from contaminating microglia cells. Whether the differences in relative hsp-encoding mRNA levels between astrocytes and oligodendrocytes represent differences in β-actin levels is unclear. Alternatively, these high levels might be interpreted as the result of chronic stress for oligodendrocytes. Consistent with this idea is that oligodendrocytes were difficult to culture *in vitro* (they did not proliferate and died within two to three weeks). In addition, they constitutively expressed high levels of activated p38-Mitogen-activated Protein Kinase (p38-MAPK), a family member of the stress-activated kinases (data not shown).
In oligodendrocytes, αB-crystallin mRNA levels were selectively upregulated by both IFN-γ and TNF-α. In astrocytes, TNF-α appeared to be the only individual cytokine tested capable of inducing upregulation of αB-crystallin mRNA levels (Table 8.2).

Table 8.2. αB-Crystallin and hsp60 mRNA stimulation indices in response to different cytokines in oligodendrocytes and astrocytes. αB-Cryst. = αB-crystallin; HS = Heat shock. Marked in bold are SI ≥ 3.

Cell type	Oligodendrocytes		Astrocytes	
Stimulus	αB-Cryst.	Hsp60	αB-Cryst.	Hsp60
HS, 30' at 43°C		*Donor: S99-86*		*S98-142*
1h	**4.1**	1.3	1.9	1.9
2h	**4.6**	0.7	**3.9**	**8.6**
4h	**7.5**	2.1	**5.3**	**5.4**
IL-1β		*S99-95*		*S97-145*
1h	n.d.	n.d.	0.7	1
2h	2.7	2.4	n.d	n.d
4h	2.2	1.6	1.2	**7.2**
24h	n.d.	n.d.	0.7	1.6
IFN-γ		*S99-86*		*S98-142*
1h	n.d.	n.d.	1	2.1
2h	**3.3**	0.9	n.d.	n.d.
4h	2.4	0.9	1	2.8
24h	**4.4**	1.7	1.2	**3.7**
TNF-α		*S99-86*		*S97-145*
2h	1.8	0.5	n.d.	n.d.
4h	2.3	1.1	0.9	2
24h	**9.1**	**3.1**	**4**	**6.3**
48h	n.d.	n.d.	**9.8**	1.4
IL-1β, IFN-γ, TNF-α		*S99-86*		*S98-142*
1h	n.d	n.d	**3.8**	**3.3**
2h	2.2	0.6	n.d	n.d
4h	0.8	0.2	2.4	2.4
24h	0.6	0.3	2.3	1.9
IL-6		*S98-290*		*S97-145*
1h	n.d.	n.d.	0.9	0.9
2h	1.4	**3.6**	n.d.	n.d.
4h	0.9	2	0.8	**8.4**
IL-2		*S98-286*		
1h	1.4	1.2	n.d.	n.d.
2h	1.3	0.9	n.d.	n.d.
4h	0.7	0.7	n.d.	n.d.

Noteworthy is the fact that both IFN-γ and TNF-α have been described as cytotoxic to oligodendrocytes (Vartanian et al., 1995; Selmaj et al., 1991). In both astrocytes and oligodendrocytes, an expected synergistic effect of stimulation with a mix of pro-inflammatory cytokines (consisting of IL-1β, IFN-γ and TNF-α) on expression levels of αB-crystallin-encoding mRNA was not found. In contrast, comparison of the αB-crystallin-inducing potential of the pro-inflammatory mix with the potential of the individual components of this mix would rather suggest an antagonistic mechanism to be involved.
Upregulation of hsp60 mRNA levels in astrocytes were enhanced by stimulation with IL-1β, IFN-γ, TNF-α, IL-6 and the mix of proinflammatory cytokines. This promiscuous pattern of induced mRNA-expression was not found in oligodendrocytes. Only TNF-α and IL-6 were capable of (moderately) upregulating hsp60 mRNA levels (Table 8.2), demonstrating that cytokine-induced upregulation of hsp60 mRNA in human oligodendrocytes appears to be a more selective event than in astrocytes. As for αB-crystallin, induction of hsp60 mRNA by the mix of pro-inflammatory cytokines suggested the involvement of an antagonistic mechanism.
It should be noted that results for oligodendrocytes might be seriously affected by the *in vivo-in vitro* difference (as decribed above) and extra care needs to be taken in extrapolating *in vitro* results for oligodendrocytes. Also, reconfirmation of these results at the protein level is required.

3.2. Stimulation of astrocytes with a mix of pro-inflammatory cytokines induces a marked upregulation of TNF-α receptor II

In order to gain insight in the observed antagonistic effect of the mix of proinflammatory cytokines on induction of hsp-mRNA levels, we analyzed cytokine receptor-mRNA levels during stimulation. The same cDNA that was used for the quantitative analyses of hsp-mRNA levels was used for these experiments. The absolute β-actin cDNA-values, as determined in these experiments, were used to spike samples for cDNA content. Using the same absolute amounts of β-actin mRNA, samples were compared for cytokine receptor mRNA levels.
Stimulation with IL-1β, IFN-γ, TNF-α or the combination of these cytokines did not alter the mRNA levels for IL-1-β receptor I or II, both chains of the IFNγ-receptor or TNF-α receptor I in astrocytes (data not shown). However, while stimulation with IL-1β, IFN- γ or TNF-α weakly induced the expression of TNF-α receptor II (TNF-α RII)-mRNA levels, the mix of these proinflammatory cytokines proved to have a potent synergistic effect on TNF-α RII-mRNA levels (Fig. 8.2). We were not able yet to investigate whether this also applies to oligodendrocytes.
To analyze whether the induced upregulation of TNF-α RII-mRNA led to enhanced protein expression as well, we used immunofluorescence. Fig 8.3 demonstrates that stimulation of astrocytes with the mix of pro-inflammatory cytokines indeed leads to detectable upregulation of TNF-α RII-levels by 24 h.

TNF-α RII does not contain the death domain that enables TNF-α receptor I to induce apoptosis. Enhanced expression of TNF-α RII might therefore exert a protective effect against TNF-α-induced apoptosis by functioning as a decoy receptor. Whether the enhanced expression of TNF-α RII explains the observed antagonistic effects on hsp-mRNA induction remains to be shown. In addition, it would be interesting to study whether oligodendrocytes and astrocytes use this same mechanism.

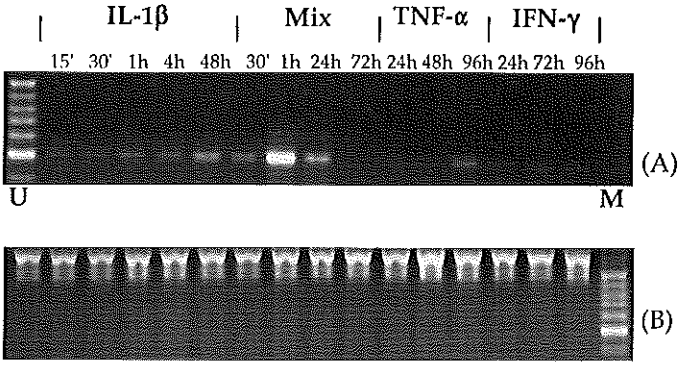

Figure 8.2. Stimulation of human astrocytes with a mix of pro-inflammatory cytokines induces a marked upregulation of TNF-α receptor II (TNF-α RII)-encoding mRNA. The enhanced increase in TNF-α RII-encoding mRNA in response to stimulation with a mix of IL-1β, IFN-γ and TNF-α when compared to induction of TNF-α RII by either of these cytokines alone suggest a synergistic effect.
(A). TNF-α RII-mRNA levels of astrocytes in response to different stimuli as determined by RT-PCR,
(B). β-actin-mRNA levels of astrocytes in response to different stimuli as determined by RT-PCR.
U = unstimulated astrocytes; M = 50-bp-spaced DNA-weight marker (Biozym, Landgraaf, The Netherlands).

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References

Aquino, D.A., Capello, E., Weisstein, J., Sanders, V., Lopez, C., Tourtellotte, W.W., Brosnan, C.F., Raine, C.S., Norton, W.T. 1997. Multiple sclerosis: altered expression of 70- and 27-kDa heat shock proteins in lesions and myelin. J Neuropathol Exp Neurol 56, 664-672.

Bajramovic, J.J., Lassmann, H., van Noort, J.M. 1997. Expression of alphaB-crystallin in glia cells during lesional development in multiple sclerosis. J Neuroimmunol 78, 143-151.

Bajramovic, J.J., Bsibsi, M., Geutskens, S.B., Hassankhan, R., Verhulst, K.C., Stege, G.J.J., de Groot C.J.A., van Noort, J.M. 2000. Differential expression of stress proteins in human adult astrocytes in response to cytokines. J Neuroimmunol *In press a*

Bajramovic, J.J., Geutskens, S.B., Bsibsi, M., Boot, M., Hassankhan, R., Verhulst, K.C., van Noort, J.M. 2000. The stress-kit: a new method based on competitive reverse transcriptase-polymerase chain reactionto quantify the expression of human αB-crystallin, hsp27 and hsp60. Cell Stress Chaperones *In press b*

Birnbaum, G., Kotilinek, L. 1997. Heat shock or stress proteins and their role as autoantigens in multiple sclerosis. Ann N Y Acad Sci 835:157-67, 157-167.

Birnbaum, G., Kotilinek, L., Schlievert, P., Clark, H.B., Trotter, J., Horvath, E., Gao, E., Cox, M., Braun, P.E. 1996. Heat shock proteins and experimental autoimmune encephalomyelitis (EAE): I. Immunization with a peptide of the myelin protein 2',3' cyclic nucleotide 3' phosphodiesterase that is cross-reactive with a heat shock protein alters the course of EAE. J Neurosci Res 44, 381-396.

Blasi, F., Riccio, M., Brogi, A., Strazza, M., Taddei, M.L., Romagnoli, S., Luddi, A., D'Angelo, R., Santi, S., Costantino-Ceccarini, E., Melli, M. 1999. Constitutive expression of interleukin-1beta (IL-1beta) in rat oligodendrocytes. Biol Chem 380, 259-264.

D'Souza, S.D., Antel, J.P., Freedman, M.S. 1994. Cytokine induction of heat shock protein expression in human oligodendrocytes: an interleukin-1-mediated mechanism. J Neuroimmunol 50, 17-24.

De Groot, C.J., Langeveld, C.H., Jongenelen, C.A., Montagne, L., Van, D., V, Dijkstra, C.D. 1997. Establishment of human adult astrocyte cultures derived from postmortem multiple sclerosis and control brain and spinal cord regions: immunophenotypical and functional characterization. J Neurosci Res 49, 342-354.

Dopp, J.M., Mackenzie-Graham, A., Otero, G.C., Merrill, J.E. 1997. Differential expression, cytokine modulation, and specific functions of type-1 and type-2 tumor necrosis factor receptors in rat glia. J Neuroimmunol 75, 104-112.

Freedman, M.S., Bitar, R., Antel, J.P. 1997a. gamma delta T-cell-human glial cell interactions. II. Relationship between heat shock protein expression and susceptibility to cytolysis. J Neuroimmunol 74, 143-148.

Freedman, M.S., Buu, N.N., Ruijs, T.C., Williams, K., Antel, J.P. 1992. Differential expression of heat shock proteins by human glial cells. J Neuroimmunol 41, 231-238.

Freedman, M.S., D'Souza, S., Antel, J.P. 1997b. gamma delta T-cell-human glial cell interactions. I. In vitro induction of gammadelta T-cell expansion by human glial cells. J Neuroimmunol 74, 135-142.

Gao, Y.L., Brosnan, C.F., Raine, C.S. 1995. Experimental autoimmune encephalomyelitis. Qualitative and semiquantitative differences in heat shock protein 60 expression in the central nervous system. J Immunol 154, 3548-3556.

Georgopoulos, C., McFarland, H. 1993. Heat shock proteins in multiple sclerosis and other autoimmune diseases. Immunol Today 14, 373-375.

Head, M.W., Corbin, E., Goldman, J.E. 1994. Coordinate and independent regulation of alpha B-crystallin and hsp27 expression in response to physiological stress. J Cell Physiol 159, 41-50.

Head, M.W., Hurwitz, L., Goldman, J.E. 1996. Transcription regulation of alpha B-crystallin in astrocytes: analysis of HSF and AP1 activation by different types of physiological stress. J Cell Sci 109, 1029-1039.

Hightower, L.E. 1991. Heat shock, stress proteins, chaperones, and proteotoxicity. Cell 66, 191-197.

Morimoto, R.I., Kline, M.P., Bimston, D.N., Cotto, J.J. 1997. The heat-shock response: regulation and function of heat-shock proteins and molecular chaperones. Essays.Biochem 32:17-29, 17-29.

Otero, G.C., Merrill, J.E. 1994. Cytokine receptors on glial cells. Glia 11, 117-128.

Otero, G.C., Merrill, J.E. 1997. Response of human oligodendrocytes to interleukin-2. Brain Behav Immun 11, 24-38.

Satoh, J., Yamamura, T., Kunishita, T., Tabira, T. 1992. Heterogeneous induction of 72-kDa heat shock protein (HSP72) in cultured mouse oligodendrocytes and astrocytes. Brain Res 573, 37-43.

Satoh, J.I., Kim, S.U. 1995. Differential expression of heat shock protein HSP27 in human neurons and glial cells in culture. J Neurosci Res 41, 805-818.

Selmaj, K., Brosnan, C.F., Raine, C.S. 1991. Colocalization of lymphocytes bearing gamma delta T-cell receptor and heat shock protein hsp65+ oligodendrocytes in multiple sclerosis. Proc Natl Acad Sci USA 88, 6452-6456.

Selmaj, K., Raine, C.S., Farooq, M., Norton, W.T., Brosnan C.F. 1991. Cytokine cytotoxicity against oligodendrocytes. Apoptosis induced by lymphotoxin. J Immunol 147:1522-1529.

Torres, C., Aranguez, I., Rubio, N. 1995. Expression of interferon-gamma receptors on murine oligodendrocytes and its regulation by cytokines and mitogens. Immunology 86, 250-255.

van den IJssel, P., Norman, D.G., Quinlan, R.A. 1999. Molecular chaperones: small heat shock proteins in the limelight. Curr Biol 9, R103-R105

van Noort, J.M., van Sechel, A.C., Bajramovic, J.J., el Ouagmiri, M., Polman, C.H., Lassmann, H., Ravid, R. 1995. The small heat-shock protein alpha B-crystallin as candidate autoantigen in multiple sclerosis. Nature 375, 798-801.

Vartanian, T., Li, Y., Zhao, M., Stefansson, K. 1995. Interferon-gamma-induced oligodendrocyte cell death: implications for the pathogenesis of multiple sclerosis. Mol Med 1:732-743.

Watanabe, D., Yoshimura, R., Khalil, M., Yoshida, K., Kishimoto, T., Taga, T., Kiyama, H. 1996. Characteristic localization of gp130 (the signal-transducing receptor component used in common for IL-6/IL-11/CNTF/LIF/OSM) in the rat brain. Eur J Neurosci 8, 1630-1640.

Welsh, M.J., Gaestel, M. 1998. Small heat-shock protein family: function in health and disease. Ann N Y Acad Sci 851:28-35, 28-35.

9.

Infection of human astrocytes with human herpesvirus 6 (HHV-6) does not directly affect the expression of the MS-associated small heat shock protein αB-crystallin[1]

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Abstract

Multiple sclerosis (MS) is a chronic demyelinating disease of the central nervous system (CNS) characterized by focal areas of demyelination (lesions or plaques). Although the exact etiology of MS is unknown, it is generally accepted that autoimmunity is involved in the pathogenesis of the disease. The autoantigen(s) probably reside in CNS myelin, the target of the immune response. In the complete collection of proteins extracted from MS-affected myelin the dominant human T-cell antigen appears to be αB-crystallin, a small heat shock protein. αB-Crystallin is expressed at enhanced levels in the cytoplasm of oligodendrocytes and astrocytes in MS lesions. The cause(s) for this enhanced expression of αB-crystallin is not known yet. Recently, human herpes virus 6 (HHV-6) has been associated with MS. In the present study, the effects of HHV-6 infection on αB-crystallin expression levels and localization in cultured adult human astrocytes were examined.
HHV-6-infected astrocytes displayed slightly enhanced nuclear expression levels of αB-crystallin when compared to non-infected astrocytes. However, this increase in nuclear αB-crystallin expression levels was not detectable in every infected cell and might be related to cell death rather than to HHV-6 infection perse. More importantly, uninfected astrocytes in close vicinity of HHV-6-infected astrocytes showed increased cytosolic staining for αB-crystallin. These results suggest that HHV-6 has an indirect rather than a direct effect on the expression levels or intracellular localization of αB-crystallin. No synergistic or antagonistic effects of IFN-γ, TNF-α or RANTES stimulation on αB-crystallin expression in HHV-6-infected astrocytes were found when compared to cytokine-stimulated non-infected astrocytes.
In summary, these data provide encouragement to examine altered behaviour and immunomodulatory potential of HHV-6-infected astrocytes. Uninfected astrocytes in the direct vicinity of infected astrocytes might be influenced by infected astrocytes. Such indirect effects could play an important role in the innate immune response of the brain responding to viral infection.

Introduction

Multiple sclerosis (MS) is a chronic demyelinating disease of the central nervous system (CNS) characterized by focal areas of demyelination (lesions or plaques). Although the etiology of MS is unknown, both genetic and environmental factors are thought to play a role (Bernard and Kerlero de Rosbo, 1992; ffrench-Constant et al., 1994). It is generally accepted that autoimmunity is involved in the pathogenesis of the disease. The autoantigen(s) probably reside in CNS myelin, the target of the immune response. In the complete collection of proteins extracted from MS-affected myelin the dominant human T-cell antigen appears to be αB-crystallin, a small heat shock protein (van Noort et al., 1995). αB-crystallin is present at enhanced levels in the cytosol of oligodendrocytes and astrocytes in MS lesions, where it is not detectable in healthy controls or normal appearing white matter (Bajramovic et al., 1997). In addition, it has recently been demonstrated that, as an early event after myelin phagocytosis in MS lesions, αB-crystallin becomes available to T cells (Bajramovic et al., submitted). Although TNF-α-induced nuclear expression of αB-crystallin has been described *in vitro* in astrocytes (Head et al., 1994; Bajramovic et al., in press), to date it is not known what causes the enhanced cytosolic expression of αB-crystallin in astrocytes and oligodendrocytes.

Many studies have been devoted to the potential role of viruses in MS (Allen and Brankin, 1993; Dalgleish, 1997; Monteyne et al., 1998; Wekerle, 1998) and several studies have suggested the involvement of human herpes virus-6 (HHV-6) in the pathogenesis of MS. HHV-6 is a β-herpesvirus that was discovered in 1986 as a human B-lymphotropic virus (Salahuddin et al., 1986) and has been identified as the causal agent for exanthem subitum in children. In later studies it was found that the virus predominantly infects $CD4^+$ T cells and monocytes/macrophages (Ablashi et al., 1987), and also has the capability to infect astrocytes, microglia and oligodendrocytes (He et al., 1996; Albright et al., 1998) *in vitro*. HHV-6 is a common virus and thought to be latently present in approximately 90% of the adult population (Luppi et al., 1994). After primary infection the virus is able to establish a persistent infection throughout life.

In MS patients, increased serum and CSF antibody responses to HHV-6 antigens have been measured as compared to healthy controls (Ablashi et al., 1998). Also, active HHV-6 infection could be demonstrated in 30% of MS patients and not in any of the controls (Soldan et al., 1997). In addition, immunohistochemical studies demonstrated the presence of HHV-6 antigens in oligodendrocytes of MS patients only (Challoner et al., 1995). Finally, the potential of HHV-6 to play a role in the demyelination proces as it occurs in MS is suggested by several case-studies (Carrigan et al., 1996; Kamei et al., 1997; Mock et al., 1999).

The reported tropism of HHV-6 for glia cells, the notion that HHV-6 was most frequently present in oligodendrocytes and astrocytes around MS lesions (Challoner et al., 1995), and our own observations on αB-crystallin upregulation in peripheral blood

mononuclear cells by HHV-6 and Epstein Barr virus (Van Sechel et al., 1999) inspired us to investigate whether infection of astrocytes with HHV-6 could affect αB-crystallin expression.
In this study immunofluorescence was used to investigate the effects of HHV-6 infection on αB-crystallin expression levels and localization in human adult astrocytes. In addition, potential synergistic or antagonistic effects of cytokine stimulation on αB-crystallin expression in HHV-6-infected astrocytes were studied.

Material and methods

Culture of human post mortem astrocytes

Human adult post-mortem astrocytes were obtained from control donors as previously described (De Groot et al., 1997). Briefly, tissue samples from subcortical white matter or corpus callosum were collected and meninges and visible blood vessels were removed before mincing the tissue into cubes of ≤2mm^3. The tissue fragments were incubated at 37°C for 20 minutes in a Hank's balanced salt solution containing 0.25% w/v porcine trypsin (Sigma, St. Louis, MO), 0.2 mg/ml EDTA, 1 mg/ml glucose and 0.1 mg/ml bovine pancreatic DNase I. After digestion cell suspensions were gently triturated, washed and taken into culture. Cells were grown in 1:1 v/v Dulbecco's Modified Eagle's Medium (high glucose; Life Technologies, Breda, The Netherlands) /HAMF10 (with L-glutamine; Life Technologies) + 10% v/v FCS (Gibco; Life Technologies) and antibiotic supplement (penicillin 100U/ml and streptomycin 0.1 mg/ml) at 37°C in a humidified atmosphere containing 5% CO_2. To avoid contamination of the astrocyte cultures with meningeal and blood monocyte derived macrophages, cell suspensions were grown overnight in uncoated tissue culture flasks allowing monocytes/macrophages to adhere. Subsequently the supernatant was transferred to poly-L-lysine (15 µg/ml; Sigma, St. Louis, MO) coated 80 cm^2 flasks and after 48 h medium was changed to remove unattached cells and myelin debris. To passage the adherent cells they were rinsed with PBS, incubated for 5-10 min with 0.25% w/v porcine trypsin at 37°C, washed once with culture medium containing 10% v/v FCS and replated in 25 cm^2 flasks (Nunc, Roskilde, Denmark) or 8 well chamberslides (Becton Dickinson, Franklin Lakes, NJ). Cells were used at early passage (not after passage 8) and experiments were performed with post confluent cultures.

Viral stocks and infectivity

The human CD4$^+$ T cell line JJHan was used a a carrier cel lline for HHV-6A (strain U1102; supplied by U. Gompels, London School of Hygiene and Tropical Medicine). Cells were grown in RPMI1640 (Gibco) + 10% v/v FCS, 1 mM pyruvate (Gibco) and

antibiotic supplement (penicillin 100U/ml and streptomycin 0.1 mg/ml) at 37°C in a humidified atmosphere containing 5% CO_2. Viral stocks were prepared by infecting 4 x 10^6 non-infected cells with HHV-6 infected cells ($TCID_{50}$= 1 x 10^6) at a multiplicity of infection (MOI = $TCID_{50}$/# target cells) of 1. Cells were harvested when maximal cytopathological effect (CPE) was observed (day 4-6 post infection) by centrifugation at 1000 rpm for 10 min. Subsequently, cells were pooled in RPMI1640 + 10% v/v FCS + 20% v/v dimethylsulfoxide (DMSO; Merck, Darmstadt, Germany) and stored at -150°C in aliquots of approximately 1 x 10^6 cells/biofreeze vial.
The infectivity of viral stocks ($TCID_{50}$) was determined by means of an immunofluorescence assay. Infected cells were added in a 2-fold serial dilution (2^0-2^4 x) to 1 x 10^6 non-infected cells in 10 ml culture medium in 25 cm^2 flasks. At day 1-6 post infection, samples were taken from each flask and spotted on a multispot microscope slide (Hendley, Essex, UK). Slides were air-dried overnight at 37°C, processed for immunofluorescence (see below), double-stained for CD3 (TRITC) and HHV-6-antigens (FITC) and analyzed under the microscope.

HHV-6 infection of post mortem astrocytes and subsequent cytokine/chemokine stimulations

Human post mortem astrocytes were infected with HHV-6 by adding 50 µl of infected JJHan cells ($TCID_{50}$ = 1 x 10^6)/chamber. Astrocytes were cultured in the presence of infected JJHan cells for 1-10 days, washed extensively with PBS to remove non-adherent JJHan cells, air-dried and processed for (double)-immunofluorescence. To study the effect of cytokines on HHV-6-infected astrocytes, astrocytes were cultured in the presence of infected JJHan cells for 18 h, washed extensively with PBS and recombinant human cytokines or chemokine were added for various timepoints at the following concentrations: IL-1β (500 U/ml; Peprotech, Rocky Hill, NJ), IFN-γ (500 U/ml; Peprotech), TNF-α (500 U/ml; Peprotech), and RANTES (100 ng/ml; Peprotech). HHV-6-infected astrocytes without cytokine-stimulation and non-infected astrocytes stimulated with the same cytokines were examined as controls on the same chamberslides.

Immunofluorescence

Immunofluorescence was performed on multispot microscope slides or on chamberslides. Samples were fixed in acetone + 0.03% v/v H_2O_2 (Merck) for 10 minutes. Following air-drying, slides were incubated overnight at 4°C with primary antibodies in PBS + 0.1% w/v BSA and rinsed extensively with PBS + 0.05% w/v Tween-20. For the detection of astrocytes rabbit polyclonal anti-glial fibrillary acidic protein (α-GFAP, Zymed, So. San Francisco, CA) was used, for the detection of the T cell line JJHan mouse monoclonal anti-CD3 was used (DAKO, Glostrup, Denmark). For detection of MHC-I and II mouse monoclonal anti-human HLA class I antigen (DAKO) and mouse

monoclonal anti-HLA class II antigen (MHC-HLA-D; Novocastra, Newcastle-upon-Tyne, UK) were used. For detection of αB-crystallin affinity-purified rabbit polyclonal anti-lap70 (Katholieke Universiteit Nijmegen, the Netherlands), directed against the C-terminal 14 amino acids of αB-crystallin, was used. For detection of HHV-6 proteins mouse monoclonal NCL-HHV-6 (Novocastra) and mouse monoclonal anti-early antigen (Chemicon, Temecula, CA) were used. Secondary antibodies used were biotinylated horse-anti-mouse (Vector, Burlingame, CA) and biotinylated donkey-anti-rabbit (Amersham Life Science, Roosendaal, The Netherlands) in PBS + 1% w/v BSA + 1% v/v Normal Human Serum (NHS) for 45 min at room temperature. After rinsing with PBS + 0.05% w/v Tween-20, FITC-conjugated streptavidin (DAKO), TRITC-conjugated donkey anti-mouse (Jackson, West Grove, PA) or TRITC-conjugated goat anti-rabbit (Jackson) in PBS + 1% w/v BSA + 1% v/v NHS were added for 45 min at 20 °C.

Results and discussion

Determination of $TCID_{50}$

The tissue culture infectious dose 50 ($TCID_{50}$) is generally used to express infectivity of HHV-6 batches, and it can be determined by several different methods. Most often, HHV-6-infected cells are added in a 10-fold ($1\text{-}10^8$) serial dilution to 1×10^6 non-infected cells and the subsequent occurence of cytopathological effects (CPE) is monitored at days 1-8 after infection. The $TCID_{50}$ is estimated as the final dilution at which CPE can be distinguished at the maximum of infection. Apart from being subjective this method has limited discriminative power. Differences in dilutions ranging from 10^4-10^8 are hard to detect and as a consequence this method is suitable only to detect large differences in infectivity between different stocks. Therefore a new, objective and more quantitative method was developed to determine the $TCID_{50}$. Infected cells were added in a 2-fold serial dilution (1-16 x) to non-infected JJHan cells and at days 1-6 after infection cells were spotted in duplo on multispot slides. The $TCID_{50}$ was then determined by counting the number of infected cells using double-immunofluorescence. Two different anti-HHV-6 antibodies were tested. Both revealed the same infection pattern but the NCL-HHV-6 antibody (Novocastra) consistently proved to detect more infected cells than the anti-early antigen antibody (Chemicon). Therefore, the anti-NCL-HHV-6 antibody was used as the standard.
Infection appeared to follow a biphasic course (Fig. 9.1). At day 2, the number of infected cells increased as compared to day 1, followed by a decrease at day 3. At day 4, maximum of infection was reached. At day 5 and 6, the number of infected cells decreased again, probably due to outgrowth of uninfected JJHan cells. The percentage of infected cells at the maximum of infection (most often day 4-5) was used to estimate the $TCID_{50}$.

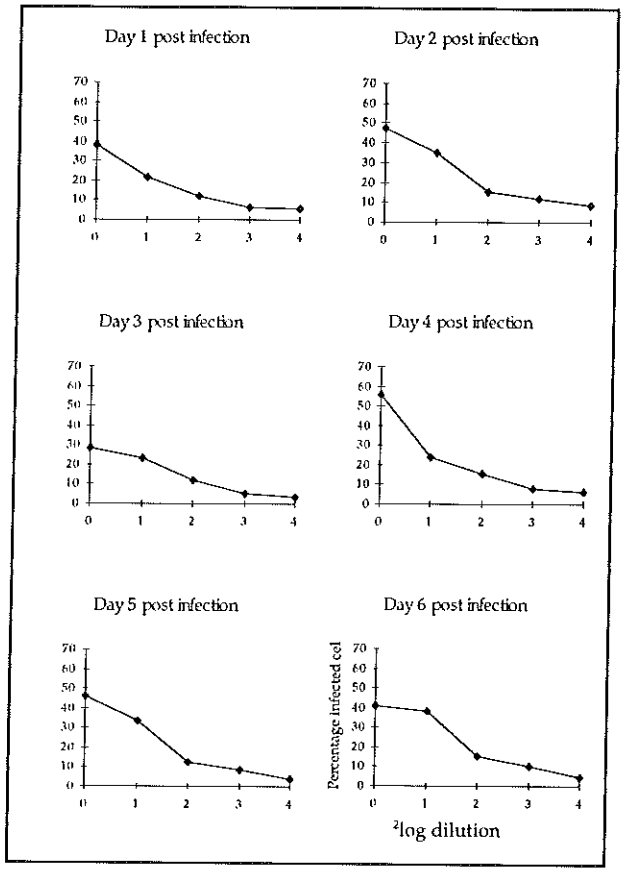

Figure 9.1. Determination of $TCID_{50}$ for JJHan batch 980922 by 2-fold titration (1-16x). At day 1-6 post infection, percentages of infected cells were counted using double-immunofluorescence. The maximum of infection was about 55% (day 4), leading to a $TCID_{50}$ of approximately $1x10^5$.

Table 9.1. Infectivity of different viral stocks as determined by limiting dilution of CPE and by immunofluorescence assay (IFA).

Virus batch #	$TCID_{50}$ (determined by CPE)	Maximum % infected cells (determined by IFA)
980729	1×10^6	37%
980922	1×10^6	55%
990309	1×10^6	28%

When the immunofluorescence assay (IFA) is compared to the method based on CPE-scores upon dilution it is evident that the IFA is better suited to objectively discriminate between different viral stocks (Table 9.1). Despite the fact that the IFA is more discriminative, its utility in determining $TCID_{50}$ is limited to viral stocks that are strong enough to yield a minimum of 50%-positive cells. However, even when this minimum rate of infection is not reached, improved characterization of different viral stocks is possible by the use of IFA.

Expression of αB-crystallin in HHV-6-infected astrocytes

Recently, it has been shown that cultured human fetal astrocytes can be infected by HHV-6 (He et al., 1996). There are no data available, however, on cultured adult human astrocytes. In this study, human post mortem astrocytes were co-cultured with HHV-6-infected JJHan cells (MOI of ± 5) for 1-10 days. By immunofluorescence, astrocytes were found to be positive for HHV-6 antigens from day 1 post infection onward (Fig. 9.2A). HHV-6 protein was detected mainly in the nucleus of infected cells. At the maximum of infection (days 2-4), about 75% of the astrocytes were positive for HHV-6. Haematoxilin-staining showed extensive fusion of infected JJHan cells with astrocytes (data not shown). Infected astrocytes showed severe CPE. Normal morphology was lost, cells were swollen and often contained more than one nucleus. At later stages of infection (days 7-10) the number of infected astrocytes decreased probably due to cell death. Since HHV-6 infection was not detectable by immunofluorescence after day 8-9 and total numbers of astrocytes at this timepoint were hardly affected, it is not likely that HHV-6 infection productively spreads in astrocytes. Astrocytes might be permissive for the HHV-6 infectious cycle to a certain stage only and as a consequence HHV-6 could persist latently. However, the extensive CPE of infected astrocytes combined with the reported production of HHV-6 virions by infected astrocytes (He et al., 1996) renders this highly unlikely. Rather, it can be hypothesized that HHV-6 infection of astrocytes occurred by cell-fusion of infected JJHan cells with astrocytes. The replicative cycle of HHV-6 would then be completed in astrocytes resulting in cell death. Subsequently, the produced virions would not (or only to a limited extent) be able to gain access to uninfected astrocytes. Such a limited accessibility of astrocytes for HHV-6 would not have been predicted from the recent identification of CD46 as a cellular receptor for HHV-6 (Santoro et al., 1999). CD46 is described to be expressed on cultured human astrocytes (Gordon et al., 1992).
Doublestaining of infected astrocytes for αB-crystallin and HHV-6 (Fig. 9.2B) showed that αB-crystallin often co-localized with HHV-6 protein in the nucleus of cells. As a consequence, comparison of expression levels of αB-crystallin between uninfected and infected astrocytes by the use of double-immunofluorescence was hampered. Since

infected astrocytes showed extensive CPE, infected astrocytes were discriminated from uninfected astrocytes on the basis of morphological criteria. Single staining for αB-crystallin was performed and infected, morphologically abnormal astrocytes were compared with morphologically normal astrocytes in the same chamber and with uninfected astrocytes from adjacent cultures. Infected astrocytes seemed to have slightly enhanced nuclear expression levels of αB-crystallin when compared to non-infected astrocytes (Fig. 9.2C-D). However, this increase in nuclear αB-crystallin expression level was not visible in every infected cell and might therefore relate to cell death rather than to HHV-6 infection perse. More importantly, uninfected astrocytes in HHV-6-infected cultures showed increased cytosolic staining for αB-crystallin (Fig. 9.2E). These results suggest that HHV-6 has an indirect rather than a direct effect on the expression levels or intracellular localization of αB-crystallin.
Such an indirect effect might be caused by soluble factors produced by HHV-6-infected astrocytes. The HHV-6 genome does contain sequences encoding a chemokine (Zou et al., 1999) and a β-chemokine receptor (Gompels et al., 1995; Isegawa, 1998) that might be expressed in infected astrocytes . In addition, it has been described that viral infection leads to enhanced production of nitric oxide (NO) by infected cells (Lopez-Guerrero and Alonso, 1997; Paludan et al., 1998). Oxidative stressors such as NO have recently been shown to induce cytosolic expression of αB-crystallin in human post mortem astrocytes (Bajramovic et al., submitted), making it tempting to speculate on oxidative stress as a cause for the appearance of cytosolic αB-crystallin in uninfected astrocytes that are present in the direct vicinity of HHV-6-infected astrocytes.
In the near future, studies on potential soluble mediators produced by HHV-6-infected astrocytes as well as studies on expression levels of immunomodulatory mediators (e.g. inducible NO-synthase) in infected astrocytes will be started. Already in this study, the influence of HHV-6 infection on the expression levels of MHC class I and MHC class II molecules was studied. *In vitro*, uninfected human post mortem astrocytes were found to express high levels of MHC class I and low levels of MHC class II. These expression levels were not altered upon infection (data not shown).
At least as interesting as the effects on astrocytes are the effects of HHV-6 infection on oligodendrocytes. Recently it has been described that oligodendrocytes are susceptible for HHV-6 infection (Albright et al., 1998) and preliminary results in our lab indeed confirm this. Noteworthy is the fact that the U373 astroglioma celline (ATCC; Manassas, VA) was not susceptible to HHV-6 infection (data not shown).

Expression of αB-crystallin in HHV-6-infected astrocytes stimulated with TNF-α, IFN-γ or RANTES

During inflammation, many different cytokines and chemokines are produced with the potential to modulate expression of αB-crystallin in astrocytes. The presence of HHV-6 in astrocytes might modulate signal transduction pathways triggered by these

mediators. For example, the genome of HHV-6 has been reported to contain a functional β-chemokine receptor. This could render infected cells responsive to chemokines that would normally not be responded to (Isegawa, 1998). To investigate whether the effect of certain cytokines or chemokines on αB-crystallin expression by astrocytes is altered upon infection with HHV-6, astrocytes were infected with HHV-6 prior to exposure to the cytokines TNF-α (500 U/ml) and IFN-γ (500 U/ml) and the chemokine RANTES (100 ng/ml). Infected astrocytes were stimulated for 4, 24 and 48 hr and slides were stained for αB-crystallin. Non-infected astrocytes, infected astrocytes without stimulation, and stimulated non-infected astrocytes were used as controls. As described previously (Bajramovic et al., in press), αB-crystallin expression levels were upregulated at 24 hr and 48 hr post stimulation with TNF-α in uninfected astrocytes. An increase in the number of cells with nuclear/cytoplasmic staining for αB-crystallin was observed after TNF-α stimulation of HHV-6-infected astrocytes similar to what is seen after HHV-6 infection alone (Fig. 9.2F). Also, stimulation of infected astrocytes with IFN-γ or RANTES did not result in altered expression levels or intracellular localization of αB-crystallin. In summary, these experiments do not provide clues for altered signal transduction pathways in astrocytes for TNF-α, IFN-γ and RANTES after HHV-6 infection. It should be kept in mind, however, that the predictive value of these experiments is limited since the read-out parameter was based on altered expression of αB-crystallin only.

Taken together, these studies do not provide much evidence for direct effects of HHV-6 infection on the expression of αB-crystallin (and hsp27 and hsp60; data not shown) in adult astrocytes. However, they do provide a strong stimulus to examine altered behaviour of infected astrocytes that might affect uninfected astrocytes in the direct vicinity. In addition, the recently acquired capability to culture adult oligodendrocytes *in vitro* in our lab allows studies on interactions of HHV-6 with the glia celltype thought to be most important in the pathogenesis of MS. Noteworthy in this aspect is the suggested MS lesion-specific association of HHV-6-infected oligodendrocytes.

Whether HHV-6 is *the* cause for the expression of αB-crystallin in glia cells as found in MS lesions might be an inappropriate question. It could well be that HHV-6 is *a* cause for the expression of αB-crystallin in glia cells as found in MS lesions. Double-staining of MS lesions for HHV-6 and αB-crystallin could give more insight, although one can argue that the initial stimulus (i.e. HHV-6 infection) might be long gone by the time a lesion develops, leaving only a footprint in the form of molecules that are affected by infectious stress. A broad *in vitro* study of altered mRNA-expression levels upon infection using DNA-microarrays combined with immunofluorescence to localize the changes in expression or localization of putative target molecules could provide valuable insight in the intruiging interactions between HHV-6 and glia cells.

References

Ablashi, D.V., Salahuddin, S.Z., Josephs, S.F., Imam, F., Lusso, P., Gallo, R.C. HBLV (or HHV-6) in human cell-lines. *Nature* 329: 207 (1987).

Ablashi, D.V., Lapps W., Kaplan, M., Whitman, J.E., Richert, J.R., Pearson, G.R. Human Herpesvirus 6 (HHV-6) infection in multiple sclerosis: a preliminary report. *Mult. Scler.* 4:490-498 (1998).

Albright, A.V., Lavi, E., Black, J.B., Goldberg, S., O'Connor, M.J., Gonzalez-Scarano, F. The effect of human herpesvirus-6 (HHV-6) on cultured human neural cells: oligodendrocytes and microglia. *J. Neurovirol.* 4: 486-492 (1998).

Allen, I., Brankin, B. Pathogenesis of Multiple Sclerosis-The Immune Diathesis and the Role of Viruses. *J. Neuropathol. Exp. Neurol.* 52 (2): 95-105 (1993).

Bajramovic, J.J., Lassman, H., van Noort, J.M. Expression of αB-crystallin in glia cells during lesional development in multiple sclerosis. *J. Neuroimmunol.* 78: 143-151 (1997).

Bajramovic, J.J., Bsibsi, M., Geutskens, S.B., Hassankhan, R., Verhulst, K.C., Stege, G.J.J., De Groot, C.J., Van Noort, J.M. Differential expression of stress proteins in human adult astrocytes in response to cytokines. *In Press: J. Neuroimmunol.* (2000).

Bajramovic, J.J., Plomp, A.C., Van der Goes, A., Koevoets, C., Newcombe, J., Cuzner, M.L., Van Noort, J.M. Presentation of αB-crystallin to T cells in active Multiple Sclerosis lesions: an early event following inflammatory demyelination. *Submitted for publication.*

Bernard, C.C.A., Kerlero de Rosbo, N. Multiple Sclerosis: an autoimmune disease of multifactorial etiology. *Curr. Opin. Immunol.* 4: 760-765 (1992).

Carrigan, D.R., Harrington, D., Knox, K.K. Subacute leukoencephalitis caused by CNS infection with human herpesvirus-6 manifesting as acute multiple sclerosis. *Neurology* 47:145-152 (1996).

Challoner, P.B., Smith, K.T., Parker, J.D., MacLeod, D.L., Coulter, S.N., Rose, T.M., Schultz, E.R., Lindsley Bennet, J., Garber, R.L., Chang, M., Schad, P.A., Stewart, P.M., Nowinski, R.C., Brown, J.P., Burmer, G.C. Plaque-associated expression of human herpesvirus 6 in multiple sclerosis. *Proc. Natl. Acad. Sci. USA* 92: 7440-7444 (1995).

Dalgleish, A.G. Viruses and Multiple Sclerosis. *Acta Neurol. Scand.* Suppl. 169: 8-15 (1997).

De Groot, C.J.A., Langeveld, C.H., Jongenelen, C.A.M., Montagne, L., Van Der Valk, P., Dijkstra, C.D. Establishment of human adult astrocyte cultures derived from postmortem multiple sclerosis and control brain and spinal cord regions: immunophenotypical and functional characterization. *J. Neurosci. Res.* 49: 342-354 (1997).

ffrench-Constant, C. Pathogenesis of Multiple Sclerosis. *Lancet* 343: 271-275 (1994).

Gompels, U.A., Nicholas, J., Lawrence, G., Jones, M., Thomson, B.J., Martin, M.E., Efstathiou, S., Craxton, M., Macaulay, H.A. The DNA sequence of human herpesvirus-6: structure, coding content, and genome evolution. *Virology* 209(1):29-51 (1995).

Gordon, D.L., Sadlon, T.A., Wesselingh, S.L., Russell, S.M., Johnstone, R.W., Purcell, D.F. Human astrocytes express membrane cofactor protei (CD46), a regulator of complement activation. *J. Neuroimmunol.* 36(2-3): 199-208 (1992).

He, J., McCarthy, M., Zhou, Y., Chandran, B., Wood, C. Infection of primary human fetal astrocytes by human herpesvirus 6. *J. Virol.* 70 (2):1296-1300 (1996).

Head, M.W., Corbin, E., Goldman, J.E. Coordinate and independent regulation of α B-crystallin and hsp27 expression in response to physiological stress. *J. Cell. Physiol.* 159, 41-50 (1994).

Isegawa, I. Human herpesvirus 6 open reading frame U12 encodes a functional beta-chemokine receptor. *J. Virol.* 72 (7): 6104-6112 (1998).

Kamei, A., Ichinohe, S., Onuma, R., Hiraga, S. Acute disseminated demyelination due to primary human herpesvirus-6 infection. *Eur. J. Pediatr.* 156: 709-712 (1997).

Lopez-Guerrero, J.A., and M.A. Alonso. Nitric oxide production induced by herpes simplex virus type 1 does not alter the course of the infection in human monocytic cells. *J. Gen. Virol.* 78:1977-80 (1997).

Luppi, M., Barozzi, P., Maiorana, A., Marasca, R., Torelli, G. Human Herpesvirus 6 infection in normal human brain tissue. *J. Infect. Dis.* 169: 943-944 (1994).

Mock, D.J., Powers, J.M., Goodman, A.D., Blumenthal, S.R., Ergin, N., Baker, J.V., Mattson, D.H., Assouline, J.G., Bergey, E.J., Chen, B., Epstein, L.G., Blumberg, B.M. Association of human herpesvirus 6 with the demyelinative lesions of progressive multifocal leukoencephalopathy. *J. Neurovirol.* 5:363-367 (1999).

Monteyne, P., Bureau, J-F., Brahic, M. Viruses and multiple sclerosis. *Curr. Opin. Neurol.* 11: 287-291 (1998).

Paludan, S.R., Ellermann-Eriksen, S., Mogensen, S.C. NF-kappaB activation is responsible for the synergistic effect of herpes simplex virus type 2 infection on interferon-gamma-induced nitric oxide production in macrophages. *J.Gen. Virol.* 79:2785-93 (1998).

Salahuddin, S.Z., Ablashi, D.V., Markham, P.D., Josephs, S.F., Sturzenegger, S., Kaplan, M., Halligan, G., Biberfeld, P., Wong-Staal, F., Kramarsky, B., Gallo, R.C. Isolation of a New Virus, HBLV, in Patients with Lymphoproliferative Disorders. *Science* 234: 596-601 (1986).

Santoro, F., Kennedy, P.E., Locatelli, G., Malnati, M.S., Berger, E.A., Lusso, P. CD46 is a cellular receptor for human herpesvirus 6. *Cell* 99(7): 817-827 (1999).

Soldan, S.S., Berti, R., Salem, N., Secchiero, P., Flamand, L., Calabresi, P.A., Brennan, M.B., Maloni, H.W., McFarland, H.F., Lin, H-C., Patnaik, M., Jacobson, S. Association of human herpes virus 6 (HHV-6) with multiple sclerosis: Increased IgM response to HHV-6 early antigen and detection of serum HHV-6 DNA. *Nat. Med.* 3 (12): 1394-1397 (1997).

Van Noort, J.M., van Sechel, A.C., Bajramovic, J.J., El Ouagmiri, M., Polman, C.H., Lassman, H., Ravid, R. The small heat-shock protein αB-crystallin as candidate autoantigen in multiple sclerosis. *Nature* 375: 798-801 (1995).

Van Sechel, A.C., Bajramovic, J.J., Van Stipdonk, M.J.B., Persoon-Deen, C., Geutskens, S.B., Van Noort, J.M. Epstein-Barr virus-induced expression and HLA-DR-restricted presentation by human B cells of αB-crystallin, a candidate autoantigen in multiple sclerosis. *J. Immunol.* 162 (1): 129-135 (1999).

Wekerle, H. The viral triggering of autoimmune disease. *Nat. Med.* 4 (7): 770-771 (1998).

Zou, P., Isegawa, Y., Nakano, K., Haque, M., Horiguchi, Y., Yamanishi, K. Human herpesvirus 6 open reading frame U83 encodes a functional chemokine. *J. Virol.* 73(7):5926-33 (1999).

10.

General Discussion

Multiple Sclerosis (MS) is a severely disabling neurological disease affecting mostly young adults. MS patients develop focal inflammatory reactions in the central nervous system (CNS) resulting in the selective breakdown of myelin. The exact etiology of MS is still unknown but the immune system is known to be involved in the pathogenesis. Currently, the most accepted hypothesis is that the clinical features of MS are due to a T cell-mediated autoimmune response that is directed against myelin antigen(s). There is, however, very little evidence for differences in the peripheral immune system of MS patients and healthy controls. Therefore it could well be that the peripheral T cell repertoire against myelin antigens that exists in both MS patients and healthy controls is largely a background feature, acted upon by other factors that are more specific for MS.
Instead of analyzing potential differences in the peripheral immune system, we choose an approach in which the T cell response profiles of MS patients and healthy controls were mapped against the complete, fractionated collection of proteins derived of MS-affected CNS myelin and were compared with responses that were measured against control CNS myelin. This unbiased approach took into account that the MS-affected tissue could be somehow different in its biochemical make-up from normal tissue. The study led to the identification of an immunodominant human T cell autoantigen in MS-affected myelin that was absent in control myelin; the small heat shock protein (hsp) αB-crystallin (chapter 2). The studies described in this thesis were designed to characterize the expression of αB-crystallin in the CNS. Major questions adressed were where, when, why and how αB-crystallin was expressed. The combination of the *in situ* and *in vitro* studies described in this thesis revealed several interesting phenomena with regard to the expression of αB-crystallin. In this chapter results of these studies as well as the implications of these findings for the potential role of αB-crystallin as autoantigen will be discussed.

Expression of αB-crystallin in glia cells

Where and when?
In MS lesions, the expression of αB-crystallin was restricted to the cytosol of oligodendrocytes and astrocytes (chapter 2). In general, relatively more αB-crystallin-expressing astrocytes could be detected than αB-crystallin-expressing oligodendrocytes. Expression of αB-crystallin in glia cells correlated with immunological activity. In immunologically active lesions the relative numbers of αB-crystallin-expressing glia cells were maximal, whereas in immunologically inactive lesions αB-crystallin-expressing oligodendrocytes were virtually absent in contrast to αB-crystallin-expressing astrocytes. In addition to the difference in kinetics and relative numbers of αB-crystallin-expressing glia cells, the distribution pattern of αB-crystallin-expressing oligodendrocytes differed markedly from that of αB-crystallin-expressing astrocytes in MS lesions (chapter 3). αB-Crystallin-expressing astrocytes showed a patchlike

distribution pattern, whereas αB-crystallin expression in oligodendrocytes showed a more individual distribution pattern. Even adjacent oligodendrocytes displayed dramatic differences in apparent αB-crystallin levels. These differences suggest either different regulation pathways for hsp in each cell type or the involvement of different types of mediators to be responsible for the altered expression of αB-crystallin in both types of glia cells (as will be discussed in detail below).
Immunologically, the enhanced or altered (see below) expression of αB-crystallin in glia cells in very early MS lesions and the demonstration of αB-crystallin in myelin-phagocytosing macrophages in actively demyelinating lesions (chapter 4) confirmed a role for αB-crystallin as autoantigen in the *progression* of a lesion. Whether αB-crystallin also is likely to play a role in the *initiation* of MS (lesions) is a question not easily answered. We propose the following series of events in the development of a lesion:

1) Local "stress" in the CNS resulting in:
   a) tissue damage,
   b) activation of local APC
2) Blood Brain Barrier (BBB) activation
3) Infiltration and inflammation
4) (Partly reversible) Demyelination
5) (Irreversible) Axonal damage

Whereas point 1 is still speculative, data are gradually accumulating on abnormalities and activation of local APC in normal appearing white matter (NAWM) of MS patients (1,2). If the CNS is thought to play an active role in the *initiation* of the immunopathological process, then αB-crystallin expression might be altered before the lesion starts to develop. Important to note in this aspect is that we never found early lesions *without* αB-crystallin expression in glia cells (chapter 3), something that would be expected if the expression of αB-crystallin would follow rather than precede the inflammatory reaction. Also, the localization of αB-crystallin-expressing glia cells at the lesional borders suggest that αB-crystallin expression either precedes or very closely follows lesional progression. Although this is circumstantial evidence, we believe that αB-crystallin also plays a role in the initiation phase of lesional development (as will be discussed in more detail below).

Why and how?
Since the expression of αB-crystallin in MS lesions appeared different in astrocytes as compared to oligodendrocytes, both cell types will be adressed separately.
The patchlike distribution pattern of αB-crystallin-expressing *astrocytes* in MS lesions suggests that soluble mediators might be responsible for the altered expression of αB-crystallin in astrocytes. In studies described in chapter 6 the influence of pro- and anti-inflammatory cytokines, known to be present in MS lesions, on the expression of αB-crystallin, hsp27 and hsp60 was investigated. The expression of αB-crystallin mRNA appeared to be regulated differently from other hsp like hsp27 and hsp60. *In vitro*

results were in line with our *in situ* observations and convincingly demonstrate that although αB-crystallin, hsp27 and hsp60 are all heat shock proteins by name, they are highly differentially regulated stress proteins by nature. The data on induced hsp expression patterns in human astrocytes in response to cytokines and RANTES showed that enhanced expression of hsp60 is a relatively aspecific event. Hsp60 mRNA- and protein-expression levels were found to be enhanced in response to ROS (chapter 8) and to both pro- and anti-inflammatory cytokines. Upregulation of hsp27 was primarily induced by immunoregulatory cytokines such as IL-4, IL-6 and TGF-β whereas enhanced αB-crystallin expression was found to be even more restricted. Only pro-inflammatory cytokines, and in particular TNF-α, were found capable of enhancing mRNA- and protein levels. These data are particularly interesting in that the selective upregulation pattern of αB-crystallin in response to cytokines when compared to the relative promiscuous pattern found for hsp60 most probably results in different frequencies with which the immune system encounters these hsp. In addition, it is likely that the immunological context in which different hsp are seen by the immune system differ, thereby possibly giving rise to different types of immune responses that are raised towards different hsp. In support of this idea it has been reported that T-cell responses to hsp60 are of the immunoregulatory (3, 4) rather than of the Th1 type. This in marked contrast to the observed type1 T-cell responses directed against the more selectively expressed αB-crystallin (5).
Surprisingly, none of the cytokines studied caused altered expression of αB-crystallin in a way that is consistent with the *in situ* situation. However, *in vitro* studies (chapter 7) demonstrated the potential of reactive oxygen species (ROS) to cause the cytosolic expression of αB-crystallin as found in MS lesions, without affecting mRNA expression levels. ROS-induced phosphorylation of αB-crystallin by p38-MAPK, and in particular the (transient) phosphorylation of serine residue 59, was demonstrated to play an important role in determining the intracellular localization of αB-crystallin. Furthermore, *in situ* analysis of MS lesions suggested that this phenomenon indeed takes place in astrocytes in MS lesions. These observations suggest that enhanced cytosolic expression of αB-crystallin in astrocytes in MS lesions as detected by immunohistochemistry may reflect changes in the intracellular distribution of the protein rather than enhanced transcription. While enhanced mRNA-transcription and protein-translation levels might affect the antigen dose, phosphorylation and changes in the intracellular localization of αB-crystallin could affect immune responses directed against αB-crystallin in a different way (Table 10.1). It has for example been shown that phosphorylation of αB-crystallin can result in the generation of neo-epitopes for T cells (6). However, the amount of phosphorylated αB-crystallin found *in situ*, the exclusive localization in astrocytes and the transient nature of the phosphorylation at serine residue 59 renders it unlikely that phosphorylated αB-crystallin will be presented to T cells in MS lesions, although one can not formally rule out this possibility. Also, the shift in intracellular localization from either the nucleus to the cytosol or from a highly

aggregated state to a less aggregated state could render αB-crystallin more readily available to APC's. One could even interprete such an enhanced visibility of αB-crystallin to the immune system as a genuine "danger signal"(7). In addition, these studies show that studies directed only at mRNA- and protein expression levels are insufficient to detect the more subtle changes that might well be relevant to the immune system. Sensitive new and fashionable techniques like DNA-microarrays and proteomics show enormous potential but are limited by the fact that both techniques are not suited to detect such differences. Therefore, supplementation of those kinds of studies with immunohistochemical or immunofluorescent detection methods remains advisable.
Whether ROS are responsible for the altered αB-crystallin expression in astrocytes in MS lesions remains to be established. The cellular sources of ROS in MS lesions can be cells of the CNS (activated microglia and possibly also activated astrocytes) as well as infiltrating macrophages (8). Comparative *in situ* studies studying the expression of αB-crystallin and e.g. iNOS should provide more insight in the role of ROS, ROS-producing cells and altered expression of αB-crystallin in astrocytes.

Table 10.1. Altered expression of αB-crystallin and the possible consequences for immune recognition.

Altered expression of αB-crystallin at the level of:	Effect on the immune system:
mRNA transcription	Antigen dose
Protein translation	Antigen dose
Protein phosphorylation	Neo-epitopes
Intracellular localization	Availability for APC

In MS lesions relatively fewer αB-crystallin-expressing *oligodendrocytes* than αB-crystallin-expressing astrocytes could be detected. Where αB-crystallin-expressing astrocytes were distributed in a patchlike pattern, αB-crystallin-expressing oligodendrocytes were distributed in a markedly different way (chapter 3). Even adjacent oligodendrocytes displayed dramatic differences in apparent αB-crystallin levels. Since the microenvironment of adjacent oligodendrocytes and astrocytes in a lesion is the same, they will have been exposed to the same set of soluble mediators. Possibly, oligodendrocytes are less sensitive to these mediators or react in a different way to them. Decreased sensitivity to soluble mediators like cytokines can be caused by differences in (cytokine) receptor occupancy or by differences in signal transduction pathways.

However, *in vitro* studies presented in chapter 8 demonstrate that oligodendrocytes respond to cytokines in a similar way as astrocytes with regard to the regulation of αB-crystallin-encoding mRNA. This does not support the idea that oligodendrocytes might use other signal transduction pathways. In addition, oligodendrocytes appeared to be even *more* sensitive than astrocytes to soluble mediators as cytokines. *In vitro* studies on the cytotoxicity of IFN-γ and TNF-α to oligodendrocytes are in line with such an enhanced sensitivity of this cell type to soluble mediators (9, 10). However, it should be kept in mind that post mortem human oligodendrocytes are difficult to culture *in vitro* (they did not proliferate and died within two to three weeks) and they persisted *in vitro* in a "stressed state". The stress-activated kinase p38-MAPK for example could be detected activated constitutively in the cytosol of unstimulated cultured oligodendrocytes as well as low levels of αB-crystallin that was phosphorylated at serine 59 (own observations). Therefore, results might be seriously affected by the *in vivo-in vitro* difference and extra care needs to be taken in extrapolating *in vitro* results for oligodendrocytes.
Yet, the tissue distribution of oligodendrocytes expressing high levels of αB-crystallin was quite different from that of astrocytes in MS lesions. The differences in αB-crystallin expression patterns in adjacent oligodendrocytes might be explained by the possibility that such altered expression of αB-crystallin in oligodendrocytes is a rapid and very transient phenomenon, thereby resulting in a diverse reaction pattern to the same stressor. Also, the tissue distribution of αB-crystallin-expressing oligodendrocytes could be influenced by contact of oligodendrocytes with degenerating neurons that are localized at some distance.
Alternatively, additional factors might be involved in the regulation of αB-crystallin expression that can be different between individual cells in the same microenvironment. The underlying reason for altered αB-crystallin expression in oligodendrocytes might therefore be found *inside* oligodendrocytes rather than *outside*. Intracellular differences between individual oligodendrocytes could include the state of oligodendrocyte maturation, apoptosis or (reactivation of a) viral infection.
The subpopulation of αB-crystallin-expressing oligodendrocytes in MS lesions could not be characterized as immature (chapter 3) and did not show the typical morphology of apoptotic oligodendrocytes. However, this last possibility can not be discarded since enhanced intracellular ceramide levels might *predispose* oligodendrocytes to undergo apoptosis thereby possibly altering their reaction pattern upon soluble mediators. In addition, ceramide has been described as a direct mediator of αB-crystallin expression (11). The induced-expression of αB-crystallin in peripheral blood mononuclear cells in response to Epstein Barr virus (EBV) infection (5) makes it tempting to speculate on the capacity of glia-tropic herpesviruses to cause this same effect in oligodendrocytes.
To date, however, we have no clear indications to prefer one of these possible explanations.

αB-crystallin as a key autoantigen in MS?

The first requirement for an antigen to be involved in a T cell-mediated autoimmune disease is the presence of autoantigen-specific T cells. As for many other self proteins, this appears to be the case for αB-crystallin; αB-crystallin-specific T cells can be obtained easily from both MS patients and healthy controls. When total T cell response profiles of MS patients and healthy controls were mapped against the complete, fractionated collection of proteins derived of MS-affected CNS myelin, αB-crystallin turned out as the immunodominant protein of this collection (chapter 2). More importantly, this T cell reactivity towards αB-crystallin was measured only when MS-affected myelin was used as an antigen source.
A second requirement for an (auto-)antigen to be involved in the pathogenesis of MS is that it must be presented to T cells during or before pathogenesis. The data in chapter 4 on local antigen presentation of αB-crystallin to T cells by macrophages shortly after myelin phagocytosis strongly suggest that this is the case for αB-crystallin. αB-Crystallin was found in intracellular vesicles of the endo-lysosomal pathway of myelin-phagocytosing macrophages, colocalizing with other myelin proteins. In addition, it was shown that when whole (MS-affected) myelin membranes were used as antigen source, αB-crystallin becomes functionally presented to T cells. Since αB-crystallin in phagocytosing macrophages colocalized with other myelin proteins it was most probably myelin-derived. Whether myelin-derived αB-crystallin is phosphorylated or not is still unknown.

With both immunological requirements fullfilled, how do the studies on the altered expression of αB-crystallin in glia cells contribute to our understanding of the immunopathology of MS?
For astrocytes, *in vitro* studies confirmed the idea that a soluble mediator might cause the altered expression of αB-crystallin (chapter 3). It was demonstrated that ROS, but not cytokines, are capable of altering the expression of αB-crystallin in a way consistent with that seen in MS lesions (chapter 6+7). In addition, it is suggested that activation of p38-MAPK and subsequent phosphorylation of αB-crystallin are a necessary event in order to obtain the cytosolic localization of αB-crystallin as observed *in situ*, thereby narrowing the list of potential mediators of αB-crystallin expression. Whether ROS cause the altered expression of αB-crystallin in astrocytes in MS lesions remains to be established. It is also not known yet whether ROS are the only stressors capable of causing the diffuse cytosolic expression of αB-crystallin.
For oligodendrocytes the nature of potential mediators of αB-crystallin expression remains more obscure. However, the distribution pattern of αB-crystallin-expressing oligodendrocytes (chapter 3) as well as the *in vitro* studies (chapter 8) do not directly support a role for a soluble mediator, but might suggest an intracellular cause (viral infection?) for the altered expression of αB-crystallin. In addition, it is unknown

whether phosphorylation of αB-crystallin plays a role, as is likely to be the cause for astrocytes, in the altered expression of αB-crystallin in oligodendrocytes.
In MS lesions, the αB-crystallin that is being presented to T cells is most probably derived of the myelin/oligodendrocyte complex (chapter 4). This leaves identification of the potential cause(s) for altered αB-crystallin expression in this cell type important. Whether the altered αB-crystallin expression as observed in astrocytes is an epiphenomenon that merely accompanies ongoing demyelination or could also be involved in the pathogenesis of MS is unknown.

How then is αB-crystallin thought to be involved in the pathogenesis of MS? Is altered expression of αB-crystallin *the cause* of MS, a cofactor or merely an epiphenomenon? Since altered expression of αB-crystallin is always the result of a previous event, αB-crystallin can, by definition, never be *the primary cause* of MS. In order to adress this issue properly it is important to try to define exactly when an inflammatory insult of the CNS becomes a lesion. In other words, what is the "MS-specific" part of a lesion? Local disturbances in the CNS as well as BBB disturbances can hardly be considered MS-specific and even inflammatory reactions that occur within the CNS are not MS-specific. The start of demyelination might be considered to be MS-specific but is also caused by an (MS-specific?) preceding event, presumably the MHC-II-restricted recognition of autoantigen(s) in the CNS by T cells. This would probably be preceded by (MS-aspecific?) tissue damage. We know that αB-crystallin can play a role once demyelination has started (chapter 4). In our opinion it is likely that expression of αB-crystallin can be altered and go accompanied by MHC-II-restricted presentation of αB-crystallin-derived peptides as a result of tissue damage. A recent study reporting on enhanced antibody titers directed against the normally intracellular αB-crystallin in MS patients (12) is consistent with the occurence of tissue-damage. The altered expression of αB-crystallin in oligodendrocytes in response to a stressor yet unknown and/or in astrocytes in response to an aspecific stressor like ROS might well represent the link between *MS-aspecific* stress in the CNS and the *MS-specific* autoimmune response. Therefore, *the cause* of MS might not exist and it could rather be that in order to develop MS a list of requirements **have** to be fulfilled, none of them being MS-specific in itself. A proposal for such a list is the following:

1) genetic predisposition (e.g. the MHC-II alleles),
2) EBV-infection and subsequent generation of an αB-crystallin-specific pool of memory T cells,
3) reactivation of the pool of αB-crystallin-specific T cells (caused by viral reactivation?),
4) local stress in the CNS leading to MHC-II restricted presentation of (oligodendrocyte/myelin-derived?) αB-crystallin,
5) local stress in the CNS leading to altered BBB characteristics thereby actively recruiting T cells.

As an extra requirement point 3, 4 and 5 should happen within a certain, overlapping, time frame. In such a list αB-crystallin would be the key antigen in the pathogenesis of this disease without being MS-specific.
We believe that αB-crystallin might be envisaged at the end of a funnel, linking (aspecific) CNS stress (viral infection?) to (specific) autoimmunity (Fig. 10.1).

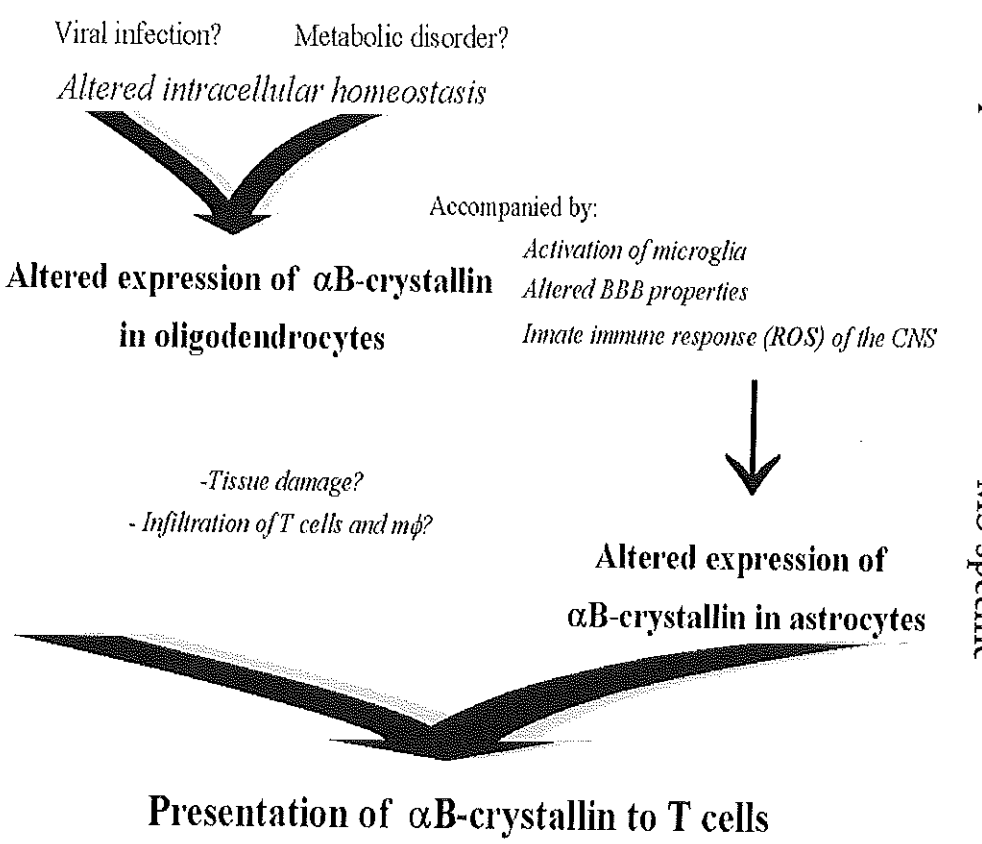

Figure 10.1. Tentative model depicting the potential causes for altered expression of αB-crystallin in glia cells resulting in antigen presentation to T cells by macrophages.

Future research

Future studies will be aimed at the elucidation of T cell epitopes that are contained within αB-crystallin. Knowledge about these peptides will allow logical predictions with regard to the influence of phosphorylation on the immune response. These studies are also crucial with regard to the potential use of (recombinant) αB-crystallin in antigen-specific therapy. If the aim of such a therapy is to induce tolerance to αB-crystallin, then one should make sure that tolerance is induced to the relevant form of αB-crystallin. At this moment it seems most likely that αB-crystallin that is being presented to T cells in MS lesions will be unphosphorylated.
In addition, it might be interesting to study whether the use of cells with induced, altered (disaggregated cytosolic) αB-crystallin distribution as an antigen source will raise different T cell responses when compared to cells with normal αB-crystallin distribution as an antigen source.
Furthermore, the combination of *in vivo* and *in vitro* approaches should be continued. Both approaches should, if possible, consist of a combination of biochemistry, mRNA- and protein expression studies and immunohistochemical methods in order to generate data that are relevant for the *in vivo* situation. For *in vitro* studies with oligodendrocytes it would be preferable to switch to a system that resembles the *in vivo* situation better. An alternative approach is to work with thin brain slices rather than with dissociated oligodendrocytes. If such a system is operational it could be studied whether soluble mediators (e.g. ROS) influence αB-crystallin expression in oligodendrocytes in the same way as it does in astrocytes in the same tissue. Also, it may be of interest to study the effects of phosphorylation of αB-crystallin on the intracellular distribution of αB-crystallin in the oligodendrocyte/myelin complex.
It would be most interesting to study the effect of viral infection or reactivation on the expression of αB-crystallin in glia cells. In addition, effects of viral infection on the expression of e.g. immunomodulatory molecules in glia cells could be studied by the use of DNA microarray technology. This should, after the initial experiments, be supplemented with immunohistochemical studies. The combination of techniques present at the lab and the frequent supply of post mortem material provides a fruitful setting to perform these *in vitro* experiments. A relevant candidate virus would be HHV-6 (chapter 9), which is described to infect both oligodendrocytes and astrocytes, but also other viruses could be tested.
Finally, the search for *one* MS-specific virus might prove to be unproductive. Another approach would be to study viral infection of the CNS, *irrespective of the virus* keeping in mind that infection with different viruses could lead to the same clinical disease via a common final mechanism. This would also apply to epidemiological studies trying to link *one* virus to MS.

References

1. Li, H., M.L. Cuzner, and J. Newcombe. 1996. Microglia-derived macrophages in early multiple sclerosis plaques. *Neuropathol Appl Neurobiol 22:207.*

2. Hayes, G.M., M.N. Woodroofe, and M.L. Cuzner. 1987. Microglia are the major cell type expressing MHC class II in human white matter. *J Neurol Sci 80:25.*

3. Van Eden, W., van der Zee, R., Taams, L.S., Prakken, A.B., van Roon, J., Wauben, M.H. 1998. Heat-shock protein T-cell epitopes trigger a spreading regulatory control in a diversified arthritogenic T-cell response. *Immunol Rev 164:* 169.

4. Van Roon, J.A., van Eden, W., van Roy, J.L., Lafeber, F.L., Bijlsma, J.W. 1997. Stimulation of suppressive T cell responses by human but not bacterial 60-kD heat-shock protein in synovial fluid of patients with rheumatoid arthritis. *J Clin Invest 100(2):* 459.

5. van Sechel, A.C., J.J. Bajramovic, M.J. van Stipdonk, C. Persoon-Deen, S.B. Geutskens, and J.M. van Noort. 1999. EBV-induced expression and HLA-DR-restricted presentation by human B cells of alpha B-crystallin, a candidate autoantigen in multiple sclerosis. *J Immunol 162:*129.

6. van Stipdonk, M.J., A.A. Willems, S. Amor, C. Persoon-Deen, P.J. Travers, C.J. Boog, and J.M. van Noort. 1998. T cells discriminate between differentially phosphorylated forms of alphaB-crystallin, a major central nervous system myelin antigen. *Int Immunol 10:*943.

7. Matzinger, P. 1994. Tolerance, danger, and the extended family. *Annu Rev Immunol 12:991.*

8. Brosnan C.F., Battistini L., Raine C.S., Dickson D.W., Casadevall A., Lee S.C. 1994. Reactive nitrogen intermediates in human neuropathology: an overview. *Dev Neurosci 16:*152.

9. Vartanian, T., Y. Li, M. Zhao, and K. Stefansson. 1995. Interferon-gamma-induced oligodendrocyte cell death: implications for the pathogenesis of multiple sclerosis. *Mol Med 1:*732.

10. Selmaj, K., C.S. Raine, M. Farooq, W.T. Norton, and C.F. Brosnan. 1991. Cytokine cytotoxicity against oligodendrocytes. Apoptosis induced by lymphotoxin. *J Immunol 147:*1522.

11. Chang, Y., A. Abe, and J.A. Shayman. 1995. Ceramide formation during heat shock: a potential mediator of alpha B- crystallin transcription. *Proc Natl Acad Sci U S A 92:*12275.

12. Agius M.A., Kirvan C.A., Schafer A.L., Gudipati E., Zhu S. 1999. High prevalence of anti-alpha-crystallin antibodies in multiple sclerosis: correlation with severity and activity of disease. *Acta Neurol Scand 100(3):*139.

Samenvatting

Multipele Sclerose (MS) is een autoimmuunziekte waarbij de myelineschedes in het centraal zenuwstelsel van patiënten het doelwit zijn van het eigen afweersysteem. De afweercellen die een belangrijke rol spelen in het ontstaan van deze ziekte zijn de ($CD4^+$) T cellen. Deze cellen lijken een myelinecomponent te herkennen (een *auto-antigeen*) en hierop te reageren, wat uiteindelijk resulteert in schade aan het myeline. Myeline is de witte stof die te beschouwen is als de isolerende laag rondom de zenuwbanen die de elektrische prikkelgeleiding in het centraal zenuwstelsel verzorgen. Gebieden in de hersenen waarin het myeline zichtbaar beschadigd is noemen wij *laesies*. Ten gevolge van deze laesies doen zich allerlei klinische verschijnselen voor waaronder verlamming, incontinentie en visuele problemen.
Een belangrijk probleem in MS-onderzoek is de vraag waarom het afweersysteem bij MS-patiënten nu op myeline reageert en tegen welke component(en) deze afweerreactie dan precies gericht is. Dit proefschrift beschrijft experimenten die tot doel hadden om inzicht te verschaffen in deze vraag. Wij hebben de reacties van het afweersysteem op myeline dat was verkregen uit de (aangedane) hersenen van MS-patiënten vergeleken met de reacties van het afweersysteem op myeline dat was verkregen uit de hersenen van niet-zieke controles. Om de reacties van het afweersysteem goed in kaart te brengen werd al het myeline in kleine fracties gescheiden. Vervolgens werden de reacties van het afweersysteem (de T cellen) tegen deze verschillende fracties gemeten. Uit deze experimenten vielen twee belangrijke conclusies te trekken. Ten eerste werden er geen verschillen gevonden tussen de responsen van T cellen afkomstig van MS-patiënten en responsen van T cellen afkomstig uit gezonde controles. Het afweersysteem van MS-patiënten was in onze experimenten dus niet meetbaar verschillend van dat van gezonde controles. Ten tweede werd er een verschil gevonden tussen MS-aangedaan myeline en gezond myeline. Eén fractie, afkomstig uit MS-aangedaan myeline, activeerde de T cellen zeer sterk. Belangrijker was dat de corresponderende fractie afkomstig uit gezond myeline deze capaciteit miste. Tezamen suggereren deze twee bevindingen dat "de oorzaak" voor MS niet een afwijkend immuunsysteem is, maar eerder te vinden zou zijn in een component die aanwezig is in MS-myeline en die afwezig is (of in mindere dan wel andere mate aanwezig is) in gezond myeline. Deze component werd uit de bewuste fractie geïsoleerd en geïdentificeerd als zijnde één eiwit. Dit eiwit bleek het kleine stresseiwit αB-crystalline te zijn, dat slechts in zeer kleine hoeveelheden te detecteren was in onaangedaan hersenweefsel.
Vervolgonderzoek aan patiëntenmateriaal maakte duidelijk dat αB-crystalline inderdaad in het aangedane hersenweefsel van MS-patiënten aan te tonen was en wel in twee gespecialiseerde typen hersencel (*gliacellen*); in oligodendrocyten (de cellen die het myeline aanmaken) en in astrocyten. Al in de vroegste stadia van laesievorming werd αB-crystalline gevonden in deze celtypen.

Het vaststellen van de sterke reacties van T cellen op αB-crystalline gecombineerd met de aanwezigheid van dit eiwit in verschillende typen gliacellen gaf echter nog geen aanwijzingen over de daadwerkelijke zichtbaarheid van het betreffende eiwit in MS-aangedaan hersenweefsel voor T cellen. Een T cel reageert op een klein stukje van een eiwit, een zogenaamd *peptide*. Om te kunnen reageren op zo'n peptide (bijvoorbeeld afkomstig van het αB-crystalline eiwit) moet een eiwit eerst door gespecialiseerde antigeen-presenterende cellen (APC) opgenomen worden, verteerd worden (*processing*) en vervolgens aangeboden worden aan T cellen. Pas bij herkenning van dit peptide, voorzien van de juiste extra signalen (*co-stimulatie*) zullen de specifieke T cellen reageren.

In beginnende MS laesies is er inderdaad soms myeline aan te tonen in macrofagen (een bepaald type APC). Dit myeline zal hoogstwaarschijnlijk verteerd worden en vervolgens zullen de verschillende eiwitten die afkomstig zijn uit dit myeline als peptiden aan T cellen aangeboden kunnen worden. Zulke beginnende MS laesies zijn gebruikt om te bestuderen of αB-crystalline in MS-aangedaan hersenweefsel gepresenteerd wordt aan T cellen.
In verschillende MS laesies werden zowel myeline als αB-crystalline in macrofagen aangetoond. Deze macrofagen brachten tevens de juiste co-stimulatoire signalen tot expressie, bevonden zich in de directe nabijheid van T cellen en bevatten (gedeeltelijk) verteerd αB-crystalline. Het bleef echter onduidelijk of T cellen nu dan ook in staat zouden zijn om het, in zeer kleine hoeveelheden in myeline aanwezige, αB-crystalline te herkennen wanneer het complete, ongefractioneerde, myeline gebruikt wordt als uitgangsmateriaal. Dit laatste reflecteert de situatie zoals die zich voordoet in een MS laesie waarschijnlijk het best. Om dit te onderzoeken werden macrofagen gevoerd met MS-aangedaan myeline en werd bestudeerd of T cellen, die specifiek αB-crystalline peptiden herkenden, hierop reageerden. Het bleek dat αB-crystalline-specifieke T cellen goed in staat waren om te reageren op de relatief kleine hoeveelheden αB-crystalline die aanwezig waren in het uitgangsmateriaal.
Deze gegevens laten zien dat αB-crystalline in MS laesies ook daadwerkelijk door APC gepresenteerd wordt aan T cellen en dat deze T cellen hier dan ook op kunnen reageren. Hiermee werd een eventuele rol van αB-crystalline als auto-antigeen in MS nogmaals bevestigd.

Naast de identificatie van αB-crystalline als auto-antigeen en het bestuderen van het voorkomen van αB-crystalline in MS laesies, is een groot deel van het in dit proefschrift beschreven werk gericht op de vraag *waarom* genoemde gliacellen in MS laesies αB-crystalline maken. Hiertoe zijn wij begonnen met het kweken van zowel astrocyten als oligodendrocyten, verkregen uit obductie materiaal van MS-patiënten en niet-zieke donoren. Verschillende testsystemen zijn opgezet om de mate van expressie te bestuderen van verschillende, verwante, stress eiwitten (waaronder αB-crystalline).

De resultaten van het onderzoek aan astrocyten laten zien dat veelvoorkomende ontstekingsmediatoren (cytokines) niet de oorzaak lijken te zijn van de verhoogde expressie van αB-crystalline zoals die gezien wordt in het hersenweefsel van MS-patiënten. Verder konden wij aantonen dat de expressie van verschillende stress eiwitten sterk differentieel gereguleerd was; verschillende stress eiwitten werden door de astrocyten verhoogd aangemaakt in respons op verschillende stimuli. Hierbij bleek dat de aanmaak van αB-crystalline, in vergelijking met de andere bestudeerde stress eiwitten hsp27 en hsp60, in astrocyten slechts zeer moeizaam te stimuleren was. Recent hebben wij aanwijzingen gevonden dat oxidatieve stress (bijvoorbeeld in de vorm van waterstofperoxide; H_2O_2) wèl in staat is om αB-crystalline door astrocyten tot expressie te laten brengen op een manier die sterk gelijkt op wat wij in MS-laesies zien. Deze veranderde expressie na H_2O_2-stimulatie blijkt afhankelijk te zijn van de phosphoryleringsstatus van αB-crystalline. Meer specifiek hebben wij aanwijzingen dat phosphorylering van αB-crystalline op het serine residue 59 (S59) door de p38-mitogeen geactiveerde eiwitkinases (MAPKs) hierbij een belangrijke rol spelen. De gevonden expressie in MS laesies van op S59-gephosphoryleerd αB-crystalline mag hierbij als ondersteunend bewijs gerekend worden.

De combinatie van beschrijvend onderzoek aan patiëntenmateriaal en van meer experimenteel onderzoek aan celkweken, heeft er toe geleid dat wij een nieuw kandidaat auto-antigeen hebben weten te identificeren met een mogelijk centrale rol in de pathogenese van MS. Deze identificatie opent mogelijk nieuwe wegen naar de toepassing van antigeen-specifieke therapie in MS-patiënten. Voorts zijn wij veel te weten gekomen over de mechanismen die de regulatie van stresseiwitten in gliacellen kunnen beïnvloeden. Met betrekking tot αB-crystalline hebben wij laten zien dat oxidatieve stress de meest waarschijnlijke veroorzaker is van de expressie zoals die gevonden wordt in MS laesies. Deze experimentele data geven aanleiding tot voorzichtige hoop op mogelijkheden om, selectief, de verhoogde expressie van αB-crystalline in MS-hersenen tegen te kunnen gaan.

Dankwoord

Hè, hè. Af.
Of althans wel bijna af, want dit laatste stukje tekst is eigenlijk het belangrijkste stukje van het proefschrift. Het zal in ieder geval het meest gelezen worden. Hiermee wil ik dan ook proberen om iedereen heel erg te bedanken die er aan heeft bijgedragen dat dit boekje er uiteindelijk ligt.

Om te beginnen iedereen binnen de afdeling Immunologische- en Infectieziekten. Het zal niemand ontgaan zijn dat ik het erg naar mijn zin heb gehad en dat iedere volgende werkplek eigenlijk alleen maar kan tegenvallen. De grote hoeveelheid borrels, de retraites, de kerstdiners en de talrijke andere etentjes droegen en dragen nog steeds bij aan de relaxede sfeer op de afdeling. Helaas waren daar ook de onvermijdelijke afscheidsborrels en een aantal vertrokken mensen wil ik toch wel met naam noemen. Marjan, jij hebt mij in Rijswijk nog wegwijs gemaakt in de illustere wereld der immunohistochemie en ik hoop dat de plaatjes in dit proefschrift je bevallen. Naast dat ik heel veel van je geleerd heb, herinner ik me vooral dat je altijd vrolijk was en dat we heel wat afgelachen hebben zowel in Rijswijk als in het mooie nieuwe Leidse lab. Ik vond het dan ook oprecht jammer dat je verkoos om in Rotterdam te gaan werken. Gelukkig heb je het daar erg naar je zin en valt je accent ook niet meer zo op. Jon, ik heb je zeer leren waarderen. Er is weinig waar jij niet over hebt nagedacht, waarmee in mijn ogen het overtuigende bewijs geleverd is dat als je ècht nadenkt over voetbal Ajax de enige club is. Wim, ook jou wil ik bedanken voor de leerzame tijd. De talloze discussies over van alles wat een mens maar dwars kan zitten, hebben, misschien onbedoeld, bijgedragen tot een wat relativerendere kijk op de wereld. Ik hoop dat je in Maxima een goede partij ziet voor Willem-Alexander, want ik weet dat je dan in ieder geval een stuk rustiger slaapt. Marie-Joan, wij kwamen als stagiares ongeveer tegelijkertijd binnen bij TNO en bleven daarna allebei. De prettige, lekker onbezorgde, tijd als stagiare heb ik voor een groot deel met jou kunnen delen en daar heb ik van genoten. Ook toen jij het grootste deel van je tijd op het CI doorbracht en uiteindelijk (voorlopig) stopte met werken is dat contact nooit verwaterd. Chantal, ik heb je pas echt goed leren kennen toen wij samen in de retraitecommissie zaten en op Texel slaperig over het eiland rondtoerden. Erg leuk en gevaarlijk, en ik heb je gemist toen je eenmaal vertrokken was! Naast dat er mensen vertrokken zijn, is er gelukkig genoeg vers bloed bij gekomen om het leuk te houden. Met name Karin, Jos en Wouter hebben het laatste deel van mijn AiO-periode het werk aanzienlijk veraangenaamd.

Het αB-clubje was een goede plaats om ideetjes te testen, onzin te spuien, Hans te pesten of afentoe ook nog eens serieus en met open vizier over de voortgang te spreken. Ieder αB-club lid heeft dan ook een (in)directe bijdrage geleverd aan dit proefschrift. Stip, opeens was daar een meisje op station Rijswijk dat bij de eerste ontmoeting al wist te vertellen dat ze later professor wilde worden. En, hoe langer ik je ken, hoe meer ik er

van overtuigd raak dat dat ook wel gaat lukken. En nee, ik lijk echt niet op René Froger! Arianne, van de eerste (illegale?) sigaretten in de zuurkast in Rijswijk tot aan de champagne in Royaal, je blijft een apart mens dat niet rust voordat dingen kloppen. Aan de ene kant is dat erg prettig als je in het onderzoek zit, aan de andere kant ook erg moeilijk. Ik heb enorm veel van je geleerd en heel veel met je gepraat, zowel onzin als zin. Ik vraag me af of er een frans equivalent bestaat van een Arianne. Carla, ondanks dat ik je vaak tot waanzin gedreven heb door het zo regelmatig met Hans oneens te zijn, hebben wij toch heel productief en prettig samen weten te werken. En ondanks dat ik er in het begin best een beetje tegenop zag om samen achter de kweek te moeten liep het allemaal gesmeerd. Ik hoop voor je dat de volgende αB-club leden wat beter naar Hans zullen luisteren. Malika, jij bent niet voor niets één van mijn paranimfen. Lia, het was vooral stimulerend om te zien hoe jij in de sera-chaos orde wist te scheppen. Richard, veel succes! Jammer genoeg zijn er ook uit het αB-clubje mensen vertrokken die ik nog wil bedanken. Margret, jammer dat het gelopen is zoals het gelopen is. Bedankt voor de hulp met de HPLC-fracties en vooral bedankt voor de gezelligheid (het vrijheidsbeeld staat toch helemaal niet in Parijs?). Frank, je bent wat dichter bij huis gaan werken maar ik weet niet of er daar ook bijzondere olympische disciplines uitgevonden worden (indoor-huldigen) en of de waardering voor uitstervende oud-hollandse gezegdes, woorden en Jiskefet daar net zo groot is als binnen TNO. Ik heb vooral heel erg veel met je gelachen en het TNO-humorgehalte nam sterk af na jou vertrek. Marit, spijtig dat onze samenwerking geen succes was. Desondanks was het niet allemaal treurnis, want er is in ieder geval nog een artikel uit geboren. Veel succes als AiO.

Het leukste onderdeel van het werken bij TNO waren toch wel de stagiares; iedere keer weer een nieuwe die weer totaal anders was als de vorige. Karien, jij was de eerste in een redelijk lange rij en was in veel opzichten een echt proefkonijn. Of je nou door ben gaan studeren doordat je het werk zo leuk vond of doordat wat ik deed er wel erg simpel uitzag weet ik nog steeds niet. Ryan, jij bent één van de meest extraverte personen die ik ken. Gespreksonderwerpen en interesses zijn bij jou zo overdadig aanwezig dat het inderdaad het verstandigste was om, als je ècht hard wilde werken, dat dan maar 's nachts te doen. Ik ben blij dat ik je nog regelmatig zie en dat ik je ondertussen tot mijn vrienden mag rekenen. Cindy, ondanks een jaar vol problemen heb je het toch volgehouden en je stage prima afgerond. Net toen ik je begon te verstaan ging je weg. Ik ben blij dat het zo goed bevalt in Amsterdam. Malika, jij kwam, zag en bleef. Simone, ik heb niet de indruk dat ik jou bijzonder veel heb kunnen leren. Toch was de stage erg succesvol, getuige ook de prijs die je ervoor hebt ontvangen. Maar ja, met een vriendje in Amerika moet je toch wàt. Veel succes op het LUMC! Inge, jij had een tough cookie. Nog geen twee maanden nadat jij begonnen was besloten we om de betreffende onderzoekslijn even in de koelkast te zetten. Jij hebt veel veerkracht getoond door je vol goede moed, en met succes, in te zetten voor een ander, heel moeilijk, stukje werk. Het gaat je ongetwijfeld lukken in Australië!

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Marc, ik ben blij dat jij één van mijn paranimfen wilde zijn. Naast een rielèkste collega met lef en hagt voâh de hele afdelink bè jû eigûluk bes wel een toffu gozàh. Ik weet zeker dat de post-moderne inrichting van onze hotelkamer in Montreal ondertussen

daar navolging heeft gevonden. Eén van de belangrijkste redenen om uit het MS-wereldje te verkassen is dat ik daarin, na de compromitterende foto's uit Basel, nooit meer serieus genomen ga worden. Ik hoop dat Aatje niet al te eenzaam wordt.
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Paris is waiting for us!

Curriculum vitae

Jeffrey Bajramović werd op 19 juli 1971 geboren te Den Haag. In 1988 behaalde hij het VWO-B diploma aan het Lodewijk Makeblijde College te Rijswijk. In datzelfde jaar begon hij aan de studie biologie aan de Rijksuniversiteit Leiden. Stages werden gelopen bij de vakgroep celbiologie en genetica aan de Rijksuniversiteit Leiden (Dr. W. de Priester), bij de divisie tumorbiologie aan het Nederlands Kanker Instituut (Dr. H. Vos) en bij de afdeling Immunologie van het Medisch Biologisch Laboratorium (TNO; Dr. J.M. van Noort). Het hoofdvaktentamen in de specialisatierichting celbiologie werd behaald bij Dr. W. de Priester in 1993. In augustus 1994 behaalde hij het doctoraal examen, waarna hij enige maanden als research medewerker werkte bij TNO. Tijdens deze periode werd een onderzoeksstage gelopen aan de universiteit van Wenen, Neurologisches Institüt, in het laboratorium van Prof.dr. H. Lassmann. In het voorjaar van 1995 werd er een aanvang gemaakt met zijn periode als assistent-in-opleiding bij de afdeling Immunologie van de Erasmus Universiteit Rotterdam. Onder begeleiding van Dr. J.M. van Noort en met steun van Prof.dr. E. Claassen werd het in dit proefschrift beschreven onderzoek uitgevoerd bij de divisie Immunologische- en Infectieziekten van TNO Preventie en Gezondheid in Leiden. Gedurende deze periode werden onderzoeksstages gelopen aan de universiteit van Londen, Institute of Neurology in het laboratorium van Prof.dr. M.L. Cuzner en aan the London School of Hygiene and Tropical Medicine in het laboratorium van Dr. U Gompels.
Aansluitend op zijn promotie zal hij, op een recent toegekend NWO-TALENT stipendium, als post-doc onderzoeker gaan werken aan het Institut Pasteur, Unité des Virus Lents, Parijs. In het laboratorium van Dr. Brahic en onder begeleiding van Dr. Gonzalez Dunia zal gewerkt worden aan opheldering van de moleculaire mechanismen die ten grondslag liggen aan, door persistente infectie met Borna Disease Virus veroorzaakte, pathologie van neuronen.

Publications

Van Noort, J.M., van Sechel, A.C., Bajramović, J.J., El Ouagmiri, M., Polman, C.H., Lassmann, H., and Ravid, R. The small heat shock protein αB-crystallin as a candidate autoantigen in multiple sclerosis. **Nature 375**, 798-801 (1995).

Bajramović, J.J., Lassmann, H., and van Noort, J.M. Selective dissapearance of αB-crystallin positive oligodendrocytes in late MS lesions. **Immunol. Lett. 86 (1)**, 114 (1995).

Bajramović, J.J., Lassmann, H., and van Noort, J.M. Expression of αB-crystallin in glia cells during lesional development in multiple sclerosis. **J. Neuroimmunol. 78**, 143-151 (1997).

Van Noort, J.M., van Sechel, A.C., van Stipdonk, M.J.B. and Bajramović, J.J. The stress protein αB-crystallin as key autoantigen in multiple sclerosis. **Progr. Brain Res. 117**, 435-452 (1998).

Van Noort, J.M., van Sechel, A.C., van Stipdonk, M.J.B. and Bajramović, J.J. Bystander T-cell activation to αB-crystallin as a mechanism for virus-induced autoimmunity in multiple sclerosis. In: Talwar, G.P., Nath, I., Ganguly, N.K., Rao, K.V.S. (eds.): **The 10th International Congress of Immunology (2)**,759-764 (1998).

Bajramović, J.J. and van Noort, J.M. Key Papers. **Int. MSJ. 5 (2)**, 42-43 (1999).

Van Sechel-Plomp, A.C., Bajramović, J.J., van Stipdonk, M.J.B., Geutskens, S.B., and van Noort, J.M. Activation of autoreactive helper T cells by virus-induced presentation of a self antigen. **J. Immunol. 162(1)**, 129-135 (1999).

Bajramović, J.J., Geutskens, S.B., Bsibsi, M., Boot, M., Hassankhan, R., Verhulst, K.C., and van Noort, J.M. The stress-kit: a new method based on competitive rt-PCR to quantify the expression of human αB-crystallin, hsp27 and hsp60. **Cell Stress and Chaperones.** ***In press*** (2000).

Bajramović, J.J., Bsibsi, M., Geutskens, S.B., Hasssankhan, R., Verhulst, K.C., Stege, G.J.J., de Groot, C.J.A., and van Noort, J.M. Differential expression of stress proteins in human adult astrocytes in response to cytokines. **J. Neuroimmunol.** ***In press*** (2000).

Van Noort, J.M., Bajramović, J.J., Plomp, A.C., and van Stipdonk, M.J.B. Mistaken self, a novel model that links microbial infections with myelin-directed autoimmunity in multiple sclerosis. **J. Neuroimmunol.** ***In Press*** (2000).

Publications

Bajramović, J.J., Plomp, A.C., van der Goes, A., Koevoets, C., Newcombe, J., Cuzner, M.L., and van Noort, J.M. Presentation of αB-crystallin to T cells in active Multiple Sclerosis lesions: an early event following inflammatory demyelination. **J. Immunol.** *In Press* (2000).

Bajramović, J.J., de Jong, I., Bsibsi, M., Quinlan, R.A., de Groot, C.J.A., and van Noort J.M. Phosphorylation of the small heat shock protein αB-crystallin at serine-59 causes a major shift in intracellular distribution of the protein in human astrocytes. *To be submitted for publication.*