

Molecular pathogenesis of neuroinflammation

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The past few years have seen significant progress towards understanding the mechanisms of immune surveillance and inflammation in the nervous system. In this review, the milestones of scientific discovery in this field are discussed, and the strengths and limitations of the different ways of examining the molecular pathogenesis of neuroinflammation examined. The review is limited to the inflammatory reactions of the central nervous system that occur in multiple sclerosis and experimental autoimmune encephalomyelitis.

Today we are well aware that there is substantial communication between the immune system and the central nervous system (CNS), and that interactions between these two systems occur both in the healthy organism and in pathological situations.¹ However, this was not always the case.

Some 20 to 30 years ago, the CNS and the immune system were considered strictly separate structures, and the CNS was viewed as the classical example of an "immunoprivileged site." This assumption was based on the prolonged survival of tissue grafts within the CNS,² the presence of the blood–brain barrier (an endothelial barrier between blood and brain, which forms tight junctions and prevents an uncontrolled influx of molecules and cells into the CNS³), the lack of classical lymphatic drainage pathways,³ the lack of professional antigen presenting cells such as dendritic cells,⁴ and the infrequent expression of major histocompatibility complex (MHC) molecules needed to present CNS antigens to infiltrating T cells.⁴

All these observations seemed to indicate that the immune system and the CNS are separate domains. However, over the years novel experimental evidence challenged this traditional viewpoint: the intact CNS parenchyma regularly contains small numbers of T cells,^{5–6} T cells may also enter the CNS in the course of CNS inflammation and degeneration,^{7–8} and interstitial/cerebrospinal fluid and proteins drain from the brain to the blood and may affect immune responses in the draining lymph nodes.⁹

It is now clear that the CNS is under constant immune surveillance, and that it interacts with cells of the immune system in both health and disease.

EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS – AN ANIMAL MODEL TO STUDY INTERACTIONS OF THE IMMUNE SYSTEM WITH THE CNS

Most of our current knowledge about neuroinflammation has been obtained from experimental autoimmune encephalomyelitis (EAE), which for decades has served as the best animal model to study the aspects of CNS inflammation and demyelination seen in human patients with multiple sclerosis. It is quite bizarre that this invaluable animal model had its origin in a series of neuroparalytic accidents in humans receiving anti-rabies treatments with killed carbolised virus isolated from infected animal brains. The discovery that brain material contaminating the vaccine was responsible for paralysis¹⁰ paved the way for pioneering experiments in which CNS inflammation and demyelination were induced in experimental animals by immunisation with brain material.¹¹ The resulting disease model was termed EAE, and has been markedly refined over the years. At the start, crude spinal cord or brain extracts were used for immunisation¹²; later, purified CNS proteins were used, such as myelin basic protein (MBP),¹³ proteolipid protein (PLP),¹⁴ myelin oligodendrocyte glycoprotein (MOG),¹⁵ S100 β ,¹⁶ and now often only small peptides (of about 20 amino acids in length) derived from CNS proteins.⁷

A breakthrough came when it was observed that EAE can be transferred between animals by lymph node cells,¹⁷ that pure CNS antigen specific T cells can be cultured and activated *in vitro*,¹⁸ and that these cells were also able to initiate EAE upon transfer in naive recipient animals.¹⁸ In fact, T cell transferred EAE has considerable advantages over immunisation induced EAE in that it starts earlier and shows less variation between animals.⁷

Other refinements of the EAE model applied to the choice of animals. As seen in human patients with multiple sclerosis, different strains of animal display different types of disease course, different symptoms, and different pathological changes within the CNS.⁷ The most recent developments in the field are:

Abbreviations: BDNF, brain derived neurotrophic factor; EAE, experimental autoimmune encephalomyelitis; EAN, experimental autoimmune neuritis; IMDS, isolated monosymptomatic demyelinating syndrome; INF γ , interferon γ ; MBP, myelin basic protein; MHC, major histocompatibility complex; MOG, myelin oligodendrocyte glycoprotein; PLP, proteolipid protein; TNF α , tumour necrosis factor α ; CNS, central nervous system; PCR, polymerase chain reaction

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- the use of genetically modified animals with defined changes in cells of the immune or central nervous systems;
- the production and transfer of fluorescent CNS antigen specific T cells to trace their migration and cellular changes in the course of the disease;
- microdissection and molecular analyses of single cells involved in the disease process;
- spectratyping of disease relevant T cell receptors;
- the search for antigens recognised by CNS infiltrating T cells in biopsy/necropsy material from patients suffering from inflammatory CNS disease;
- the use of DNA microarrays to study simultaneously the interactions between many different genes in CNS inflammation;
- the development of new treatments based on the unique property of activated CNS antigen specific T cells to cross the blood–brain barrier.

HOW MODERN TECHNIQUES CONTRIBUTED TO OUR UNDERSTANDING OF NEUROINFLAMMATION IN EAE AND MULTIPLE SCLEROSIS

Genetically manipulated animals

Probably the most fruitful—and currently most popular—technology is the genetic manipulation of experimental animals so that factors or even entire cell populations are missing (“knock-out animals”), or so that proteins under investigation are produced in a temporally or spatially controllable way (conditionally “transgenic” animals). Such strategies have furthered our understanding of the different phases of autoimmune CNS inflammation.

The initiation of inflammatory lesions in the CNS

It is well established that CNS antigen specific T cells are normal components of the immune system of every healthy organism.^{19, 20} However, spontaneous CNS inflammation provoked by these cells is exceedingly rare. Why? To answer this question, transgenic mice were constructed which carry receptors for MBP—a major component of the myelin sheath in the CNS—on essentially all their T cells. Furthermore, the (rearranged) genes for these particular T cell receptors had been isolated from T cells that were found to be highly pathogenic in EAE experiments.²¹ The first surprising observation was that the animals did not get sick as long as they were kept under specific pathogen-free conditions. However, they did develop spontaneous autoimmune CNS inflammation when they were exposed to the ubiquitous pathogens of the environment. These experiments clearly showed that it is the activation and not the mere presence of CNS antigen specific T cells in the immune system that provokes the induction of neuroinflammation. As these animals had not been immunised with the CNS antigen recognised by the transgenic T cell receptor, T cell activation must have resulted from the contact with bacterial or viral proteins. Four recent observations suggest that this observation is not just intrinsic to the animal model of EAE, but may also be highly significant for human patients with multiple sclerosis. Firstly, and perhaps trivially, the onset of disease or exacerbations of its course in human patients with multiple sclerosis or other disorders with an autoimmune component is sometimes associated with infections.²² Secondly, some human MBP specific T cell clones can be activated by peptides derived from herpes simplex virus, Epstein-Barr virus, or influenza virus, which are presented by the same MHC molecule.²² Thirdly, even different MHC molecules loaded with different peptides may show such a high degree of structural equivalence that they are recognised by the same T cell receptor of a patient with multiple sclerosis (for

example DRB1*1501 loaded with an MBP peptide and DRB5*0101 loaded with an Epstein-Barr virus peptide).²³ Finally, a “humanised mouse model” of multiple sclerosis has been developed using multiply transgenic mice.²⁴ These mice express three human components involved in T cell recognition of the multiple sclerosis related autoantigen MBP: HLA-DR2, an MHC class II antigen associated with multiple sclerosis in north Europeans; a T cell receptor from a T cell clone specific for the MBP peptide 84–102 derived from a multiple sclerosis patient; and the human co-receptor CD4. When backcrossed to Rag2 deficient mice, these transgenic humanised mice spontaneously develop inflammatory disease of the CNS.²⁴

The EAE experiments described above clearly show that CNS antigen specific T cells which had been activated somewhere in the body—whether by direct antigen contact in the course of immunisation or by the antigen independent action of certain bacterial or viral proteins—are able to cross the intact blood–brain barrier and enter the CNS parenchyma. It is assumed that these cells find “their” specific antigen upon entry into the CNS, are further activated, and then interact with local glial cells, leading to a locally confined release of chemokines and cytokines. These proteins activate the blood–brain barrier and attract further cells from the immune system. A second wave of immune cell infiltration into the CNS follows, which seems to be independent of the antigen recognition or the activation status of the recruited cells. Inflammatory lesions develop.¹

Progression of CNS inflammation and the resulting tissue damage

Like multiple sclerosis, EAE can take different clinical courses. In some animal strain or antigen combinations the course is monophasic; in others it is fulminant/lethal, relapsing/remitting, or chronically progressive. In animals with relapsing/remitting or chronically progressive types of EAE, and in human patients with isolated monosymptomatic demyelinating syndrome (IMDS), the proliferative response of T cells to the “priming” myelin protein often declines with time, while T cells with specificities for other parts of the same myelin protein or even for other myelin proteins increase in numbers. This effect is called “epitope spreading” and has been held responsible for the progression of chronic CNS inflammation in animals with EAE and for the progression of IMDS to clinically definite multiple sclerosis.²⁵ Moreover, the phenomenon of epitope spreading could present an obstacle to experimental “T cell vaccination treatments” aiming at the selective elimination of certain CNS antigen specific T cell clones from the immune repertoire of patients with multiple sclerosis.²⁶ However, a more recent study suggests that the possible relation between epitope spreading and disease progression may be more complicated.²⁷ To test whether progressive courses of EAE can also occur in the absence of epitope spreading, transgenic mice were created which carried T cells with only one defined CNS antigen specificity—which effectively excluded T cell reactivity to other CNS proteins. Despite a monospecific T cell response, these mice developed progressive and relapsing/remitting disease courses. This indicates that under certain conditions disease initiating T cells may also drive the subsequent progressive phase.²⁷

CNS inflammation is commonly associated with some degree of tissue damage—that is, loss of myelin sheaths or loss of axons. This is observed in animal models of EAE and is a central theme in human patients with multiple sclerosis. Again, EAE models were instrumental in elucidating the different cellular and molecular pathways resulting in demyelination. One pathway was discovered in knock-out mice which had been rendered deficient in B cells by genetic

disruption of the membrane bound antibody μ chain. When these animals were immunised with a myelin protein to induce EAE, they developed both inflammatory lesions and primary demyelination.²⁸ These data indicated that in mice, antibodies and B cells are not necessary for myelin loss, or that T cells and their products are sufficient for demyelination. What are these T cells products?

It is well established that not only macrophages but also T cells can produce tumour necrosis factor α (TNF α), and that activated T cells may also secrete interferon γ (IFN γ). Both these cytokines are found in inflammatory lesions of EAE animals. To learn more about the role of these cytokines in myelin loss, transgenic mice were created which over-expressed either TNF α ²⁹ or IFN γ ³⁰ in their CNS. Both types of transgenic animal developed demyelination in the absence of immunisation, which clearly showed that the presence of these cytokines in the CNS of mice is sufficient to trigger myelin loss.^{29, 30}

Another pathway leading to demyelination in the course of CNS inflammation was identified in rats. In these animals, T cells themselves are not capable of causing noticeable damage to myelin sheaths. Instead, loss of myelin is only observed when activated CNS antigen specific T cells open the blood–brain barrier and antibodies specifically recognising epitopes of proteins on the surface of myelin sheaths gain access to the inflammatory lesions.³¹

Yet another pathway to demyelination could be initiated by primary changes in the myelin sheaths themselves, as seen in transgenic mice and rats overexpressing PLP in their myelin forming oligodendrocytes.^{32–34} When the PLP overdose exceeded a certain threshold, myelin sheaths were not produced at all owing to early oligodendrocyte death. Below the threshold, myelin sheaths were formed but were unstable. The resulting subclinical myelin degeneration paved the way for spontaneous demyelination in transgenic mice,^{32, 33} and for an increase in demyelination in EAE induced by T cells and antibodies directed against myelin proteins in transgenic rats (Bradl M, unpublished data). In summary, demyelination in the course of CNS inflammation may be caused by T cells and T cell products, by antibodies, and by an instability of myelin sheaths.

As mentioned above, multiple sclerosis and EAE are not only characterised by loss of myelin sheaths, but also by damage to axons. Transections of axons, axonal swellings, and spheroid formations in the CNS had already been described at the turn of last century, but were neglected for many decades and only recently attracted renewed attention.³⁵ These pathological changes are held responsible for the permanent clinical deficits in multiple sclerosis.³⁶ They can be caused by cytotoxic T cells³⁷ or by the action of inflammatory mediators such as nitric oxide.³⁸

The resolution of inflammatory lesions within the CNS

Genetically modified animals are also essential for studying the termination of CNS inflammation—that is, the resolution of inflammatory lesions. Studies in conventional EAE models revealed unusually large numbers of apoptotic T cells in the late phases of CNS inflammation and it was concluded from these observations that inflammatory CNS lesions may resolve through the induction of apoptosis in T cells.^{39, 40} However, it remained unclear whether the induction of programmed cell death only affects autoantigen specific and hence disease initiating T cells, or whether it also affects recruited T cells non-specifically, and whether it occurs at some sites rather more often than at others. To address these questions, EAE was induced by injections of CNS antigen specific transgenic T cells into wild-type (non-transgenic) recipients. The transgenic T cells carried a nuclear marker and

could therefore be easily identified in histological specimen of inflamed CNS tissue. It was found that apoptotic T cells are preferentially located within the CNS parenchyma, and almost never in the meninges or perivascular spaces. Moreover, all T cells are cleared from the CNS parenchyma, no matter whether they were disease initiating or non-specifically recruited, or whether they had previously been activated or were resting.⁴¹

Fluorescent T cells

Based on the experiments described above it became firmly established that CNS antigen specific T cells have to be maximally activated before their transfer into naive recipients in order to induce EAE. Nevertheless, there was a need to explain the curious time window of about 72 hours between T cell transfer and clinical disease, and consequently to address the following questions. What happens to the disease inducing T cells during that time? Where do they reside? Is there a way of tracing and characterising such cells, and of discriminating them from lymphocytes with other antigen specificities which will never participate in the disease process?

In brief, there is indeed an elegant way to follow the fate of autoreactive T cells *in vivo*. This relies on the introduction of the gene for a green fluorescent protein into CNS antigen specific T cells which can be achieved by retroviral gene transfer (fig 1) and which leads to highly fluorescent T cells with a stable, self replenishing, and selectable marker.⁴² When such cells were injected into recipient animals to cause EAE, they could be reisolated for further studies at any time between disease induction (that is, T cell transfer) and CNS inflammation. These analyses provided very interesting information: it turns out that in the preclinical phase of EAE, freshly injected CNS antigen specific T cells migrate first to the perithymic lymph nodes and then to the spleen, where they acquire a new “migratory” phenotype, characterised by downregulation of activation markers, upregulation of chemokine receptors, and an increase in MHC class II molecules on the cell surface. These T cells then change phenotype a second time when they enter the CNS. Here they are reactivated on encountering their specific antigen, which is presented by activated antigen presenting cells of the CNS.⁴⁴

MICRODISSECTION AND MOLECULAR ANALYSES OF SINGLE CELLS INVOLVED IN THE DISEASE PROCESS

As mentioned above, T cells injected to induce EAE can easily be reisolated from the CNS or from lymphoid organs of the recipient animals. Their phenotype can be determined and their encephalitogenic potential can even be tested by further transfer into naive recipients. In humans the situation is quite different, for obvious reasons. Here, information must be gathered from the few T cells found in inflammatory lesions in necropsy or biopsy material, which demands more refined techniques such as microdissection and molecular analysis of individual T cells. The feasibility of this approach was recently demonstrated by Babbe and coworkers, when they analysed multiple sclerosis plaques.⁴⁵ They first stained frozen tissue sections for the presence of CD4 or CD8 positive T lymphocytes, and then mobilised and isolated single T cells from the surrounding tissue with the help of a micromanipulator. Afterwards, they analysed the T cell receptors of these cells by polymerase chain reaction (PCR). This approach revealed interesting differences between CD8⁺ and CD4⁺ T cells. CD8⁺ T cells were the dominant T cell population in multiple sclerosis lesions but represented the descendants of only a few different T cell clones. In contrast, CD4⁺ T cells

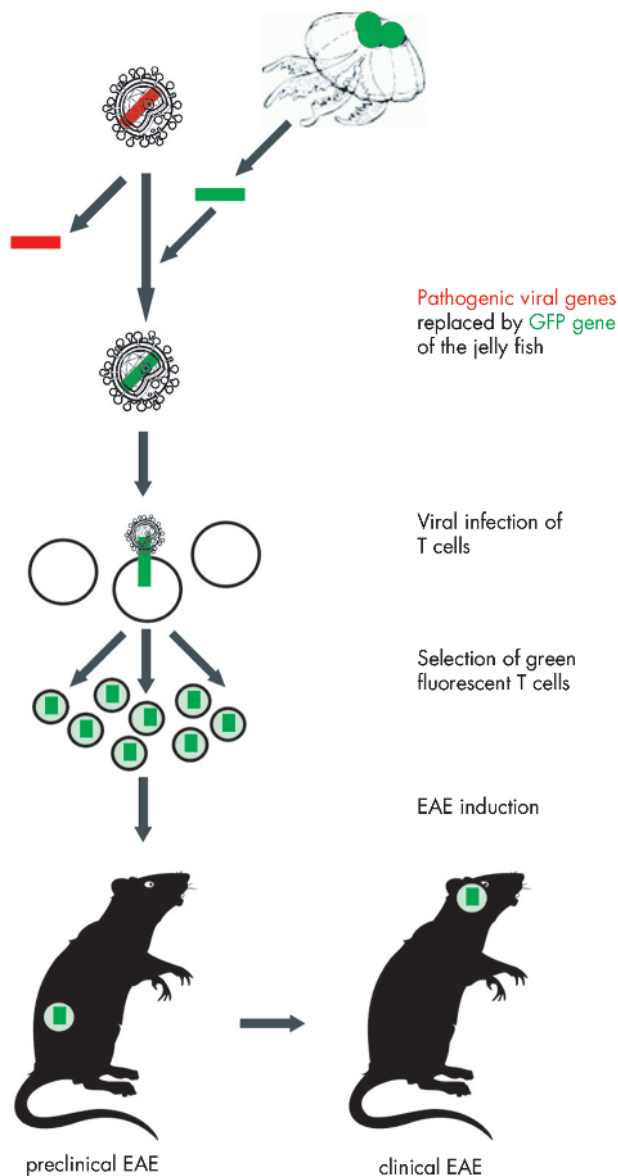


Figure 1 Fluorescent T cells.^{42–43} Viruses are genetically modified so that disease inducing viral genes are replaced by the gene encoding the green fluorescent protein (GFP) derived from the jellyfish.⁴³ These viruses are then used to infect T cells and to introduce the GFP gene. In some cells the gene is expressed, the fluorescent protein produced, and the T cells turn green. Green fluorescent CNS antigen specific T cells can be selected, activated, and transferred to naive recipient animals. At different time points after T cell transfer, they can be isolated from the CNS, spleen, or lymph nodes for further analyses. EAE, experimental autoimmune encephalitis.

were much less numerous but much more heterogeneous, representing progeny of many different T cell clones.⁴⁵

The dominance of T cell clones in inflammatory lesions could have at least two different causes. It could reflect a preferential infiltration of certain clones into the affected area, or a random recruitment of clones which are over-represented in the peripheral immune repertoire. One way to address this problem involves the isolation, cellular cloning, and characterisation of T cells *in vitro*, and the molecular cloning and characterisation of their receptors by sequencing analyses, which would be a rather laborious task. Instead, it is significantly more efficient to use a different technique that is especially suited to screening T cell receptor repertoires. This is called “CDR3 spectratyping.”

CDR3 spectratyping

This technique is based on the fact that individual T cells and their clonal descendants carry unique T cell receptors with which they recognise “their” specific antigen. The high degree of diversity of T cell receptors is caused by a random combination of gene segments and by inaccuracy in the joining process of these segments. This leads to the arbitrary addition or deletion of nucleotides and to the formation within each T cell receptor gene of areas which are hypervariable both in their length and in their molecular sequence. One of these regions of the T cell receptor is called the complementary determining (CDR)-3 region. For each T cell receptor gene segment, amplification of this region by PCR yields a spectrum of PCR products of differing lengths (the “spectratype”), which reveals the expansion of T cell clones in the peripheral blood,⁴⁶ or the persistence of CNS antigen specific T cells in blood⁴⁷ and cerebrospinal fluid of patients as strong signals (“peaks”).

NOVEL TECHNIQUES AND THEIR CONTRIBUTION TO CLINICAL NEUROLOGY—A FUTURE PERSPECTIVE

The search for the autoantigen of CNS inflammation

As mentioned above, in the animal model of EAE, activated T cells with specificity for neural antigens were shown to initiate CNS inflammation and autoimmune disease. However, the antigen specificity of CNS infiltrating T cells in multiple sclerosis lesions remains largely unknown. What do these T cells recognise?

If the (auto)antigens are unknown, the unique (clonotypic) T cell receptor of the presumably autoaggressive T cells might provide a clue. A potentially promising approach combines the cloning of T cell receptors found on individual cells within inflammatory lesions, and the expression of the cloned receptors in cells which contain the complete machinery for signalling through T cell receptors, but do not themselves express full receptors. If such cells are grafted with a cloned receptor derived from an autoaggressive T cell, they will secrete cytokines, and possibly even proliferate upon recognition of an antigen for which the T cell receptor is specific. The feasibility of this approach was recently demonstrated with autoreactive T cells derived from polymyositis lesions.⁴⁸ Once the antigen is known, antigen specific T cells can be tracked with soluble MHC–peptide tetramers. These synthetic structures behave in a similar way to the natural MHC–peptide complexes presented on the surface of antigen presenting cells: they are recognised by T cells carrying the corresponding specific T cell receptors, and bind only to these specific cells. Principally, tetramers can be constructed using MHC class I⁴⁹ or class II⁵⁰ molecules and peptides, thus allowing the tracking of CD8⁺ and CD4⁺ T cells. This approach works in fluorescent activated cell sorting analyses of peripheral blood cells, where it was used to visualise antigen specific T cells, for example in Epstein-Barr virus infected⁴⁹ or HIV infected patients,⁵¹ or in cancer patients.^{52–55} Unfortunately, it does not yet work reliably on histological sections. However, as there are ongoing efforts in many different laboratories to optimise the MHC–peptide tetramer techniques (see for example^{54–55}), it is probably just a matter of time until this problem is solved.

The search for therapeutic target structures with DNA microarrays

As stated earlier, inflammation of the CNS is a very complex process, requiring the spatial and temporal regulation of T cell activation, coordinated interaction between T lymphocytes and other components of the immune system, and intercellular interactions between T cells and local glial cells within the CNS parenchyma. Hence it is not surprising that

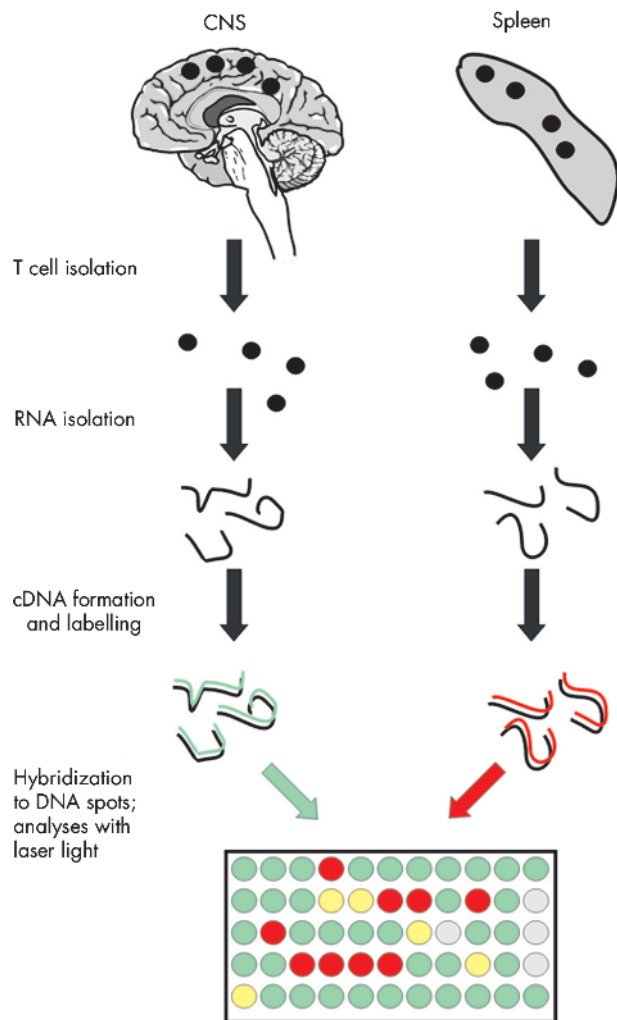


Figure 2 DNA microarrays.⁵⁶ Messenger RNA is isolated from T cells found within the CNS or the spleen and is converted to fluorescence labelled, and hence coloured, cDNA. The cDNA of CNS derived T cells is labelled green, the cDNA of spleen derived T cells red. The two different sets of cDNA are mixed and hybridised to the microarray. A microarray (also called DNA chip) contains spots of thousands of DNA sequences which all represent different genes and which can be simultaneously tested with the labelled cDNA. If the cDNA finds corresponding sequences on the microarray, it will bind to it. Unbound cDNA is washed off. The microarray is analysed with laser light (red and green). The resulting images are analysed. Green spots represent genes that are expressed in CNS derived T cells only; red spots represent genes that are only expressed in spleen resident T cells; yellow spots represent genes that are expressed in both sets of T cells, and the absence of coloured spots indicates that a particular gene is not expressed at all.

there is also a complex interaction between many different genes and gene products of individual cell populations. To study these interactions and to identify novel molecules involved in the different phases of neuroinflammation, DNA microarrays (also called DNA chips) are increasingly used. They consist of hundreds or thousands of DNA sequences representing defined genes, which are attached to a glass surface and can be hybridised to the complete RNA (or cDNA) from cells of interest (fig 2). The RNA (or cDNA) probes bind to matching gene spots on the DNA microarray and identify all expressed genes of a given sample. To date, microarrays have been used to investigate multiple sclerosis or EAE tissue samples^{57–59} to characterise molecules involved in specific biological processes (such as cellular activation, intercellular signalling, cell adhesion, transmigration, or cell-

cell interactions), with the aim of ultimately identifying new targets for therapeutic intervention of CNS inflammation.

T cells as therapeutic agents

All the scenarios described above clearly show that T cells and their products may provoke damage or even loss of myelin sheaths and axons. Other observations, however, indicate that the role of inflammatory cells, especially T cells, might be considerably more complex. For example, T cells were found to produce neuroprotective molecules such as neurotrophin-3⁶⁰ or brain derived neurotrophic factor (BDNF).⁶¹ Neurotrophins and neuroprotective factors promote neuronal survival and may also mediate anti-inflammatory effects.⁶² In addition, depending on the subset studied, T cells may also produce cytokines such as interleukin-4 or interleukin-10, which can also suppress autoimmune responses.⁶³

These examples clearly show that T cells may also have beneficial functions in CNS inflammation.¹ Under natural conditions this “beneficial” side of inflammation might be too weak to outweigh the harmful and toxic effects. In certain experimental systems, however, the neuroprotective and anti-inflammatory effects of inflammation can be strengthened so that they become clinically apparent. An especially appealing approach is based on the following reasoning. If activated T cells can cross the endothelial blood–brain barrier whereas other factors are efficiently excluded from the CNS,³ and if these cells accumulate at sites of neuronal degeneration⁸ and can easily be manipulated to express foreign genes while maintaining all other cellular functions^{42 44 64 65}, then why not use them as vehicles to transport therapeutic agents across the blood–brain barrier?⁶⁶ The feasibility of such an approach was demonstrated in animal models of EAE and experimental autoimmune neuritis (EAN). The gene for nerve growth factor was retrovirally transduced into neural antigen specific T cells. When these T cells were transferred to experimental animals, an attenuation of the disease process was observed in the animal models of both EAE⁶⁵ and EAN.⁶⁴ These promising results encourage further efforts to refine and optimise this technique and to create better tools for the treatment of inflammatory CNS disease. Furthermore, it appears that some of the existing treatments⁶⁷ might—at least in part—even rely on the beneficial role of inflammation. For example, it is known that Copaxone (glatiramer acetate (GA)), which is approved for the treatment of multiple sclerosis,⁶⁷ induces a Th1 to Th2 cytokine shift in GA reactive T cells,⁶⁸ possibly because of its action on dendritic cells.⁶⁹ These findings indicate that activated GA-reactive T cells migrate into the CNS and produce immunomodulatory cytokines⁶⁸ or even BDNF⁷⁰ locally.

CONCLUSION

As we have discussed in this brief review, our understanding of the molecular pathogenesis of neuroinflammation is growing steadily. Several recent studies have revealed unexpected insights—for example, by providing hints of a potentially protective role of inflammation. New techniques are providing increasingly powerful research tools. Progress in different areas of basic research will help further to improve the treatment of patients with neuroimmunological diseases.

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