Bacterial peptidoglycan and immune reactivity in the central nervous system in multiple sclerosis

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Summary

Multiple sclerosis is believed to result from a CD4+ T-cell response against myelin antigens. Peptidoglycan, a major component of the Gram-positive bacterial cell wall, is a functional lipopolysaccharide analogue with potent proinflammatory properties and is conceivably a mediator of sterile inflammation. Here we demonstrate that peptidoglycan is present within antigen-presenting cells in the brain of multiple sclerosis patients. These cells have macrophage and dendritic cell characteristics, and are immunocompetent as evidenced by co-expression of inflammatory cytokines and co-stimulatory molecules. In addition, intrathecal plasma cells specific for peptidoglycan are present in multiple sclerosis brain tissue, and antibodies binding peptidoglycan are present in CSF during active disease. Peptidoglycan may thus contribute to T- and B-cell activity during brain inflammation without a requirement for local bacterial replication.

Keywords: multiple sclerosis; peptidoglycan; cytokines; antigen-presenting cells; antibodies

Abbreviations: ELISA = enzyme-linked immunosorbent assay; LPS = lipopolysaccharide; Mab = monoclonal antibody; NAMLAA = *N*-acetyl muramyl L-alanine amidase; PG = peptidoglycan

Introduction

According to prevailing dogma, multiple sclerosis is caused by autoreactive CD4+ T cells specific for myelin components with a proinflammatory T helper phenotype (Lassmann, 1998). Since healthy donors have similar numbers of myelin-reactive T cells to patients, additional factors are clearly needed to promote (auto)-reactivity of T cells within the CNS (Lassmann and Wekerle, 1998). Recent studies in several experimental models, including the experimental autoimmune encephalomyelitis model for multiple sclerosis, have shown that various infectious and proinflammatory agents such as lipopolysaccharide (LPS) are capable of reversing the tolerant state of CD4+ and CD8+ T cells (Röcken *et al*., 1992; Vella *et al*., 1995; Segal *et al*., 1997; Ehl *et al*., 1998). Activation of these cells is crucially dependent on the presence of both the stimulatory agent and the specific (auto)-antigen (Ehl *et al*., 1998). Research on inflammatory properties of bacterial antigens in brain tissue has shown that bacteria injected into the brain parenchyma are able to induce inflammatory responses only after peripheral sensitization of T cells (Matyszak, 1998). However, there is no clear evidence to date that bacterial agents or components derived thereof, such as LPS, are functionally involved in initiation or perpetuation of multiple sclerosis disease activity. In addition, studies attempting to link infectious agents to (autoimmune) inflammation are often based on the premise that replication occurs within the affected organ (Relman, 1999), but this is not necessarily the case.

Peptidoglycan (PG) is a major constituent of the cell wall of Gram-positive bacteria, which are abundantly present at all mucosa, and most prominently in the gut as part of the normal microbial flora (for review, see Hamann *et al*., 1998). PG is composed of long sugar chains of alternating *N*-acetyl glucosamine and *N*-acetyl muramic acid residues, which are interlinked by peptide bridges, resulting in a large complex macromolecular structure (Schleifer and Kandler, 1972). PG is only a very minor constituent of the cell wall of Gram-negative bacteria. PG can be regarded as a functional analogue of LPS, and both molecules activate innate immune defence mechanisms (Hoffmann *et al*., 1999). First, both molecules use CD14 on monocytes/macrophages and granulocytes as their cellular receptor, differentially engaging the recently identified Toll-like receptors (Medzhitov *et al*., 1997;

Schwandner *et al.*, 1999; Ulevitch, 1999). Secondly, the intracellular signalling pathways employed by both molecules are highly similar if not identical (Mattsson *et al.*, 1996; Hamann *et al.*, 1998; Gupta *et al.*, 1999). Thirdly, both LPS and PG induce strong proinflammatory responses *in vitro*, including production of cytokines such as IL-1 and TNF-α (Weidemann *et al.*, 1994; Gupta *et al.*, 1999; Le Roy *et al.*, 1999). LPS and PG are complex macromolecules which are difficult to digest by phagocytes (Forestier *et al.*, 1999). Macrophages lack amidase (*N*-acetyl muramyl L-alanine amidase, NAMLAA) expression (Hoijer *et al.*, 1996, 1997*a*) which is required for full degradation of PG in joint action with lysozyme (Hoijer *et al.*, 1997*b*). This resistance to degradation explains, at least in part, the persistence of microbial cell walls, which may be a pivotal factor in chronic (autoimmune) inflammation (Relman, 1999).

Antigen-presenting cells containing PG are present in the joints of patients with rheumatoid arthritis, possibly contributing to the local inflammatory environment (Hazenberg, 1995; Melief *et al.*, 1995). Interestingly, PG retaining proinflammatory capacity such as the induction of TNF-α and IL-1, can be fractionated from sterile human spleen (Timmerman *et al.*, 1993; Weidemann *et al.*, 1994; Schrijver *et al.*, 1999), suggesting that continuous redistribution of PG occurs from the mucosa to the secondary lymphoid organs, as well as to other anatomical sites in the absence of sepsis, and without requirement for local bacterial replication. This is fully consistent with the presence of circulating leucocytes carrying PG in human healthy subjects (Lehtonen *et al.*, 1995) and distinct patient categories (Lehtonen *et al.*, 1997).

In view of the strong influx of macrophages into the CNS of multiple sclerosis patients facilitated by severely impaired blood–brain barrier function (de Vries *et al.*, 1997), we hypothesized that phagocytic antigen-presenting cells carrying PG gain access to the CNS and can locally promote immune reactivity of T and B cells. Here we show that PG-containing antigen-presenting cells are present in human multiple sclerosis brain tissue, and that they express co-stimulatory molecules and immunomodulatory cytokines. In addition, antibody forming cells specific for PG are present in human multiple sclerosis brain tissue and patients with active disease have antibodies against PG in the CSF. These findings suggest that PG affects immune reactivity in the brain.

Patients and methods
Patient samples
Human brain tissue taken at autopsy was provided by the Netherlands Brain Bank in Amsterdam (Coordinator Dr R. Ravid). Average post-mortem delay was 6 h (range 4–9 h) for multiple sclerosis patients and 8 h (range 6–10 h) for control patients. No abnormalities could be detected at autopsy in the brain tissues of the control subjects who died owing to different causes, e.g. cardiac arrest, cancer and pneumonia. Patient features are shown in Table 1.

CSF samples of 10 multiple sclerosis patients and 10 control patients were obtained from the Department of Neurology of the Erasmus University and University Hospital Rotterdam-Dijkzigt. CSF samples of relapsing–remitting multiple sclerosis patients were taken prior to immunomodulating therapy during a relapse of multiple sclerosis. Control subjects were patients who presented with severe headache for which no cause could be determined.

Immunohistochemistry
Immunohistochemical procedures used for detection of cellular subsets, antigens, antigen specific plasma cells, co-stimulatory molecules and cytokines in frozen sections have all been described in detail previously (Claassen and Jeurissen, 1998; Schrijver *et al.*, 2000). The frozen sections were fixed in acetone with 0.5% H_2O_2 for 10 min. Antibodies used are shown in Table 2. To detect specific antibody forming cells, peptidoglycan–polysaccharide isolated from human faeces (Hazenberg *et al.*, 1989) was labelled with biotin (Laman *et al.*, 1990; Claassen *et al.*, 1992). Sections from reactive human tonsils were included on each individual object slide to provide internal positive control tissue. Sections were evaluated by two independent observers blinded to the staining procedure.

Monoclonal antibody (Mab) against peptidoglycan (2E9)
2E9 is a murine Mab (IgG3) raised against a pure fraction of peptidoglycan-polysaccharides isolated from normal human faeces (Kool *et al.*, 1994). The specificity of this Mab has been extensively investigated and confirmed. First, inhibition studies using enzyme-linked immunosorbent assay (ELISA) show that 2E9 recognizes cell wall fragments, muramyl dipeptide and lysozyme-solubilized cell walls of different Gram-positive bacteria (Kool *et al.*, 1994). These experiments suggest that it binds to the glycan backbone of PG since muramic acid forms part of the structure recognized. Secondly, spleen sections of germ-free rats and of neonatal rats in which the gut flora has not been established yet, display no PG-containing phagocytes, whereas spleens of adult rats do. In addition, intravenous injection of PG into neonatal rats results in binding of 2E9 to phagocytes in spleen sections (Kool *et al.*, 1994). Thirdly, immunoelectron microscopy shows that Mab 2E9 binds to cell walls of *Staphylococcus epidermidis* surviving intracellularly in macrophages around infected biomaterial. Fourthly, 2E9 displays no detectable reactivity against >10 000 random dodecapeptides and 8000 tripeptides in Pepscan approaches (Slootstra *et al.*, 1997), confirming this Mab has very low crossreactivity with non-PG epitopes.

An isotype-matched control antibody (NS7, IgG3) of

Table 1 *Clinical and neuropathological data of multiple sclerosis cases*

Case	Sex	Age (years)	pm time (h:min)	Clinical diagnosis; type of multiple sclerosis; staging of lesions	Neuropathology	Cause of death
96–104	M	72	4:45	Disease duration: 22 years Progressive phase: 1993 1 chronic inactive lesion	MS with preactive plaques (MS + P)	Carcinoma of bladder and intestine
96–121	F	53	7:16	Disease duration: 16 years Progressive phase: 1980 Number of lesions: 4 Lesion stage: 4 active	MS with active plaques (MS + A)	Pneumonia
97–006	F	62	6:45	Disease duration: 25 years Number of lesions: 2 Lesion stage: 2 active Lesions	MS with active plaques (MS + A)	Cardiac asthma
97–070	F	82	4:30	Disease duration: 15 years Progressive phase: 1975	MS without active lesions (MS–). Most plaques are chronic inactive, some appear remyelinated	Cachexia
97–077	M	50	5:40	Disease duration: 17 years 'highly progressive MS' Number of lesions: 3 Lesion stage: 2 chronic active, 1 chronic inactive	MS, the plaques are more chronic inactive than active	Pneumonia/ cachexia
96–040	F	35	5:45	Disease duration: 15 years 'Very aggressive course' Number of lesions: 5 Lesion stage: 1 active, 4 chronic active, 1 chronic inactive	MS with active lesions (MS + A). All plaques contain an extremely active phagocytic component	General decline
95–095	M	56	5:24	Disease duration: 15 years Secondary progressive	MS with subacute, partly chronic plaques (MS + P). Developing cerebral deterioration (senile plaques)	Respiratory insufficiency
96–026	F	69	6:40	Disease duration: 19 years progressive form Number of lesions: 3 Lesion stage: 3 (P) reactive	MS with perivascular infiltrates and leptomeningeal infiltrates (MS + P)	Respiratory insufficiency
96–076	F	81	4:15	Disease duration: 49 years Secondary progressive Number of lesions: 2 Lesion stage: 1 normal, 2 (P) reactive	MS with small plaques (MS + P). Slight senile involutive cortical changes.	Cachexia
96–039	F	57	5:45	Disease duration: 19 years Primary progressive Number of lesions: 5 Lesion stage: 1 active, 4 chronic inactive	MS with few active plaques and many subcortical plaques (MS + A)	Sepsis
96–074	F	40	7:00	Disease duration: 14 years Progressive phase: 1991 Number of lesions: 8 Lesion stage: 4 active, 4 chronic inactive	MS with extensive affliction of the white matter and many perivascular phagocytes (MS + A)	Dehydration

pm time = post-mortem delay until autopsy; MS = multiple sclerosis; MS + A = active plaques; MS + P and (P) = reactive plaques; MS– = no active plaques.

Table 2 *Reagents used for the detection of various determinants*

Marker	Antibody clone	Expressed by
CD1a	OKT6	Dendritic cells, B cells
CD3	A452	Pan T cells
CD14	MY-4	Monocytes, granulocytes
CD68	KP-1	Monocytes, macrophages
CD40	5D12	Macrophages, B cells
CD80/B7.1	M24	Antigen-presenting cells
CD83	HB15a	Mature dendritic cells
CD86/B7.2	1G10	Antigen-presenting cells
HLA-DR	L243	Antigen-presenting cells
IL-1	VMP18	Monocytes, macrophages, dendritic cells, T/B cells, NK cells
IL-1	VMP20	Monocytes, macrophages, dendritic cells, T/B cells, NK cells
IL-4	Anti-IL-4 Genzyme	T cells, mast cells
IL-6	Anti-IL-6 Genzyme	T/B cells, macrophages
IL-10	B-S10	T cells, monocytes, macrophages
IL-12	Pharmingen	Monocytes, macrophages, B cells
IFN-γ	MD-2	T cells, NK cells
TNF-α	MC16	Monocytes, macrophages, T cells
Peptidoglycan	2E9	Present in some antigen-presenting cells

irrelevant specificity (sheep red blood cells) was used throughout the studies and displayed no binding activity in brain tissue of multiple sclerosis patients or control subjects.

Statistical analysis

The number of PG-containing cells and plasma cells binding PG present in multiple sclerosis brain tissue and control brain tissue were compared using the Mann-Whitney rank-sum test. A *P* value <0.05 was considered to be significant.

PG ELISA

To measure anti-PG antibodies in CSF, an ELISA, extensively validated and used before, was performed as described (Hazenberg *et al.*, 1990). Briefly, 50 µl of peptidoglycan–polysaccharides (10 µg/ml) was coated overnight at 50°C onto 96-well polystyrene microtitre plates. Aliquots (100 µl) of 1 : 2, 1 : 4 and 1 : 8 dilutions of CSF were incubated during 1 h at 37°C. As detecting antibody, peroxidase conjugated rabbit anti-human IgM, IgG or IgA (Jackson Immunoresearch, Inc., Westgroove, Pa., USA) was used for 1 h at 37°C. The development of the colorimetric assay took place at 37°C for 30–45 min after the addition of 100 ml of ortho-phenylenediamine/H_2O. The reaction was stopped by 4 M H_2SO_4 and the optical density was measured at a wavelength of 492 nm with a Titertek Multiskan (Flow Laboratories, Irvine, Scotland). Between all incubation steps the plates were washed three times with PBS (phosphate-buffered saline)–0.02% Tween and CSF and conjugates were diluted in PBS–0.2% Tween.

Results

PG-containing cells are present in multiple sclerosis brain tissue

The presence of PG was analysed in cryo-sections of brain white matter of multiple sclerosis patients and control subjects using a monoclonal antibody against PG (Kool *et al.*, 1994). The numbers of PG-containing cells were significantly higher in tissue from multiple sclerosis brains compared with control patients (Fig. 1) (*P* = 0.001). In multiple sclerosis patients, PG-containing cells were mainly present on the edge of active lesions (Lassmann *et al.*, 1998) (Fig. 2A), as defined by Oil-red O staining for myelin breakdown products ingested by macrophages. Furthermore, PG-containing cells were present around blood vessels, or scattered in the normal appearing white matter (Fig. 2B). Only rarely could PG-containing cells be found within the fully demyelinated foci forming the centre of lesions. To further analyse the role of PG-containing cells in inflammation of the brain, double labelling was performed on brain tissue of multiple sclerosis patients selected for high numbers of PG-containing cells, to determine the phenotype and activation state of the PG-containing cells.

PG is present in macrophages and dendritic cells

To determine the phenotype of PG-containing cells, co-expression of PG with CD68, CD83, CD14, 27E10, CD1a and CD3 was analysed. Figure 3 shows that PG is mostly present in macrophages expressing CD68 and dendritic cells expressing CD83. CD14, which is the cellular receptor for both PG and LPS present on relatively immature phagocytes,
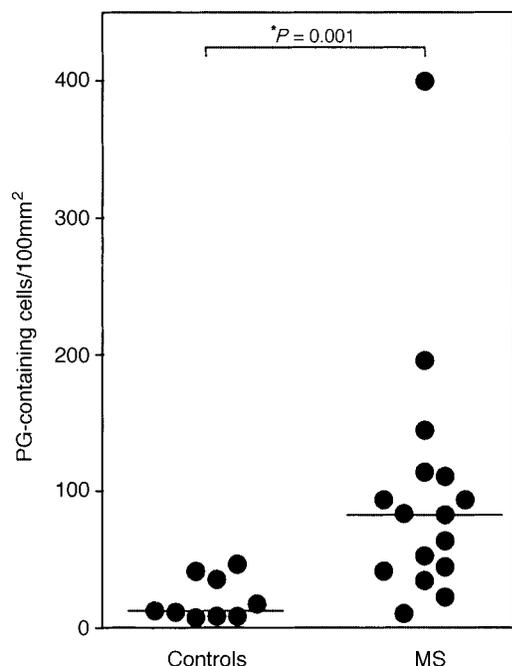
Fig. 1 Peptidoglycan is present in brain white matter of multiple sclerosis patients. Immunohistochemistry was performed on brain tissue of 17 multiple sclerosis patients (MS) and 10 control subjects using Mab 2E9 raised against PG isolated from human faeces. The median (horizontal bar) of the number of positive cells was $82/100\ mm^2$ in multiple sclerosis patients compared with $12/100\ mm^2$ in the control subjects. $*P = 0.001$ using the Mann–Whitney rank-sum test.

was expressed by 17% (range 0–27%) of the PG-containing cells (Weidemann *et al.*, 1997). The majority of the PG-containing cells (mean 62%), and in two out of nine patients all of these cells (range 33–100%), expressed 27E10, an acute stage inflammatory macrophage marker characteristic for active multiple sclerosis lesions (Brück *et al.*, 1995).

Although PG is most likely to be presented by HLA molecules, the possibility cannot be excluded that CD1a is involved in the presentation of the carbohydrate molecules present in PG. However, only very few PG-containing cells co-expressed CD1a, suggesting that this mechanism of antigen presentation is highly unlikely for PG.

PG-containing cells co-express co-stimulatory molecules

The capability of PG-containing cells to present antigen was examined by double staining for HLA-DR, CD80/B7–1, CD86/B7–2 and CD40, which are centrally involved in (co)-stimulation of CD4+ T cells. Co-expression with PG occurred for all four molecules to varying extents, in particular for CD80 (Fig. 2C), suggesting that PG-containing cells are mainly functionally competent, active antigen-presenting cells (Fig. 3).

Peptidoglycan containing cells co-express different cytokines

To assess whether PG-containing cells can contribute to CNS inflammation in multiple sclerosis by producing cytokines, double labelling was performed for proinflammatory cytokines (IL-1α and β, IL-6, IL-12, TNF-α and IFN-γ) and immunoregulatory cytokines (IL-4 and IL-10). Except for IL-1α, all cytokines analysed were expressed by PG-containing cells, to varying extents. Of the proinflammatory cytokines, co-expression was most frequently found for TNF-α (mean 44% of PG-containing cells, range 9–100%). In one patient, all PG-containing cells produced the strongly proinflammatory and myelotoxic cytokine TNF-α. In addition, TNF-α was also often strongly expressed by cells directly adjacent to PG-containing cells (Fig. 2E). Co-expression of PG was also found for IL-1β (mean 30%, range 14–52%), IL-6 (mean 11%, range 0–22%), IL-12 (mean 18%, range 0–65%) and IFN-γ (mean 11%, range 10–73%), although the latter cytokine was mostly expressed by cells directly adjacent to PG-containing cells. Immunoregulatory cytokines were prominently produced by PG-containing cells. IL-4 was detected in 58% (range 13–93%) of PG-containing cells. IL-10 was mainly produced by cells directly juxtaposed with PG-containing cells (Fig. 2D), but co-expression was found in 40% (range 0–83%) of all PG-containing cells.

Antibody forming cells specific for peptidoglycan are present in multiple sclerosis brain tissue

Healthy human subjects have systemic IgM, IgG and IgA levels against PG (Hazenberg *et al.*, 1990), reflecting continuous exposure to Gram-positive bacteria at the mucosa. To determine whether intrathecal antibodies against PG were produced in multiple sclerosis brain tissue, plasma cells specific for PG were visualized *in situ* using biotin-labelled PG (Laman *et al.*, 1990; Claassen *et al.*, 1992). Plasma cells specific for PG could be detected in seven out of 13 multiple sclerosis patients and in two out of seven controls (Fig. 4). The numbers of plasma cells specific for PG were limited in control patient tissues and three of the multiple sclerosis patients; however, four multiple sclerosis patients had high numbers of PG-specific antibody forming cells. In multiple sclerosis patients, the number of antibody forming cells against PG was directly correlated with the number of PG-containing cells ($P < 0.01$). Antibody forming cells specific for PG were scattered throughout the tissue (Fig. 2F) and could often be found near blood vessels. Pre-incubation of tissue sections with unlabelled PG resulted in a dose dependent reduction of the number of PG-binding cells, confirming the specificity of staining. Double labelling for CD20, a surface plasma cell marker, IgM, IgG and PG-biotin
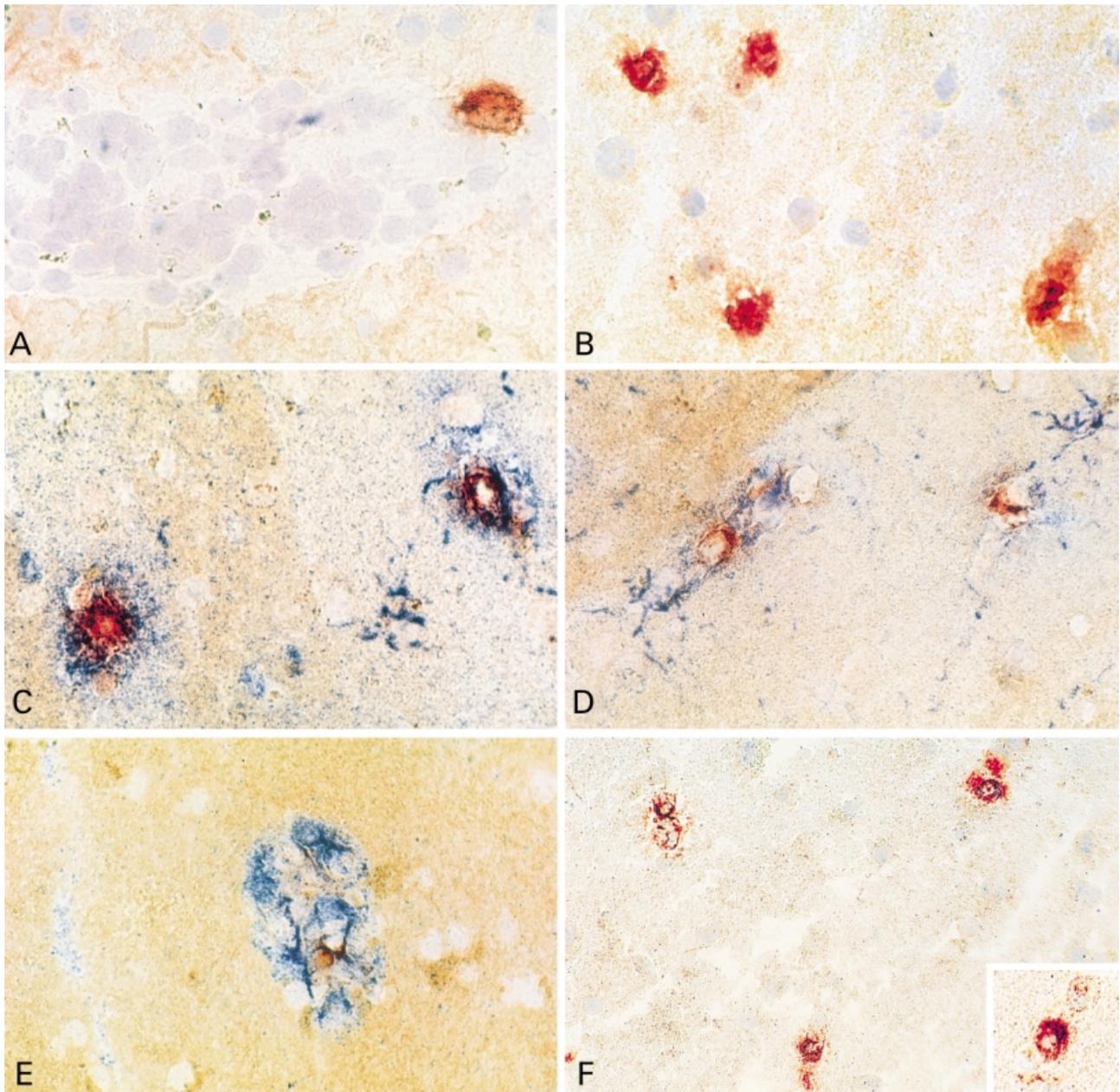
Fig. 2 Peptidoglycan promotes immune reactivity in multiple sclerosis brain tissue. Peptidoglycan is present in the white matter of multiple sclerosis patients on the edge of infiltrates or active lesions (**A**), or distributed in normal appearing white matter (**B**). Co-expression was found with CD80 (**C**) and co-localization with other cells expressing IL-10 (**D**). Both co-expression and co-localization were found with TNF-α (**E**). Using biotin-labelled PG, antibody forming cells specific for PG could be detected in multiple sclerosis brain tissue (**F** + inset). Magnification: **A–E**, ×630; **F**, ×400; inset, ×630.

confirmed that PG-binding cells were indeed plasma cells containing high levels of cytoplasmic immunoglobulin.

Antibodies specific for peptidoglycan are present in CSF of patients with active multiple sclerosis

To confirm our finding of intrathecal production of antibodies against PG *in situ* in multiple sclerosis autopsy brain tissue, ELISA was used to determine IgM, IgG and IgA antibody levels against PG in CSF samples. Samples taken during relapse from relapsing–remitting multiple sclerosis patients not treated with immunosuppressive drugs were compared with samples from patients with severe headache for which no cause could be determined. Anti-PG antibodies could not be detected in any of the control patients. Using conservative cut-off values, six out of 10 multiple sclerosis patients displayed considerable levels of anti-PG antibodies of the IgG and/or IgA isotypes (Fig. 5).
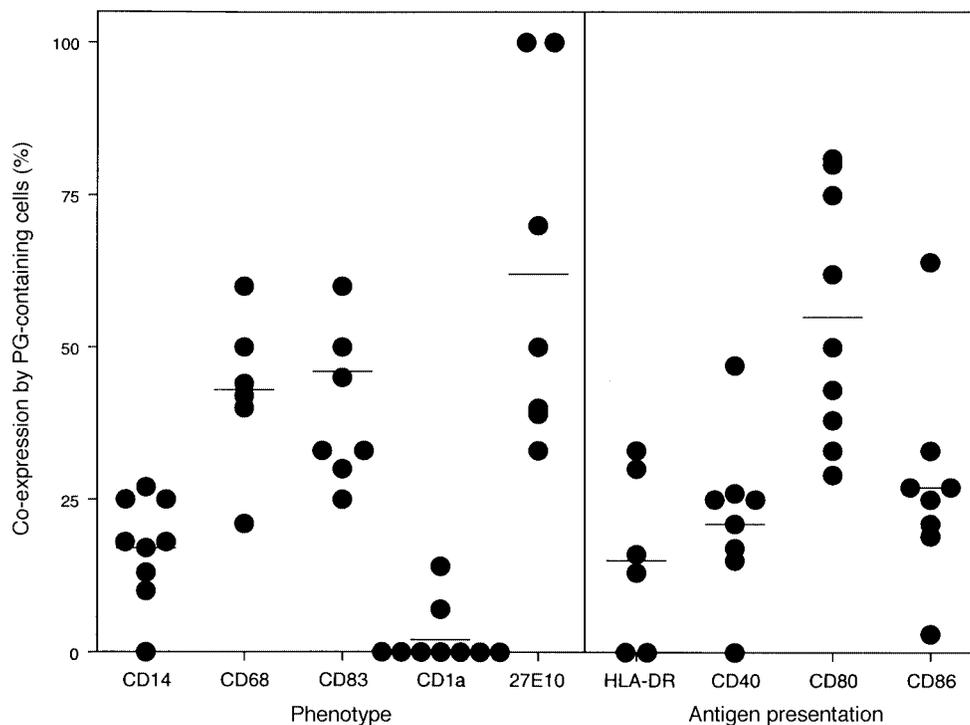
Fig. 3 Peptidoglycan is present in macrophages and dendritic cells capable of antigen presentation. Using immunohistochemistry, double labelling was performed with the Mab 2E9 raised against peptidoglycan (PG) isolated from human faeces and various subset markers and functional markers of antigen-presenting cells. PG is present in both macrophages (CD68/27E10) and dendritic cells (CD83), which are capable of antigen presentation and co-stimulation as reflected by the expression of HLA-DR, CD40, CD80 and CD86. Horizontal bars represent mean numbers of PG-containing cells co-expressing the analysed marker.
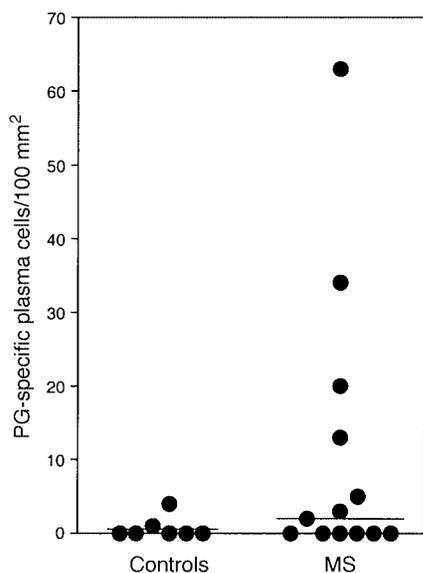
Fig. 4 Peptidoglycan-specific plasma cells are present in multiple sclerosis brain tissue. Using biotin-labelled PG, antibody forming cells could be detected in seven of 13 multiple sclerosis patients and only in two of seven control subjects. In four of the multiple sclerosis patients (MS) relatively high numbers of antibody forming cells could be detected. Horizontal bars represent the median of the number of PG specific plasma cells in both groups.

Discussion

The current study shows that antigen-presenting cells in the CNS of multiple sclerosis patients contain the Gram-positive cell wall constituent peptidoglycan, which is abundantly present at all mucosal surfaces. In the CNS, PG may contribute to inflammation by induction of cytokine secretion, modulating T-cell function and stimulating antibody formation.

The presence of bacterial peptidoglycan in CNS tissue sheds a different light on the search for infectious agents involved in multiple sclerosis, where the emphasis has been on identification of viruses (e.g. HHV-6) and not on bacteria (Soldan *et al.*, 1997; Noseworthy, 1999). The implicit assumption of such approaches is that local replication of viruses or bacteria is essential. In sharp contrast, we show that peptidoglycan may stimulate CNS-inflammation in the apparent local absence of viable bacteria and replication, implying that this antigen can be a microbial mediator in sterile inflammation. Thus, our data support novel emerging insights that persistence of bacterial cell wall antigens can contribute to autoimmune diseases (Relman, 1999).

Studies of autopsy brain material and multiple sclerosis routinely and consistently reveal a partial overlap of pathological characteristics between patient and control subject tissues (Ravid *et al.*, 1995). For example, activated macrophages can be found in control subject tissues (Purba
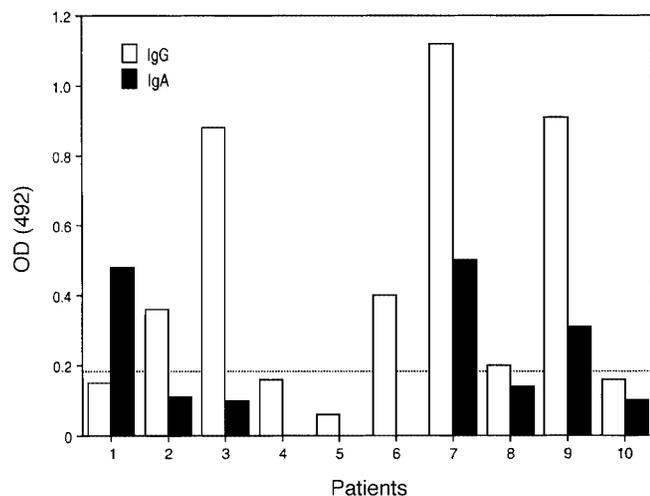
Fig. 5 IgA and IgG antibodies against PG are present in multiple sclerosis CSF. Using ELISA, IgM, IgG and IgA levels against PG were measured in CSF samples of 10 relapsing–remitting multiple sclerosis patients taken during a relapse, and in 10 control patients with severe headache for which no cause could be determined. Using a CSF dilution of 1 : 2, anti-PG IgG and/or IgA antibodies could be detected in seven of 10 multiple sclerosis patients. Antibodies against PG could not be detected in any of the control patients. The dashed line at 0.19 shows the mean OD (optical density) + 3 SD of the control patients, which represents a conservative cut-off value. IgM against PG was not detected in any of the donors, showing that antibody levels against PG in multiple sclerosis patients do not simply result from an indiscriminate rise of all isotypes.

et al., 1993). This also explains why limited numbers of PG-positive cells could be detected in control tissues in the current study.

Peptidoglycan is present in functionally competent antigen-presenting cells

Characterization of PG-containing cells showed that macrophages containing PG were mostly mature, while only a minority had an immature phenotype. The persistence of PG in macrophages can be explained by the fact that amidase cannot be detected in macrophages (Hoijer *et al.*, 1997*a*). High expression of NAMLAA (Hoijer *et al.*, 1996, 1997*a*) is required for full degradation of PG in joint action with lysozyme (Hoijer *et al.*, 1997*b*). The high percentage of 27E10 expression by PG-containing cells indicated that PG is present in active lesions (Brück *et al.*, 1995), of which 27E10 is a reliable marker. This is consistent with the concept that antigen-presenting cells mature upon uptake of antigen and migration (Mellman *et al.*, 1998), where migration through vascular endothelium is a crucial step promoting functional maturity and loss of surface markers such as CD14 (Randolph *et al.*, 1998). CD14+ monocytes can also mature into dendritic cells, a transition enhanced by bacterial stimuli (Rescigno *et al.*, 1999), which may explain the presence of PG in a limited number of dendritic cells.

The expression of HLA-DR, CD40, CD80 and CD86 by PG-containing cells indicates that these cells may be potent stimulators of antigen-specific T-cell activation. *In vitro* analysis of PG isolated from human tissues has shown that PG is able to induce T-cell proliferation (unpublished results). In addition, PG-containing cells synthesized cytokines involved in regulation of inflammatory activity (e.g. IL-1β, IL-6, IL-12, TNF-α, IFN-γ, IL-4 and IL-10), demonstrating that they produce soluble mediators directly affecting T-cell proliferation, activation and survival (Vella *et al.*, 1995, 1997). PG isolated from sterile human spleen is capable of inducing IL-1, IL-6 and TNF-α (Schrijver *et al.*, 1999) by macrophages *in vitro*; IL-10, IFN-γ and IL-12 and gelatinase B are also induced upon PG stimulation *in vitro* (Schrijver *et al.*, 2001*a*). This confirms that PG can indeed be responsible for the cytokine production by the PG-containing cells in the CNS.

Peptidoglycan and T-cell tolerance

We hypothesize that PG in the CNS may contribute to breaching T-cell tolerance and promotes T-cell activity by antigen presentation and induction of cytokine production (Röcken *et al.*, 1992; Vella *et al.*, 1995; Segal *et al.*, 1997; Ehl *et al.*, 1998). This is supported by the expression of HLA-DR, co-stimulatory molecules and cytokines by PG-containing cells in multiple sclerosis brain tissue. It has been shown in animal models that inflammatory processes created by bacterial infections or LPS lead to activation of autoreactive CD4+ cells (Röcken *et al.*, 1992) or CD8+ cells (Ehl *et al.*, 1998) which otherwise die by apoptosis. The major mechanism leading to breaking of tolerance is cytokine induction by infectious agents leading to T-cell survival (Vella *et al.*, 1995, 1998). This view is supported by the finding that LPS induces experimental autoimmune encephalomyelitis in mice injected with myelin basic protein-specific T cells through induction of IL-12 (Segal *et al.*, 1997). Therefore, the presence and persistence of PG in brain tissue may promote loss of tolerance against autoantigens present in the brain, e.g. myelin basic protein, proteolipid protein and others.

Intrathecal antibody synthesis against peptidoglycan

Plasma cells producing antibody specific for PG were also present *in situ* in multiple sclerosis brain tissue. This demonstrates that intrathecal antibody production against PG occurs. To confirm this finding, CSF of multiple sclerosis patients and control patients was analysed, showing that none of the control patients had specific antibody titres, whereas the majority of multiple sclerosis patients had detectable specific IgG and IgA levels. The presence of antibodies in the CSF may be partly due to leakage from the blood, but the presence of plasma cells specific for PG within the brain

tissue unequivocally demonstrates that antibodies specific for PG are also produced intrathecally. The specificity and pathological relevance of plasma cells in the brain and oligoclonal immunoglobulins in multiple sclerosis has been highly obscure thus far (see Cortese *et al.*, 1998). Our finding that PG evokes specific intrathecal antibody production *in situ* identifies a hitherto unknown specificity of local plasma cells.

Perspective

In conclusion, this study suggests that immunocompetent PG-containing cells are able to reach the brain in multiple sclerosis and may contribute to local immune reactivity, by expression of co-stimulatory molecules and cytokines, and by stimulating intrathecal antibody formation. We do not propose a single antigen–disease link. Instead we suggest that PG may be a crucial mediator in polymicrobial involvement during chronic inflammatory disease (see Relman, 1999). Redistribution of PG from mucosal sites to the brain depends on the intricate interplay between several factors such as the permeability of the intestine, activity of enzymes specifically involved in PG degradation (Hoijer *et al.*, 1997a) and mucosal and systemic antibody responses against PG (Schrijver *et al.*, 2001b). Restricting the access of PG to non-mucosal sites could therefore be of clinical benefit to patients, as this may lead to lower levels of inflammation.

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