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FAST TRACKS

New Molecular Markers of Early and Progressive CJD Brain Infection

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Abstract Transmissible spongiform encephalopathies (TSEs), including human Creutzfeldt–Jakob disease (CJD), are caused by a related group of infectious agents that can be transmitted to many mammalian species. Because the infectious component of TSE agents has not been identified, we examined myeloid cell linked inflammatory pathways to find if they were activated early in CJD infection. We here identify a specific set of transcripts in CJD infected mouse brains that define early and later stages of progressive disease. Serum amyloid A3 and L-selectin mRNAs were elevated as early as 20 days after intracerebral inoculation. Transcripts of myeloid cell recruitment factors such as MIP-1α, MIP-1β, and MCP1, as well as IL1α and TNFα were upregulated >10 fold between 30 and 40 days, well before prion protein (PrP) abnormalities that begin only after 80 days. At later stages of symptomatic neurodegenerative disease (100–110 days), a selected set of transcripts rose by as much as 100 fold. In contrast, normal brain inoculated controls showed no similar sequential changes. In sum, rapid and simple PCR tests defined progressive stages of CJD brain infection. These markers may also facilitate early diagnosis of CJD in accessible peripheral tissues such as spleen and blood. Because some TSE strains can differentially target particular cell types such as microglia, several of these molecular changes may also distinguish specific agent strains. The many host responses to the CJD agent challenge the assumption that the immune system does not recognize TSE infections because these agents are composed only of the host's own PrP. J. Cell. Biochem. 93: 644–652, 2004.

Key words: host recognition; innate immunity; RT-PCR; diagnosis; TSE agents; prion

The spread of transmissible bovine spongiform encephalopathy (BSE), chronic wasting disease (CWD) of cervids, and human Creutzfeldt–Jakob disease (CJD), have become major public health issues. These infections can be inapparent for years, and a case of BSE-linked variant CJD (vCJD) now appears to have been transmitted from an asymptomatic person via blood transfusion [Pincock, 2004]. Because white blood cells carry the CJD agent [Manuelidis et al., 1978, 1985], such transmissions were predictable [Manuelidis, 1994, 1997b]. Early diagnosis of these infections has been limited because behavioral disturbances and neuropathologic sequelae occur only late in the course of the infection. Current diagnostic tests are based on detection of prion protein (PrP), a host molecule that shows resistance to limited proteolysis in a test-tube assay using detergents. Resistant PrP (PrP-res) is typically not detectable until high levels of infectivity accumulate [Manuelidis and Fritch, 1996]. The finding of PrP-res in diseased brain led to the development of the prion hypothesis, which states that PrP-res itself is the infectious entity [Prusiner, 1982]. This hypothesis remains formally unproven, however, because no purified, recombinant, or amplified PrP has been able to transmit infection [reviewed in Manuelidis, 2003], and contamination has been apparent in transmissions from PrP modified mice [Manuelidis, 1994]. In contrast, infectious particles with a single homogeneous viral size and density separate from most abnormal PrP [Manuelidis et al., 1995; Manuelidis, 2003]. These agents also breed true even when propagated in cells with markedly different PrP patterns, and thus strain characteristics are not "enciphered" by the different forms of PrP [Arjona et al., 2004].

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The recent epidemic spread of BSE and CWD additionally underscores an exogenous source of infection, rather than a "spontaneous" infection arising from host PrP protein [Prusiner et al., 1995; Manuelidis, 1997a]. Because the infectious component of these agents has not yet been identified, we examined host inflammatory pathways that might be recruited in response to a foreign infectious agent. Inflammatory pathways have been overlooked, largely because the host's own PrP should not elicit an immune response [Gajdusek, 1977; Prusiner, 1995; Prusiner et al., 1995]. Nevertheless, many latent and persistent viruses can evade adaptive immune defenses of antibody recognition, yet still evoke innate immune responses [Furman and Ploegh, 2002].

Recent studies have shown some transmissible spongiform encephalopathies (TSE) agents can be carried by several types of antigen-processing myeloid cells, including peripheral dendritic cells and macrophages, as well as brain microglia [Manuelidis et al., 2000; Aucouturier et al., 2001; Baker et al., 2002]. Dapsone, a drug that targets infections of myeloid cells, can significantly delay clinical disease in a CJD rodent model marked by an intense and prolonged microglial reaction [Manuelidis et al., 1998]. Myeloid cells are classically Trojan horses for the spread of viruses to different tissues, and can also participate in viral clearance [Manuelidis et al., 1997; Manuelidis, 2003]. These cells are also central players in any immune response, and infectious microglia mount a complex transcriptional response to CJD infection [Baker and Manuelidis, 2003]. This response includes elaboration of mRNAs related to innate immunity, and indicates the host recognizes the CJD agent as a foreign entity [Baker et al., 2004]. To find if inflammatory markers might be of diagnostic value at both early and progressive stages of disease, we evaluated CJD brain long before the appearance of PrP-res or clinical signs [Baker et al., 2004]. Since we were able to identify several positive molecular markers for early infection, we investigated an even broader group of myeloid cell transcripts that could be of diagnostic value in assessing sequential stages of CJD brain infection. We here demonstrate profound induction (sometimes up to 100-fold) of transcripts related to proteolysis, inflammatory cell recruitment, and cell–cell interactions. Remarkably, a specific set of transcripts was significantly elevated at early stages of disease when no abnormal PrP or neuropathologic changes were detectable. Thus these changes can warn of infection in apparently healthy mammals. The sequential development of specific patterns of host responses also defines different stages of brain infection using a relatively simple, rapid, and inexpensive RT-PCR test.

METHODS

Inoculation and Sample Preparation

CD-1 mice were intracerebrally inoculated with 30 μl of a 1% brain homogenate containing the Asiatic FU strain of CJD [Manuelidis, 1998]. In parallel, animals were inoculated with normal brain homogenate to control for non-specific effects of intracerebral inoculation. To further avoid non-specific effects of inoculation, the inoculated half or region of CJD brain samples were excluded and used for Western blot and histologic studies. For each 10–50 day time point, four CJD brains were pooled to yield a typical or average profile for each time point. All other samples represented pools of 2–3 brains, and asymptomatic mice (those with an incubation time of <90 days) were randomly selected for cDNA preparation. RNA was isolated from brains by homogenization in Trizol (Invitrogen, Carlsbad, CA). Microglia (95% $CD11b^+$ cells) were isolated from the brains of mice with clinical signs of CJD, or age-matched controls, using previously established protocols [Baker et al., 2002]. Microglia were maintained in vitro for 16–18 h at 37°C and 5% CO_2 in microglial medium (RPMI-1640 with 5% fetal bovine serum, 10 mM HEPES, 1 mM sodium pyruvate, 50 U/ml penicillin, 50 μg/ml streptomycin). Following this brief culture period, cells were quickly rinsed with microglial medium to remove any remaining debris before RNA extraction with Trizol. For stimulation of the TLR9 receptor [Dalpke et al., 2002], unmethylated CpG DNA oligonucleotides (TCCATGACGTTCCTGATGCT) or oligonucleotides with an inverted CpG motif (TCCATGAGCTTCCTGATGCT) were included in the overnight culture at a final concentration of 3 μM.

RT-PCR Analysis

Digestion of RNA samples with DNAse I, reverse transcription, and PCR amplification

with biotinylated nucleotides were done as described previously [Baker et al., 2002]. The number of PCR cycles required for detection within the linear range of amplification was determined empirically for each product. In the linear PCR range, the amplification per cycle was $2^{1.54}$ as determined from over 500 independent PCR experiments using varying amounts of input DNA. Optimal PCR conditions and primer sequences used are listed in Table I. Other RT-PCR controls included the following: (1) every cDNA (CJD and control sample) was first normalized to give the same amount of GAPDH product (~5 ng of cDNA per 20μl reaction); (2) dilution of cDNA input was reduced to confirm the fold increase (multiplication factor) in the CJD brain relative to normal brain (taken as 1×); (3) RT-PCR products were diluted for gel blot analysis, where appropriate, to further confirm the relative fold change; (4) films for quantitative analysis were exposed in the linear range; (5) film exposure times were systematically varied to confirm quantitative differences; and (6) controls for standardizing dose and film intensity were included. PCR products were separated on agarose gels, transferred to Biodyne B nylon membranes (Pierce, Rockford, IL), and then visualized with the BrightStar Bio-Detect kit (Ambion, Austin, TX) and Biomax MR film (Kodak, New Haven, CT). Densitometry was conducted using NIH Image (with density standards), with final normalization to glyderaldehyde-3-phosphate dehydrogenase (GAPDH) to control for slight differences in starting RNA quantity.

RESULTS

Figure 1 shows upregulation of inflammation-linked transcripts in mouse CJD brain at early time points after inoculation. These alterations occurred long before any PrP-res or clinical disease signs (shown by arrows), and often persisted throughout the course of disease. L-selectin and serum amyloid A3 (SAA3) exhibited some of the earliest significant changes taken as >4 fold (i.e., >4×) the normal brain control. Significant induction started as soon as 20 days after inoculation. Notably, after intracerebral inoculation, infectious agent begins to replicate exponentially in brain only 20 days after injection in experimental CJD [Manuelidis and Fritch, 1996]. In contrast, an acute microglial response to needle injury typically appears much earlier, during the first 48 h, and then subsides to negligible levels by 10 days. Normal brain inoculum did not induce these responses at 20–50 days (Fig. 1B), further indicating the response was specific for infected brain. Indeed, SAA increased only 2

TABLE I. PCR Conditions

Gene	Anneal (°C)	No. cycles	Forward primer	Reverse primer
C1qα	65	21	GGACAGCGGCCCCCAAGGACT	CAGGCCGAGGGGAAAATGAGGAATC
C1qα	65	22	CTACGGGGCTACACAGAAAGTCG	CAGGGAAAAGCAGAAAGCCAGTGAAGA
Cathepsin D	60	19	GGCAACCCGGAGGAGAACTAA	CCACTGGGAGGGGGTATGTC
Cathepsin L	65	20	CAGGGCCAGTGCGGGTCTTGTT	GTTGTCCCGGTCTTTGGCTATTTTGATGTA
CD14	59	25	CTTCCTCAGATCCGAAGCCAGATTG	TCGCCCAATTCAGGATTGTCAG
CD48	63	26	ACCACCGGCAGCAATGTAACCCTG	GTCGTTCTTGCTGCTTACAGGATTGC
CD68	60	26	CAACAAAACCAAGGTCCAGGGA	CCAATGATGAGAGGCAGCAAGA
CX3CR1	60	24	AGCTGCTCAGGACCTCACCAT	GTCATATGCAGGAACTCTGGG
Cystatin F	65	28	CAGCCATGTGGCTGGCCATTCTGCTTG	ACTTCAGAGTAGCAATATAGAGTCCGC
GAPDH	60	20	GACCTCAACTACATGGTCTACAT	TGGTTCACACCCATCACAAACAT
IL-1α	63	29	TGCAAGTGTCTGAAGCAGCTATGG	GGTGGGTGTGCCGTCTTTCATTACA
L-selectin	65	31	ACTCTGGGAAATGGAACGATGAC	AATGAAGAGGGGGTTGTAGTCACC
Mac2	72	26	TATCCTGCTGCTGGCCCTTATGGTGTCC	CGTGGTTAGCGCTGGTGAGGGTTATGTC
MCP1	60	28	CCACTCACCTGCTGCTACTCATTC	GTCACTCCTACAGAAGTGCTTGAGG
MIP-1α	60	28	GAAGAGTCCCTCGATGTGGCTA	CCCTTTTCTGTTCTGCTGACAAG
MIP-1α	60	32	CCACAATAGCAGAGAAACAGCAAT	AACCCCGAGCAACACCATGAAG
Properdin	72	25	AGAGACATCAGGGTAGAAGACTGCTG	ATAGGCTGGTCCTGAGCAGGGTTTC
SAA3	67	34	ATGAAGCCTTCCATTGCCATCATTC	TCAGTATCTTTTAGGCAGGCCAGC
TLR2	68	25	ACAGTAGAGAACAGCAAGGTCTTCC	GCTCTTGCAGCCGAGGCAAGAAC
TLR3	65	28	GCAGTTTCCAACTCTGGATCTACC	GTGTTTGCAAAGACATTTGAAAGGGTG
TLR4	68	25	TCGTTCAGTGAGCTACCACAGTTGC	TGCCATTAGGCAGGGTGCCATTGG
TLR9	68	31	TTTCTCTTGGTTAGGCCAACTCAGG	ACTGGGATTTCATCTAAGCCGTTGG
TNFα	60	32	GCTGGAAGACTCCTCCCAGGTA	ATGATCCGCGACGTGGAACTG

Conditions for RT-PCR experiments, including primer sequences, annealing temperature, and number of PCR cycles.

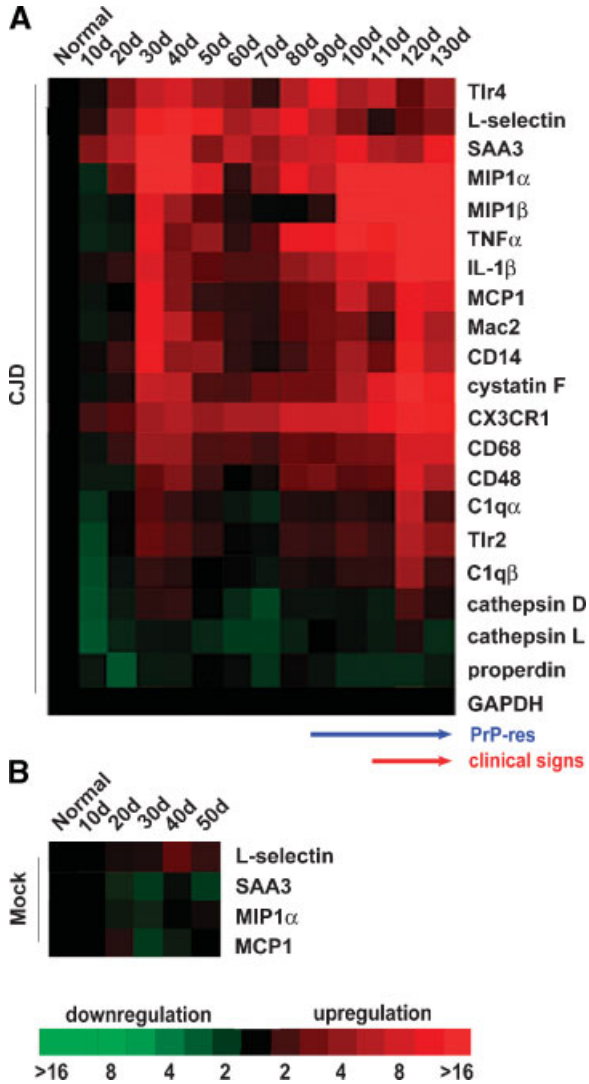

Fig. 1. **A**: Quantitative changes in inflammation-linked brain transcripts at 10 day intervals after intracerebral inoculation of CJD homogenate. Representative RT-PCR blots, quantified by densitometry, with normalization to GAPDH (to compensate for slight differences in cDNA input). Colorimetric scale indicates fold change relative to uninfected samples. Transcripts arranged top to bottom from those showing earliest changes to those with predominantly later changes. Brain accumulation of PrP-res during the course of disease indicated by a blue arrow, and appearance of clinical signs is indicated by a red arrow. **B**: Mock inoculation with normal brain homogenate did not similarly affect inflammation-linked transcripts, as shown for representative markers. The SAA elevation in mock controls at 40 days was only 2 fold greater than normal, and other representative transcripts were actually downregulated (green). In contrast, all the CJD transcripts (more intensely red, see color bar for fold change equivalents) were elevated >4 fold at multiple sequential time points (also representatively graphed in Fig. 2).

fold at 40 days (an insignificant change) in these mock controls, and many transcripts that were elevated in CJD were instead downregulated in mock controls (as representatively shown in Fig. 1B). The temporal expression pattern of SAA and L-selectin transcripts was unlike that of any other genes evaluated below because upregulation was higher at 20–50 days than at later stages of disease. Peak levels of SAA3 occurred at 30 days (22× the normal value) and subsided by 50 days, whereas L-selectin levels reached their peak (38× the normal value) at 40 days, and decreased somewhat thereafter (Fig. 2A). These declines were not secondary to neurodegeneration, because PrP and other pathological changes in the brain begin only after 90 days by both Western blot and histologic assessments (see legend Table II). The significant sequential elevation of these transcripts at a defined series of time points from a pool of four randomly selected asymptomatic mice also further validated their reproducibility. Additionally, the design of the experiment, where the inoculated portion of CJD brain was excluded from the cDNA pool (see "Methods"), also makes non-specific local injury responses a most unlikely cause of these global changes.

We next considered the possibility that early increases in L-selectin mRNA reflected a transient influx of T lymphocytes positive for L-selectin. Infiltration of T cells into the brain has recently been observed in several mouse scrapie models [Betmouni et al., 1996; Lewicki et al., 2003]. However, flow cytometry analysis of leukocytes from CJD brain revealed no increase in cells positive for both cell-surface L-selectin and the T cell marker CD3 (data not shown). Therefore, upregulation of L-selectin mRNA likely reflects a response of resident glial cells to CJD infection.

At 30 days after inoculation, the myeloid cell recruitment factors MIP-1α, MIP-1α, and MCP1 were also significantly induced, as well as IL-1α and TNFα proinflammatory activators. Each of these transcripts was upregulated at least tenfold, and then decreased somewhat prior to massive upregulation of 20–100 fold during neurodegenerative stages of disease (Fig. 2B,C). The temporal profile of these genes was, therefore, distinct from the pattern of L-selectin and SAA3. This biphasic inflammatory response did not coincide with a detectable change in exponential agent replication since CJD, as well as other TSE agents, show continuous exponential replication by bioassay, even when PrP-res is arrested [reviewed in Manuelidis and Fritch, 1996]. Nevertheless, since the bioassay

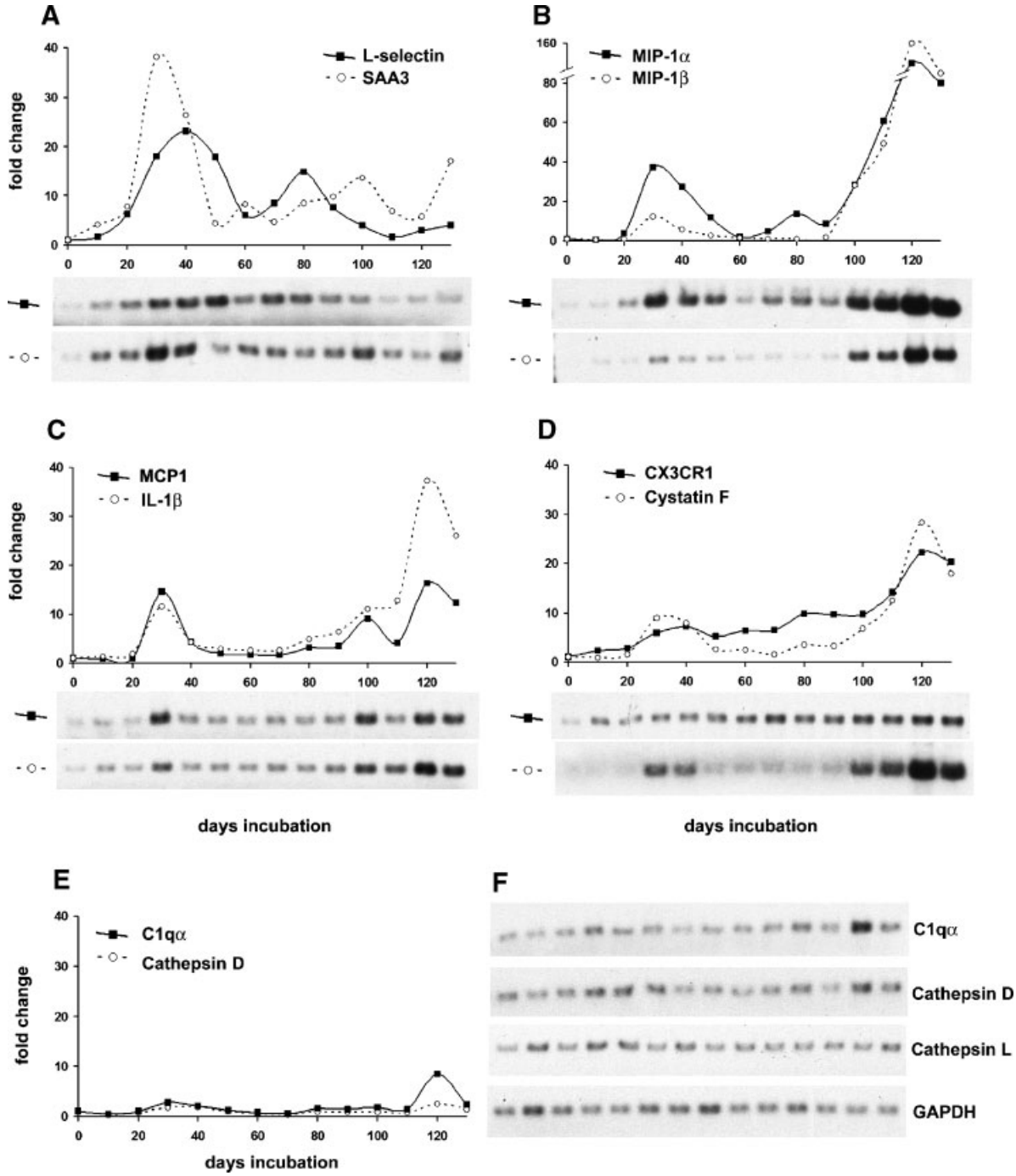

Fig. 2. Different genes show distinct kinetic expression patterns during CJD progression. Representative RT-PCR blots graphed to show the fold-change versus normal brain. **A**: L-selectin and SAA3 showed most pronounced changes during early asymptomatic phase (20–40 days). **B**, **C**: Other transcripts exhibited substantial increases during both early and terminal stages of disease (e.g., MIP-1α, MCP1). **D**: Cystatin F and CX3CR1 displayed more gradual increases throughout infection, whereas other transcripts showed little or no effect of CJD infection (**E**, **F**). Note the different scale in (B).

for infectivity cannot discriminate a <10 fold change, some suppression of agent replication cannot be ruled out. However, the transient nature of these early responses suggests that infectious agent replication directly or indirectly induces, and then suppresses, complex host transcriptional responses.

A third pattern of more gradual progressive transcript elevation was exemplified by CX3CR1, a receptor thought to mediate microglial–

TABLE II. Matrix of Diagnostic Markers for Different Stages of CJD

Marker	10d	20d	30d	40d	50d	60d	70d	80d	90d	100d	110d	120d	130d
SAA3	+	+	+++	+++	+	+	+	+	+	++	+	+	++
L-selectin	−	+	++	++	++	+	+	++	+	+	−	−	+
MIP-1α	−	−	+++	+++	++	−	+	++	+	+++	++++	++++	++++
ImmResG1	−	−	++	++	+	+	+	+	++	++	++	+++	++
MIP-1α	−	−	++	+	−	−	−	−	−	+++	+++	++++	++++
IFI202	−	−	++	+	+	−	−	+	+	++	++	+++	+++
IFI204	−	−	++	+	−	−	−	+	+	++	+	++	++
CD84	−	−	++	++	+	−	−	+	+	+	+	++	++
CD72	−	−	++	++	−	−	−	−	+	++	+	++	+
IL-1α	−	−	++	+	−	−	−	+	+	++	++	+++	+++
MCP1	−	−	++	+	−	−	−	−	−	+	+	++	++
LY9	−	−	++	+	−	−	−	−	−	−	−	++	+
TNFα	−	−	++	−	+	−	−	++	++	++	++	++++	+++
CX3CR1	−	−	+	+	+	+	+	+	+	+	++	++	++
Cathepsin S	−	−	+	++	+	−	−	+	+	+	+	++	++
CD68	−	−	+	+	−	−	−	−	+	++	−	++	+
Cystatin F	−	−	+	+	−	−	−	−	−	+	++	+++	++
CXCL13 (BLC)	−	−	+	−	−	−	−	−	+	++	++	+++	+++
LY86 (MD-1)	−	−	+	−	−	−	−	−	−	+	+	+++	++
CXCL10 (IP-10)	−	−	+	+	−	−	−	+	−	+	+	++	++
RANTES	−	−	+	+	−	−	−	−	−	+	−	+	−
C1qα	−	−	−	−	−	−	−	−	−	−	−	+	−
PrP-res	−	−	−	−	−	−	−	−	+	++	++	+++	+++
Clinical signs	−	−	−	−	−	−	−	−	−	−	+	++	+++
			Early				Middle				Late		

This matrix depicts mRNA levels for various genes at different stages of CJD, incorporating current data and several other markers documented previously [Baker et al., 2004]. −, no significant upregulation; +, greater than 4-fold change; ++, greater than 10-fold change; +++, greater than 25-fold change; ++++, greater than 50-fold change. PrP-res and clinical signs indicate subtle (+) to maximal (+++). Western blot analyses of brain from these same brains (the inoculated portion) showed detectable PrP-res only at ≥100 days, even using 20× gel loads at earlier time points (data not shown). Furthermore, as in previous studies, very infrequent and tiny PrP-res deposits could only be found at 90 days using immunocytochemical detection [Baker et al., 1999].

neuronal interactions [Harrison et al., 1998]. The protease inhibitor cystatin F, which may be involved in maturation of antigen-presenting cells [Hashimoto et al., 2000], also rose, though less progressively. Nevertheless, significant induction of these two mRNAs were detected as early as 30 days after inoculation, with peak increases of >20-fold at the terminal stage of disease (Fig. 2D). All the forgoing patterns contrasted with transcripts exhibiting little or no response to CJD infection, such as the complement cascade components C1qα and C1qα (Fig. 2E,F). Even at terminal stages of disease, these genes showed only very small changes, consistent with secondary responses to progressive neurodegenerative events. Similarly, we found the proteases cathepsin D and cathepsin L were not altered over the entire course of infection (Fig. 2E,F), whereas another lysosomal protease, cathepsin S, was significantly induced in the same CJD infectious model [Baker et al., 1999]. Therefore, specific lysosomal proteases seem to be elicited by infection, and may play a role in agent processing.

We also evaluated several members of the Toll-like receptor (TLR) family of proteins involved in host recognition of viruses and other pathogens. TLRs have also been linked to inflammation-induced neurodegenerative cascades [Lehnardt et al., 2003]. Microglia treated with particular TLR agonists exhibit transcriptional changes that partially overlap with those observed in microglia isolated from CJD brain [Baker and Manuelidis, 2003; Baker et al., 2004]. Peripherally inoculated scrapie can also be retarded by injection of unmethylated CpG oligonucleotides that are known to bind TLR9 [Sethi et al., 2002].

Although TLR9 mRNA was detectable in spleen cells by RT-PCR, no transcripts could be detected in whole brain or adult microglia using this sensitive assay (data not shown). Additionally, analysis of cDNA expression arrays revealed microglia isolated from adult CNS were unresponsive to CpG oligonucleotides (data not shown). This may suggest that CpG exerts its effects in scrapie by a non-TLR9 mechanism. We were, however, able to detect transcripts for TLR2 and TLR3 in brain, but they remained unchanged over the course of CJD infection (Fig. 1 and data not shown). In contrast, TLR4 mRNA levels increased 10-fold at 40 days, and 15-fold at 90 days (Fig. 1).

DISCUSSION

The present results demonstrate induction of a specific pattern of inflammation-linked transcripts in mouse CJD brain well before the onset of neurodegeneration, behavioral changes, or PrP pathology. Although each transcript alone may not be specific for CJD in mice, the entire set of genes depicted here provide a reasonable assessment of early infection. It is also likely that some strains of CJD, BSE, and scrapie may evoke similar early changes in other species, including humans, further extending the use of these diagnostic markers. In human vCJD linked to infection by the UK strain of BSE, there is also a similarly strong recruitment of microglia [Manuelidis et al., 1997] as seen in the murine CJD model studied here.

L-selectin, SAA3, and TLR4 elevations occurred very early after intracerebral inoculation, closely followed by transient induction of MIP1α, MIP1α, IL1α, and TNFα. Progressive upregulation of CX3CR1, and terminal increases in many other transcripts provided indices for later stages of infection. In combination with other diagnostic markers previously identified in murine CJD, such as CD72, CD84, and LY-9 [Baker et al., 2004], our RT-PCR assays indicate how far infection has progressed in the brain of a given animal (Table II). This composite set of genes may also distinguish CJD infection from non-transmissible neurodegenerations such as Alzheimer's disease. For example, cathepsin D mRNA is elevated in Alzheimer's disease brains [Cataldo et al., 1995; Callahan et al., 1999], but not in our murine CJD model. Although it remains to be tested, more natural and longer peripheral infections will probably evoke similar host responses once the infectious agent establishes itself in the brain.

The transcriptional increases identified here further implicate host recognition of a foreign agent early in CJD infection. These host responses include several molecules linked to inflammatory cell recruitment, such as CXCL10 (also known as IP-10) and RANTES [Baker et al., 2004], as well as MIP-1α, MIP-1α, and TNFα (this study). Increases in all these transcripts are also observed in myeloid dendritic cells exposed to the MMTV retrovirus [Burzyn et al., 2004]. Obvious microglial recruitment is found in rat CJD before neurodegenerative changes [Manuelidis et al., 1997], and microglia can migrate to the eye in as little as two days after intravitreal scrapie but not normal brain inoculation [Marella and Chabry, 2004]. Because these host responses can vary with different TSE agent strains [Manuelidis and Fritch, 1996; Baker et al., 1999], RT-PCR tests may yield sufficient information to identify specific agent strains in a single host. Such an approach would be useful for distinguishing sporadic human CJD from variant CJD linked to BSE exposure [Bruce et al., 1997; Hill et al., 1997]. Many independent transmissions from sporadic human CJD to mice all show very long (>350 day) incubation times even after serial passage in mice [Manuelidis et al., 1988], and pathology as well as microglia activation is largely inapparent in brains infected with the SY sporadic agent strain, even at terminal stages of disease [Manuelidis and Lu, 2003; Arjona et al., 2004]. Preliminary sequential studies of SY infected brains show no similar elevation of early transcripts as those provoked by the more virulent FU agent studied here (L. Manuelidis, unpublished data). Such data further indicates different agent strains may be discriminated by the set of host responses they induce.

Because the molecular markers here were associated with activation of myeloid microglia, similar studies of peripheral myeloid cells in spleen, lymph nodes, and blood are likely to be fruitful. We presume such markers will provide sensitive diagnostic indicators of infection before these agents invade the brain. This is important because therapy can be curative at this stage [Ehlers and Diringer, 1984]. Markers for infection in blood would be most valuable for starting early therapy, for culling infected but asymptomatic animals, and for preventing person to person spread by transfusion, organ transplantion, or other means [Manuelidis, 1997b]. CJD agents have already been documented in circulating rodent and human white blood cells [Manuelidis et al., 1978, 1985; Tateishi, 1985], infection of gut by blood-borne agent occurs early after inoculation [Radebold et al., 2001], and vCJD has apparently been transmitted inadvertently by transfusion [Pincock, 2004]. Obviously, identifying the infectious molecules that specify each agent strain is still needed. Only an agent-specific test will be adequate for evaluating low levels of infection in sera, hormones, or vaccines. Notably, ~1,300 sheep became infected with scrapie from

an experimental louping ill vaccine [Gordon, 1946]. The inadvertent spread of TSE agents in humans and other animals is no longer a theoretical issue [Manuelidis, 1994]. Although abnormal PrP is a reasonable indicator of late infection and pathology, it is foolhardy to look at nothing else.

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