Journal of NeuroVirology, 10: 29–40, 2004

ISSN: 1355-0284 print / 1538-2443 online
DOI: 10.1080/13550280490261761

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Early induction of interferon-responsive mRNAs in Creutzfeldt-Jakob disease

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Foreign infectious agents typically evoke a host immune response. In scrapie and Creutzfeldt-Jakob disease (CJD), no immune response has been detectable. However, many latent or persistent viruses evade immune recognition but still activate inflammatory pathways. Unique microglial responses in late CJD infection that could be part of a host defense mechanism were previously delineated, although changes secondary to neurodegeneration could not be excluded. Data here show these microglial transcriptional changes are detectable in CJD brain beginning at 30 days after innoculation. In addition, 10 other interferon-sensitive genes were similarly upregulated at very early stages of infection. These responses occurred well before abnormal prion protein (PrP) and clinical signs of CJD were detectable. Further analyses in very pure microglia from CJD brain suggested the CJD agent activated signaling pathways distinct from those induced by amyloidogenic proteins (including abnormal PrP). Although increases in interferon-α or -β transcript levels were not seen in cultures or in whole brain, CJD microglia exhibited a potentiated interferon response when challenged with double-stranded RNA. The induction of interferon-sensitive genes without appreciable interferon synthesis was strikingly similar to that seen in some viral infections. These data suggest the CJD agent is recognized as a foreign virus-like entity. Moreover, the early reactive gene expression profiles described here may be useful in preclinical diagnosis. *Journal of NeuroVirology* (2004) **10,** 29–40.

Keywords: amyloid; immunity; interferon; microglia; prion

Introduction

According to the prion hypothesis, the infectious agent in scrapie and Creutzfeldt-Jakob disease (CJD) is composed of host prion protein (PrP) that forms beta-pleated amyloid and thereby becomes infectious (Prusiner *et al*, 1998). Thus, no inflammatory or immune responses to a foreign agent or virus would be expected in these infections. However, infectivity can be high when abnormal forms of PrP are undetectable (Lasmezas *et al*, 1997; Manuelidis and Fritch, 1996; Manuelidis *et al*, 1995, 1997; Xi *et al*, 1992), and to date no purified, recombinant, or amplified PrP has demonstrated significant infectivity (Barron *et al*, 2001; Hill *et al*, 1999; Hsiao *et al*, 1994; Saborio *et al*, 2001). Furthermore, the existence of many diverse CJD and scrapie strains is difficult to reconcile with multiple hypothetical conformations of this small protein. In contrast, these agents exhibit many viral properties, such as latency, exponential replication, ability to evolve with serial passage (Manuelidis, 1997; Manuelidis *et al*, 1997), as well as homogenous virus-like physical characteristics of size and density (Manuelidis *et al*, 1995). We therefore examined various inflammatory responses to CJD infection that might indicate host recognition of a foreign agent. Recently, we have also tested serum for agent-induced antibodies and found no effect of serum in interference assays (Manuelidis and Lu, 2003).

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This work was supported by grants NS12674 and NS34569 from the National Institutes of Health.

Received 17 September 2003; revised 29 September 2003; accepted 8 October 2003.

Instead, evidence suggested soluble brain factors can participate in the suppression of a more virulent CJD agent. To test if soluble factors of the innate immune system were elaborated early in response to infection, and prior to PrP pathology, we developed additional microglial markers to delineate progressive changes during CJD.

Microglia, which reside in the central nervous system (CNS) but are of myeloid origin, contain substantial levels of CJD infectivity despite their extremely low PrP expression (Baker *et al*, 2002). These results extend previous findings of infectivity in other myeloid cells such as macrophages (Manuelidis *et al*, 2000) and dendritic cells (Aucouturier *et al*, 2001). Microglia isolated from the brains of mice with terminal CJD exhibit altered levels of about 40 genes relative to microglia from normal brain (Baker and Manuelidis, 2003; Baker *et al*, 2002). Interestingly, many of these mRNA changes in infectious CJD microglia strongly resemble the innate immune response of macrophages and dendritic cells to invading bacteria and viruses (Baker and Manuelidis, 2003). Some of these changes have also been observed in scrapie brain (Riemer *et al*, 2000) and include the up-regulation of several chemokines. These host responses to infection continue to raise the possibility that CJD is caused by a more conventional infectious agent, such as a virus, that is recognized by the host as foreign.

Innate immune responses to foreign invaders can be initiated by cell surface molecules of the toll-like receptor (TLR) family, and thus we dissected these pathways in the present study. TLRs recognize various types of foreign antigens, including bacterial cell wall components, viral envelope glycoproteins, and viral replication intermediates (Takeuchi and Akira, 2002). The various intracellular signaling cascades used by certain TLRs eventually converge to increase the synthesis of type I interferons (IFNα and IFNβ). These interferons are then secreted, and act in both an autocrine and paracrine manner to up-regulate various interferon-sensitive transcripts. These transcripts encode chemoattractants that recruit cells to sites of active infection, proteins that directly interfere with viral replication, and molecules whose functions remain to be fully determined. Microglia isolated from CJD brain exhibit increased expression of several interferon-sensitive transcripts (Baker and Manuelidis, 2003; Baker *et al*, 2002), and therefore further studies of how the CJD agent might invoke an interferon response were warranted.

Because interferon responses are usually produced during the early acute phase of viral infection, we chose to examine these pathways in both microglia and CJD brain at progressive stages after inoculation. We here demonstrate early induction of various interferon-sensitive transcripts in CJD brain that persisted throughout the course of disease. To further characterize how these mRNA changes fit into known innate immunity signaling cascades, we compared the expression of interferon-sensitive transcripts in CJD microglia and normal microglia stimulated with the TLR ligand poly I:C. These studies demonstrate an innate immune response of CJD microglia in the absence of detectable interferon synthesis. This atypical response in CJD may have implications for the host control of infection, and may also be useful for diagnosis of infection during the early asymptomatic phases of disease.

Results

Early induction of interferon-sensitive genes in CJD brain

Previous *in vitro* studies in microglia derived from CJD brain demonstrated the induction of various interferon-sensitive genes, including CXCL10 (IP-10), CXCL13 (BLC), immunoresponsive gene 1 (ImmResG1), interferon-induced protein 202 (IFI202), interferon-induced protein 204 (IFI204), 47-kDa interferon-responsive protein, primary response gene B94, and glucocorticoid attenuated response gene 39 (Baker and Manuelidis, 2003; Baker *et al*, 2002). To confirm these changes *in vivo* and determine whether they respresented an early response to the CJD agent itself, or instead were associated with the onset of pathologic PrP accumulation and neurodegenerative disease, we examined transcripts at regular intervals throughout the course of CJD infection in whole brain. We also assayed other interferon-linked transcripts that have not yet been studied in CJD or scrapie. These genes included the known inhibitors of viral replication 2′,5′-oligoadenylate synthase (OAS) and double-stranded RNA–dependent protein kinase (PKR), as well as the chemoattractant RANTES and the 15-kDa interferon-sensitive gene (ISG15). Our survey also examined the interferon-responsive factor (IRF) family of transcription factors, which regulate the expression of other interferon-sensitive genes (Eklund *et al*, 1998; Marecki *et al*, 2001; Tamura *et al*, 2002) we previously showed were up-regulated in CJD microglia.

Ten interferon-sensitive transcripts were up-regulated more than 10-fold in CJD brain. The chemokine CXCL13 displayed the greatest increase, with a 40-fold up-regulation at the terminal stage of CJD (Figure 1A). Remarkably, mRNA increases of >5-fold for these 10 genes were seen as early as 30 to 40 days after inoculation, ~50 days before the appearance of detectable accumulation of protease-resistant PrP (PrP-res), clinical symptoms, and neuropathological changes. This 30- to 40-day time point also coincides with early replication of the CJD agent that starts after a ~25-day eclipse phase (Manuelidis and Fritch, 1996). Elevations for CD72 (see Figure 1B), CD84, and IFI202 began even earlier, starting at 20 days. None of the 10 transcripts shown were elevated at parallel times in control mice inoculated with equivalent amounts of uninfected brain (mock-inoculated).

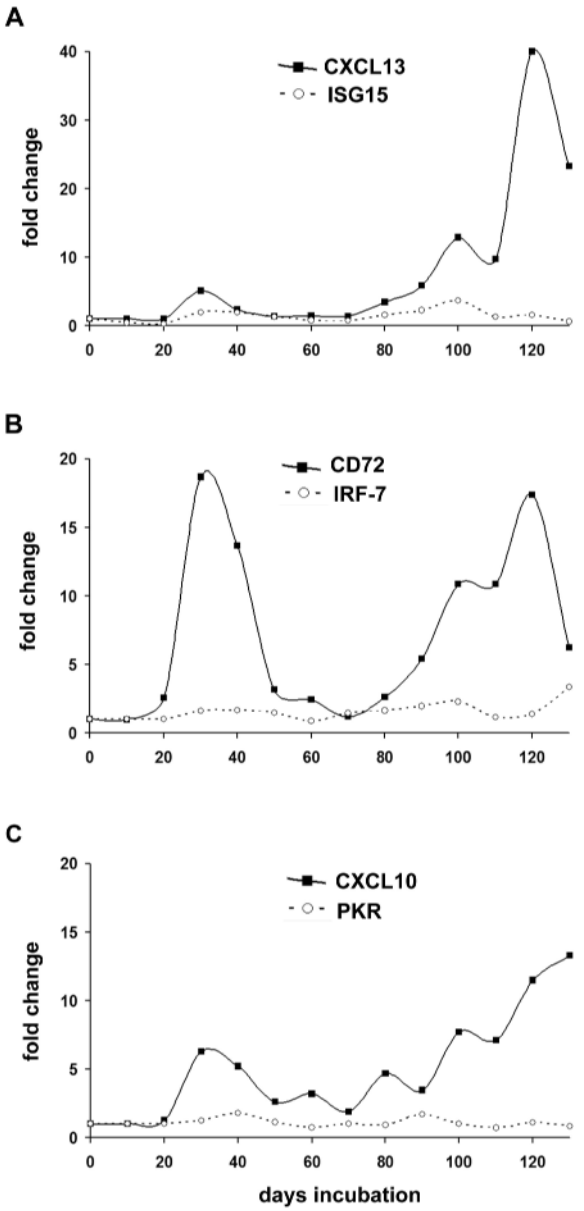

Figure 1 Quantitative plots of representative transcripts up-regulated in brain during the asymptomatic phase of CJD. Results are expressed in terms of fold change from normal brain after normalization based on GAPDH expression. CXCL13 exhibited the greatest increase of more than 40-fold at 120 days (**A**). Several transcripts displayed a biphasic pattern of induction (**B**), whereas others exhibited a more gradual increase (**C**). For comparison, each graph includes a transcript that remained relatively unaltered throughout the disease course (*open circles*). Note the different scale used in **A**.

Furthermore, mock-inoculated and uninoculated mice showed comparable expression profiles (data not shown). Therefore, the transcriptional response in CJD could not be correlated with, and did not reflect, a response to either trauma or the brain inoculum. For 7 of the 10 genes, these early increases subsided toward normal levels prior to a resurgence at the more terminal stages of disease (as in Figure 1B). In contrast, ImmResG1, and IFI202, and CXCL10 (shown in Figure 1C) exhibited a more continuous up-regulation throughout the asymptomatic CJD incubation period. These plots make it easier to see the greater than 16-fold changes documented for many transcripts in the cluster analysis (see below).

Cluster analysis summarizing transcriptional expression patterns in CJD brain

Similar temporal expression patterns among the transcripts were identified by hierarchical cluster analysis of reverse transcriptase–polymerase chain reaction (RT-PCR) results (Figure 2). Raw density measurements from films were first normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels to control for differences in RNA loading, and then represented in terms of fold difference relative to uninoculated mouse brain. To assess any correlation between interferon-sensitive gene up-regulation and astrocyte activation, we also analyzed the astrocytic marker glial fibrillary acidic protein (GFAP). Early up-regulation of GFAP in CJD brain revealed activated astrocytes that might contribute to changes in interferon-sensitive gene expression (Figure 2). However, many of the transcripts analyzed are undetectable in cultured astrocytes (Baker *et al*, 2002, and unpublished observations). On the other hand, all the mRNAs in the present study were detected in very pure microglia (see below), indicating these cells were the most likely source of up-regulated interferon-sensitive transcripts.

The cluster algorithm roughly divided the gene expression results into four groups (Figure 2B; clusters I to IV). Cluster I contained transcripts with a biphasic expression pattern in CJD brain. This biphasic response was inconsistent with evocation by PrP-res, because PrP-res was not detectable at these early time points even with high gel loads and ultrasensitive chemiluminescent Western blotting. Moreover, PrP-res has never been shown to decline in a similar fashion. Up-regulation of these genes was quite robust, with peak increases of >25-fold for CD84, LY86, and CXCL13. In contrast, cluster II defined three transcripts (exemplified by ImmResG1) exhibiting a more sustained increase in CJD brain. Induction of these genes was again profound, reaching peak increases of greater than 10-fold. Transcripts in cluster IV also exhibited the biphasic response pattern seen in cluster I, but with less intense peak increases of about five- to eightfold.

In contrast to the early and profound induction of the transcripts in clusters I, II, and IV, cluster III delineated a subset of interferon-sensitive genes with little or no up-regulation in CJD brain (Figure 2B). These mRNAs included ISG15, the transcription factors IRF-2 and IRF-7, the double-stranded RNA-dependent protein kinase (PKR), and IFNβ. Similarly, no isoforms of IFNα were detected in

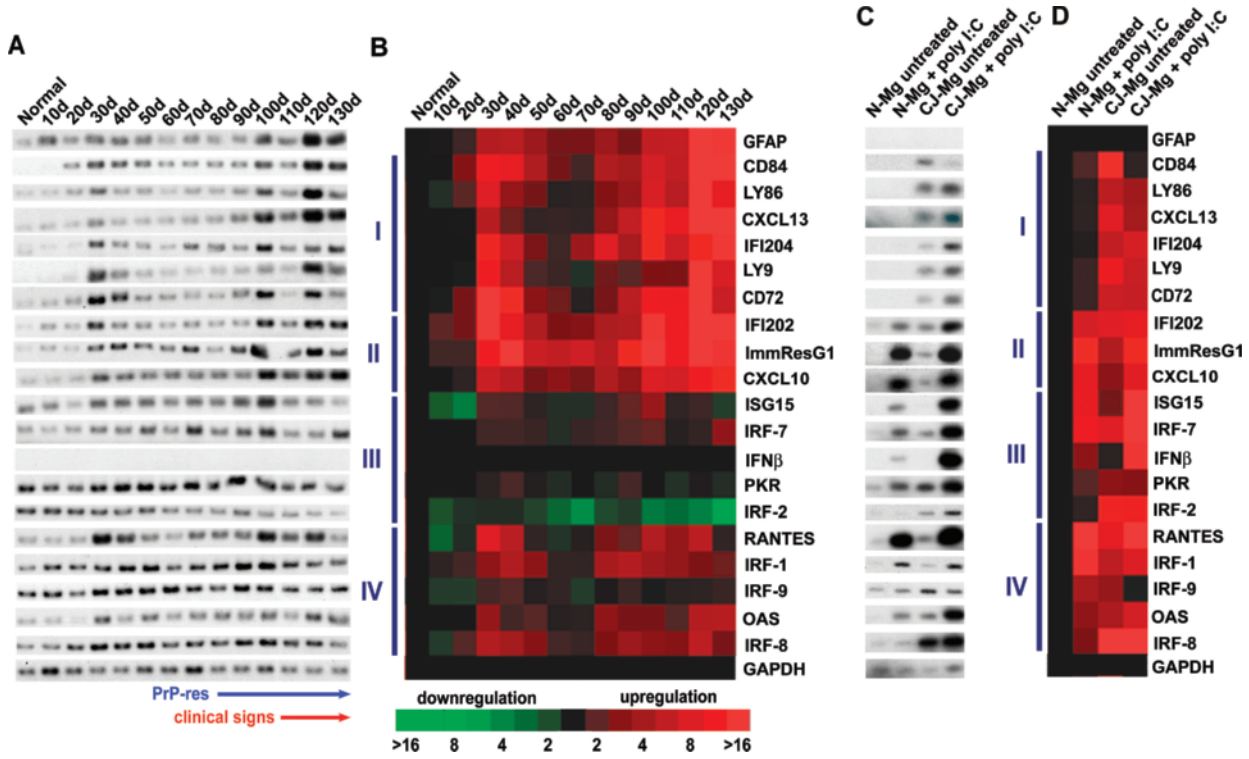

Figure 2 Early induction of innate immune responses in CJD brain at 10-day intervals, and in microglia. (**A**) Representative RT-PCR blots from normal and CJD brains. Brain accumulation of PrP-res during the course of disease is indicated by the blue arrow, and the appearance of clinical symptoms is indicated by the red arrow. (**B**) Quantitative cluster analysis of transcriptional responses in brain. Vertical bars with roman numerals delineate the four gene clusters so identified. The colorimetric scale indicates fold change (up-regulation or down-regulation) relative to control uninfected samples. (**C**) Blots representative of triplicate RT-PCR experiments in microglia from normal brain (N-Mg) or end-stage CJD brain (CJ-Mg), either with or without stimulation by the double-stranded RNA analog poly I:C. (**D**) Quantitative representation of microglial RT-PCR results, expressed relative to normal untreated microglia (leftmost column). Genes were grouped into the same four clusters defined in the brain analysis and use the same colorimetric scale. All results were normalized to GAPDH to control for differences in original RNA quantity between samples.

CJD brain (data not shown). These observations suggested the up-regulations we did observe were part of a specific response to CJD infection and not simply the result of general microglial activation. Furthermore, the absence of detectable interferon synthesis suggested the induction of interferon-sensitive genes in CJD brain occurred via an alternate mechanism. Thus, further analyses were needed to explore the up-regulation of interferon-sensitive transcripts and to differentiate CJD-specific responses from general inflammatory activation of microglial cells.

Interferon-sensitive gene expression in CJD brain is distinct from that of stimulated microglia

To determine whether the interferon-sensitive gene responses to CJD infection could be mimicked by a less-specific inflammatory signal, we stimulated cultures of normal microglia with the double-stranded RNA analog poly I:C. Extracellular poly I:C engages TLR3 at the cell surface, eventually leading to increased transcription of interferons and interferon-sensitive genes (Alexopoulou *et al*, 2001; Doyle *et al*, 2002). Microglia isolated from normal adult mouse brain responded to poly I:C with the induction of various interferon-sensitive transcripts (Figure 2C, compare first and second columns). Contaminating astrocytes are highly unlikely to be responsible for this effect, because GFAP transcripts were undetectable in the microglial cultures (Figure 2C, top row). When compared to the profiles of CJD brain, genes in clusters II and IV were sensitive to poly I:C stimulation in the microglial culture system. However, poly I:C treatment failed to induce any transcripts in cluster I. Combined with our previous demonstrations that these transcripts are unaffected by treatment of microglia with lipopolysaccharide (LPS) or IFNγ (Baker and Manuelidis, 2003; Baker *et al*, 2002), up-regulation of cluster I genes continues to be relatively specific for CJD infection. In particular, up-regulation of CD84 and LY9 may facilitate the spread of the CJD agent (see Discussion).

CJD microglia exhibit enhanced responsiveness to double-stranded RNA

Poly I:C–treated normal microglia also up-regulated most of the transcripts in cluster III, which were unaltered in CJD brain. To test the possibility that CJD infection prevented the induction of these transcripts,

microglia derived from CJD brain were also exposed to poly I:C. Even in the absence of stimuation, all the interferon-sensitive transcripts up-regulated in CJD brain were still increased in CJD microglia relative to cells from normal brains (Figure 2C, compare first and third columns). Stimulation of CJD microglia with poly I:C also up-regulated cluster III genes as well (Figure 2C, compare third and fourth columns), demonstrating that CJD infection did not prevent microglia from mounting an interferon response. Interestingly, CJD microglia exhibited a potentiated reaction to poly I:C stimulation for IFNβ, ISG15, OAS, and IFI202 (Figure 2C, compare second and fourth columns). For example, the poly I:C–induced up-regulation of IFNβ was 10-fold greater in CJD microglia relative to normal cells. These results indicated CJD microglia were primed for induction of interferon-sensitive genes by proinflammatory stimuli such as poly I:C. This primed state might occur through increased levels of the IRF transcription factors (Antalis *et al*, 1998; Sato *et al*, 2000). Consistent with this hypothesis, levels of IRF-1, -2, -7, -8, and -9 were elevated in CJD microglia compared to normal cells (Figure 2C).

Signal transduction pathways in CJD microglia

To examine the signal transduction pathways mediating the induction of interferon-sensitive genes in CJD brain, we again took advantage of our microglial culture system. In typical innate immune reactions, up-regulation of these transcripts is mediated in part by phosphorylation and nuclear translocation of mitogen-activated protein kinases (MAPKs) (Combs *et al*, 1999, 2001; Fiebich *et al*, 2002; Johnson *et al*, 2000; Navarro and David, 1999) or IRF-3 (Doyle *et al*, 2002; Lin *et al*, 1998; Sato *et al*, 2000; Wathelet *et al*, 1998). IRF-3 was not activated in CJD microglia (Figure 3A and data not shown) as determined by three different methods: hyperphosphorylation-induced shifts in IRF-3 mobility on Western blots (Servant *et al*, 2001), nuclear translocation by immunofluorescence (Ruvolo *et al*, 2003), and presence of IRF-3 protein in the nuclear fraction of microglial lysates (Doyle *et al*, 2002). These results were consistent with the lack of induction in CJD microglia of IFNβ and ISG15, which are known transcriptional targets of IRF-3. Similarly, CJD microglia exhibited no increase in the active phosphorylated forms of p44, p42, and p38 MAPKs relative to normal cells (Figure 3B and data not shown). These observations contrast with previous studies depicting MAPKs as important mediators of microglial responses to amyloidogenic proteins such as PrP (Combs *et al*, 1999). This should not be surprising, because the gene expression profile of normal microglia exposed to PrP amyloid is very different from that of microglia infected with the CJD agent (Baker and Manuelidis, 2003).

In contrast to these negative phosphorylation findings, we found increased activation of the STAT1

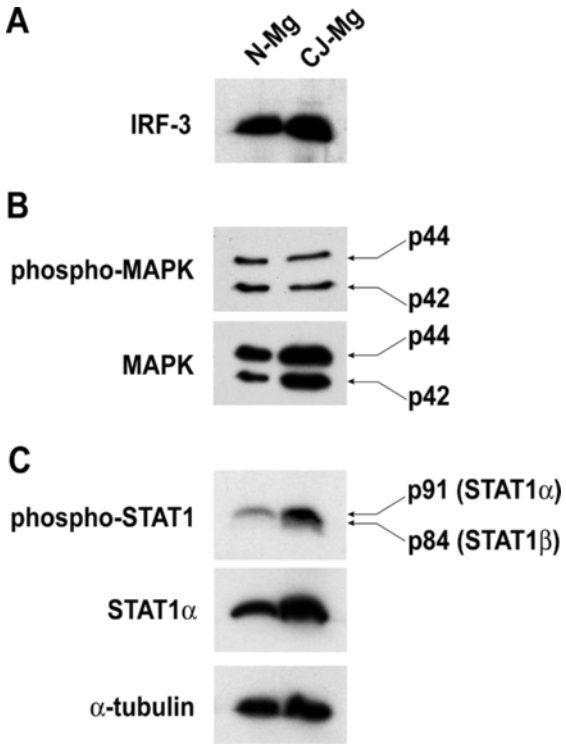

Figure 3 Activation of kinases and transcription factors in CJD microglia. Representative western blots for IRF-3 (**A**), total and phosphorylated forms of the p44 and p42 MAP kinases (**B**), as well as phosphorylated STAT1 and total STAT1α (**C**). The phospho-STAT1 antibody recognizes both the STAT1α (p91) and STAT1β (p84) forms of the molecule, which are generated by alternative splicing. Normalization to α-tubulin protein was used to control for differences in protein loading on the blots. Equivalent levels of total p44/p42 MAP kinases and STAT1α in normal and CJD microglia confirm that any changes in the phospho-specific forms of these molecules are indeed the result of differential phosphorylation between these cell populations.

transcription factor in CJD microglia. Activation of STAT family members occurs via tyrosine phosphorylation, after which they translocate to the nucleus along with IRF-9 to increase transcription of interferon-sensitive genes such as OAS and PKR (Nakaya *et al*, 2001). Using an antibody specific for phosphorylated STAT1, we found ~3-fold higher levels of the phosphorylated STAT1α isoform and ~10-fold higher levels of phosphorylated STAT1β in CJD microglia relative to normal cells (Figure 3C). This differential phosphorylation was probably not due to increased STAT1 availability, because amounts of STAT1α protein were less than twofold different between CJD and normal microglia when normalized to α-tubulin expression (Figure 3C). Furthermore, RT-PCR experiments showed mRNA levels for STAT1α and STAT1β were unaltered in CJD microglia (data not shown). Although the exact cause of this increased phosphorylation remains unknown, these results indicate activation of the STAT pathway in CJD-infected microglia. The presence of binding sites for the STAT1/STAT2/IRF-9 transcriptional activator complex in many of the up-regulated genes in CJD

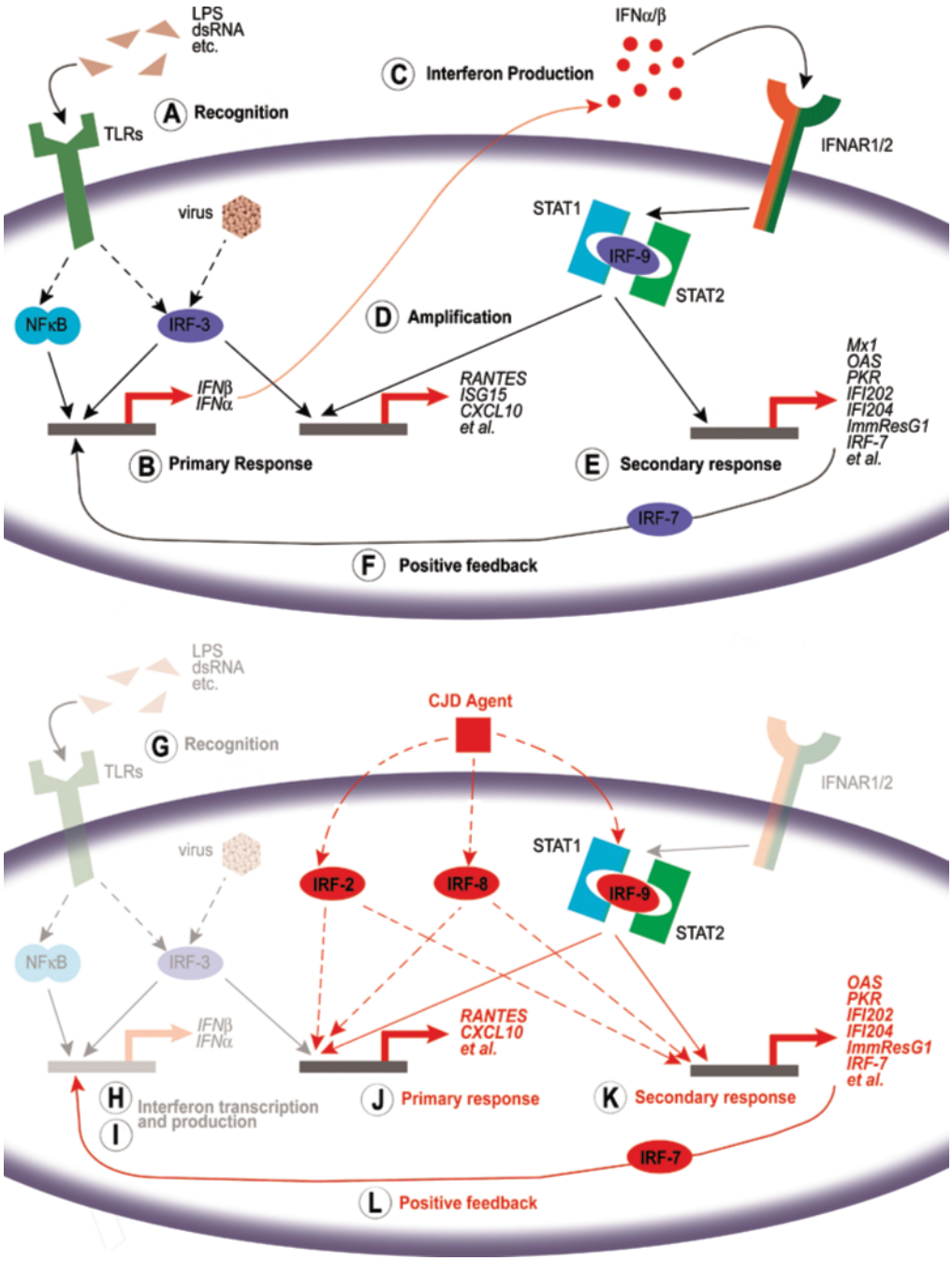

Figure 4 Comparison of typical innate immune signaling cascades (*top panel*) with those activated by CJD infection (*bottom panel*). The pathway diagram has been adapted from Taniguchi and Takaoka (2002). Dotted lines indicate interactions that are indirect or hypothetical. Typical innate immune responses are initiated by recognition of certain foreign protein motifs, mediated by toll-like receptors (TLRs) or other molecules (at A). This leads to the activation of NFκB and IRF-3 transcription factors, which induce the expression of interferons, RANTES, ISG15, CXCL10, and other genes as part of a primary response (at B). Newly synthesized interferons (at C), acting through the type I interferon receptor (IFNAR1/2), activate the STAT1/STAT2/IRF-9 transcription factor complex and instigate two major effects. First, this complex amplifies the expression of some primary response genes (at D). Second, this complex stimulates the expression of a distinct set of transcripts, termed secondary response genes (at E). One of these secondary response genes, the IRF-7 transcription factor, augments transcription of type I interferons and thus completes a positive feedback loop in the innate immune response (at F). In comparison to nonspecific stimuli, the CJD agent does not appear to follow the same recognition pathway (G) leading to increased interferon transcription (H) or synthesis (I). The CJD agent instead elevates certain primary (J) and secondary (K) response genes in an interferon-independent manner, which may involve IRF family transcription factors. The induction of IRF-7 in CJD microglia in principle should result in positive feedback onto the IFNβ promoter (L), priming the cells to exhibit a potentiated response to a challenge stimulus such as poly I:C.

microglia suggests STAT activation may mediate CJD-induced transcriptional changes in microglia.

Comparison of CJD-induced interferon-sensitive gene expression with the typical innate immune response

The robust up-regulation of interferon-sensitive genes in CJD brain at early stages of infection suggest host recognition of a foreign agent through pathways of innate immunity. Several observations further distinguished CJD-induced changes from typical innate immune responses: (i) no IFNα or IFNβ species were detected; (ii) no activation of IRF-3 or MAPKs was observed; and (iii) the CJD response was different from that elicited by poly I:C. In contrast, innate immune responses (outlined in top panel of Figure 4) are typically initiated by ligand binding to toll-like receptors or viral activation of the IRF-3 transcription factor (Taniguchi and Takaoka, 2002) (Figure 4, at A). Eventually this leads to increased transcription of several primary response genes, including chemokines and the antiviral cytokine IFNβ (Figure 4, at B). In turn, newly synthesized IFNβ (Figure 4, at C) signals via type I interferon receptors to activate the STAT1/STAT2/IRF-9 transcription factor complex. This complex then translocates to the nucleus and amplifies the transcription of some primary response genes such as RANTES (Figure 4, at D). Additionally, this STAT/IRF complex induces a distinct set of secondary response genes (Figure 4, at E) to produce an antiviral state. All of these processes are modulated by positive feedback via the transcription factor IRF-7, which further stimulates interferon transcription (Figure 4, at F).

The pathways activated during CJD infection differ from typical innate immune responses, as outlined in the bottom panel of Figure 4. Activation of TLRs and IRF-3 (Figure 4, at G), leading to production of type I interferons (Figure 4, at H and I), does not appear to be induced. However, mRNA levels for the primary response genes RANTES and CXCL10 were elevated (Figure 4, at J), as were those of various secondary response genes (Figure 4, at K). The exact mechanisms by which the CJD agent alters gene expression remain unknown, but may relate to microglial increases shown herefore IRF-2 and IRF-8. These two IRFs heve been linked to altered gene expression in other myeloid cells (Eklund *et al*, 1998; Marecki *et al*, 2001; Tamura *et al*, 2002). Increased phosphorylation of STAT1 isoforms in CJD microglia also suggests the CJD agent influences transcription, either directly or indirectly. The interactions of several viral proteins with IRF and STAT family members provides a precedent for a direct effect (Baca *et al*, 1994; Gotoh *et al*, 2003; Parisien *et al*, 2001; tenOever *et al*, 2002).

Another common denominator of innate immunity is the nuclear factor (NF) NFκB family of transcription factors, and NFκB activation has been reported in astrocytes from end-stage scrapie brain (Kim *et al*, 1999). We did not find two major consequences of NFκB activation in myeloid cells, the synthesis of type I interferons and the degradation of the inhibitory IκB subunit (Alexopoulou *et al*, 2001), in CJD microglia (Figure 2 and data not shown). Thus, NFκB may participate in astrocytic responses to the CJD agent, whereas STAT transcriptional activity probably underlies the CJD microglial response.

Discussion

We have shown specific up-regulated interferon pathways in CJD-infected microglia as well as in CJD brain at very early stages of infection. In both microglia and whole brain, this response included up-regulation of multiple interferon-sensitive genes without increased interferon synthesis. Using highly purified isolated CJD microglia, we were able to further demonstrate that CJD infection activated STAT1 transcription factors and potentiated induction of IFNβ. Furthermore, dramatic increases in interferon-related transcripts in brain occurred long before the onset of abnormal PrP accumulation and clinical disease. These transcriptional changes were robust, with initial increases of 5- to 10-fold, often reaching >30-fold during terminal disease.

A subset of interferon-sensitive genes displayed a biphasic expression pattern in CJD brain. Strong up-regulation of interferon-sensitive transcripts occurred between 30 and 40 days after inoculation, when agent titers begin to rise exponentially. The transcriptional response then tended to subside somewhat prior to a second wave of induction during the final ~35 days of the disease course, when agent titers plateau and the brain is degenerating (Manuelidis and Fritch, 1996). The early rise and fall of this transcript subset was incompatible with the typically progressive rise in PrP-res as the cause of these changes. This pattern suggests that the CJD agent evokes a transient but robust innate immune response that is attenuated during the intermediate phase of exponential agent replication. The biphasic pattern of interferon-related responses may signify the ability of the host to control infection initially, but not during the later phase of neurodegeneration.

In the typical innate immune response, recognition of a foreign agent leads to increased synthesis of interferons and chemokines. This primary response is followed by the induction of various secondary response genes whose transcription is usually dependent on intact interferon signaling (Doyle *et al*, 2002; Sato *et al*, 2000). We found various secondary response genes were up-regulated in CJD without detectable interferon production, a somewhat atypical situation. However, several viruses have been shown to up-regulate secondary response genes in an interferon-independent manner. For example, cytomegalovirus (CMV) infection induces IFI204 and OAS by a mechanism independent of protein

synthesis or interferon receptors (Boyle *et al*, 1999; Rolle *et al*, 2001). Furthermore, the Tat protein from human immunodeficiency virus (HIV) and the SM protein from Epstein-Barr virus (EBV) both up-regulate various interferon-sensitive genes without triggering interferon production (Izmailova *et al*, 2003; Ruvolo *et al*, 2003). Similar gene expression profiles elicted by CMV, human immunodeficiency virus (HIV), EBV, and the CJD agent likely reflect a host response program to a foreign infection that induces interferon-sensitive transcripts through an alternate pathway bypassing interferon signaling. This might explain why administration of anti-interferon antibodies has little effect on scrapie pathogenesis (Gresser *et al*, 1983). On the other hand, we cannot rule out low interferon production in CJD, because secondary response genes can be induced by very low levels of interferon signaling that are below the threshold of detection in common assays (Antalis *et al*, 1998; Belardelli *et al*, 1984; Sato *et al*, 2000). Although some of these early transcripts can also be elicited by tissue damage, the lack of pathology in CJD at 30 to 40 days favors the infectious agent itself as the instigator of these responses.

In our previous studies of microglia, only CD84 and CD48 showed induction by both PrP-res exposure and CJD infection (Baker and Manuelidis, 2003). CD84 influences cell interactions in the immune system (Martin *et al*, 2001) and is part of a larger protein family that plays a role in the pathogenesis of several viral infections (Schneider-Schaulies *et al*, 2001; Sidorenko and Clark, 2003). CJD brain also exhibited early up-regulation of LY9, another member of this protein family, and other family members can act as receptors for viral infection, and can also mediate the immunosuppressive effects of particular viruses. It is therefore possible that up-regulation of CD84 facilitates immune response evasion and spread of the CJD agent.

Because myeloid cells are long-lived and can traffic between the nervous system and various peripheral tissues, they may provide a vehicle for dissemination of the CJD agent (Manuelidis *et al*, 1997). Consistent with this hypothesis, multiple cell types of myeloid origin have been shown to tranmit CJD and scrapie infection (Aucouturier *et al*, 2001; Baker *et al*, 2002; Manuelidis *et al*, 2000). However, we cannot exclude the contribution of other cell types in agent spread. For example, the up-regulation of GFAP mRNA observed in the current study, as with other CJD models (Manuelidis *et al*, 1997), indicates an early astrocytic response to the CJD agent. Interestingly, early astrocyte responses to infection have been documented in a hamster CJD model that lacks profound microglial activation (Manuelidis and Fritch, 1996). Although the host species may be partially responsible for this blunted microglial response, particular CJD agent strain target specific cell types that are activated during the infection.

Are the interferon-linked responses evoked by the CJD agent detrimental or protective for the host? Early increased levels of chemoattractant molecules such as CXCL10, CXCL13, and RANTES could serve to recruit other cell types to areas of active infection. We have also found the same early induction of other chemoattractants in CJD brain (Baker *et al*, in preparation). This response may be exploited by the CJD agent to spread infectivity, as has recently been proposed for HIV (Izmailova *et al*, 2003). Although some of these chemoattractants can be produced by lymphocytes and other cells, our results with highly purified cells unequivocally demonstrate microglial expression of these molecules. The up-regulation of chemoattractants early in CJD can further mediate the recruitment of myeloid as well as lymphoid cells to the brain. The migration of T lymphocytes to the CNS has been documented with two different mouse scrapie strains (Betmouni *et al*, 1996; Lewicki *et al*, 2003). Chemoattraction and cell recruitment could also be important in the periphery, where both myeloid and lymphoid cells can carry and disseminate the CJD agent to other tissues such as peripheral nerves. In the spleen, agent spread can also involve follicular dendritic cells, which are a potential source for infection of lymphocytes and other transiting cells (Brown *et al*, 1999; Manuelidis *et al*, 2000).

The innate immune responses shown here could also be part of a host defense mechanism that is eventually subverted by the CJD agent. For example, many conventional infectious agents interfere with proteins involved in innate immunity (Burysek and Pitha, 2001; Gotoh *et al*, 2003; Parisien *et al*, 2001, 2002; Rodriguez *et al*, 2002; Xiang *et al*, 2002). It will be of interest to determine which pathways of the innate immune response are defensive, and which collaborate in agent propagation. Innate immune responses are likely key to the profound virus-like interference effects observed when an attenuated CJD strain suppresses subsequent infection by a virulent agent (Manuelidis, 1998; Manuelidis and Lu, 2000, 2003). The prevention of peripheral scrapie infection by unmethylated CpG DNA, an activator of innate immunity pathways, is also consistent with a defensive role of the innate immune system in these diseases (Sethi *et al*, 2002).

In summary, the robust responses to CJD infection suggest the CJD agent is recognized by the host as a foreign entity. This recognition may be mediated in part by direct interactions between the CJD agent and microglia myeloid. Coupled with the complex molecular expression profile previously documented in end-stage CJD microglia (Baker and Manuelidis, 2003), the specific pattern of interferon-related genes shown here may be used to diagnose infected myeloid cells throughout the body. The demonstration of specific genes induced in whole brain at very early stages of disease further suggests accessible peripheral tissues may similarly display

definitive markers of early infection. Few other studies have examined very early timepoints (less than 40 days after inoculation), and the transient nature of some of the mRNA changes shown here increases the likelihood that they may have been overlooked. The present studies provide an approach to preclinical diagnosis, including the use of easily obtained white blood cells that are infectious in both rodent and human CJD (Manuelidis *et al*, 1978, 1985).

Materials and methods

Brain and microglia sample preparation
CD-1 mice were intracerebrally inoculated with the FU strain of CJD (Manuelidis, 1998), using 30 μl of a 1% brain homogenate per mouse. Other animals inoculated with normal brain homogenate served to control for any nonspecific effects of brain inoculum. Brains were collected at 10-day intervals and homogenized in Trizol (Invitrogen, Carlsbad, CA) for RNA extraction according to the manufacturer's instructions. In addition, brains from mice with clinical signs of end-stage CJD were used for preparation of microglia as previously described (Baker *et al*, 2002). As a control, microglia were isolated from normal uninoculated mice in parallel with the CJD preparations. This method yields microglia of 95% purity, with no astrocyte contamination detectable by western blotting, immunofluorescence, or RT-PCR assays for the astrocytic marker GFAP. Microglia were cultured at 37°C and 5% CO_2 16 to 18 h in microglial medium (RPMI-1640 with 5% fetal bovine serum, 10 mM HEPES, 1 mM sodium pyruvate, 50 units/ml penicillin, 50 μg/ml streptomycin) either with or without 100 μg/ml poly I:C (Roche Molecular Biochemicals, Indianapolis, IN). Following this brief *in vitro* maintenance, cultures were quickly rinsed with microglial medium to remove any remaining debris before RNA extraction with Trizol or protein isolation (see below).

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. These optimal PCR conditions and primer sequences are listed in Table 1. PCR products were separated on agarose gels, transferred to Biodyne B nylon membranes (Pierce, Rockford, IL), 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 normalization to GAPDH to control for differences in starting RNA quantity. Results were expressed in terms of fold change relative to control samples (either normal brain or normal untreated microglia). To identify similar patterns of expression across the genes analyzed, the base 2 logarithms of these values were used for hierarchical clustering with Cluster and TreeView software (Eisen *et al*, 1998) (available from http://rana.lbl.gov/EisenSoftware.htm). The results from both microglia and brain were grouped together in the same cluster analysis and were weighted equally when computing

Table 1 PCR conditions

Gene	*Anneal (°C)*	*No. cycles (microglia)*	*No. cycles (brain)*	*Forward primer*	*Reverse primer*
CD72	72	24	28	CTGTCATCTGCCTGGGAGTTCGCTATCTGC	TGCCTCCACTTCTTGCTCATCTGTATCCA
CD84	61	21	26	ATGGGATTCTTGGGGAGTCAGT	TGGCAACACAGCATGGCGAGGAT
CXCL10	60	25	32	GCTGCAACTGCATCCATATCGA	TTGGCTAAACGCTTTCATTAAATTC
CXCL13	60	25	31	TCAGCACAGCAACGCTGCTTCT	CTGGAGCTTGGGGAGTTGAAGA
GAPDH	60	20	20	GACCTCAACTACATGGTCTACAT	TGGTTCACACCCATCACAAACAT
GFAP	64	28	25	GAGGGCCAAAGCCTCAAGGAGGAGA	CTTTACCACGATGTTCCTCTTGAGGTG
IFI202	63	22	28	ATGAAACTGCCACTGTGTCAGAGGC	TCTGAAGAATTCTGTATCAGTAGCCAC
IFI204	63	22	28	CATGATGGAAGAGAAATTTCCAGC	TGCAGTGAGCACCATCACTGT
IFNβ	54	24	31	CAGCCTGGCTTCCATCATGAAC	GCAGTAGATTCACTACCAGT
ImmResG1	69	23	31	ATATGCTGCTTTTGTTTAATGGTGTTGC	AAGGTCTTCGGGGGAGTAGTTGG
IRF-1	65	22	25	ATGCGGATGAGACCCTGGCTAG	TCTGGTTCCTCTTTGCAGCTGAAG
IRF-2	65	25	21	TCCAATACGATACCAGGGCTAAAGTG	GGTGACCTGCAGATCTGGCTTG
IRF-3	55	22	22	GATCCTTCTCAACAACTGCCAA	GTAGGCCTTGTACTGGTCAGA
IRF-7	63	22	26	CGCTCCCGCTGCATCTGTAGT	GCCGCTGCTGACCTCCCCCAATAG
IRF-8	68	28	24	AAAAGGGTCTCTGGTGTGAGGTAC	AGAGATTGAGGAGCTGATCAAGGAAC
IRF-9	65	21	25	AGACTTCCGAGAGACCAGGATG	CAGAAGTAGGTGGTCTTGAAGAGCT
ISG15	56	23	27	GAAGCAGACTCCTTAATTCC	CCAATGCTATCCCAAAAGTC
LY86	68	23	23	GAAAATGGTTGGCCCAAGCACACG	TGGCATTGGCACAAGCCACAGTAGC
LY9	72	26	27	AGGGATGCTAGGGGGTTCTGTGACTTTCTC	GGATTCCAGGCTTTGCAGGTATAGGGTAGG
OAS	67	22	27	GCGCCCAACCAAGCTGAAGAGT	GAACCACCGTCGGCACATCC
PKR	67	26	28	GTACAAGCGCTGGCAGAACTCAAT	GTACAAGCGCTGGCAGAACTCAAT
RANTES	56	23	27	GGGTACCATGAAGATCTCTG	GAGTAGGGGATTACTGAGTG
STAT1	55	22	23	CATACGGAAAAGCAAGCGTA	CTGTCGTTCTACCACGAAGGA

the correlations within the genes analyzed. Depicting changes graphically with a logarithmic scale was useful for the simultaneous display of mild (2- to 4-fold) and robust (10-fold) changes in gene expression, but tended to underemphasize the large changes of 10- to 40-fold seen for many genes.

Western blotting

Normal or CJD microglia were collected directly in 50 μl of lysis buffer (10 mM KCl, 10 mM HEPES, 0.1 M EDTA, 0.5% NP-40, 0.5 mg/ml dithiothreitol [DTT], 2 μg/ml leupeptin, 2 μg/ml pepstatin, 1 mM phenylmethylsulfonyl fluoride [PMSF]) per 3×10^6 cells. Genomic DNA was sheared by several passages through a 26-gauge needle. Samples were mixed with an equal volume of loading buffer (0.3 M Tris pH 6.8, 1.5 M β-mercaptoethanol, 20% glycerol, 5% sodium dodecyl sulfate [SDS], 0.2 mg/ml bromophenol blue), and boiled for 5 min prior to loading 40 μl per lane on 13% polyacrylamide gels. Electrophoresis and Western blotting were conducted according to established protocols (Manuelidis and Fritch, 1996). Solutions for blocking and antibody incubation were 1× Tris-buffered saline (TBS)/0.1% Tween-20/1% nonfat dry milk/50 mM NaF (for phospho-specific antibodies) or 1× TBS/0.1% Tween-20/5% nonfat dry milk (for other antibodies). Rabbit antibodies against p44/p42 MAPK (1:2000), phospho-p44/p42 MAPK (1:2000), and phospho-p38 (1:2000) were purchased from Promega (Madison, WI). Mouse antibodies against STAT1α (1:500) and phospho-STAT1 (1:500) were purchased from Zymed (South San Francisco, CA), as was a rabbit antibody against IRF-3 (1:500). Mouse anti–α-tubulin (1:2000) was purchased from Sigma (St. Louis, MO).

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