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RESEARCH ARTICLE

Transcriptional Regulation of Caspases in Experimental Pneumococcal Meningitis

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Apoptosis and necrosis in brain account for neurological sequelae in survivors of bacterial meningitis. In meningitis, several mechanisms may trigger death pathways leading to activation of transcription factors regulating caspases mRNA synthesis. Therefore, we used a multiprobe RNA protection assay (RPA) to examine the expression of 9 caspase-mRNA in the course of experimental *Streptococcus pneumoniae* meningitis in mouse brain. Caspase-6, -7 and -11 mRNA were elevated 6 hours after infection. 12 hours after infection caspases-1, -2, -8 and -12 mRNA rose. Caspase-14 mRNA was elevated 18 h and caspase-3 mRNA 24 h after infection. *In situ* hybridization detected caspases-3, -8, -11 and -12 mRNA in neurons of the hippocampal formation and neocortex. Development of sepsis was paralleled by increased transcription of caspases mRNA in the spleen.

In TNFα-deficient mice all caspases examined were less upregulated, in TNF-receptor 1/2 knockout mice caspases-1, -2, -7, -11 and -14 mRNA were increased compared to infected control animals. In caspase-1 deficient mice, caspases-11, and -12 mRNA levels did not rise in meningitis indicating the necessity of caspase-1 activating these caspases. Hippocampal formations of newborn mice incubated with heat-inactivated *S. pneumoniae R6* showed upregulation of caspase-1, -3, -11 and -12 mRNA.

These observations suggest a tightly regulated caspases network at the transcriptional level in addition to the known cascade at the protein level.

Introduction

Bacterial meningitis continues to be associated with a high mortality and a high incidence of neurological sequelae including cognitive impairment in survivors (6, 9). Especially in children one-third of survivors develop learning disabilities and memory loss (10, 52).

The mechanisms that lead to neuronal injury in meningitis are not fully understood. In a rabbit model of *S. pneumoniae* infection, neuronal apoptosis is consistently present in the dentate gyrus of the hippocampal formation 24 h after infection (64). Neuronal apoptosis in the dentate gyrus is also regularly observed in infant rat models of *Streptococcus* group B, *S. pneumoniae* and *Listeria monocytogenes* meningitis (19, 24, 31). In mice with experimental *S. pneumoniae* meningitis, necrotic and apoptotic injury occurs in the Formatio hippocampi and neocortex. The neuronal damage found is similar to the lesions observed in human meningitis (34) and ranges from necrosis to apoptosis.

Apoptosis requires tightly regulated death pathways and gene transcription, including activation of cysteine proteases of the caspase family (44, 47, 50). Caspases are synthesized as zymogens and remain inactive in most healthy cells. Upon activation they are cleaved at specific aspartic acid residues to generate two distinct subunits that assemble into a heterotetramer to form the active protease.

Cleavage experiments on tetrapeptides divide these enzymes into three major groups (53): Group I (mediators of inflammation: caspases-1, -4, -5, -11, -12, -14), group II (effectors of apoptosis: caspases-2, -3, -7), group III (activators of apoptosis: caspases-6, -8, -9, -10). Caspases play an important role in development as well as in pathological processes such as stroke (16), epilepsy (2), inflammatory diseases and cytokine maturation (17, 58, 60, 64).

Caspases can be activated by *e.g.* lipopolysaccarides (63), TNFα (5), cytochrome c in mitochondrial damage (47), receptor activation (*e.g.* CD95/Fas/APO-1, TNF-receptor, TRAIL-R1/DR4)(12, 51), or by granzyme B (liberated by natural killer cells and T-cells) and direct

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cleavage of apical caspases (62). Diverse stimuli such as drugs, radiation, and heat shock seem to converge on the mitochondria/cytochrome c pathway (20).

Caspases appear to be involved in both the effector phase of apoptosis and in at least some of the upstream signaling pathways, where they become activated and, in turn, activate the caspases involved in the effector phase. Strong evidence for the crucial role of caspases in bacterial meningitis is the observation that a pan-caspase inhibitor reduced the neuronal apoptosis in rabbit *S. pneumoniae* meningitis (8).

Caspases operate in a network-like fashion, in which initial activation of one caspase can lead to activation of multiple other family members, resulting in cleavage of many different substrates and integrating the different apoptotic stimuli to one pathway (55). The role of a particular caspase within the activation cascade is likely to be partially context-dependent. Caspases that are apical in the context of certain pro-apoptotic stimuli may participate in amplification in other conditions (48).

Transcriptional events may contribute to de novo synthesis supplementing the pool of pre-existing procaspases (46). In support of this concept, transient global brain ischemia induces the caspase-3 mRNA expression (35).

Expression of the transcriptional factors c-jun and c-fos, their phosphorylation, and formation of the AP-1 factor appear to be essential for neuronal apoptosis (36) as they, as well as nuclear factor-κB (32), coordinate gene expression. The activation of these transcriptional factors can be induced by NMDA, glutamate, and other excitotoxic amino acids (13) as well as by TNFα or Fas-ligand via Fas-associated death domain protein (FADD), TNF-receptor-associated death domain protein (TRADD) or fas-binding-protein Daxx (1, 35).

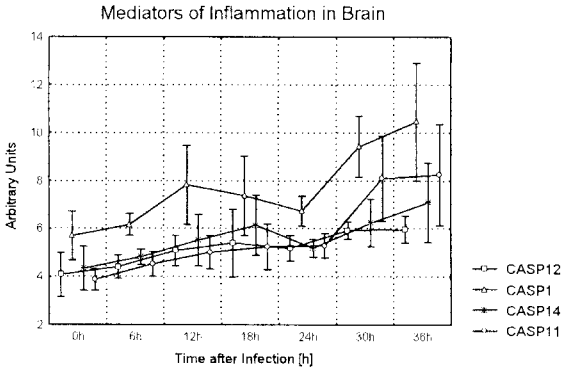
In the present study, the expression patterns of caspases-1, -2, -3, -6, -7, -8, -11, -12, -14 mRNA were examined with a multiprobe RNA protection assay in experimental *S. pneumoniae* meningitis in wild-type mice and knock-out-mice for caspase-1 (ICE), TNFα, and TNF-receptor1/2 to assess whether mRNA transcription contributes to caspase activation in meningitis and to evaluate regulatory mechanisms.

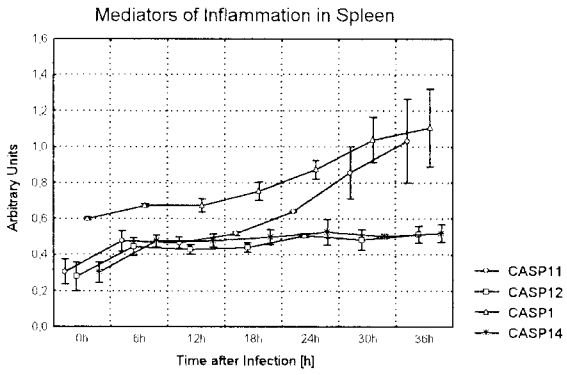
Methods

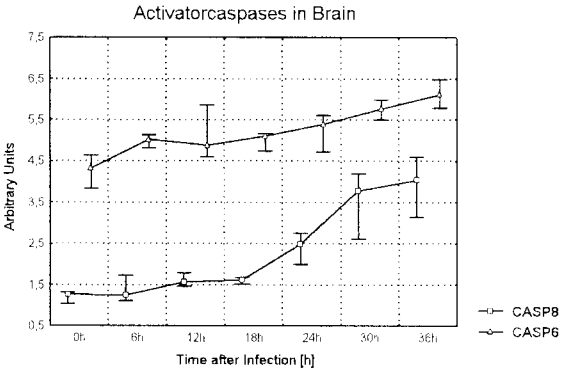
Mice. Knock-out mice were kindly provided by: BASF Res. Corp, Worcester, USA (caspase-1 (ICE) -/- and heterozygous controls (C57BL/6 background) (27)), Hellenic Pasteur Institute, Athens, Greece (TNFα -/-, C57BL/6 background (37)), Hoffmann-La Roche, Basel, Switzerland; (TNF-Receptor1/2 -/- and heterozygous controls (C57BL/6 × 129/Ola background) [11, 42]). NMRI and C57BL/6 wild-type mice were bred at the animal care facilities of the Max-Planck-Institute for Biophysical Chemistry and University Hospital Goettingen.

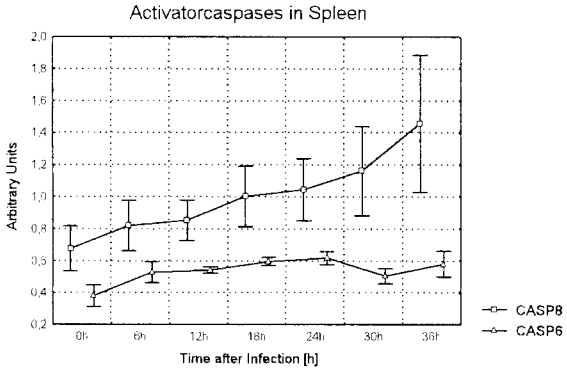
Mouse Meningitis Model. Mice (2-3 months old) with free access to food and drinking water were anaesthetized with ketamine 100 mg/kg and xylazine 20 mg/kg and infected by injecting approximately 10^4 colony-forming units (cfu) of a *S. pneumoniae* type 3 strain (MIC/ MBC: 0.03/ 0.06 μg/ml for ceftriaxone) into the right forebrain (i.c.). The health status of the mice was assessed by weighing and observation of spontaneous activity. Animals (n=3) were killed by decapitation 6, 12, 18, 24, and 36 hours after infection, and blood was sampled. Then, the whole brain and spleen were removed. The cerebellum and one half of the spleen were homogenized in saline. Serial dilutions of blood, cerebellum and spleen homogenates were plated on blood agar plates. C57BL/6 mice injected once with 25 μl NaCl i.c. served as 0 hours control animals. Tissues for *in situ* hybridization and immunohistochemistry were fixed in 4% formalin for 48 h and then embedded in paraffin, or frozen in liquid nitrogen for RNA preparation. The animal experiments were approved by the District Government Braunschweig, Germany.

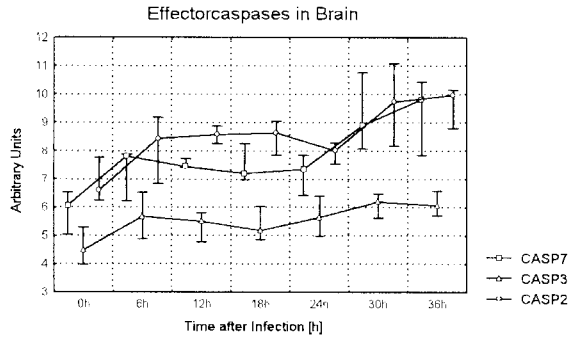
Organotypical Hippocampal Cultures (OTC)(14). 5 to 7 day-old NMRI mice were decapitated; the hippocampal formations were prepared under sterile conditions, and cut transversely with a McIlwain tissue chopper into slices of 400 μm thickness. Slices were kept in Grey's balanced salt solution (GBSS) supplemented with 36 mM D-glucose at 4°C for 30 min. Subsequently, slices were embedded in plasma clots on glass coverslips, which were then coagulated by the addition of thrombin. Coverslips were transferred to plastic culture tubes containing 750 μl culture medium composed of 50% Eagles' basal medium (BME), 25% Hanks' balanced salt solution (HBSS), 25% heat-inactivated horse serum supplemented with glutamine (1 mM), and D-glucose (36 mM). Culture tubes were placed in a roller device rotating at 10 revolutions/h in an air-ventilated incubator at 36°C. The medium was changed twice and after 7 days half of the cultures were exposed to heat-inactivated *S. pneumoniae* R6 (hiR6) for 24 h the other half served as controls. For RNA isolation 20 slices of hiR6 exposed hippocampi or untreated negative controls were pooled.

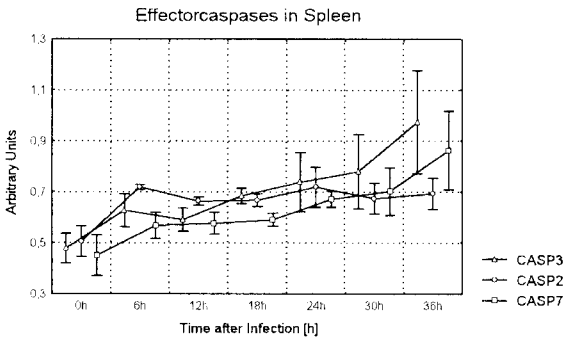

Mediators of Inflammation in Brain
Arbitrary Units
Time after Infection [h]
CASP12
CASP1
CASP14
CASP11

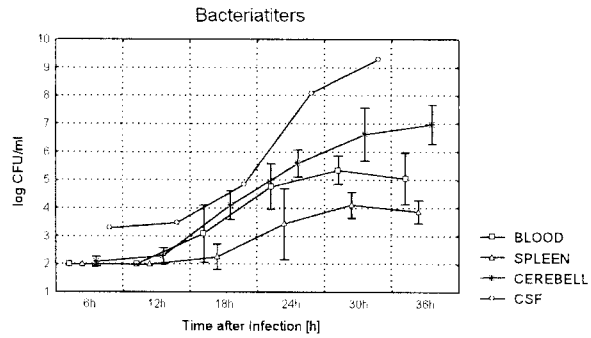

Mediators of Inflammation in Spleen
Arbitrary Units
Time after Infection [h]
CASP11
CASP12
CASP1
CASP14

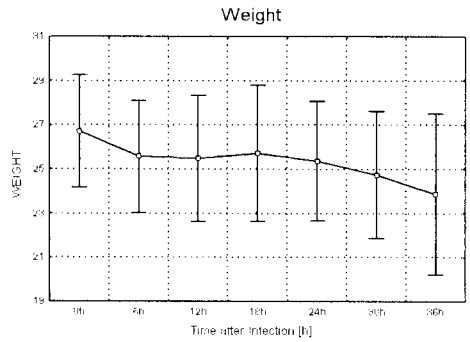

Activatorcaspases in Brain
Arbitrary Units
Time after Infection [h]
CASP8
CASP6


Activatorcaspases in Spleen
Arbitrary Units
Time after Infection [h]
CASP8
CASP6


Effectorcaspases in Brain
Arbitrary Units
Time after Infection [h]
CASP7
CASP3
CASP2


Effectorcaspases in Spleen
Arbitrary Units
Time after Infection [h]
CASP3
CASP2
CASP7


Bacteriatiters
log CFU/ml
Time after Infection [h]
BLOOD
SPLEEN
CEREBELL
CSF


Weight
WEIGHT
Time after Infection [h]

hiR6 preparation: The non-encapsulated apathogenic *S. pneumoniae* strain R6 was grown overnight at 37°C in tryptic soy broth (TSB) to mid-logarithmic phase. Cells were harvested by centrifugation, washed with sterile saline (0.9% NaCl), and then resuspended in 1 ml saline. The tubes were heated in a water bath (80°C) for 20 min to kill the bacteria and to inactivate the autolysin. The heat-inactivated pneumococci (hiR6) were used at concentration equivalents of 10^8 cfu/ml.

Propidium iodide (PI) staining: The vital dye PI was used to determine cell membrane injury *in vitro*. At the end of the treatment period, the medium was removed and replaced by medium containing PI (25 μg/ml). Cultures were then incubated for 30-60 min at 36°C in the roller tube apparatus. Thereafter, cultures were removed, examined by fluorescence microscopy and photographed (Zeiss Axiophot, Oberkochen, Germany; Kodak Elite 400 ASA) with a ×2.5 objective (Zeiss, Oberkochen, Germany). With loss of cell membrane integrity PI enters the cell and binds to DNA. PI fluorescence is therefore related to necrotic or late apoptotic cell damage.

Annexin V staining: In the normal cell, phosphatidyl-serine is located on the inner part of the plasma membrane. During the early stage of apoptosis, phosphatidyl-serine is translocated to the cellular surface and can be labeled by Annexin V that binds preferentially to phosphatidyl-serine (57). We used a fluorochrome-labeled Annexin V kit (R&D systems, Abingdon, UK). In contrast to the proposed protocol adapted to cell cultures, we used the tenfold concentration of Annexin V dissolved directly in the culture medium. The cultures were set back into the roller tube apparatus for one hour and examined by fluorescence microscopy.

Nissl staining: Cultures for Nissl staining were kept in 4% buffered formaldehyde at 4°C. The morphology of cultures was determined qualitatively following staining with cresyl violet. Fixated cultures were shortly washed in 0.1 M phosphate-buffered Triton (0.5%), then in distilled water. Subsequently, they were submerged in 0.5% buffered cresyl violet for 2-3 min. Stained cultures were differentiated in 0.5% and 1% acetic acid until a homogeneous staining of the whole tissue was achieved and then washed in distilled water.

Multiplex S1-RNA Protection Assay (RPA). RNA was isolated from brain tissue (left and right cerebral hemisphere) or OTC slices with Trizol-Reagent (Gibco/BRL-LifeTechnologies, Karlsruhe, Germany) according to the manufacturers' protocol. Integrity was confirmed by agarose gel electrophoresis and the RNA quantitated by optical density measurement (260 nm).

For *in vitro* transcription the multiprobe RNA protection assay (RPA) (Pharmingen, San Diego, USA, mAPO-1, #45358P) was used following the manufacturers' instructions (T7 polymerase: Promega, Heidelberg, Germany; ^{32}P UTP (20 μCi/μl) from Amersham Buchler, Braunschweig, Germany). The radioactively labeled cRNAs were resuspended in a phosphate buffer (0.05 M HPO_4^{2-}/ $H_2PO_4^-$, 0.7% sodiumdodecylsulfate (SDS), 0.1 mM EDTA and 0.1% BSA fraction V to a final concentration of 350.000 cpm/μl. The probe was diluted 1:10 with hybridization buffer (150 μl 0.5 M PIPES pH 6.8, 120 μl 5 M NaCl, 6 μl 0.5 M EDTA and 624 μl H_2O).

20 μg of total RNA or yeast tRNA (as negative controls, Boehringer, Mannheim, Germany) were dissolved in 20 μl H_2O, and then 10 μl of hybridization buffer including the labeled cRNAs (35.000 cpm/μl) were added to each RNA tube. The hybridization mix was gently vortexed, centrifuged and two drops of mineral oil (Sigma) were added to each sample to avoid evaporation during the hybridization. The tubes were heated to 100°C for three minutes and then immediately transferred to the incubator; hybridization was performed at 80°C overnight. 470 μl of digestion buffer (per sample: 5 μl 5 M NaCl, 5 μl 3 M Na-acetate pH 4.6, 5 μl herring sperm DNA (10 μg/μl)(Type XIV, Sigma, Deisenhofen, Germany), 20 μl 0.1 M $ZnSO_4$, 434 μl H_2O and 350 U S1 nuclease (Pharmacia, Freiburg, Germany) were added to each sample. The tubes were gently vortexed and centrifuged. Digestion was performed in a water bath for 60 min at 37°C. The reaction was stopped by adding 100 μl 4 M ammonium acetate/0.1 M EDTA.

Figure 1. (Opposing page) Caspase mRNA levels in the course of meningitis in brain and spleen (means ± SD). Caspases are grouped into mediators of inflammation, activator and effector caspases. The densitometric measurements of signals from the RNA protection assays are expressed in arbitrary units in control-animals (0h) and animals 6, 12, 18, 24, 30 and 36 hours after i.c. infection. Different patterns of increased mRNA during meningitis were found in brain and spleen paralleled by increasing bacterial titers in cerebellum, CSF, spleen, and blood and worsening of the clinical condition as indicated by weight loss. Caspases in brain: significantly increased after 6 h: caspases-6, -7 and –11 ($p<0.05$); after 12 h: caspases-1, -2, -6, -7, -8, -11, -12 ($p<0.05$); after 18 h: caspases-1, -2, -6, -7, -8, -11, -12, -14 ($p<0.05$); after 24 h: caspases-3, -6, -7, -8, -11, -12, -14 ($p<0.05$); 36h: all 9 caspases examined ($p<0.05$). Caspases in spleen: significantly increased after 6h: caspases-1, -2, -3, -6, -11, -12, -14 ($p<0.05$); after 18 h: all 9 caspases examined ($p<0.05$).

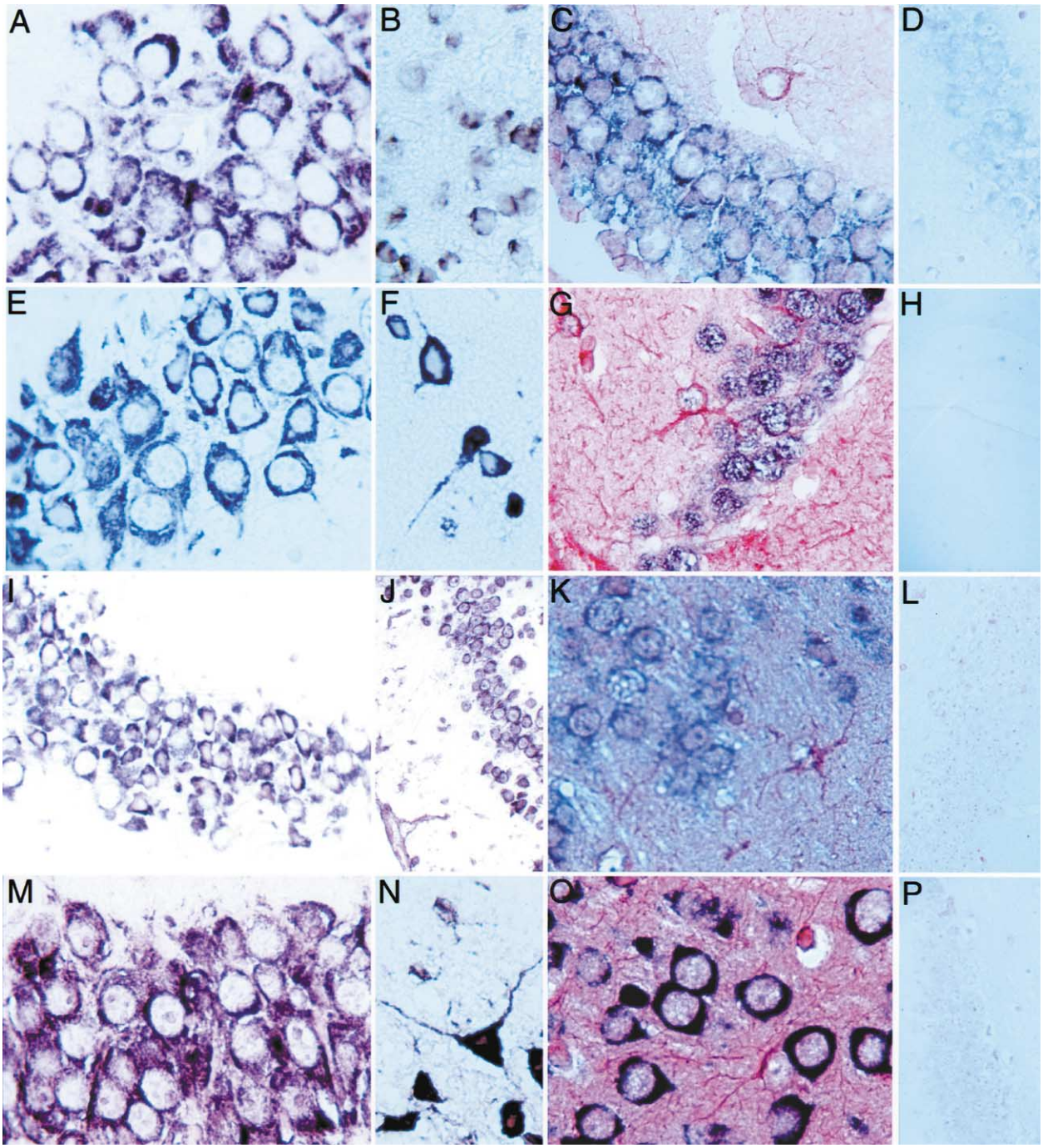


Figure 2. *In situ*-hybridization of adult C57BL/6 mouse brain 36 h after intracerebral infection with caspases-3 (**A**-**D**) as an effector-caspase, -8 (**E**-**H**) as an activator-caspase, -11 (**I**-**L**) and -12 (**M**-**P**) as mediators of inflammation. Positive staining of the caspases was found in neurons of the hippocampal formation (**A**, **E**, **I**, **M**) and neo-cortex (**B**, **F**, **J**, **N**). In GFAP immunohistochemistry and caspase *in situ* hybridization double-stained slices astrocytes did not express any caspases -3, -8, -11, -12 mRNA(**C**, **G**, **K**, **O**). **D**, **H**, **L**, **P** are hybridized with sense-transcripts as negative controls counter-stained with nuclear fast red. (**A**-**C**, **E**-**G**, **K**, **M**-**O** ×100; **D**, **H**-**J**, **L**, **P** ×40)

Each sample was precipitated with 700 µl of isopropanol containing 1 µl dextran blue (20 µg/µl) per sample and 20 µg yeast tRNA and washed with 500 µl of 70% ethanol. The addition of dextran blue to the isopropanol resulted in a solid blue-colored pellet, which minimized the loss of pellets during the precipitation step. The samples were resuspended in 5 µl TE (10mM Tris, 1 mM EDTA, pH 8) plus 4 µl loading buffer (80% formamide; 10 mM EDTA pH 8; 1 mg/ml xylene cyanol and 1 mg/ml bromphenol blue). Samples were heated for 3 min to 90°C and kept on ice for another 3 min. Thereafter 2.5 µl aliquots were loaded on denaturing acrylamide gels (7% acrylamide/ 8 M urea). Autoradiography was performed on X-ray films with intensifying screens at -80°C for varying time spans. Autoradiographs were scanned and RNA bands were quantitated by densitometric analysis with NIH Image 1.62 (National Institute of Health, Bethesda, Maryland, USA), and results were normalized for L32 and GAPDH gene expression.

In situ hybridization, in situ tailing (IST) and immunohistochemistry. *In situ* cDNA templates were amplified and cloned in pCRII-TOPO (Invitrogen, Groningen, Netherlands) as follows: caspase-3: 325 bp insert (GenBank Y13086, nt 411-735), caspase-8: 405 bp insert (GenBank AF007749, nt 751-1157), caspase-11: 253bp insert (GenBank Y13089, nt 893-1145), caspase-12: 226 bp insert (GenBank NM009808, nt 1050-1275). The sequences were flanked by SP6/T7-Promotors for *in vitro* transcription according to the manufacturers' protocol (Boehringer Mannheim, Germany). Immunohistochemistry, *in situ* tailing and *in situ* hybridization were performed on 1-2 µm thick sections of paraffin-embedded tissues mounted on poly-L-lysine-coated glass slides. The sections were deparaffinised in xylene (two times 15 min) and hydrated in descending ethanol concentrations.

For *in situ* hybridization sections were incubated in 4% paraformaldehyde for 20 min at 4°C and washed 3 times in TBS. After incubation in 0.2 M HCl for 10 min at room temperature (RT) and washing again 3 times in TBS, sections were incubated in 0.5% acetic anhydride (in 100 mM TRIS pH 8) for 10 min at RT with gentle shaking and washed 3 times in TBS. Then sections were treated with proteinase K (50 µg/ml in TBS containing 2 mM $CaCl_2$) for 20 min at 37°C in a water-saturated atmosphere. After washing the sections 3 times in TBS at RT and once in ice-cold TBS they were dehydrated with ascending ethanol concentrations. Hybridization was performed overnight at 60°C in a water-saturated atmosphere in a solution containing 2-fold SSC, 50% deionised formamide, 10% dextran sulfate, 0.01% salmon sperm DNA, and 0.02% SDS, as well as either the sense or antisense digoxigenin-labelled cRNA probe specific for mouse caspases-3, -8, -11 and -12. Sections were then washed twice in wash buffer I (1-fold SSC containing 0.1% SDS) for 10 min at 60°C and twice in wash buffer II (0.2-fold SSC containing 0.1% SDS) for 10 min at 60°C. Before incubation of the sections with the alkaline phosphatase-labeled anti-digoxigenin antibody (AP-Fab) (diluted 1:250 in Boehringer blocking reagent containing 10% FCS and 3% mouse serum) for 2 h at RT in a water-saturated atmosphere, unspecific binding of the Fab fragments was blocked by treating the sections with blocking reagent containing no Fab fragments for 15 min at RT in a water-saturated atmosphere. Then, sections were washed 5 times in TBS at RT and incubated in 4-nitroblue-tetrazolium-chloride/5-bromine-4-chloride-3-indolyl-phosphate (NBT/BCIP) chromogenic substrate up to 24 h in a dark chamber at 4°C. Slices were repeatedly controlled by microscopy until the specific dark-violet signals were visible. Finally, control sections were counterstained with nuclear fast red (0.1% in 5% aluminium sulfate) for 5 min at RT resulting in a light-red background and dark-red nuclei.

For *in situ* tailing (IST) the sections were treated with 50 µg/ml proteinase K (Sigma, Deisenhofen, Germany) for 15 min at 37°C. The sections were incubated for 1 h at 37°C in a reaction mixture containing 10 µl of 5x tailing buffer, 1 µl digoxigenin DNA labeling mix, 2 µl cobalt chloride, 12.5 U terminal transferase (all reagents from Boehringer, Mannheim, Germany) and the necessary amount of distilled water to give a volume of 50 µl. After washing, the sections were incubated with 10% FCS for 15 min at RT and then washed again. A solution of anti-digoxigenin AP-Fab in 10% FCS (1:250) was placed on the sections for 60 min at 37°C. The color reaction was developed with NBT/BCIP. The sections were counterstained with nuclear fast red-aluminiumhydroxide.

For immunohistochemistry sections were heated 5 times in a microwave for 3 min in citrate buffer (10 mM citric acid monohydrate pH 6.0) and incubated for 20 min in 10% FCS in TBS. Sections were then incubated with the polyclonal rabbit antibody against Glial Fibrillary Acidic Protein (GFAP, DAKO #Z0334) at a dilution of 1:50 in TBS (25 mM Tris and 150 mM NaCl pH 7.5) or CM1 (acitvated caspase-3) for 2 h at RT in a water-saturated atmosphere. The sections were then incubated with mouse anti-rabbit immunoglobulins (diluted 1:40 in TBS, DAKO #0737) for 30 min, with rabbit-anti-

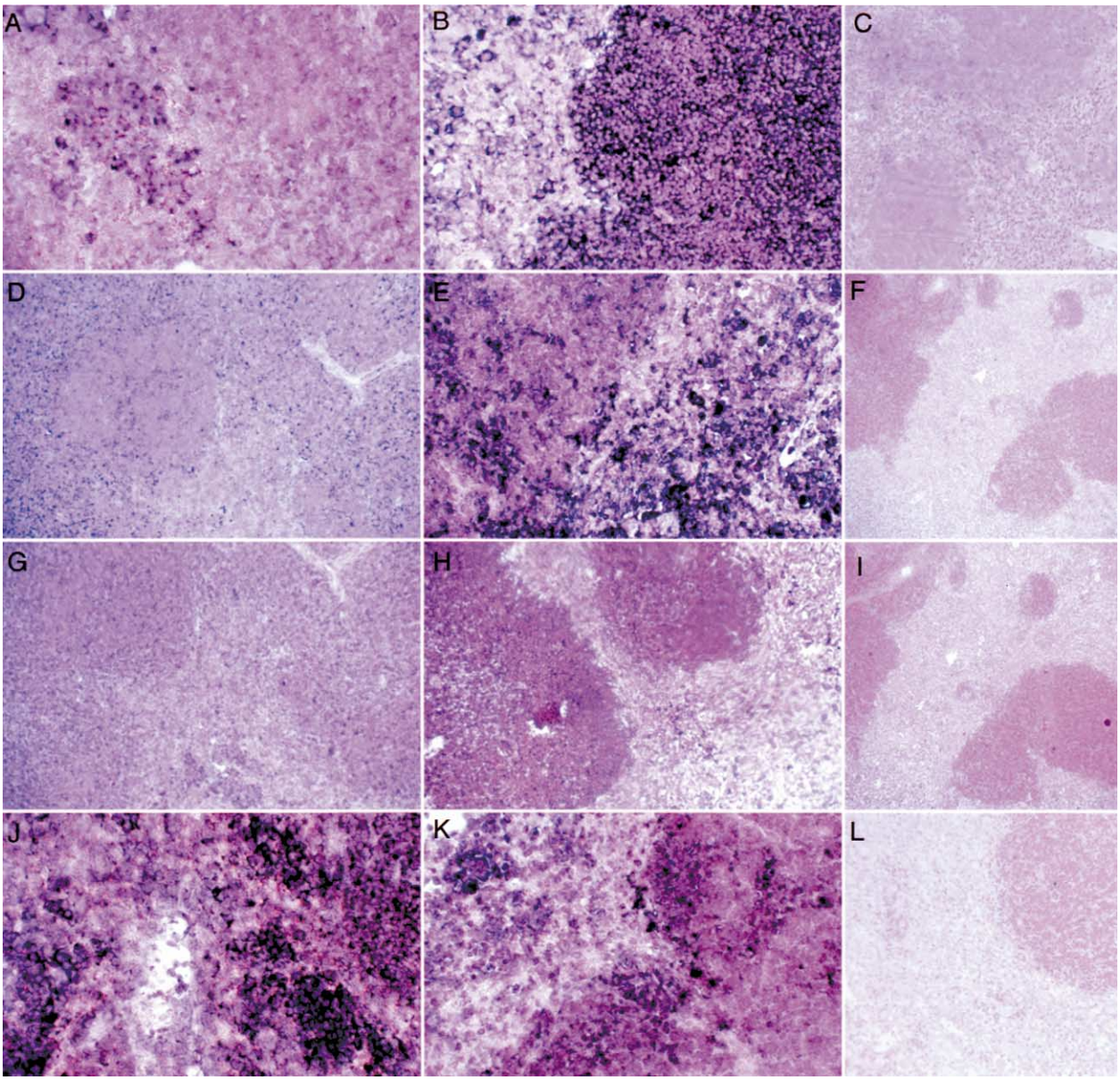


Figure 3. *In situ* hybridization of spleen with caspases-3 (**A**-**C**), -8 (**D**-**F**), -11 (**G**-**I**), and -12 (**J**-**L**). Uninfected control animals (**A**, **D**, **G**, **J**) were compared with septicemic animals 36 hours after intracerebral infection (**B**, **E**, **H**, **K**). As negative controls spleens of meningitic animals were hybridized with the respective sense cRNA (**C**, **F**, **I**, **L**). Caspase-3 mRNA was strongly upregulated, especially within the white pulp in T cells and B cell regions. Similar observations were made with caspase-8 mRNA. Caspase-11 and -12 mRNA changes were not detectable in *in situ* hybridization.

mouse immunoglobulins (diluted 1:50 in TBS, DAKO #Z0259) and alkaline phosphatase anti-alkaline phosphatase (diluted 1:50 in TBS, APAAP; DAKO #D0651) for 1 h at RT in a water-saturated atmosphere. After each incubation, sections were washed three times in TBS. Sections were then incubated in neufuchsin substrate under visual control for varying times at RT. Specific binding of the GFAP antibody was visible as red staining.

Statistics. Data are expressed as means ± standard deviations (SD). Groups were compared by Student-t-tests performed with Statistica 5.1 (StatSoft, Inc. (1996), Tulsa, USA).

Results

Course of Meningitis in Wild-Type Mice. The amount of caspase mRNA transcribed in brain and

spleen was measured by RNA protection assay in mice with meningitis and healthy control mice (0h, 25 µl NaCl i.c.) (Figure 1). 6 hours after infection, caspases -6, -7, and -11 had significantly higher mRNA levels in brain (left and right hemisphere showing the same pattern) than controls ($p<0.05$). 12 hours after induction of meningitis, caspases -2, -8, -1, -12 were also elevated ($p<0.05$). After 18 hours caspase-14 transcript had increased ($p<0.05$), and after 24 hours finally an upregulation of the main effector caspase-3 mRNA level was observed ($p<0.05$).

The mice lost weight in parallel to rising bacterial titers in CSF, cerebellum, spleen, and blood (Figure 1).

As the intracerebrally infected mice developed sepsis, caspases in spleen were also determined. After 6 hours all caspases except -8 and -7 were increased ($p<0.05$). Caspase-8 and caspase-7 induction followed 12 hours later ($p<0.05$) (Figure 1).

To determine the neuronal damage occurring in meningitis in mice, brain slices from brains after 36 h of meningitis were examined by haematoxalin/eosin-staining (HE) and IST. Severe neuronal injury with necrotic and apoptotic neurons in various regions of the hippocampus and in the granular cell layer of the dentate gyrus was visible.

To localize caspase-producing cells, we examined sections of mouse brain after 36 h of meningitis with *in situ* hybridization for caspase-3, -8, -11 and –12 (Figure 2). *In situ* hybridization detected gene transcription of the 4 caspases mainly in neurons of the Formatio hippocampi and in the neocortex.

In situ hybridization for caspases in the spleen of non-infected control animals revealed the following mRNA distribution (Figure 3): Caspase-3 mRNA showed a weak signal predominantly in the red pulp within the sinus. Positive cells mainly had a macrophage-like morphology. Caspase-8 mRNA also was expressed in the white pulp, mainly in the peripheral T cell regions of the germinal centers. A similar distribution was found for caspase-11 and -12 mRNA with a slightly stronger signal for caspase-12. In meningitis animals, caspase-3 mRNA was strongly upregulated, especially within the white pulp in T cell and B cell regions. Similar observations were made with caspase-8 mRNA. In *in situ* hybridization the signals for caspase-11 and -12 mRNA remained unchanged.

TNFα and TNF-receptor 1/2 Knockout Mice. TNFα deficient mice and C57BL/6 wild-type controls were compared for their caspases mRNA expression 36 hours after intracerebral infection. In TNFα-deficient mice upregulation of 8 of the 9 examined caspase mRNA was less intense than in infected wild-type mice ($p<0.005$) (Figure 4a). Caspase 12 transcription levels were at the limit of detection. TNFα deficient mice had significantly increased bacterial titers in spleen ($p<0.05$) and died earlier than control mice indicating a less efficient host defense against pneumococci.

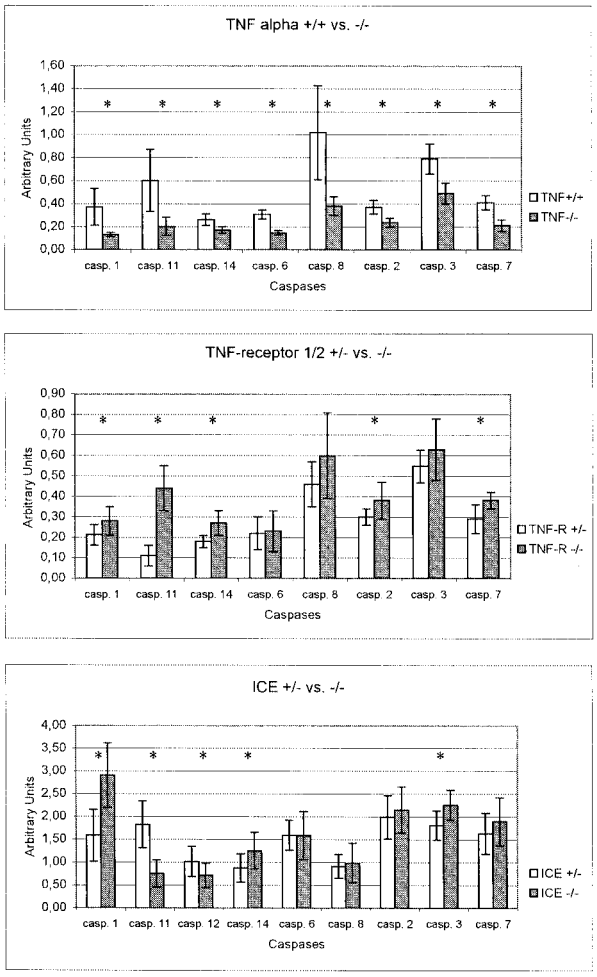


Figure 4. Caspase-mRNA in knock-out mice. (**a**) I.c. infected TNFα-deficient (-/-) mice showed a weaker increase of transcription of caspases-1, -2, -3, -6, -7, -8, -11, and -14 compared to C57BL/6 mice with meningitis (+/+) (mean ± SD; $p< 0.005$). (**b**) Comparing knock-out mice for TNF-receptor 1 and 2 (-/-) with heterozygous controls (+/-), an increased mRNA transcription was found mainly for caspase-11 (mean ± SD, $p<0.00005$), as well as for caspases-1, -2, -7, and -14 (mean ± SD, $p<0.05$). (**c**) Caspase-1 deficient mice (ICE -/-) showed a marked decrease of caspase-11 transcriptional activation during meningitis (mean ± SD, $p< 0.00005$) as well as a decrease of caspase-12 mRNA ($p<0.005$). mRNA of the nonfunctional caspase-1 ($p<0.00005$), and caspases-3, and -14 ($p<0.005$) were increased compared to controls.

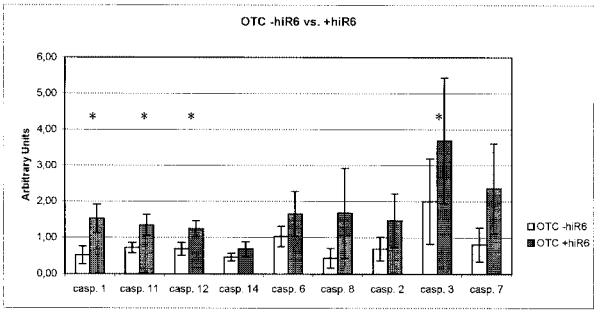


Figure 5. mRNA levels of Casapses-1, -2, -3, -6, -7, -8, -11, -12, -14 in organotypical hippocampal cultures (OTC) were measured by RPA and are expressed in arbitrary units (mean ± SD). Caspases-1, -3, -11, and -12 were significantly increased in OTC challenged by heat-inactivated *S. pneumoniae* (+hiR6) compared to controls (-hiR6). Mean caspase-2, -6, -7, -8 and -14 mRNA expression was higher in OTC cultures after bacterial challenge than in untreated cultures but the difference did not reach statistical significance (n=3).

Knockout mice for TNF-receptor 1 and 2 showed a different pattern of transcription compared to heterozygous controls (Figure 4b): The knockout mice had an increased mRNA-level mainly of caspase 11 ($p<0.00005$). Furthermore caspases-1, -2, -7, and -14 were transcribed on higher levels than in control mice ($p<0.05$). Increased bacteria titers were detected in spleen in comparison to heterozygous controls ($p<0.05$). The animals did not differ in severity of the disease.

Caspase-1 (ICE) Knockout Mice. In the ICE-deficient mice, the neomycin cassette is inserted in exon 6 producing an out-of-frame shift beyond this residue. A compensatory increase of this non-functional mRNA was detected by the RPA in knock-out mice infected with *S. pneumoniae* compared to heterozygous controls ($p<0.0005$, Figure 4c). Elevated levels of mRNA were also shown for caspase-3 and -14 ($p<0.005$).

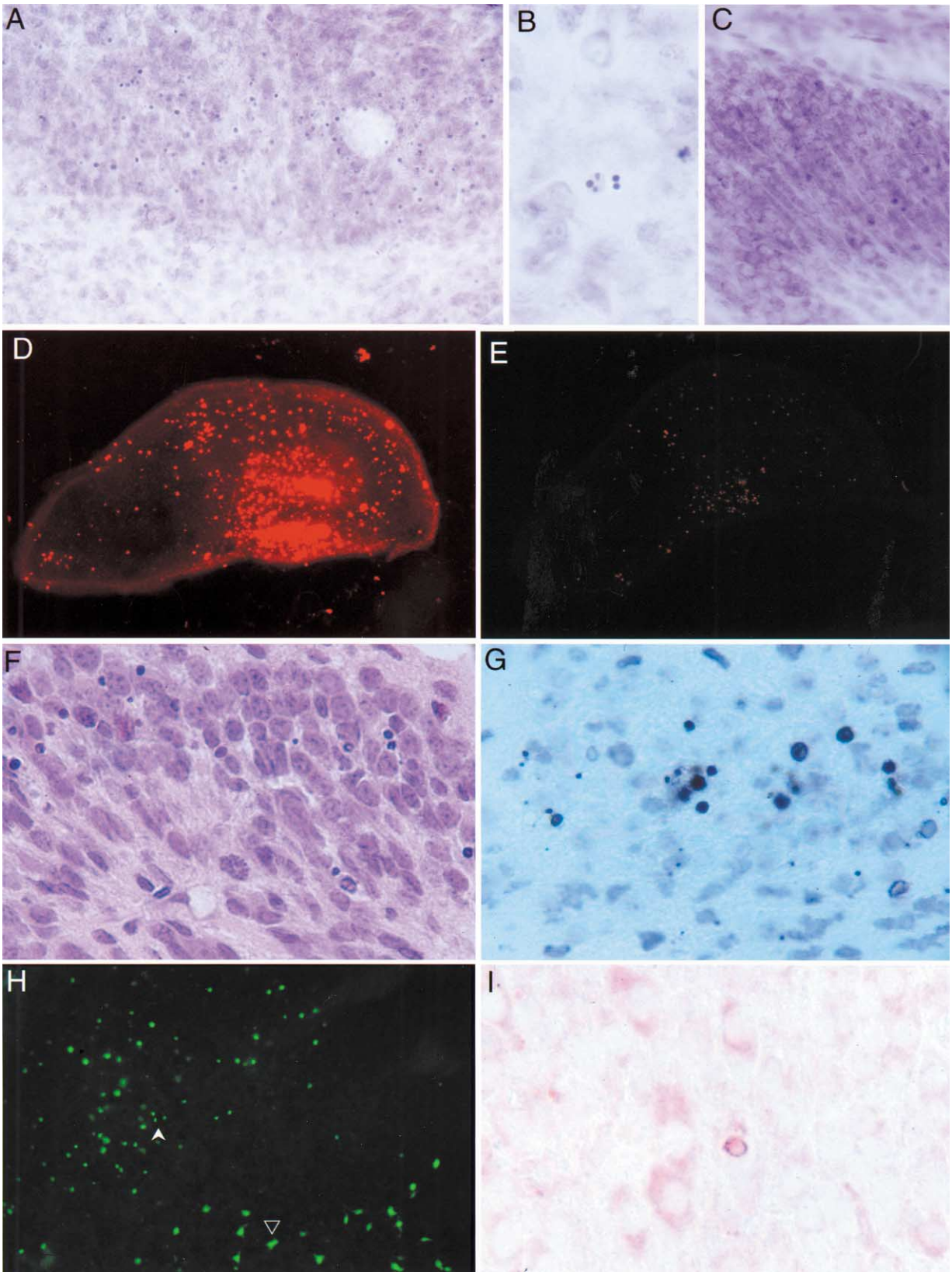
A dramatic decrease was found in activation of caspase-11 in ICE-deficient mice compared to controls ($p<0.0005$). A decrease was also demonstrated for caspase-12 ($p<0.005$). There was no difference in bacterial titers of blood, spleen or cerebellum and of the severity of disease.

Organotypic Hippocampal Cultures. To exclude that caspase mRNA upregulation was a consequence of leukocyte infiltration of the brain during meningitis, the *in vivo* results were completed by comparing cultured slices of hippocampal formations exposed to heat-inacitvated *S. pneumoniae* and unchallenged control slices. Caspases-1, -3, -11, and -12 were significantly increased in infected OTC compared to controls (Figure 5). Mean caspase-2, -6, -7, -8 and -14 mRNA expression was also higher in OTC cultures after bacterial challenge than in untreated cultures, but the difference did not reach statistical significance (n=3). To determine the neuronal damage occurring, the OTC were examined by propidium-iodide, Nissl and HE-staining (Figure 6). In OTC exposed to hiR6, the hippocampus and dentate gyrus showed an increase in neuronal damage visualized by PI-staining in contrast to controls (Figure 6D, E). In Nissl- and HE-stained OTC apoptotic neurons characterized by shrunken cytoplasma, condensed nuclei, and apoptotic bodies were found in the dentate gyrus and in the pyramidal cell layer of the hippocampus (Figure 6 A-C, F). Annexin V staining detected apoptotic cells in the granule and pyramidal cell layers (Figure 6H). Activated caspase-3 was detected by CM1 antibody immunohistochemistry in neurons of the dentate gyrus of preapoptotic and apoptotic morphology (Figure 6I).

Discussion

Neuronal damage in meningitis is believed to originate from brain edema and vasculitis leading to ischemia, the host's immune response and direct toxicity of bacterial components. Several mechanisms appear to be ultimately involved in the generation of neuronal damage: excitatory amino acids (25, 54), cytokines (45) and oxygen radicals (21). Oxidative injury and excitotoxicity are linked closely: stimulation of neurons with excitatory amino acids leads to the production of reactive oxygen species (ROS) (23, 40), exposure of the hippocampus to ROS generating substrates leads to the release of excitatory amino acids (38), and glutamate uptake into rat cortical astrocytes is inhibited by ROS (56). This implies that both mechanisms may potentiate each other in bacterial meningitis.

Figure 6. (Opposing page) Cell damage in organotypical hippocampal cultures (OTC). Nissl-staining of OTC (thickness: 200 μm) detected an increased level of apoptotic neurons in OTC cultured with heat-inactivated *S. pneumoniae* R6 (**A** ×20, **B** ×100) compared to uninfected controls (**C** ×20). Propidium-iodide staining, detecting necrotic and late apoptotic neuronal damage, was increased mainly in dentate gyrus of co-cultured OTC (**D**, ×2.5) in comparison to controls (**E**, ×2.5). HE-staining of thin sections (1-2 μm) of OTC detected neurons with clear apoptotic morphology (**F**, dentate gyrus, ×100). IST-positive neurons with apoptotic morphology in dentate gyrus (**G**, ×100). Annexin V-staining showed positve fluorescent pyramidal and granular cells of hippocampus and dentate gyrus (**H**, ×10). CM1-immunhistochemistry detecting activated caspase-3 showed staining in neurons of the dentate gyrus of preapoptotic and apoptotic morphology (**I**, ×100).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In OTC challenged with hiR6 pneumococci (Figure 6) we found severe apoptotic and necrotic changes in the hippocampal formation. The damage was visualized by PI uptake, IST, Annexin V-staining and by morphologic changes in Nissl- and HE-staining typical for apoptosis such as shrinkage, condensation of nuclei and apoptotic bodies. In adult mice the predominant morphology of damaged neurons was necrotic, but additionally an increased density of apoptotic neurons was detected in the dentate gyrus. Occasionally neurons of necrotic morphology stained positive by IST reaction, indicating that morphologically necrotic neurons shared some features of apoptosis. In accordance with these observations it has been shown that cell death following target deprivation, excitotoxicity and ischemia can coexist as apoptosis, necrosis, and hybrid forms along an apoptosis-necrosis continuum. Whether apoptosis or necrosis predominates, depends on the noxious agent, the strength of the injury, the cell population involved and maturity of the cells (44, 30). Apoptosis and necrosis share common initiation pathways and can be elicited by the same stimuli at different intensities (7, 15, 29). The importance of caspases in this context is emphasized by the observation that inhibition of caspases causes a switch from apoptosis to necrosis in cultured B-lymphocytes (26). As morphology may not be sufficient to define the exact mode of cell death, caspases furthermore provide a valuable tool for monitoring the mechanisms of neuronal loss.

To examine the gene regulation of the most important regulators and executors of apoptosis, RNA steady-state levels of various caspases were quantified by RPA during the course of meningitis. The first caspases transcribed at higher levels were -6, -7 and -11. Caspase-6 belongs to the activator group, caspase-7 to the effector group and caspase-11 is a mediator of inflammation. This is a very early increase in transcription caused by only 2×10^2 cfu/ml bacteria in cerebellum and CSF. Caspase-1 and the other caspases increased at later timepoints. The caspase activated last was caspase-3, the main effector caspase. Our results point to a regulation of caspases on transcriptional level additionally to the known network on protein level.

Caspase-11 rose very early during infection. In inflammation caspases-11 and -1 can trigger the cascade and initiate the effector-caspases via caspase-8 on protein level (18). A connection between protein- and mRNA-levels was demonstrated, since caspase-3 can activate transcriptional factor c-jun in ceramide activated HL-60 cells (22). For this reason it can be speculated that caspase -1 can activate mRNA transcription of caspase-11 in this model, too.

In spleen the caspases mRNA rose also, however, in a different sequence. The mRNA of all caspases but -8 and -7 were elevated 6 hours after infection. Caspase-8 and caspase-7 were elevated 18 hours after infection. Thus, the transcription pattern in spleen differed from brain and showed that the caspase transcription is dependent on the cell type. In spleen activated and caspase-mRNA positive follicles were shown by *in situ* hybridization. A possible explanation could be apoptosis of leucocytes after ingestion of pneumococci.

TNFα is an essential initiator and regulator of host defense against pathogenic bacteria and coordinates a variety of cellular enzymes and transcription factors leading to activation, differentiation or death of the target cell (4, 28). It was found to be produced in CNS during experimental meningitis (3) or after injection of lipopolysaccarides (39). Receptors of the TNF-receptor family appear to activate the JNK/c-jun pathway and NFκB/AP1 (transcription factors that mediate survival and death) by different cellular proteins that are recruited by the receptors following their oligomerization (TRADD, FADD/MORT1, RIP, TRAF) (1).

We therefore investigated meningitis in knockout mice for TNFα and TNF-receptor 1 (p55) and 2 (p75). To evaluate the interaction among the caspases, caspase-1 deficient mice (ICE) were also examined. Caspase-1 deficient mice almost lacked transcription of caspase-11 (Figure 3c) indicating the importance of caspase-1 as an activator of caspase-11 transcription in inflammation. This corresponds to observations in which caspase-11 is necessary to activate caspase-1 by forming complexes together on protein level (59). It shows, that caspase-11 transcription is not always upstream of caspase-1 activation.

In TNFα knockout mice a marked decrease of all caspases examined (Figure 3a) indicated the dependence of caspase transcriptional activation in pneumococcal meningitis on TNFα. The host defense against pneumococcal infections was disturbed in TNFα-deficient mice: All TNFα-deficient animals died after intraperitoneal challenge with 10^2 *S. pneumoniae*, whereas all wild-type littermates survived (33). The clearance of *S. pneumoniae* from the blood stream was affected in TNFα-deficient mice, resulting in bacterial titers in blood and spleen, which were 2 orders of magnitude higher than in wild-type controls 36 h after intracerebral infection. This observation was confirmed in this study as TNFα knockout mice infected intracerebrally showed significant higher bacterial titers in spleen as their wild-type controls.

In knockout mice for TNF-receptors 1 and 2 a different pattern was found (Figure 4b). The most prominent changes were the increased transcription of caspase-11 and -1 mRNA in knockout mice during meningitis. A possible cause of this increased host reaction might be that the lacking TNF-receptor 2 normally limits the inflammatory host response in wild-type mice by binding soluble TNFα in blood (57). Increased bacterial titers in spleen indicated a disturbed bacterial clearance from blood corresponding to the results in TNFα -/- mice.

In conclusion, caspase activation has emerged as the central molecular event leading to apoptosis, preceding DNA degradation and the development of apoptotic morphology (43). Our results suggest a tightly regulated cascade on RNA level for caspases in neurons in addition to the known proteolytic cascade. The transcription might be regulated by the transcription factors AP-1 and NF-κB as demonstrated earlier for chemokine expression (41). By binding to promotor regions this could provide a critical regulatory mechanism for caspases to be selectively expressed in a cell type-specific and stimulus-specific manner. On mRNA level, the caspase-network appears to be influenced by various factors such as TNFα/TNF-receptors, transcription factors and by interacting caspases themselves.

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