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Olfactory receptor neurons prevent dissemination of neurovirulent influenza A virus into the brain by undergoing virus-induced apoptosis

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Olfactory receptor neurons (ORNs) were infected upon intranasal inoculation with the R404BP strain of neurovirulent influenza A virus. Virus-infected neurons and a small fraction of neighbouring uninfected neurons displayed apoptotic neurodegeneration substantiated by the immunohistochemistry for activated caspase-3 molecules and the terminal deoxynucleotidyl transferase-mediated dUTP nick end-labelling method. However, virus infection was restricted within the peripheral neuroepithelium and all mice survived the infection. Virus-infected ORNs revealed upregulated expression of the Fas ligand molecules, activating the c-Jun N-terminal kinase signal transduction pathway. In addition, Iba1-expressing activated microglia/macrophages appeared to partake in phagocytic activities, eventually clearing apoptotic bodies. These results raise the possibility that induction of apoptosis in olfactory receptor neurons at an early stage of infection may provide protective effects against invasion of the neurovirulent virus from the peripheral to the CNS.

Introduction

A variety of viruses, including herpes simplex virus, poliovirus, vesicular stomatitis virus, Borna disease virus and parainfluenza type 1 virus, take advantage of the olfactory pathway of mammals to gain access to the CNS (Kristensson, 1996; Mohammed *et al.*, 1993). The olfactory receptor neurons (ORNs) are exposed directly to the external environment, i.e. the nasal cavity, providing a pathway for virus invasion into the brain (Kristensson, 1996). These viruses infect ORNs at the first step, travel to the olfactory bulb and target the brain structures anatomically connected to the olfactory bulb, such as the limbic system and reticular core neurons (i.e. cholinergic diagonal band, serotonergic raphe and noradrenergic locus coeruleus). Exceptionally, parainfluenza type 1 virus infection remains in ORNs and persists in the nerve tissue (Mori *et al.*, 1995, 1996). To date, no virus has been shown to induce apoptotic death of ORNs. Some reports, however, suggest the occurrence of apoptosis in the neuroepithelium during olfactory neurogenesis and after the bulbectomy *in vivo* and in a dopamine-treated olfactory neuronal cell line *in vitro* (Coronas *et al.*, 1997; Farbman *et al.*, 1999; Voyron *et al.*, 1999). As for the neuronal death following bulbectomy, the Fas/Fas ligand (Fas L) system most likely mediates activation of the apoptosis cascade (Farbman *et al.*, 1999).

Neurovirulent influenza A virus infection induces neuronal apoptosis in the mouse CNS following direct introduction of the virus into the olfactory bulb, depending on T cell-mediated mechanisms, including the perforin/granzyme and Fas/Fas L systems (Mori *et al.*, 1999; Mori & Kimura, 2000, 2001).

Here we present evidence that ORNs die through apoptosis following intranasal infection of the recombinant R404BP strain of neurovirulent influenza A virus with upregulated expression of the Fas L molecules and activation of the c-Jun N-terminal kinase (JNK) cascade. The virus grown in ORNs did not invade the CNS or kill animals. The R404BP virus carries the matrix and neuraminidase genes of the neurovirulent WSN

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strain (H1N1) and other genes of the non-neurovirulent A/Aichi/2/68 strain (H3N2), showing an increased neurovirulent phenotype compared with the parental WSN virus. The virus, when introduced directly into the mouse CNS, spreads widely in the brain and kills 100% of the mice (Takahashi *et al.*, 1995), suggesting a highly neurovirulent potential. We further show dynamic reactions of microglial cells in the olfactory epithelium, partaking in phagocytic activities for eventual clearing of apoptotic bodies.

Methods

■ **Experimental infection of animals.** The R404BP and WSN viruses (kindly supplied by Dr S. Nakajima, The Institute of Public Health, Japan) were propagated as described elsewhere (Mori *et al.*, 1999). R404BP virus (10^6 p.f.u.) in 10 μl sterile PBS was instilled into the right nostril of specific-pathogen-free female C57BL/6 mice (Clea) at 4 weeks of age. Stereotaxic microinjection of the virus into the olfactory bulb was carried out as reported previously (Mori *et al.*, 1999).

■ **UV-inactivation of the virus.** Stock virus suspension was exposed to a 15 W UV lamp at a distance of 30 cm for 30 min at 4 °C with continuous and gentle stirring. After irradiation, infectivity was reduced to $< 10^{-7}$ of the original.

■ **Tissue processing.** Under deep anaesthesia by the intraperitoneal administration of 7·2% chloral hydrate in PBS (0·05 ml/g body weight), mice were transcardially perfused with 3·7% formaldehyde in PBS. The nose was decalcified in 4 % ethylenediaminetetraacetic acid at room temperature for 7 days. The nose and brain were soaked in 20% sucrose in PBS at 4 °C overnight and frozen at −80 °C. Coronal sections of 14 μm thickness of the nose and brain tissues were cut on a cryostat.

■ **Immunohistochemistry.** Immunohistochemistry was performed as described previously (Mori & Kimura, 2000). Primary antibodies included rabbit polyclonal anti-WSN virus antibody (a generous gift from Dr S. Nakajima), rabbit anti-inducible nitric oxide synthase (iNOS) antibody (working concentration of 5 μg/ml; Upstate Biotechnology), anti-nitrotyrosine (NT) antibody (working concentration of 20 μg/ml; Upstate Biotechnology) and rabbit polyclonal anti-Iba1 antibody (Imai *et al.*, 1996). The Iba1 molecules are expressed selectively in microglia/macrophages (Mori *et al.*, 2000).

■ **Dual immunofluorescent labelling.** Tissue slices were incubated in 5% donkey serum (Chemicon International) containing 0·3% Triton-X in PBS for 20 min and reacted in a mixture of two primary antibodies diluted in 2% donkey serum containing 0·3% Triton-X in PBS at 4 °C overnight. The primary antibodies used were goat polyclonal anti-influenza A virus antibody (working dilution of 1:400; Chemicon International), rabbit polyclonal anti-neural cell adhesion molecule (NCAM) antibody (working concentration of 2·0 μg/ml; Chemicon International; Calof *et al.*, 1996), rabbit polyclonal anti-cleaved caspase-3 antibody (working dilution of 1:50; Cell Signalling Technology), rabbit polyclonal anti-Fas antibody (working dilution of 1:400; Wako Pure Chemical Industries), rabbit polyclonal anti-Fas L antibody (working dilution of 1:200; Wako Pure Chemical Industries), rabbit polyclonal anti-Iba1 antibody (working concentration of 2·0 μg/ml), goat polyclonal anti-Fas L antibody (working dilution of 1:100; Santa Cruz Biotechnology), rabbit polyclonal anti-phospho-JNK antibody (Thr^{183}/Tyr^{185}; working dilution of 1:200; Cell Signalling Technology) and anti-phospho-c-Jun antibody (Ser^{63}; working dilution of 1:200; Cell Signalling Technology). Then, tissue sections were incubated in secondary antibodies affinity-purified and absorbed for dual immunolabelling (all diluted 1:100 in PBS supplemented with 0·3% Triton-X; Chemicon International), including fluorescein-labelled donkey anti-rabbit immunoglobulin, rodamine-labelled donkey anti-goat immunoglobulin, fluoroscein-labelled donkey anti-goat immunoglobulin and rodamine-labelled donkey anti-rabbit immunoglobulin. Binding was visualized under a confocal laser scanning microscope.

■ ***In situ* detection of DNA fragmentation.** DNA fragmentation was detected by the terminal deoxynucleotidyl transferase-mediated dUTP nick end-labelling (TUNEL) method, using an ApopTag Direct *In Situ* Apoptosis Detection kit (Intergen). Dual imaging for virus antigens and TUNEL reaction was carried out as described in a previous report (Mori & Kimura, 2000).

■ **Detection of influenza virus matrix protein 1 (M1) mRNA.** Viral mRNA encoding influenza virus M1 was extracted from the nasal tissue and olfactory bulb by using an mRNA Isolation kit (Loche). cDNA synthesis and PCR were carried out with the One-Tube RT–PCR system (Takara), according to the manufacturer's instructions, with the sense primer 5′ GAGATCGCACAGAGA 3′ and the antisense primer 5′ TCGTTGCATCTGCAC 3′ (Urabe *et al.*, 1993). The programme conditions were as follows: 50 °C for 30 min and 94 °C for 2 min, followed by 30 cycles of 94 °C for 15 s, 50 °C for 30 s and 72 °C for 45 s. The final products were differentiated on 1% agarose gel, stained with ethidium bromide and visualized under a UV lamp. The expected size of the product was 684 bp.

Results

Survival of mice after intranasal infection with the R404BP virus

Mice were intranasally inoculated with the R404BP strain of influenza A virus at a dose of 10^6 p.f.u. per mouse. All mice ($n = 5$) survived through the course of infection. When the virus (10^5 p.f.u.) was stereotaxically microinjected into the olfactory bulb, the virus infection spread to the anatomically connected brain area (including the anterior olfactory nucleus, piriform cortex, diagonal band, dorsal raphe and locus coeruleus) and killed 100% of the mice ($n = 5$) within 14 days, indicating the potentiality of the virus to cause lethal encephalitis (Takahashi *et al.*, 1995; Mori *et al.*, 1999). The survival of intranasally infected mice exhibited statistical significance on the Fisher's exact probability test ($P < 0·005$).

Infection of ORNs

The immunohistochemical study showed that infected cells appeared in the olfactory epithelium as early as day 1 after intranasal inoculation with the R404BP virus (Fig. 1c). Dual staining for viral antigens and the NCAM antigen, a marker of ORNs, proved that infected cells were identical primarily to the ORN (Fig. 2). Clusters of infected ORNs and a small number of sustentacular cells, a kind of supporting cells in the olfactory epithelium, became infected on day 3 after infection (Fig. 1e). On day 5, virally infected neurons began to shrink and split into small bodies (Fig. 1g). On day 7, a virus antigen-immunopositive substance composed of small bodies appeared

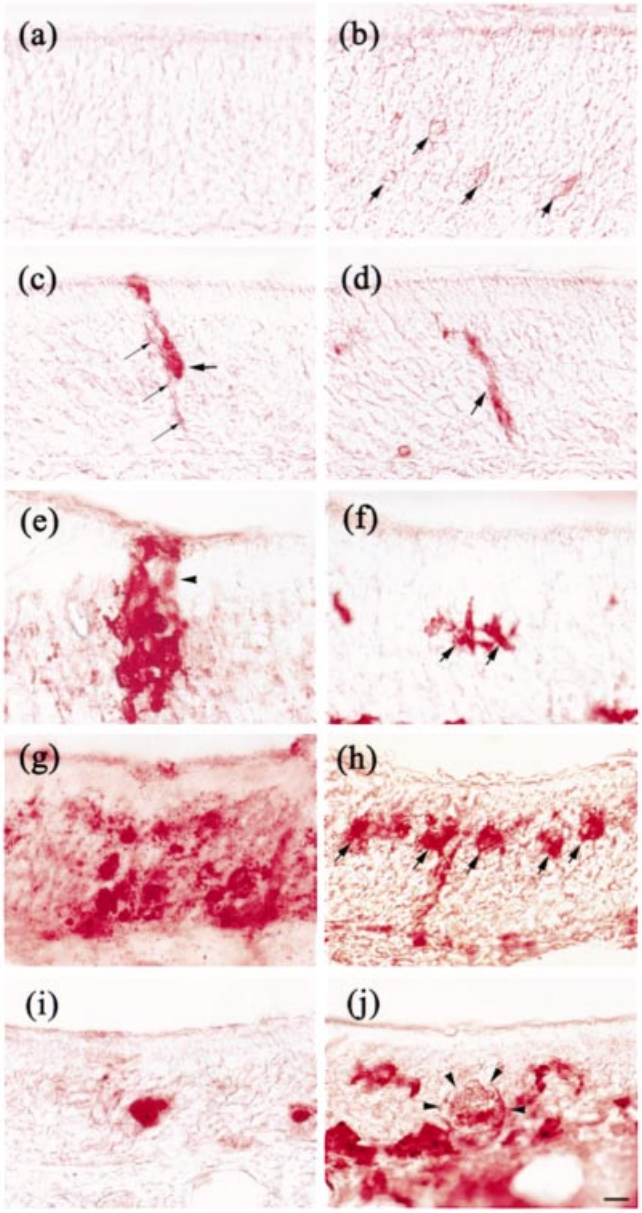

Fig. 1. Kinetics of expression of virus antigens and Iba1 molecules in the nose upon intranasal infection with the neurovirulent R404BP strain of influenza A virus. Slices of the nose tissue were immunohistochemically strained with rabbit polyclonal anti-influenza A/WSN virus antibody (a, c, e, g and i) and with rabbit polyclonal anti-Iba1 antibody (b, d, f, h and j). Before infection (a, b) and on days 1 (c, d), 3 (e, f), 5 (g, h) and 7 (i, j). In the normal olfactory neuroepithelium, Iba1-immunoreactive microglia-like cells of a resting phenotype were observed (arrows in b). On day 1 after infection, ORNs became infected sporadically and singly (arrow in c) and neurites were occasionally stained (thin arrows in c). In the consecutive section, a rod-shaped cell with Iba1-immunoreactivity was observed (arrow in d). On day 3, a cluster of infected ORNs appeared (e) and a sustentacular cell also became infected (arrowhead in e). In the consecutive slice, cells with strong Iba1-immunoreactivity were observed (arrows in f). On day 5, infected cells became shrunken and fissured into small bodies (g). In the consecutive section, Iba1-immunopositive macrophage-like cells appeared (arrows in h). On day 7, a virus antigen-positive substance composed of small bodies appeared (i) and seemed to be phagocytosed by an Iba1-immunopositive huge cell (surrounded by arrowheads in j). Bar, 5 μm.

(Fig. 1i), which vanished by day 14 (data not shown). The virus infection also occurred restrictedly in some ciliated respiratory epithelial cells facing the nasal cavity but did not reach the lung. RT–PCR detected viral M1 mRNA in the nose from days 1 to 5 and proved negative on day 7 (Fig. 3). Lymphocytic infiltration was not obvious in the neuroepithelium (Fig. 4). The immunohistochemistry detected no iNOS or NT expression, indicators of inflammatory response, in virally attacked areas in the olfactory epithelium.

In contrast, no viral antigens were detected in the olfactory bulb or in other brain slices tested that encompassed the levels of the anterior olfactory nucleus, diagonal band, hypothalamus, amygdala, substantia nigra and dorsal raphe. No viral mRNA could be detected in the olfactory bulb by RT–PCR (Fig. 3). Following infection of UV-inactivated virus into the nostril, no trace of infection was detected in the olfactory and respiratory epithelium.

The response of Iba1-immunopositive cells in the olfactory epithelium

Immunohistochemistry for Iba1 molecules, a specific marker of a monocytic-macrophage lineage, was performed in the consecutive nasal tissue slices. In the normal olfactory epithelium, small round cells with thick processes were observed (Fig. 1b). On day 1 after infection, rod-shaped cells that were immunopositive for Iba1 appeared in the corresponding area where infected ORNs were observed (Fig. 1d). On day 3, cells presenting strong Iba1-immunoreactivity with thick processes were found (Fig. 1f). On day 5, macrophage-like cells with prominent Iba1-immunoreactivity were detected (Fig. 1h). On day 7, phagocyte-like cells (around 15 μm in diameter) with Iba1-immunoreactivity appeared in the corresponding regions where the virus antigen-immunopositive substance composed of small bodies emerged (Fig. 1j). Such microglial activation did not take place in mice intranasally infected with UV-inactivated virus.

Demonstration of apoptosis in infected ORNs

Shrinkage of infected ORNs and appearance of small bodies were highly indicative of the occurrence of classical apoptosis. Cytoplasmic accumulation of cleaved caspase-3 in infected ORNs verified the occurrence of apoptosis (Fig. 4). A small number of uninfected cells with immunoreactivity for cleaved caspase-3 was also detected in areas surrounding a cluster of infected ORNs. The TUNEL-specific signal appeared at first in a nuclear staining pattern of infected ORNs on day 3 after infection (data not shown).

Activation of the Fas/Fas L system and JNK signal transduction pathway

In the normal olfactory neuroepithelium, Fas molecules were detected through the olfactory layer in a cytoplasmic

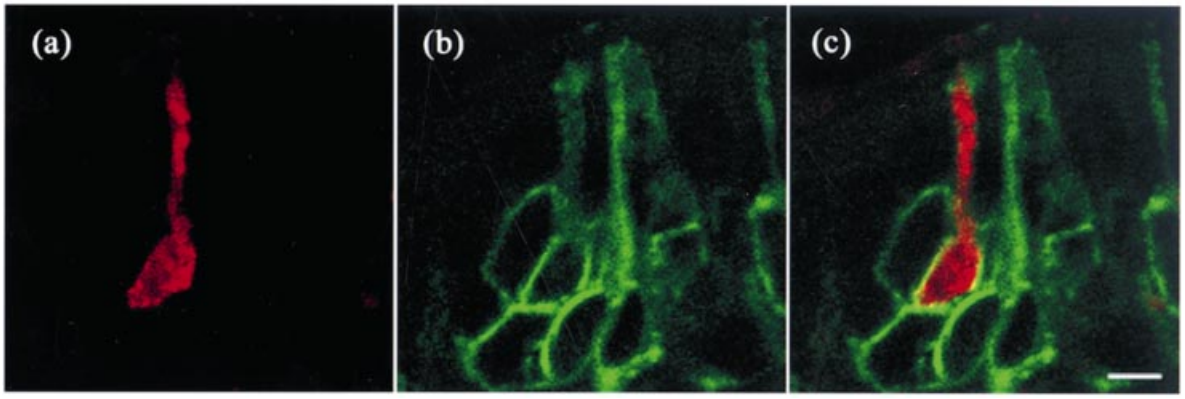

Fig. 2. Dual immunolabelling of the olfactory neuroepithelium for the influenza A virus and NCAM on day 1 after infection. Virus antigens (a), NCAM (b) and their merged confocal image (c). Bar, 5 μm.

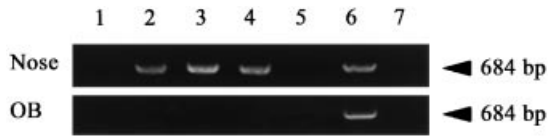

Fig. 3. Detection of influenza A virus M1-specific mRNA in the nose and olfactory bulb tissues by RT–PCR. Lanes: 1, before infection; 2, day 1; 3, day 3; 4, day 5; 5, day 7; 6, positive control (infected cells); 7, negative control (water). OB, olfactory bulb.

staining fashion. Occasionally, the cell surface showed stronger Fas-specific signals typically as a dotted pattern (Fig. 5a). In sharp contrast, Fas L molecules were barely detectable through the layer of the olfactory epithelium (Fig. 5b).

Upregulation of Fas molecules was evident in infected ORNs on day 3 after infection (Fig. 5d). Upregulated expression of Fas L molecules appeared equivocal on day 1 after infection but became evident in infected ORNs on day 3 (Fig. 5g). A small number of infected sustentacular cells also expressed Fas L molecules (Fig. 5j). Upregulated expression of Fas L was confined to virally attacked areas. Microglia-like cells found on days 1 and 3 did not present Fas L-specific signals (data not shown).

Since the stress-activated signalling pathway appeared to be highly involved in the process of neuronal apoptosis (Putcha *et al.*, 2001; Whitfield *et al.*, 2001), we investigated the expression of JNK (phosphorylated at Thr^{183} and Tyr^{185}) and c-Jun (phosphorylated at Ser^{63}) in infected ORNs. The immunofluorescence assay detected phosphorylated JNK and c-Jun in infected ORNs firstly on day 3, suggesting that the JNK/c-Jun signalling pathways become activated during the process of apoptosis induced by the R404BP virus infection (Fig. 6).

Discussion

The present data provide morphological and molecular evidence that ORNs die through mechanisms associated with apoptosis upon infection with the neurovirulent influenza A virus. To our knowledge, this is the first report concerning

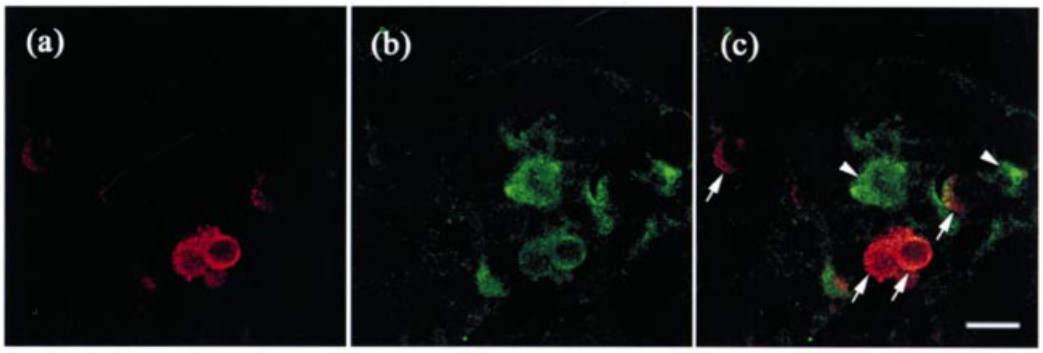

Fig. 4. Activated caspase-3 detected in infected ORNs on day 3 after infection. Virus antigens (a), cleaved caspase-3 (b) and their merged confocal image (c). The arrows in (c) point to infected ORNs and the arrowhead indicates an uninfected ORN. Note the cytoplasmic staining pattern of activated caspase-3. Bar, 10 μm.

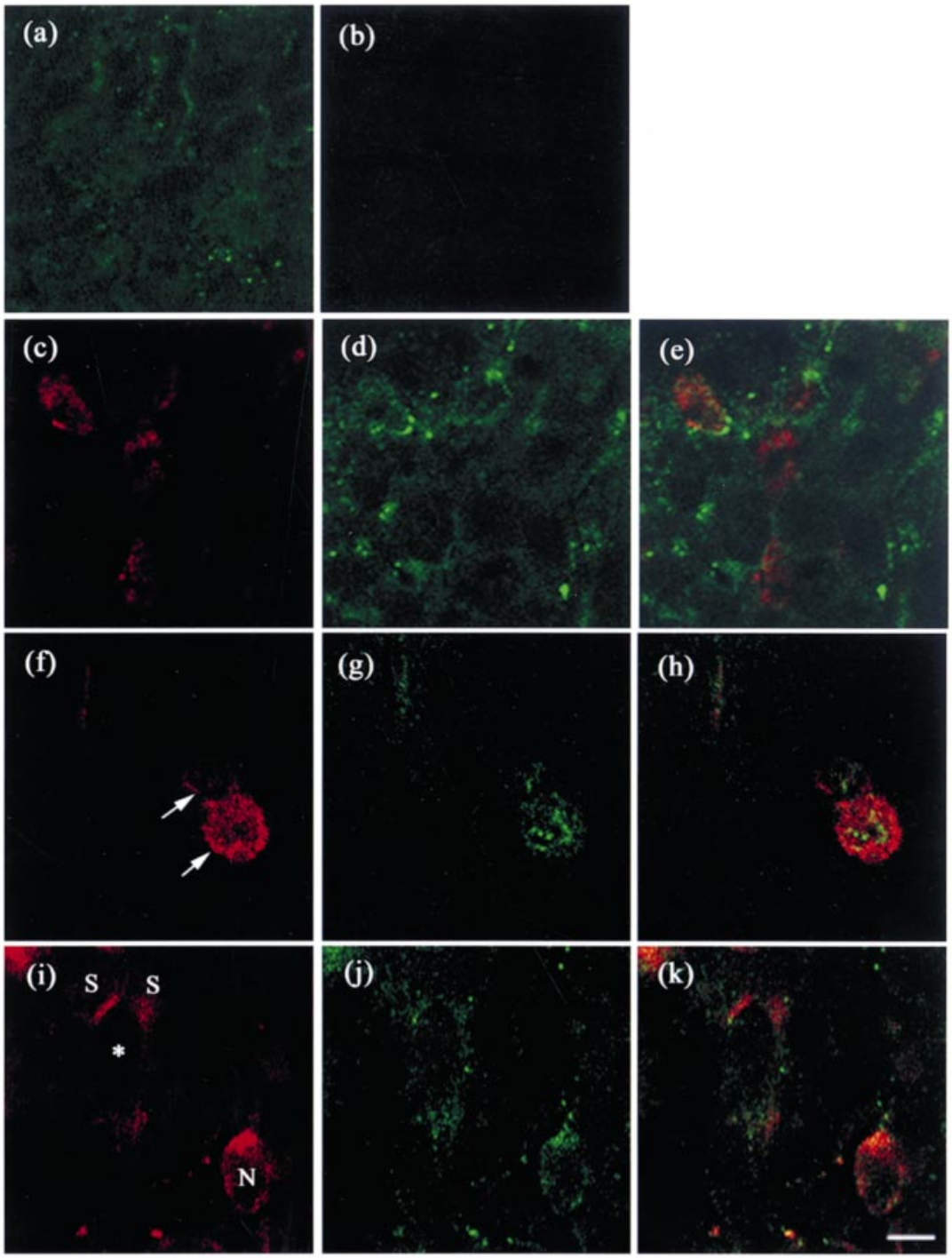

Fig. 5. Expression of Fas and Fas L molecules in infected cells on day 3 after infection. Fas (a) and Fas L (b) molecules in the non-infected control olfactory neuroepithelium. Note that Fas molecules are constitutively expressed, whereas Fas L molecules are barely expressed. Virus antigens (c), Fas (d) and their merged confocal image (e) in infected ORNs. Virus antigens (f), Fas L (g) and their merged confocal image (h) in infected ORNs. Virus antigens (i), Fas L (j) and their merged confocal image (k) in infected sustentacular cells (S) surrounding an uninfected ORN (asterisk) and an infected ORN (N). Upregulation of Fas L was confined to virally attacked areas. Bar, 5 μm.

virus-induced apoptosis in ORNs. Infected ORNs become degenerated, fissured into apoptotic bodies and are eventually cleared out by phagocytic activities of Iba1-positive microglia-like cells. Lymphocytic infiltration is not evident through the course of infection, suggesting that cellular immunity does not play a major role in the process of apoptosis under the present

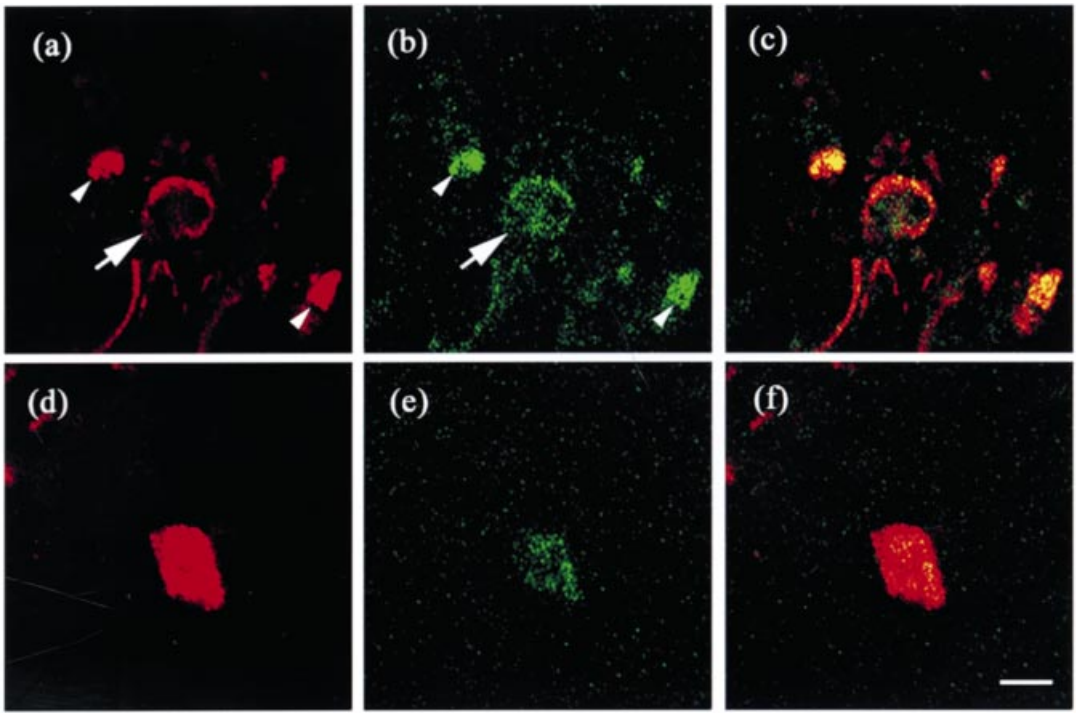

Fig. 6. Activation of the JNK/c-Jun signal transduction pathway in infected ORNs on day 3 after infection. Virus antigens (a), phosphorylated JNK (b) and their merged confocal image (c). Note the phosphorylated JNK-specific signals accumulating in the nucleus. An arrow points to an infected ORN and arrowheads indicate degenerating ORNs presenting stronger signals of phosphorylated JNK. Virus antigens (d), phosphorylated c-Jun (e) and their merged confocal image (f). Note the nuclear staining pattern of phosphorylated c-Jun. Bar, 5 μm.

experimental conditions. Lack of inflammatory response in the olfactory epithelium is also exemplified by detecting no appreciable level of iNOS induction: apoptosis incites neither discharge of cytoplasm contents into the extracellular space nor inflammatory response (Bredesen, 1995). Importantly, early induction of apoptosis of ORNs upon infection blocks an access of the virus to the olfactory bulb. It should be noted that only 1 p.f.u. of the R404BP virus kills mice when microinjected into the olfactory bulb (unpublished data). Thus, the present data may provide the novel concept that virus-induced apoptosis in ORNs gives rise to protective effects against CNS invasion by the potentially neurovirulent virus. Of interest, some viruses spread along the developing postnatal olfactory system into the CNS. This type of spread is curtailed as the nervous system matures (Oliver & Fazakerley, 1998; Fazakerley & Allsopp, 2001), which might be explained by virus-induced apoptosis of ORNs.

Recent studies underscore the importance of the Fas/Fas L system in influenza virus-induced apoptosis in cultured cells. Influenza A virus infection upregulates the expression of Fas and Fas L molecules on the surface of HeLa cells, leading to apoptosis when infected cells come into contact with each other (Fujimoto *et al.*, 1998). Furthermore, the Fas/Fas L signalling pathway has also been implicated in the occurrence of human lymphocyte apoptosis after influenza A virus exposure (Nichols *et al.*, 2001). Once upregulated, Fas L activates the apoptosis cascade in an autocrine or paracrine fashion by stimulating its receptor, Fas (Morishima *et al.*, 2001). We have shown that the neurovirulent influenza A virus induces Fas L in infected ORNs as well as sustentacular cells. Thus, it is possible that these infected cells and, at least in part, uninfected neighbouring cells, which are densely in contact with each other, undergo apoptosis through activation of the Fas/Fas L system. The JNK/c-Jun signal transduction pathway plays a critical role in the process of apoptosis of the nervous system, such as in cerebellar granule neurons, sympathetic neurons after withdrawal of nerve growth factor, hippocampal neurons in response to the excitotoxin kainic acid and cortical neurons exposed to β-amyloid (Morishima *et al.*, 2001; Whitfield *et al.*, 2001). Activation of the JNK/c-Jun increases expression of the BH3-only protein BIM, which promotes BAX-dependent cytochrome c release from the mitochondria to the cytoplasm, leading to the formation of apoptosome (Putcha *et al.*, 2001; Whitfield *et al.*, 2001). On the other hand, activated JNK phosphorylates c-Jun, which stimulates transcription of several key molecules including Fas L. The JNK/c-Jun–Fas/Fas L signalling pathway is highly implicated in dopamine- and β-amyloid-induced apoptosis in cellular models of Parkinson's and Alzheimer's diseases, respectively (Luo *et al.*, 1998; Morishima *et al.*, 2001). In our

experimental system, the JNK cascade also becomes activated in infected ORNs, suggesting the importance of this signal transduction system in the induction of neuronal apoptosis, which appears to be mediated by both the 'intrinsic' (i.e. apoptosome) and the 'extrinsic' (i.e. death receptor) pathways (Putcha *et al.*, 2001). Another mechanism might also be involved in the process of apoptosis of ORNs induced by the R404BP virus, which remains open for further studies. In cultured cortical neurons, β-amyloid activates calpain I, which cleaves p35, the regulatory subunit of cyclin-dependent kinase 5 (cdk5), to p25, leading to constitutive activation of cdk5 and, ultimately, neuronal apoptosis (Lee *et al.*, 2000).

Iba1 is an EF hand calcium-binding protein, specifically expressed in a monocytic cell line, including microglia (Imai *et al.*, 1996; Ito *et al.*, 1998; Mori *et al.*, 2000). Iba1 has been proven to be an actin cross-linking protein involved in membrane ruffling and phagocytosis of macrophages/microglia (Ohsawa *et al.*, 2000; Sasaki *et al.*, 2001). We have found dynamic reactions of Iba1-expressing microglia-like cells in the olfactory epithelium in response to infection of ORNs with the neurovirulent influenza A virus. Microglial activation in the CNS takes place in a stereotypic and graded fashion irrespective of the cause of the lesion (Kreutzberg, 1996; Mori *et al.*, 2000; Mori & Kimura, 2001). In the first stage, when neuronal injury is sublethal, resting microglia become activated and produce trophic factors, contributing to tissue repair. In the second stage, when neuronal injury is lethal, activated microglia further transform themselves into phagocytic cells, known as microglia-derived brain macrophages. Although macrophage-like cells are detected in the rat olfactory epithelium after bulbectomy by using the OX 42 antibody (Suzuki *et al.*, 1995), the present paper is the first study showing such morphological transformation of microglia-like cells in the peripheral neuroepithelium upon virus infection.

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