2. Hidaka H, Watanabe H, and Gotoh K. Travel clinic in Kurume university hospital, Japan. 8th Asia-Pacific Travel Health Conference, Nara, 2010.
3. Hara K, Nakazono Y, Kashiwagi T, and Watanabe H. The N-terminal region of the PA subunit of influenza virus RNA polymerase contributes to the functional RNA formation. Options for the Control of Influenza VII, Hong Kong, 2010.
4. Kashiwagi T, Hara K, Nakazono Y, Hamada N, and Watanabe H. PA subunit of Influenza A virus RNA polymerae is involved in the stable RNA replication at high temperature. Options for the Control of Influenza VII, Hong Kong, 2010.
5. Watanabe H. Travel medicine and travel clinic. Joint Meeting of the 19th National Conference on Nosocomial Infection of Chinese Preventive Medicine Association (CPMA) and the 6th Shanghai International Forum of Infection Control (SIFIC). Nanjing, China, 2010.
6. Liang Qin and Watanabe H. Bacterial biofilms and hospital-acquired infection. Joint Meeting of the 19th National Conference on Nosocomial Infection of Chinese Preventive Medicine Association (CPMA) and the 6th Shanghai International Forum of Infection Control (SIFIC). Nanjing, China, 2010.
7. Qin L, Goto K, and Watanabe H. Concurrent administration of antibiotics, the role of antimicrobial effect of imipenem for the nontypeable *Haemophilus influenzae* biofilms. 110th American Society for Microbiology General Meeting. San Diego, USA, 2010.
8. Watanabe H, Kashiwagi T, Hara K, Masunaga K, Kawano S, Miura M, Iwahashi J, Qin L, Imamura Y, and Hamada N. Infection control for the pandemic (H1N1)2009 in Kurume university hospital. Meeting of the global outbreak alert and response network (GOARN) partners from the western pacific region. Siem Reap, Cambodia, 2010.
9. Watanabe H, Goto K, and Hara K. Clinical study concerning the relationship between community-acquired pneumonia and influenza in northern Thailand. US-Japan Cooperative Medical Science Program Acute Respiratory Infections Panel. San Francisco, USA, 2010.

国内学会

1. 渡邊　浩　「久留米大学病院におけるパンデミックインフルエンザ流行時の感染対策」　第51回日本熱帯医学会大会、仙台、2010.
2. 三浦美穂、升永憲治、渡邊　浩「ICD・ICP講習会、日本の深在性真菌症感染対策、病院建物解体工事に伴う院内での感染対策について」第58回日本化学療法学会西日本支部総会、大分、2010.
3. 秦　亮、渡邊　浩　「中国における市中気道感染症病原体についての臨床的調査」第58回日本化学療法学会西日本支部総会、大分、2010.

4. 濱田 信之、原 好勇、今村 宜寛、柏木 孝仁、後藤憲志、大津 寧、渡邊 浩「新規ヒトメタニューモウイルス迅速診断キットの有用性の検討」第80回日本感染症学会西日本地方会学術集会、松山、2010.
5. 渡邊 浩「教育講演2、海外渡航関連感染症とトラベルクリニック」第80回日本感染症学会西日本地方会学術集会、松山、2010.
6. 原 好勇、渡邊 浩「PAサブユニットはA型インフルエンザウイルスRNAポリメラーゼの亜型間での親和性に重要である」第80回日本感染症学会西日本地方会学術集会、松山、2010.
7. 上村勇作、秦 亮、後藤憲志、渡邊 浩「The role of antimicrobial effect of imipenem for the nontyple *Haemophilus influenzae* biofilms in vitro」第80回日本感染症学会西日本地方会学術集会、松山、2010.
8. 濱田 信之、原 好勇、柏木 孝仁、今村 宜寛、中園 陽子、千々和 勝己「オセルタミビル耐性株と感受性株が同時に検出された新型インフルエンザウイルスA(H1N1/2009)の変異解析」第58回日本ウイルス学会学術集会、徳島、2010.
9. 原 好勇、中園 陽子、柏木 孝仁、今村 宜寛、濱田 信之、渡邊 浩「A型インフルエンザウイルスRNAポリメラーゼサブユニットの亜型間での親和性解析-PA(H3N2)の親和性決定部位について-」第58回日本ウイルス学会学術集会、徳島、2010.
10. 中園 陽子、原 好勇、柏木 孝仁、今村 宜寛、濱田 信之、渡邊 浩「A型インフルエンザウイルスRNAポリメラーゼサブユニットの亜型間での親和性解析-PB2(H5N1)の親和性決定部位について-」第58回日本ウイルス学会学術集会、徳島、2010.
11. 柏木 孝仁、原 好勇、中園 陽子、濱田 信之、岩橋 潤、今村 宜寛、渡邊 浩「A型インフルエンザウイルス遺伝子複製酵素の温度変化への適応」第58回日本ウイルス学会学術集会、徳島、2010.
12. 渡邊 浩「Nontypable *Haemophilus influenzae* が産生したバイオフィルムに対する抗生物質の効果についての基礎的検討」第24回 Bacterial Adherence and Biofilm 学術集会、東京、2010.
13. 秦 亮、渡邊 浩「*Haemophilus influenzae* が産生したバイオフィルムに対する imipenem の抑制効果に関する基礎的検討」第58回日本化学療法学会総会、長崎、2010.
14. 渡邊 浩「レクチャー3、呼吸器感染症のABC、細菌性肺炎の診断と治療」第58回 日本化学療法学会総会、長崎、2010.
15. 原 好勇、渡邊 浩「インフルエンザウイルスの遺伝子再集合における親和性について」第84回日本感染症学会総会、京都、2010.
16. 秦 亮、木田 豊、渡邊 浩「ワークショップ29、バイオフィルム、Impaired *Streptococcus pneumoniae* capsular polysaccharide promotes biofilm formation」第84回日本感染症学会総会、京都、2010.
17. 矢野寿一、山崎善隆、沖津尚弘、後藤憲志、新井和明、平潟洋一、賀来満夫、渡邊浩「ワークショップ29、バイオフィルム、急性中耳炎患児から分離されたインフルエンザ菌のバイオフィルム産生能と細胞内侵入性の検討」第84回日本感染症学会総会、京都、2010.

18. 真崎宏則、秦　亮、渡邊貴和雄、渡邊　浩「家庭における *Moraxella catarrhalis* 保菌状況についての追跡的調査」　第 84 回日本感染症学会総会、京都、2010.
19. 秦　亮、渡邊　浩　「中国上海における市中気道感染症についての臨床的調査」日本国際保健医療学会 第 28 回西日本地方会. 長崎、2010.
20. 稗田文代、加納昭子、田中真由美、坂井渚、酒井義朗、川野佐百里、大塚まり子、安達康子、升永憲治、渡邊　浩　「消毒薬・外用薬取り扱いマニュアルの改訂」第 25 回日本環境感染学会総会、東京、2010.
21. 渡邊　浩「タイ国北部地域における HIV 陽性患者に合併した市中肺炎の臨床的検討」第2回福岡県医学会総会、福岡、2010.

G．知的財産権の出願・登録状況

特になし

III　研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Tsuge M, Yasui K, Ichiyawa T, Saito Y, Nagaoka Y, Yashiro M, Yamashita N, Morishima T.	Increase of tumor necrosis factor-alpha in the blood induces early activation of matrix metalloproteinase-9 in the brain.	Microbiol Immunol.	54(7)	417-24	2010
Ichinohe, T., Ainai, A., Nakamura, T., Akiyama, Y., Meyama, J., Odagiri, T., Tashiro, M., Takahashi, H., Sawa, H., Tamura, S., Chiba, J., Kurata, T., Sata, T., Hasegawa, H.	Induction of cross-protective immunity against influenza A virus H5N1 by an intranasal vaccine with extracts of mushroom mycelia.	J. of Med. Virology	82	128-137	2010
Nakajima, N., Hata, S., Sato, Y., Tobiume, M., Katano, H., Kaneko, K., Nagata, N., kataoka, M., Ainai, A., Hasegawa, H., Tashiro, M., Kuroda, M., Odani, T., Urasawa, N., Ogino, T., Hanaoka, H, Watanabe, M., Sata, T.	The First Autopsy Case with Pandemic Influenza(A/H1N1pdm) Virus Infection in Japan: Detection of high copy number of the virus in type II alveolar epithelial cells by pathological and virological examination.	Jpn. J. Infect. Dis.	63	67-71	2010
Ujike, M., Shimabukuro, K., Mochizuki, K., Shimabukuro, K, Mochiuki, K., Obuchi, M., Kageyama, T., Shirakura, M., Kishida , N., Yamashita, K, Horikawa, H, Kato, Y., Fujita, N., Tashiro, M., Odagiri, T., Working Group for Influenza Virus Suveillance in Japan.	Oseltamivir-Resistant Influenza Viruses A (H1N1) during the 2007-2009 Influenza Seasons, Japan.	Emerging Infectious Diseases	16(6)	926-935	2010

Ogami M, Hotomi M, Togawa A, Yamanaka N.	A comparison of conventional and molecular microbiology in detecting differences in pneumococcal colonization in healthy children and children with upper respiratory illness.	Eur J Pediatr	169(10)	1221-5	2010
Kawakami K, Ohkusa Y, Kuroki R, Tanaka T, Koyama K, Harada Y, Iwanaga K, Yamaryo T, Oishi K.	Effectiveness of pneumococcal polysaccharide vaccine against pneumonia and cost analysis for the elderly who receive seasonal influenza vaccine in Japan.	Vaccine	28	7063-7069	2010
Kuroda M, Katano H, Nakajima N, Tobiume M, Ainai A, Sekizuka T, Hasegawa H, Tashiro M, Sasaki Y, Arakawa Y, Hata S, Watanabe M, Sata T.	Characterization of quasispecies of pandemic 2009 influenza A virus (A/H1N1/2009) by de novo sequencing using a next-generation DNA sequencer.	PLoS One.	5(4)	e10256	2010
Sakoda, Y., Sugar, Sengee., Batchluunn, D., Erdene-Ochir, T., Okamatsu, M., Isoda N, Soda, K., Takakuwa, H., Tsuda, Y., Yamamoto, N., Kishida, N., Matsuno, K., Nakayama, Eri., kajihara, M., Yokoyama, A., Takada, A., Sodnomdarjaa, R., Kida, H.	Characterization of H5N1 highly pathogenic avian influenza virus strains isolated from migratory waterfowl in Mongolia on the way back from the southern Asia totheir northern territory.	Virology	Vol.406	p.88-94	2010
Sawada A, Komase K, Nakayama T.	AIK-C measles vaccine expressing fusion protein of respiratory syncytial virus induces protective antibodies in cotton rats.	Vaccine	29	1481-1490	2011

Hishiki H, Ishiwada N, Fukasawa C, Abe K, Hoshino T, Aizawa J, Ishikawa N, Kohno Y	Incidence of bacterial coinfection with respiratory syncytial virus bronchopulmonary infection in pediatric inpatients.	J Infect Chemother.	11	Epub	2010
Mori T, Morii M, Terada K, Wada Y, Kuroiwa Y, Hotsubo T, Fuse S, Nishioka S, Nishioka T, Tsutsumi H.	Clinical characteristics and computed tomography findings in children with 2009 pandemic influenza A (H1N1) viral pneumonia.	Scand J Infect Dis	43	47-54	2011

IV　研究成果の刊行物・別刷

Microbiology and Immunology

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ORIGINAL ARTICLE

Increase of tumor necrosis factor-α in the blood induces early activation of matrix metalloproteinase-9 in the brain

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ABSTRACT

Increases of cytokine in the blood play important roles in the pathogenesis of influenza-associated encephalopathy. TNF-α was administered intravenously to wild-type mice, after which blood, CSF and brain tissue were obtained, and changes in BBB permeability, the amounts of MMP-9 and TIMP-1, and the localization of activated MMP were assessed. There was a significant increase in BBB permeability after 6 and 12 hr. MMP-9 was increased after 3 hr in the brain and cerebrospinal fluid, which was earlier than in the serum. TIMP-1 protein in the brain increased significantly after MMP-9 had increased. Activation of MMP-9 was observed in neurons in the cerebral cortex and hippocampus, and in vascular endothelial cells. These findings suggest that an increase in blood TNF-α promotes activation of MMP-9 in the brain, and may also induce an increase in permeability of the BBB. Early activation of MMP-9 in the brain may contribute to an early onset of neurological disorders and brain edema prior to multiple organ failure in those inflammatory diseases associated with highly increased concentrations of TNF-α in the blood, such as sepsis, burns, trauma and influenza-associated encephalopathy.

Key words blood-brain barrier, influenza associated encephalopathy, matrix metalloproteinase-9, tumor necrosis factor-alpha.

TNF-α is a pleiotrophic pro-inflammatory cytokine which is produced by various cells including activated monocytes, macrophages, B and T cells and fibroblasts. TNF-α plays a role in the induction of septic shock, autoimmune diseases, rheumatoid arthritis, inflammation and diabetes. TNF-α can change the permeability of the BBB where there is inflammation in the brain, as observed in meningitis (1), brain abscess (2) or brain ischemia (3).

MMP belong to a family of zinc-dependent proteolytic enzymes which are capable of degrading the components of the extracellular matrix in a variety of physiological and pathological conditions including embryogenesis, cell migration, tissue modeling, wound healing, and inflammation (4, 5). MMP are secreted as inactive pro-forms which are activated in proteolytic cascade reactions to yield active MMP. An uncontrolled increase in active MMP can result in tissue injury and persistent inflammation. MMP-9, a member of this enzyme family, is capable of degrading collagen IV, a major component of the basal membrane of the cerebral vascular endothelium, while also promoting a disruption of the BBB. MMP-9 is involved in acute brain injury such as cerebral ischemia (6–9), intracerebral hemorrhage (10, 11), neurodegenerative diseases (12, 13) and bacterial infections (14–16). In addition, MMP-9 is

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List of Abbreviations: BBB, blood-brain barrier; CSF, cerebrospinal fluid; DQ, dye-quenched; GFAP, glial fibrillary acidic protein; FITC, fluorescein isothiocyanate; IL-6, interleukin-6; IL-1β, interleukin-1β; MMP, matrix metalloproteinase; OCT, optimal cutting temperature; TIMP, tissue inhibitor of matrix metalloproteinase; TNF-α, tumor necrosis factor-alpha.

produced in the brain by several cell types, including endothelial cells, microglia, astrocytes, and neurons. MMP-9 activity is strictly regulated via gene transcription and dynamic inhibition by TIMP-1.

Acute encephalopathy, a severe complication of influenza infection in children, often results in severe brain edema and a grave prognosis (17). Several studies have demonstrated that concentrations of inflammatory cytokines, such as TNF-α, interleukin-6 and interleukin-1β, are increased in the serum or CSF of these patients (18–20). Interestingly, although neurological symptoms can occur at an early stage and a severe prognosis can easily follow, no influenza virus is found in the brain. Although these facts suggest that inflammatory cytokines are involved in the development of neurological injury in influenza-associated encephalopathy, the mechanism is still unclear. In the acute phase, serum concentrations of MMP-9 are much greater in influenza-associated encephalopathy than in influenza infection with no neurologic complications (21).

Although *in vitro* TNF-α can induce MMP-9 activation in various cells, such as macrophages, granulocytes, astrocytes, neurons, microglial cells, brain endothelial cells and choroid plexus epithelial cells, there have so far been no reports describing whether, *in vivo*, MMP-9 is actually activated in the brain in response to an increase of serum TNF-α. The current study evaluated time-dependent changes in the amount of MMP-9 in brain tissue, serum and CSF to determine the relationships between increases in serum TNF-α and activation of MMP-9 in the brain. In addition, under conditions of experimental hypercytokinemia with TNF-α *in vivo*, this study also showed changes in the brain in both TIMP-1 protein and localization of increased MMP.

MATERIALS AND METHODS

Animals

Eight-week-old male C57BL/6 mice weighing 25–30 g were obtained from Japan SLC, Shizuoka, Japan. The animals were housed in an air-conditioned room with a 12-hr light/dark cycle and free access to food and water. All procedures involving the animals were conducted in accordance with the Guidelines for Animal Experiments at Okayama University Advanced Science Research Center.

Treatment of animals and tissue preparation

One hundred μg/kg of murine recombinant TNF-α, IL-6 or IL-1β (Pepro Tech EC, London, UK) was injected intravenously into mice. Some mice were treated with saline as a vehicle control using the same volume and time schedule as for the cytokine treatments. The mice were deeply anesthetized with 1% halothane in 30% oxygen and 70% nitrous oxide using a face mask at 0, 3, 6, 12, 24 and 48 hr after the intravenous injection, and both blood and CSF quickly collected. They were then perfused transcardially with ice-cold PBS, after which their brains were quickly removed, divided into hemispheres, immediately frozen in liquid nitrogen, and stored at −80°C. After complete coagulation the blood samples were centrifuged at 3000 *g* for 10 min. The supernatants were collected and stored at −80°C. CSF samples were directly stored at −80°C.

Quantitative evaluation of extravasation of Evans blue

Vascular permeability was quantitatively evaluated using Evans blue dye. 2% Evans blue (Wako, Osaka, Japan) in saline was injected intravenously (4 ml/kg) as a BBB permeability tracer 1 hr prior to the termination of each time point ($n = 5$ per time point). The mice were killed one hr after this injection and then transcardially perfused with 100 ml of ice-cold PBS to remove the intravascular dye. The brains were removed, homogenized in 1 ml of 50% trichloroacetic acid, and then centrifuged (10 000 *g*, 20 min). A spectrophotometer was used to quantify the dye concentrations at 605 nm. The calculations were based on external standards (50–1000 μg/ml) dissolved in the same solvent. The amount of extravasated Evans blue was quantified as one microgram per gram of brain.

MMP inhibition *in vivo*

Intraperitoneal GM6001 (Calibiochem, San Diego, CA, USA) at 65 mg/kg or vehicle (1.5 ml of 10% dimethyl sulfoxide) was injected 1 hr before administration of TNF-α. Six hours after the administration of TNF-α, 2% Evans blue dye was injected intravenously and the brains dissected out 1 hr later.

Protein extraction

All procedures were carried out at 4°C. The brains were homogenized in a Polytron homogenizer (Paterson, NJ, USA) in 2 ml of working buffer (50 mM Tris-HCl, 150 mM NaCl, 5 mM $CaCl_2$, 1% Triton X-100, 0.05% BRIJ-35) and centrifuged, then the supernatants were stored at −80°C for further analysis. The total protein concentration was measured using the BCA protein assay kit (Pierce Biotechnology, Rockford, IL, USA). The total protein concentration of the serum samples was also measured using the same method.

ELISA

ELISA was used to measure the amounts of MMP-3, MMP-9 and TIMP-1 in order to detect the proteins that were expressed in the brain ($n = 5$ per time point). The MMP-9 assay recognizes both the pro- and active forms of MMP-9. Each capture antibody, the biotinylated detection antibodies, protein standards, and detection reagents for the ELISA were obtained from R&D Systems (Minneapolis, MN, USA).

Gelatin zymography

Equal amounts of prepared protein samples were diluted in twice the volume of sample buffer (125 mM Tris-HCl, 20% glycerol, 4% SDS, 0.003% bromophenol blue) without β-mercaptoethanol and were then loaded onto 10% SDS-PAGE gels containing 1% gelatin under non-reducing conditions. The gels were separated by electrophoresis at 4°C. After electrophoresis, the gels were washed twice for 20 min each in renaturing buffer containing 2.5% Triton X-100 at room temperature to remove any SDS, and were then incubated for 24 hr in developing buffer (10 mM CaCl2, 50 mM Tris, 50 mM NaCl) at 37°C. After incubation, the gel was stained for 1 hr with 0.5% Coomassie blue G-250 in 40% methanol and 10% acetic acid and was then destained in the same buffer without dye.

In situ gelatin zymography

In situ gelatin zymography was performed with DQ-gelatin-FITC (Molecular Probes, Eugene, OR, USA) on frozen fresh brain slices obtained from the mice. The brains were dissected out, immediately embedded in Tissue-Tek OCT compound (Sakura Finetechnical, Tokyo, Japan) and frozen on dry ice. Ten μm thick coronal sections were cut using a cryostat, and then air-dried for 30 min at room temperature, re-hydrated in PBS and incubated overnight at 37°C with 40 μg/ml of DQ-gelatin-FITC in PBS. Excess fluorogenic substrate was removed by washing three times in PBS. The brain slices were incubated with DQ-gelatin-FITC including the MMP inhibitor GM6001 (Calibiochem) to determine whether the gelatinolytic activity was due to matrix metalloproteinases. After *in situ* gelatin zymography had been done, immunohistochemistry was performed on the same brain sections to assess the cellular localization of gelatinases. Neurons were stained with NeuroTrace fluorescent Nissl stain solution (1:100; Molecular Probes), astrocytes with anti-GFAP antibody (1:200; Molecular Probes) and cerebral vascular endothelial cells with anti-claudin-5 H-52 antibody (1:100; Santa Cruz Biotechnology, Santa Cruz, CA, USA). After blocking with 2% BSA the sections were incubated for 20 min at room temperature with NeuroTrace fluorescent Nissl stain solution to stain the neurons. After washing in PBS, the sections were incubated for 2 hr at room temperature. The sections were treated with primary antibodies for astrocytes or vascular endothelial cells for 2 hr at 37°C after blocking. The sections were then washed in PBS, and incubated with secondary antibody solutions (anti-rabbit Alexa Fluor 594, 1:200; Molecular Probes) for 30 min at 37°C. Finally, the slides were washed in PBS and mounted on Vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA). The tissue sections were photographed by fluorescence microscopy (Keyence BZ-9000) using the Keyence BZ-2 system (Keyence, Osaka, Japan).

Statistical analysis

All analyses were performed using the SPSS for Windows software package (SPSS, Chicago, IL, USA). Quantitative data were expressed as the mean ± d standard deviation. Statistical comparisons were conducted using ANOVA followed by the Tukey–Kramer tests for multiple comparisons. Differences with a P value of < 0.05 were considered to be statistically significant.

RESULTS

Quantitative evaluation of Evans blue extravasation

Changes in vascular permeability of the BBB were observed by measuring the amount of Evans blue leaking into the brain from the blood. Intravenous murine TNF-α induced a significant increase in Evans blue leaking outside the blood vessels 6, 12 and 24 hr later, in comparison to that found at the time of TNF-α injection ($P < 0.05$; Fig. 1). Intravenous saline as control produced no change in the amount of extravasated Evans blue up until 48 hr later. In comparison to the controls, significant increases in Evans blue extravasation were also seen both 6 and 12 hr after TNF-α injection ($P < 0.05$). Murine recombinant IL-6 and IL-1β administered in the same way had no effect on the amount of extravasated Evans blue into the brain 24 and 48 hr later (Fig. 2a, b).

Effect of MMP inhibition on Evans blue extravasation

GM6001 is a potent broad-spectrum inhibitor of matrix metalloproteinases and has been used in a number of animal models of disease where matrix metalloproteinases are thought to be involved. GM6001 solution was administered intraperitoneally prior to intravenous injection of TNF-α. Extravasated dye in the brain was measured 6 hr after injection of TNF-α. The amount of extravasated

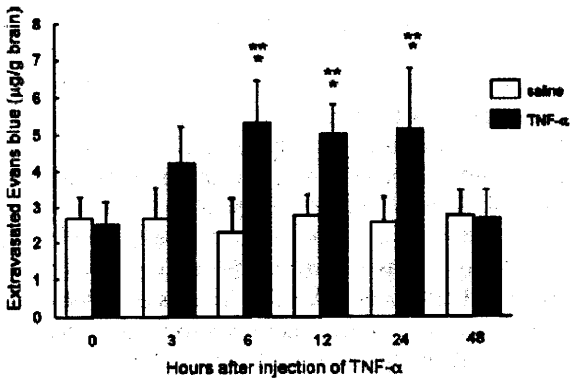

Fig. 1. Quantitative evaluation of Evans blue extravasation in the brain after intravenous injection of TNF-α (100 μg/kg). Extravasated Evans blue is expressed as μg/g of brain tissue. The values represent the mean ± standard deviation ($n = 5$ per time point). *$P < 0.05$, in comparison to 0 hr after TNF-α administration; **$P < 0.05$, in comparison to saline administration at the each time point.

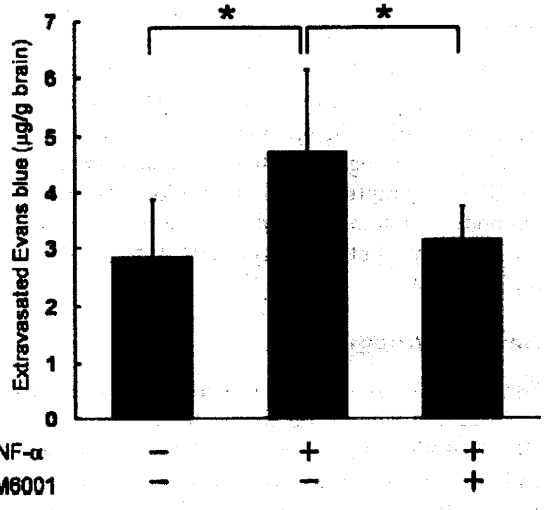

Fig. 3. Inhibition of the increase in Evans blue extravasation after intravenous injection of TNF-α (100 μg/kg) by intraperitoneal administration of an MMP inhibitor (GM6001). The values represent the mean ± standard deviation ($n = 8$ per time point). *$P < 0.05$.

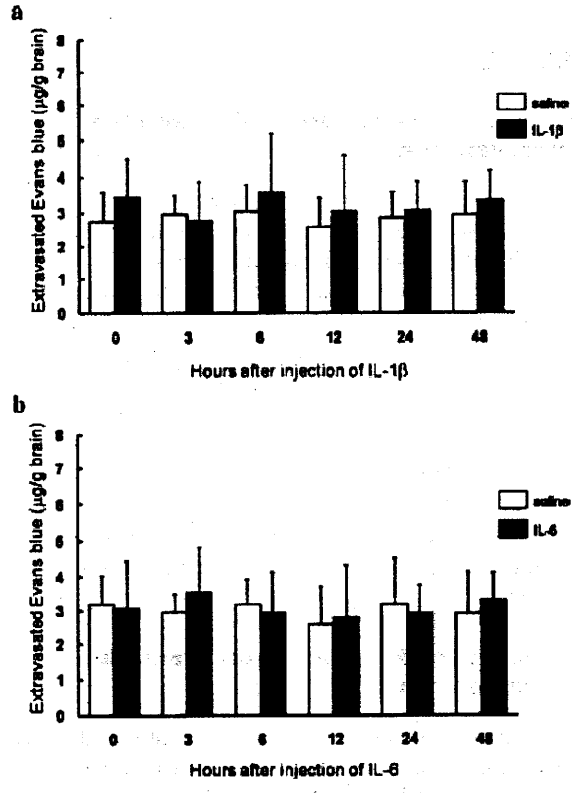

Fig. 2. Quantitative evaluation of Evans blue extravasation after intravenous injection of (a) IL-6 (100 μg/kg) and (b) IL-1β (100 μg/kg). The amount of extravasated Evans blue dye had not changed by 48 hr after the injection ($n = 5$ per time point).

dye was found to be significantly less in the brains of mice treated with GM6001 solution than in mice treated with solvent solution only ($P < 0.05$; Fig. 3).

MMP-3, MMP-9 and TIMP-1 protein in the brain

Time-dependent changes in MMP-9 and TIMP-1 proteins in the brain after TNF-α injection were evaluated using ELISA. The assay for MMP-9 was designed to measure total mouse MMP-9 (pro-MMP-9, active MMP-9 and TIMP-complexed MMP-9). MMP-9 protein in the brain was increased significantly at 3 hr post injection ($P < 0.01$), and gradually decreased thereafter (Fig. 4a). TIMP-1 protein in the brain was significantly increased at 12 hr post injection ($P < 0.01$), but had decreased significantly by 24 hr post injection (Fig. 4b). Time-dependent changes in MMP-3 protein in the brain were also evaluated using ELISA. MMP-3 protein was not detected in the brain (Fig. 4c). MMP-3 protein was detected from 0 to 48 hr in the serum, but was not significantly increased.

MMP-2 and MMP-9 in the brain, serum and CSF

After electrophoresis under non-reducing conditions, gelatin zymography classically produces two bands for murine MMP-2 (proenzyme and active forms; 72 and 65 kDa, respectively) and murine MMP-9 (proenzyme and active forms; 105 and 97 kDa, respectively) on the basis of molecular weight. Time-dependent changes in MMP-9 in the brain, serum and CSF were evaluated using

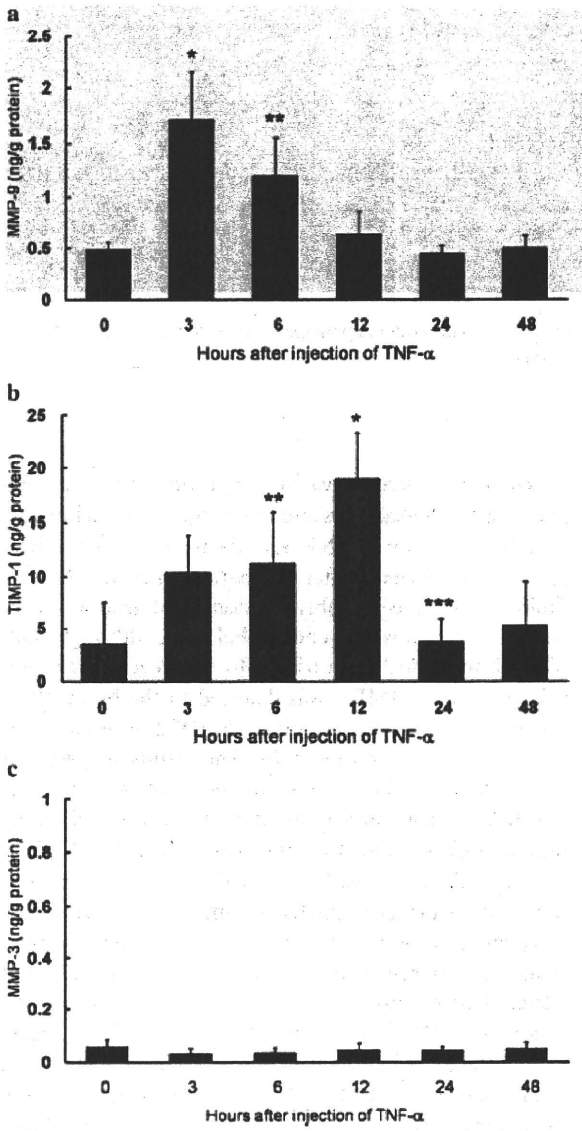

Fig. 4. Time-dependent changes in the amounts of (a) MMP-9, (b) TIMP-1 and (c) MMP-3 protein in the brain after intravenous injection of TNF-α as quantified by ELISA. The amount of each protein is expressed as ng/g of brain tissue. The values represent the mean ± standard deviation ($n = 5$ per time point). *$P < 0.01$, in comparison to at the time of TNF-α administration; **$P < 0.05$, in comparison to at the time of TNF-α administration; ***$P < 0.01$, in comparison to 12 hr after TNF-α administration.

gelatin zymography. MMP-9 increased predominantly in the active form in the brain, serum and CSF. The amounts of active MMP-9 in the brain and CSF peaked 3 hr after injection (Fig. 5). In comparison, the amount of active-MMP-9 in the serum was increased at 6 hr after injection. MMP-2 degrades gelatin as well as MMP-9. Active MMP-2 was continuously detectable in the brain and CSF from

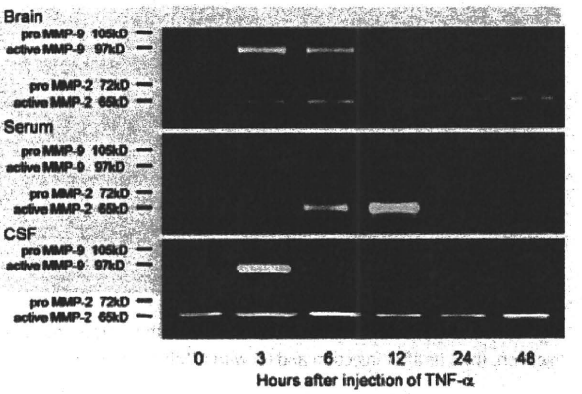

Fig. 5. Gelatin zymography after intravenous injection of TNF-α (100 μg/kg). Active MMP-9 was detectable in the brain, serum and CSF. MMP-9 increased transiently after the injection, and active MMP-9 in the brain and CSF were detected earlier than in the serum. MMP-2 was continuously detectable and demonstrated no significant change in the brain and CSF until 48 hr later, but MMP-2 increased in the serum at 6 and 12 hr post injection.

0 to 48 hr and did not seem to change significantly after administration of TNF-α. In contrast, both forms of MMP-2 were strongly detectable in the serum at 12 hr post injection. No other bands were detected by gelatin zymography.

Localization of gelatinases in the brain

MMP-9 can degrade not only collagen VI, but also gelatin. *In situ* gelatin zymography was used to determine in which parts of the brain gelatinases increased after TNF-α injection. After the brain had been put on gelatin labeled with FITC and incubated, the sites where there were activated gelatinases could be detected by the presence of digestion of the gelatin. We found that gelatinolytic activity was increased in the brain 3 hr later. In addition, *in situ* gelatin zymography with MMP inhibitor showed a decrease in most FITC signals (Fig. 6). Double staining with Nissl dye demonstrated that gelatinolytic activation was localized to the neurons of the cerebral cortex and hippocampus (Fig. 7). Activation was also detected in vascular endothelial cells by double staining with claudin-5. Astrocytes were found to come into contact with, or be surrounded by, cells that were positive for gelatinase activity, rather than being colocalized with this activity.

DISCUSSION

An increase in the vascular permeability of the BBB was induced by intravenous administration of murine recombinant TNF-α *in vivo*. Intracranial administration of TNF-α increases the permeability of the BBB *in vivo* (22). In

Fig. 6. *In situ* gelatin zymography of the brains after intravenous injection of TNF-α. *In situ* gelatin zymography was performed (a) at the time of injection, (b) 3 hr after injection and (c) with MMP inhibitor at 3 hr after the injection.

addition, an increase in BBB permeability was visualized by electron microscopy after intravenous administration of TNF-α (23). Our results are consistent with those of previous reports, suggesting that increased serum TNF-α can affect the permeability of the BBB.

An increase in MMP-9 protein, but not other MMP, in the brain was observed after intravenous administration of TNF-α, while MMP-9 gradually decreased after this injection. In addition, TIMP-1 protein, which can deactivate the gelatinase activity of MMP-9, increased in the brain after intravenous administration of TNF-α, the increase in TIMP-1 occurring later than that in MMP-9. TIMP-1 is produced in macrophages, brain endothelial cells, astrocytes and microglia *in vitro*, and TIMP-1 protein has been reported to be increased in the brain in studies of bacterial meningitis model mice (24) and herpes simplex virus encephalitis (25). The current results are consistent with those of previous reports, supporting the view that TIMP-1 protein is produced in the brain in response to a prior increase in MMP-9 protein.

An increase in serum TNF-α in promoted a transient increase in active MMP-9 in the brain, serum and CSF, and this active MMP-9 increased earlier in the brain and CSF than in the blood. Intracranial injection of TNF-α or the TNF superfamily can promote an increase in MMP-9 in the brain *in vivo* (22, 26). The current study suggests that active MMP-9 can increase in the brain in response to TNF-α transferred from blood vessels across the BBB. The time-lag of the increase in MMP-9 between brain and serum has not previously been estimated. This result may be due to differences between the cell types in response to stimulation by TNF-α. It is known that neurological symptoms can occur rapidly prior to multiple organ failure in influenza-associated encephalopathy. An early increase in active MMP-9 in the brain might therefore contribute to the induction of early neurological symptoms prior to the development of systemic inflammation.

Gelatinases were activated in neurons of the cerebral cortex and hippocampus and in vascular endothelium after administration of TNF-α. A decrease in gelatinolytic activity was observed after incubation with an MMP inhibitor; MMP are probably the dominant gelatinases activated in neurons and endothelial cells. Although both MMP-2 and MMP-9 contribute to *in situ* gelatin zymography, whereas MMP-2 was detected in the brain, there was no change in the amount of MMP-2 until 48 hr according to gelatin zymography. The results suggest that the increase in FITC signals in the brain at 3 hr post injection in comparison to that at the time of injection is probably due to activation of MMP-9. MMP-9 is activated in the walls of blood vessels and in neurons and astrocytes of the hippocampus in a mice model of cerebral ischemia (27), and in intracerebral hemorrhage (28), herpes simplex virus encephalitis (25) and bacterial meningitis (14). It is not clear whether activation of MMP-9 in the neurons led directly to digestion of the basal lamina of the BBB in the current study. Strong activation of MMP-9 in neurons may contribute to neuronal dysfunction rather than breakage of the basal lamina.

In summary, an increase in serum TNF-α can promote early activation of MMP-9 in the brain. This report supports the hypothesis that an increase in serum TNF-α can alter the permeability of the BBB by activating MMP-9 in the brain. The acute onset of neurological symptoms, such as influenza-associated encephalopathy, can often be seen in human patients. Although the influenza virus can result in inflammation in the lung separately from that in the brain, severe neurological symptoms can subsequently be observed in the absence of direct viral invasion of the brain. Our results have led us to believe that an increase in serum TNF-α can contribute to the development of neurological symptoms in influenza encephalopathy.

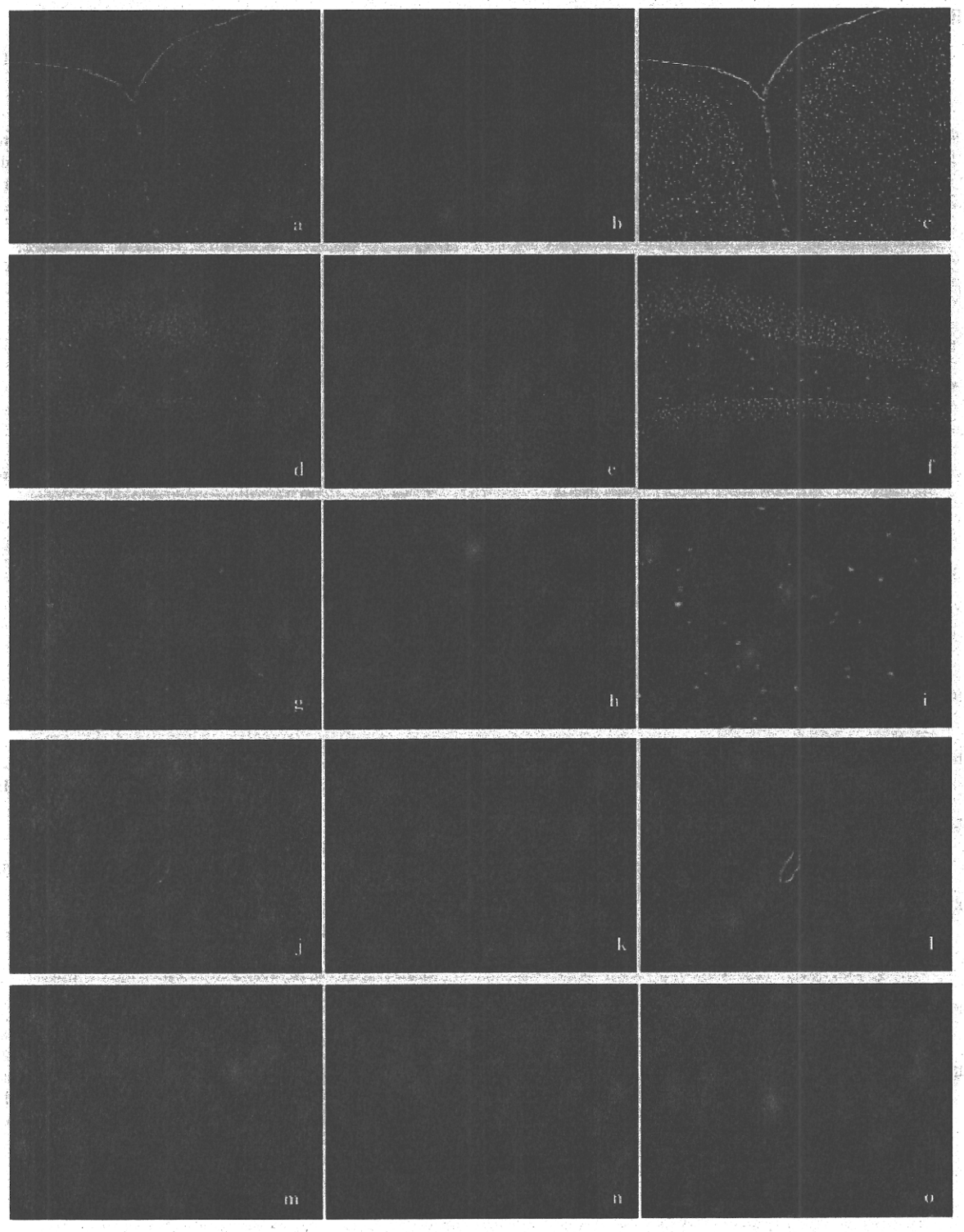

Fig. 7. (a, d, g, j, m) *In situ* gelatin zymography of the brains 3 hr after injection. (b, e, h) Doublestained with fluorescent Nissl stain, (k) with claudin-5 antibody and (n) with GFAP antibody. (c, f, i, l, o) Merged imaging. Co-localization of gelatinolytic activation in Nissl-stained neurons was observed (a–c) in the cerebral cortex and (d–f) hippocampus. Panels g–i show high magnifications of panels d–f respectively. Gelatinolytic activation was also recognized (j–l) in vascular endothelial cells, but (p–r) no co-localization was observed with GFAP-positive astrocytes.

REFERENCES

1. Kieseier B.C., Paul R., Koedel U., Seifert T., Clements J.M., Gearing A.J., Pfister H.W., Hartung H.P. (1999) Differential expression of matrix metalloproteinases in bacterial meningitis. *Brain* **122:** 1579–87.
2. Baldwin A.C., Kielian T. (2004) Persistent immune activation associated with a mouse model of Staphylococcus aureus-induced experimental brain abscess. *J Neuroimmunol* **151:** 24–32.
3. Yang G.Y., Gong C., Qin Z., Liu X.H., Lorris Betz A. (1999) Tumor necrosis factor alpha expression produces increased blood-brain barrier permeability following temporary focal cerebral ischemia in mice. *Brain Res Mol Brain Res* **69:** 135–43.
4. Yong V.W., Power C., Forsyth P., Edwards D.R. (2001) Metalloproteinases in biology and pathology of the nervous system. *Nat Rev Neurosci* **2:** 502–11.
5. Rosenberg G.A. (2002) Matrix metalloproteinases in neuroinflammation. *Glia* **39:** 279–91.
6. Romanic A.M., White R.F., Arleth A.J., Ohlstein E.H., Barone F.C. (1998) Matrix metalloproteinase expression increases after cerebral focal ischemia in rats: inhibition of matrix metalloproteinase-9 reduces infarct size. *Stroke* **29:** 1020–30.
7. Fujimura M., Gasche Y., Morita-Fujimura Y., Massengale J., Kawase M., Chan P.H. (1999) Early appearance of activated matrix metalloproteinase-9 and blood-brain barrier disruption in mice after focal cerebral ischemia and reperfusion. *Brain Res* **842:** 92–100.
8. Lee S.R., Tsuji K., Lee S.R., Lo E.H. (2004) Role of matrix metalloproteinases in delayed neuronal damage after transient global cerebral ischemia. *J Neurosci* **24:** 671–8.
9. Lee H., Park J.W., Kim S.P., Lo E.H., Lee S.R. (2009) Doxycycline inhibits matrix metalloproteinase-9 and laminin degradation after transient global cerebral ischemia. *Neurobiol Dis* **34:** 189–98.
10. Rosenberg G.A., Navratil M. (1997) Metalloproteinase inhibition blocks edema in intracerebral hemorrhage in the rat. *Neurology* **48:** 921–6.
11. Horstmann S., Su Y., Koziol J., Meyding-Lamadé U., Nagel S., Wagner S. (2006) MMP-2 and MMP-9 levels in peripheral blood after subarachnoid hemorrhage. *J Neurol Sci* **251:** 82–6.
12. Lorenzl S., Albers D.S., Narr S., Chirichigno J., Beal M.F. (2002) Expression of MMP-2, MMP-9, and MMP-1 and their endogenous counterregulators TIMP-1 and TIMP-2 in postmortem brain tissue of Parkinson's disease. *Exp Neurol* **178:** 13–20.
13. Kiaei M., Kipiani K., Calingasan N.Y., Wille E., Chen J., Heissig B., Rafii S., Lorenzl S., Beal M.F. (2007) Matrix metalloproteinase-9 regulates TNF-alpha and FasL expression in neuronal, glial cells and its absence extends life in a transgenic mouse model of amyotrophic lateral sclerosis. *Exp Neurol* **205:** 74–81.
14. Leib S.L., Leppert D., Clements J., Täuber M.G. (2000) Matrix metalloproteinases contribute to brain damage in experimental pneumococcal meningitis. *Infect Immun* **68:** 615–20.
15. Leib S.L., Clements J.M., Lindberg R.L., Heimgartner C., Loeffler J.M., Pfister L.A., Täuber M.G., Leppert D. (2001) Inhibition of matrix metalloproteinases and tumour necrosis factor alpha converting enzyme as adjuvant therapy in pneumococcal meningitis. *Brain* **124:** 1734–42.
16. Lee K.Y., Kim E.H., Yang W.S., Ryu H., Cho S.N., Lee B.I., Heo J.H. (2004) Persistent increase of matrix metalloproteinases in cerebrospinal fluid of tuberculous meningitis. *J Neurol Sci* **220:** 73–8.
17. Morishima T., Togashi T., Yokota S., Okuno Y., Miyazaki C., Tashiro M., Okabe N.; Collaborative Study Group on Influenza-Associated Encephalopathy in Japan. (2002) Encephalitis and encephalopathy associated with an influenza epidemic in Japan. *Clin Infect Dis* **35:** 512–7.
18. Kawada J., Kimura H., Ito Y., Hara S., Iriyama M., Yoshikawa T., Morishima T. (2003) Systemic cytokine responses in patients with influenza-associated encephalopathy. *J Infect Dis* **188:** 690–8.
19. Ichiyama T., Isumi H., Ozawa H., Matsubara T., Morishima T., Furukawa S. (2003) Cerebrospinal fluid and serum levels of cytokines and soluble tumor necrosis factor receptor in influenza virus-associated encephalopathy. *Scand J Infect Dis* **35:** 59–61.
20. Ichiyama T., Morishima T., Isumi H., Matsufuji H., Matsubara T., Furukawa S. (2004) Analysis of cytokine levels and NF-kappaB activation in peripheral blood mononuclear cells in influenza virus-associated encephalopathy. *Cytokine* **27:** 31–7.
21. Ichiyama T., Morishima T., Kajimoto M., Matsushige T., Matsubara T., Furukawa S. (2007) Matrix metalloproteinase-9 and tissue inhibitors of metalloproteinases 1 in influenza-associated encephalopathy. *Pediatr Infect Dis J* **26:** 542–4.
22. Rosenberg G.A., Estrada E.Y., Dencoff J.E., Stetler-Stevenson W.G. (1995) Tumor necrosis factor-alpha-induced gelatinase B causes delayed opening of the blood-brain barrier: an expanded therapeutic window. *Brain Res* **703:** 151–5.
23. Abraham C.S., Deli M.A., Joo F., Megyeri P., Torpier G. (1996) Intracarotid tumor necrosis factor-alpha administration increases the blood-brain barrier permeability in cerebral cortex of the newborn pig: quantitative aspects of double-labelling studies and confocal laser scanning analysis. *Neurosci Lett* **208:** 85–8.
24. Sellner J., Leib S.L. (2006) In bacterial meningitis cortical brain damage is associated with changes in parenchymal MMP-9/TIMP-1 ratio and increased collagen type IV degradation. *Neurobiol Dis* **21:** 647–56.
25. Sellner J., Simon F., Meyding-Lamade U., Leib S.L. (2006) Herpes-simplex virus encephalitis is characterized by an early MMP-9 increase and collagen type IV degradation. *Brain Res* **1125:** 155–62.
26. Polavarapu R., Gongora M.C., Winkles J.A., Yepes M. (2005) Tumor necrosis factor-like weak inducer of apoptosis increases the permeability of the neurovascular unit through nuclear factor-kappa B pathway activation. *J Neurosci* **25:** 10094–100.
27. Asahi M., Wang X., Mori T., Sumii T., Jung J.C., Moskowitz M.A., Fini M.E., Lo E.H. (2001) Effects of matrix metalloproteinase-9 gene knock-out on the proteolysis of blood-brain barrier and white matter components after cerebral ischemia. *J Neurosci* **21:** 7724–32.
28. Copin J.C., Merlani P., Sugawara T., Chan P.H., Gasche Y. (2008) Delayed matrix metalloproteinase inhibition reduces intracerebral hemorrhage after embolic stroke in rats. *Exp Neurol* **213:** 196–201.

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Induction of Cross-Protective Immunity Against Influenza A Virus H5N1 by an Intranasal Vaccine With Extracts of Mushroom Mycelia

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The identification of a safe and effective adjuvant that is able to enhance mucosal immune responses is necessary for the development of an efficient inactivated intranasal influenza vaccine. The present study demonstrated the effectiveness of extracts of mycelia derived from edible mushrooms as adjuvants for intranasal influenza vaccine. The adjuvant effect of extracts of mycelia was examined by intranasal co-administration of the extracts and inactivated A/PR8 (H1N1) influenza virus hemagglutinin (HA) vaccine in BALB/c mice. The inactivated vaccine in combination with mycelial extracts induced a high anti-A/PR8 HA-specific IgA and IgG response in nasal washings and serum, respectively. Virus-specific cytotoxic T-lymphocyte responses were also induced by administration of the vaccine with extract of mycelia, resulting in protection against lethal lung infection with influenza virus A/PR8. In addition, intranasal administration of NIBRG14 vaccine derived from the influenza A/Vietnam/1194/2004 (H5N1) virus strain administered in conjunction with mycelial extracts from *Phellinus linteus* conferred cross-protection against heterologous influenza A/Indonesia/6/2005 virus challenge in the nasal infection model. In addition, mycelial extracts induced proinflammatory cytokines and CD40 expression in bone marrow-derived dendritic cells. These results suggest that mycelial extract-adjuvanted vaccines can confer cross-protection against variant H5N1 influenza viruses. The use of extracts of mycelia derived from edible mushrooms is proposed as a new safe and effective mucosal adjuvant for use for nasal vaccination against influenza virus infection. ***J. Med. Virol. 82:128–137, 2010.*** © 2009 Wiley-Liss, Inc.

KEY WORDS: avian influenza; adjuvant; immunoglobulin A; hetero-subtypic immunity

INTRODUCTION

When developing a vaccine, both prophylactic effectiveness and safety must be considered. The mucosal immune system of the respiratory tract, which is a primary site of influenza infection, is usually the first immunological barrier against influenza virus infection. The influenza virus is able to cause annual epidemics of influenza by altering the antigenic properties of its surface hemagglutinin (HA), the antigenic glycoprotein that is responsible for binding of the virus to sialic acids

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on the surface of susceptible cells. Inactivated vaccines against the influenza virus are administered parenterally to induce the production of anti-HA IgG antibodies that are highly protective against homologous virus infection, but less effective against heterologous virus infection [Ichinohe et al., 2008]. In contrast, a number of studies have shown that the mucosal immunity acquired through natural infection, which is mainly due to the secreted form of IgA in the respiratory tract, is more effective and provides greater cross-protection against virus infections than systemic immunity induced by parenteral vaccination in humans and mice [Asahi et al., 2002]. In this regard, induction of secreted IgA in the respiratory tract has a stronger potential to confer protection against unpredictable epidemics of influenza.

In the effort to develop effective intranasal vaccines, cholera toxin and *Escherichia coli* heat-labile toxin have been used as adjuvants to enhance the mucosal immune response [Tamura et al., 2005]. Although these toxins effectively provoke mucosal immune responses, they elicit adverse clinical side effects, such as nasal discharge and the facial paralysis of Bell's palsy [Mutsch et al., 2004]. Therefore, other adjuvants that are both effective and safe for human use have been developed for clinical application with intranasal influenza vaccine [Coulter et al., 2003; Hasegawa et al., 2005; Ichinohe et al., 2005, 2006, 2007a,b; Asahi-Ozaki et al., 2006].

It has been reported that extracts derived from certain mushrooms can elicit an innate immune response, resulting in activation of NF-κB, and strongly stimulate cellular and humoral immunity [Kim et al., 2003; Kuo et al., 2006]. These mushroom extracts induce phenotypic and functional maturation of dendritic cells, tumoricidal activity in macrophages, and augmentation of natural killer cell activity [Sorimachi et al., 2001; Kodama et al., 2005; Kim et al., 2006]. It has also been shown that oral administration of mushroom extracts has an anti-inflammatory effect [Bernardshaw et al., 2006] and decreases IgE levels through modulation of the Th1/Th2 balance [Inagaki et al., 2005; Lim et al., 2005]. In an experimental peritonitis model, mice that were treated orally with edible mushroom (*Agaricus blazei*) extracts prior to bacterial challenge showed significantly lower levels of septicemia and improved survival rates [Bernardshaw et al., 2006]. Extracts from these mushrooms also have been used in immunotherapy to prevent tumor growth and metastasis [Ukawa et al., 2000; Sanzen et al., 2001]. These findings prompted an investigation into whether the administration of intranasal influenza vaccine in combination with mushroom extracts would induce a protective immune response against a lethal and heterologous virus challenge. To accomplish this, the effectiveness of 12 mycelial extracts as an immune-enhancing adjuvant was assessed by comparison with the effects of the adjuvant, poly(I:C). The results of the present study demonstrate for the first time that intranasal administration of inactivated influenza virus vaccine in combination with mycelial extracts as a mucosal adjuvant induces cross-protective immune responses against homologous and heterologous variant influenza viruses, including highly pathogenic influenza A H5N1 virus isolates.

MATERIALS AND METHODS

Mice

Six- to 8-week-old female BALB/c mice were purchased from Japan SLC (Hamamatsu, Shizuoka, Japan). MyD88-deficient mice were kindly provided by Dr. Shizuo Akira (Osaka University, Osaka, Japan) [Adachi et al., 1998]. Mice were kept under specific pathogen-free conditions approved by the Institutional Animal Care and Use Committee of the National Institute of Infectious Diseases.

Vaccines and Influenza Viruses

HA vaccine (split-product virus vaccine) was prepared at the Kitasato Institute (Saitama, Japan) from members of the family Orthomyxoviridae, genus *Influenzavirus A, B*, species *Influenzavirus A*, including influenza A/PuertoRico/8/34 (A/PR8; H1N1). The virus was grown in allantoic cavities of 10- to 11-day-old fertile chicken eggs, purified and disintegrated with ethyl ether. The vaccines contained all proteins from the virus particle; however, the major component of the vaccine was HA (about 30% of the total protein). The A/PR8 virus used for the challenge experiments was adapted for use in mice by subculturing 148 times in ferret, 596 times in mouse, and 73 times in 10-day-old fertile chicken eggs.

The strains of influenza A virus H5N1 used in this study were A/Vietnam/1194/2004 and A/Indonesia/6/2005 [Gao et al., 1999]. The influenza A/Vietnam/1194/2004 virus and influenza A/Indonesia/6/2005 virus obtained from patients with H5N1 disease were propagated in 10-day-old embryonated chicken eggs for 2 days at 37 C. These viruses were stored at −80 C and viral titers were quantified by plaque assay using MDCK cells. The H5N1 vaccine used in these studies was NIBRG14, a formalin-inactivated whole virus vaccine derived from a recombinant avirulent avian virus containing modified HA and neuraminidase from the highly pathogenic avian influenza A/Vietnam/1194/2004 virus and other viral proteins from influenza A/PR/8/34 (H1N1) [Nicolson et al., 2005]. Modified HA lacks the multibasic amino acids at the cleavage site.

Preparation of Adjuvants

The mycelia extracts of *Phellinus linteus*, *Cordyceps militaris*, *Lyophyllum decastes*, *Macrolepiota gracilenta*, *Naematoloma sublateritium*, *A. blazei*, *Grifola frondosa*, *Ganoderma lucidum*, *Hericium erinaceum*, *Inonotus obliquus*, *Lentinula edodes*, and *Pleurotus nebrodensis* were kindly provided by Intelligence Biological Institute Co., Ltd (Nirasaki, Yamanashi, Japan). The extracts of mycelia were prepared as described previously [Inagaki et al., 2005]. Synthetic double-stranded RNA poly(I:C) was kindly provided by Toray

Industries, Inc. (Kamakura, Kanagawa, Japan). Lipopolysaccharide and Zymosan A from *Saccharomyces cerevisiae* were purchased from Sigma (St. Louis, MO).

Immunization and Infection

Five mice from each experimental group were anesthetized with diethyl ether and primarily immunized by dropping 1 μg of vaccine per mouse with various adjuvants into both nostril. Four weeks later, they were re-immunized in the same manner with the same adjuvant. For A/PR8 virus infection, two different infection protocols were used. Under the first protocol, each mouse was anesthetized and infected by intranasal application of 20 μl of virus suspension (1,000 PFU in PBS; 40 LD_{50}). This procedure induced total respiratory tract infection, which resulted in virus shedding from the nose and lungs, and led to death from viral pneumonia about 7 days later. Under the second protocol, anesthetized mice were infected by dropping 2 μl of virus suspension (1,000 PFU in PBS) into each nostril. The nasal-restricted volume (4 μl) of virus suspension induced nasally localized infection, which was not lethal. The nasal and lung wash virus titers were used as indices of protection in the upper and lower respiratory tracts of immunized mice, respectively. For infection with influenza A H5N1 virus, each mouse was anesthetized and 4 μl of PBS containing virus suspension with 1,000 PFU of H5N1 was administered intranasally (2 μl/nostril). The virus suspension remained in the local nasal area and could not enter the lung tissue, and the initial viral infection was limited to the nasal area, leading to death about 8 days later. H5N1 infection experiments were carried out in Biosafety Level 3 containment facilities, approved by the Guides for Animal Experiments Performed at National Institute of Infectious Diseases.

Measurement of Virus Titer and Antibody Titer

Serum, nasal washings, and bronchoalveolar washings were collected for measurement of virus titer and antibody titer from mice euthanized under anesthesia with chloroform. To collect nasal washings, a hypodermic needle was inserted into the posterior opening of the nasopharynx and 1 ml of PBS containing 0.1% bovine serum albumin was injected three times (1 ml total). Bronchoalveolar washings were collected by washing the trachea and lungs twice by injection of 1 ml PBS containing 0.1% BSA (2 ml total). The levels of IgA and IgG antibodies versus HA molecules purified from the A/PR8 viruses or NIBRG14 vaccine were determined by ELISA as described previously [Ichinohe et al., 2005, 2007a]. Briefly, ELISA was performed sequentially from the solid phase (EIA plates; Costar, Cambridge, MA) with a ladder of reagents as follows: first, HA molecules purified from influenza A/PR8 virus or NIBRG14; second, nasal washings, bronchoalveolar washings, or serum; third, either goat anti-mouse IgA antibody (α-chain specific; Amersham Biosciences, Piscataway, NJ) or goat anti-mouse IgG antibody (γ-chain-specific; Amersham Biosciences) conjugated with biotin; fourth, streptavidin conjugated with alkaline phosphatase (Life Technologies, Rockville, MD); and fifth, *p*-nitrophenylphosphate. The amount of chromogen produced was determined by measuring the absorbance at 405 nm using an ELISA reader. A twofold serial dilution of either purified A/PR8 HA-specific IgA (320 ng/ml) or A/PR8 HA-specific monoclonal IgG (160 ng/ml) was used as a standard, as described previously [Asahi et al., 2002]. The binding kinetics of the standard A/PR8 HA-specific monoclonal IgG was comparable with A/PR8 HA-specific IgG obtained from immunized mice. The A/PR8 HA-specific antibody concentration of each sample was determined from standard regression curves constructed for each assay with a programmed SJeia Autoreader (Model ER-8000; Sanko Junyaku, Tokyo, Japan). Standards for NIBRG14-reactive IgA and IgG antibody titration were prepared from the nasal washings or serum of survived mice after H5N1 virus challenge, and expressed using the same arbitrary units (160-unit). The NIBRG14-reactive antibody titer of each sample was determined from the standard regression curve constructed by twofold serial dilution of the 160-unit standard for each assay.

Before the hemagglutination inhibition tests, receptor-destroying enzyme (RDE II; Denka Seiken Co., Ltd, Tokyo, Japan) was added to the RBC-treated sera at 37°C overnight to inactivate non-specific hemagglutination inhibitors, followed by incubation at 56°C for 1 hr to inactivate RDE. Briefly, hemagglutination inhibition tests were performed by mixing 25 μl aliquots of serial twofold dilutions of the treated serum samples with four HA units of virus in microtiter plates and incubating them at room temperature for 30 min. Then, 50 μl of 0.5% chicken RBCs were added to each well and incubated at room temperature for 30–40 min. The hemagglutination inhibition titer was expressed as the reciprocal of the highest serum dilution that completely inhibited hemagglutination of four HA units of the virus.

The virus titer was measured as follows: 200 μl aliquots of serial 10-fold dilutions of the nasal washings were inoculated into MDCK cells in six-well plates. After incubation for 1 hr, each well was overlaid with 2 ml of agar medium. The number of plaques in each well was counted 2 days after inoculation. All experiments were repeated independently at least three times, and the data are presented as means ± SD.

Antigen-Specific T-Cell Response

Antigen-specific T-cell responses were measured as described previously [Ichinohe et al., 2005]. Spleens were harvested from mice 1 week after booster vaccination. After preparation of a single-cell suspension, T-cells were purified by depletion of $CD11b^+$ (Mac-1), $CD45R^+$ (B220), $DX5^+$, and $Ter\text{-}119^+$ cells using a magnetic cell sorter (MACS: Militenyi Biotec, Bergisch, Germany). To prepare antigen-presenting cells, splenocytes from normal BALB/c mice were depleted of

CD90 (Thy1.2)$^+$ cells by MACS and irradiated at 2,000 cGy.

T-cells were purified from the spleen (1×10^5 cells/well) and cultured with irradiated antigen-presenting cells (5×10^5 cells/well) in the presence or absence of A/PR8 vaccine (0.1, 1, or 10 μg/ml). After 4 days of culture, the cytokine concentration in the culture supernatant was measured by ELISA using a Mouse interferon-γ Immunoassay Kit (Biosource International, Camarillo, CA) according to the manufacturer's instructions.

Bone Marrow-Derived Dendritic Cell Preparation and Mycelial Extract Sensitivity Analysis

Bone marrow cells were isolated from the femurs and tibiae of wild-type or MyD88-deficient mice and bone marrow-derived dendritic cells were prepared as described [Inaba et al., 1992]. Lipopolysaccharide (1 μg/ml), Zymosan (2 μg/ml), *P. linteus* (5 μg/ml), *M. gracilenta* (5 μg/ml), *L. edodes* (5 μg/ml), or *G. frondosa* (5 μg/ml) was added on day 5 after cultivation with granulocyte–macrophage colony stimulating factor (Wako, Tokyo, Japan). On day 6, culture supernatants were collected for tumor necrosis factor (TNF)-α titration. Concentrations of TNF-α were determined by ELISA using a Mouse TNF-α Immunoassay Kit (Biosource International) according to the manufacturer's instructions.

Statistical Analysis

Comparisons between experimental groups were performed by Student's *t*-test. $P < 0.05$ was considered significant.

RESULTS

Local and Systemic Antibody Responses in BALB/c Mice Immunized Intranasally With the Hemagglutinin Vaccine and Mycelia Extracts as Adjuvants

The immune-enhancing effects of 12 varieties of mycelia extracts as mucosal adjuvants for intranasal influenza vaccine were investigated in BALB/c mice (Fig. 1). The mice were immunized twice intranasally with 1 μg of HA vaccine in combination with various adjuvants and the activities of the mycelial extracts were compared. Mice treated with mycelia extracts from *P. linteus*, *M. gracilenta*, *G. frondosa*, and *L. edodes*-adjuvanted vaccines developed sufficient levels of both HA-specific IgAs in the nasal washings and IgGs in the serum (Fig. 1). No specific antibodies were detected in the nasal washings or serum from control mice immunized with non-adjuvanted vaccine (Fig. 1).

Intranasal Immunization With the Hemagglutinin Vaccine Combined With Mycelia Extracts Protects Against Lethal Influenza Virus Lung Infection in Mice

Next, the protective effects of intranasal immunization with HA vaccine combined with mycelia extracts against lethal influenza virus lung infection were examined (Fig. 2). Mice immunized with *P. linteus*-adjuvanted vaccine showed equivalent amounts of IgG in the lung washings and serum when compared with those immunized with poly(I:C)-adjuvanted vaccine, and the viral titer of the lung washings was remarkably decreased compared with that of the control group (Fig. 2A). *M. gracilenta*- or *L. edodes*-adjuvanted

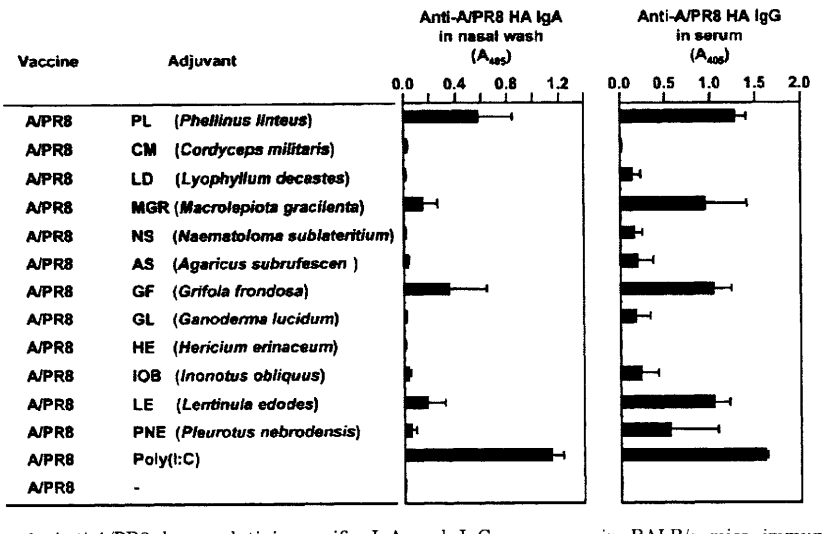

Fig. 1. Anti-A/PR8 hemagglutinin-specific IgA and IgG responses in BALB/c mice immunized intranasally with hemagglutinin vaccine alone, or in combination with various mycelia extracts or poly(I:C). Nasal washings and serum samples were collected 14 days after the final immunization. Antibody titers were measured by ELISA. Data represent the means ± SE of three mice per group.