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Induced eosinophilia and proliferation in *Angiostrongylus cantonensis*-infected mouse brain are associated with the induction of JAK/STAT1, IAP/NF-κB and MEKK1/JNK signals

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Abstract

Eosinophilic meningitis or meningoencephalitis caused by *Angiostrongylus cantonensis* is endemic to the Pacific area of Asia, especially Taiwan, Thailand, and Japan. Although eosinophilia is an important clinical manifestation of *A. cantonensis* infection, the role of eosinophils in the progress of the infection remains to be elucidated. In this experiment, we show that *A. cantonensis*-induced eosinophilia and inflammation might lead to the induction of IAP/NF-κB, JAK/STAT1 and MEKK1/JNK signals. The phosphorylation levels of JAK and JNK, STAT1, IAP, NF-κB and MEKK1 protein products were significantly increased after 12 days or 15 days of *A. cantonensis* infection. However, no significant differences in MAPKs such as Raf, MEK-1, ERK1/2 and p38 expression were found between control and infected mice. The activation potency of JAK/STAT1, IAP/NF-κB and MEKK1/JNK started increasing on day 3, with significant induction on day 12 or day 15 after *A. cantonensis* infection. Consistent results were noted in the pathological observations, including eosinophilia, leukocyte infiltration, granulomatous reactions, and time responses in the brain tissues of infected mice. These data suggest that the development of brain injury by eosinophilia of *A. cantonensis* infection is associated with activation of JAK/STAT1 signals by cytokines, and/or activation of MEKK1/JNK by oxidant stress, and/or activation of NF-κB by increasing IAP expression.

Introduction

Angiostrongylus cantonensis, which causes eosinophilic meningitis and eosinophilic meningoencephalitis in humans, has been found throughout South-east Asia and the Pacific area (Alicata, 1969), especially in Thailand (Punyagupta *et al.*, 1970), Taiwan (Yii, 1976), and Japan (Sato & Otsuru, 1983). Human angiostrongyliasis is usually related to eating habits, general socioeconomic status and the health status of the population. Humans acquire the infection by ingesting infected molluscs with third-stage infective larvae, which migrate to the brain, spinal cord, and eyes, causing severe clinical manifestations. In addition to changes in the central nervous system (CNS), eosinophilia in the cerebrospinal fluid (CSF) is another important clinical manifestation of the disease (Yoshimura *et al.*, 1984, 1994). Although pathological changes have been observed mainly in the CNS of

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infected patients, pulmonary injury, including pneumonia, granulomatous reactions of pulmonary vessels, and pulmonary haemorrhage, have also been reported (Normura & Lin, 1945; Yii *et al.*, 1968). Lesions of granulomatous inflammatory reactions are composed of mononuclear cells, lymphocytes, plasma cells, macrophages, and eosinophils. In previous studies, the accumulation of L-ferritin in macrophages in the lungs of rats infected with *A. cantonensis* has been related to the proliferation of connective tissue elements and an inflammatory response (Lee *et al.*, 1996). However, the mechanisms of this phenomenon in the CNS and lung remain unclear.

Among mitogen-activated protein kinases (MAPKs), a well-defined MAPK subfamily, the stress-activated protein kinases (SAPK)/ Jun N-terminal kinases (JNK) are members of the MAPK family that are activated by a variety of environmental stresses, inflammatory cytokines (Davis, 1999; Ichijo, 1999). Signal transducer and activator of transcription (STATs) are phosphorylated by Janus tyrosine kinase (JAKs), dissociate from the receptor, dimerize, and translocate into the nucleus. In addition to activating STATs, JAK kinases phosphorylate other signalling/adaptor proteins, linking JAK signalling to other pathways such as the MAP kinases. These parallel regulatory pathways are important in shaping the specificity of cellular responses to various stresses (Heim, 1999; Starr & Hilton, 1999). Recently, it has been suggested that the development of brain injury by eosinophilia of *A. cantonensis* infection is associated with nuclear factor-kappa binding protein (NF-κB) and/or nuclear protooncogene expression, which is activated by the tyrosine phosphorylation pathway (Lee *et al.*, 2000).

Therefore, the aim here is to reveal the signal transduction mechanism that might be involved with the pathogenesis of *A. cantonensis*-caused brain injury, in other words, whether *A. cantonensis* causes mouse eosinophilia and inflammation through induction of JAK/STAT1 and MEKK1/JNK signals.

Material and methods

Chemicals

Tris-HCl, EDTA, EGTA, 2-mercaptoethanol, Triton X-100, phenylmethylsulphonyl fluoride (PMSF), leupeptin, glycerol and bromophenol blue were purchased from Sigma Chemical Co. (St Louis, Missouri, USA). The protein assay kit was purchased from Kenlor Industries, Inc. (Costa Mesa, California, USA). Polyclonal antibodies against NF-κB, STAT1, IAP, MEKK1, Raf and MEK-1 were obtained from Signal Transduction Lab. (Kentucky, USA), and polyclonal antibodies against phospho-JAK, -JNK, -ERK1/2 and p38 were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, California, USA).

Parasitological procedures

Third-stage (infective) larvae of *A. cantonensis* were obtained from naturally infected snails (*Achatina fulica*) collected from fields in Pingtung County, southern Taiwan. Larvae were liberated from the minced snail tissues by pepsin digestion.

Male BABL/c mice (5–6 weeks old), purchased from the National Laboratory Animal Center, Taipei, Taiwan, were provided with Purina Laboratory Chow and water *ad libitum* and kept in the laboratory for more than 1 week before experimental infection. All procedures were performed in accordance with the guidelines for the care and use of laboratory animals, and approved by the Institute of Animal Care and Use Committee (IACUC).

Mice were divided into six groups, six mice per group, including one uninfected control group. Experimental groups of D_3, D_6, D_9, D_{12} and D_{15} were infected by oral inoculation with 60 *A. cantonesis* larvae on day 0 post-inoculation (PI) and mice were sacrificed on days 3, 6, 9, 12 and 15 PI. The mouse brains were excised and collected for histopathological study and immunological analyses.

Histopathology

Brain tissues were fixed in 10% neutral buffered formalin for 24 h, dehydrated in a graded ethanol series (50%, 75%, 100%) and xylene, then embedded in paraffin at 55°C for 24 h. Serial sections were cut at a 5 μm thickness for each organ from each mouse. Paraffin was removed by heating the sections for 1 h at 65°C. These sections were dewaxed by washing three times for 5 min each in xylene, then rehydrated through 100, 95, and 75% ethanol for 5 min each, and finally rinsed with distilled water. After staining with haematoxylin and eosin, pathological changes were examined under a light microscope.

Western blot analysis

Brain tissues were homogenized in buffer containing 0.1% Triton X-100, 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.5 mM KH_2HPO_4. Homogenates were centrifuged at 12000 rpm at 4°C for 10 min, and the protein contents of the supernatants were determined with protein assay kits using bovine serum albumin as the standard. An equal volume of loading buffer (62.5 mM Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 5% 2-mercaptoethanol, 0.05% bromophenol blue) was added to the samples, which contained 30 μg of brain tissue protein. The mixure was boiled for 5 min before being subjected to polyacrylamide gel electrophoresis. Samples were submitted to SDS-polyacrylamide gel under non-reducing conditions and electrotransferred to a nitrocellulose membrane at a constant current of 190 mA for 90 min. The membrane was then saturated with PBS containing 0.3% Tween 20 for 30 min at room temperature and allowed to react with primary antibody (β-actin, NF-κB, STAT1, IAP, MEKK1, Raf, MEK-1, phospho-JAK, -JNK, -ERK1/2 and p38) at 4°C overnight. The membrane was washed three times with PBS containing 0.05% Tween 20 (PBS-T), followed by incubation with HRP-conjugated secondary antibody for 60 min at room temperature to detect the bound primary antibody. The reactive protein was detected by enhanced chemiluminescence (Amersham, UK). To confirm an equivalent protein loading, membranes were stripped by incubation in 62.5 mM of Tris-HCl (pH 6.8), 2% SDS, and 100 mM 2-mercaptoethanol at 55°C, subsequently washed with PBS-T, and reprobed with anti-β-actin antibody (dilution 1:500).

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Results were reported as mean ± SD and statistical analysis was obtained using an unpaired *t*-test. A value of $P < 0.05$ was considered statistically significant.

Results

Western blot autoradiography and densitometric quantitation showed that the phosphorylation of JAK was significantly increased, with inductions of 4.4-, 3.8-, and 6.8- fold, respectively after 6, 9 and 12 days of infection. The increasing phosphorylation of JAK progressed from days 6 to 15 (fig. 1). In addition, the same potency existed not only in the STAT1 (fig. 2) and MEKK1 (fig. 3) protein expression but also in the time response after the *A. cantonensis* infection. The amounts of STAT1 and MEKK1 proteins were significantly elevated 13.4- and 3.2- fold on day 15 compared to those in uninfected mice.

The JNK phosphorylation progressed from days 6 to 15, with an induction of 13- fold after 15 days of infection (fig. 4). Moreover, a similar potency existed in the expression of NF-κB transcription factor and apoptosis protein inhibitor (IAP) in the time response after the *A. cantonensis* infection. The amounts of NF-κB and IAP were significantly elevated 10.3- and 11.3- fold on day 15 compared with uninfected mice (figs 5 and 6). The protein levels of MAPKs such as Raf, MEK-1 and ERK1/2 and phosphorylation of ERK1/2 and p38 underwent no changes after 15 days in *A. cantonensis*-infected mice (data not shown). Acute inflammatory reactions consisting mainly of eosinophilic and neutrophilic polymorphonuclear leukocytes were observed in the brain tissue of infected mice (fig. 7). Granulomata were found on the surface of cerebral or cerebellar hemispheres. These lesions were formed by epithelial cells, remnants of

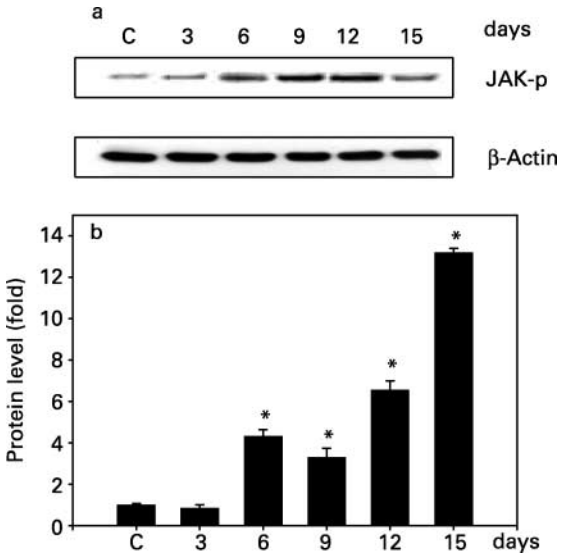

Fig. 1. (a) Western blot of the brain tissue of *Angiostrongylus cantonensis*-infected mice showing phosphorylation of JAK and (b) protein levels on days 3, 6, 9, 12 and 15 post-infection. Error bars indicate the standard deviations and the asterisks indicate significant differences from control (C, at 0 days time point), $^*P < 0.05$.

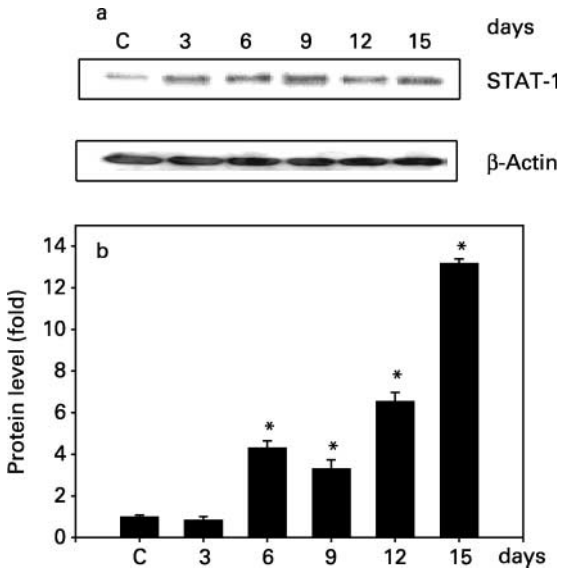

Fig. 2. (a) Western blot of the brain tissue of *Angiostrongylus cantonensis*-infected mice showing STAT1 and (b) protein levels on days 3, 6, 9, 12 and 15 post-infection. Error bars indicate the standard deviations and the asterisks indicate significant differences from control (C, at 0 days time point), $^*P < 0.05$.

A. cantonensis larvae, and mononuclear cells. However, larvae were also present in some areas of the brain without any surrounding inflammatory reaction. Macrophages containing haemosiderin pigment were found particularly in the meninges. All pathological alterations gradually increased after infection, reaching a high level on day 15. These results suggest that *A. cantonensis*-caused brain injury was associated with MEKK1/JNK,

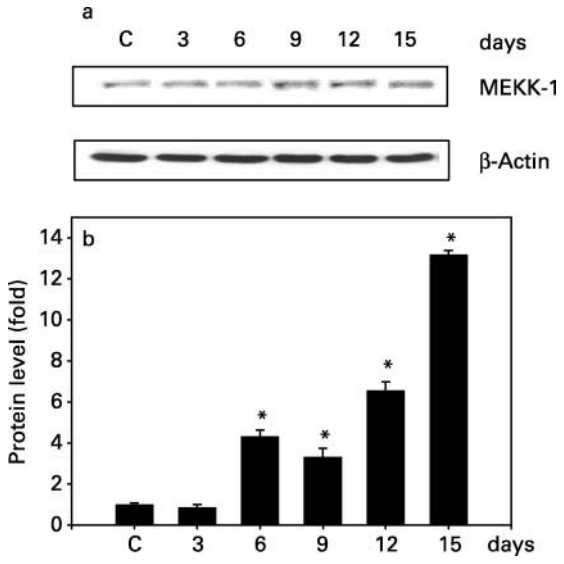

Fig. 3. (a) Western blot of the brain tissue of *Angiostrongylus cantonensis*-infected mice showing MEKK1 and (b) protein levels on days 3, 6, 9, 12 and 15 post-infection. Error bars indicate the standard deviations and the asterisks indicate significant differences from control (C, at 0 days time point), $^*P < 0.05$.

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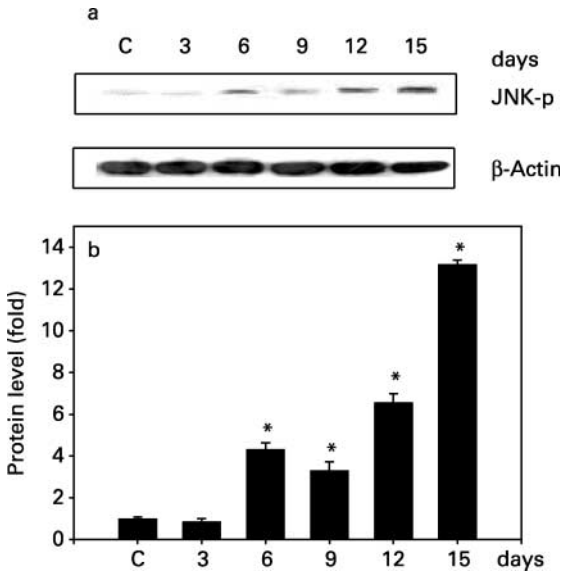

Fig. 4. (a) Western blot of the brain tissue of *Angiostrongylus cantonensis*-infected mice showing phosphorylation of JNK and (b) protein levels on days 3, 6, 9, 12 and 15 post-infection. Error bars indicate the standard deviations and the asterisks indicate significant differences from control (C, at 0 days time point), $^*P < 0.05$.

JAK/STAT1 and IAP/NF-κB activation, but not through a Raf/MEK signalling pathway.

Discussion

Angiostrongylus cantonensis is well known as a major causative agent of human eosinophilic meningoencephalitis. This neurotropic metastrongyloid nematode exhibits some remarkable and unique adaptations. In non-permissive hosts (e.g. mice and man), however, worms will invariably die in the CNS after reaching the subadult stage. Very little is known about the biochemical basis of such successful neurotropism and the mechanisms involved in this host–parasite relationship. Recently, it has been demonstrated that expressions of NF-κB and iNOS significantly increase in the mouse brain following *A. cantonensis* infection (Lee *et al.*, 2000). In previous studies, the accumulation of L-ferritin in macrophages in the pulmonary arteries of rats infected with *A. cantonensis* was considered to be an inflammatory response (Lee *et al.*, 1996).

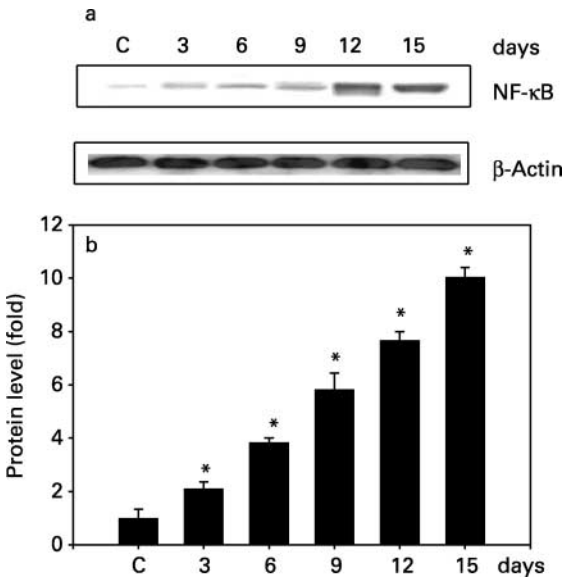

Fig. 5. (a) Western blot of the brain tissue of *Angiostrongylus cantonensis*-infected mice showing NF-κB and (b) protein levels on days 3, 6, 9, 12 and 15 post-infection. Error bars indicate the standard deviations and the asterisks indicate significant differences from control (C, at 0 days time point), $^*P < 0.05$.

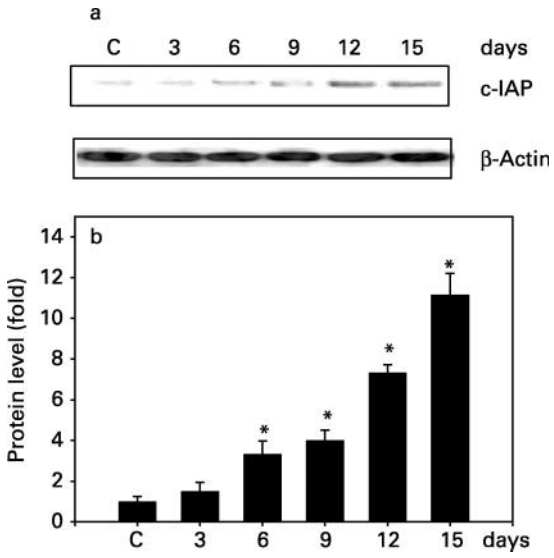

Fig. 6. (a) Western blot of the brain tissue of *Angiostrongylus cantonensis*-infected mice showing IAP and (b) protein levels on days 3, 6, 9, 12 and 15 post-infection. Error bars indicate the standard deviations and the asterisks indicate significant differences from control (C, at 0 days time point), $^*P < 0.05$.

The NF-κB protein has been demonstrated to be an important mediator for the genetic programmes underlying inflammation in the CNS (Grilli & Memo, 1997). This mediator activates genes involved in the immune and inflammatory responses. The target genes include cytokines, chemokines, MHC class I, iNOS, COX-2, P53 and IAP (Grilli *et al.*, 1993; Wu & Lozano, 1994; Baldwin, 1996; Uberti *et al.*, 1998). The IL-5 is a most important cytokine in provoking eosinophilia in *A. cantonensis* infection in mice (Sasaki *et al.*, 1993). Another major function of NF-κB is the regulation of programmed cell death (Baichwal & Baeuerle, 1997; Wang *et al.*, 1998). Nuclear factor-kappa binding protein (NF-κB) plays a protective role in the context of TNF-α-mediated apoptosis by regulating the expression of anti-apoptotic genes. The anti-apoptotic pathway leads to the activation of the transcription factor NF-κB that regulates gene expression such as the IAP gene family which protect cells from TNF-α-mediated apoptosis (Hu *et al.*, 1998; Wu *et al.*, 1998). In turn, some IAPs have been shown to modulate NF-κB activity (Baichwal & Baeuerle, 1997;

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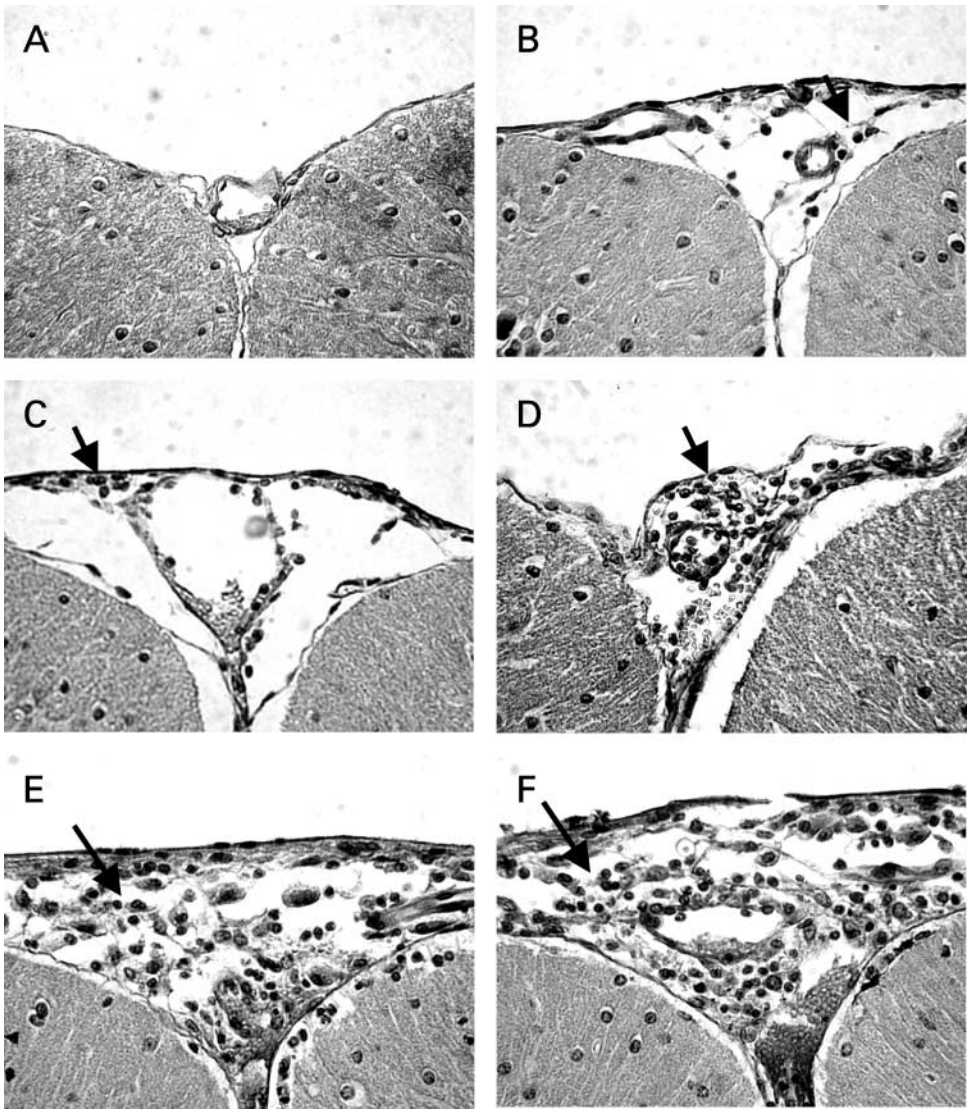

Fig. 7. Sequential pathogenesis of the brain tissues of *Angiostrongylus cantonensis*-infected mice; sections from control mice (A) and from mice after 3 (B), 6 (C), 9 (D), 12 (E), and 15 (F) days post-infection. Arrows indicate acute inflammatory reaction consisting of neutrophilic and eosinophilic polymorphonuclear leukocytes. B and C show mild leukocyte infiltration, D shows moderate leukocyte infiltration, E and F show severe inflammatory reaction (haematoxylin and eosin staining, 400 ×).

Hofer-Warbinek *et al.*, 2000). On the other hand, previous data showed that IAP inhibition of apoptosis depends on the selective activation of JNK1 (Sanna *et al.*, 1998; Stehlik *et al.*, 1998; Hofer-Warbinek *et al.*, 2000). The expression of catalytically inactive JNK1 blocks IAP protection against ICE- and TNF-α-induced apoptosis, indicating that JNK1 activation is necessary for the anti-apoptosis effect of IAP (Sanna *et al.*, 2002).

In the present study, it has been shown that the expression of NF-κB significantly increases from days 3 to 15 (fig. 5). In addition, the same potency exists in the IAP protein expression following *A. cantonensis* infection (fig. 6). These findings suggest that damage to brain tissue by *A. cantonensis* may be associated with induction of NF-κB and JNK phosphorylation via IAP expression, which lead to brain tissue injury by preventing damaged cells undergoing apoptosis.

Jun N-terminal kinase (JNK) are members of MAPK family that are activated by a variety of environmental stresses and inflammatory cytokines. In previous studies, the accumulation of ferritin in *A. cantonensis*-infected rats (Lee *et al.*, 1996), is considered to be a protection process against tissue damage, including lysosomal membrane fragility and peroxidation of mitochondria and microsomes, which may be linked with inflammation (Hoy & Jacobs, 1981; Bacon *et al.*, 1983). The activation of JNK in *A. cantonensis*-infected mouse brain might be partially induced by reactive oxygen species (ROS) generated from oxidant stress. In addition, the ROS has the capability of stimulating MEKK1 to phosphorylate JNK. In the present study, the expression of MEKK1 increased and the

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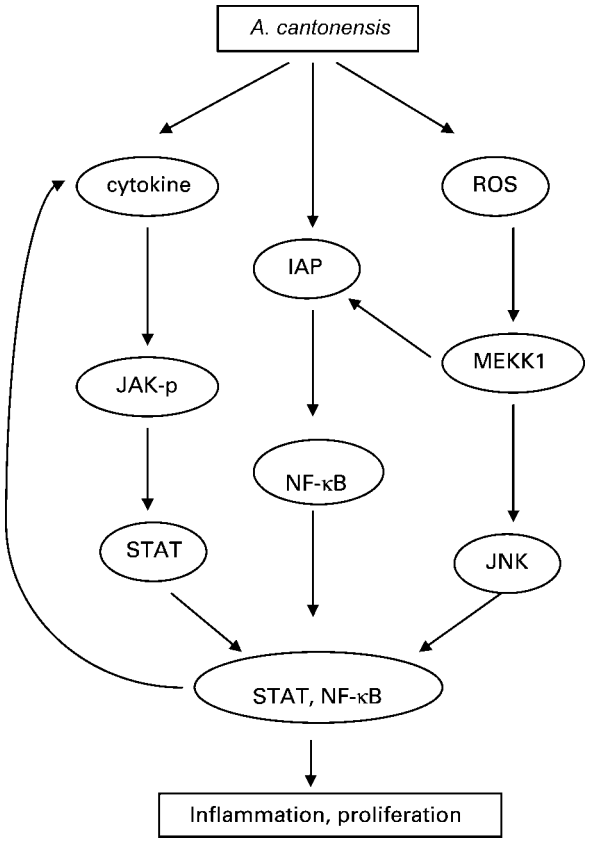

Fig. 8. Schematic illustration of the activation of MEKK1/JNK, IAP/NF-κB and JAK/STAT1 signals in *Angiostrongylus cantonensis*-infected mouse brain.

activation of JNK was observed in the mouse brain during *A. cantonensis* infection. This implies that MEKK1/JNK signalling might be involved in the pathogenesis of *A. cantonensis*-infected mouse brain. Much current research is focused on understanding the role of the JAK/STAT pathway in oncogenesis, tumour progression, angiogenesis, cell motility and the immune response (Bromberg *et al.*, 1999; Dentelli *et al.*, 1999; Lim & Cao, 1999). Janus kinases (JAKs) and STATs are utilized by receptors for a wide variety of ligands including cytokines, hormones, growth factors and neurotransmitters. Receptor-bound STAT are phosphorylated by JAK, dissociate from the receptor, dimerize and translocate into the nucleus. However, there is still no information concerning the relationship between brain injury caused by *A. cantonensis* infection and JAK phosphorylation, MEKK1, or downstream mitogenic signallings. In the present study, the stimulation of MEKK1 is activated by JAK phosphorylation and hence a JAK/STAT pathway might be involved in the pathogenesis of *A. cantonensis*-infected mouse brain.

Brain tissue damage, including eosinophilia, neutrophilia, and granulomas, increased from days 3 to 15 in infected mice. The effect of JAK/STAT1, IAP/NF-κB and MEKK1/JNK signalling was observed in parallel with a increase in brain damage, suggesting that multiple signalling pathways may be involved in the pathogenesis of *A. cantonensis*-infected mouse brain.

In conclusion, these results suggest that brain damage effects caused by *A. cantonensis* infections may be due to the generation of ROS and cytokines, which alternatively lead to the activation of MEKK1/JNK and JAK/STAT1 pathways. Another effect caused by *A. cantonensis* infections may be due to the induction of IAP, which alternatively leads to the activation of NF-κB and JNK. Nuclear factor-kappa binding protein translocates to the nucleus and activates cytokine which, in turn, activates the JAK/STAT pathway. On the other hand, the activation of JNK is triggered by the proliferation and inflammation of brain tissue during *A. cantonensis* pathogenesis. These findings suggest that the signalling pathways of JAK/STAT1, IAP/NF-κB, IAP/JNK and MEKK1/JNK might be important in initiating and/or enhancing brain injury asociated with *A. cantonensis* infections. The proposed hypothesis for *A. cantonensis* pathogenesis is summarized in fig. 8. In the animal model, a direct pathogenesis role of ROS and cytokines generated from *A. cantonensis* in infected mice brain has not yet been defined. Further experiments are needed to confirm direct evidence to support our hypothesis using a STAT-or JNK-deficient animal model.

Acknowledgements

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