

Original Article

Histopathological features of central nervous system in pediatric cases of fatal hand, foot and mouth disease

Wen-Ting Huang^{1*}, Wei-Jia Mo^{1*}, Yu-Yan Pang¹, Li-Jie Zhang¹, Hong-Bo Pan¹, Yi-Wu Dang¹, Lu Huang², Shu-Wen Bi^{1,3}, Chun-Qin Huang¹, Gang Chen¹, Zhen-Bo Feng¹, Dian-Zhong Luo¹

¹Department of Pathology, First Affiliated Hospital of Guangxi Medical University, Nanning 530021, Guangxi, People's Republic of China; ²Guangxi Jingui Judicial Expertise Center, Nanning 530022, Guangxi, People's Republic of China; ³Department of Pathology, Beihai People's Hospital (Ninth Affiliated Hospital of Guangxi Medical University), Beihai 536000, Guangxi, People's Republic of China. *Equal contributors.

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Abstract: Background and objective: Hand, foot and mouth disease (HFMD) is an acute infectious disease caused by enterovirus infection, which breaks out frequently in China in recent years. Previously, we have reported that striking inflammatory change was visible in the central nervous system (CNS) of fatal pediatric HFMD cases. There are a few studies all over the world that HFMD virus infection may lead to immune dysfunction. However, studies on local immune response in tissues are scarce. Thus, in this study, we investigated the detailed histopathological features of CNS in pediatric cases of HFMD, and further clarified the distribution, quantity and significance of various immunocytes in CNS tissues of HFMD children. Materials and methods: Tissues of CNS were gathered from seven autopsies of fatal HFMD, as well as three children of accidental suffocation. The histopathological features were observed in all samples by using hematoxylin-eosin (HE) staining. Immunohistochemistry (IHC) was performed to examine the expression of CD3, CD4, CD8 for T lymphocytes, CD20 for B lymphocytes, CD57 for natural killing (NK) cells and CD68 for macrophages in all groups. Results: As could be seen from HE staining, in HFMD patients' mid-brain/pons, medulla oblongata and spinal cord, pathological changes included a wide range of vascular dilatation and congestion, perivascular cuffings formed by inflammatory cells (mainly lymphocyte-like and monocyte-like cells). And microglia nodules and scattered infiltration by lymphocyte-like and monocyte-like cells were also observed in the parenchyma. Besides, neuronophagia, cribriform malacoplakia and nerve cells degeneration and necrosis were noted as well. All these pathological changes were relatively minor in the brain and cerebellum. Nevertheless, these pathological changes were not found in the control group. Compared with control group, the counting was higher of CD3 positive (CD3+), CD4+, and CD8+T lymphocytes, B lymphocytes and macrophages/microglia cells in the midbrain/pons, medulla oblongata and spinal cord of HFMD patients ($P < 0.05$). In spinal cord, the ratio of CD4+ to CD8+ was remarkably higher in HFMD group ($P < 0.05$). In HFMD group, the counting was noticeably higher of CD3+, CD4+, and CD8+T lymphocytes, B lymphocytes and macrophages/microglia cells in the midbrain/pons, medulla oblongata and spinal cord than in brain and cerebellum ($P < 0.05$). In addition, the counting of CD3+T lymphocytes in the brain was significantly higher than that in cerebellum ($P < 0.05$). Conclusions: Presence of lesions is mostly recorded in lower parts (midbrain/pons, medulla oblongata and spinal cord) than in upper parts (brain and cerebellum) in HFMD patients' CNS. T lymphocytes-mediated immunity, B lymphocytes-mediated humoral immunity and macrophages/microglial cells-executed innate immunity may be involved in the local immune response of the CNS lesions of HFMD cases. Additionally, cellular immunity and innate immunity may play a more vital role in local immune response than humoral immunity. No CD57 + NK cells were found in any CNS tissue of HFMD patients. It was indicated that NK cells might not be involved in local immune response of advanced CNS lesions of HFMD patients.

Keywords: Hand, foot and mouth disease (HFMD), histopathology, central nervous system, T lymphocyte, B lymphocyte, naturel killing (NK) cell, macrophage

Introduction

Hand, foot and mouth disease (HFMD), which prevails among preschooler, is an acute infectious disease caused by enterovirus infection

[1-3]. In China, recent years have witnessed a sharp increase in the morbidity of HFMD. The main symptoms of HFMD include fever and skin rashes on hands, feet and mouth [1-3]. Previously, we have reported that in some

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severe cases, patients died of brainstem encephalitis and neurogenic pulmonary edema which were results of central nervous system (CNS) damage [4]. As a global epidemic disease, HFMD has now become a growing threat to children's health and lives. So far, the pathogenesis of HFMD has not been fully elucidated. Insufficient domestic and international studies have shown that HFMD virus infection can cause immune dysfunction, and abnormal immune response may be involved in the incidence of HFMD. However, the specific role of the immune response in the pathogenesis of severe HFMD remains unclear.

Previous studies on HFMD-induced immune status change focused on the detection of immune cells and cytokines in peripheral blood and cerebrospinal fluid [5-8]. However, there were rare reports available on local immune response in the tissues. In this study, by immunohistochemical staining, we detected the expression of the T lymphocyte markers CD3, CD4, CD8, B lymphocyte marker CD20, NK cell marker CD57 and macrophage/microglia cell marker CD68 in CNS tissues (brain, midbrain/pons, medulla oblongata, spinal cord and cerebellum) in seven autopsies of HFMD cases, and investigated the distribution, quantity and significance of various immunocytes in CNS tissues of HFMD patients.

Materials and methods

Materials

The paraffin-embedded tissues of brain, mid-brain/pons, medulla oblongata, spinal cord and cerebellum from seven autopsies of HFMD confirmed by pathological examination at the Pathology Department of the First Affiliated Hospital of Guangxi Medical University in 2010 were collected, including six cases of males and one case of female. The age ranged from eight months to two years old, with an average of 0.86 years. All the samples were pathologically confirmed as severe HFMD in accordance with the diagnostic criteria implemented by Ministry of Health (<http://www.nhfpc.gov.cn/zyygj/s3593g/201306/6d935c0f43cd4a1fb46f8f71acf8e245.shtml>) [1]. Three autopsies of accidental suffocation from 2011 to 2012 were chosen as the control (two cases of males and one case of female). The age ranged from two months to one year old, with an average of 0.53 years. The Ethical Committee of the First Affiliated

Hospital of Guangxi Medical University approved the current study and written informed consents were obtained by the families and clinicians to permit the usage of the samples for research.

Morphology and immunohistochemistry

In this study, we used FFPE sections on polylysine-coated slides for the morphology and IHC evaluation, and applied hematoxylin-eosin (HE) staining to detect the pathological morphology in all samples. Immunohistochemical staining was conducted with Supervision™ detection system (Long Island, Shanghai, China) as previously reported [9]. The monoclonal antibodies were incubated overnight in a humidity chamber at 4°C, involving CD3, CD4, CD8, CD20, CD57 and CD68 (Zhongshan Jinqiao Corp, Beijing, China). Blank control sections were incubated with phosphate buffer solution (PBS) alone, instead of primary antibody. Tissues of acute tonsillitis were taken as positive controls for CD3, CD4, CD8, CD20 and CD57, and normal hepatic tissues were employed as positive controls for CD68.

The IHC positivity was evaluated as specified according to the immunodetection of stain intensity. The staining pattern appeared yellow or brown in the cytomembrane for CD3, CD4, CD8, CD20, and CD57, and in the cytoplasm for CD68. The positive signaling could be focal, fine granular, linear or diffuse. The positive cells were recognized, counted in 10 high magnification (HPF 400x), and randomly selected under a light optic microscope. The mean number of 10 visual fields was presentative for the result of a subtype of immunocytes, i.e., CD3, CD4 and CD8 for T lymphocytes; CD20 for B lymphocytes, CD57 for natural killing (NK) cells, and CD68 for the macrophages. All IHC staining was examined independently by three pathologists (HBP, SWB and GC).

Statistical analyses

SPSS 20.0 for Windows was used to assist statistical analysis. The number of the positive cells was expressed as Mean ± Standard deviation (Mean ± SD) if the variable complied with normal distribution; Or else the data was presented as Median (Inter-Quartile Range (IQR)) [M (Q)]. Student *t* test was used to measure the difference of immunocytes among a variety of groups if the data were in accordance with nor-

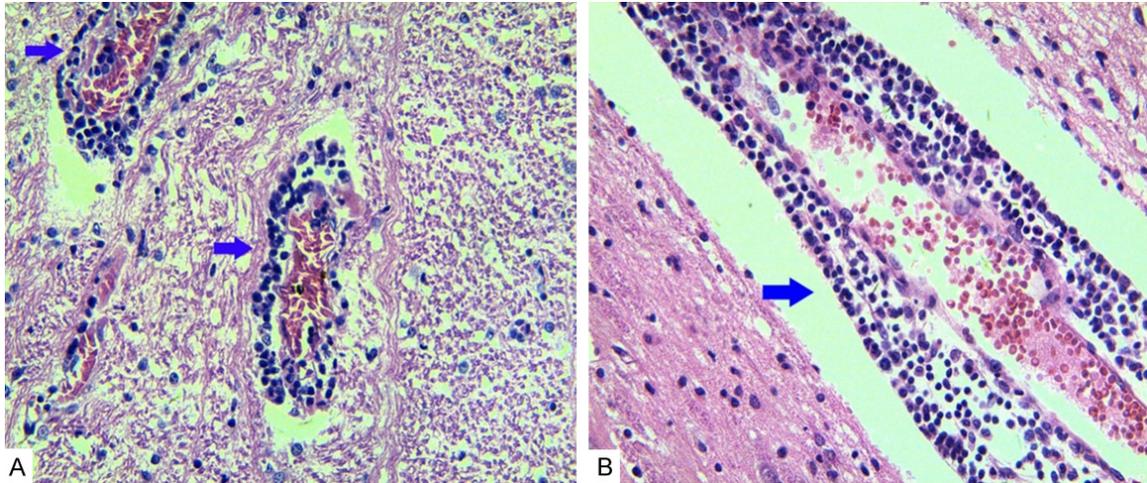


Figure 1. Perivascular cuffings in medulla oblongata of fatal Hand, Foot and Mouth Disease. HE staining, $\times 400$. Arrow: perivascular cuffing.

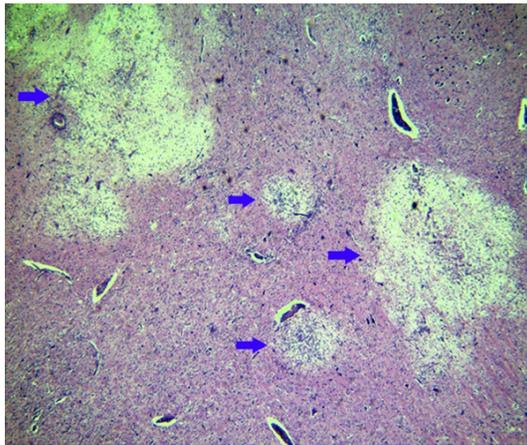


Figure 2. Reticular softening lesions of necrosis in mesencephalon of fatal Hand, Foot and Mouth Disease. HE staining, $\times 40$. Arrow: cribriform malacoplakia.

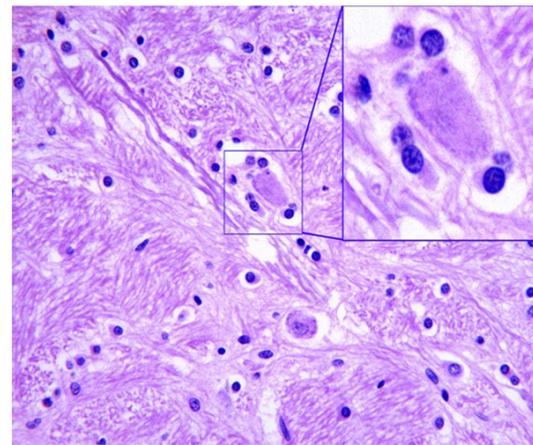


Figure 3. Neuronophagia in medulla of fatal Hand, Foot and Mouth Disease. HE staining, $\times 400$. Square: Neuronophagia.

mal distribution and homogeneity of variances; otherwise, Mann-Whitney U test would be employed. A value of $P < 0.05$ was considered statistically significant.

Results

Pathological morphology of CNS in fatal HFMD

As can be seen from HE staining, in HFMD patients' midbrain/pons, medulla oblongata and spinal cord, pathological changes included a wide range of vascular dilatation and congestion, perivascular cuffings formed by inflammatory cells (mainly lymphocyte-like and monocyte-like cells, **Figure 1A** and **1B**). Scattered

reticular softening lesions of necrosis (cribriform malacoplakia) could be observed (**Figure 2**). Microglia nodules and scattered infiltration by lymphocyte-like and monocyte-like cells were observed in the parenchyma. Apart from that, some neurons swelled and Nissl bodies became invisible; deviation of cell nucleus, pyknosis, karyolysis and apoptosis were noticed as well. It was also discovered that a small number of macrophages/microglia formed clusters, i.e., neuronophagia and glial nodule (**Figures 3** and **4**). All these pathological changes were relatively minor in the brain. Vascular dilatation was occasionally observed, and about 10 lymphocyte-like and monocyte-like cells formed clus-

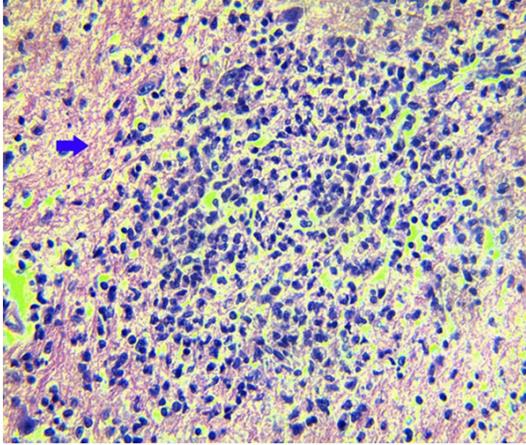


Figure 4. Glial nodule in spinal cord of fatal Hand, Foot and Mouth Disease. HE staining, $\times 400$. Arrow: glial nodule.

ters. In the parenchyma was present a small range of scattered infiltration by lymphocyte-like and monocyte-like cells. However, no obvious microglia nodules were discovered. No clearly visible degeneration and necrosis of neurons were detected either. In addition, no neuronophagia and cribriform malacoplakia were found. There was no easily noticed dilatation and congestion in cerebellar interstitial vessels, around which no more than 10 lymphocyte-like and monocyte-like cells clustered. Morphological abnormality was not found in the parenchyma. All these pathological changes mentioned above were not observed in the control groups.

The comparison of distribution and quantity of various immunocytes in the CNS between HFMD group and the control group

In addition to NK cells, CD3+, CD4+, CD8+T lymphocytes, B lymphocytes and infiltration by macrophages/microglia were discovered in CNS tissues in both HFMD group and controls. However, the distribution of cells varied in these groups.

In HFMD patients' midbrain/pons, medulla oblongata and spinal cord, CD3 positive (CD3+), CD4+, and CD8+T lymphocytes were largely distributed around vessels and in where microglia nodules were intensive in the parenchyma; B lymphocytes' distribution was the same as T lymphocytes'. Macrophages/microglia cells were mostly seen around vessels and microglia nodules. The distribution was also observed in the parenchyma-macrophages/

microglia clustered tightly around some necrotic neutrons in the form of neuronophagia. The distribution pattern of various immunocytes was similar both in the brain and in the mid-brain/pons, medulla oblongata and spinal cord, but the cell number was rather smaller. Similarly, the distribution in cerebellum was alike, but around vessels were discovered several positive cells which were not found in the parenchyma. In control groups, the distribution pattern of immunocytes was similar in CNS tissues; however, only a few positive cells, which were not noted in the parenchyma, were occasionally distributed around vessels. No CD57 + NK cells were found in tissues in either HFMD or control groups (**Figure 5**). In terms of lesion regions, there were more CD3+, CD4+, CD8+T lymphocytes, B lymphocytes and macrophages/microglia cells in HFMD sufferers' brain, midbrain/pons, medulla oblongata and spinal cord than in controls' ($P < 0.05$). Nevertheless, in cerebellar tissues, there was no statistical significance in lymphocytes counting between these two groups ($P > 0.05$). In brain, midbrain/pons, medulla oblongata and cerebellar tissues, the ratio of CD4+ to CD8+ had no statistical significance both in HFMD and control groups ($P > 0.05$); however, in spinal cord, the ratio was higher in HFMD group ($P < 0.05$, **Tables 1 and 2**).

The comparison of quantity of various immunocytes between different parts of CNS tissues in HFMD group

In HFMD cases, the counting of CD3+, CD4+, CD8+T lymphocytes, B lymphocytes and macrophages/microglia cells was remarkably higher in the midbrain/pons, medulla oblongata and spinal cord than that in brain and cerebellum ($P < 0.05$). No statistical significance was found in medulla oblongata and spinal cord. The counting of CD3+T lymphocytes in the brain was noticeably higher than that in cerebellum ($P < 0.05$). No statistical significance was found in the counting of CD4+, CD8+T lymphocytes, B lymphocytes and macrophages/microglia in brain and cerebellum ($P > 0.05$, **Table 3**).

The comparison of quantity between different types of immunocytes in HFMD group

In HFMD group, the counting of CD3+T lymphocytes and macrophages/microglia cells were remarkably higher than that of B lymphocytes ($P < 0.05$). There was no significant difference

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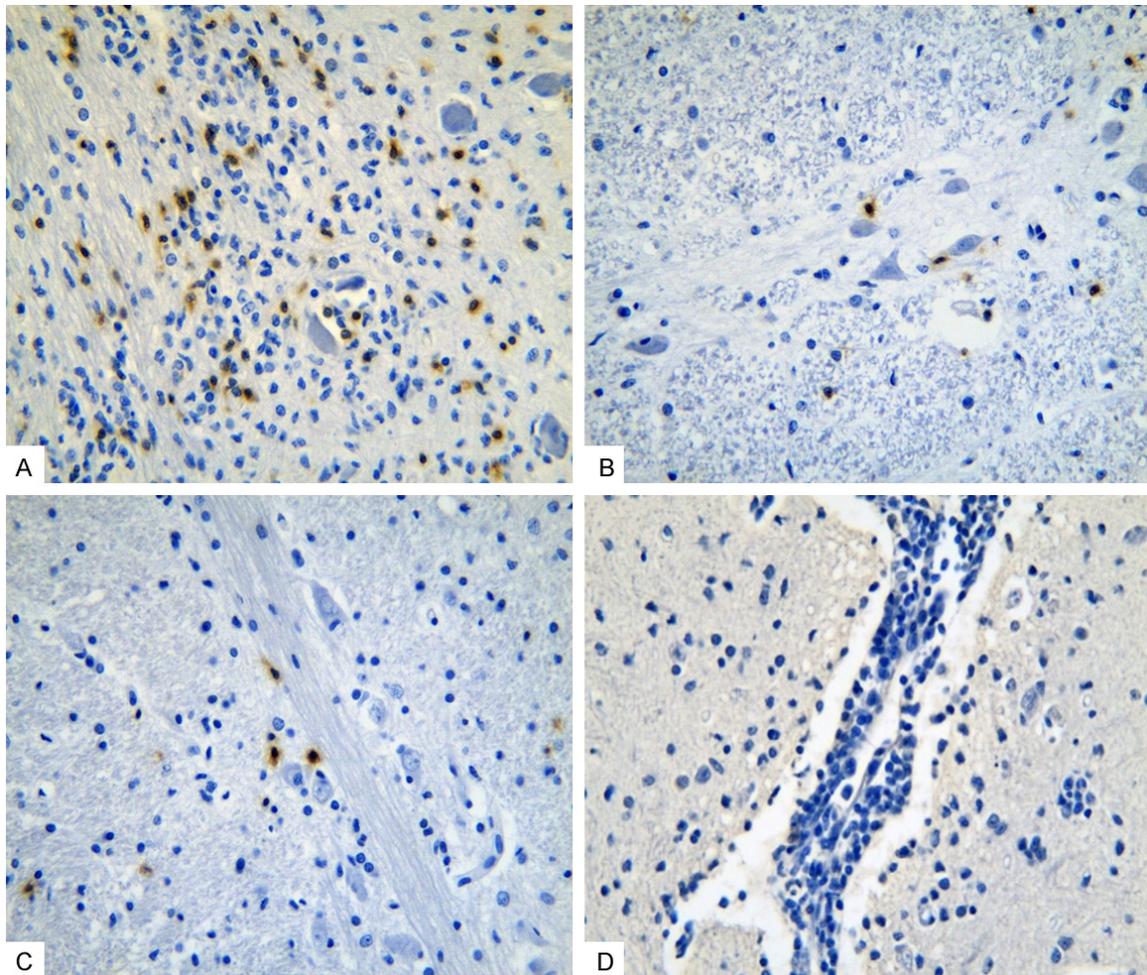


Figure 5. Immunohistochemical staining of various immunocytes in central nervous system of fatal Hand, Foot and Mouth Disease. A: CD3+ cells in the glial nodule in medulla oblongata, B: CD3+ cells around the neurons in medulla oblongata, C: CD8+ cells around the neurons in medulla oblongata, D: No CD57+ natural killer cell was observed around blood vessels in spinal cord. Immunohistochemical staining, Supervision method, $\times 400$.

Table 1. The comparison of the numbers of CD3+, CD4+, CD8+T cells in various tissues in central nervous system of Hand, Foot and Mouth Disease [$\bar{x}\pm s/M(Q)$]

Sites	CD3+			CD4+			CD8+		
	HFMD (n=7)	Controls (n=3)	P	HFMD (n=7)	Controls (n=3)	P	HFMD (n=7)	Controls (n=3)	P
Brain	2.69 \pm 0.77	0.20 (0.10)	0.017	0.99 \pm 0.65	0.10 (0.10)	0.033	2.60 (2.00)	0.20 \pm 0.10	0.017
Midbrain/pons	50.26 \pm 10.73	1.00 \pm 0.26	<0.001	49.20 (19.60)	0.40 \pm 0.20	0.017	26.40 \pm 11.47	0.77 \pm 0.57	0.017
Medulla oblongata	63.34 \pm 13.62	0.23 \pm 0.21	<0.001	55.56 \pm 18.61	0.23 \pm 0.15	0.001	28.36 \pm 7.91	0.10 (0.10)	0.017
Spinal cord	62.67 \pm 20.87	0.57 \pm 0.25	0.017	50.61 \pm 8.94	0.20 \pm 0.10	<0.001	27.33 \pm 14.36	0.57 \pm 0.25	0.017
Cerebellum	1.26 \pm 0.62	0.57 \pm 0.32	0.113	0.79 \pm 0.45	0.43 \pm 0.25	0.247	0.84 \pm 0.36	0.40 (0.20)	0.067

between the CD4+ and CD8+T lymphocytes counts ($P>0.05$, **Tables 4** and **5**).

Discussion

HFMD was first reported in Shanghai in 1981 and its widespread outbreaks took place in

2008, which plagued many parts of China. Since then, there has been a rise in HFMD cases all over China [1, 10-15]. HFMD is highly infectious and can spread rapidly among people, especially children. And HFMD has current lyposed a major threat to children's health and lives due to its high mortality and deformity

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Table 2. The comparison of the numbers of B, macrophages/microglial cells and ratio of CD4+/CD8+ in various tissues in central nervous system of Hand, Foot and Mouth Disease [$x\pm s/M(Q)$]

Sites	CD20+			CD68+			CD4+/CD8+		
	HFMD (n=7)	Controls (n=3)	P	HFMD (n=7)	Controls (n=3)	P	HFMD (n=7)	Controls (n=3)	P
Brain	0.50±0.24	0.10±0.10	0.026	3.49±1.51	0.83±0.55	0.021	0.80±0.72	0.44±0.51	0.466
Midbrain/pons	20.94±10.63	0.00 (0.20)	0.017	61.54±14.90	1.17±0.55	0.017	1.92±1.08	0.94±0.96	0.211
Medulla oblongata	19.40±6.37	0.10 (0.10)	0.017	59.97±4.92	0.33±0.15	<0.001	2.13±1.09	1.00 (3.00)	0.667
Spinal cord	17.01±3.34	0.13±0.15	0.017	60.40±4.98	0.80±0.20	0.017	2.52±1.68	0.33 (0.04)	0.017
Cerebellum	0.31±0.20	0.10±0.10	0.116	2.79±1.27	2.30±1.28	0.594	0.94±0.34	1.00 (0.75)	0.667

rates. As a result, it is imperative that pathogenesis and pathological features of HFMD be fully explored in order to provide theoretical base for vaccination research and more effective clinical treatment, thus reducing HFMD mortality.

Clinical symptoms of HFMD are almost the same regardless of pathogens. In some fatal HFMD cases, children deceased from serious complications like respiratory failure, which resulted from CNS damage [4]. Fatal HFMD is mainly caused by EV71 infection, which is a highly neurotropic virus that is different from other HFMD viruses like Cox A16. And EV71 has been proved to be directly linked with HFMD patients' CNS damage. In previous studies, we morphologically observed cerebral tissues of 14 HFMD autopsies by means of HE staining, and found that CNS pathological changes of HFMD patients were characterized by brain stem encephalitis. Mononuclear lymphocyte perivascular cuffing was observed in the brain-stem; besides, neuronal degeneration and necrosis were noticed; additionally, cribriform malacoplakia, neuronophagia and microglia nodules were found [4]. In this study, CNS tissues of HFMD cases were categorized into brain, midbrain/pons, medulla oblongata, spinal cord and cerebellum. Also we meticulously observed pathological changes in HFMD patients' CNS, and analyzed the distribution and quantity of immunocytes (mainly T-lymphocytes, B-lymphocytes, NK cells and macrophages/microglia cells) in CNS of patients who died of HFMD. This study also explored the local immune response of immunocytes in HFMD sufferers' CNS and ascertained the role of immunocytes in occurrence and development of CNS pathological alterations.

Firstly, we were interested in the morphological changes and significance of HFMD patients' CNS. In the current study, we discovered that in

HFMD patients' midbrain/pons, medulla oblongata and spinal cord were seen pervasive vascular dilatation and congestion, perivascular cuffings formed by inflammatory cells (mainly lymphocyte-like and monocyte-like cells). And microglia nodules and scattered infiltration by lymphocyte-like and monocyte-like cells were also observed in the parenchyma. Moreover, some neurons degenerated and deceased; neuronophagia and cribriform malacoplakia also appeared. These pathological changes were not obvious in brain and cerebellum. It was indicated that lesions of HFMD patients' CNS were mainly midbrain/pons, medulla oblongata and spinal cord, whereas no pathological alterations were seen in brain and cerebellum. It was of paramount importance to clarify pathological features of different parts in HFMD children's CNS, whereby imaging examination could be conducted to facilitate fatal HFMD diagnosis and to assist prognosis. Furthermore, it provided more reliable evidence for clinicopathological diagnosis of HFMD.

Next, we continued with the investigation of the distribution, quantity and significance of various immunocytes in CNS of HFMD patients and healthy children. Immune responses can be classified as innate immunity and adaptive immunity. Cells which are involved in innate immunity include mononuclear macrophages, dendritic cell, NK cells and neutrophil granulocytes, etc. Adaptive immunity was divided into two types: T lymphocytes-mediated cellular immunity and B lymphocytes-mediated humoral immunity.

CD3 is a vital surface marker in mature T cells. CD3+T cells can be divided into two types: CD4+T cells and CD8+T cells. In CD4+T cells, CD4+ effector T cells are the major components, which consist of Th1 cells and Th2 cells. In this study, compared with control groups, counting of CD3+, CD4+ and CD8+T cells was

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Table 3. The numbers of T, B and macrophages/microglial cells in various tissues in central nervous system of Hand, Foot and Mouth Disease [x±s/M (Q)]

Sites	n	CD3+		CD4+		CD8+		CD20+		CD68+						
		$\bar{X} \pm s$	P	$\bar{X} \pm s/M(Q)$	P	$\bar{X} \pm s/M(Q)$	P	$\bar{X} \pm s$	P	$\bar{X} \pm s$	P					
brain	7	2.69±0.77	<0.001 ^a	0.001 ^b	0.99±0.65	0.001 ^a	0.001 ^b	2.60 (2.00)	0.001 ^a	0.001 ^b	0.50±0.24	0.001 ^a	0.001 ^b	3.49±1.51	0.001 ^a	<0.001 ^b
midbrain/pons	7	50.26±10.73	0.069 ^c	0.259 ^d	49.20 (19.60)	0.165 ^c	0.805 ^d	26.40±11.47	0.717 ^c	0.896 ^d	20.94±10.63	0.747 ^c	0.805 ^d	61.54±14.90	0.902 ^e	1.000 ^d
medulla oblongata	7	63.34±13.62	0.944 ^e	0.001 ^f	55.56±18.61	0.538 ^e	0.001 ^f	28.36±7.91	0.871 ^e	0.001 ^f	19.40±6.37	0.398 ^e	0.001 ^f	59.97±4.92	0.874 ^e	<0.001 ^f
spinal cord	7	62.67±20.87	0.001 ^g	0.001 ^h	50.61±8.94	0.001 ^g	0.001 ^h	27.33±14.36	0.001 ^g	0.001 ^h	17.01±3.34	0.001 ^g	0.001 ^h	60.40±4.98	0.001 ^g	0.001 ^h
cerebellum	7	1.26±0.62	0.020 ⁱ	<0.001 ^j	0.79±0.45	0.516 ⁱ	0.001 ^j	0.84±0.36	0.073 ⁱ	0.001 ^j	0.31±0.20	0.136 ⁱ	0.001 ^j	2.79±1.27	0.366 ⁱ	0.001 ^j

PS a: brain VS midbrain/pons, b: brain VS medulla oblongata, c: midbrain/pons VS medulla oblongata, d: midbrain/pons VS spinal cord, e: medulla oblongata VS spinal cord, f: medulla oblongata VS cerebellum, g: spinal cord VS cerebellum, h: spinal cord VS brain, i: cerebellum VS brain, j: cerebellum VS midbrain/pons.

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Table 4. Comparison of the numbers of T, B and macrophages/microglial cells in central nervous system of Hand, Foot and Mouth Disease

	CD3+	CD20+	CD68+
M (Q)	43.60 (57.60)	13.10 (19.50)	54.20 (56.90)
P	<0.001 ^a	<0.001 ^b	0.275 ^c

PS a: CD3+ vs CD20+; b: CD20+ vs CD68+; c: CD68+ vs CD3+.

Table 5. Comparison of the numbers of CD4+ and CD8+T cells in central nervous system of Hand, Foot and Mouth Disease

	CD4+	CD8+
M (Q)	39.20 (49.00)	16.90 (28.90)
P	0.078	

remarkably higher in HFMD patients' cerebrum, mesencephalon/pons, medulla oblongata and spinal cord. And CD3+, CD4+ and CD8+T cells not only existed in perivascular cuffing but also dispersed in parenchyma, especially in where glial nodule accumulated. It was indicated that T lymphocytes-mediated cellular immunity played a significant role in local immune response of HFMD patients' CNS lesion. CD4+Th1 cells functioned efficiently mainly by secreting various cytokines to stimulate macrophages, inducing and recruiting macrophages and lymphocytes to infected parts of CNS. Also cytokines like IL-2 were produced to facilitate activation, proliferation and differentiation of T cells. And B cells were assisted to produce antibodies to enhance phagocytosis of HFMD-related viruses by macrophages. Thus, cellular immunity was mediated. In addition, CD4+ Th2 cells regulated B cells activation and Ig class switch. CD8+T cells were cytotoxic T cells, releasing toxic granules and death receptor pathway to mediate target cells death, thus efficiently and specifically eliminated virus-infected host cells such as neurons. As was revealed in the study, the ratio of CD4+ to CD8+ in CNS tissues (except spinal cord) had no statistical significance between HFMD and control groups. It was indicated that CD4+T cells and CD8+T cells might infiltrate proportionally in cerebrum, mesencephalon/pons, medulla oblongata and cerebellum; however in spinal cord, more CD4+ penetrated into local pathological tissues and therefore produced immunological effect.

Furthermore, a large number of CD3+, CD4+ and CD8+T oozed out of vessels in certain parts, which might explain the loss of CD3+, CD4+ and CD8+T cells in HFMD patients' peripheral blood.

CD20 expressed in pre-B and mature B cells, regulating B cells activation and proliferation. It was discovered in this study that compared with control group, counting of CD20+B cells were notably higher in HFMD patients' cerebrum, mesencephalon/pons, medulla oblongata and spinal cord tissues. And distribution of CD20+B cells in nervous tissue was the same as that of T cells. It was inferred that B lymphocytes-mediated humoral immunity also participated in local immune response in CNS lesion of HFMD patients. With the help of specific antigens, B cells were activated, proliferated and differentiated into plasmacyte which produced specific antibody and mediated humoral immunity. Despite the evidence, its role in local CNS lesions of HFMD patients has remained unknown. According to our analysis, in local lesions, B cells might absorb and process HFMD-related viral antigens to produce pMHC-II molecular complexes. Moreover, B cells presented antigen for antigen-specific CD4+ Th cells whose involvement assisted B cells in activation, proliferation and differentiation into plasma cells. As a result, antiviral capsid protein was produced to neutralize virus in order to prevent virus from absorbing permissive cells and to curb the spread of virus in tissues.

CD68 is the best marker for macrophages, which could act as an activation marker for microglial cells as well. This study found that the counting of macrophages/microglial cells was significantly higher in HFMD patients' nervous tissue (except cerebellum) compared with control group. And these cells mostly existed in perivascular cuffing and glial nodules, surrounded particular necrotic neurons, and also scattered in parenchyma. It was suggested that innate immunity also engaged in local immune response of CNS lesions of HFMD sufferers. Macrophages, produced by the differentiation of monocytes, were essential effector cells which were involved in innate immune late response. Activated macrophages could produce TNF- α or utilize iNOS-dependent pathway to mediate antiviral effect. As important antigen presenters, macrophages could also absorb, process and present viral antigens to

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CD4+T cells to initiate adaptive immune response. Apart from that, macrophages secrete types of cytokines to improve the effect of Th1 cells and to stimulate local inflammatory reaction. Microglia, a special type of mononuclear phagocyte, was similar to macrophage in functions; they were widely seen in CNS, accounting for 5%-20% of all glial cells in CNS. In this study, CD68+ microglial cells were absent in control group, while CD68+ macrophages/microglia abounded in parenchyma of mesencephalon/pons, medulla oblongata and spinal cord in HFMD group. There were also a few CD68+ macrophages/microglia in cerebrum parenchyma. Healthy children's CNS microglia failed to express CD68 probably because these cells were in resting state. When the host infected HFMD-related viruses, microglia were activated, proliferated and expressed its activation marker CD68. Morphologically, microglial cells took the form of neurite retraction, soma enlargement, even resemblance to macrophages, which made it difficult to distinguish microglia from invasive macrophages via blood circulation. Activated microglial cells functioned as antigen presenter, producing inflammatory factors and initializing immune response; microglia then assembled around necrotic and apoptotic neurons and phagocytized degenerated nerve tissues debris with invasive macrophages through blood circulation.

CD57, a type of glycoprotein, is expressed in leukocytes and neuroendocrine cells and its anti-CD57 is used to detect NK cells in lymphocytes subgroups. In this study, NK cells were not observed in HFMD group. Generally, NK cells would congregate at infected sites by chemotaxis in around three days after infection. NK cells play a vital role in early phase of immune system against viruses since NK cells take effect before adaptive immune response even replication of viruses. Therefore, NK cells were not likely to participate in local immune response of advanced lesions in HFMD patients' CNS.

In local immune system of HFMD patients' CNS lesions, T lymphocytes, B lymphocytes and macrophages/microglia interacted to regulate occurrence, development and prognosis of HFMD. These immunocytes targeted CNS lesions, theoretically inhibiting virus invasion and postponing development of HFMD. However, immunocytes in HFMD patients' CNS

were not able to reverse into disease progression and advanced stage, despite their large quantities. It was indicated that there were many factors contributing to HFMD patients' CNS damage. We hypothesized that viruses probably infected nervous tissue cells by NGFR (neuron growth factor receptor) or by SCARB2 (scavenger receptor class B2), directly destroying neurons [16-20]. Therefore, immune response alone could not curb disease progression. Moreover, immunocytes infiltrated in large numbers but failed to trigger anti-viral immune response as viruses infected leucocytes through PSGL-1 (P-selectin glycoprotein ligand-1) and then prevented leucocytes from acting [21-23]. In addition, progression of CNS lesions might be accelerated by immune injury of local nervous tissues which resulted from immunological dysfunctions, different types of proinflammatory cytokines and increased expression of metabolite immunotoxicity.

We further explored the quantity and significance of different types of immunocytes in CNS tissues of HFMD patients. This study compared the quantity of immunocytes in tissues of HFMD patients and found that a large number of T lymphocytes, B lymphocytes and macrophages/microglial cells existed in midbrain/pons, medulla oblongata and spinal cord rather than in cerebrum and cerebellum. And the study further proved that inflammatory lesions of HFMD cases' CNS were mainly observed in midbrain/pons, medulla oblongata, spinal cord while lesions in cerebrum and cerebellum were rarely seen, because EV71 often invaded brainstem and spinal cord in CNS. It was deduced that local inflammatory response of HFMD patients' CNS and response intensity might be associated with invasion by viruses into local tissues. Some research results showed that EV71 infected vascular smooth muscle cells (VSMCs) and induced expression of VCAM-1 (vascular cell adhesion molecule-1) via activating PDGFR, PI3K/Akt, p38 MAPK, JNK and NF-kappaB [24]. A possible mechanism that EV71 caused local inflammatory response in CNS was that EV71 infected VSMCs in order to stimulate VCAM-1 expression, inducing monocytes and lymphocytes to adhere to vascular endothelial cells which would migrate to infected sites in parenchyma.

Finally, we studied the quantity and significance of different types of immunocytes in HFMD

patients' central nervous tissue. As this study discovered, in CNS lesions of HFMD patients, the quantity of T lymphocytes was almost the same as that of macrophages/microglia whereas B lymphocytes were present in smaller quantities. Also, CD4+ cells and CD8+T cells were similar in numbers. It was suggested that in local immune response of HFMD patients' CNS lesions, T lymphocytes-mediated cellular immunity and macrophages/microglia-executed innate immunity were more essential than B cells-mediated humoral immunity. Besides, CD4+ and CD8+T cells were equally important.

In summary, the lesions seemed more seriously in the lower parts (midbrain/pons, medulla oblongata and spinal cord) than in the upper parts (brain and cerebellum) in the CNS of HFMD patients. Infiltration of CD3+, CD4+, CD8+T lymphocytes, B lymphocytes and macrophages/microglia cells could be observed in all tissues of the CNS, especially in the midbrain/pons, medulla oblongata and spinal cord, in HFMD cases. Cellular immune mediated by T lymphocytes, humoral immune mediated by B lymphocytes and innate immune executed by macrophages/microglial cells are involved corporately in the local immune response of the CNS lesions of HFMD cases. Cellular immune and innate immune may play a more important role than the humoral immune, and CD4+ and CD8+T lymphocytes may be of equal importance in the local immune response of the CNS lesions of HFMD. No CD57+ NK cells were found in any CNS tissue of HFMD patients. NK cells may not be involved in the later phase of local immune response of the CNS lesions of HFMD patients.

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Disclosure of conflict of interest

None.

Address correspondence to: Gang Chen and Zhen-Bo Feng, Department of Pathology, First Affiliated Hospital of Guangxi Medical University, Nanning 530021, Guangxi, People's Republic of China. Tel:

0086-771-5356534; Fax: 0086-771-5356534;
E-mail: chen_gang_triones@163.com (GC); guanghu1963@126.com (ZBF)

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