Expression of T subsets and mIL-2R in peripheral blood of newborns with hypoxic ischemic encephalopathy

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Original article

Background: **Infantile and undifferentiated immune cells in the pathogenesis of neonates with HIE have been studied in recent years. This study was undertaken to observe the expression level of T subsets and membrane interleukin-2 receptor (mIL-2R) in the peripheral blood of newborns with hypoxic ischemic encephalopathy (HIE) and its clinical manifestations.**

Methods: **The peripheral blood mononuclear cells (PBMCs) of newborns with HIE and normal controls were isolated by the routine Ficoll-Hypaque method, and the rates of CD_3^+, CD_4^+, CD_8^+, CD_4^+/CD_8^+ and mIL-2R induced and not induced by phytohemagglutinin (PHA) were detected by biotin-streptavidin (BSA) at the first, third and seventh day after birth.**

Results: **At the first day after birth, the positive rates of CD_3^+, CD_4^+, CD_8^+, CD_4^+/CD_8^+ and mIL-2R induced and not induced by PHA were (37.4±6.7)%, (29.4±6.9)%, (16.7±3.3)%, 1.8±0.5, (3.6±1.1)% and (20.9±4.8)%, respectively. Significant differences were observed between the HIE group and the normal controls (P<0.01-P<0.05). At the third day after birth, the positive rates of CD_3^+, CD_4^+, CD_8^+, CD_4^+/CD_8^+ and mIL-2R induced and not induced by PHA were (41.0±7.4)%, (35.8±6.9)%, (22.6±4.5)%, (1.7±0.5), (3.9±1.2)%, and (22.8±5.1)%, respectively. There were significant differences between the HIE group and the normal controls (P<0.05). At the seventh day after birth, the positive rates of CD_3^+, CD_4^+, CD_8^+ were (41.8±6.1)%, (36.4±5.1)% and (25.6±4.3)%, respectively. There was significant difference between the HIE group and the normal controls (P<0.05). The ratio of CD_4^+/CD_8^+ and the expression level of mIL-2R induced and not induced by PHA were 1.5±0.3, (4.1±1.2)% and (23.8±5.2)%, respectively. There was no significant difference between the HIE group and the normal controls (P>0.05).**

Conclusions: **Peripheral blood mononuclear cells of newborns are immature and undifferentiated with a very low expression level of surface markers. The changes of cell immunity involve in the pathogenesis of HIE. The disorder of cellular immune function exists in newborns with HIE. Cell immunity and immune regulative response in newborns are gradually improved or mature during the period of growing, facilitating the recovery from brain injury caused by HIE.**

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Key words: **biotin-streptavidin;**
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T subsets

Introduction

Hypoxic ischemic encephalopathy (HIE), a severe complication which causes significant mortality and long-term morbidity, is easily induced by asphyxiation in newborns.[1] Hypoxic-ischemic cerebral injury during the perinatal period is one of the most commonly recognized causes of severe, long-term neurologic deficits including cerebral palsy in children.[2] Peripheral blood mononuclear cells (PBMCs) have a large number of different active immune cells such as T lymphocytes, and play a key role in releasing various cytokines and regulating immune response to inflammation in the host.[3-5] The membrane interleukin-2 receptor (mIL-2R), an important symbol of active T cells, can be evaluated as an indicator for the cellular immune function of newborns during the growth of life. In order to highlight the relationship between HIE and cellular immune function, the expression of T subsets and mIL-2R was detected by immunohistochemistry in the present study.

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Methods

Patients

Thirty-two newborns with HIE were recruited from the Maternity and Infant Health Institute of Huainan, Anhui Province from May 2002 to October 2003. They were 22 males and 10 females, with the gestational age ranging from 37 to 42 weeks and birth weight from 2500 to 4000 g. The criteria for diagnosis were based on the guidelines formulated at Chinese Hypoxic Ischemic Encephalopathy Conference held in Hangzhou in 1996. The 32 newborns were confirmed to have a history of asphyxiation, and they were divided into three groups: mild (15 newborns), moderate (9), severe (8) according to clinical manifestations such as conscious disturbance, muscular tension, dysreflexia, convulsion, as well as results of CT. Another 30 newborns (19 males, 11 females) with a normal full-term delivery of 37 to 42 weeks, birth weight of 2500 to 4000 g, were regarded as controls. The inclusion criteria included no intrauterine growth retardation, history of asphyxiation, intrauterine infection, and other complications less than seven days after birth. Excluded were mothers who had a history of pathogenic infection during pregnancy, injection of blood preparation and immunodepressant or immunoenhancer.

Detection of T lymphocyte subsets

Peripheral venous blood (2 ml) was taken from the newborns and then distributed in a sterile Eppendorf tube and an anticoagulant tube (heparin), respectively. After mixing the heparin anticoagulant blood with an equal volume of Hank's solution without Ca^{2+} and Mg^{2+}, separation medium for lymphocytes (Sichuang Biochemistry Limited Company, Shanghai) was used to separate PBMCs. The cells were washed twice with Hank's solution without Ca^{2+}, Mg^{2+} and diluted to (1-3)× 10^6/ml with RPMI 1640 (Gibco, USA) complete culture solution. About 10 μl PBMCs suspension was smeared on the slide, dried naturally and fixed with acetone for 15 or 20 minutes. Different monoclonal antibody (10 μl) against anti-CD_3, anti-CD_4, anti-CD_8 with biotin and SA-HRP was smeared on different sheet glasses. The cells were incubated by continuous culture (37, 50 ml/L CO_2) for 30 minutes. The immune sheet glass pores were measured after staining with the color-developing agent and several washings with tris-buffer solution (TBS). The total number of 200 PBMCs was counted and positive cells were statistically analyzed under a microscope. The cells with membrane stained brown were regarded as positive.

Detection of mIL-2R in silence and inducement states

Ten μl PBMCs suspension was smeared on the slide, dried naturally and fixed with acetone for 15 or 20 minutes. Ten μl of anti-Tac antibodies was mixed with smears. The cells were grown in continuous culture (37ºC, 50 ml/L CO_2 in atmosphere) for 30 minutes and the immune sheet glass pores were measured after staining with the color-developing agent and washing with TBS for several times. About 0.5 ml PBMCs suspension was mixed with RPMI 1640 culture liquid containing phytohemagglutinin (PHA) 200 mg/L. The cells were grown in the continuous culture (37ºC, 50 ml/L CO_2) for 72 hours and its mIL-2R induced by PHA could be measured by antibodies against the membrane of T cells. The anti-Tac antibodies with biotin and SA-HRP were smeared on different sheet glasses. The immune sheet glass pores were measured after staining with the color-developing agent and several washings with TBS. The total number of 200 PBMCs was counted and its positive cells were statistically analyzed under a microscope. The cells with membrane stained brown was regarded as positive.

Treatment modality

All the newborns with HIE were treated by sufficient ventilation and air exchange, correction of acid base disorder and electrolyte disturbance, systematic hemoperfusion, improvement of heart function, degradation of convulsion, and acute intracranial hypertension.

Statistical analysis

All the data were expressed as means ± SD. Student's *t* test was performed for comparison of parameters between the HIE group and the normal controls. Statistical significance was considered when a *P* value was lower than 0.05.

Results

The expression levels of CD_3^+, CD_4^+, CD_8^+ and mIL-2R were lower in newborns with HIE at the first day after birth than that in the controls ($P<0.01$-$P<0.05$, Table 1), so did at the third day after birth in this study (Table 2). Interestingly, induced reaction of mIL-2R in newborns with HIE against PHA was still high, although the expression level of mIL-2R was low in HIE ($P<0.01$). At the seventh day after birth, the expression level of T subsets and mIL-2R was obviously increased after symptomatic and supportive treatments comparing with the normal controls (Table 3).

Discussion

The mechanism of HIE includes energy dysmetabolism, neurotoxic injury caused by excitatory amino acids,

internal flow of Ca^{2+}, reperfusion injury, free radical injury, capillary injury, etc. Transcellular ion-pump failure results in the intracellular accumulation of Na^{+}, Ca^{2+}, and water (cytotoxic edema). Ca^{2+} ions accumulate within the cytoplasm as a consequence of increased cellular influx and decreased efflux across the plasma membrane combined with release from mitochondria and endoplasmic reticulum.[5-7] Typical examples of focal injuries are arterial or venous infarctions, whereas the classical example of diffuse injury is HIE. In recent years, the progress of HIE has been found to be associated with the cellular immune function of the host.[8-9] Abundant naive lymphocytes can be identified in most of newborns but few of differentiaed mature T cells (CD_3^+), T helper cells (CD_4^+), suppression or cytotoxic T cells (CD_8^+) in newborns with HIE, which is intimately related to the state of an illness. In our study, the disordered cellular immunity, inducing disorder of T lymphocytes subsets, and low level of immunoglobulin and complement could be induced by asphyxiation and hypoxia.[10] Conversely, anoxia neonatorum ischemia brain injury could be induced by dysfunction of cellular immunity.T lymphocytes, a group of complicated multifunctional cell colonies, include many subsets of CD_3^+, CD_4^+, CD_8^+ and others. The T subset positive for CD_3^+, usually represents T lymphocytes and can reflect the condition of cellular immunity and immune response to different exogenous

Table 1. The expression levels of CD_3^+, CD_4^+, CD_8^+ and mIL-2R in PBMCs of the newborns with HIE (*n*, means±SD; %, first day)

Group	*n*	CD_3^+	CD_4^+	CD_8^+	CD_4^+/CD_8^+	mIL-2R	
						Silence	Inducement
First day							
Control	30	42.6±7.3	33.3±5.6	24.1±4.5	1.6±0.4	4.3±1.3	24.1±5.5
HIE	32	37.4±6.7	29.4±6.9	16.7±3.3	1.8±0.5	3.6±1.1	20.9±4.8
t		2.9246	2.4338	7. 4171	1.7316	2.2936	2.4450
P		<0.01	<0.05	<0.01	>0.05	<0.05	<0.05
Mild	15	42.6±7.0	33.5±7.2	18.8±3.6	1.8±0.6	3.9±1.2	23.2±5.0
Moderate	9	35.2±6.6*	28.2±6.8	15.5±3.2*	1.9±0.4	3.4±1.2	20.0±4.8
Severe	8	30.3±6.2†	23.1±6.3†	14.0±3.0†	1.5±0.3	3.1±0.9*	17.6±4.5†

*: *P*<0.05, †: *P*<0.01; compared with HIE group (mild).

Table 2. The expression levels of CD_3^+, CD_4^+, CD_8^+ and mIL-2R in PBMCs of the newborns with HIE (*n*, means±SD; %, third day)

Group	*n*	CD_3^+	CD_4^+	CD_8^+	CD_4^+/CD_8^+	mIL-2R	
						Silence	Inducement
Third day							
Control	30	44.9±7.6	39.7±6.1	25.5±4.7	1.8±0.4	4.6±1.2	24.9±5.7
HIE	32	41.0±7.4	35.8±6.9	22.6±4.5	1.7±0.5	3.9±1.2	22.8±5.1
t		2.0469	2.3517	2.4820	0.8658	2.2951	1.5307
P		<0.05	<0.05	<0.05	>0.05	<0.05	>0.05
Mild	15	43.6±7.3	37.8±7.1	25.6±4.7	1.6±0.5	4.1±1.3	23.8±5.2
Moderate	9	40.8±7.6	35.4±6.9	21.5±4.4*	1.7±0.5	3.8±1.2	22.5±5.1
Severe	8	36.4±7.2*	32.4±6.5*	18.2±4.1†	1.7±0.6	3.5±1.0	21.1±4.9

*: *P*<0.05, †:*P*<0.01; compared with HIE group (mild).

Table 3. The expression level of CD_3^+, CD_4^+, CD_8^+ and mIL-2R in PBMCs of the newborns with HIE (*n*, means±SD; %, seventh day)

Group	*n*	CD_3^+	CD_4^+	CD_8^+	CD_4^+/CD_8^+	mIL-2R	
						Silence	Inducement
Seventh day							
Control	30	45.8±5.7	40.3±6.6	26.1±4.6	1.6±0.4	4.7±1.0	25.3±5.8
HIE	32	41.8±6.1	36.4±5.1	25.6±4.3	1.5±0.3	4.1±1.2	23.8±5.2
t		2.6633	2.6131	0.4424	1.1186	2.1314	1.0735
P		<0.01	<0.05	>0.05	>0.05	<0.05	>0.05
Mild	15	43.5±6.1	38.0±5.2	26.4±4.3	1.5±0.3	4.4±1.3	24.7±5.3
Moderate	9	41.7±6.2	36.5±5.0	25.4±4.3	1.5±0.3	4.0±1.2	23.5±5.2
Severe	8	38.9±6.0†	33.3±5.1*	24.4±4.5	1.5±0.4	3.8±1.0	22.4±5.0

*: *P*<0.05, †: *P*<0.01; compared with HIE group (mild).

antigens. The T subset positive for CD_4^+, however, is regarded as T helper cells/T inducible cells that can help B cells to promote the function of various antibodies and IL-2. The T subset positive for CD_8^+ or T suppression cells/T cytotoxic cells can help B cells to restrain various antibodies and exert cytotoxic effect on MHC-I antigen at the membrane of target cells. The ratio of CD_4^+/CD_8^+, an important marker in immunoregulation in the host, is usually recognized as a manifestation of concordance, enhancement and immune balance in various immune cells. Biotin-streptavidin staining, a new method of immunohistochemistry staining, has a low isoelectric point, few glycon, low nonspecific adsorption, high specificity and sensitivity,[11] and its results can exactly reflect the cellular immune function in newborns with HIE.

The low expression levels of CD_3^+, CD_4^+, CD_8^+ in our study were major cellular immune manifestations of the newborns with HIE at the first day after birth. The symptoms of HIE would disappear gradually after prolonged comprehensive treatment. At the third day and seventh day after birth, despite the increase of the levels of CD_3^+, CD_4^+, CD_8^+ in the newborns with HIE, differences between the experimental group and controls still exsited. This finding was disagreed by Liu et al.[12] Interestingly, we found the severity of HIE was associated with cellular immunity of newborns. This is possibly due to the following reasons: 1) synthesis and secretion of multiple neuroendocrine hormones decreased after hypoxic-ischemia and brain injury so as to restrain the immune response;[13] 2) apoptotic pathways were abnormally activated and contributed to the apoptosis of T lymphocytes;[14-15] 3) overall, the accumulation of sIL-2R and decrease of mIL-2R after hypoxia-ischemia was detrimental to neuronal cells, leading to reversible brain damage and decrease of CD_4^+ T lymphocytes.[16-19] Although the total cellular immunity is lower in newborns, the capability of immunoregulation has been developed after birth, as was illustrated by the normal ratio of CD_4^+/CD_8^+ and expression of mIL-2R. Our results also confirmed that Th1, Th2 cells could play a key role in the newborns with HIE via the pathway of secreting IFN-γ, IL-2, IL-5, IL-6, etc.

mIL-2R, an important symbol of active T cells, plays an important role in biologic effect of IL-2, and its expression reflects the activity of T cells and state of immunity.[20-21] If T lymphocytes are destroyed to some extent, the expression of mIL-2R would be decreased obviously. The results of our study demonstrated that the expression levels of mIL-2R in silence and inducing stage were lower in the newborns with HIE than in the normal controls. Interestingly, at the seventh day after birth, the expression level of mIL-2R was increased or approached normal. We confirmed in the present study that the better immune reaction against different inflammation is caused by hypoxia and ischemia via the pathway of regulation of different cytokines, such as releasing IL-1β, IL-6, IL-8 to the site of inflammation, or releasing IL-10, inhibiting inflammatory transmitters, and down-regulating local immunologic injury.[22]

In conclusion, cellular immunity may take part in the course of HIE. Cellular immunity disorder has been confirmed in newborns with HIE and more common in those with severe HIE. The better immune regulation of newborns with HIE has higher effect of repairment on brain injury. Clinical trials should be continued to improve the cure rate of HIE and various treatments are needed to prevent aggravation of HIE in newborns.

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