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Effect of human cytomegalovirus on proliferation of hematopoietic progenitor cells of cord blood

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Abstract: Objective This study was designed to investigate the effect of human cytomegalovirus (HCMV) on the proliferation of colony forming unit granulocyte-macrophage (CFU-GM), CFU-erythroid (CFU-E), burst forming unit-erythroid (BFU-E), CFU-multipotential (CFU-Mix) and CFU-megakaryocytic (CFU-Mk) progenitor cells of cord blood *in vitro* as well as the possible mechanism. **Methods** Twenty cord blood specimens were collected from the umbilical vein of normal full-term neonates delivered spontaneously. This study consisted of five groups: 3 Infection groups in which 0.1 mL 10^3, 10^4 and 10^5 plague forming unit (PFU) HCMV-AD_{169} virus solution was added to the culture system, an Inactivated control group in which the equal volume of inactivated virus solution was added, and a Blank control group (normal progenitor cells culture system without HCMV virus infection). Colony forming unit-assay was applied to detect the effects of HCMV-AD_{169} strain on the colony formation, inhibition rate and colony-maintaining duration of CFU- GM, CFU-E, BFU-E, CFU-Mix and CFU-Mk of cord blood. PCR technique was used to demonstrate the existence of HCMV-DNA in the colony cells of cultured CFU-GM, CFU-E, CFU-Mix and CFU-Mk. **Results** HCMV-AD_{169} (10^3 PFU) in low concentration had inhibition effects on colony formation of the CFU-Mix and CFU-MK ($P < 0.05$), whereas 10^5 PFU and 10^4 PFU HCMV-AD_{169} lead to decreased colonies in CFU-GM, CFU-E, BFU-E, CFU-Mix and CFU-MK compared with the Blank control and the Inactivated control groups ($P < 0.05$). The suppression effect of HCMV on the colony formation was dose-dependant. The colony-maintaining duration of the CFU-GM, CFU-E, BFU-E, CFU-Mix and CFU-Mk in the 10^5 PFU and 10^4 PFU HCMV infection groups was significantly shorter than that in the two control groups ($P < 0.01$). The low concentration of HCMV-AD_{169} (10^3 PFU) infection resulted in a shortened colony-maintaining duration of the CFU-Mix and CFU-Mk ($P < 0.01$), but had no effects on the colony-maintaining duration of CFU-GM, CFU-E and BFU-E. PCR amplification demonstrated the existence of HCMV-AD_{169} DNA in the colony cells of the three Infection groups. **Conclusions** HCMV-AD_{169} strain can infect hematopoietic progenitors of cord blood and inhibit the proliferation of hematopoietic progenitors, associated with anemia, neutropenia and thrombocytopenia in HCMV patients.

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Key words: Cytomegalovirus; Hematopoietic stem cells; Cell proliferation; Cord blood

人类巨细胞病毒感染对脐血造血祖细胞增殖的影响

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[**摘　要**] **目的**　探讨人类巨细胞病毒(HCMV)感染对脐血造血祖细胞(CFU-GM、CFU-E、BFU-E、CFU-Mix及CFU-Mk)体外增殖的抑制作用及其机制。**方法**　20例脐血标本收集于正常足月顺产新生儿。实验共分5组:(1)3个HCMV感染组,每个感染组分别加入0.1 mL的10^3、10^4及10^5空斑形成单位(PFU)HCMV-AD_{169}病毒液于培养体系中;(2)灭活对照组,加入同体积灭活HCMV病毒液;(3)空白对照组,不加HCMV病毒液,代之以同体积的IMDM。采用造血祖细胞体外半固体培养技术,培养、观察、计数HCMV-AD_{169}株对脐血CFU-GM、CFU-E、BFU-E、CFU-Mix及CFU-Mk集落数、抑制率和集落维持时间;并用聚合酶链反应(PCR)技术检测集落细胞内HCMV-DNA。**结果**　(1)在造血祖细胞培养体系中加入不同滴度的HCMV-AD_{169}后,10^4和10^5 PFU滴度感染对CFU-GM、CFU-E、BFU-E、CFU-Mix及CFU-Mk集落形成均有显著的抑制作用,10^3 PFU滴度感染对CFU-Mix及CFU-

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Mk 集落形成有显著的抑制作用,与空白对照组和灭活对照组比较,差异有显著性($P<0.05$)。病毒滴度越高,抑制程度越明显($P<0.05$)。(2) 10^4 和 10^5 PFU 滴度感染组 CFU-GM、CFU-E、BFU-E、CFU-Mix 及 CFU-Mk 集落维持时间较对照组明显缩短($P<0.01$),10^3 PFU 滴度感染组 CFU-Mix 和 CFU-Mk 集落维持时间较对照组明显缩短($P<0.01$)。(3) PCR 显示 3 个感染组的 CFU-GM、CFU-E、CFU-Mix 及 CFU-Mk 集落细胞内均有 HCMV-AD_{169} DNA 存在。**结论** HCMV-AD_{169}能直接感染 CFU-GM、CFU-E、BFU-E、CFU-Mix 及 CFU-Mk 造血祖细胞,并抑制造血祖细胞的增殖,这可能与 HCMV 感染患儿出现粒细胞减少、血小板减少和贫血等造血功能紊乱有关。

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Human cytomegalovirus (HCMV), a member of the herpes virus family, infects the majority of the population by adulthood [1]. Acute HCMV infections in immunocompromised individuals may cause severe hematological disorders. Clinically, in patients undergoing hematopoietic stem cell transplantation (HSCT) HCMV can be associated with delayed platelet engraftment, phenotypically abnormal peripheral blood leukocytes, and graft rejection [2,3], possibly through a direct viral effect on hematopoietic progenitor cells [4]. However, the mechanism of the suppression effect of HCMV on colony-forming has not been completely identified. Recently, considerable interest has arisen as to use cord blood as a source of hematopoietic stem cells for allogenic transplantation. Goodrum's study [5] has shown the suppression effects of HCMV on colony forming of hematopoietic precursors of bone marrow (BM). It is important to examine whether a similar effect is also observed in HCMV-infected hematopoietic progenitor cells of cord blood. In this study we investigated the effect of HCMV on proliferation of colony forming unit granulocyte-macrophage (CFU-GM), CFU-erythroid (CFU-E), burst forming unit-erythroid (BFU-E), CFU-multipotential (CFU-Mix) and CFU-megakaryocytic (CFU-Mk) progenitor cells of cord blood *in vitro* as well as the possible mechanism.

Materials and methods

Cord blood specimens

Twenty cord blood specimens were provided by the Department of Obstetrics of the Affiliated Hospital of Luzhou Medical College. They were collected from the umbilical vein of normal term neonates delivered spontaneously.

HCMV strains

HCMV-AD_{169} strains were obtained from the Institute of Virology, Chinese Academy of Preventive Medicine. The initial concentration of HCMV-AD_{169} was 10^6 plague forming unit (PFU)/mL, which was diluted into three kinds of concentration (10^5, 10^4 and 10^3 PFU/mL) before use. A part of initial HCMV-AD_{169} solution was inactivated by exposing to ultraviolet lamp for 15 minutes. These were preserved at -70℃ for use.

Main reagents

Iscove's Modification of Dulbecco's Medium (IMDM) and methylcellulose were provided by Sigma Company (USA), and recombinant human granulocyte/monocyte colony stimulating factor (rhGM-CSF), by Kirin Company (Japan). Recombinant human thrombop-oietin (rhTPO), recombinant human interleukin-3 (rhIL-3) and recombinant human interleukin-6 (rhIL-6) were obtained from Pepro Tech EC (England). Recombinant human erythropoietin (rhEPO), and 2-mercap-toethanol and L-glutamine (L-Glu), as well as HCMV PCR test kit, were provided by Shandong Kexin Biological Products Company, Shanghai Huashun Biological Products Company and Shanghai Fuxin Biological Products Company of China, respectively.

Methods

Separation of cord blood mononuclear cells (CBMC)

The cord blood sample was firstly diluted with 1640 medium at 1:1 before centrifugation (1 500 rpm, 10 minutes). Supernatant fluid was discarded, followed by addition of equal volume of 1640 medium. After thorough mixing, the solution was transferred onto the surface of 3 mL lymphocyte separation solution (Hypague-Ficoll, specific weight = 1.077). Thereafter, centrifugation at 1 800-2 000 rpm for 20 minutes was carried out. The supernatant fluid was then added into 1640 medium. After mixing, they were centrifuged at 1 800-2 000 rpm for 10 minutes and were washed twice. Finally, the precipitation was diluted with IMDM to dispense the mononuclear cell suspension.

Experimental grouping

There were 5 experimental groups: (1) 10^3, 10^4

and 10^5 PFU three infection groups: 0.1 mL 10^3, 10^4 and 10^5 PFU HCMV-AD_{169} virus solution was added to the culture system to each respective group; (2) Inactivated control group: equal volume of inactivated virus solution was added; (3) Blank control group: no HCMV virus solution was added and equal volume of IMDM was added instead.

Culture of hemopoietic progenitor cells

The culture system consisted of 0.9 g/L methylcellulose, 30% FCS, CBMC suspension (final concentration: 2×10^5/mL), IMDM, and HCMV-AD_{169} solution. Stimulation factors needed for the growth of hemopoietic progenitor cells were added into various culture systems to establish an *in vitro* hemopoietic microenvironment similar to the one under physiological conditions. See Table 1. The specimens were incubated in a 37℃ and 5% CO_2 saturated humidity atmosphere for 14-30 days[6].

Table 1 Stimulation factors added into the various culture systems

	Stimulation factors
CFU-GM	rhGM-CSF
CFU-E	2-mercaptoethanol, L-Glu, rhEPO
CFU-Mk	IL-3, IL-6, rhTPO, 2-mercaptoethanol
CFU-Mix	IL-3, IL-6, rhTPO, rhGM-CSF, rhEPO, 2-mercaptoethanol, L-Glu

Identification and counting of the colony

The colony was counted under an inverted microscope. The mean value of two wells was adopted. The CFU-GM colony, the BFU-E colony, and the CFU-Mix colony (including erythroid, granulocyte, macrophage and /or megakaryocyte systems) meant a cell mass which contained over 50 cells and was counted at the 7th day, 10th-14th day and 14th day of culture, respectively. The CFU-E colony meant a cell mass which contained 8-50 cells and was counted at the 5th-7th day of culture. The CFU-Mk colony which contained more than 3 cells megalokaryocyte mass was counted at the 18th day of culture.

Morphological analysis of colony cells

In order to identify the cell component of colonies routine Wright-Giemsa staining was applied in the analysis of CFU-GM, CFU-Mix and CFU-Mk colony cells, and biphenylamine staining was used for the analysis of BFU-E and CFU-E colony cells.

Detection of the HCMV-DNA in the colony cells

HCMV-DNA in the colony cells of CFU-GM, CFU-E, CFU-Mix and CFU-Mk groups was detected using PCR. Colony cells were washed with PBS buffer solution (0.1 M, PH 7.2) under low speed centrifugation twice, and then washed with ddH_2O once. Supernatant was discarded to remove the residual virus attached to cell surface. PCR was performed according to the kit instruction. The appearance of a 420 bp specific amplification band indicated positive results.

Statistical analysis

Statistical analysis was performed by statistical software SPSS 10.0. Data were presented as means ± SD ($\bar{x} \pm s$). ANOVA and SNK were applied to test the differences between groups. Percent of inhibition = (colonies of the Inactivated control group - colonies of the HCMV infection group)/(colonies of the Inactivated control) ×100%.

Results

Effects of HCMV on colony formation of hematopoietic progenitor cells

Low concentration of HCMV-AD_{169} (10^3 PFU) was found to have inhibition effects on the colony formation of the CFU-Mix and CFU-Mk. 10^5 PFU and 10^4 PFU HCMV-AD_{169} were shown to decrease colonies of CFU-GM, CFU-E, BFU-E, CFU-Mix and CFU-Mk compared with the Blank control and the Inactivated control groups. The suppression effect of HCMV on the colony formation was dose-dependant. See Table 2.

Table 2 Effects of HCMV on colony formation of hematopoietic progenitor cells ($\bar{x} \pm s$, $n = 20$)

Group	CFU-GM		CFU-E		BFU-E		CFU-Mix		CFU-Mk	
	Colonies (/2×10^5CBMC)	Inhibition rate (%)	Colonies (/2×10^5CBMC)	Inhibition rate (%)	Colonies (/2×10^5CBMC)	Inhibition rate (%)	Colonies (/2×10^5CBMC)	Inhibition rate (%)	Colonies (/2×10^5CBMC)	Inhibition rate (%)
Blank	90.4 ± 12.2	—	814.4 ± 211.8	—	37.2 ± 4.0	—	129.3 ± 21.2	—	90.1 ± 10.9	—
Inactivated	90.9 ± 9.4	—	799.3 ± 209.6	—	36.6 ± 4.6	—	126.8 ± 18.1	—	86.5 ± 10.1	—
10^5PFU HCMV	53.7 ± 9.7[a,b]	40.9	416.3 ± 114.4[a,b]	47.9	21.2 ± 2.6[a,b]	53.3	56.7 ± 11.9[a,b]	55.2	47.0 ± 13.1[a,b]	42.1
10^4PFU HCMV	61.2 ± 7.5[a,b]	32.7	558.3 ± 114.4[a,b]	30.2	23.4 ± 5.1[a,b]	36.1	82.3 ± 12.6[a,b]	35.1	60.8 ± 11.3[a,b]	29.7
10^3PFU HCMV	83.6 ± 7.5[b]	8.0	744.2 ± 180.6[b]	6.9	33.6 ± 4.4[b]	8.2	101.1 ± 13.4[a,b]	20.3	74.0 ± 8.1[a,b]	14.5

a compared with the Blank and the Inactivated groups, $P < 0.05$; b comparison among the three Infection groups, $P < 0.05$

Effects of HCMV on the colony-maintaning duration of hematopoietic progenitors cells

The colony-maintaning duration of the CFU-GM, CFU-E, BFU-E, CFU-Mix and CFU-Mk in the 10^5 PFU and 10^4 PFU HCMV infection groups was significantly shorter than that in the two control groups. Low concentration of HCMV-AD_{169} (10^3 PFU) infection resulted in a shortened colony-maintaining duration of the CFU-Mix and CFU-Mk, but had no effects on the colony-maintaining duration of CFU-GM, CFU-E and BFU-E. See Table 3.

Table 3 Effects of HCMV on colony-maintaining duration of hematopoietic progenitors ($\bar{x} \pm s$, $n = 20$)

Group	Colony-maintaining duration (day)				
	CFU-GM	CFU-E	BFU-E	CFU-Mix	CFU-MK
Blank	17.6 ± 4.1	10.5 ± 2.5	22.4 ± 3.6	23.4 ± 2.9	28.9 ± 3.2
Inactivated	18.2 ± 3.4	11.7 ± 1.3	23.8 ± 3.2	23.9 ± 3.1	27.6 ± 2.9
10^5PFU HCMV	12.5 ± 2.4[a]	7.4 ± 1.6[a]	17.1 ± 3.2[a]	14.1 ± 2.3[a]	20.8 ± 2.5[a]
10^4PFU HCMV	13.1 ± 2.6[a]	8.9 ± 2.1[a]	18.6 ± 2.8[a]	15.7 ± 2.8[a]	22.5 ± 1.7[a]
10^3PFU HCMV	16.9 ± 4.7	9.1 ± 2.8	22.4 ± 2.6	18.5 ± 4.8[a]	23.8 ± 4.9[a]

a compared with the Blank and the Inactivated groups, $P < 0.01$

HCMV-DNA

The colony cells (CFU-GM, CFU-E, CFU-Mix and CFU-Mk) were collected at the peak time of colony formation and the HCMV-DNA of the colony cells was detected by PCR. As a result a specific 420 bp amplification band appeared in the three HCMV infection groups, while the Blank control and the Inactivated control groups presented negative results (Figure 1).

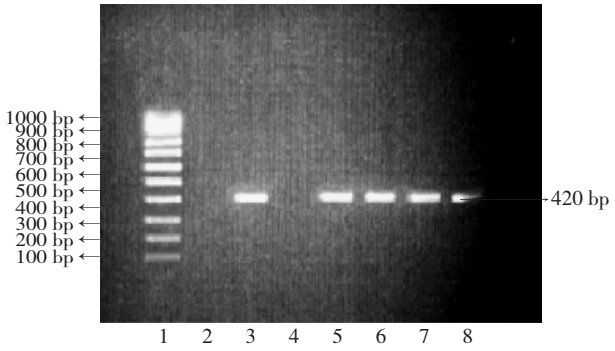

Figure 1 HCMV-DNA PCR products of colony cells.
1. DNA molecular weight standard; 2. Blank control group; 3. Positive control group; 4. Inactivated control group; 5. HCMV group: CFU-GM; 6. HCMV group: CFU-E; 7. HCMV group: CFU-Mix; 8. HCMV group: CFU-Mk.

Discussion

It was reported that many diseases were related to hematopoietic suppression induced by virus infection[7]. HCMV may cause hemopoietic system diseases such as mononucleosis, thrombocytopenia, leukopenia and hemolytic anemia[2]. However, its mechanism is still unclear. In this study, cord blood CFU-GM, CFU-E, BFU-E, CFU-Mix and CFU-Mk were cultured *in vitro* using a hemopoietic progenitor cell culture system. Hemopoietic progenitor cells were continuously infected by different concentrations of HCMV solution. Samples were cultured until the peak time of colony formation. Thereafter, the colony number of CFU-GM, CFU-E, BFU-E, CFU-Mix and CFU-Mk as well as the colony-maintaining duration were measured. In this study, an obvious suppression effect of HCMV on the CFU-GM, BFU-E, CFU-E, CFU-Mix and CFU-Mk colony formation was observed, and the suppression effect was dose-different, i. e. the colony number decreasing with the virus concentration increasing. It was also observed that the colony-maintaining duration was significantly shortened in the HCMV infected groups than that of the Control group. These results suggest that HCMV infection can remarkably suppress the proliferation of cord blood hemopoietic progenitor cells.

We collected the colony cells (CFU-GM, CFU-E, CFU-Mix and CFU-Mk) at the peak time of colony formation and the HCMV-DNA of the colony cells was tested by PCR. As a result the HCMV groups presented positive but negative in the Blank control and the Inactivated control groups. This suggests showed that HCMV was present in progenitor cells.

Without support of stromal cells, B lymphocytes can not further mature in cord blood, which means the influences of stromal cells and immune cells were relatively less in this experiment. This study showed that, comparing with the Blank control and the Inactivated control groups, the colony number was significantly decreased, and the HCMV-AD_{169} DNA could be detected in hemopoietic progenitor cells in the HCMV infection groups. These results indicated that the suppression of hemopoietic progenitor cell colony formation might result from direct infection of HCMV, which may be related to the haematopoiesis disorders such as anemia, granulocytopenia and thrombocytopenia after HCMV infection.

This is a preliminary *in vitro* study on the suppression of HCMV on cord blood hemopoietic progenitor cells. Further investigation is needed to elucidate its mechanism and the suppression effect of HCMV on hemopoietic system *in vivo*.

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· 消息 ·

《中国当代儿科杂志》被 MEDLINE 收录

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2005 年 11 月 30 日

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