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Propagation of CAR Bacillus in Artificial Media

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CAR bacillus propagated successfully in an artificial medium, and the number of CAR bacillus was about 30 times the original number after 8 days of cultivation. The medium consisted of Eagle's minimum essential medium supplemented with 10% fetal calf serum and 20% hamster tracheal organ culture soup. By intranasal inoculation to mice, two strains of the CAR bacillus passaged 5 and 6 times in this artificial medium produced the same lung lesions as natural CAR bacillus infection. — KEY WORDS : CAR bacillus, culture, medium

Cilia-associated respiratory (CAR) bacillus is a newly recognized respiratory pathogen in laboratory animals including mice and rats [1~5]. This organism can grow in the allantoic cavity of embryonated hen's eggs [1] and also by overlay cultivation on the 3T3 cells, mouse fibroblast cell line [Waggie, K. S. Personal communication], but not in cell-free artificial media [1,2]. This brief communication describes the propagation of CAR bacillus in cell-free artificial media and the pathogenicity of the propagated bacilli to mice.

To examine the growth of CAR bacillus in culture media, the CBM strain [5] of CAR bacillus of mouse origin passaged in the allantoic cavity of 7-day-old chick embryos was used. The examination media were prepared with Eagle's minimum essential medium without antibiotics (MEM, Flow lab., USA) or Dulbecco's phosphate buffered saline (PBS, Nissui, Tokyo) as basal media, and with fetal calf serum (FCS, GIBCO, USA) and hamster tracheal organ culture soup (HTS) as supplements. The HTS was prepared as follows. The tracheas of 4-week-old Syrian hamsters were taken aseptically, cut about 5 pieces in each and cultured with MEM supplemented with 10% FCS in

5% CO₂ atmosphere for one month in a culture plate with 48 wells (Corster, USA). The culture medium was changed twice a week. Then, the culture soup was pooled and stored at -20°C. After filtration using a millipore filter (pore size : 0.2 µm), the soup was used as HTS. To examine the growth of the CAR bacillus, 0.1ml of the CBM-infected allantoic fluid containing 2.0×10^7 bacilli/ml was inoculated in triplicate in a culture plate with 48 wells filled with 0.5 ml of each examination medium. Then, the inoculated culture plate was incubated at 37°C in 5% CO₂ atmosphere. After 7 days, the number of CAR bacillus in 5 µl of the culture medium spread over a 1cm² square area was estimated by a microscopic count of preparations stained with immunofluorescent staining.

As shown in Table 1, the CBM strain was successfully propagated in the both of MEM and PBS supplemented with 10% FCS and 20% HTS. However, the growth ratios in the respective media were 11 and 19 times, and lower than that of the chick embryos. Although there was no clear difference in the CBM strain counts between MEM and PBS, the CBM strain propagated with MEM as basal medium showed higher motility and firmer stainability than

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Table 1. Propagation of the CBM strain of CAR bacillus in artificial media

Media	Mean bacterial count after cultivation (/ml)	Growth rate*
MEM without supplements	8.0×10^6	2.5
MEM + FCS(10%)	5.0×10^6	1.6
+ HTS(20%)	5.6×10^6	1.8
+ FCS(10%) + HTS(20%)	60.0×10^6	18.8
PBS without supplements	9.5×10^6	3.0
PBS + FCS(10%)	9.9×10^6	3.1
+ HTS(20%)	25.0×10^6	7.8
+ FCS(10%) + HTS(20%)	36.0×10^6	11.3
(Original bacterial count :	3.2×10^6)	
Chick embryo	95.0×10^6	79.2
(Original bacterial count :	1.2×10^6)	

* : Mean bacterial count / Original bacterial count

Fig. 1. Growth curve of the CBM strain of CAR bacillus in the medium

that propagated with PBS, suggesting their higher viability.

The growth curve of the CBM strain propagated in the present artificial medium (MEM supplemented with 10% FCS and 20% HTS) is shown in Fig. 1. After 8 days of culturing, the number of the CAR bacillus reached about 30 times the original number. The

numbers decreased gradually during the 10 days following cultivation, and declined to the starting number at the end of the observation for 20 days. By microscopic observation as shown Fig. 2, the CAR bacillus propagated in this medium tended to aggregate easily in the medium, and propagated on the bottom of the well, which seemed like extension from the aggregates.

In order to elucidate the pathogenicity of the CAR bacillus propagated in this artificial medium, experimental infection to mice was examined. Two strains of CAR bacillus, mouse origin CBM and rat origin CBR strains [2], passaged in this medium 6 and 5 times in the same manner contained 0.9×10^7 and 1.6×10^7 bacilli/ml, respectively. Ten microliters of the passaged medium or the bacteria-free medium was inoculated intranasally under ether anesthesia into 4-week-old female Jcl : ICR mice (CLEA Japan, Tokyo) which had been confirmed free from Sendai virus, mouse hepatitis virus, mouse adenovirus, mouse poxvirus, *Mycoplasma pulmonis*, *Pasteurella pneumotropica*, Tyzzer's organism, *Corynebacterium kutscheri* and *Salmonella typhimurium*. Mice were housed in autoclaved cages with wood shavings as bedding and given γ ray-irradiated pellets (CE-2, CLEA Japan, Tokyo) and tap water *ad libitum*. The animal room was controlled at 20 to 24°C and a 12hr light/dark cycle. After 8 weeks, these inoculated mice were sacrificed and subjected to pathological, bacteriolo-

Fig. 2. Phase contrast photomicrograph of CAR bacillus in the medium $\times 100$ A : 6 days after cultivation B : 9 days after cultivation

Fig. 3. Lung lesion of a mouse inoculated with CBM (6 th passage) H-E stain $\times 10$

Table 2. Pathogenicity of CAR bacillus strains passaged in the medium to mice

Inoculum strains	Lung lesion	Antigen* in lungs	Serum** antibody	CAR bacillus*** isolation
CBM (6th passage)	5/5****	5/5	5/5	5/5
CBR (5th passage)	5/5	5/5	5/5	5/5
Medium	0/4	0/4	0/4	0/4

* : Immunofluorescent method

** : ELISA method

*** : Inoculation

to chick embryo with the lung washout

**** : No. positive / No. examined

gical and serological examinations. The lung tissues were fixed in formalin, embedded in Paraplast+ (Monoject, USA), and stained with hematoxylin and eosin for histopathological examination. Isolation of CAR bacillus from the lung washout was performed by the method using chick embryos described previously [2, 5]. Detection of serum antibody against CAR bacillus was performed by ELISA as described previously [6]. These results are summarized in Table 2. Histopathologically, severe peribronchial lymphoid cell accumulation was observed in all of the inoculated mice (Fig. 3), and CAR bacillus was detected in the cilia of the bronchial epithelium by the indirect immunofluorescent staining described previously [2]. These lung lesions and the distribution of the pathogen were identical to natural and experimental CAR bacillus infection in mice and rats

[1~5]. Moreover, CAR bacillus was reisolated from the lung washouts of all mice. All of the inoculated mice were seroconverted. These findings indicate that CAR bacillus propagate successfully in artificial medium and the CAR bacillus passaged in this medium are pathogenic for mice.

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人工培地による CAR バチルスの培養

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CAR バチルスが MEM 培地に牛胎仔血清とハムスター気管の培養上清をそれぞれ10%と20%添加した人工培地で増殖することを明らかにした。菌数は培養8日後に

接種時の30倍になった。さらに、本培地で5から6代継代した CAR バチルスのマウスに対する病原性も確認された。