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Characteristics of recombinant human butyrylcholinesterase[1]

YUAN Hui-Jun[2], YU Wei-Yuan[3], SHI Cheng-Hua[3], SUN Man-Ji[4]
(*Institute of Pharmacology and Toxicology*, [3]*Institute of Biotechnology*, *Academy of Military Medical Sciences*, *Beijing 100850*, *China*)

KEY WORDS butyrylcholinesterase; gene expression; recombinant proteins; succinylcholine; drug metabolic detoxication; CHO cells; enzyme-linked immunosorbent assay; Western blotting; physostigmine

ABSTRACT

AIM: To study the biochemical-pharmacological properties of the recombinant human butyrylcholinesterase (rhBChE) and thereby to size up the potential possibility of using it as a detoxifying agent in succinylcholine intoxication. **METHODS:** CHO-dhfr$^-$ cells were transfected with plasmids by electroporation. BChE activity was determined colorimetrically by 5, 5′-dithiobis-(2-nitrobenzoic acid) (DTNB) method. Antigenicity was estimated by enzyme-linked immunosorbent assay and Western blot. **RESULTS:** The maximal expression amounted to 25.83 ng · h^{-1}/10^6 cells. The rhBChE was highly similar to the native human BChE (nhBChE) in terms of its catalytic property, substrate affinity, inhibitor sensitivity, reactivation, stability, and immunoreactivity with anti-nhBChE antibodies. Mice challenged with 1.5 lethal dose of succinylcholine preincubated with rhBChE survived without any symptoms of intoxication. **CONCLUSION:** The rhBChE and nhBChE exhibit similar biochemical-pharmacological features. It is of potential value in practical use.

INTRODUCTION

Many organophosphate (OP) compounds have been developed as farm pesticides and chemical warfare agents for their strong toxicity. A new approach of protection against OP intoxication is to pretreat potential subjects with cholinesterase preparations[1,2] which rapidly "neutralize" and thereof remove OP from the circulation before they reach the targets. Preinjection of butyrylcholinesterase (BChE) prevents animals from intoxication of multiple lethal doses of soman without any symptoms. BChE is naturally present in human blood in a soluble form with a half-life of approximately 1 wk[3], so a single injection will have a long-term effect. In Europe, human BChE has also been an approved drug used in human for reversion prolonged apnea caused by muscle relaxant succinylcholine in surgery[4]. BChE may be also useful in therapy of intoxication of cocaine and heroin. BChE extracted from outdated plasma is limited in resources, and is fraught with danger of infection of diseases such as hepatitis and AIDS. It therefore may be more desirable to use the recombinant human butyrylcholinesterase (rhBChE) instead. However it has not yet been proved to be identical to the native human BChE (nhBChE) in biochemical-pharmacological properties. The aim of this paper was to study the biochemical-pharmacological properties of the rhBChE and thereby to size up the potential possibility of using it as a

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[2] Now in *Institute of Otolaryngology, PLA General Hospital, Beijing 100853, China*.
[4] Correspondence to Prof SUN Man-Ji. Phn 86-10-6693-0691. Fax 86-10-6821-1656. E-mail sunmj@nic.bmi.ac.cn
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detoxifying agent in succinylcholine intoxication.

MATERIALS AND METHODS

Plasmid Expression plasmid pReCMV-BCHE carried full-length BChE and its signal peptide gene (1.8 kb). The expression vector pReCMV was approximately 5.5 kb in length containing 655 bp of cytomegalovirus (CMV) promoter, neomycin and ampicilin resistant genes.

Reagents Human serum BChE (9 kU · g^{-1}, Boehringer Mannheim, Germany); polyclonal antibody against human serum BChE (Dako, Denmark); serum-free medium (SFM) and Dulbecco's modified Eagle medium (DMEM, Gibco, USA); methotrexate (MTX), hypoxanthine, and thymine (Sigma, USA); physostigmine (Wellcome, UK). 5,5′-dithio-bis-(2-nitrobenzoic acid) (DTNB) and reactivator 1-(2-hydroxyimino methyl-1-pyridinio)-3-(4-carbamoyl-1-pyridinio)-2-oxapropane dichloride (HI-6) were synthesized by Department of Chemical Synthesis in our Institute.

Identification of expression plasmid pReCMV-BCHE Expression plasmid pReCMV-BCHE was digested at 37 ℃ for 1 h with restriction endonucleases Sac Ⅰ, Pst Ⅰ + Bgl Ⅱ, Apa Ⅰ, EcoR Ⅰ, BamH Ⅰ, and Hind Ⅲ + Apa Ⅰ, and analysed by 1% agarose gel electrophoresis.

Cell culture CHO cells containing zero copy of the dihydrofolate reductase (dhfr) gene were cultured in the growth medium DMEM plus glysine, *L*-proline, hypoxanthine, thymine, antibiotics, and 10 % fetal calf serum (FCS). Having been transfected with plasmid carrying DHFR gene, CHO cells were cultured in the selective medium (growth medium lacking of hypoxanthine and thymine).

Transformation and selection of CHO cells Half confluent CHO cells (8×10^6) were mixed with plasmids pReCMV-BCHE and pSV_2-dhfr (10:1). The plasmids were introduced into CHO cells by electroporation (capacitance 760 μF, voltage 240 V, discharge time 1000 ms). Cells that had taken up foreign DNA were selected by growing them in selective medium. Colonies appeared in 2 – 3 wk after transfection. Single colonies were isolated and grew in 96-well plate. When the cells were confluent, the culture media were replaced with SFM and the rhBChE activity was assayed in 24 h. The positive clones were treated with MTX 0.05 – 3 μmol · L^{-1} for elevating expression level of rhBChE.

Secreted and intracellular rhBChE To find the relative amounts of active rhBChE in the culture medium and in the cell, activity was tested in both compartments. Secreted activity was determined by adding the cultured medium directly to the assay mixture. Intracellular BChE activity was determined after lysing cells by freeze-thawing. The soluble form of BChE was extracted with low salt buffer by freezing the pellet in the presence of 100 mL of phosphate buffer 0.1 mol · L^{-1} (pH 7.0), followed by thawing of the lysed cell, centrifuging and collecting the supernatant. The remaining pellet was extracted with high salt buffer by freezing the pellet in the presence of 100 mL of NaCl 1 mol · L^{-1}, egtazic acid 1 mmol · L^{-1}, Tris · Cl 10 mmol · L^{-1} (pH 7.6), 1 % Triton X-100, followed by thawing, centrifuging, and collecting the supernatant. Each supernatant was used in the activity assay.

BChE activity assay BChE activity and/or its residual activity after inhibition and reactivation were measured by the DTNB method[5]. Final substrate concentration was butyrylthiocholine iodide 20 mmol · L^{-1}.

Recognition by anti-nhBChE antibody The rhBChE was tested for immunoreactivity with antibody against human serum BChE by sandwich ELISA and Western blot[6].

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Detoxification of succinylcholine by rhBChE *in vitro* Twenty Kunming mice (♂, 18 – 22 g) obtained from Animal Centre of the Academy of Military Medical Sciences (Grade Ⅱ, Certificate № 01-3023) were equally divided into 2 groups. Concentrated culture medium (1 mL) containing rhBChE 25 U was mixed with 1 mL succinylcholine solution (3 $g \cdot L^{-1}$). After incubation at 37 ℃ for 30 min, each mouse in the test group was injected ip the mixed solution 10 $mL \cdot kg^{-1}$ (corresponding to succinylcholine 15 $mg \cdot kg^{-1}$, ca 1.5 lethal dose). In the control group, rhBChE solution was replaced by concentrated culture medium of untransfected CHO-dhfr$^-$ cells. The survival and dead mice were examined after injection in the following 24 h. The experiment was repeated 3 times.

RESULTS

Identification of expression plasmid pRcCMV-BCHE The number and size of restriction fragments were exactly the same as that of expected after digestion with restriction endonucleases (Fig 1). BChE gene (1.8 kb) was cut off by digestion with Hind Ⅲ + Apa Ⅰ (Fig 1, lane 8).

Expression of rhBChE and gene amplification Fifty-four CHO-dhfr$^+$ colonies with BChE activity were detected by DTNB and sandwich ELISA. After amplifying the BCHE gene copies in CHO cells for 4 months, the rhBChE activity in 5 representative colonies raised up to various extents (Tab 1). The highest expression level of rhBChE came up to 25.83 $ng \cdot h^{-1}$ per 10^6 cells (Tab 1, D6).

Recognition of rhBChE by antibodies All colonies expressing BChE activity showed positive reaction in ELISA. Western blot showed that there was only a single band of the SFM of D6 cells whereas no band was found with SFM of the non-transfected CHO-dhfr$^-$ cells (Fig 2). However, the molecular weight of rhBChE

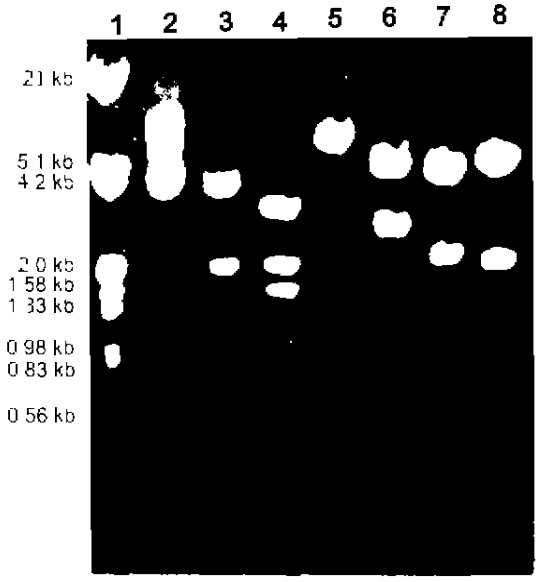

Fig 1. Agarose gel electrophoresis of restriction fragments of recombinant plasmid pRcCMV-BCHE. 1) DNA markers (λDNA/EcoR Ⅰ + Hind Ⅲ, 21 kb, 5.1 kb, 4.2 kb, 2.0 kb, 1.58 kb, 1.33 kb, 0.98 kb, 0.83 kb, 0.56 kb). 2) Plasmid pRcCMV-BCHE. 3) Plasmid pRcCMV-BCHE digested with Sac Ⅰ (4.5 kb, 1.9 kb, 0.8 kb). 4) Plasmid pRcCMV-BCHE digested with Pst Ⅰ and Bgl Ⅱ (3.0 kb, 1.8 kb, 1.4 kb, 0.9 kb). 5) Plasmid pRcCMV-BCHE digested with Apa Ⅰ (7.3 kb). 6) Plasmid pRcCMV-BCHE digested with EcoR Ⅰ (4.7 kb, 2.5 kb). 7) Plasmid pRcCMV-BCHE digested with BamH Ⅰ (4.9 kb, 2.0 kb, 0.3 kb). 8) Plasmid pRcCMV-BCHE digested with Hind Ⅲ and Apa Ⅰ (5.5 kb, 1.8 kb). The 1.8 kb DNA band was the BChE gene.

Tab 1. rhBChE expression levels subjected to MTX selection.

Cell colony	rhBChE expression levels under MTX pressure /$ng \cdot h^{-1}$ per 10^6 cells					
	0.05	0.1	0.5	0.8	1.5	3 $\mu mol \cdot L^{-1}$
D6	3.38	7.17	16.54	18.42	24.29	25.83
D8	2.71	5.00	11.83	3.38	–	–
C6	1.63	6.25	7.63	–	–	–
C10	1.38	2.45	10.42	16.83	15.17	17.92
D10	1.08	3.88	7.58	11.58	–	–

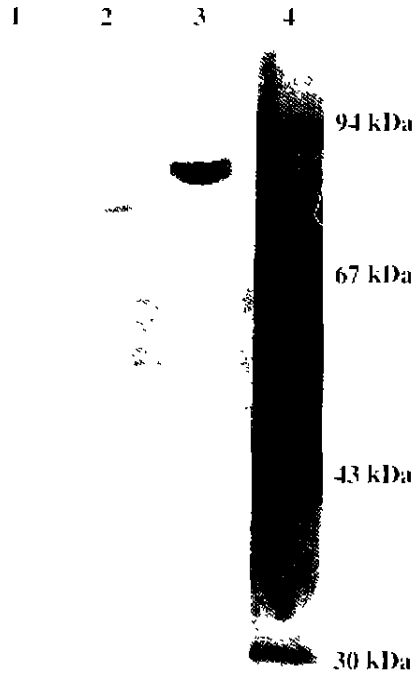

Fig 2. Western blot of BChE. 1) Concentrated SFM from CHO-dhfr⁻ cells. 2) Concentrated SFM from cells secreting recombinant BChE in 60 ng. 3) Standard native BChE in 200 ng. 4) protein molecular weight markers in 500 ng.

(approximately 81 kDa) was 4 kDa less than that of the nhBChE (85 kDa).

Secretion of rhBChE A 93 % of the rhBChE activity was secreted into the culture medium, 4 % in soluble forms extractable with low salt buffer, and 3 % in membrane bound forms extractable with high salt buffer (Tab 2).

Tab 2. Distribution of rhBChE activity. n = 3 experiments in duplicate. $\bar{x} \pm s$.

Distribution of rhBChE	Enzyme activity / nmol·min^{-1}	Enzyme activity /%
Culture medium	3.81 ± 0.07	93
Soluble form in cell	0.163 ± 0.020	4
Membrane bound form	0.120 ± 0.010	3

K_m value The Lineweaver-Burk reciprocal plot showed that the K_m value of rhBChE was 1.72 mmol · L^{-1} when butyrylthiocholine was used as the substrate, similar to that of nhBChE (1.14 mmol·L^{-1}) (Fig 3).

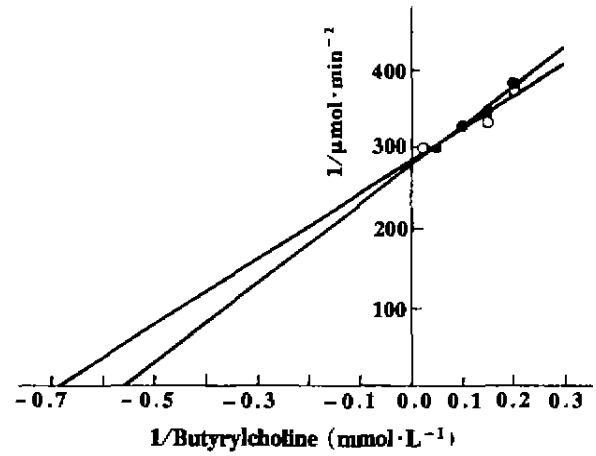

Fig 3. Linweaver-Burk reciprocal plot of nhBChE (○) and rhBChE (●). n = 3 experiments in duplicate.

Inhibitor sensitivity of rhBChE About 85 % activities of rhChE and nhBChE were inhibited by physostigmine 10 μmol·L^{-1} (pI_{50} = 7.2, pI_{90} = 4.4) (Fig 4).

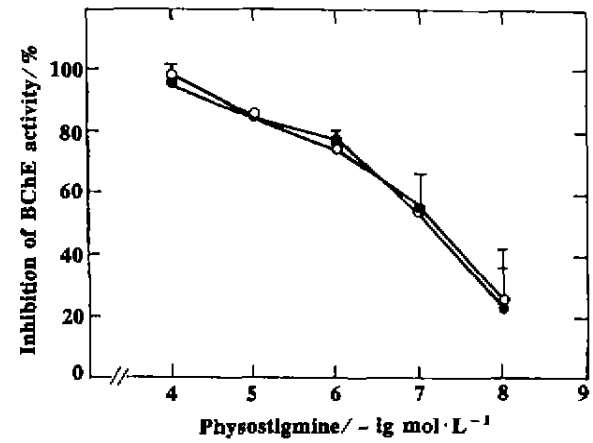

Fig 4. Physostigmine inhibition on nhBChE (○) and rhBChE (●). n = 3 experiments in duplicate, $\bar{x} \pm s$.

Reactivation of inhibited BChE by HI-6 The carbamylated and phosphonylated BChE reactivated spontaneously or by HI-6 along with the time. The reactivation rates of inhibited

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rhBChE and nhBChE were comparable with each other in spontaneous and HI-6 reactivation (Tab 3).

Tab 3. Reactivition of physostigmine- and sarin-inhibited BChE by HI-6. $n = 3$ **experiments in duplicate.** $\bar{x} \pm s$. **E: normal BChE activity. EI: inhibited-BChE activity. EIR: inhibited BChE activity reactivated by HI-6.**

Reactivation time/h	Enzyme activity /nmol·min^{-1} E	EI	EIR	Reactivation /% Spontaneous	by HI-6
Physostigmine-inhibited BChE					
nhBChE					
2	3.03 ± 0.10	0.30 ± 0.10	0.87 ± 0.13	0	21
5	3.03 ± 0.10	0.40 ± 0.17	1.23 ± 0.13	1	32
9	3.03 ± 0.10	0.67 ± 0.03	1.57 ± 0.17	11	38
13	3.03 ± 0.10	1.07 ± 0.13	1.90 ± 0.10	26	42
24	3.03 ± 0.1	1.23 ± 0.17	2.47 ± 0.30	30	69
rhBChE					
2	3.60 ± 0.13	0.40 ± 0.10	1.07 ± 0.10	0	21
5	3.60 ± 0.13	0.73 ± 0.13	1.30 ± 0.27	6	23
9	3.60 ± 0.13	0.80 ± 0.03	1.70 ± 0.10	8	32
13	3.60 ± 0.13	1.37 ± 0.03	2.23 ± 0.17	26	39
24	3.60 ± 0.13	1.70 ± 0.17	2.90 ± 0.27	36	63
Sarin-inhibited BChE					
nhBChE					
1	3.97 ± 0.27	0.17 ± 0.03	0.87 ± 0.13	2	28
4	3.97 ± 0.27	0.13 ± 0	1.23 ± 0.13	1	42
7	3.97 ± 0.27	0.17 ± 0.10	1.57 ± 0.17	2	43
10	3.97 ± 0.27	0.57 ± 0.13	1.90 ± 0.10	12	55
24	3.97 ± 0.27	0.40 ± 0.27	2.47 ± 0.30	8	73
rhBChE					
1	5.1 ± 0.5	0.17 ± 0	1.07 ± 0.10	1	24
4	5.1 ± 0.5	0.23 ± 0.03	1.40 ± 0.27	2	42
7	5.1 ± 0.5	0.17 ± 0.03	1.70 ± 0.10	1	51
10	5.1 ± 0.5	0.83 ± 0.10	2.23 ± 0.17	16	58
24	5.1 ± 0.5	0.80 ± 0.03	2.90 ± 0.27	15	72

Stability of rhBChE The rhBChE lost 7 % activity after incubation at 37 ℃ for 24 h and 9 % activity at 4 ℃ in 6 months. The stability was similar to that of nhBChE (5 % and 10 %).

Inhibition of BChE by polyclonal antibodies Equal aliquots (50 μL) of rhBChE and 1 : 5000 nhBChE were reacted with diluted polyclonal antibody against human serum BChE at 4 ℃ for 24 h. The enzyme activities decreased with the increase of the rabbit anti-human BChE IgG (Tab 4).

Tab 4. BChE activity inhibited by polyclonal antibody. $n = 3$ **experiments in duplicate.** $\bar{x} \pm s$. **nhBChE and rhBChE normal activities: butyrylthiocholine 3.29 ± 0.53 and 4.93 ± 0.29 nmol·min^{-1}, respectively.**

Dilution of antibody	Enzyme activity/nmol·min^{-1} nhBChE	rhBChE	Inhibition/% nhBChE	rhBChE
1:100	3.03 ± 0.30	4.27 ± 0.23	8	13
1:50	2.87 ± 0.27	3.90 ± 0.13	13	21
1:10	2.60 ± 0.47	3.47 ± 0.29	21	30
1:5	2.43 ± 0.43	3.33 ± 0.13	26	32
1:1	1.93 ± 0.43	2.73 ± 0	41	45

***In vitro* detoxification of succinylcholine by rhBChE** In the test group mice survived without any neuromuscular symptoms, none of the 30 mice died in 24 h, whereas all of the 30 mice in the control group rapidly exhibited convulsion and suffocation, and died in 2 – 5 min.

DISCUSSION

Full-length hBChE cDNA was successfully cloned in 1987[7,8]. It has been expressed in *E coli*, *Xenopus* oocytes, and CHO expression systems. The rhBChE from *E coli* expression system existed in inclusion body without enzyme activity. After renaturation by unfolding and refolding, the total activity of rhBChE was 0.13 U per 100 mL culture medium, equaled to 0.7 μg active enzyme per 100 mL culture medium[9]. The maximal yield of rhBChE from *Xenopus* oocytes expression system was 58 pg per oocyte. This system only produced membrane bound rhBChE whose subunits were assembled into dimers[10]. In CHO expression system, Lockridge has given the maximal expression level of 125 μg·L^{-1} (personal communication). In the present study, two CHO positive colonies which

were co-transfected with human BChE gene and DHFR gene and followed by gene amplification in exposure to MTX for 4 months secreted active human BChE. The maximal expression level came up to 25.83 $ng \cdot h^{-1}$ per 10^6 cells (266 $\mu g \cdot L^{-1}$). It was the highest level reported so far.

All colonies expressing BChE activity showed positive reaction in ELISA. It suggests that the activity in DTNB assay comes from BChE other than other esterases that might be present. Secretion experiment of rhBChE indicated that the 28-amino acid-signal peptide was sufficient for direct secretion of rhBChE into the culture medium.

We conclude that the rhBChE produced by CHO expression system is highly similar to nhBChE in terms of catalytic activity, substrate affinity, inhibitor sensitivity, reactivation, stability, and recognition by anti-human BChE antibodies. However the molecular weight of rhBChE is a bit less than that of the nhBChE, it might be due to the different modification of carbohydrate structure in CHO cells and human cells. Kaetzel obtained bovine luteinizing hormone with a higher molecular weight and attributed it to the modification of carbohydrate structure by CHO cell[11]. Although the alternation of carbohydrate structure of rhBChE does not influence the property of the enzyme and manifested remarkable detoxification effects on succinylcholine *in vitro*, the rhBChE should be used carefully in repeated injections.

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重组人丁酰胆碱酯酶的特性[1]

袁慧军[2], 俞炜源[3], 石成华[3], 孙曼霖[4]
(军事医学科学院毒物药物研究所,
[3]生物工程研究所, 北京100850, 中国)

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关键词 丁酰胆碱酯酶；基因表达；重组蛋白；琥珀酰胆碱；药物代谢解毒；CHO 细胞；酶联免疫吸附试验；蛋白质印迹；毒扁豆碱

目的：研究重组人丁酰胆碱酯酶(rhBChE)的生化药理学性质，探讨其做为琥珀酰胆碱中毒解毒剂的可行性. **方法**：中国仓鼠卵巢细胞用电击法转染质粒，DTNB 法测定 BChE 活性，酶联免疫吸附试验和蛋白质印迹检测抗原性. **结果**：rhBChE 最高表达水平为每 10^6 细胞 25.83 $ng \cdot h^{-1}$. rhBChE 的底物亲和力、对抑制剂的敏感性、被抑制后的可重活化性、稳定性及与抗体的反应性等性质与天然 BChE(nhBChE)极为相似. 1.5 个致死量的琥珀酰胆碱经 rhBChE 体外解毒后，注射小鼠未出现任何中毒症状. **结论**：rhBChE 和 nhBChE 的生化药理学性质极为相似，rhBChE 具有潜在实用价值.

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为何许多药厂在竞相生产"Carticel"?

为何美国最看好的生物工程制药公司之一：Genzyme (http://www.genzyme.com)的利润不断上扬?

美国宇航局(NASA)生命中心通过因特网：http://microgravity.msfc.nasa.gov 和 www.synthecon.com 正式公布：一项拯救生命的、培养组织和器官来替代实验动物和人的技术

——细胞组织在微重力环境下进行的三维的高分化度培养技术 已进入临床和生产

新药"Carticel(软骨细胞)"，用来生成软骨. 患者自身正常的极少软骨细胞可培养出大量的高分化的软骨细胞，这是普通培养装置所做不到的，Genzyme 用的就是三维的高分化度的旋转式细胞培养系统(Rotary Cell Culture System 即 RCCS). Carticel 拯救了无数的患者，给公司带来了极大的利润.

● **重大意义** **肿瘤**：**有效杀灭肿瘤**：对肿瘤组织活体取样、与其自身的白细胞或淋巴细胞在 RCCS 中混合培养，刺激或训练识别和攻击肿瘤组织、再把经训练后有杀伤力的细胞直接注入病人的肿瘤中、这些经感化的淋巴细胞彻底杀灭了肿瘤. **肿瘤模型**：人体肿瘤组织的培养有利于为测试化疗药在病人自身的经培养和分化的肿瘤上的疗效，决定其成分的有效性、避免盲目使用. 以前这些试验是在鼠的组织模型上进行的，但由于物种差异、人的许多肿瘤在鼠中的生长并不好. 而在 RCCS 中、所有的肿瘤组织都能得到很好的生长、且避免了原先鼠蛋白的干扰. **软骨再生**：经培养的软骨密度高可用在损伤和关节炎的治疗中. **AIDS/HIV、肾病和心脏病模型**：RCCS 可培养正常人的器官、腺体和淋巴结. 如用 HIV 来感染这些类器官，跟踪 HIV 的生长. 把 HIV 药物用在被 HIV 感染的 RCCS 培养的组织模型上，研究其对抗 HIV 的效果及其对抗方式. **疫苗生产**：RCCS 培养的肝和正常人的肝没有什么区别、使产生肝炎疫苗的病毒生长在人的肝脏成为现实. 在美国已广泛用于生产. **激素、酶和其它只有由人体组织产生的蛋白以及基因工程**：培养出分化的人体组织，其被刺激后能分泌治疗用的蛋白. 经培养的神经组织产生的神经生长激素能修复脊椎损伤. **骨髓再生**：RCCS 中骨髓生长情况极佳，且可连续进行增生和低温冷冻保藏、以建骨髓库. **糖尿病**：胰岛素培养后能插入病人体内继续生长、无数患者有望免去长期注射胰岛素. **组织移植**：RCCS 培养的肝和正常人的肝在整体上无区别、使局部的肝脏组织移植成为可能. **皮肤移植**：以前培养的皮肤在肌理、灵巧度和肤色上，与真皮都有很大的差距. 现在培养的皮肤具有极高分化度、其外观和感觉可以乱真. ● **与类似产品根本区别** RCCS 具有**一般的培养装置**无可比拟的三大特点：(1) **高分化度**. 分化(如胰岛产生胰岛素等)的人体组织能在实验室中生长，模仿器官和肿瘤. (2) **模拟微重力**. 因重力将分裂原本应在一起的细胞组织成分，而 RCCS 中无重力. (3) **完善的三维细胞培养**. 而一般的生物反应器因保持细胞的悬浮而导致剪切力的产生. 这剪切力破坏了细胞间和细胞与基质间的稳定性、使组织和细胞集中于自身的修复，大大影响其生长和其它的正常生理功能. 还有**发孝罐**主要培养大量细菌、不适于大量培养哺乳动物细胞组织、因很难把大量的细胞移植其中，即使行、所得的细胞数也有限且很难生成组织. 而 RCCS 能行且可用于大规模生产、因其细胞成活率平均为 97% 且分化度极高. ● **系统介绍** RCCS 中无气泡和破坏应力使生成的三维组织具有与父系相同的结构和功能. 其培养的组织密度为 10^{10}至 10^{11} 个细胞/ml；细胞密度为 10^8. 至今她能培养所有的细胞或组织. 2000 多台 RCCS 在用，包括在中国.

美国 VWR-SYNTHECON 公司 http://www.synthecon.com

【上海】021-59916760，1331806815，jasongl@usa.net、201800 上海市城中路 20 号 上海大学 607# 刘 闪

【北京】010-63032704、63170894，1381374172，yuyikj@public.bta.net.cn，100050 北京南纬路 29 号 中国预医科技中心 丁宏斌