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Suppressive effect of kanglemycin C on T- and B-lymphocyte activation

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KEY WORDS kanglemycin C; cyclosporine; concanavalin A; phytohemagglutinins; tetradecanoylphorbol acetate; ionomycin; mixed lymphocyte culture test; B-lymphocytes; T-lymphocytes; T-lymphocyte subsets

ABSTRACT

AIM: To elucidate the suppressive effect of kanglemycin C (Kan) on lymphocyte proliferation and T-lymphocyte subsets. **METHODS**: Splenocyte proliferation was quantified with [^{3}H]thymidine ([^{3}H]TdR) pulsing method or 3-(4,5- dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) colorimetery. L3T4$^+$ and Lyt2$^+$ T-cell subsets were measured with fluorescence-activated cell sorter (FACS). Splenocyte viability was assessed with trypan blue exclusion. **RESULTS**: Like ciclosporin (Cic), Kan 8, 40, 80, and 400 nmol·L^{-1} inhibited the proliferation of 20 % – 80 % incubated mouse splenocytes stimulated by concanavalin A (Con A) 5 mg·L^{-1}, phytohemagglutinin (PHA) 5 mg·L^{-1}, tetradecanoylphorbol acetate (TPA) 10 μg·L^{-1} + ionomycin (IM) 0.5 mg·L^{-1}, and alloantigen (mixed lymphocyte reaction). Kan had no toxicity to the splenocytes at the treated doses. Suppression by Kan was declined with addition time of Kan after culture onset. Furthermore, the suppressive effect of Kan on splenocyte proliferation stimulated by lipopolysaccharides (LPS) 10 mg·L^{-1} was similar to that on splenocyte proliferation mediated by Con A. Unlike Cic, Kan reversed the ratio of L3T4$^+$/Lyt2$^+$ T-cell subsets. **CONCLUSION**: Kan had a suppressive action on proliferation of T- and B-lymphocytes and had a selective effect on helper-inducer T-lymphocyte (T_h) subset from Cic. Suppression by Kan was time-dependent and not associated with toxicity of Kan.

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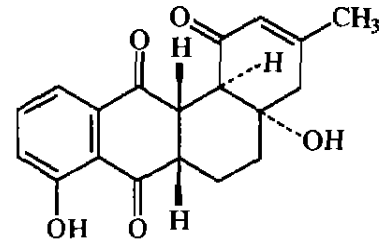
INTRODUCTION

An immune response can be divided into several overlapping phases. Cell activation and proliferation represent the cardinal events of the immune response. Immune precursor cell differentiated and matured into different cell subsets, different T-cell subsets are involved in delayed hypersensitivity (DH), cytotoxicity, the regulation of B- and T-cell functions, and the control of many other cell types[1,2]. Both kanglemycin C (Kan) ($C_{19}H_{18}O_5$) and ciclosporin (Cic) ($C_{59}H_{110}N_{11}O_{12}$) were isolated from the fungal metabolites. They are soluble in methanol, ethanol, and insoluble in water. Although their chemical structures were quite different, both had potent immunosuppressive actions[3,4].

Our previous study[5] uncovered that Kan, like Cic, markedly inhibited mouse DH and cyclophosphamide-potentiated DH induced by DNFB, prolonged the survival time of mouse skin and heart allografts, and suppressed hemolysin production of mouse sensitized splenocyte.

The study here further compared the immunosuppressive effect of Kan *in vitro* with Cic.



Kanglemycin C

MATERIALS AND METHODS

Reagents and drugs RPMI-1640 medium was purchased from Gibco. Complete medium was supplemented with *L*-glutamine 2 mmol · L^{-1} (Kaibiomed Scientific Co. Beijing), 10 % fetal bovine serum (FBS, Gibco), 2-mercaptoethanol 0.5 nmol · L^{-1} (Shanghai № 4 Chemical Reagent H V Co Ltd), benzylpenicillin 100 kU · L^{-1}, streptomycin 100 mg · L^{-1}. Con A (Sigma Chemical Co.), and PHA (Institute of Basic Medicine, Chinese Academy of Medical Sciences. Beijing) were dissolved at 2 g · L^{-1} and LPS (Sigma) was at 10 g · L^{-1} in RPMI-1640. TPA (Sigma) and IM (Sigma) were prepared as 5 g · L^{-1} in Me_2SO. Kan, yellow pin-shaped crystal, purity 99.5 %, and mp 170 ℃ was isolated by Institute of Medical Biotechnology, Chinese Academy of Medical Sciences, a solution of 10 g · L^{-1} was prepared in ethanol and protected from light until use. Cic (Sandoz Pharmaceuticals, E Hanover, NJ, USA), was kindly given by Dr M A Evans (Indiana University, USA). Cic 10 mg was dissolved in ethanol 1 mL. The reagents stated above were diluted freshly to the desired concentration in complete RPMI-1640. Anti-L3T4$^+$ monoclonal-antibody (Mab), anti-Lyt2$^+$ Mab, and fluorescein-isothiocyanate (Fitc)-conjugated rabbit anti rat-IgG antibody were purchased from Department of Immunology, Beijing Medical University, China, diluted as the protocol provided.

Mice Inbred BALB/c (Grade Ⅱ, Certificate № 01-3046), C57BL/6j (Grade Ⅱ, Certificate № 01-3044) mice, ♀, 8 – 12 wk, 20 – 22 g, were purchased from the Department of Experimental Animals, Beijing Medical University, Beijing.

Cell separation[6] Splenocyte was prepared in a general way, and the concentration and viability of the cells were determined by trypan blue exclusion.

Cell proliferation[6-8] Splenocytes (2×10^5) were incubated with one of the mitogens (Con A 5 mg · L^{-1}, PHA 5 mg · L^{-1}, TPA 10 μg · L^{-1} + ionomycin 0.5 mg · L^{-1} or LPS 10 mg · L^{-1}) alone, or with Kan or Cic of 8, 40, 80, and 400 nmol · L^{-1} in flat-bottom microtiter plates in 0.2 mL of complete media for 48 h. The mixed lymphocyte reaction (MLR) was performed by culturing 1×10^5 responding cells (BALB/c splenocytes) with 2.5×10^4 stimulating cells (C57BL/6j 1×10^{10} · L^{-1} splenocytes dealt with mitomycin C 25 mg · L^{-1} at 37 ℃ for 20 min) for 5 d. The cultures were triplicate and incubated at 37 ℃ in a humidified atmosphere of 5 % CO_2 in air. The proliferation was measured by [^{3}H]TdR (920 GBq · mol^{-1}, 37 GBq · L^{-1}, Shanghai Institute of Nuclear Research, Chinese Academy of Sciences) or 3-(4, 5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method.

Flow cytometry determination of L3T4$^+$ and Lyt2$^+$ T-cells Splenocytes (1×10^6) were cultured in 24-well plate for 48 h, centrifuged, and washed twice with PBS. The cells were incubated with an appropriate dilution of anti-L3T4$^+$ Mab or anti-Lyt2$^+$ Mab in 0.2 mL for 30 min on ice and washed 3 times with PBS. Fluorescein-isothiocyanate (Fitc)-conjugated rabbit anti-rat-IgG antibody was then added at a saturated concentration. After 30-min incubation, cells were washed 3 times, and analyzed on a fluorescence-activated cell sorter (FACS, Becton Dickinson Co).

Statistics Data were shown as $x \pm s$ and compared by *t*-test.

RESULTS

Effect of Kan on lymphocyte proliferation mediated by mitogens and alloantigen Mouse splenocyte proliferation induced by T-cell mitogen Con A and PHA, and alloantigen in the one-way mixed lymphocyte reaction (MLR) were respectively suppressed 30.8 % – 82.7 %, 20.8 % – 76.3 %, 54.2 % – 87.2 % by Kan, at 8 – 400 nmol · L^{-1}. In the same doses, suppressive effect of Kan on proliferation induced by Con A and alloantigen (MLR) was milder than that of Cic ($P < 0.05$). Whereas, inhibitory effect of Kan on activation induced by PHA was stronger than that of Cic ($P < 0.05$). Mouse splenocyte proliferation induced by the polyclonal B-cell mitogen LPS and a combination of TPA and IM was also inhibited by Kan in a dose-dependent manner ($P < 0.05$ *vs* Kan of the higher dose), respectively to 57.9 % – 11.7 % and 75.3 % – 22.9 % of without drugs. But Cic had no significant effect on mouse splenocyte proliferation mediated by LPS (Tab 1, 2).

Time-kinetics of effect of Kan on splenocyte proliferation mediated by Con A and LPS By addition of Kan from 3 h after cultures onset, the suppressive effect of Kan declined with time of Kan

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Tab 1. Effect of Kan and Cic on splenocyte proliferation induced by mitogen. $n = 4$ wells. $\bar{x} \pm s$. $^bP < 0.05$, $^cP < 0.01$ *vs* control. $^dP > 0.05$, $^eP < 0.05$, $^fP < 0.01$ *vs* Cic of the same dose.

Groups/nmol·L^{-1}		Proliferation Con A (A_{570})	PHA (A_{570})	LPS/Bq	TPA + IM/Bq
Media		0.30 ± 0.06	0.28 ± 0.03	41 ± 11	40 ± 7
Control		0.82 ± 0.08	0.74 ± 0.06	533 ± 60	554 ± 53
Kanglemycin C	8	0.66 ± 0.07^{ce}	0.64 ± 0.06^{bd}	326 ± 50^c	427 ± 15^c
	40	0.56 ± 0.04^{cf}	0.51 ± 0.03^{cf}	306 ± 21^c	303 ± 41^{ce}
	80	0.52 ± 0.06^{cf}	0.41 ± 0.04^{cf}	243 ± 47^c	226 ± 22^c
	400	0.39 ± 0.02^{cf}	0.39 ± 0.03^{ce}	98 ± 18^c	158 ± 19^c
Ciclosporin	8	0.54 ± 0.10^c	0.69 ± 0.06^c	506 ± 48	419 ± 25^c
	40	0.40 ± 0.02^c	0.60 ± 0.04^c	494 ± 60	228 ± 16^c
	80	0.34 ± 0.04^c	0.56 ± 0.06^c	483 ± 97	144 ± 16^c
	400	0.32 ± 0.03^c	0.46 ± 0.05^c	438 ± 51	45 ± 9^c

Tab 2. Effect of Kan on one-way MLR. $n = 6$ wells. $\bar{x} \pm s$. % = ($\bar{x}$ of every dose agents − $\bar{x}$ of media)/($\bar{x}$ of control − $\bar{x}$ of media). $^cP < 0.01$ *vs* control. $^eP < 0.05$, $^fP < 0.01$ *vs* Cic of the same dose.

Groups/nmol·L^{-1}		Proliferation Bq	%
Media		40 ± 7	–
Control		475 ± 22	–
Kanglemycin C	8	240 ± 23^{ce}	45.8
	40	184 ± 18^{cf}	33.1
	80	125 ± 12^{cf}	19.5
	400	96 ± 15^{cf}	12.7
Ciclosporin	8	213 ± 18^c	39.6
	40	118 ± 17^c	17.7
	80	92 ± 23^c	10.7
	400	52 ± 7^c	2.6

added. Kan was effective when added within the first 24 h after the onset of Con A-induced splenocyte proliferation. However, Cic was almost not effective to splenocyte proliferation by addition of Cic at 6 h after cultures began. The suppressive effect of Kan on splenocyte proliferation induced by LPS was similar to that on splenocyte proliferation mediated by Con A (Fig 1).

Effect of Kan on T-lymphocyte subsets

Kan decreased the amount of L3T4$^+$ T-cell and Lyt2$^+$ T-cell, and L3T4$^+$ cells were decreased more. Thus the ratio of L3T4$^+$/Lyt2$^+$ was inversed, whereas Cic did not (Tab 3).

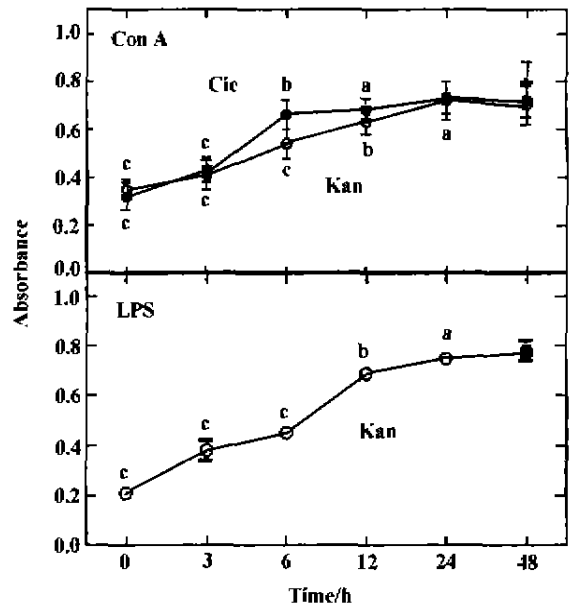

Fig 1. Suppressive time-kinetics of Kan on mouse splenocyte proliferation induced by mitogen. Con A, $n = 4$ wells. LPS, $n = 3$ wells. $^aP > 0.05$, $^bP < 0.05$, $^cP < 0.01$ *vs* control.

Effect of Kan on splenocyte viability

Splenocyte was respectively incubated with Kan and Cic 400 nmol·L^{-1} for 24, 48, and 72 h and viability was determined by trypan blue exclusion at the end of culture. Kan as high as 400 nmol·L^{-1} had no significant effect on mouse splenocyte viability compared with control.

Tab 3. Effect of Kan on mouse spleen T-lymphocyte subsets *in vitro*. $n=3$ wells. $\bar{x} \pm s$.
$^aP>0.05$, $^cP<0.01$ *vs* control.

Groups/ nmol·L^{-1} × 48 h	L3T4$^+$ / %	Lyt2$^+$ / %	L3T4$^+$ / Lyt2$^+$
Control	43.0 ± 2.0	36.1 ± 1.2	1.19 ± 0.02
Vehicle	41.8 ± 3.8^a	34.4 ± 0.8^a	1.22 ± 0.09^a
Ciclosporin 80	39.6 ± 0.8^a	30.6 ± 2.7^a	1.30 ± 0.13^a
Kanglemycin C 80	28.4 ± 1.2^c	29.9 ± 0.8^c	0.95 ± 0.07^c

DISCUSSION

Immune cell activation and proliferation are the main events of the immune response. Kan markedly inhibited splenocyte proliferation induced by mitogens and alloantigen. It indicated that the immunosuppression by Kan came true through the inhibition of cell activation course. Kan effectively inhibits splenocyte proliferation until Kan added at 24 h, though the suppressive effect of Kan declined with time of Kan added from 3 h on after cultures onset. However, Cic was almost not effective to splenocyte proliferation by addition of Cic at 6 h after cultures initiated. It suggested that Kan was not only effective at early phase but also at late phase of lymphocyte activation. Whereas, Cic, consisted with the previous research, acted the early phase of cell activation. Furthermore, Kan markedly inhibited splenocyte proliferation mediated by LPS; whereas Cic did not. It was shown that Kan had an influence on lymphocyte activation in a different mode by comparison with Cic. Moreover, the suppressive time-kinetics of Kan on proliferation induced by Con A and by LPS were similar. It suggested that Kan might have some effects on the processes of some common signal molecules or the same target points of T- and B-cell activation courses. Kan did not interfere in the mouse splenocyte viability. Thus, the ability of Kan to suppress splenocyte proliferation was not associated with decreased cell viability as a result of toxicity. L3T4$^+$ T-cells recognize antigen in the context of class Ⅱ major histocompatibility complex molecules, whereas Lyt2$^+$ T-cells recognize antigen in conjunction with class I major histocompatibility complex molecules[9]. Kan depressed the amount of L3T4$^+$ and Lyt2$^+$. It was indicated that Kan suppressed T_h cells more potently than cytotoxic-inducer T-lymphocytes (T_c).

In a summary, the study demonstrated that Kan strongly suppressed T- and B-lymphocyte proliferation induced by mitogens and alloantigen. Kan suppressed T_h cells more potently than T_c cells. Kan inhibited mouse splenocyte proliferation in a different way by comparison with Cic.

REFERENCES

1 Nossal GJ. Current concepts: immunology, the basic components of the immune system. N Engl J Med 1987; 316: 1320 – 5.
2 Ivan RI. Essential immunology. 8th ed. Oxford: Blackwell Scientific Publications; 1994. p 312 – 36.
3 Han BL, Zhou JQ, Sun CH, Wang NJ, Kyuichi N. A novel immunosuppressant, kanglemycin C, its fermentation, isolation, physico-chemical and biological properties. Chin J Antibiot 1995; 20: 273 – 7.
4 Borel JF, Feurer C, Gubler HU, Stahelin H. Biological effect of cyclosporin A: a new antilymphocytic agent. Agents Actions 1976; 6: 468 – 75.
5 Li JM, Lin ZB. Kanglemycin C *vs* ciclosporin on immunosuppression in mice. Acta Pharmacol Sin 1999; 20: 65 – 8.
6 Chen MS, Wei W, Zhang H, Liang JS. The experimental methods of immunosuppressive agents and immunostimulants. In: Xu SY, Bian YN, Chen X, editors. The experimental methodology of pharmacology. 2nd ed. Beijing: People's Medical Publishing House; 1994. p 1208 – 45.
7 Szamel M, Bartels F, Resch K. Cyclosporin A inhibits T cell recepter-induced interleukin-2 synthesis of human T lymphocytes by selectively preventing a transmembrane signal transduction pathway leading to sustained activation of a protein kinase C isoenzyme, protein kinase C-β. Eur J Immunol 1993; 23: 3072 – 81.
8 Bach FH, Voynow NK. One-way stimulation in mixed leukocyte cultures. Science 1966; 153: 545 – 7.
9 Sprent JM, Schaefer DL, Korngold R. Functions of purified L3T4 and Lyt-2 cells *in vitro* and *in vivo*. Immunol Rev 1986; 91: 195 – 218.

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康乐霉素 C 对 T-和 B-淋巴细胞活化的抑制作用

R379.5

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关键词 康乐霉素 C；环孢素；刀豆球蛋白 A；植物血凝素；十四酰佛波醇乙酯；伊屋诺霉素；混合淋巴细胞培养试验；B-淋巴细胞；T-淋巴细胞；T-淋巴细胞亚类

免疫

目的：阐明康乐霉素 C (Kan)对脾细胞增殖和 T-细胞亚型的作用. **方法**：氚掺入法或噻唑蓝(MTT)比色法测定细胞增殖；用荧光激活细胞分选仪(FACS)测定细胞亚型；曲利苯蓝排斥法测定细胞存活率. **结果**：Kan 8、40、80 和 400 nmol·L^{-1}，除抑制丝裂原(Con A，PHA 和 TPA + IM)和同种异型抗原刺激的小鼠脾细胞增殖外；与 Cic 不同，抑制 LPS (10 mg·L^{-1})刺激的脾细胞增殖；使 L3T4$^+$/Lyt2$^+$ T-细胞亚型比值倒置；Kan 于 Con A (5 mg·L^{-1})刺激后 24 h 内加入，仍抑制脾细胞增殖. Kan 8-400 nmol·L^{-1}不影响脾细胞存活率. **结论**：与 Cic 的作用方式不同，Kan 抑制 T-和 B-细胞活化的早期和晚期时相，抑制细胞增殖，对 T_h-细胞有选择性.

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