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Vinblastine Determination Measured by a Sensitive ELISA Inhibition Assay

Jae-Wha Kim†, Mi-Young Han, Hee-Gu Lee, Eun-Young Song Tai-Wha Chung, Kyung-Soo Nam[1] and In-Seong Choe

Molecular and Cellular Biology Research Group, Korea Research Institute of Bioscience and Biotechnology, KIST, P. O. Box 115, Yusung, Taejon, 305-600, Korea [1]Department of Pharmacology, College of Medicine, Dongguk University, Kyongju 780-714, Korea

Abstract: Specific monoclonal antibodies(mAbs) against bis-indole alkaloids related to vinblastine were established to develop a simple and specific immunoassay system for the quantitation of *Vinca* alkaloids. Vinca alkaloids were extracted from tissue cultured cells of periwinkle plant (*Vinca rosea L.*). Spleen cells from Balb/c mice immunized with vinblastine-bovine serum albumin(VBL-BSA) conjugate as immunogen were fused with myeloma cells(Sp2/0-Ag.14) in the presence of polyethylene glycol. In the preliminary experiments, 32 clones which highly reacted with VBL-BSA conjugate were selected by ELISA(Enzyme-linked immunosorbent assay). These clones were further analyzed by inhibition assay of ELISA. The results obtained with two typical monoclonal antibodies, KN-1 and KN-2, were described. KN-1 exhibited considerable reactivities with soluble dimeric bis-indole alkaloids, whereas no cross reacted with monomeric bis-indole alkaloids. However KN-2 showed cross reactivity with mono- and di-meric bis-indole alkaloids. Furthermore, KN-1 was applied to the immunoassay system for determining the VBL amounts of *in vitro* cultured cell extracts. This assay system could detect dimeric vinca alkaloid as low as 0.05 nM.

Key Words: Vinblastine, Enzyme-linked immunosorbent assay(ELISA), Inhibition assay of ELISA.

INTRODUCTION

Vinca alkaloids, vinblastine(VBL) and vincristine(VCR), have been used effective anticancer agents for several types of cancer such as malignant lymphoma and acute leukemia. These compounds are alkaloids isolated from the periwinkle plant, *Vinca rosea Linn.* The studies of VBL and VCR for elucidation of pharmacokinetic and biosynthetic mechanism had been carried out for many years[1-4]. Until recently, pharmacokinetic investigation of Vinca alkaloids have been limited severely by the lack of sufficiently sensitive analytical techniques. Furthermore, these compounds are extracted with extremely low concentration from *in vitro* cultures. Their isolation and screening from the extracts of cultured cells are very tedious and expensive procedure.

Radioimmunoassay(RIA), a very sensitive method to measure antigen concentration and to monitor vinca alkaloids used in chemotherapy, has been described[5,6]. Although RIA is an extremely sensitive way to measure antigen concentration, this method can be costly and hazardous. As well, quantitation of RIA requires

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†Corresponding author

the use of expensive measuring equipments. Therefore, a relatively new and simple analytical techniques was needed. ELISA enables one to measure antigen levels without using radiolabeled antigen. This assay is not only sensitive as RIA but also have distinct advantages of being inexpensive and nonbiohazardous.

In this study, we report the production and characterization of one high affinity monoclonal antibody(mAb) directed against bis-indole alkaloids related to VBL and its use in sensitive and specific ELISA inhibition methods. Furthermore, the application to the extracts of *in vitro* cultured cell is discussed.

ATERIALS AND METHODS

Animals

Balb/c mice(female, 6 to 8 wks old) were purchased from Korea Research Institute of Chemical Technology, Taejeon, Korea. They were fed standard rodent chow and water *ad libitum* until used in this study.

Chemicals

VBL, VCR and other derivatives[Fig. 1] were purchased from Sigma Chemical Co. (St. Louis, MO.). Polyvinyl chloride microtiter plates(Immulon 2) were from Dynatech Laboratories, Inc.. RPMI 1640, Hypoxanthine, thymidine and aminopterine were also purchased from Sigma Chemical Co.. Polyethylene glycol 4000 was bought from Merck (Darmastadt, F.R.G.). Fetal bovine serum was obtained from GIBCO Laboratories(Grand Island, N.Y.).

Preparation of extract from in vitro cultured cells

The extraction of crude alkaloids was performed by a modified method of Renaudin[7]. For each sample, 1.0 g(fr.wt) of cells was extracted three times with 10 ml of 100% methanol for 30 min in an ultrasonic bath (50℃). The residues obtained by removal of methanol *in vaccuo* were extracted with 1 N HCl and ethyl acetate(1/1, v/v). Acidic solution was adjusted to pH 10 with 5 N NaOH and extracted 3 times with ethyl acetate. The combined ethyl acetate phase was then evaporated under reduced pressure to give the crude alkaloid extract.

Preparation of VBL-BSA conjugate

Carboxylic acid derivative in VBL was prepared according to oxidation methods[5]. In brief, 10 mg of VBL dissolved in 1.0 ml water was added 3 mg of potassium carbonated, precipitated VBL was resolved by addition of 0.35 ml acetnitrile. Sodium periodate was added followed by 0.2 mg of potassium permanganate. The reaction mixture was allowed to stir at ambient temperature for 5 hr. Before 20 mg BSA was added by 10 mg of 1-ethyl-3-(3-dimethylamino propyl)-carbodiimide. After an additional stirring for 17 hr at 20℃, the cloudy brown solution was chromatographed on a column of Sephadex G-50 wet packed and eluted with PBS buffer.

Preparation of monoclonal antibodies

The antigen was coated on *Salmonella* minesota, strain R595 which was kindly provided by Dr. M. Umeda(The Pharmaceutical Science, The University of Tokyo). The *Salmonella* were coated with VBL-BSA and the suspension containing 10 ug of VBL and 50 ug of *Salmonella* in 0.2 ml of 20 mM phosphate buffer (pH 7.4) and 150 mM NaCl (PBS) were prepared as described previously[8]. Immunization of mice was performed as described previously[9]. One week after the intrasplenic injection of the antigen complex, the second intrasplenic injection was performed and the fusion with Sp2/0-Ag.14 mouse myeloma cells was performed three days later as described previously[10]. Two weeks after the fusion, the supernatants of hybridoma were tested for production of anti-VBL-BSA antibodies by ELISA. Ultimately, positive cells were cloned

three times by limiting dilution and resulting mAbs were injected into pristan-treated Balb/c mice to obtain increased production of mAbs in the ascites fluid. The IgM mAbs in the ascites fluid were purified by 50% ammonium sulfate precipitation and purified HPLC with TSKG-4000SWXL column(Tosoh Co., Tokyo, Japan). The heavy chain classes of the mAbs were determined by using double immunodiffusion.

Binding of mAbs to VBL-BSA

The binding of the mAbs to VBL-BSA was measured by ELISA[11]. In brief, the wells of the microtiter plate were coated with 50 ul of the VBL-BSA antigen in coating buffer (carbonate buffer, pH 9.5). The wells were blocked with 200 ul solution containing BSA (30 mg/ml) and incubated with 50ul of the hybridoma supernatant. The antibody bound was detected by biotinylated anti-mouse Igs (ZYMED laboratories, San Francisco, CA) followed by incubation with peroxidase-conjugated streptavidin(ZYMED laboratories). Optical density at 490 nm was determined by the addition of o-phenylendiamine substrate in ELISA reader(Titertek Multisan MCC/340, Labsystems, Finland). Throughout this study, we used phosphate-buffered saline(PBS) containing 0.05% Tween 20.

Inhibition assay of ELISA

Inhibition of the binding of anti-VBL-BSA mAb by bis-indole alkaloid derivatives related to VBL was performed as follows. 50 ul of the mixture containing monoclonal antibody and the various derivatives were preincubated for 1 hr at room temperature and the mixture was transferred to the microtiter wells coated with VBL-BSA. The amounts of antibody bound to the plate were measured as described above. All bis-indole alkaloid derivatives containing VBL were dissolved in PBS and used for the experiment.

RESULT AND DISCUSSION

Specificities of mAbs against bis-indole alkaloids related to VBL

In our preliminary experiments, the binding of the specific mAbs to VBL was analyzed by direct binding of the mAb to the VBL-BSA coated on the microtiter plates(ELISA)[Fig. 2]. Among 32 mAbs obtained, results obtained two typical mAbs named KN-1(IgM, *k* chain) and KN-2(IgM, *k* chain), are described in this paper. Both mAbs had high affinity to VBL-BSA and no cross reaction with carrier protein (BSA). KN-1 showed cross reactivity with soluble VBL and VCR, whereas weakly reacted with other monomeric VBL derivatives[Fig. 3]. In order to further analyze the interaction between the mAb and soluble VBL derivatives, an inhibition analysis of the ELISA by VBL derivatives was performed. The inhibitory ratio of soluble VBL derivatives was designated as a binding activity(% binding activity, B/Bo). The binding activity of KN-1 was considerably inhibited with dimeric compounds which contained VBL and VCR, whereas weakly inhibited with monomeric compounds which contained ajamalicine and vindoline. This specificity study allowed us to deduce

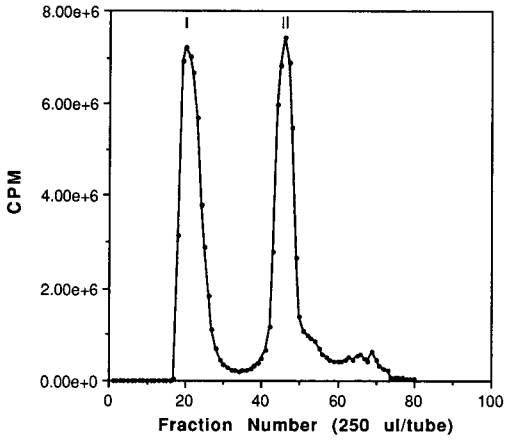

Fig. 1. Chemical sturctures of bis-indole alkaloids related to VBL. Ⅰ, vinblastine; Ⅱ, vincristine; Ⅲ, ajamalicine; Ⅳ, vindoline.

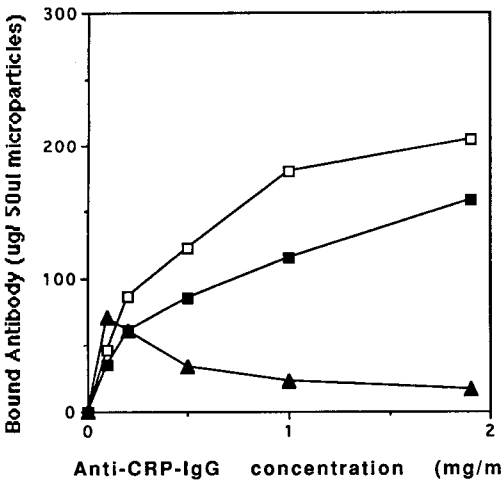

Fig. 2. Reacitivity of KN-1 and KN-2 with VBL-BSA. Microtiter plate was coated with 2 ug of VBL-BSA. KN-1(○) and KN-2(●) were detected with biotinylated anti-mouse Igs and streptaviding-conjugated peroxidase.

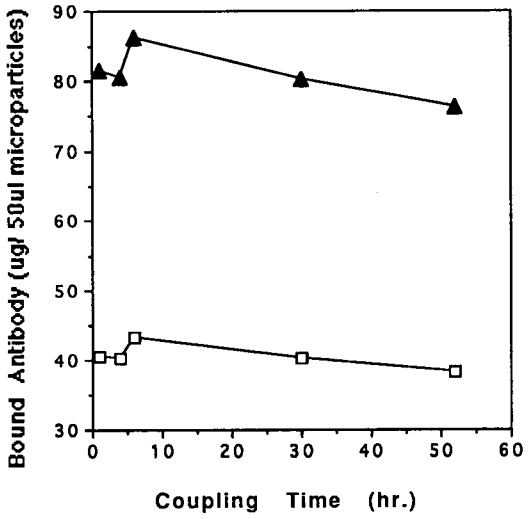

Fig. 3. Cross-reaction of KN-1 with VBL, VCR and other bis-indole alkaloids related to VBL. Cross-reaction was indicated by ELISA inhibition assay. KN-1 was preincubated with water soluble VBL(●), VCR(□), ajamalicine(○) and vindoline(■). The mixture were transferred to the microtiter wells coated with VBL-BSA. After incubation, the mAb bound was detected with biotinylated anti-mouse Igs and streptavidine-conjugated peroxidase.

that KN-1 is specific for dimeric structures related to VBL. The cross reactivity assay with modified cleavamine moieties on VBL molecule is necessary to clarify the recognition pro-

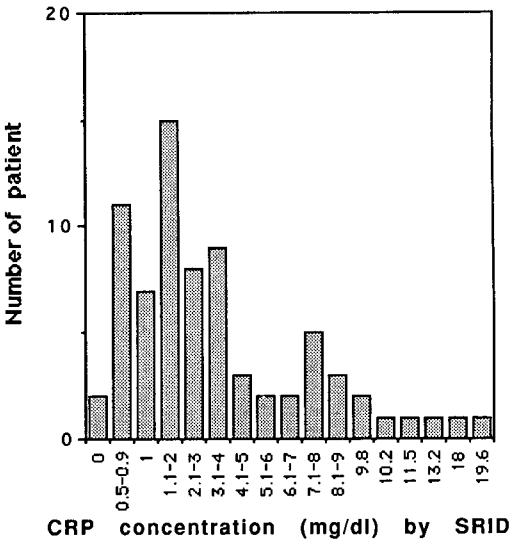

Fig. 4. Caliberation curve of KN-1 for VLB and VCR. B and B_0 are the percentage of binding in the presence and absence of VBL(●) or VCR(○) respectively, B/B_0 indicates the ratio of percentage binding of KN-1 to coated VBL-BSA. The mAb bound was detected as described in Fig. 3.

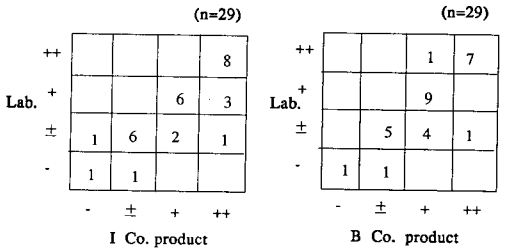

Fig. 5. Determination of bis-indole alkaloids related to VBL in the *in vitro* cultured cells. B and B_0 are the percentage of binding in the presence and absence of extract I (●) or extract II (○) respectively. The amounts of bis-indole alkaloids related to VBL from the two cultured cell extracts can be detected in the caliberation curve. The mAb bound was detected as discribed in Fig. 3.

file of KN-1 in detail. Similar results were also obtained with KN-2 (data not shown). Standard curves covering a range of 0-5 n moles (ca. 0-4 ug) of VBL and VCR were obtained [Fig. 4].

Intrasplenic immunization technique, in which the antigen is deposited into the spleen tissue, is appropriated when small amount of immunogen is available[12]. Although the effective activation of the spleen B cells was

achieved by injection of the antigen directly into the spleen, this method was not generalized. Actually, we tried to produce anti-VBL mAb using an usual immunization protocol several times but failed. However, we were able to establish a series of mAbs that reacted with VBL and other bioactive chemical compounds by the intrasplenic immunization method[10,13-15]. On the other hand, further studies will be necessary for understanding the precise mechanisms of the interaction between KN-1 and bis-indole alkaloids related to VBL.

Application of the KN-1 to in vitro cultured cells

We applied this mAb to the extracts from *in vitro* cultured cells. These extracts have been assayed by previously explained inhibition assay of ELISA system. The binding ratio of KN-1 decreased with the concentration of each cultured cell extract and 50% binding activity of KN-1 was respectively observed at 3 ul(0.2 nM) of extract I and 4 ul(0.3 nM) of extract II [Fig. 5]. This evidence will be used in assay system and evaluation for *Vinca* alkaloid amounts extracted from *in vitro* cultured cells. The lower level of *Vinca* alkaloids sensitivity on our assay system ranged over 0.05 nM.

In conclusion, in order to detect minute quantities (about 100 ng) of bis-indole alkaloids related to VBL, we have developed highly sensitive ELISA inhibition assay. This assay is specific for natural bis-indole alkaloids, such as VBL and VCR. However, the monomeric alkaloids, such as ajamalicine and vindoline, precusors of the dimeric molecules are not recognized. This ELISA inhibition assay system is available to the measurement of bis-indole alkaloids related to VBL, and its specificity and sensitivity are similar to previously reported radioimmunoassay[16,17]. Work is now underway to test our cell culture and a further analysis of the variety of bis-indole alkaloids produced.

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=국문초록=

ELISA Inhibition Assay에 의한 Vinblastine의 측정

생명공학 연구소 분자세포생물그룹, 동국대학교 약리학교실[1]

김재화 · 한미영 · 이희구 · 송은영 · 최용경 · 정태화 · 남경수[1] · 최인성

Vinblastine을 포함하는 bis-indole alkaloids에 대한 단일클론 항체를 생산하여 *Vinca* alkaloids의 양을 측정할수 있는 간편한 immunoassay 체계를 확립하였다. *Vinca* alkaloids는 periwinkle식물체의 배양된 세포로 부터 추출하여 BSA와 접합한 후 Balb/c생쥐에 면역시켜 얻은 비장세포와 골수종양세포의 융합을 유도하여 VBL-BSA에 반응하는 클론을 ELISA 방법으로 분석하였으며 이들 클론 중 bis-indole alkaloids 와 특이적으로 반응하는 항체는 inhibition assay를 통하여 분리할 수 있었고 그 결과 두 개의 단일클론 항체를 형성하는 세포주(KN-1과 KN-2)를 확립하였다. KN-1의 경우 dimeric bis-indole alkaloids 와는 상당한 교차반응을 나타낸 반면 monomeric bis-indole alkaloids 와는 교차반응을 나타내지 않았으며 이 클론의 항체를 이용하여 배양된 세포 추출물에 포함된 *Vinca* alkaloids의 양을 측정한 결과 0.05 nM정도의 dimeric *Vinca* alkoloids까지도 측정할 수 있었다.

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