ARISING PROBLEMS IN CIGUATOXIN DETECTION

Serge PAUILLAC, H . LABROUSSE, C. JEHL-MARTINEZ, A-M. LEGRAND*
and S. AVRAMEAS

Laboratoire d'immunocytochimie, Institut Pasteur de Paris, 28 rue du Dr Roux, F - 75724 Paris cedex 15:
** Unité d'océanographie médicale, Institut Territorial de Recherches Médicales Louis Malardé, BP 30, Papeete, Tahiti, Polynésie Française.*

Résumé : *la divergence et la faible sensibilité des tests de toxicité pour la détection en routine de la ciguatoxine (CTX) ont conduit au développement d'une approche immunoenzymatique. En raison du manque de CTX purifiée, nous avons choisi de travailler avec la monensine, un antibiotique polyéther de faible masse moléculaire et de structure chimique proche à celle de certaines toxines marines. Nous avons tout d'abord défini les conditions optimales de préparation de conjugués haptène-protéine en grosse quantité et, ensuite établi une méthode requièrant seulement 100 µg d'haptène. Des anticorps polyclonaux de lapins et monoclonaux de souris de hautes spécifité et affinité ont pu ainsi être produits. Un microtest ELISA de type compétitif a été mis au point sur plaque de Térasaki, permettant d'abaisser le seuil de détection de la monensine libre à 75 pg. Aucune réaction croisée avec la CTX libre n'a été décelée.La microméthode de préparation des conjugués est actuellement appliquée à une brévétoxine (BTX), autre toxine marine de structure chimique semblable à la CTX mais commercialement disponible.*

Abstract : *the discrepancy and low sensitivity of bioassays for routine detection of ciguatoxin (CTX) had led to the development of an immunoassay procedure. Because of the lack of purified CTX, we choose to work with monensin, a low dalton lipid polyether antibiotic chemically related to some marine toxins. We first defined the conditions for the optimal preparation of hapten-protein conjugates in bulk quantities and then, establish a procedure requiring only 100 µg of hapten. The conjugates obtained were used for immunization of rabbits and mice. Polyclonal and monoclonal antibodies of high specificity and affinity were produced.In a miniaturized competitive ELISA procedure using Terasaki microtiter plates, the minimum detection limit for free monensin was 75 pg. No cross-reactivity was detected against free CTX. These procedures are now under current investigation with brevetoxin (BTX), another marine toxin with a similar polyether backbone structure to CTX, but commercially available.*

Introduction

Marine toxins of the ciguatoxin (CTX) family, produced by the benthic dinoflagellate *Gambierdiscus toxicus* and accumulated in tropical reef fishes, are responsible for ciguatera, a human food poisoning causing gastrointestinal, neurological and cardiovascular disorders. Until now the most commonly used detection tests for CTX in fish tissues have been bioassays in animals such as mongoose, cat, chick, mouse and mosquito (1, 2, 3). The disadvantages of these assays (4) combined with their moderate correlation at low CTX level (2, 5) had led to the development of a number of more practical and specific detection procedures based upon immunological, cellular and physico-chemical techniques. Four directions have been explored: i) the polyclonal and monoclonal antibodies production; ii) the interaction with cellular receptors; iii) the cytotoxic or the mitogenic effect on cellular targets; vi) more recently the fluorimetric HPLC detection after derivatizing CTX into an anthroyl ester (6). These three last approaches still require an extraction procedure in organic solvents followed by several steps of

chromatographic purification, efficient but constraining. For this reason the development of enzyme- and radioimmunoassays (EIA and RIA) for the detection of CTX has been emphasized. Since 1977, Pr Hokama and his coworkers reported the use of an RIA procedure with a sheep antiserum to distinguish toxic from nontoxic fishes (7). In spite of the improvements afforded later to their studies, by means of monoclonal antibodies and a number of different tests based on RIA (5, 8) and EIA (9, 10), these results are still much debated (11, 12). First, the antigens used for animals immunization and antibodies detection were not characterized, secondly these antibodies seemed to be poorly specific. This paper presents the use of monensin, a low dalton lipid polyether antibiotic, as a model for the immunochemical approach of CTX detection.

Materials and methods

Synthesis and purification of monosuccinyl monensin : 500 mg (0.721 moles) of monensin sodium salt (Sigma) were recrystallised from anhydrous pyridine then redissolved in 5 ml of the same solvent. 721.5 mg (7.21 mmoles) of cold succinic anhydride and 5 mCi of ^{14}C-succinic anhydride were added. Succinylation was checked by thin layer chromatography (TLC). The Rf values found for monensin and succinyl monensin were 0.54 and 0.41 respectively. After evaporation, the product was dissolved in 3 ml dichloromethane and purified by flash chromatography. The fractions eluted from the column were checked for the presence of monosuccinyl monensin, pooled and rechromatographied. The structure of monosuccinyl monensin was confirmed by proton NMR spectrum performed on a Bruker MSL 300 (300 Mhz) spectrometer.

Preparation of the monensin-protein conjugate : the conjugates were prepared according to the mixte anhydride method of Erlanger *et al.* (13). The resultant solution was freeze-dried and stored at 4° C until used. The protein concentration of the conjugates was measured by the microassay procedure using the Bio-Rad protein assay and their hapten content estimated by three different ways. A direct estimation of the extent of substitution in the conjugates was made either by measuring their monensin content with vanillin (14) or by counting undialysable radioactive material. In addition, the procedure of Habeeb (15) in which trinitrobenzene sulfonic acid is used as reagent for estimation of free amino groups in the conjugate was also performed.

Immunization procedure : for the production of polyclonal and monoclonal antibodies, 2 adult male white rabbits (103 and 104) and 6 adult female Balb/c mice were immunized with $(MON)_{21}$-BSA. The animals received subcutaneously 3 injections of conjugate at intervals of 2-3 weeks, the first emulsified in FCA, the following in FIA. Each dose corresponded to 16 and 3 µg monensin for rabbits and mice respectively. Rabbits were bled from the central ear artery and mice from the retroorbital venous plexus 3 weeks after the last booster and their sera examined for the presence of antibody activity to monensin.

Hybridoma fusion : a mouse was given a fourth injection and 6 days after was bled under ether anaesthesia. Node cells were fused in presence of polyethylene glycol 1500 with the X63 Ag8.653 Balb/c myeloma cells, according to the protocol of Kohler and Milstein (16). Fused cells resuspended in the selective medium were dispensed in 96-well tissue culture plates.

Antibody screening assay : sera from rabbits or mice and hybridoma supernatants were analysed for reactivity to monensin by ELISA.The following buffers were used throughout enzyme immunoassays: phosphate buffered saline (PBS) pH 7.2 for coating, PBS containing 0.1% Tween 20 (PBS-T) for washing, PBS containing either 0.5% gelatin (PBS-G) or the same plus 0.1% Tween 20 (PGT) for dilutions. In order to use small quantities of reactants the classical procedure was modified according to Labrousse et al (17) . 60-well Terasaki microtiter plates were coated with 2 µl MON-BSA at 1-2 µg/ml (overnight at 4° C, then 2h at

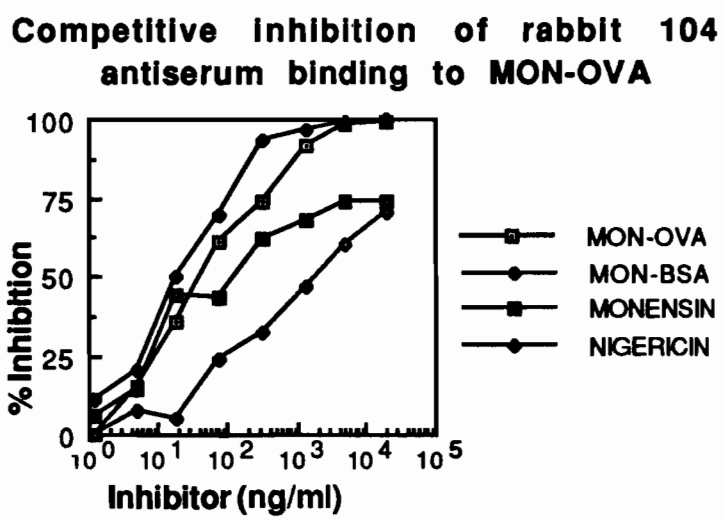
room temperature: RT). Following washings, 15 µl of serum or hybridoma supernatant dilutions were added (2h, RT). The plates were washed again and incubated with 5 µl of the corresponding ß-galactosidase-conjugated sheep antibody directed against the first antibody. After washings, 15 µl of saturated 4-methyl umbelliferyl β-D-galactoside solution were added and allowed to incubate (1h, RT). After this time the whole content of the Terasaki plates wells were transferred to 96-well microtiter plates containing 40 µl of 2N Na_2CO_3. The 4-methyl umbelliferone released was measured with a Dynatech fluoroscan and expressed as relative fluorescence units. Wavelenghts used were 365 nm for excitation and 450 nm for emission.

Further studies of antibodies : antibodies or sera at appropriate dilutions were mixed (3h, RT) with an equal volume of various concentrations of inhibitors (MON-BSA, MON-OVA, BSA, monensin, nigericin, lasalocid acid, or CTX). The purification procedure of CTX from moray eels have been previously described (18). Samples of these reaction mixtures were transferred onto antigen coated plates, allowed to incubate and after washings the antibodies associated with the plates revealed as above. Determination of the dissociation constants (KD) of rabbit polyclonal and mouse monoclonal antibodies was performed according to the method of Friguet et al (19).

Results

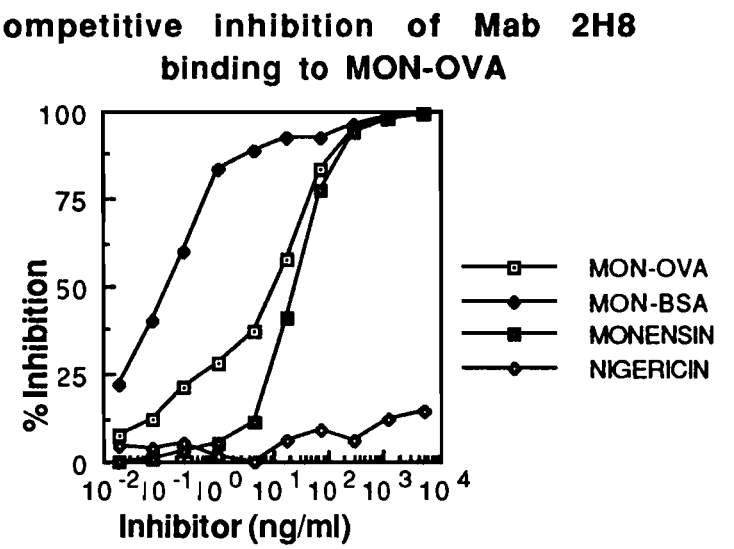
Preparation of monensin-protein conjugates were attempted following the procedure described in materials and methods. After the purification procedure, the final yield of succinyl monensin was 73%. The subsequent reaction of the hemisuccinate with isobutyl chlorocarbonate led to a mixte anhydride with a yield greater than 90%. For coupling to the proteins the best medium was found to be 0.1 M carbonate buffer, pH 9.5-dioxane (1:1) which gave an epitope density of 21 hapten molecules per molecule of BSA and 10 per molecule of OVA. These conjugates were subsquently used for the immunization of animals and for coating the microtitration plates.

The sera of the two rabbits (103 and 104) immunized with multiple injections of $(MON)_{21}$-BSA were found to contain high titers of anti-monensin antibodies when examined on plates coated with MON-OVA comparatively to the preimmune sera (data not shown). That these antibodies were specific for monensin was further assessed by performing competitive ELISA using MON-OVA coated Terasaki plates (Fig.1). MON-BSA, MON-OVA, free monensin and nigericin, an antibiotic structurally related to monensin, were found to inhibit the binding while lasalocid acid and CTX were not effective.



Six mice were immunized with multiple injections of $(MON)_{21}$-BSA and their sera examined for the presence of anti-monensin antibodies comparatively to their preimmune sera (data not shown). The specificity of these antibodies was clearly demonstrated in competitive tests (data not shown) and led us to the opportunity of performing the hybridoma fusion experiment.

The mouse showing the higher titer of anti-monensin antibody was sacrified and hybridomas were prepared from its two popliteal lymph nodes. Supernatants from 75 hybridomas cultured in selective medium were screened for the presence of anti-monensin antibodies. 8 hybridoma supernatants were selected on the basis of their reactivity against MON-OVA and the absence of cross-reactivity with non related hapten-protein conjugates. The corresponding hybrids were cloned by limiting dilution. From 18 positive clones, 3 were selected and one more particularly studied (2H8). This clone was grown up in bulk culture and injected i.p. into pristane-treated histocompatible mice to collect ascites fluid. This monoclonal antibody (MAb) was shown to be an IgG γ_1, κ subclass by ELISA. The high specificity of this MAb is shown in Fig. 2.



No inhibition was recorded with free lasalocid acid, CTX and nigericin. With monensin as inhibitor, assuming that a decrease of 3 standard deviation of the mean signal without inhibitor is significative for detection, the sensibility of this assay allows 5 ng/ml (7.5×10^{-9} nM) monensin to be detected. Considering the total volume of the sample deposited onto the Terasaki wells equals to 15 µl, it appears that 75 pg of monensin (110 fmoles/well) is the smallest quantity clearly detectable in this assay.

Discussion

In this study, we employed monensin, an polyether antibiotic having ionophoric properties and chemically related to some marine toxins structures, for the preparation of hapten-protein conjugates. To obtain an efficient immunogen, we tested several methods and various coupling reagents to covalently bind monensin to BSA or OVA. The best procedure was shown to be the successive formation of an hemisuccinate and an mixte anhydride which can readily react with the amino groups of the protein leading to amide linkages and a 4 carbones spacer. The miniaturization of the hemisuccinate formation was succesfully performed using only 100 µg of CTX with a yield greater than 95%. However the subsequent

reaction with the protein led to a poor epitope density which failed to induce specific antibodies to mice. Nevertheless the successfull development of an enzyme immunoassay for monensin based on a monoclonal reagent and the minimum use of material during the microELISA procedure provide the basis for the development of similar assays for other rare lipid haptens which pose analytical problems at low levels of detection. In order to save further some very precious material, the immunogen doses will be decreased and the best route of injection must be searched (20, 21, 22, 23). The availability of commercial BTX makes it possible to prepare, in future, BTX-BSA conjugates in order to produce anti-BTX MAb which could also react with CTX.

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Troisième Symposium sur les substances naturelles d'intérêt biologique de la région Pacifique-Asie

Nouméa, Nouvelle-Calédonie, 26-30 Août 1991

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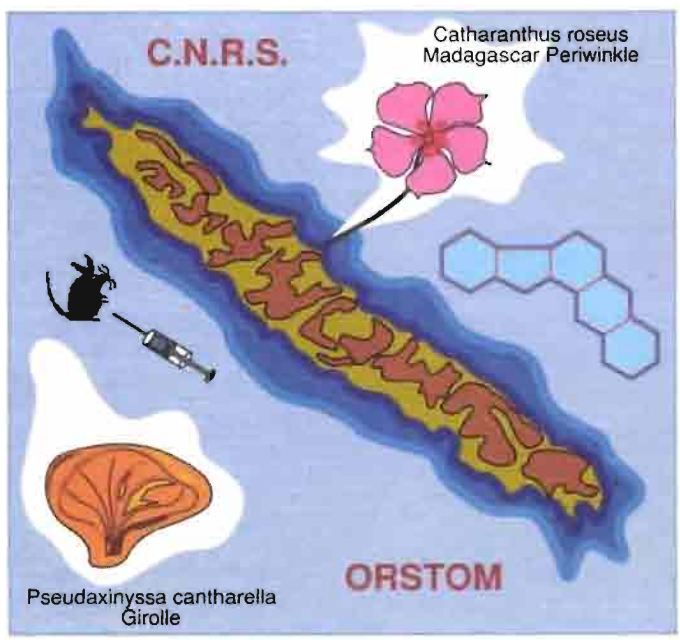

Editeurs : Cécile DEBITUS, Philippe AMADE,
Dominique LAURENT, Jean-Pierre COSSON