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AN ANTI-OKADAIC ACID-ANTI-IDIOTYPIC ANTIBODY BEARING AN INTERNAL IMAGE OF OKADAIC ACID INHIBITS PROTEIN PHOSPHATASE PP1 AND PP2A CATALYTIC ACTIVITY[1]

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SUMMARY: Okadaic acid (OA), produced by marine phytoplankton, is the parent compound of a family of marine toxins responsible for diarrhetic shellfish poisoning (DSP). A monoclonal antibody to OA (6/50) (Ab1) has been raised and in turn used for immunization of syngeneic animals. Mice inoculated with the 6/50 idiotype produced both anti-idiotypic antibodies (Ab2) and OA binding antibodies (Ab3). The selected anti-idiotypic antibody 1/59 bound to the immunizing 6/50 idiotype but not to $F(ab')_2$ fragments of pooled normal mouse Ig. It inhibited the binding of OA to solid-phase attached $F(ab')_2$ of 6/50 IgG as well as the binding of 6/50 IgG to a solid-phase bound OA. Like OA, 1/59 anti-idiotypic antibody inhibited protein phosphatase 1 and 2A catalytic subunits in a ^{32}P-phosphorylase *a* phosphatase radioassay. Thus, 1/59 IgG is a novel internal image anti-idiotypic antibody (Ab2β) and can serve as a surrogate of OA in biological assays.

Okadaic acid (OA) was first identified as a cytotoxic agent from the sponge *Halichondria okadaii* (1). OA and the related compounds DTX-1 and DTX-3 were later isolated from toxic mussels and scallops and identified as the principal agents responsible for diarrhetic shellfish poisoning (DSP) (2,3). Later work established that the DSP toxins are products of certain marine dinoflagellates (phytoplankton or microalgae) that can be ingested and stored by filter feeding animals in the ocean. DSP does not appear to be fatal, but its high morbidity rate and worldwide occurrence have made it a serious threat to the shellfish industry and to public health in general (3,4,5).

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Abbreviations used in this paper: BCG, Bacillus Calmette-Guerin; DSP, diarrhetic shellfish poisoning; DTX-1, dinophysistoxin-1; EDC, ethyl dimethylamenopropyl carbodiimide; FBS, fetal bovine serum; HRP, horse-radish peroxidase; HT, hypoxanthine, thymidine; IMDM, Iscove's Modified Dulbecco's Medium; Me ester OA, methyl ester of OA; MeOH, methanol; OA, okadaic acid; OPD, o-phenylenediamine dihydrochloride; PPD, tuberculin purified protein derivative; PP1 and 2A, protein phosphatase 1 and 2A; Sulfo-NHS, N-hydroxysulfosuccinimide; TS, Tris-buffered saline.

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In addition, it has been found that OA is a potent inhibitor of protein phosphatases PP1 and PP2A (4). Like the phorbol esters, OA displays tumor promoting activity (5), but unlike the phorbol esters, tumor promotion is believed to be caused by the inactivation of the phosphatases rather than activation of protein kinase C.

Immunization of animals with foreign immunoglobulins results in the production of anti-allotypic and anti-idiotypic antibodies (anti-Id). Anti-idiotypic antibodies (Ab2) represent a heterogeneous population displaying various specificities that can be classified into three major categories:

(1) Ab2α, which recognize the framework segments of variable region of Ab1. The binding of Ab2α to Ab1 does not alter the binding of Ab1 to antigen and thus is not hapten inhibitable;

(2) Ab2γ, which recognize combining site-associated idiotypes. Such antibodies can inhibit the binding of Ab1 to antigen but cannot elicit an Ab3 response (Ab1-like antibody that binds the nominal antigen) when injected into an animal;

(3) Ab2β, which mimic the antigen (internal image anti-idiotypic antibodies). Such antibodies bind to the antigen combining site of Ab1 and inhibit the binding of Ab1 to the nominal antigen. Ab2β idiotype is an antigen surrogate that is able to produce Ab3 antibodies specific for nominal antigen in the absence of the immunization with antigen itself (6,7).

As a result of two separate immunization protocols, an idiotypic anti-OA monoclonal antibody (mAb), 6/50, and an anti-Id mAb to OA, 1/59 have been produced. In a series of competitive ELISAs we have demonstrated that 1/59 (anti-Id) competes with OA for binding to the paratope of 6/50 (IgG). Therefore, the anti-Id mAb has properties of an Ab2β or "internal image" antibody in this system since it also induced the production of anti-OA-binding Ab3 antibody in the absence of immunization with OA (data not presented here). Using this antibody, a competitive ELISA for quantitation of OA has been developed in which free OA competes with the solid-phase bound 1/59 $(Fab')_2$ for limited binding sites on 6/50 antibody (8). 1/59's mimicry of OA is also biologically relevant in that it inhibits protein phosphatase catalytic activity in a ^{32}P-phosphorylase *a* phosphatase radioassay.

MATERIALS AND METHODS

1. Isolation of OA and its analogues from marine specimens

OA, DTX-1 and the diol ester were isolated from large-scale laboratory cultures of the marine dinoflagellates *Prorocentrum concavum* and *P. lima* as already described (9). DTX-2 was isolated from contaminated Irish mussels and the structure was identified as reported (10). The methyl ester of okadaic acid was prepared by standard laboratory procedures using diazomethane. Reduction of the methyl ester with sodium borohydride gave okadaic acid alcohol. The tetra-acetate was prepared by overnight treatment of okadaic acid with acetic anhydrid/ pyridine. All these synthetic okadaic acid derivatives were characterized by nuclear magnetic resonance spectroscopy and mass spectrometry (J.L.C. Wright *et al.*, manuscript in preparation).

2. Production and purification of anti-OA 6/50 antibody

Okadaic acid was coupled to protein carriers BSA and ovalbumin (OVA) using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and N-hydroxysulfosuccinimide (Sulfo-NHS) (Pierce Chemicals) according to the methods of Richardson et al. (11) and Staros et al. (12).

Balb/c females were immunized subcutaneously (s.c.) at two-week intervals, with 2 μg OA-BSA conjugate emulsified with Freund's adjuvant and then boosted intraperitoneally (i.p.) with 5 μg OA-BSA in PBS three days prior to fusion.

Myeloma P3X63.Ag8.653 cells were fused with spleen cells in the presence of 50% polyethylene glycol 4000 (Merck, Darmstadt, Germany) solution containing 5% DMSO at pH 7.4 and plated in IMDM-10% FBS (HyClone, Logan, UT) containing 1 x 10^{-4} M hypoxanthine, 4 x 10^{-5} amino-pterin, and 1.6 x 10^{-5} M thymidine (IMDM-HAT) (Gibco, Grand Island, NY) (13). Supernatants collected from growing hybrids were screened against OA-OVA in ELISA and hybrids secreting anti-OA antibodies were further expended and subcloned. The clone designated 6/50 was selected and grown as ascites in Balb/c mice.

Ascitic fluid was obtained by injecting $5x10^6$ cells i.p. into pristane-treated Balb/c mice. IgG antibodies were purified from ascites by affinity chromatography on Protein-A Sepharose (Pharmacia, Dorval, Québec). $F(ab')_2$ fragments of 6/50 IgG were prepared by limited proteolysis with pepsin (Worthington Biochemical, New Jersey, USA) for 24 hours at pH 3.9 using a pepsin-IgG ratio of 1:50. Undigested IgG and Fc fragment were removed by passage through Protein A-Sepharose column.

3. Production and purification of anti-OA-anti-idiotypic antibody 1/59

The anti-OA monoclonal antibody was conjugated to PPD according to the method developed by Cambridge Research Biochemicals Ltd. (Cambridge, UK). Briefly, 5 µl of commercial high-grade glutaraldehyde was added to a glass vial containing 10 mg of PPD dissolved in 920 µl of sodium hydrogen carbonate buffer (pH 8.4) and 2 mg of anti-OA monoclonal antibody prepared in 275 µl of the same buffer. The contents were stirred for 12-18 hours at room temperature. The conjugate was then dialyzed against two changes of 0.9% sodium chloride solution for 24-48 hours at 4°C.

To produce anti-idiotypic monoclonal antibodies, Balb/c mice were first inoculated s.c. with 25 µg Bacillus Calmette-Guerin (BCG) vaccine three weeks prior to the first immunization with the 6/50 antibody-PPD conjugate. At week 3, 87 µg of the 6/50 mAb-PPD conjugate in a 1:1 emulsion of incomplete Freund's adjuvant was administered s.c. On the seventh week, they were inoculated s.c. with the same emulsion followed by another i.p. injection on the tenth week. Finally, two weeks later, mice with high sera titers were boosted i.p. with 5 µg of anti-OA mAb-PPD in sterile PBS three days prior to fusion.

Fusion was carried out as above. Hybridoma supernatants were screened for antibody secretion in ELISA where microtitration wells were coated with $F(ab')_2$ fragments of 6/50 IgG_1, incubated with culture supernatants collected from growing hybrids and bound antibodies detected with peroxidase anti-mouse Ig Fc fragment-specific conjugate (Sigma Chemical Co., St-Louis, MO) as described above. The selected clone was designated as 1/59.

4. Competitive ELISAs

Each of the specific ELISAs described below followed a general method. Briefly, 96-well Immulon 1 microtiter plates (Dynatech Laboratories, Chantilly, VA) were coated with 10 µg/ml of OA-OVA in 0.05 M sodium carbonate/bicarbonate buffer, pH 9.6 overnight at 4°C. The unbound antigen was washed off with Tris-buffered saline (TS)-Tween and remaining binding sites saturated with 1% powdered-milk in TS (TS-milk) for one hour at 37°C. After washing, the plates were incubated with the tested antibody for one hour at 37°C. After the unbound antibody was washed off, a peroxidase-conjugated goat anti-mouse IgG (H+L) (Jackson Laboratories, West Grove, PA) or Fc-fragment specific conjugate was added and incubated for an additional 1 hour at 37°C. The colorimetric reaction was developed upon the addition of 0.03% H_2O_2 in 0.1 M sodium citrate buffer, pH 5.0, containing 0.1% OPD. The reaction was stopped with 3 N H_2SO_4 and the color intensity measured at a wavelength of 492 nm using a BioRad ELISA reader.

a) Competition between coupled and uncoupled OA for binding to 6/50 IgG when OA is fixed to a solid phase and 6/50 is measured in ELISA

Briefly, microtitration plates were coated with OA-OVA and incubated simultaneously with increasing amounts of OA and 6/50 IgG. Bound 6/50 IgG was detected with peroxidase-anti-mouse IgG conjugate antiserum.

b) Competition between anti-idiotypic 1/59 IgG and OA for binding to 6/50 idiotype when OA is fixed to a solid-phase and 6/50 is measured in ELISA

Microtitration plates were coated with OA-OVA and incubated simultaneously with increasing amounts of anti-idiotype and a fixed amount of 6/50 IgG. Bound 6/50 IgG was detected as above.

c) Competition between anti-idiotypic 1/59 IgG and uncoupled OA for binding to 6/50 $F(ab')_2$ fixed to a solid-phase when anti-Id is measured in ELISA

Microtitration wells coated with $F(ab')_2$ fragments of 6/50 IgG were incubated simultaneously with a fixed amount of anti-idiotypic IgG 1/59 and uncoupled OA at increasing concentrations. Bound anti-Id was detected with peroxidase-anti-mouse IgG Fc fragment specific antiserum.

d) Competition between uncoupled OA and anti-idiotypic 1/59 $F(ab')_2$ fixed to a solid-phase for binding to 6/50 IgG

Microtitration wells coated with $F(ab')_2$ fragments of 1/59 IgG and incubated simultaneously with a fixed amount of 6/50 IgG and free OA at increasing concentrations. Bound 6/50 IgG was detected with peroxidase-anti-mouse IgG Fc fragment specific antiserum (8). Additionally, in this ELISA OA analogues and DTX derivatives were tested in place of free OA.

5. Anti-Id (1/59) mediated inhibition of protein phosphatases PP1/2A catalytic activity

The biological activity of OA was assayed by the ability of this toxin to inhibit dephosphorylation of ^{32}P-radiolabelled glycogen phosphorylase *a* (E.C. 2.4.1.1) by protein phosphatases (E.C. 3.1.3.16) in the standard phosphorylase *a* phosphatase assay (14,15).

Using a standard commercial preparation of okadaic acid (Moana Bioproducts, Hawaii) and purified anti-Id, the IC_{50} for PP1 and PP2A inhibition was determined (15).

RESULTS AND DISCUSSION

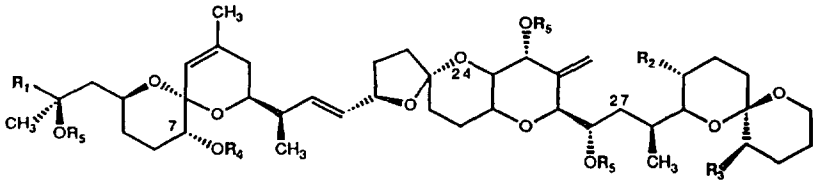
1. Characterization of anti-OA monoclonal antibody (6/50)

Monoclonal antibody 6/50 is IgG_1 κ. It binds to OA but not to the carrier protein or unrelated toxin such as domoic acid.

A number of naturally occurring OA-related compounds and synthetic derivatives were used in the assessment of the specificity of the antibody in the competition ELISA described above where microtitration wells were coated with $F(ab')_2$ fragments of 1/59 antibody and incubated with 6/50 antibody in the presence of escalating amounts of competing OA or OA derivatives (Figure 1). The cross reactivity of 6/50 antibody with these compounds is demonstrated in Figure 2. OA derivatives such as the methyl ester, the diol ester, and okadaic acid alcohol reacted almost as well as OA with 6/50 antibody. On the other hand, OA derivatives such as DTX-1 and DTX-2, with varying methyl group substitution on the spiro ring system most remote from the carboxyl group (Figure 1) are bound to 6/50 IgG with significantly lower affinity. The least effectively bound derivatives are the tetra-acetate, in which all the free hydroxyl groups of okadaic acid have been esterified with acetate, and DTX-3 which contains the DTX-1 skeleton linked to a fatty acid chain through the C-7 hydroxyl group. Only OA, DTX-1, DTX-2 and DTX-3 are known to be present in seafood. Except for alcohol-OA (a synthetic compound), all other OA-derivatives tested have been detected in minute amounts in microalgae but not in seafood.

2. Characterization of a mouse monoclonal anti-idiotypic antibody (1/59)

All mice immunized with the syngeneic anti-OA antibody 6/50 exhibited anti-6/50 titer in ELISA. One of the obtained hybridomas, designated 1/59, was selected for propagation and further characterization. It reacted with



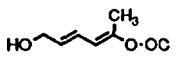
	R_1	R_2	R_3	R_4	R_5
OA	HOOC	CH_3	H	H	H
DTX-1	HOOC	CH_3	CH_3	H	H
DTX-2	HOOC	H	CH_3	H	H
DTX-3	HOOC	CH_3	CH_3	acyl	H
OA methyl ester	CH_3OOC	CH_3	H	H	H
Diol ester		CH_3	H	H	H
OA alcohol	$HOCH_2$-	CH_3	H	H	H
OA tetraacetate	HOOC	CH_3	H	CH_3CO-	CH_3CO-

Figure 1. Structures of okadaic acid derivatives.

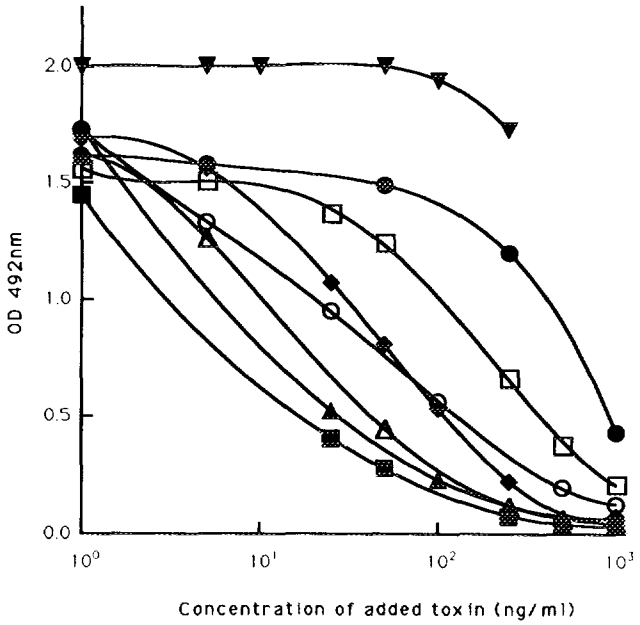


Figure 2. Cross-reactivity of mAb 6/50 with OA (■), DTX-1 (□), DTX-2 (○), DTX-3 (▼), and various analogues of OA [OA tetraacetate (●), OA diol ester (◆), OA methyl ester (△), and OA alcohol (▲)] tested in a competitive ELISA.
Assay wells were coated with purified anti-Id (1/59) $F(ab')_2$ (5 µg/ml) and incubated with a fixed concentration of mAb 6/50 (100 ng/ml) and varying amounts of OA analogues in 40% MeOH. Bound 6/50 IgG was detected with a HRP-conjugated anti-mouse IgG Fc fragment specific antiserum. Each point is the mean of duplicate values.

$F(ab')_2$ fragments of the immunizing idiotype (6/50) but not with $F(ab')_2$ fragments of normal pooled mouse IgG. To determine whether 1/59 binds 6/50 IgG at the paratope (antigen-binding site), a series of competitive ELISAs was developed.

When free 1/59 IgG was competing with solid-phase bound OA for a limited number of binding sites on anti-OA 6/50 IgG, a marked decrease in bound 6/50 was observed (Figure 3a) indicating that 1/59 anti-Id binds to the same binding site on 6/50 idiotope as does the nominal antigen, OA. However, the affinity of anti-Id for 6/50 Id is nearly 300-fold lower than OA for 6/50 IgG, since as much as 10 µg/ml of anti-Id versus 30 ng/ml of OA was needed to completely inhibit the binding of 6/50 (results not shown).

In the reverse assay, unbound OA clearly inhibited the binding of anti-idiotypic 1/59 Ig to solid phase bound 6/50 IgG $F(ab')_2$. The results presented in Figure 3b reconfirm the previous finding that OA and anti-Id compete for the same binding sites on the variable region of the 6/50 IgG.

3. Inhibition of protein phosphatase PP1/2A activity by anti-idiotypic Ab 1/59

In order to determine whether anti-Id 1/59 could mimic OA in biological assays, the antibody was tested in the standard phosphorylase *a* phosphatase assay. Complete titration curves for PP1/PP2A inhibition with OA, 1/59 IgG, and $F(ab')_2$ fragments of 1/59 IgG are presented in Figure 4. OA inhibited PP2A and PP1 catalytic activity very effectively at concentrations as low as 0.2 nM and 19 nM respecitvely. Anti-idiotypic IgG is less effective. The $IC_{50's}$ for inhibition of PP1 and PP2A by 1/59 IgG are 1100 nM and 145 nM, respectively and by the $F(ab')_2$

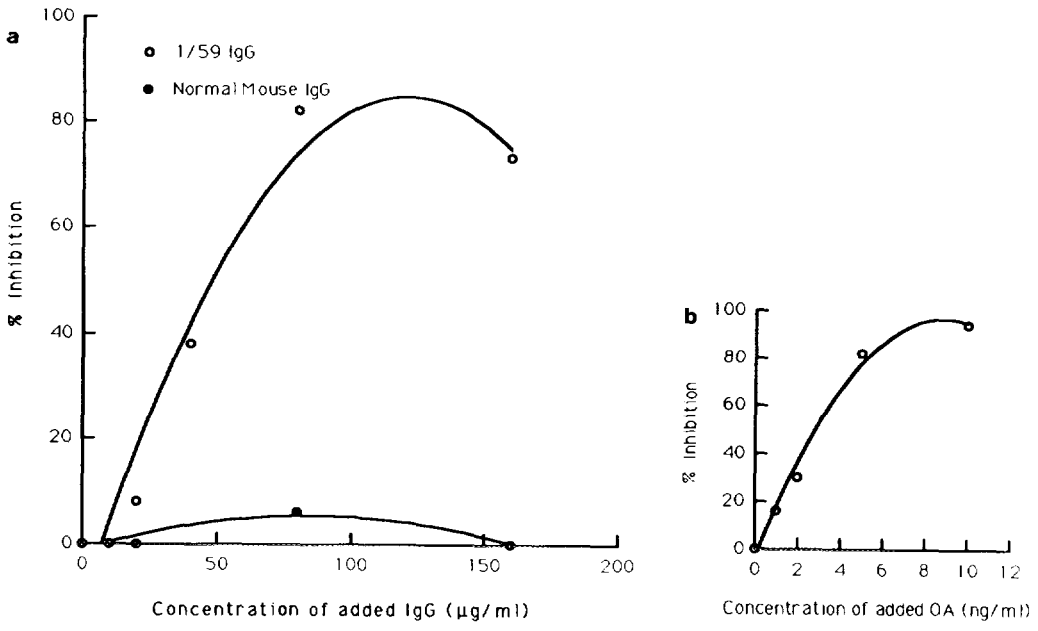


Figure 3a. Inhibition of anti-OA 6/50 IgG binding to OA by anti-anti-OA 1/59 IgG in ELISA. Microtitration wells coated with OA-OVA (10 µg/ml) were incubated simultaneously with a fixed concentration of purified 6/50 IgG (30 ng/ml) and increasing concentrations of 1/59 IgG. Bound 6/50 IgG was detected with a HRP-anti-mouse IgG. Each point is the mean of duplicate values.

Figure 3b. Competition between anti-Id (1/59) IgG and uncoupled OA for binding to mAb 6/50 in ELISA. ELISA plates were coated with purified $F(ab')_2$ fragments of 6/50 IgG and incubated with purified 1/59 IgG and uncoupled OA at increasing concentrations. Bound 1/59 IgG was detected with HRP-anti-mouse IgG Fc fragment specific antiserum. Each point is the mean of duplicate values.

fragments of 1/59 over 2200 nM and 445 nm respectively. An irrelevant monoclonal IgG_1 (anti-*Pseudomonas* peptide) did exhibit some inhibitory activity against PP1/2A in the assay, however, 1000 times less than 1/59 IgG (in micromolar concentrations).

Anti-idiotypic antibody inhibited phosphatase activity in a manner similar to OA in that it was more active towards PP2A than PP1, but 725 and 55 times, respectively, less active than OA.

We have successfully selected, cloned and produced a monoclonal antibody, 6/50, to okadaic acid, the parent member of the DSP toxins. The antibody binds several OA derivatives almost as well as OA itself (Figure 2). All these derivatives possess changes or modifications to the carboxyl group of okadaic acid, suggesting that 6/50 antibody recognizes the portion of the molecule most distant from the carboxyl group. For example, the methyl ester is efficiently recognized and so too is the OA alcohol in which the carboxyl group has been changed to a primary alcohol function. These results are not entirely surprising since the method of immunogen preparation would likely result in esterification of, and linkage through, the free carboxyl group of OA. Further support for this comes from the observation that the derivatives DTX-1 and DTX-2, which have varying degrees of methyl substitution on the terminal spiro ring system, are less well bound to 6/50 IgG. Interestingly we have observed in the NMR data of DTX-1 and DTX-2 that varying methyl substitution also results in significant conformational changes in this portion of the molecule (J.A. Walter, J.L.C. Wright and T. Hu, unpublished results).

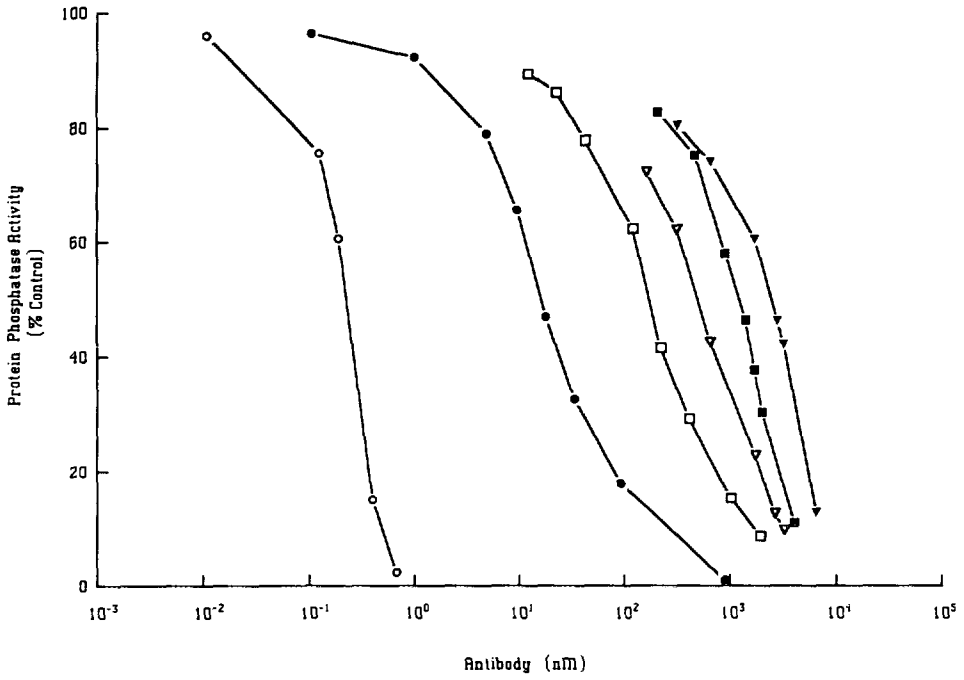


Figure 4. Inhibition of protein phosphatases 1 and 2A catalytic subunits (PP1, closed symbols; PP2A, open symbols) by OA (●;○) and anti-OA anti-idiotypic mAb 1/59 IgG (■;□) and its F(ab')$_2$ fragments (▼;▽),respectively, in a standard ^{32}P-radiolabelled phosphorylase *a* phosphatase assay.

The OA derivative described as DTX-3 is a mixture of compounds which possess the DTX-1 skeleton and carry a variety of unsaturated fatty acyl side chains (C_{16}-C_{18}) at position C-7. The DTX-3 group show a significant reduction of binding to 6/50 IgG which is partly accounted for by the DTX-1 skeleton itself, and compounded by the presence of the bulky acyl side chain. Acetylation of the four hydroxyl groups of okadaic acid to form the tetra-acetate derivative also has a profound effect upon binding to 6/50 IgG, though the reason for reduced binding is different in this case. Firstly, the attachment of acetate groups, particularly those at C-24 and C-27 would result in a substantially different shape at the portion of the molecule distant from the carboxyl group, the portion which the 6/50 antibody recognizes. Secondly, it has been reported that the conformation of okadaic acid glycol in solution is dictated by intra-molecular hydrogen bonding between the carboxyl carbonyl group and the C-hydroxyl group. Acylation of this hydroxyl would preclude the formation of such an intramolecular hydrogen bond and consequently the tetra-acetate would be expected to assume an entirely different conformation.

The obtained 6/50 antibody was in turn used for the production of anti-OA anti-idiotypic antibodies in a mouse syngeneic system. The selected anti-Id (1/59), when examined in a competitive ELISA, exhibited typical "internal image" anti-idiotypic antibody (Ab2β) behaviour in that it inhibited the binding of both OA to 6/50 F(ab')$_2$ and 6/50 IgG to solid phase bound OA (Figure 3). An ELISA kit for quantitation of OA in seafood was developped using F(ab')$_2$ fragments of 1/59 anti-idiotype as a capture antibody and 6/50 IgG as a detection antibody in a competitive assay as described in detail elsewhere (8).

Anti-Id antibodies to hormone or hormone receptor antibodies have been shown to stimulate a physiological action of ligand or exhibit receptor-like activities, such as binding the ligand or eliciting the

production of antireceptor antibodies when injected into animals. This has been found to be true for such ligands as insulin, retinol-binding protein, alprenolol, formylpeptide chemoattractant, thyroid-stimulating hormone (TSH), acetylcholine, prolactin, substance P, angiotensin II, etc. (7).

Reversible protein phosphorylation is widely recognized as an important mechanism for the control of a wide variety of cellular processes (16,17). Phosphorylation of key proteins with associated changes in their biological activity accounts for their physiological response. The phosphate content of these proteins reflects a net balance of the protein kinases and protein phosphatases acting on them. Mammalian serine/threonine protein phosphatases have been classified by Ingebritsen and Cohen (18) into type-1 (PP1) and type-2 (PP2) phosphatases.

Characterization of protein phosphatases is largely based on the use of various activators and inhibitors. It has been shown recently that okadaic acid binds to PP1 and PP2A (19,20) and inhibits their activities (21,22) inducing hyperphosphorylation of proteins (23) resulting in the accumulation of the phosphorylated proteins in larger amounts than in the usual state, which is reflected as the apparent "activation" of protein kinases and tumor promotion (24).

1/59 antibody was found to mimic the biological function of OA (Figure 4) in that it inhibited protein phosphatase 1 and 2A catalytic activity in a protein phosphatase bioassay developed by Cohen and Holmes (14,15). Analogously to OA, anti-idiotypic antibody inhibited the activity of PP2A more strongly than that of PP1 (145 versus 1100 nM). If indeed the obtained data were artifactual, identical inhibition profiles could be expected for both enzymes. These results are consistent with competitive ELISA data (Figure 3) in that the anti-Id displaces OA from binding to anti-OA antibody. Anti-Id, however, is not as potent a protein phosphatase inhibitor as OA. This may be due in part to steric or conformational hindrance since the anti-Id is approximately 200 times the size of OA (M.W. 808).

Thus, the results presented here demonstrate that 1/59 anti-idiotypic IgG mimics OA properties in both immunological and biological assays. Although the interactions between the anti-idiotypic antibody and its receptor, protein phosphatase, are still not fully understood, warranting further investigation, 1/59 may serve as a surrogate of OA in marine and protein chemistry research, in purification of protein phosphatases by affinity chromatography, as well as in elucidation of tumor promotion mechanism.

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