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Antigenic relatedness between phospholipases A_2 from *Naja naja* venom and from mammalian cells

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Polyclonal antiserum was prepared against phospholipase A_2 from *Naja naja* and used to prepare a purified antibody. It cross-reacted with the antigen, and with intracellular mammalian PLA_2. This antibody was immunoreactive and inhibited the PLA_2 activity of *Naja naja* and of guinea pig alveolar macrophages or rat lymphocytes. By immunoblotting, this antiserum revealed one band of PLA_2 from *Naja naja* (14 kDa) and 3 bands for guinea pig alveolar macrophages and rat lymphocytes (30, 45 kDa and a minor band of 14 kDa). These results show an antigenic relatedness between an extracellular PLA_2 and membrane-bound PLA_2 from two different mammalian species and cell types.

Phospholipase A_2; Intracellular enzyme; Antibody; Cross-reactivity; (*Naja naja*)

1. INTRODUCTION

Phospholipases A_2 (EC 3.1.1.4) are present in most cells. Membrane-bound phospholipases A_2 are involved in phospholipid turnover and in the release of arachidonic acid which is the limiting step of the arachidonate cascade [1–3]. These intracellular phospholipases A_2, in contrast with lysosomal phospholipases, are hydrophobic, require calcium and have a neutral or alkaline optimum pH [1]. As they represent a very small part of the cell proteins, only few of them have been purified to homogeneity [4]. Therefore, contradictory data have been reported about cell membrane phospholipases A_2, whereas extracellular enzymes such as phospholipase A_2 from snake venom or pancreatic juice are well characterized in terms of amino acid sequence, 3-dimensional structure [2,5] and recently mRNA sequence [6].

Secreted phospholipases A_2 share common properties with membrane-bound intracellular phospholipases A_2 (calcium and pH requirement) although their biological functions are completely different. Contradictory results have been reported about their antigenic relatedness. Meijer et al. [7] failed to show any detectable cross-reactivity between polyclonal antibodies directed against pancreatic phospholipase A_2 and venom phospholipases. No immunological relationship was found by Wurl and Kunze [8] between human seminal and pancreatic phospholipases A_2. Conversely, Okamoto et al. [9] observed an antigenic cross-reactivity between an antiserum prepared against rat pancreatic phospholipase A_2 and rat spleen microsomal phospholipase A_2. Using several monoclonal antibodies directed against rat liver mitochondrial phospholipase A_2, De Jong et al. [10] observed a cross-reactivity with rat liver cytosol or rat platelet phospholipase A_2 but not with rat or pig pancreatic phospholipase A_2 nor with *Crotalus atrox* phospholipase A_2. Recently, Khalil et al. [11] reported the inhibition of snake venom and porcine pancreatic phospholipases A_2 by a monoclonal antibody generated against human plasma lecitin-cholesterol acyltransferase,

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which acts both as phospholipase A_2 and as acyltransferase.

The present study reports the cross-reaction of a polyclonal antibody prepared against *Naja naja* phospholipase A_2 with membrane-bound phospholipases A_2 from different mammalian cell types and species.

2. MATERIALS AND METHODS

2.1. *Anti*-Naja naja *phospholipase A_2 antibody*

Naja naja venom phospholipase A_2 (grade V, Sigma, St. Louis, MO) was purified by polyacrylamide gel electrophoresis in the presence of SDS and β-mercaptoethanol according to Laemmli [12]. Phospholipase A_2 bands were cut, pooled and homogenized. After emulsification with Freund's complete adjuvant, they were injected into rabbit. Booster injections were administered at 2, 4, 6 and 8 weeks. Antiserum samples were titrated by ELISA procedure. In all experiments we used the material collected after 9 weeks and stored at $-20°C$. Immunoglobulin G (IgG) was purified from the antiserum by gel exclusion chromatography on a column of DEAE cellulose (DE 52 Whatman). A further purification on a Trisacryl-Blue column (Pharmacia) was performed to eliminate the albumin-bound phospholipase A_2 inhibitor described in rabbit plasma [13]. IgG purification was controlled by electrophoresis and IgG was concentrated in dialysis tubing embedded in polyethylene glycol 20 M.

2.2. *Phospholipase A_2 activity*

Phospholipase A_2 from *Naja naja* was tested using di-[1-^{14}C]palmitoyl phosphatidylcholine (95 mCi/mmol, Amersham) diluted with dipalmitoyl phosphatidylcholine (Sigma) as substrate. Incubations were carried out for 20 min at 37°C under agitation in 1 ml of 50 mM Tris buffer (pH 9) containing 16 μM substrate, 10 mM calcium and 6 ng of phospholipase.

Membrane-bound phospholipase A_2 from guinea pig pulmonary alveolar macrophage (PAM) was solubilized by 1 M NaCl as described by Colard-Torquebiau et al. [14] and measured using dioleyl phosphatidyl [1-^{14}C]ethanolamine (60 mCi/mmol, Amersham) diluted with phosphatidylethanolamine (Sigma) as substrate. NaCl extracts were prepared from PAM harvested as described [15]. After differential adhesion, the cells were scraped in 50 mM Tris buffer (pH 8) containing 1 M NaCl, then sonicated twice for 15 s using a MSE sonifier. After a 30 min incubation at room temperature, the lysate was centrifuged at 30000 $\times$ *g* for 20 min. The supernatant was recovered and used as NaCl extract. PAM phospholipase activity was assayed by incubating, for 15 min at 37°C, 60 μg of NaCl extract proteins in 1 ml of 50 mM glycine-NaOH buffer (pH 8.5) with 8 μM substrate and 2 mM calcium. NaCl extract from rat lymphocytes was prepared and its phospholipase activity assayed as described [16].

In order to test the inhibition of phospholipase A_2 activities by the anti-*Naja naja* phospholipase A_2 antibody, enzyme samples from *Naja naja* or macrophage and lymphocyte NaCl extracts were preincubated with the appropriate dilution of antibody for 10 min at 37°C. The phospholipase activity was then assayed as described above.

At the end of the incubation, lipids were extracted [17] and separated by thin layer chromatography on silica gel plates using chloroform:methanol:water (65:25:4, v/v). Spots were revealed by iodine vapor, scraped off and radioactivity measured with a Kontron scintillation counter.

2.3. *Western blotting*

Electrophoresis was performed according to Laemmli [12]. Samples of *Naja naja* phospholipase A_2 or of macrophages and lymphocyte NaCl extracts were preincubated for 1 h at 37°C in 0.5 M Tris buffer (pH 6.8) containing 20% sucrose, 0.05% bromophenol blue and 1.8% SDS. Standards were preincubated for 2 min at 95°C in the same buffer containing 5% β-mercaptoethanol. Western immunoblotting was done according to Burnette. [18]. Electrophoretic transfer onto the nitrocellulose papers was carried out for 90 min at a constant voltage of 40 V in 25 mM Tris base buffer (pH 8.3) containing 0.192 M glycine. The antigen-antibody complex was revealed with ^{125}I-protein A (700000 cpm/ml) for 1 h.

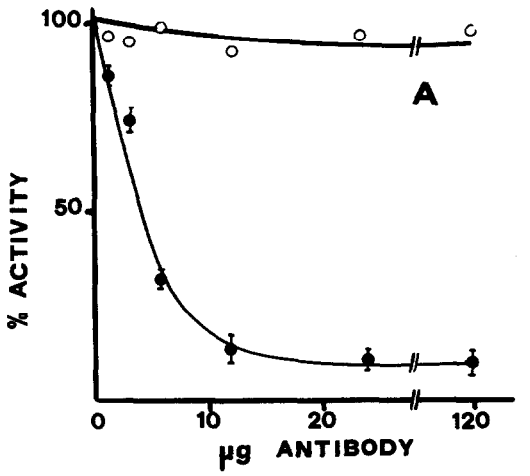
3. RESULTS AND DISCUSSION

As shown in fig.1A, the polyclonal antibody

generated against the *Naja naja* phospholipase A_2 inhibited the activity of its own antigen. The 50% inhibition of the activity (IC_{50}) was obtained with 5 μg of antibody; 20 μg induced an almost complete inhibition. We could exclude any effect of the albumin-bound phospholipase A_2 inhibitor previously described in rabbit serum [13] because the antibody was purified to eliminate albumin. Furthermore, the preimmune rabbit antibody prepared in the same conditions did not inhibit *Naja naja* phospholipase A_2 activity (fig.1A).

Immunoblotting of *Naja naja* phospholipase A_2 showed a single band of apparent molecular mass of 14 kDa revealed by the anti-*Naja naja* phospholipase A_2 antiserum (fig.1B, lane a). This band was detected neither by the antiserum preincubated with an excess of antigen (fig.1B, lane b) nor by the preimmune serum (fig.1B, lane c). Taken together, these data established the specificity of the polyclonal antibody that we prepared against *Naja naja* phospholipase A_2.

In a second step, we studied the cross-reactivity of this antibody towards mammalian phospholipase A_2 preparations. We found that the anti-*Naja naja* phospholipase A_2 antibody inhibited the phospholipase A_2 activity of NaCl extracts from guinea pig PAM (fig.2A). The IC_{50} was reached for 13 μg of antibody whereas preimmune antibody did not decrease the phospholipase A_2 activity. A similar inhibition curve was observed with



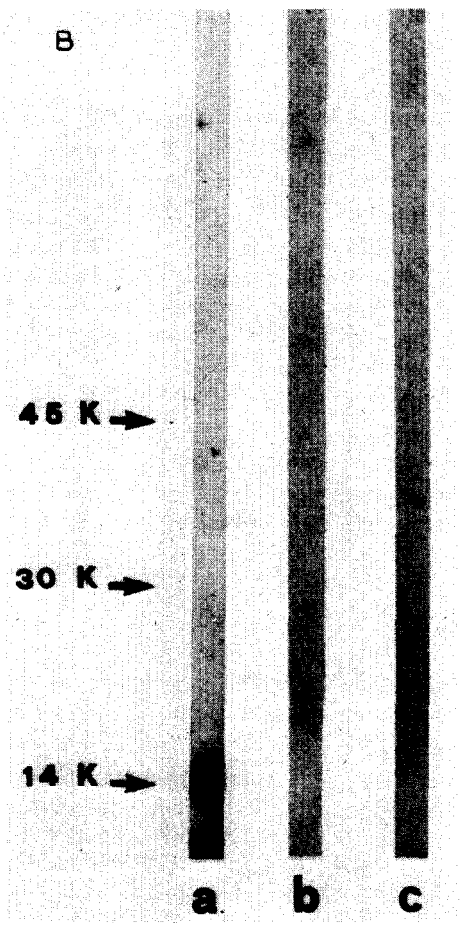
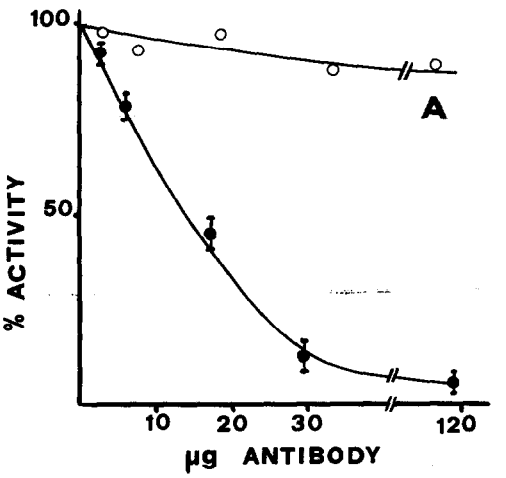
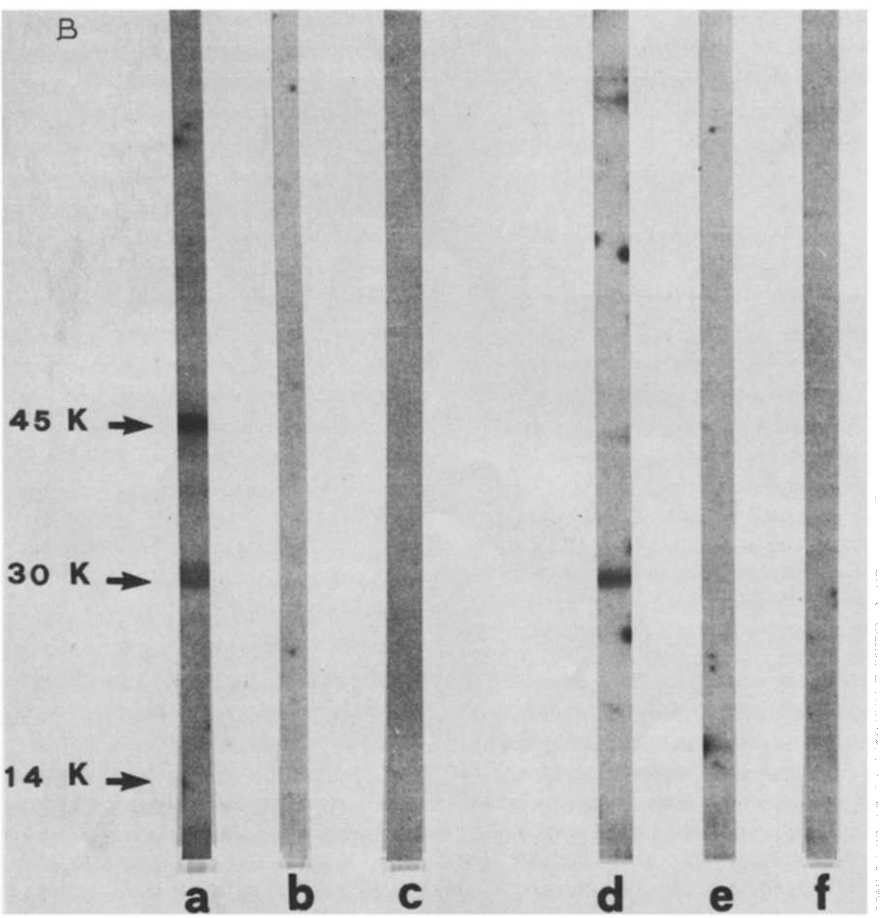


Fig.1. Inhibition and immunoblotting detection of *Naja naja* phospholipase A_2. (A) Inhibition of *Naja naja* phospholipase A_2 activity as a function of antibody concentration: after preincubation for 10 min at 37°C with the anti-*Naja naja* phospholipase A_2 antibody (close circles) or preimmune antibody (open circles), phospholipase A_2 was assayed as described in the text. Results are expressed as percentages of the initial activity of the enzyme (34.6 ± 2.7 μmol of fatty acids released per min and mg protein of *Naja naja* phospholipase). (B) Immunoblotting detection of phospholipase A_2 from *Naja naja* (0.1 μg) with the anti-*Naja naja* phospholipase A_2 antiserum (lane a), the same antiserum pretreated with a large excess of antigen (lane b) or preimmune serum (lane c). After the 12% polyacrylamide gel electrophoresis and the transfer procedure, nitrocellulose bands were revealed with 500-fold diluted antiserum. Molecular masses were determined by comparison with standards.


100
50
% ACTIVITY
A
10
20
30
120
µg ANTIBODY


B
45 K
30 K
14 K
a
b
c
d
e
f

NaCl extracts from rat lymphocytes (not shown).

By immunoblotting of NaCl extracts from either guinea pig PAM or rat lymphocytes, the antiserum revealed two major bands of apparent M_r 30000 and 45000 and a minor band of M_r 14000 (fig.2B, lanes a and d). These three bands were only weakly stained with Coomassie blue, indicating that they did not correspond to major proteins of the NaCl extract. As for *Naja naja* phospholipase A_2, these bands were not revealed neither with the antiserum previously incubated with a large excess of its antigen (fig.2B, lanes b and e) nor with the preimmune serum (fig.2B, lanes c and f). These results meant that the antiserum reacted with these three bands. A molecular mass of 14 kDa has been reported for cellular phospholipase A_2 isolated from different tissues and species [1–3] but Apitz-Castro et al. [19] found a 44 kDa phospholipase A_2 in human platelets. Furthermore, dimeric and monomeric phospholipases A_2 have been evidenced in *Crotalus* snake venom [20]. As we obtained the same migration with NaCl extracts treated with or without β-mercaptoethanol before electrophoresis, the question remains of whether these bands correspond to different polymeric forms of the same enzyme or to different cellular phospholipases A_2.

In table 1 we have compared the IC_{50} and the half-maximal reactivity by ELISA for *Naja naja* phospholipase A_2 and NaCl extracts of guinea pig PAM and rat lymphocytes. We observed that the three preparations displayed the same ratio between their IC_{50} and half-maximal reactivity. In addition our antibody cross-reacted with a phospholipase A_2 purified from human placenta (Jordan, L., personal communication).

Previous data demonstrated either an intraspecies relatedness between a secretory and a cellular phospholipase A_2 [9] or an interspecies relatedness between secretory enzymes [11]. Recently, De Jong et al. [10], using monoclonal antibodies directed against a rat liver mitochondrial phospholipase A_2, showed an intraspecies cross-reactivity with cellular phospholipases A_2 but neither intraspecies nor interspecies reactivity with extracellular phospholipases A_2. Moreover these antibodies had no inhibitory effect on any of the phospholipases A_2 they tested.

Our results clearly demonstrated an interspecies relatedness between a polyclonal antibody prepared against a secretory phospholipase A_2 and mammalian cellular phospholipase A_2 from guinea pig PAM and rat lymphocytes. This relatedness is expressed by immunoreactivity as well as by inhibition of the phospholipase A_2 activity.

Table 1

Interspecies reactivity of the anti-*Naja naja* phospholipase A_2 antibody

	Naja naja phospholipase A_2	Guinea-pig PAM (NaCl extract)	Rat lymphocyte (NaCl extract)
50% inhibition of PLA_2 activity	5	13	24
50% of maximal reactivity by ELISA	8	34	60

Results are expressed as μg of antibody responsible for the 50% inhibition of phospholipase A_2 activity or giving the half of maximal immunoreactivity assayed by ELISA. Phospholipase A_2 activity was measured with 6 ng of *Naja naja* phospholipase A_2 or 60 μg of guinea-pig PAM and rat lymphocyte NaCl extract. ELISA was performed with 10 μg of *Naja naja* phospholipase A_2 or 50 μg of cobaye macrophage and rat lymphocyte NaCl extracts

Fig.2. Inhibition and immunoblotting detection of phospholipase A_2 from guinea-pig PAM and rat lymphocytes. (A) Inhibition of the phospholipase A_2 from guinea-pig PAM as a function of antibody concentration by polyclonal antibody against *Naja naja* (closed circles) or preimmune antibody (open circles). The initial activity was 0.49 ± 0.06 nmol of fatty acids released per min and mg proteins of 1 M NaCl extract from guinea-pig PAM. For procedure, see fig.1. (B) Immunoblotting detection of phospholipase A_2 from guinea-pig PAM (50 μg proteins of NaCl extract, lanes a, b and c) and rat lymphocyte (50 μg proteins of NaCl extract, lanes d, e and f) by the polyclonal antiserum prepared against *Naja naja* phospholipase A_2 (lanes a and d), the same antiserum pretreated with a large excess of *Naja naja* phospholipase A_2 (lanes b and e) or preimmune serum (lanes c and f). The procedure is described in fig.1.

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