Quantification of the organophosphorus nerve agent soman by competitive inhibition enzyme immunoassay using monoclonal antibody

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Received 23 September 1982

Soman *Monoclonal antibody* *Enzyme immunoassay* *Detection*

1. INTRODUCTION

The organophosphorus compound soman (methylphosphonofluoridic acid 1,2,2-trimethylpropyl ester) is a potent cholinesterase inhibitor [1,2] and a chemical warfare agent. The rapid identification and characterization of nerve agents such as soman would facilitate the diagnosis and treatment of poisoned humans, and provide a more useful means of environmental surveillance. Presently, soman is routinely identified by physicochemical techniques such as gas chromatography [3–5]. Though sensitive and specific, these techniques are time consuming and require expensive equipment and highly trained operators. Here, we discuss the production of a mouse monoclonal antibody that binds to soman and further describe the use of this antibody in a competitive inhibition enzyme immunoassay (CIEIA) capable of quantifying levels of soman as low as 1.0×10^{-6} M (200 ppb). The specificity of this antibody is demonstrated by its ability to distinguish between soman and the structurally similar organophosphorus nerve agent sarin (methylphosphonofluoridic acid, 1-isopropyl ester).

2. EXPERIMENTAL

2.1. *Chemicals*

Crystallized bovine serum albumin (BSA) was obtained from Miles Labs. (Kankakee IL) and hemocyanin from giant keyhole limpets (KLH) was purchased from Schwarz-Mann (Orangeberg NY). Soman and sarin (>95% pure) were obtained from Chemical Systems Labs. (Aberdeen Proving Ground MD). All other chemicals were reagent grade and obtained commercially.

2.2. *Preparation of immunogenic soman–protein conjugates*

Soman is non-immunogenic by virtue of its very low M_r-value. To induce antibody production, soman was conjugated to BSA and KLH by diazotization as in [6]. The method in [7] was used to determine the epitope density (mol soman bound/mol protein). For soman–KLH and soman–BSA the values were 700 and 15, respectively. The structure of the immunogen is shown in fig. 1.

2.3. *Hybridomas*

Adult female BALB/c mice (Jackson Labs., Bar Harbor ME) were immunized subcutaneously with 100 μg (protein) of soman–BSA emulsified in complete Freund's adjuvant. Fourteen days later the mice received a booster immunization of 100 μg soman–BSA in normal saline by the intraperitoneal route. After 3 days, spleens were removed and splenic lymphocytes fused with the hypoxanthine–guanine phosphoribosyltransferase deficient mouse plasmacytoma line P3-X63-Ag8.653 [8] as in [9], with some modifications. Fu-

Published by Elsevier Biomedical Press
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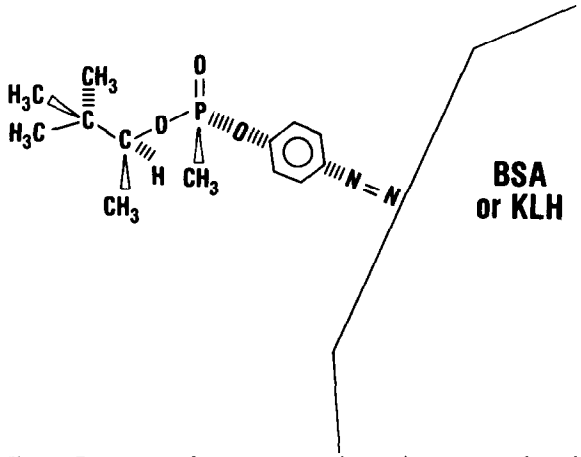

Fig. 1. Structure of soman–protein conjugates employed for immunization and/or immunoassay.

sion was accomplished with 35% polyethylene glycol (M_r 1400, American Type Culture Collection, Rockville MD), and fused cells planted in 96-well, sterile, flat-bottomed microtiter plates at 5.0×10^5 cells/well. Hybridomas were selected in hypoxanthine–aminopterin–thymidine medium for the first week, with changes of culture medium on alternate days.

2.4. *Enzyme immunoassay and cloning*

Supernatants from 14-day hybridoma cultures were analyzed for anti-soman antibody production by enzyme immunoassay [10,11]. Wells of flat-bottomed polystyrene microtiter plates (Dynatech, Alexandria VA) were incubated with 100 μl soman–KLH (10 μg/ml in 0.1 M $NaHCO_3$, pH 9.6) overnight at 4°C. These plates were washed with Dulbecco's phosphate-buffered saline (Flow Labs., Vienna VA) containing 0.5% Tween 20 (PBS-T) by 5 repetitions of filling and emptying each well. Aliquots of hybridoma supernatants (100 μl) were added to soman–KLH coated wells in duplicate and incubated for 30 min at room temperature. Unbound antibody was washed away as above and 100 μl aliquots of rabbit anti-mouse κ light chain antiserum (1:300 in PBS-T, Miles Labs., Elkhardt IN) was added and incubated for 30 min at room temperature. This reagent binds to virtually all mouse immunoglobulin classes and subclasses. Unbound rabbit antibody was washed away and 100 μl aliquots of alkaline phosphatase-conjugated goat anti-rabbit IgG (1:500 in PBS-T, Sigma Chemical Co., St Louis MO) was added. Following a 30 min incubation at room temperature and a final wash, 100 μl enzyme substrate (p-nitrophenylphosphate, 1 mg/ml in 10% diethanolamine buffer (pH 9.6), Sigma Chemical Co., St Louis MO) was added. The enzymatic reaction was allowed to proceed for 30 min at room temperature, then the colored reaction product was measured spectrophotometrically at 405 nm in a Titertek® Multiscan Micro-ELISA Reader (Flow Labs., Vienna VA). Duplicate determinations >4-times background (absorbance of tissue culture medium on antigen-coated wells) were considered to be positive.

Positive supernatants were further screened for reactivity to free soman by a modified CIEIA [12]. An aliquot of 100 μl positive supernatant was incubated for 1 h with 100 μl PBS-T or PBS-T containing 2.0×10^{-3} M soman. Of this mixture, 100 μl was tested for antibody binding exactly as above. Inhibition of >50% of the control absorbance was considered indicative of reactivity with free soman. Hybridoma cultures with supernatants that tested positive by CIEIA were cloned by limiting dilution (0.5–1 cell/well) on irradiated mouse tumor macrophage (P388D1) feeder layers. Clones were further tested by enzyme immunoassay and positive clones recloned as before.

One clone designated A10 was chosen for detailed analysis. A10 was found to be of the IgG1 subclass by Ouchterlony double diffusion against mouse immunoglobulin class- and subclass-specific reagents (Miles Labs., Elkhardt IN). The cloned hybridomas were cryopreserved in 15% dimethylsulfoxide, 85% RPMI 1640 medium (containing 10% fetal bovine serum) and stored in liquid nitrogen vapor phase. Long term cultures were established and antibody harvested by protein A–Sepharose affinity chromatography [13].

2.3. *CIEIA*

An assay system similar to that in [12] was employed. In the CIEIA, a concentration of monoclonal anti-soman antibody that bound to <100% of the available site on the solid phase bound soman–KLH was mixed with equal volumes of soman in log molar dilutions, then incubated for 1 h at room temperature. The remainder of the assay was exactly as above for hybridoma screening.

3. RESULTS

3.1. *Generation of hybridomas secreting antisoman antibodies*

In the initial screen, supernatants from all wells with growing hybridomas were tested for antibody that bound to soman conjugate. For the fusion that yielded A10, 24 of 384 wells (6%) were positive for anti-soman antibody. However, when these positives were further tested for reactivity to free soman (by CIEIA), only 4 were positive. This discrepancy has now been noted in several fusion experiments, and suggests that antibodies directed against the spacer unit or the diazo bond occur with some regularity. Since reactivity with unconjugated soman is critical for developing an immunoassay system, the CIEIA screen is all important.

3.2. *Titration of anti-soman antibody on soman–KLH*

To determine the non-saturating concentration of anti-soman antibody for use in the CIEIA, the binding of various concentrations of anti-soman to soman–KLH was assessed (fig. 2A). The concentration chosen for routine assay was 2 μg/ml, a value that was near the midpoint of the linear segment of the dilution curve.

3.3. *Detection limits in the CIEIA*

In the CIEIA, the inhibition of binding of anti-soman antibody to soman–KLH by various concentrations of free soman was used to generate a standard curve from which unknown concentrations of soman could be quantified. Fig. 2B illustrates a typical soman inhibition curve. Note that the curve is linear from 1.0×10^{-3} M to $\sim 1.0 \times 10^{-6}$ M, thus allowing the quantification of soman in test samples over a 3 log range. The maximum sensitivity of the CIEIA would be defined by the lowest soman concentration that gives a linear inhibition.

The specificity of the anti-soman antibody was assessed by comparing the inhibition induced by soman with a related organophosphorus nerve

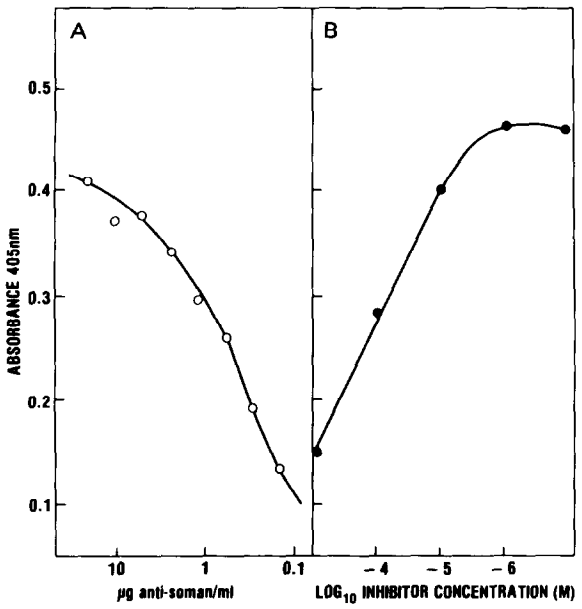

Fig. 2. (A) Titration of the binding of anti-soman to soman–KLH. (B) Inhibition of the binding of anti-soman antibody to soman–KLH by various concentrations of free soman.

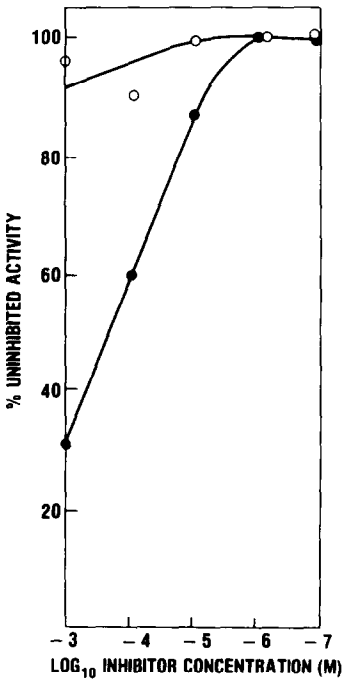

Fig. 3. Specificity of binding of anti-soman antibody to soman–KLH. The ability of soman (●—●) to inhibit binding of anti-soman antibody to soman–KLH was compared with a related organophosphorus nerve agent, sarin (○—○). The absorbance generated in the absence of either inhibitor represents 100%.

agent, sarin. Fig. 3 shows that sarin failed to inhibit anti-soman binding to soman–KLH.

4. DISCUSSION

This study describes the preparation of the first monoclonal antibody to an organophosphorus nerve agent. The feasibility of preparing immunogenic conjugates between small chemicals and carrier proteins is well documented [14]; the conjugates used here had been shown to be immunogenic in rabbits [6].

The use of two immunologically distinct carrier proteins for immunization and screening circumvented the problem of anti-carrier antibody, but did not conclusively identify antibodies reactive with free soman. The most likely explanation for this result is the induction of antibodies that recognize all or part of the *p*-aminophenyl spacer or the diazo bond. Nevertheless, subsequent screening of conjugate reactive antibodies by CIEIA identified those with exclusive soman reactivity. We also found that a non-toxic soman analogue DPMP (methylphosphonic acid, di-[1,2,2-trimethylpropyl] ester) could be substituted for soman in the CIEIA screen. However, some caution should be exercised in chosing monoclonal antibodies on the basis of analogue screening, in that antibodies may be selected that have higher affinity for the analogue than for the compound of interest.

There are advantages of monoclonal over polyclonal antibodies in immunoassay procedures. The immortality of the hybridoma creates a continuous supply of monoclonal antibodies that are homogenous and facilitate precise quality control. Polyclonal antibodies, on the other hand, tend to change with each immunization, and such antisera decline in antibody activity between immunizations. Monoclonal antibodies also exhibit high specificity in their binding as evidenced by the failure of monoclonal anti-soman antibody to bind sarin (fig. 3). In our earlier experiments, antisera raised in rabbits against the same soman–protein conjugates cross-reacted with a variety of organophosphorus compounds including sarin (unpublished).

The CIEIA system is particularly well suited to detecting nerve agents. Unlike other immunoassay methods such as radioimmunoassay, CIEIA does not require maintaining a supply of very toxic and labile radioactive organophosphonates. The soman–protein conjugates appear to be quite stable and are entirely non-toxic. The actual determination of antibody binding can be done colorimetrically, thus avoiding the use of organic scintillation fluids and expensive counters.

The CIEIA detected soman concentration as low as 1.0×10^{-6} M (200 ppb), and the standard curve generated was linear over 3 orders of magnitude. This allows for the preparation of dilutions of test samples over a wide concentration range with assurance that one or more values will fall on the standard curve. The assay was accomplished rapidly (2.5 h) and was highly reproducible.

ACKNOWLEDGEMENTS

This work was supported by Chemical Systems Laboratory, Aberdeen Proving Ground, MD. We thank Dominique M. Nau for editorial assistance.

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