A Competitive Inhibition Enzyme Immunoassay for Detection and Quantification of Organophosphorus Compounds

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A sensitive and specific method for detection and quantification of methyl phosphonic acid, *p*-aminophenyl 1,2,2,-trimethyl-propyl diester (MATP) as a model substance for organophosphorus compounds is described.

Different procedures for coupling the haptenic group for immunization, purification and immobilization allowed the detection of hapten-specific antibodies.

The competitive inhibition enzyme immunoassay (CIEIA), using purified chicken and rabbit IgG-antibodies, was able to detect MATP-concentrations as low as 10^{-10} mol/l.

Based upon our results, we postulate that the CIEIA represents a good alternative to the customary diagnosis of organophosphate intoxications, measuring blood cholinesterase activity.

Introduction

Application of organophosphates has strongly increased since their first synthesis by De Clermont in 1854.

They are used technically as softeners, lubricants and in the plastics industry, particularly in the preparation of polyvinylchloride. Due to their strong insecticide effects they are applied as pesticides and insecticides in agriculture and veterinary medicine. Some of the very toxic organophosphorous compounds like Tabun, Sarin, and Soman are produced as chemical warfare agents.

Every year more than 1000000 tons of organophosphates are produced, whereas the use uf chlorinated hydrocarbon insecticides is more and more restricted because of their longlasting residual effects [1].

Intoxication by organophosphates has resulted from absorption of toxic amounts of organophosphorous anticholinesterase compounds by careless handling during production, storage, distribution and application of these substances [2–4]. Several instances of intoxication have also occurred following exposure to organophosphorous warfare agents [5].

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Clinical diagnosis of such intoxications is currently based on estimation of blood cholinesterase activity [5–9]. Although the specific methods for detecting organophosphate substances such as mass spectrometry and gas- or gas-liquid chromatography, are very sensitive, preparation of the samples is very time consuming, equipment is very expensive, personnel must be highly trained and the results are sometimes difficult to interpret [10–12].

Enzyme-immunologic methods have been approved for the detection and quantification of toxins and low molecular substances like hormones, drugs and chemical haptens [13–16]. Here, a specific ELISA-system for the detection of methyl phosphonic acid, *p*-aminophenyl 1,2,2-trimethyl-propyl-diester (MATP) as a model substance for organic phosphorus agents will be described.

Materials and Methods

Chemicals

MATP was synthesized by the TNO, Rijswijk (Netherlands). Bovine serum albumin (BSA), human serum albumin (HSA), Borax and AMP-buffer solution were purchased from Sigma, La Jolla (USA).

Complete Freund's adjuvant (CFA) was obtained from DIFCO, Detroit (USA), 4-nitrophenylphosphate from Serva, Heidelberg, and CNBr-activated Sepharose 4B from Pharmacia, Uppsala (Sweden). All other reagents were purchased from Merck, Darmstadt.

Preparation of immunogenic MATP-protein conjugates

MATP was coupled to BSA and HSA by means of diazobinding [17]. Briefly, MATP was diazotized by addition of 1% $NaNO_3$-solution. The reaction was checked by potassium iodide starch paper. Appropriate proteins dissolved in saturated borax solution were added dropwise. The reaction was stopped by dialysis against 0.15 mol/l NaCl after 18 h. Epitope densities were determined by the method of Fenton and Singer [18].

Immunization of animals

White Leghorn chickens (LSL) and dysgammaglobulinemic UM-B 19 chickens [19] were given 20 mg/kg bodyweight $MATP_{17}$-HSA (17 MATP molecules coupled per 1 HSA molecule) i.v. on day 0 (= day of first immunization) and on days 14, 25 and 29 and 20 mg/kg bodyweight $MATP_{17}$-HSA emulsified in 0.5 ml CFA/kg bodyweight i.m. on days 21 and 33.

Three rabbits (dt. Riese) were immunized as follows:

Rabbit X (RX) was given 0.7 mg $MATP_{20}$-BSA i.m. on days 0, 21, 42, 63 and 148.

Rabbit Y (RY) was given 0.5 mg $MATP_{20}$-BSA emulsified in 1 ml CFA i.m. on days 0 and 28, and 0.5 mg $MATP_{20}$-BSA in 1 ml NaCl i.v. on days 14, 42, 63, 77, 88 and 119.

Rabbit Z (RZ) was given 5 mg $MATP_{20}$-BSA emulsified in 1 ml CFA i.m. on days 0 and 56.

Antibody purification

Isolation of anti-MATP antibodies was accomplished by affinity chromatography on antigen bound sepharose 4B. MATP was directly coupled to the sepharose gel by the method recommended by the supplier. The coupled MATP concentration was 1 umol MATP per g sepharose.

Estimation of the concentration of the purified rabbit antibodies was done by protein measurement following Bradford's method [20].

Concentrations of affinity-purified chicken anti-MATP antibody standards were determined according to the method of Laurell [21] modified as described by Schranner *et al.* [22].

ELISA for estimation of antibody concentration

96-microwell titerplates (Nunc Immunoplate II) were reacted with uncoupled MATP by a modified method of Suter [23]. Briefly, the ELISA-plates were activated for 4 h at room temperature with 0.2% glutaraldehyde in 0.1 mol/l phosphate buffer pH 5.0 (0.2 ml per well).

After washing with 0.1 mol/l phosphate buffer pH 5.0, 0.2 ml MATP in 0.1 mol/l phosphate buffer pH 8.0 (0.015 mg/ml) were added to each well. The plates were incubated for 3 h at 37 °C and overnight at 4 °C.

Determination of antigen-specific antibodies was performed as described by Kühlmann-Rabens *et al.* [24].

Anti-MATP antibody concentrations were expressed as ELISA units (U) [24] using purified anti-MATP antibodies as standard (see above).

CIEIA for MATP-detection

The polystyrene plates were coated as described above. After washing with PBS-Tween, 0.05 ml of MATP dissolved in PBS-Tween were added to each well of rows 3–12, 0.09 ml to the wells of row 2 to which 0.01 ml of the related antibody solution were finally added.

Beginning at row 2 a $\log_2$ serial dilution was accomplished by pipetting 0.05 ml from row 2 to row 3 ending at row 12.

After incubation at 37 °C for 1.5 h the plates were washed with PBS-Tween.

After this stage the tests performed with chicken antibodies differed from those performed with rabbit antibodies.

Chicken: Isotype specific enzyme-immunoglobulin-conjugates (0.1 ml; 1:500 dilution) were added to all wells of the plates. Affinity-purified rabbit anti-chicken IgG and IgM antibodies were labelled with alkaline phosphatase according to the method described by Engvall and Perlmann [25].

Rabbit: 0.1 ml goat anti-rabbit IgG-peroxidase (Paesel, Frankfurt, 1:1000) were added to each well.

The plates were then incubated for 90 min at 37 °C.

After washing with PBS-Tween, either 0.2 ml alkaline phosphatase (AP) substrate (2 mg 4-Nitrophenylphosphate per ml AMP-buffer) or 0.1 ml peroxidase (POD) substrate (0.08% 5-aminosalicylic acid with 0.005% H_2O_2) were added to each well. After 10 min the enzyme reaction was stopped by adding 0.05 ml 1 mol/l NaOH and optical density (OD) was measured by a Titertek Multiscan ELISA-

plate reader (Flow Laboratories, Meckenheim) at 405 nm (AP) respectively 492 nm (POD). Wells of row 1 served to adjust the zeropoint. Antibody dilutions giving an OD of 0.1 were defined as positive titers. Exact titers were calculated by interpolation following the method of Caulfield and Shafer [26].

Establishing a standard curve for detection of MATP, concentrations of 10^{-2}–10^{-12} mol/l MATP were used. Titers generated in the absence of either inhibitor represent 100% of activity.

Results

To obtain the best coupling conditions, various MATP/carrier ratios were tested. As shown in Fig. 1, the MATP/carrier ratio was increased by increasing hapten-concentration and decreasing protein concentration during the coupling procedure.

For immunization either $MATP_{20}$-BSA or $MATP_{17}$-HSA were applied.

As shown in Fig. 2 and 3 chickens did not attain considerable serum antibody concentrations before

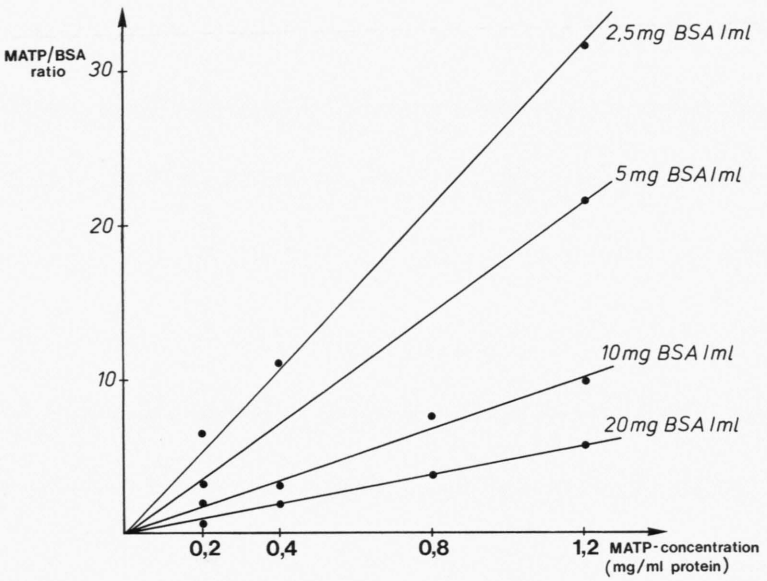

Fig. 1. Dependence of MATP/BSA ratio on the MATP and BSA concentration during the coupling procedure.

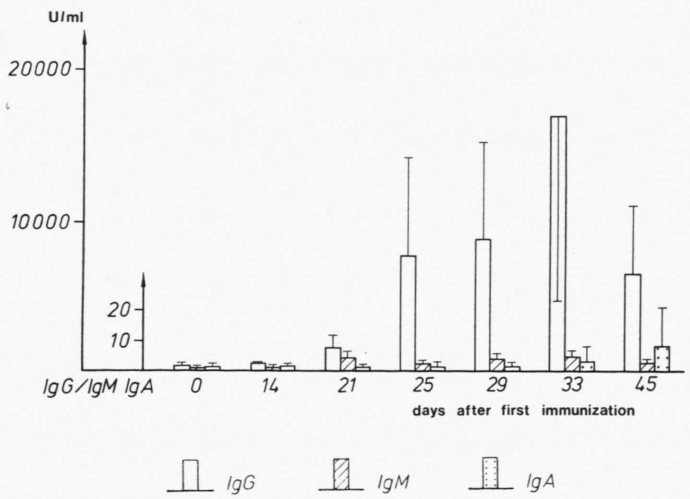

Fig. 2. Anti-MATP titers (U/ml) of LSL-chickens after immunization with MATP-HSA and CFA.

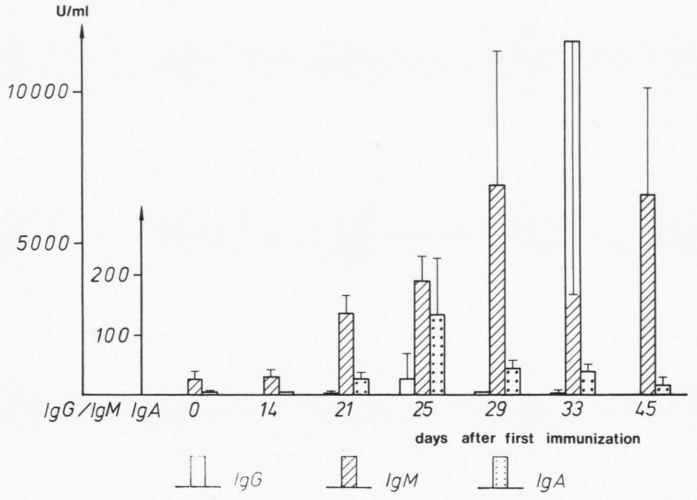

Fig. 3. Anti-MATP titers (U/ml) of dysgammaglobulinemic chickens after immunization with MATP-HSA and CFA.

the second immunization. Application of CFA however induced a much stronger elevation. In LSL-chickens these antibodies represented mainly IgG-isotype and in UM-B 19 chickens IgM-isotype.

Antibody synthesis in the rabbits (Fig. 4) showed the immunostimulating effect of CFA in a similar manner. RX, immunized without CFA showed only a slight production of specific antibodies despite frequent boosters. RY, frequently immunized with low antigen doses using CFA on days 0 and 28 reached much higher antibody concentrations than RZ, immunized only twice using the tenfold higher antigen dose together with CFA.

Neither rabbit Y nor Z showed significant antibody concentration before the second application of CFA.

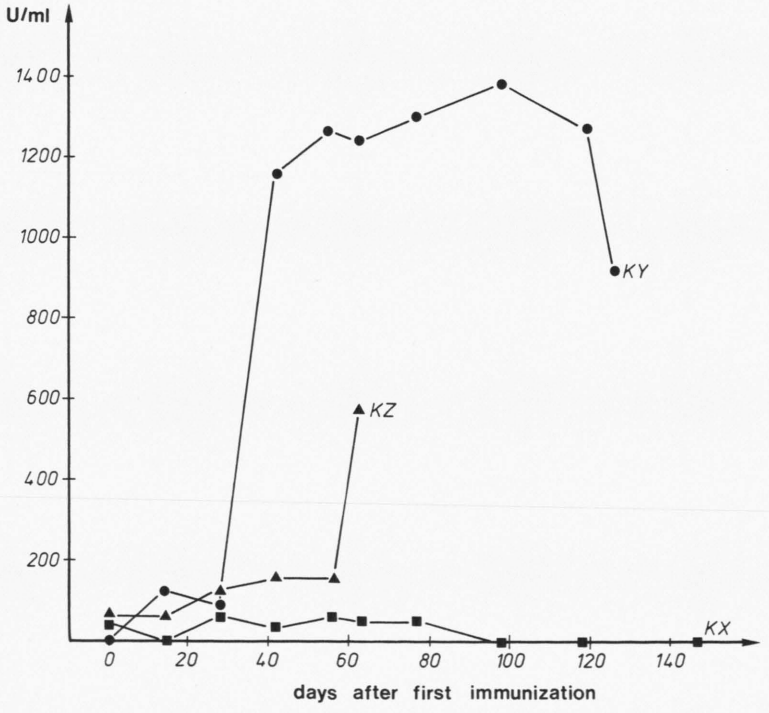

Fig. 4. Anti-MATP titers (U/ml) of rabbits after immunization with MATP-BSA. (K X, Y, Z in the text: R X, Y, Z).

In CIEIA anti-MATP antibody solutions were inhibited by various MATP-concentrations (see above). A positive correlation was seen between MATP-concentration and inhibition of antibody activity (Fig. 5 and 6). The CIEIA detected MATP-concentrations as low as 10^{-10} mol/l and the standard curve generated was nearly linear between $10^{-8}-10^{-4}$ mol/l MATP if using rabbit antibodies or chicken IgG-antibodies.

In contrast, a standard curve established with chicken IgM-antibodies was less sensitive (Fig. 5). Complete inhibition was not possible at all.

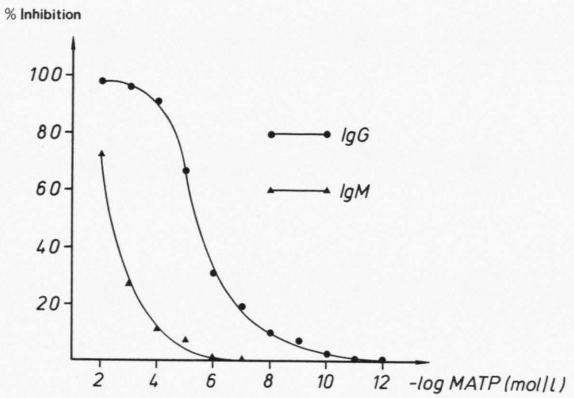

Fig. 5. ELISA for detection of MATP: Inhibition of chicken anti-MATP IgG and IgM antibodies by various MATP concentrations.

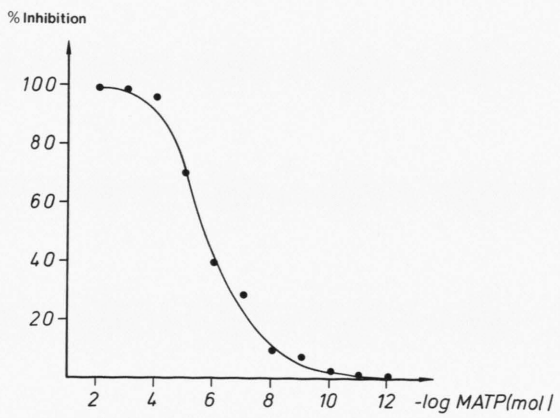

Fig. 6. ELISA for detection of MATP: Inhibition of rabbit anti-MATP antibodies by various MATP concentrations.

Discussion

This study describes a simple and highly sensitive immunological method to determine an organophosphorus nerve agent substance.

In order to induce specific antibodies in animals, it is necessary to couple the haptenic MATP to protein carriers. For optimal immunization it is recommended to bind 10–30 hapten molecules per 1 carrier molecule [27]. Diazobinding provides a good method to get suitable hapten/carrier ratios by choosing optimal coupling conditions (Fig. 1).

Since its introduction by Engvall and Perlmann [28] in 1971 the enzyme linked immunosorbent assay (ELISA) has gained a wide application for detecting specific antibodies as well as antigens. In haptenic systems, immunologically distinct carrier proteins are usually employed in immunization and testing, in order to circumvent the problem of detection of anti-carrier antibodies. However, detection of nonspecific antibodies directed against the diazobound occur in such systems as shown by Hunter and Lenz [14].

In the present study, therefore, a modified technique of Suter [23] was used to bind the hapten MATP directly to the polystyrene plate *via* Schiff's base. This method is also known to provide higher sensitivity and specificity [23]. In the same manner, coupling of MATP directly to CNBr-sepharose 4B should improve the specificity of the test system.

As shown in the present results, CFA was able to induce high antibody levels in chickens as well as in rabbits if administered together with antigen. These results agree with Tam and Benedict [29] who recommend the application of CFA especially for chickens.

In the present study the competitive inhibition enzyme immunoassay (CIEIA) was capable of quantifying levels of MATP as low as 10^{-10} mol/l in buffer. A quantitative estimation of unknown MATP-concentrations should be possible in the linear range of the standard curve between 10^{-8} and 10^{-4} mol/l. The standard curves obtained with chicken IgG antibodies and with rabbit antibodies are nearly identical. Obviously, the rabbit antibodies represent also mainly IgG-antibodies due to the long lasting immunization procedure.

The relative affinity of hapten-specific antibodies is expressed as the molar concentration of hapten which inhibited 50% of the binding of anti-hapten antibody to immobilized hapten (IC_{50}). As shown in Fig. 5, IC_{50} for the anti-MATP IgM-antibodies of the

chickens was nearly 1000-fold higher than for IgG-antibodies due to their lower affinity.

It is emphasized that corresponding methods are practicable for the detection of all organophosphates if suitable coupling procedures are available.

Moreover, blood cholinesterase activity is also depressed by agents other than organophosphates, like the carbamate insecticides. Application of oximes may then cause further inhibition of cholinesterase [7]. The very specific gas-chromatographic methods are mainly applied in the detection of trace amounts of organophosphate residues [30, 31]. These very expensive and complicated procedures are not useful for clinical diagnosis, especially in veterinary medicine. Thus, it seems desirable to develop specific immunologic methods for the determination of all common organophosphates.

Chicken represents a special producer of specific antibodies. Large amounts of IgG antibodies are transferred into the egg yolk and therefore can be gained in a simple manner and be purified [32].

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[1] M. Daunderer, D. Kirsch, S. Schäfer, L. Szinicz, and N. Weger, Dt. Ärztebl. **50,** 2975 (1980).
[2] R. C. Duncan, J. Griffith, and J. Konefal, J. Toxicol. Environ. Health **18,** 1 (1986).
[3] S. Leuzinger and A. Pasi, Schweiz. Arch. Tierheilkd. **112,** 269 (1970).
[4] R. L. Reece and P. Handson, Vet. Rec. **111,** 453 (1982).
[5] D. Grob, in: Handbuch der experimentellen Pharmakologie, Ergänzungswerk XV, p. 990, Springer Verlag, Berlin, Heidelberg, New York 1963.
[6] D. W. Jolly, Vet. Rec. **69,** 796 (1957).
[7] F. Matsumura, Plenum Press, New York and London 1985.
[8] F. Rappaport, J. Fischl, and N. Pinto, Clin. Chim. Acta **4,** 227 (1959).
[9] R. Silvestri, Am. J. Vet. Res. **38,** 659 (1977).
[10] H. P. Benshop, C. A. G. Konings, and L. P. A. de Jong, J. Am. Chem. Soc. **103,** 4260 (1981).
[11] P. A. D'Agostino and L. R. Provost, Biomed. Environ. Mass Spectrom. **13,** 231 (1986).
[12] S. Sass, T. L. Fisher, R. J. Steger, and G. A. Parker, J. Chromatog. **238,** 445 (1982).
[13] K. W. Hunter and D. E. Lenz, Life Sciences **30,** 355 (1982).
[14] K. W. Hunter, D. E. Lenz, A. A. Brimfield, and J. A. Naylor, FEBS Lett. **149,** 147 (1982).
[15] H. Peters, M. P. Dierich, and K. Dose, Hoppe-Seyler's Physiol. Chem. **363,** 1437 (1982).
[16] B. K. Van Weemen and A. H. W. M. Schuurs, in: Immunoenzymatic Techniques (G. Feldmann, P. Druet, J. Bignon, and S. Avrameas, eds.), p. 125, American-Elsevier Publishing Company, Inc.-New York 1976.
[17] J. Suttnar, Z. Hrkal, and Z. Vodrazka, J. Chromatog. **131,** 453 (1977).
[18] J. W. Fenton and S. J. Singer, Biochem. **10,** 1429 (1971).
[19] U. Lösch, I. Kühlmann, W. Klumpner, and H. Fiedler, Immunbiol. **158,** 416 (1981).
[20] M. M. Bradford, Anal. Biochem. **72,** 248 (1976).
[21] C. B. Laurell, Anal. Biochem. **15,** 45 (1966).
[22] I. Schranner, W. Dorn, and U. Lösch, J. Vet. Med. B **34,** 407 (1987).
[23] M. Suter, J. Immunol. Methods **53,** 103 (1982).
[24] I. Kühlmann-Rabens, R. Wanke, F. Storandt, B. Altmann, U. Lösch, and M. Merkenschlager, Vet. Immunol. Immunopathol. **14,** 123 (1987).
[25] E. Engvall and P. Perlmann, in: Automation in microbiology and immunology (C. G. Heden and T. Illeni, eds.), p. 542, Wiley and Sons, New York 1973.
[26] M. J. Caulfield and D. Shafer, J. Immunol. Methods **74,** 205 (1984).
[27] R. N. Pinckard, in: Handbook of Experimental Immunology (D. M. Weir, ed.), p. 171, Blackwell Scientific Publications, Oxford, London, Edinburgh, Melbourne 1978.
[28] E. Engvall and P. Perlmann, Immunochem. **8,** 871 (1971).
[29] L. Q. Tam and A. A. Benedict, Proc. Soc. Exp. Biol. Med. **150,** 340 (1975).
[30] F. B. Fishwick, Analyst **110,** 765 (1985).
[31] J. Kjolholt, J. Chromatogr. **325,** 231 (1985).
[32] U. Lösch, I. Schranner, R. Wanke, and L. Jürgens, J. Vet. Med. B **33,** 609 (1986).