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ORIGINAL PAPER

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Characterization of a monoclonal TNT-antibody by measurement of the cross-reactivities of nitroaromatic compounds

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Abstract The characterization of a commercially available monoclonal antibody directed against the explosive 2,4,6-trinitrotoluene (TNT) is reported. The cross-reactivities of various nitroaromatic compounds have been determined by competitive enzyme-linked immunosorbent assay (ELISA). Byproducts and metabolites of TNT were examined as well as the azo dye Disperse Blue 79 and its major metabolites (2-bromo-4,6-dinitroaniline and 2-chloro-4,6-dinitroaniline, respectively). By investigation of the cross-reactivities of different spacer derivatives of TNT it could be demonstrated that the bridge-recognition of the antibody is not very pronounced. N-(2,4,6-Trinitrophenyl)-methylamine shows the highest cross-reactivity (240%) of the examined compounds. Additionally, affinity constants of several nitroaromatic compounds have been determined. The affinity constant of TNT has been calculated to 1.3×10^9 L/mol from the minimal midpoint (IC50 value) of the standard curve. The detection limit achieved for TNT was 0.06 μg/L; the midpoint of the optimized assay was 0.34 μg/L.

Introduction

Explosives and other warfare agents which have been produced and used in large amounts before and during World War II are still sources of water and soil contamination [1]. Particularly the areas of former ammunition plants are contaminated with explosives and by- or breakdown products mainly as a result of improper handling during the manufacturing process [2–4]. 2,4,6-Trinitrotoluene (TNT), the main component of these products serves as an indicator for environmental contamination with nitroaromatic compounds. Rapid, inexpensive, reliable and sensitive methods with high sample throughput for the detection of TNT and related compounds are therefore needed. Analytical methods based on immunological reactions such as immunoassays or immunosensors are well suited for these purposes. Several immunological techniques for detection of free or bound TNT have been published until now [5–12]. One of the most sensitive monoclonal TNT antibodies is commercially available [13, 14]. In this paper, we report on the structure-activity relationship of this antibody. The affinity constant and the cross-reactivities of various structurally related nitroaromatic compounds have been determined by competitive enzyme-linked immunosorbent assay (ELISA). It could be shown that the monoclonal TNT antibody is not only useful for the detection of explosives, but also for other environmental pollutants such as the widely used azo dye Disperse Blue 79. This compound is one of the highest volume dyes manufactured worldwide [15] and its environmental fate has become part of the testing program from the U. S. Environmental Protection Agency EPA [16]. We investigated the cross-reactivities of the bromo/ethoxy analogue of the azo dye and of the toxic and mutagenic synthesis intermediates and degradation products of the bromo- and chloro-analogues of Disperse Blue 79 (2-bromo-4,6-dinitroaniline and 2-chloro-4,6-dinitroaniline).

Besides the study of the cross-reactivities of potential environmental pollutants we have been interested in the bridge-recognition of the antibody. For this purpose we have synthesized a homologous series of 2,4,6-trinitrophenyl-alkylamines and have investigated their cross-reactivities relative to TNT.

Experimental

Abbreviations

AcOH	Acetic acid
EDC	N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride
EtOAc	Ethylacetate
HRP	Horseradish peroxidase

Dedicated to Professor Dr. Karl Cammann on the occasion of his 60th birthday

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NHS	N-Hydroxysuccinimide
PBS	Phosphate buffered saline
RT	Room temperature
TMB	3,3′,5,5′-Tetramethylbenzidine
TNT	2,4,6-Trinitrotoluene

Reagents

2,4-Dinitrotoluene (purity 99.3%) was purchased from Dr. Ehrenstorfer (Augsburg, Germany). 2,4-Dinitroaniline (≥ 99%) was supplied by Fluka (Neu-Ulm, Germany). The following nitroaromatic compounds were purchased from Promochem (Wesel, Germany): 2,4,6-trinitrotoluene, 2-amino-4,6-dinitrotoluene, 4-amino-2,6-dinitrotoluene, 1,3-dinitrobenzene, 2,6-dinitrotoluene (> 99%), N-methyl-N,2,4,6-tetranitroaniline and 1,3,5-trinitrobenzene (> 97%). 2-Amino-4,6-dinitrobenzoic acid was a donation from Dr. Steinbach (University of Marburg, Germany). 2-Bromo-4,6-dinitroaniline and 6-chloro-2,4-dinitroaniline (> 97%) were supplied by Acros (Schwerte, Germany) and 2,2-diphenyl-1-picrylhydrazyl radical hydrate (95%, water content determined by drying at 97 °C for 7 h: 2.4%) from Aldrich (Steinheim, Germany). The (2,4,6-trinitrophenyl)-amino acids were purchased from Research Organics (Cleveland, USA) except for N-(2,4,6-trinitrophenyl)-6-aminohexanoic acid, which was synthesized. The (2,4-dinitrophenyl)-amino acids were supplied from Sigma (Deisenhofen, Germany) except for N-(2,4-dinitro)-4-aminobutanoic acid, which was purchased from ICN Pharmaceuticals (Costa Mesa, USA). Disperse Blue 79 (5′-(bis(2-acetoxyethyl)amino)-2′-(2-bromo-4,6-dinitrophenylazo)-4′-ethoxyacetanilide) was supplied by Sigma-Aldrich Library of Rare Chemicals (Milwaukee, USA). Tween 20 and TMB (3,3′,5,5′-tetramethylbenzidine) were purchased from Merck (Darmstadt, Germany), goat anti-mouse IgG from ICN Pharmaceuticals and horseradish peroxidase (HRP) from Boehringer Mannheim (Germany). Chemicals for the preparation of the buffers were supplied in the highest purity available from Fluka (Neu-Ulm, Germany).

The monoclonal antibody against trinitrotoluene (clone TNT A/1.1.1; stock solution 0.217 g/L) was made available by Strategic Diagnostics Inc., Newark, USA. The antibody belongs to the isotype IgG_1 (mouse) and as immunogen a conjugate of a TNT derivative with keyhole limpet hemocyanin (KLH) was used. Recently we received the information that the hapten for immunization was TNP-glycine (Research Organics, Cleveland, USA), which was conjugated through the carboxyl function to KLH with no additional spacer atoms[1].

Apparatus

Flat bottom polystyrene 96-well immunoplates were purchased from Greiner (Nürtingen, Germany). A Columbus washer for microtiter plates, an Easyshaker EAS 2/4 and a Reader 340 ATTC for microtiter plates controlled by a personal computer containing the standard software package EasySoftware from SLT (Gröding/Salzburg, Austria) were used. All data processing was done with ORIGIN 4.0 (Microcal Software Inc., Northhampton, USA). ^{1}H-NMR spectra were obtained on a Jeol GSX-400 NMR (Akishima, Japan) and are reported in parts per million (δ) relative to $SiMe_4$ (0.00 ppm) as internal reference, with coupling constants (J) reported in Hertz. High-resolution mass spectra were recorded on a Micromass VG Autospec mass spectrometer (Manchester, UK); ion source temperature: 270 °C; ionization energy: 40 eV. Analytical thin-layer chromatography was performed on Merck silica gel 60 F_{254} plates (0.2 mm). Liquid column chromatography was performed using the indicated solvents on Merck silica gel 60.

[1] J. Stave (Strategic Diagnostics Inc.), personal communication

ELISA protocol

Microtiter plates were coated with 250 µL/well of goat anti-mouse IgG diluted 1 : 3,000 in coating buffer (40 mmol/L sodium carbonate, pH 9.6) by incubating overnight at room temperature. Plates were washed three times with washing solution (7 mmol/L phosphate buffered saline (PBS), pH 7.6, containing 15 mmol/L sodium chloride and 0.05% v/v Tween 20). Then the plates were incubated for one hour with 200 µL/well of 0.271 mg/L monoclonal TNT antibody diluted in phosphate buffered saline (PBS, 80 mmol/L sodium phosphate, pH 7.6) containing 8.5 g/L sodium chloride. After washing the plates three times with washing solution, 200 µL of standard solutions were pipetted into the wells and pre-incubated for 30 min. The peroxidase tracer (concentration of HRP ~3 g/L; hapten density ~1 mol/mol) was diluted 1 : 100,000 in PBS (pH 7.6) and 50 µL were added to each well. After a further incubation of 15 min, the plates were washed as described above. 200 µL of a freshly prepared substrate solution (hydrogen peroxide/TMB in citrate buffer, 0.2 mol/L citrate, 0.01% sorbic acid potassium salt, pH 3.8) were added. The enzyme reaction was stopped by adding 100 µL of 5% v/v H_2SO_4, and the absorbance at 450 nm was measured on a microtiter plate reader. Standards were prepared in purified water (Milli-Q plus 185 from Millipore) and their concentration levels ranged from 1,000 to 0.001 or 10,000 to 0.01 µg/L.

Evaluation of standard curves

Each calibration point of standard curves and each sample value was determined by calculation of the median of the data (n = 3 or 4). Standard curves of different nitroaromatic compounds were obtained by fitting calculated medians to a four-parameter function (1).

$$Y = \frac{(A - D)}{\left[1 + \left(\frac{X}{C}\right)^B\right]} + D \qquad (1)$$

X: concentration of analyte [µg/L]
Y: absorbance at 450 nm
A: maximum absorbance (upper asymptote)
D: minimum absorbance (lower asymptote)
C: midpoint (IC_{50} value) [µg/L]
B: slope parameter

The detection limit was defined as concentration of analyte yielding 15% inhibition, which corresponds to a 3s-definition [17] assuming a 5% relative standard deviation of the maximum absorbance. The standard deviations of the midpoints used to determine the errors of the affinity constants and the cross-reactivities were given by the fitting program. The standard deviations of the affinity constants and the cross-reactivities were calculated according to the law of propagation of error from the standard deviations of the involved midpoints.

Determination of cross-reactivities

For the determination of cross-reactivities, each standard concentration was measured at least in triplicate. Cross-reactivities were related to TNT (= 100%), therefore a standard of TNT was included in each microtiter plate and all cross-reactivities were determined in relation to this standard curve. The cross-reactivity was calculated using the analyte concentration yielding 50% inhibition according to formula (2):

$$CR = \left(\frac{C^*}{C}\right) \cdot 100\% \qquad (2)$$

CR: cross-reactivity [%]
C*: concentration of standard at 50% inhibition [mg/L]
C: concentration of cross-reacting analyte at 50% inhibition [µg/L]

For molar cross-reactivities, all concentrations are calculated in mol/L.

Synthesis of N-(2,4,6-trinitrophenyl)-6-aminohexanoic acid

To a well-stirred solution of 336 mg (2.6 mmol) 6-aminocaproic acid in 5 mL 0.1 M borate buffer solution pH 9.2, 500 mg (1.7 mmol) trinitrobenzene sulfonic acid, dissolved in 5 mL water, was added. The solution was stirred at room temperature for 16 h. The mixture was filtered and the precipitate was washed with water and recrystallized from water/ethanol (1 : 1). The product was isolated as yellow crystals (136 mg, 23%). M.p. 143–144 °C; TLC cyclohexane : EtOAc : AcOH = 10 : 5 : 1, R_f = 0.48; ^{1}H NMR: δ (ppm) 1.47 (2*H*, m, C*H*$_2$-CH$_2$-CH$_2$-COOH), 1.67 (2H, ps-qui, J = 7.2 Hz, C*H*$_2$-CH$_2$-COOH), 1.79 (2*H*, ps-qui, J = 7.2 Hz, NH-CH$_2$-*CH*$_2$), 2.39 (2*H*, t, J = 7.2 Hz, C*H*$_2$-COOH), 3.12 (2*H*, dt, J = 7.2 Hz, J_1 = 5.2 Hz, NH-C*H*$_2$), 8.99 (1*H*, br-s, NH), 9.05 (*2H*, s, ar-H).

Synthesis of 2,4,6-trinitroaniline

2,4,6-Trinitrobenzene sulfonic acid (167 mg, 0.57 mmol) was dissolved in 2.5 mL water and the pH was adjusted to 10 with diluted sodium hydroxide. To the well-stirred solution 130 µL (1.73 mmol) ammonium hydroxide solution 25% were added. After 30 min stirring at room temperature, the solution was acidified with 6 mol/L hydrochloric acid to precipitate the product. The mixture was filtered and the precipitate was washed with water and recrystallized from ethanol. The product was isolated as yellow crystals (28 mg, 21%). M.p. 188 °C (188–190 °C [18, p. 763]); TLC cyclohexane : EtOAc : AcOH = 10 : 1 : 1, R_f = 0.18; MS (40 eV, EI): *m/z* (%): 228 (91) [M$^+$], 198 (66) [(M-NO)$^+$], 90 (100) [(M-3NO$_2$)$^+$].

Synthesis of N-(2,4,6-trinitrophenyl)-alkylamine (alkyl: methyl, ethyl, propyl and butyl)

2,4,6-Trinitrobenzene sulfonic acid (400 mg, 1.36 mmol) was dissolved in 5 mL water and the pH was adjusted to 10 with diluted sodium hydroxide. The amine was added to the well-stirred solution in threefold molar excess. After 2 h stirring at room temperature the solution was acidified with 6 mol/L hydrochloric acid to precipitate the product. Then the mixture was filtered, the yellow precipitate was washed several times with water and recrystallized from water/methanol.

N-(2,4,6-Trinitrophenyl)-methylamine: 76 mg (23% yield); yellow crystals; m.p. 114 °C (110–115 °C [19]); TLC cyclohexane : EtOAc : AcOH = 10 : 1 : 1, R_f = 0.20; MS (40 eV, EI): *m/z* (%): 242 (51) [M$^+$], 194 (91), 71 (100); ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) 3.02 (3*H*, d, J = 5.6 Hz, NH-C*H*$_3$), 9.05 (2*H*, s, arom. *H*), 9.14 (1*H*, br s, N*H*).

N-(2,4,6-Trinitrophenyl)-ethylamine: 66 mg, (19% yield); yellow crystals; m.p. 82 °C (81–82 °C [19]); TLC cyclohexane : EtOAc : AcOH = 10 : 1 : 1, R_f = 0.26; MS (40 eV, EI): *m/z* (%): 257 (82) [(M+H)$^+$], 78 (100); ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) 1.42 (3*H*, t, J = 7.2 Hz, CH$_2$-C*H*$_3$), 3.18 (2*H*, dq, J = 7.2 Hz, J_1 = 5.2 Hz, NH-C*H*$_2$-CH$_3$), 8.92 (1*H*, br s, N*H*), 9.04 (2*H*, s, arom. *H*).

N-(2,4,6-Trinitrophenyl)-propylamine: 73 mg (20% yield); yellow crystals; m.p. 56 °C (59 °C [18, p. 764]); TLC cyclohexane : EtOAc : AcOH = 10 : 1 : 1, R_f = 0.36; MS (40 eV, EI): *m/z* (%): 270 (6) [M$^+$], 241 (100) [(M-C$_2$H$_5$)$^+$], 105 (75); ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) 1.05 (3*H*, t, J = 7.2 Hz, CH$_2$-C*H*$_3$), 1.79 (2*H*, tq, J = 7.2 Hz, J_1=7.2 Hz, CH$_2$-C*H*$_2$-CH$_3$), 3.08 (2*H*, dt, J_1 = 7.2 Hz, J_2 = 5.2 Hz, NH-C*H*$_2$-CH$_2$), 9.01 (1*H*, br s, N*H*), 9.04 (2*H*, s, arom. *H*).

N-(2,4,6-Trinitrophenyl)-butylamine: 81 mg (21% yield); yellow crystals; m.p. 80 °C (80.5–81 [20]); TLC cyclohexane : EtOAc : AcOH = 10 : 1 : 1, R_f = 0.38; MS (40 eV, EI): *m/z* (%): 284 (5) [M$^+$], 241 (100) [(M-C$_3$H$_7$)$^+$], 225 (45); ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) 0.97 (3*H*, t, J = 7.2 Hz, CH$_2$-C*H*$_3$), 1.45 (2*H*, ps-sext, J = 7.2 Hz, CH$_2$-C*H*$_3$), 1.73 (2*H*, ps-qui, J = 7.2 Hz, NH-CH$_2$-C*H*$_2$), 3.11 (2*H*, ps-qua, NH-C*H*$_2$); 9.00 (1*H*, s, N*H*); 9.04 (2*H*, s, arom. H).

Synthesis of N-(2,4,6-trinitrophenyl)-pentylamine and N-(2,4,6-trinitrophenyl)-hexylamine

To a mixture of 474 µL (4.09 mmol) pentylamine or 542 µL (4.09 mmol) hexylamine respectively and 5 mL water, glyme was added to dissolve the amine in the aqueous phase. 2,4,6-Trinitrobenzene sulfonic acid (400 mg, 1.36 mmol) was added in portions to the well-stirred solution. After several hours (overnight) stirring at room temperature the mixture was acidified with 6 mol/L hydrochloric acid to precipitate the product. Then the mixture was centrifuged, the yellow precipitate was washed several times with water and recrystallized from water/methanol.

N-(2,4,6-Trinitrophenyl)-pentylamine: 85 mg (21% yield); yellow crystals; m.p. 69 °C; TLC cyclohexane : EtOAc : AcOH = 10 : 1 : 1, R_f = 0.39; MS (40 eV, EI): *m/z* (%): 241 (100) [(M-C$_4$H$_9$)$^+$], 225 (66) [(M-C$_4$H$_9$-O)$^+$]; HRMS calc. (C$_{11}$H$_{14}$N$_4$O$_6$) 298.0913; found 298.1024; ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) 0.93 (3*H*, t, J = 7.2 Hz, CH$_2$-C*H*$_3$), 1.36–1.39 (4*H*, m, CH$_2$-C*H*$_2$-CH$_3$), 1.75 (2*H*, ps-qui, J_1 = 7.2 Hz, NH-CH$_2$-C*H*$_2$), 3.10 (2*H*, dt, J_1 = 6.8 Hz, J_2 = 5.2 Hz, NH-C*H*$_2$), 9.00 (1*H*, br s, N*H*), 9.04 (2*H*, s, arom. *H*).

N-(2,4,6-Trinitrophenyl)-hexylamine: 75 mg (17% yield); yellow crystals; m.p. 66 °C (70 °C [18, p. 764]); TLC cyclohexane : EtOAc : AcOH = 10 : 1 : 1, R_f = 0.49; MS (40 eV, EI): *m/z* (%): 241 (100) [(M-C$_5$H$_{11}$)$^+$], 225 (88) [(M-C$_5$H$_{11}$-O)$^+$]; ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) 0.90 (3*H*, t, J = 6.8 Hz, CH$_2$-C*H*$_3$), 1.30–1.33 (4*H*, m, C*H*$_2$-C*H*$_2$-CH$_3$), 1.37–1.44 (2*H*, m, NH-CH$_2$-CH$_2$-C*H*$_2$), 1.74 (2*H*, ps-qui, J = 7.2 Hz, NH-CH$_2$-C*H*$_2$), 3.10 (2*H*, dt, J = 6.8 Hz, J_1 = 5.6 Hz, NH-C*H*$_2$), 9.00 (1*H*, br s, N*H*), 9.04 (2*H*, s, arom. *H*).

Synthesis of 2-(2,4,6-trinitrophenyl)-ethanol

A mixture of 1 g (4.40 mmol) TNT, 0.01 g (0.09 mmol) sodium carbonate and 0.46 mL (6.66 mmol) aqueous formaldehyde solution (37%) was heated to 90–100 °C. At 70 °C the mixture liquefied and the color became dark red. After 90 min stirring at 90–100 °C 50 mL water were added. Then the solution was decanted, freed from solvent and redissolved in 3 mL ethanol. Silica gel chromatography with cyclohexane/ethyl acetate (3:1) as eluent resulted in 348 mg (31%) of the product in form of light yellow crystals. M.p. 112 °C (112 °C [21]); TLC cyclohexane : EtOAc = 3 : 1, R_f = 0.39; ^{1}H NMR (400 MHz, CD_3OD): δ (ppm) 3.34 (2*H*, t, J = 6.2 Hz, C*H*$_2$-CH$_2$-OH), 3.80–3.83 (2*H*, t, J = 6.2 Hz, CH$_2$-C*H*$_2$-OH), 8.93 (2*H*, s, arom. *H*).

Synthesis of N-(2,4,6-trinitrophenyl)-glycinamide

To a well-stirred solution of 500 mg (1.44 mmol) trinitrobenzene sulfonic acid in 6 mL 0.1 M borate buffer solution pH 9.2, 160 mg (1.44 mmol) glycinamide, dissolved in 1 mL 0.1 M borate buffer solution pH 9.2, were added. After 4 h stirring at RT the mixture was centrifuged, the yellow precipitate was washed several times with water and recrystallised from acetonitrile/water. The product was isolated as yellow crystals (approx. 100 mg, 24%). M.p. 208–209 °C; TLC cyclohexane : EtOAc = 3 : 10, R_f = 0.59; MS (40 eV, EI): *m/z* (%): 241 (100) [(M-CONH$_2$)$^+$]; HRMS calc. (C$_8$H$_7$N$_5$O$_7$) 285.0345; found 285.0242; ^{1}H NMR: δ (ppm) 3.71 (2*H*, d, J = 4.2 Hz, C*H*$_2$-CONH$_2$), 7.56 (1*H*, s, -CON*H*$_2$), 7.81 (1*H*, s, -CONH$_2$), 8.97 (2H, s, ar-*H*), 9.74 (1H, t, J = 4.2 Hz, N*H*).

Synthesis of the trinitrophenyl enzyme tracer

30 mg (88 µmol) of N-(2,4,6-trinitrophenyl)-6-aminohexanoic acid and 13 mg (111 µmol) of NHS were dissolved in 1 mL of dry glyme. 50 mg (352 µmol) of sodium sulfate were added and the mixture was cooled to 0 °C. 34 mg (177 µmol) EDC were added, the reaction mixture was allowed to warm to RT and stirred overnight. 10 mg of horseradish peroxidase were dissolved in 1 mL of borate buffer pH 8 (Merck). In intervals of 30 min, three portions

of the NHS-ester solution (62 µL) were given slowly to the reaction mixture under intense stirring. The enzyme tracer was centrifuged and purified by gel chromatography (Sephadex G-25 M).

Results and discussion

Standard curve and affinity constant

Figure 1 shows the optimized calibration curve of the monoclonal TNT antibody obtained for TNT. In the diagram, the absorbance is plotted against the log of the TNT concentration (µg/L). The error bars correspond to the range of the threefold determination (approx. 2 s). The detection limit for TNT was estimated to be about 60 ng/L and the midpoint was determined to 340 ng/L.

According to Weller [22], the direct determination of affinity constants by competitive ELISA is possible, if the concentrations of tracer and antibody are infinitesimally low. In this case the midpoint of the standard curve reaches a minimum, which corresponds to the reciprocal affinity constant. Figure 2 shows a numerical simulation for an affinity constant of 10^8 L/mol.

As shown in the figure, the midpoint converges towards a minimum for low values of the tracer concentration. Simultaneously the relative signal intensity (maximum absorbance A minus minimum absorbance D) decreases with decreasing tracer concentration. Similar curves were found for the dependence of the midpoint or the assay signal, respectively, from the antibody dilution. In practice the minimum of the midpoint is achieved and the affinity constant can directly be determined, if the value of the midpoint does not change, when antibody and tracer concentrations are lowered. Figure 3 shows the standard curves obtained by using two different antibody concentrations and two different tracer concentrations.

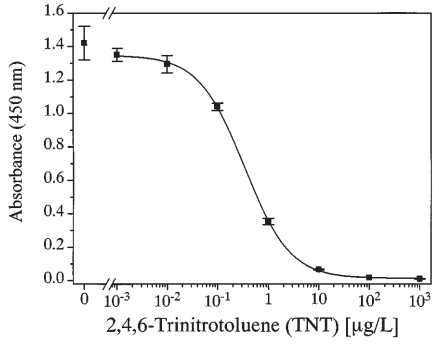

Fig. 1 Standard curve obtained with a monoclonal TNT antibody (error bars: range of threefold determination, approx. 2 s, A = 1.35 ± 0.01, B = 0.98 ± 0.07, C = 0.34 ± 0.01, D = 0.013 ± 0.006). For experimental conditions see section "ELISA protocol"

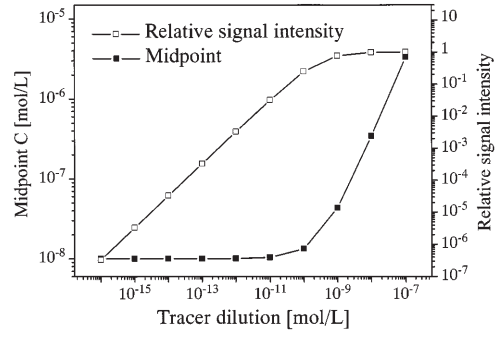

Fig. 2 Simulation of the midpoint and of the signal intensity in dependence of the tracer dilution (relative signal intensity of 1 corresponds to 3×10^{-15} mol/L antibody-tracer complex; antibody dilution: 10^{-14} mol/L; tracer: $k_1 = 10^7$ L/mol · s, $k_{-1} = 10^{-2}$ 1/s; analyte: $k_2 = 10^7$ L/mol · s, $k_{-2} = 10^{-1}$ 1/s; details in [23]). For an affinity constant of 10^8 L/mol the midpoint C converges to 10^{-8} mol/L

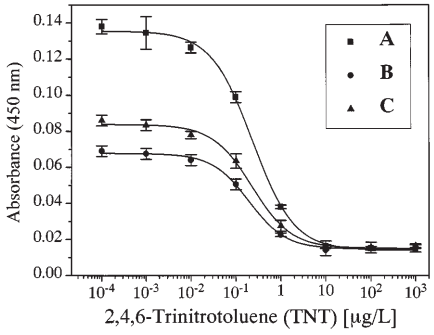

Fig. 3 TNT standard curves obtained with a monoclonal TNT antibody with different antibody and tracer dilutions (development: 30 min; error bars: range of the fourfold determination, approx. 2 s). **A:** antibody dilution 1:15,000, tracer dilution 1:400,000, midpoint 0.23 ± 0.02 µg/L; **B:** antibody dilution 1:30,000, tracer dilution 1:400,000, midpoint 0.19 ± 0.02 µg/L; **C:** antibody dilution 1:15,000, tracer dilution 1:800,000, midpoint 0.22 ± 0.03 µg/L

While the midpoint does not change significantly at lower antibody or tracer concentrations, respectively, the assay signal decreases as predicted in the simulation. Therefore, the minimum value of the midpoint has been reached and the affinity constant can be determined to $(1.3 \pm 0.1) \times 10^9$ L/mol after a volume correction due to sample dilution by tracer.

Specificity

The antibody was examined for its reaction with other nitroaromatic compounds with environmental relevance, such as explosives or the azo dye Disperse Blue 79 (Fig. 4) and their major metabolites. Midpoints and detection limits are listed in Table 1. Table 2 contains the cross-reactivities and the affinity constants obtained by multiplication of the cross-reactivities with the affinity constant determined for TNT. As shown by the midpoints and the cross-reactivities, structurally related compounds with three nitro groups, as 1,3,5-trinitrobenzene and tetryl (N-methyl-2,4,6,N-tetranitroaniline), show high cross-reactivities. Aromatics with two nitro groups have intermediate to very low cross-reaction. The formal loss of one nitro group results in a decrease of the affinity constant of two or more decades. An exception are dinitroaromatic compounds, which possess a halogen atom instead of the third nitro group, like 2-chloro-4,6-dinitroaniline or 2-bromo-4,6-dinitroaniline. These compounds show quite high cross-reactivities.

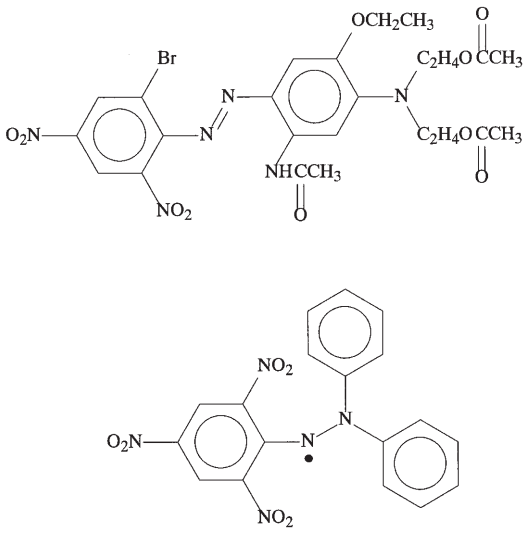

Fig. 4 Disperse Blue 79 (5′-(bis(2-acetoxyethyl)amino)-2′-(2-bromo-4,6-dinitrophenylazo)-4′-ethoxyacetanilide) and 2,2-diphenyl-1-picrylhydrazyl radical, nitroaromatic compounds with bulky substituents replacing the methyl moiety of TNT

In order to study the effect of structural variation of the methyl group of 2,4,6-trinitrotoluene on the affinity to the antibody, cross-reactivities of different trinitro- and dinitrophenyl derivatives have been examined. Based on the former assumption that the TNT antibody was raised against a 2,4,6-trinitrophenyl derivative coupled *via* spacer to a carrier protein, the compounds listed in Table 3 can be seen as spacer derivatives of the immunogen. A classification of the compounds according to the presence of heteroatoms, the length of the spacer and the presence of charged or bulky groups seems convenient to study the spacer recognition (bridge recognition).

Figure 5 shows an arrangement of TNT-derivatives without spacer (1,3,5-trinitrobenzene) or with a spacer length of formally one atom, which is attached to position one of the 2,4,6-trinitrophenyl ring. A comparison of the molar cross-reactivities of these compounds (Table 3) demonstrates that picric acid has the lowest cross-reactivity even in relation to the compound without spacer, 1,3,5-trinitrobenzene. This can be explained with the negative charge due to the high acidity of picric acid (pk_a = 1.02

Table 1 Midpoints (IC_{50}) and detection limits

Analyte	Midpoint ± s [µg/L]	Detection limit [µg/L]
2,4,6-Trinitrotoluene (TNT)	0.34 ± 0.01	0.06
2-Chloro-4,6-dinitroaniline	0.84 ± 0.12	0.12
2-Bromo-4,6-dinitroaniline	0.86 ± 0.04	0.13
Tetryl	1.3 ± 0.1	0.2
1,3,5-Trinitrobenzene	2.8 ± 0.3	0.4
2,4-Dinitroaniline	5.6 ± 0.6	0.8
2-Amino-4,6-dinitrotoluene	8.0 ± 1.6	0.6
2,4-Dinitrotoluene	22 ± 2	3
3,5-Dinitroaniline	38 ± 6	4
1,3-Dinitrobenzene	110 ± 10	10
2-Amino-4,6-dinitrobenzoic acid	120 ± 10	20
Disperse Blue 79[a]	160 ± 40	20
2,6-Dinitrotoluene	260 ± 30	30
4-Amino-2,6-dinitrotoluene	490 ± 90	80

[a] Due to the low water solubility of Disperse Blue 79 (4.2–4.6 µg/L [15]) the stock solution was prepared in DMSO (0.01 g/L) and diluted 1:10 in purified water to obtain the standard with the highest concentration (1,000 µg/L)

Table 2 Cross-reactivities[a, b] and affinity constants K

Analyte	Cross-reactivity [%]	Cross-reactivity [%] (molar)	K ± s [L/mol]
2,4,6-Trinitrotoluene (TNT)	100	100	$(1.3 \pm 0.1) \times 10^9$
2-Bromo-4,6-dinitroaniline	37 ± 6	43 ± 7	$(5.6 \pm 0.9) \times 10^8$
2-Chloro-4,6-dinitroaniline	42 ± 12	40 ± 11	$(5.2 \pm 1.4) \times 10^8$
Tetryl	22 ± 2	28 ± 3	$(3.7 \pm 0.4) \times 10^8$
1,3,5-Trinitrobenzene	17 ± 6	16 ± 6	$(2.1 \pm 1.0) \times 10^8$
2-Amino-4,6-dinitrotoluene	6.4 ± 1.9	5.6 ± 1.6	$(7.3 \pm 2.1) \times 10^7$
2,4-Dinitroaniline	6.3 ± 1.1	5.1 ± 0.9	$(6.7 \pm 1.2) \times 10^7$
2,4-Dinitrotoluene	2.1 ± 0.4	1.7 ± 0.3	$(2.2 \pm 0.4) \times 10^7$
3,5-Dinitroaniline	1.4 ± 0.3	1.1 ± 0.2	$(1.4 \pm 0.3) \times 10^7$
2-Amino-4,6-dinitrobenzoic acid	0.83 ± 0.16	0.83 ± 0.16	$(1.1 \pm 0.3) \times 10^7$
Disperse Blue 79	0.20 ± 0.08	0.56 ± 0.23	$(7.3 \pm 3.0) \times 10^6$
1,3-Dinitrobenzene	0.45 ± 0.11	0.33 ± 0.08	$(4.3 \pm 1.0) \times 10^6$
2,6-Dinitrotoluene	0.18 ± 0.04	0.14 ± 0.03	$(1.8 \pm 0.4) \times 10^6$
4-Amino-2,6-dinitrotoluene	0.10 ± 0.03	0.09 ± 0.03	$(1.1 \pm 0.4) \times 10^6$

[a] 2,4-Dinitrophenol, 2-methyl-5-nitroaniline, 2-methyl-3-nitroaniline, 4-amino-2-nitrotoluene, hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX), 1,3,5,7-tetranitro-1,3,5,7-tetraazocyclooctane (HMX), nitrobenzene, 2-nitrotoluene, 4-nitrotoluene and toluene have cross-reactivities below 0.1% [8]

[b] As the midpoint differs slightly from assay to assay, standards of TNT were included in each plate and all cross-reactivities were determined in relation to this standard curve. Therefore, the cross-reactivities should not be calculated from Table 1

Table 3 Cross-reactivities of spacer derivatives

Compound	Cross-reactivity [%]	Cross-reactivity [%] (molar)
2,4,6-Trinitrotoluene (TNT)	100	100
N-(2,4,6-Trinitrophenyl)-methylamine	240 ± 30	260 ± 30
N-(2,4,6-Trinitrophenyl)-ethanoic acid amide	150 ± 20	200 ± 30
N-(2,4,6-Trinitrophenyl)-ethylamine	170 ± 30	190 ± 30
2,4,6-Trinitroaniline	184 ± 8	180 ± 10
N-(2,4,6-Trinitrophenyl)-butylamine	108 ± 8	140 ± 10
N-(2,4,6-Trinitrophenyl)-propylamine	110 ± 20	130 ± 30
N-(2,4,6-Trinitrophenyl)-hexylamine	79 ± 6	110 ± 10
N-(2,4,6-Trinitrophenyl)-pentylamine	73 ± 18	100 ± 20
2-(2,4,6-Trinitrophenyl)-ethyl alcohol	75 ± 6	85 ± 7
N-(2,4,6-Trinitrophenyl)-6-aminohexanoic acid	47 ± 5	71 ± 7
N-(2,4,6-Trinitrophenyl)-4-aminobutanoic acid	43 ± 6	59 ± 8
N-(2,4,6-Trinitrophenyl)-aminoethanoic acid	19 ± 4	24 ± 5
N-(2,4,6-Trinitrophenyl)-3-aminopropanoic acid	6.2 ± 1.3	8.2 ± 1.7
2,2-Diphenyl-1-picrylhydrazyl radical	2.9 ± 0.5	5.0 ± 0.9
N-(2,4-Dinitrophenyl)-ethylamine	4.1 ± 0.5	3.8 ± 0.5
N-(2,4-Dinitrophenyl)-6–aminohexanoic acid	2.4 ± 0.5	3.2 ± 0.7
N-(2,4-Dinitrophenyl)-methylamine	3.1 ± 0.5	2.7 ± 0.4
2,4,6-Trinitrophenol (Picric acid)	2.6 ± 0.2	2.6 ± 0.2
N-(2,4-Dinitrophenyl)-3-aminopropanoic acid	2.2 ± 0.4	2.4 ± 0.5
N-(2,4-Dinitrophenyl)-4–aminobutanoic acid	1.8 ± 0.5	2.2 ± 0.5
N-(2,4-Dinitrophenyl)-2-aminoethanoic acid	0.23 ± 0.03	0.24 ± 0.03

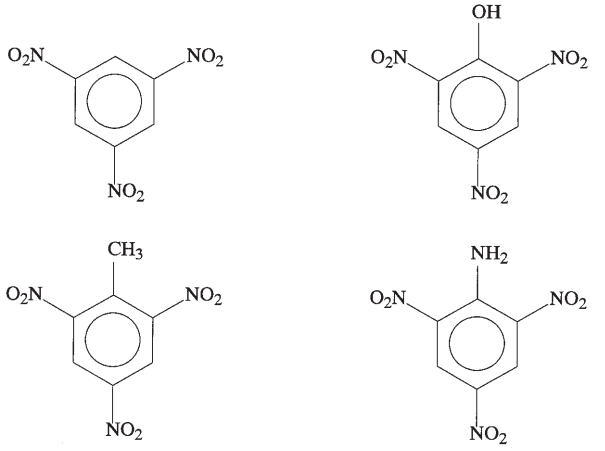

Fig. 5 TNT and derivatives with different functional groups on position 1

[23]), which influences the recognition by the antibody. A comparison of the molar cross-reactivities of 1,3,5-trinitrobenzene with TNT leads to the conclusion that the antibody binds to the methyl group. A further improvement of the affinity can be achieved by introducing an amino group instead of the methyl group. 2,4,6-Trinitroaniline has nearly twice the molar cross-reactivity of TNT, which indicated that a nitrogen-containing linkage was used for immunization.

A systematic study of the spacer recognition can provide information about the dimensions of the antibody binding site. Figure 6 shows TNT-derivatives with spacers of increasing lengths. It can be expected that the affinity and therefore the cross-reactivity increases with increasing length of the chain until the end of the antibody binding site is reached. A further extension of the chain-length should not produce a strong change of the cross-reactivity. The variation of the molar cross-reactivity with the spacer length is shown in Fig. 7. The shape of the curve exhibits a maximum at a spacer length of two formal atoms ((2,4,6-trinitrophenyl)-methylamine). The molar cross-reactivities of TNT-derivatives with longer spacers are lower and approach the cross-reactivity of TNT. This result leads to the conclusion that the TNT antibody recognizes only spacer lengths of formal two atoms. Considering the bulky substituent containing two phenyl groups, 2,2-diphenyl-1-picrylhydrazyl radical (Fig. 4) shows a relatively high molar cross-reactivity of about 5%, which also supports the assumption of low spacer recognition.

In Fig. 8 spacer derivatives with a formal chain length of three or four atoms are shown. A comparision of the molar cross-reactivities of these compounds (Table 3) shows that N-(2,4,6-trinitrophenyl)-ethylamine and N-(2,4,6-trinitrophenyl)-aminoethanoic acid amide have similar and relatively high cross-reactivities. Considering the glycine amide (aminoethanoic acid amide) structure, which was used for immunization, it is surprising that this compound does not show even a higher cross-reactivity. The replacement of the sp^3-hybridized carbon atom on the end of the spacer with a sp^2-hybridized atom has no influence on the affinity towards the antibody. This is in agreement with the assumed recognition of only two spacer atoms by the antibody.

In contrast to the cross-reactivities of the mentioned compounds, N-(2,4,6-trinitrophenyl)-ethanol and N-(2,4,6-trinitrophenyl)-aminoethanoic acid have lower cross-reactivities than TNT. The difference of the molar cross-reac-

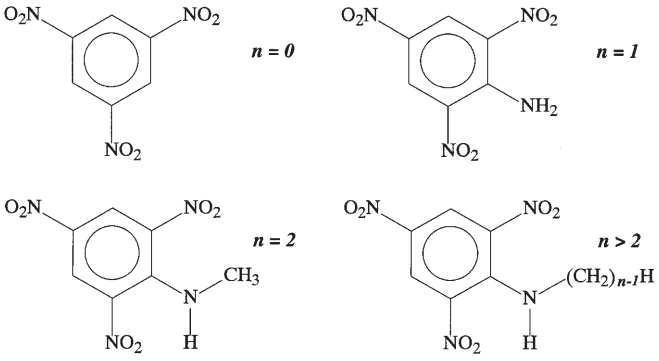

Fig. 6 Homologous series of spacer derivatives of 2,4,6-trinitrobenzene

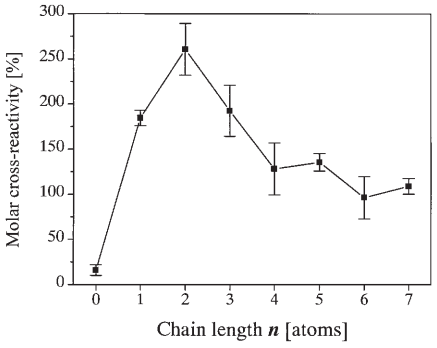

Fig. 7 Molar cross-reactivities of the homologous series of alkyl amine derivatives of 2,4,6-trinitrobenzene; error bars: calculated according to the law of propagation of error from the standard deviations of the midpoints of the standard curves; chain length *n* see Fig. 6

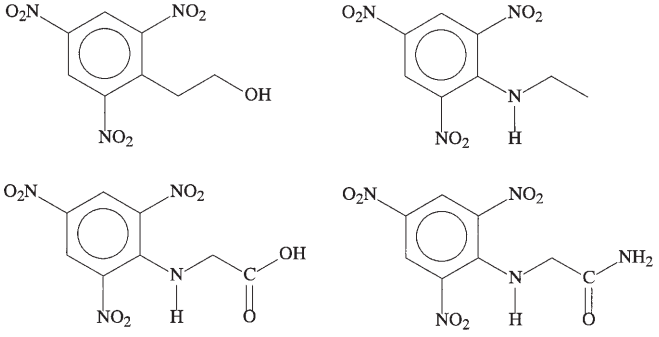

Fig. 8 Derivatives of TNT with spacer length of formal three or four atoms. 2-(2,4,6-Trinitrophenyl)-ethanol, N-(2,4,6-trinitrophenyl)-ethylamine, N-(2,4,6-trinitrophenyl)-aminoethanoic acid, N-(2,4,6-trinitrophenyl)-aminoethanoic acid amide

tivities between N-(2,4,6-trinitrophenyl)-ethylamine, a nitrogen-containing spacer derivative of TNT, and 2-(2,4,6-trinitrophenyl)-ethanol, which has a carbon spacer terminated by a hydroxyl group, is very similar to the difference of the molar cross-reactivities between 2,4,6-trinitroaniline and TNT. The difference can be mainly attributed to the replacement of the NH moiety with a CH_2 moiety. The difference between the cross-reactivities of N-(2,4,6-trinitrophenyl)-aminoethanoic acid and the corresponding amide can be attributed to the negative charge of the acid anion. The influence of the negative charge is noticeable even at a spacer length of seven atoms as the molar cross-reactivity of N-(2,4,6-trinitrophenyl)-aminohexanoic acid demonstrates (Table 3), but the difference between the molar cross-reactivities of the alkylamine and the corresponding ω-aminocarboxylic acid spacer derivatives of TNT decreases with increasing spacer length.

Conclusion

We report on the structural characterization of the antigen binding-site of a monoclonal TNT antibody by investigating the cross-reactivity of various structurally related nitroaromatic compounds. The cross-reactivity pattern can serve for the identification of an antibody as shown by Winklmair et al. [24]. In general, aromatic compounds with three nitro groups are well recognized, if there are no negatively charged substituents replacing the methyl group of TNT. Derivatives with short aminoalkyl residues at position 1 of the aromatic ring show cross-reactivities above 100%. Nevertheless, bridge recognition of the antibody is not very pronounced, N-(2,4,6-trinitrophenyl)-methylamine having the highest measured cross-reactivity of 240%. As N-(2,4,6-trinitrophenyl)-aminoethanoic acid amide, which is homologous to the immunogen structure, has no higher cross-reactivity as the methylamine derivative, the antibody recognition seems to end after the NH-CH_2-moiety. Only charged compounds have a long distance influence.

In addition to the trinitroaromatic compounds, substances with a bromine or a chlorine instead of a nitro group show remarkable high cross-reactivities and low detection limits, such as 2-bromo-4,6-dinitroaniline with a detection limit below 0.2 µg/L. These findings offer new applications for the antibody in the environmental analysis of metabolites of the azo dye Disperse Blue 79.

The affinity constant of TNT was determined to 1.3×10^9 L/mol by dilution of antibody and tracer. To our knowledge, this antibody is the most sensitive monoclonal TNT antibody at present time.

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