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Preparation of a Polyclonal Antibody and a Bioassay for Nitroaromatic Compounds by an Enzyme-Linked Immunosorbent Assay Technique and a Surface Plasmon Resonance Biosensor

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Sensitive and selective detection of 2, 4, 6-trinitrophenyl derivatives (TNP derivatives) based on surface plasmon resonance (SPR) was performed using polyclonal anti-TNP antibody and *N*-(TNP)-ovalbumin (TNP-OVA) conjugate. TNP-bovine serum albumin (TNP-BSA) conjugate was injected into the mice, and polyclonal anti-TNP antibody was gained after purification of the sera using protein G. TNP-OVA was immobilized on the Au film of the SPR sensor chip by physical adsorption. The incidence angle shift of the TNP-OVA immobilized sensor increased steeply with increasing concentration of anti-TNP antibody up to 20 μg/ml and increased only slightly above this concentration. The additions of TNP derivatives into the anti-TNP antibody solution were found to decrease the incidence angle shift because of the inhibition effects of TNP derivatives. The lowest detection limit for trinitrotoluene (TNT) by SPR was 1×10^{-7} g/ml, whereas for TNP-6-aminohexanoic acid (TNP-aha) it was 3×10^{-9} g/ml. Evaluations of affinity constants of anti-TNP antibody were performed. Analyses of SPR data were carried out by assumptions of the Langmuir isotherm and equilibrium state of immunoreaction. The value of association constant between anti-TNP antibody and immobilized TNP-OVA (K_1) was 6.4×10^6 M^{-1}. The values of association constants between antibody and TNP-aha (K_2) were 2.7 and 8.5×10^6 M^{-1} when 20 and 10 μg/ml antibody were used, respectively.

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1. Introduction

There are more than 100 million landmines buried in the world, and three million landmines are newly buried every year. It is a serious problem that antipersonnel landmines cause extensive damage to more than twenty thousand people a year. However, only 100 thousand landmines are removed a year.[1]

It is very difficult to detect the buried landmines. For their detection, therefore, dogs with special training are used. Dogs, however, cannot concentrate on detection for more than two hours.[2] Thus, it is very important to develop a novel technology for detecting landmines effectively. In recent years, various kinds of detection technologies based on physical sensors have been developed, such as ground penetrating radar (GPR);[3,4] however, it is difficult to detect landmines using only a physical sensor.

TNT detection methods using fluorescence quenching, capillary electrochromatography and HPLC were reported,[5–7] and detection methods using the antigen-antibody reaction such as enzyme-linked immunosorbent assay (ELISA), fluorescent and chemiluminescent immunosensors are more useful and are able to detect ppb levels of TNT.[8–10] These methods using the antigen-antibody reactions are sensitive, but are not suitable for on-site detection where the landmines are burried.

On the other hand, a surface plasmon resonance (SPR) sensor is small and portable, hence suitable for on-site detection. One of the most sensitive and promising sensors for detecting landmines is an immunosensor using a portable SPR sensor.[11,12] It might be possible to achieve high sensitive on-site detection of TNT and its related compounds using this method.

In the present study, we prepared a ploy clonal antibody for trinitrophenyl derivatives and applied the antibody to detect trinitrophenyl derivatives using ELISA and SPR.

2. Experimental

2.1 *Materials*

Alkaline phosphatase (ALP) and horseradish peroxidase (HRP) were obtained from Roche Diagnostics Japan, Co., Ltd. (Tokyo, Japan). Bovine serum albumin (BSA), *p*-nitrophenyl phosphate disodium salt (*p*-NPP), 2, 4, 6-trinitrobenzene sulfonate sodium salt (TNBS) and gelatin were obtained from Nacalai Tesque, Inc. (Kyoto, Japan). Ovalbumin (OVA), ALP labeled anti-mouse IgG, 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide (EDC), methylamine, ethylamine and butylamine were obtained from Sigma (St. Louis, MO, USA). 2, 4, 6-Trinitrophenyl-BSA conjugate (TNP-BSA) and trinitrophenyl-glycine (TNP-gly) were obtained from Cosmo Bio, Co., Ltd. (No. LG-1117, Tokyo, Japan) and Research Organics (Cleveland. OH., USA), respectively. 2, 4, 6-Trinitrotoluene (TNT) was supplied by Chugoku Kayaku, Co., Ltd as a 10.7 ppm aqueous solution. 2, 4, 6-Trinitrophenol (TNP-OH) and 1, 3-dinitrobenzene (DNB) were purchased from Kishida Chemical Co., Ltd (Osaka, Japan). 2, 4-Dinitrotoluene (DNT) was obtained from Wako Pure Chemicals Ind., Ltd. (Osaka, Japan). 2-amino-4, 6-dinitrotoluene (2-amino-DNT) and 4-amino-2 ,6-dinitrotoluene (4-amino-DNT) were purchased from Supelco (PA, USA). Freund's complete adjuvant was obtained from Difco. BALB/c mice (6 weeks old,

female) were purchased from Charles River. All other reagents were of analytical-reagent grade. All buffer solutions were prepared using water purified with a Milli-Q (Millipore, Bedford) system.

2.2 *Apparatus*

SPR measurements were performed using the Spreeta™ evaluation module kit (Texas Instruments, Inc., Texas, USA) attached to a sensor chip, a flow-through cell (18×2×0.2 mm) and a microtube pump (EYELA, SMP-23, Tokyo, Japan). ELISA measurements were performed using 96-well immunoplates (NUNC, No. 446612, Roskilde, Denmark) and a microplate reader (Spectra 1, Wako, Osaka, Japan). Spectrophotometric measurements were performed by the use of Shimadzu Multi Spec 1500 (Kyoto, Japan).

2.3 *Preparation of TNP-OVA conjugate*

TNBS (1 mg/ml H_2O) was reacted with 1 ml of 480 mM $NaHCO_3$ solution (pH 8.5) containing 10 mg OVA for 2 h at 40°C. After the reaction, the preparation was dialyzed against five changes of H_2O at 4°C for 3 days, and then lyophilized to produce TNP-OVA conjugate (9.4 mg yield). The molar ratio of the combined TNP moieties on OVA was estimated as approximately 7.2 mol-TNP/mol-OVA using the molar absorption coefficient of TNP-NH- (1.1×10^4).[13]

2.4 *Immunization*

BALB/c mice were immunized with TNP-BSA conjugate according to the following procedure. Conjugate dissolved in PBS (1 mg/ml) was emulsified with an equal volume of Freund's complete adjuvant. On days 0, 14, 28 and 42, 200 μl of the prepared mixture was intraabdominally injected into the mice. On days 7, 21, 28, 35 and 42, the mice were bled from the caudal veins, and the antisera were collected by centrifugation of the blood samples. The antisera were tested by direct enzyme immunosorbent assay (direct-ELISA). 96-well immunoplates were coated with 100 μl of TNP-OVA conjugate (10 μg/ml in 50 mM carbonate buffer, pH 9.6) overnight at room temperature. The following day, the plates were washed three times with PBS containing 0.05% Tween 20 (PBST), and treated with 150 μl of 1% gelatin for 1 h at room temperature. The plates were washed three times with PBST and reacted with antisera at nine different dilutions (1/8 to 1/1024 diluted with PBS) which were added to the wells (50 μl/well) and incubated for 2 h at room temperature. The plates were washed again three times with PBST, and a solution of ALP labeled anti-mouse IgG (1000-fold diluted with PBS) was added (100 μl/well) and incubated for 1 h at room temperature. The plates were washed again, and the substrate solution (2 mg/ml of *p*-NPP in 50 mM carbonate buffer, pH 9.8 containing 1 mM $MgCl_2$ and 0.1 mM $ZnCl_2$) was added and incubated for 30 min at room temperature. Then absorbance at 405 nm was measured.

2.5 *Preparation and purification of polyclonal antibody (anti-TNP antibody)*

On day 50, the mice were finally immunized intravenously *via* the tail vein with a final dose of 50 μg of TNP-BSA conjugate in 50 μl of PBS. The mice were bled through their aorta 3 days after the last injection. To the sera prepared from their whole blood, 0.2 M

phosphate buffer (pH 7.0) was added (one-tenth volume of the sera). An equal volume of saturated ammonium sulfate was added to the mixture and stirred for 1 h at 4°C. The mixture was centrifuged at 10000 *g* for 20 min and the precipitate was re-dissolved in a small amount of 50% saturated ammonium sulfate solution. After centrifugation (10000 *g*, 10 min), the precipitate was dissolved with 2 ml of 20 mM phosphate buffer (pH 7.0), and the mixture was filtered with a 0.45 μm-membrane filter (mixed cellulose ester type, ADVANTEC, Tokyo, Japan). The filtrate containing antisera was passed through Hi Trap Protein G column (Amersham Bioscience Corp., New Jersey, USA) pre-equilibrated with 20 mM phosphate buffer (pH 7.0) at a flow rate of 1 ml/min, and then rinsed with 5 ml of 20 mM phosphate buffer. The antisera were eluted with 5 ml of 0.1 M glycine-HCl buffer (pH 2.7) and 0.5 ml/fraction of the eluates were collected in the test tubes, which contained 50 μl of 1.0 M Tris-HCl buffer (pH 9.0). After the measurements of absorbance at 280 nm, the eluates with high absorbance were treated with Econo-Pac column (Bio-Rad Laboratories, California, USA) in order to exchange the buffer to PBS. For the affinity purification, EDC/diaminodipropylamine immobilization kit (Pierce, IL, USA) was used. Purification procedures were performed according to the method proposed by the manufacturer, except when using TNP-gly as a ligand. The eluates from Protein G treatment were loaded on the column of the kit, and the bound antibody was eluted with glycine-HCl (pH 2.5), and 1 ml/fractions of the eluates were collected in the test tubes, which contained 50 μl of 1.0 M Tris-HCl buffer (pH 9.5). After the measurements of absorbance at 280 nm, the eluates with high absorbance were treated with Econo-Pac columns as before.

2.6 *Syntheses of TNP-derivatives*

Syntheses of TNP-derivatives were performed according to the method reported by Zeck *et al.*[14] The outlines are shown below.

Synthesis of *N*-(2, 4, 6-trinitrophenyl)-6-aminohexanoic acid (TNP-aha): To a well-stirred solution of 336 mg 6-aminocaproic acid in 5 ml of 0.1 M borate buffer (pH 9.2), 500 mg TNBS 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.

Synthesis of 2, 4, 6-trinitroaniline (TNA): TNBS (167 mg) was dissolved in 2.5 ml water and the pH was adjusted to 10 with dilute sodium hydroxide. To the well-stirred solution, 130 μl of ammonium hydroxide solution (25%) was added. After 30 min of stirring at room temperature, the solution was acidified with 6 M 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.

Synthesis of *N*-(2, 4, 6-trinitrophenyl)-alkylamine: TNBS (400 mg) was dissolved in 5 ml water and the pH was adjusted to 10 with dilute sodium hydroxide. The amine was added to the well-stirred solution in three-fold molar excess. After 2 h stirring at room temperature, the solution was acidified with 6 M hydrochloric acid to precipitate the product. Then the mixture was filtered, and the yellow precipitate was washed several times with water and recrystallized from water/methanol. *N*-(2, 4, 6-trinirophenyl)-methylamine, *N*-(2, 4, 6-trinitrophenyl)-ethylamine and *N*-(2, 4, 6-trinitrophenyl)-butylamine are, hereafter, abbreviated as TNP-ma, TNP-ea, and TNP-ba, respectively.

2.7 *Indirect competitive ELISAs for TNP-derivatives*

ELISAs for TNP-derivatives were performed as follows. 96-well immunoplates were coated with 100 μl of TNP-OVA conjugate (10 μg/ml) in 50 mM carbonate buffer (pH 9.8) overnight at room temperature. The following day, the plates were washed three times with PBST and treated with 1% gelatin for 1 h at room temperature. The plates were washed three times with PBST and reacted with 100 μl of the equivalent mixtures of anti-TNP antibody (10 μg/ml) and serial diluted TNP-derivative antigens for 1 h at room temperature. The plates were washed three times with PBST, and then reacted with 100 μl of ALP labeled anti-mouse IgG (1000-fold diluted with PBS) for 1 h at room temperature. After washing three times with PBST, the substrate solution (2 mg/ml *p*-NPP in 50 mM carbonate buffer, pH 9.8, containing 1 mM $MgCl_2$ and 0.1 mM $ZnCl_2$) was added to each well and incubated for 30 min at room temperature. The absorbance at 405 nm was measured using a microplate reader.

2.8 *Association properties of anti-TNP antibody to various kinds of nitroaromatic compounds*

The association properties of the raised antibody to nitroaromatic compounds were investigated by indirect competitive ELISAs. Each inhibition curve was measured at least in triplicate. The value of IC_{50} was defined as the concentration of the added TNT derivative, which yields 50% inhibition compared with no inhibition (100%). Molar cross-reactivities were related to TNT (=100%); namely all molar cross-reactivities were determined in relation to the TNT standard inhibition curve. The molar cross-reactivity was calculated using the IC_{50}-value of each derivative according to formula (1):[15]

$$CR = (IC_{50}^*/IC_{50}) \times 100 \quad (1)$$

where CR is molar cross-reactivity [%], IC_{50}^* is IC_{50} of TNT standard [M], and IC_{50} is IC_{50} of derivatives [M].

2.9 *Indirect competitive SPR measurements for TNT and TNP-aha*

For highly sensitive detection, we used an indirect competitive method, which was effective for detecting chemical substances with low molecular weight.[16–18] The refractive index (or resonance angle) of Au thin films modified with antigen-protein conjugate depends on the concentration of the antigen, and this small change can be detected using the SPR. The schematic diagram of the indirect inhibition SPR measurement is shown in Fig. 1. In this method, antigen proteins were immobilized on the sensor chip (Au thin film) by circulating the TNP-OVA solution (1 mg/ml in PBS) for 1 h (physical adsorption). The sensor chip was washed by circulation of the carrier buffer (PBS) for 5 min. A BSA solution (2 mg/ml in PBS) was circulated for 30 min to reduce nonspecific adsorption of the antibody. After washing with carrier buffer (State A in Fig. 1), the anti-TNP antibody (appropriate concentration) solution was allowed to flow for 3 min, and the change in incidence angle shift ($\Delta\theta_0$) caused by the association of the anti-TNP antibodies to the immobilized antigens (TNP-OVA) was measured (State B). After 3 min, the flowing solution was changed to carrier buffer and allowed to flow for another 3 min, and then the

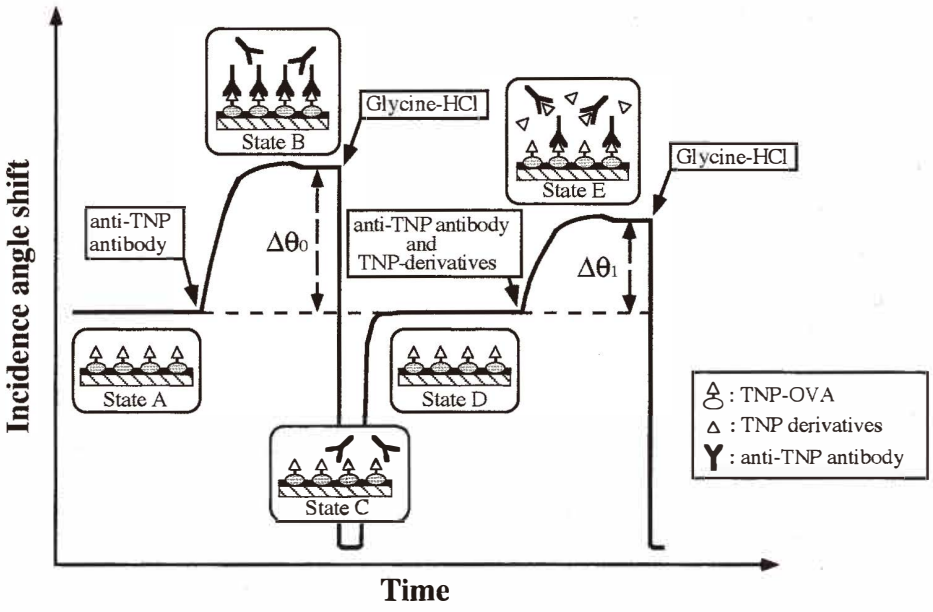


Fig. 1. Response transients of the TNP-OVA immobilized sensor to the anti-TNP antibody without antigen ($\Delta\theta_0$) and with antigen ($\Delta\theta_1$).

sensor chip was regenerated by circulation of glycine-HCl buffer (10 mM, pH 2.2) for 2 min (State C). After the chip was washed with carrier buffer and the signal base line was recovered (State D), 1 ml of an equivalent mixture of anti-TNP antibody (20, 10, or 5 μg/ml depending on the condition) and an appropriate amount of TNP derivative was allowed to flow to realize a decreased incidence angle shift ($\Delta\theta_1$) with inhibition by TNP derivative (State E). This cycle was performed for the mixture of anti-TNP antibody and serial diluted TNP derivative. Quantitative determination of the antigen concentration can be made from the difference of $\Delta\theta_0$ and $\Delta\theta_1$. The flow rate was constantly maintained at 300 μl/min, and all the procedures were carried out at room temperature.

3. Results and Discussion

3.1 *Immunization property*

The results of the immunization of the mice with TNP-BSA conjugate are shown in Fig. 2. Though the antisera titers of the control (non-immunized) mouse were almost stable, those of immunized mice increased especially after booster immunization. On the basis of these results, at day fifty the mice were finally immunized intravenously *via* the tail vein. The whole blood samples from mice were collected at day fifty-three.

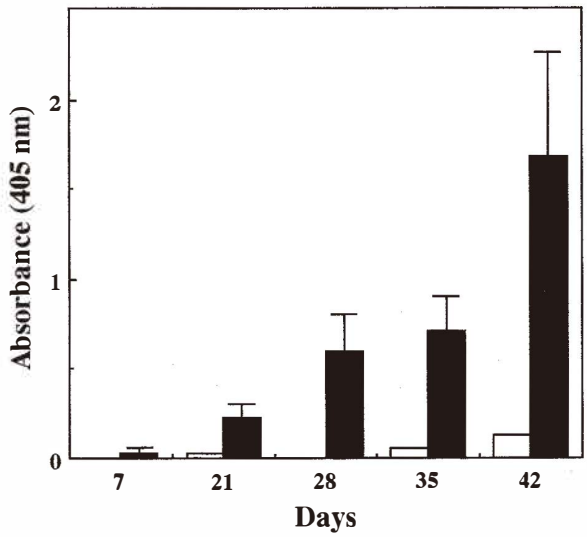


Fig. 2. Results of the immunization of the mice with TNP-BSA conjugate. The antisera 640-fold diluted with PBS were measured. Open columns and closed columns indicate the results from control mouse and immunized mice, respectively.

3.2 *Purification of antisera*

The effect of purification of antisera on the response profile of ELISA was investigated. Figure 3 shows the response profiles for TNP-aha and TNP-ea used by the protein G purified antibody (a) and affinity purified antibody after protein G purification (b). On comparison between Fig. 3(a) and 3(b), not much difference between the two treatments was observed. We decided to use the protein G purified antibody for convenience.

3.3 *Association properties of anti-TNP antibody to various kinds of nitroaromatic compounds*

First, the association properties of the raised antibody to nitroaromatic compounds were evaluated by the data of indirect competitive ELISAs. Midpoints (IC_{50}) and molar cross-reactivities are listed in Table 1. As shown in Table 1, 2, 4, 6-trinitroaniline and TNP-OH showed very low cross-reactivities of 19% and 0.8%, respectively, when the cross-reactivity of TNT was set as 100%. *N*-(2, 4, 6-TNP)-alkylamines showed relatively near molar cross-reactivities with TNT with increasing the alkylchain length. TNP-aha showed much higher association with anti-TNP antibody compared with TNT: the molar cross-reactivity was 1130%. However, TNP-gly showed low association with the antibody having a molar cross-reactivity of 44%. It is assumed that some length of the alkylchain as a spacer would be required to bind to the antibody. Nitroaromatics with two nitro groups had very low cross-reactivities, in particular, amino-DNTs showed no association with the antibody. The formal loss of one nitrogroup results in a decrease of the affinity effectively.

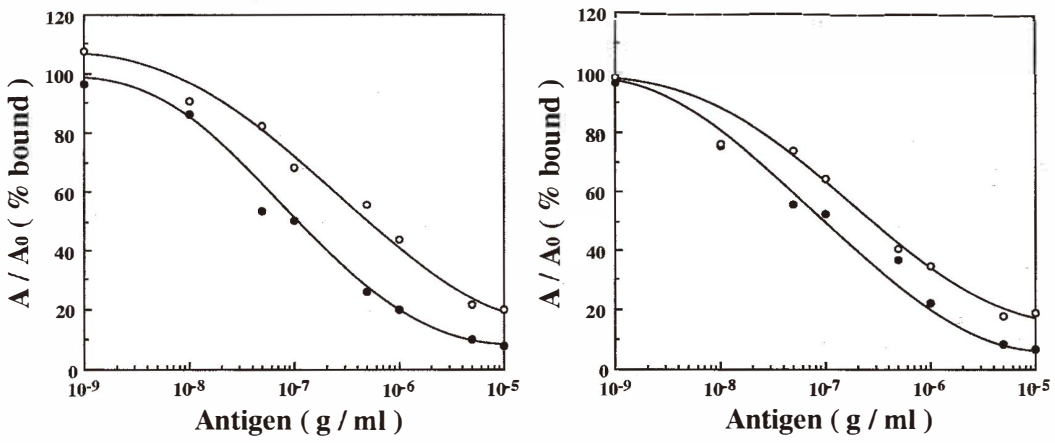


Fig. 3. Response profiles of indirect competitive ELISA using the protein G purified antibody (a) and affinity purified antibody after protein G purification (b). ●, TNP-aha ; ○, TNP-ea

Table 1
Association properties of the anti-TNP antibody to the nitroaromatic compounds.

Nitroaromatic Compounds	MW	IC_{50}(g/ml)	IC_{50}(M)	Mola Cross -reactivity(%)
2, 4, 6-Trinitrotoluene	227	6.0×10^{-7}	2.6×10^{-6}	100
N-(2, 4, 6-Trinitropheny1)-hexanoic acid	342	8.2×10^{-8}	2.3×10^{-7}	1130
N-(2, 4, 6-Trinitropheny1)-buty lamine	284	3.0×10^{-7}	1.1×10^{-6}	236
N-(2, 4, 6-Trinitropheny1)-ethy lamine	256	5.6×10^{-7}	2.2×10^{-6}	118
N-(2, 4, 6-Trinitropheny1)-gly cine	286	1.7×10^{-6}	5.9×10^{-6}	44.1
N-(2, 4, 6-Trinitropheny1)-methylamine	242	1.8×10^{-6}	7.4×10^{-6}	35.1
N-(2, 4, 6-Trinitropheny1)-hexanoic acid	228	3.2×10^{-6}	1.4×10^{-5}	18.6
2, 4, 6-Trinitroaniline	182	1.0×10^{-5}	1.4×10^{-5}	18.6
2, 4-Dinitrotoluene	168	2.0×10^{-5}	1.2×10^{-4}	2.17
2. 4, 6-Trinitrophenol	229	7.0×10^{-5}	3.1×10^{-4}	0.84
2-Amino-4, 6-dinitrotoluene	197	N.I.	N.I.	—
4-Amino-2, 6-dinitrotoluene	197	N.I.	N.I.	—

3.4 *Detection of TNT and TNP-aha by indirect competitive ELISA*

Figure 4 shows the standard curves of inhibition by TNT and TNP-aha using anti-TNP antibody in indirect competitive ELISAs. As shown in Fig. 4, TNP-aha was detected even at the concentration of 1.5×10^{-8} g/ml (about a 15% decrease). Dynamic ranges, defined by the analyte concentrations that inhibited maximum signals by 15% and 80%, were comprised over a concentration range between 1.5×10^{-8} g/ml (15 ppb) and 2×10^{-6} g/ml (2 ppm). The curve for TNT decreased slowly over a concentration range between 2×10^{-8} g/ml (20 ppb) and 10^{-6} g/ml (1 ppm).

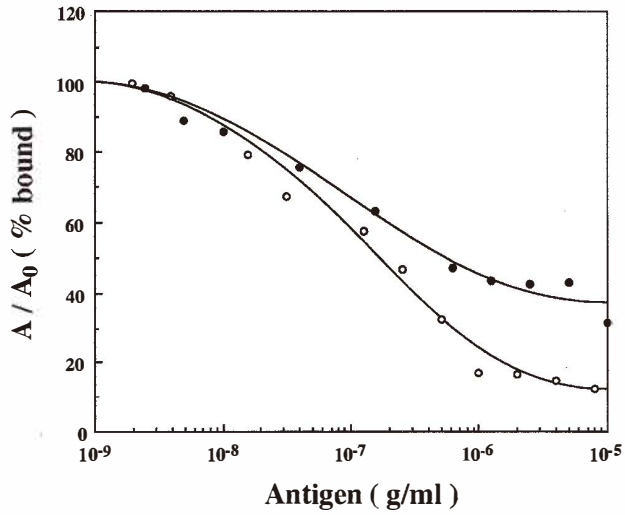


Fig. 4. Standard curves of inhibition by TNT and TNP-aha in indirect competitive ELISAs. ●, TNT ; ○, TNP-aha

3.5 *Detection of TNT and TNP-aha by SPR*

The TNP-OVA immobilized chip was exposed to the flow of anti-TNP antibody of various concentrations (5–40 μg/ml). The incidence angle shifts were plotted against the concentration of the anti-TNP antibody and the plots are shown in Fig. 5. The incidence angle shift increased rapidly with increasing concentration of the anti-TNP antibody up to 20 μg/ml and then increased slowly above this concentration. The anti-TNP antibody solutions (10, 20, and 40 μg/ml) were mixed with equal volumes of TNT of various concentrations and were incubated for 5 min at room temperature prior to flowing over the TNP-OVA immobilized sensor chip. The inhibition curves expressed as incidence angle shifts were plotted against the concentration of TNT and the plots are shown in Fig. 6(a). The RSDs (n=3) of each point were less than 4%. The inhibition curves expressed as $100\times(\Delta\theta/\Delta\theta_0)$, where $\Delta\theta$ and $\Delta\theta_0$ are the incidence angle shifts observed with TNT standard and blank samples, respectively, are shown in Fig. 6(b). The concentrations of anti-TNP antibody were described as final concentrations. The same procedures were performed for TNP-aha and the results are shown in Fig. 7(a) and 7(b). As shown in Fig. 7(b), the sensitivity for TNP-aha was apparently better when low concentrations of anti-TNP antibody were used. The lowest detection limit is defined as the concentration where the percent inhibition is increased by 15%, which is approximately three SDs, from the mean incident angle shift observed in the absence of TNP-aha. The lowest detection limit for TNP-aha was approximately 3×10^{-9} g/ml, when 10 μg/ml of anti-TNP antibody was used. It is estimated that TNT vapor concentrations from soil residues are about 0.1 ppt,[19] but there is no report that TNP-aha exists in the vapors above the buried landmines. If the

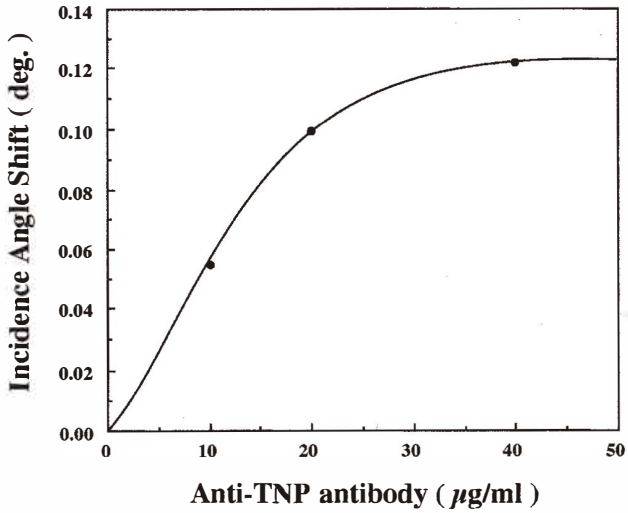


Fig. 5. Dependence of anti-TNP antibody concentration on the incidence angle shift of the TNP-OVA immobilized sensor.

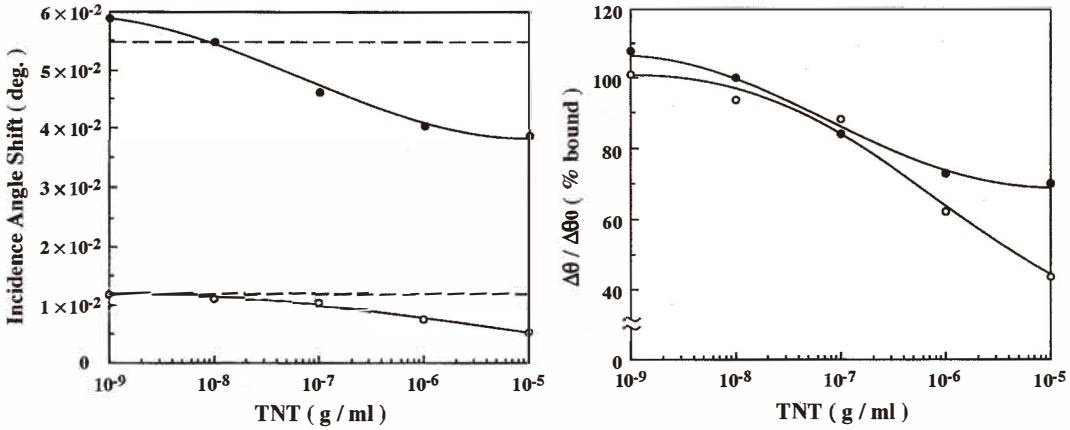


Fig. 6. Inhibition curves of TNT by indirect competitive SPR method. Open circles and closed circles indicate the results obtained using 5 μg/ml antibody and 10 μg/ml antibody, respectively. Dashed lines indicate the incident angle shifts observed without TNT. (a) the incidence angle shift ($\Delta\theta$) against TNT concentration (b) $\Delta\theta/\Delta\theta_0 \times 100$ (% bound) against TNT concentration.

monoclonal antibody specific to TNT will be produced, however, detection of sub-ppb levels of TNT will be promised using this method. To detect TNT contained in the vapors above the landmines, the vapors must be concentrated 10000 times. These vapor concentrating devices are now under construction by other members of our project team.

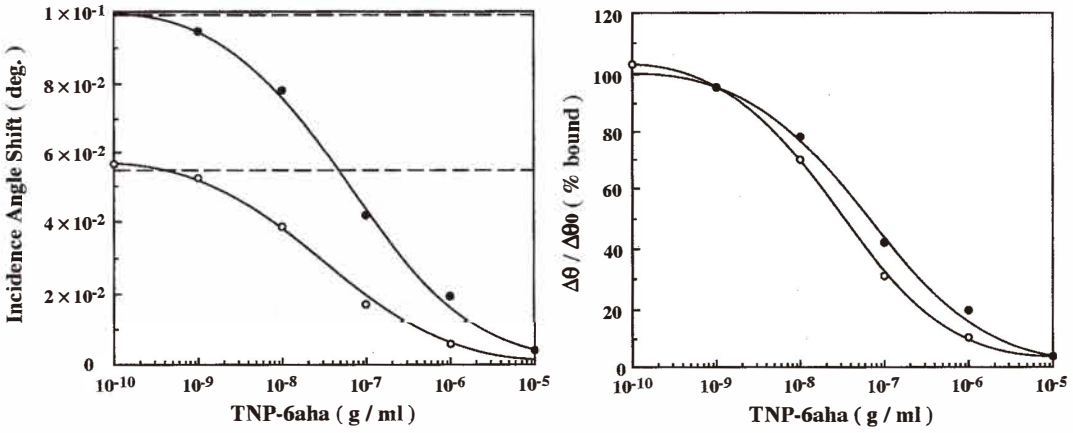


Fig. 7. Inhibition curves of TNP-aha by indirect competitive SPR method. Open circles and closed circles indicate the results obtained using 10 μg/ml antibody and 20 μg/ml antibody, respectively. Dashed lines indicate the incidence angle shifts observed without TNP-aha. (a) the incidence angle shift ($\Delta\theta$) against TNP-aha concentration (b) $\Delta\theta/\Delta\theta_0\times100$ (% bound) against TNP-aha concentration.

3.6 *Evaluation of affinity constants*

The determination of an affinity constant, K_A, or its reciprocal, the dissociation constant, K_D, is frequently useful in the study of antigen-antibody interactions. Evaluation of K_D, however, is an afflictive process because of the different values obtained by the method used for analysis. A convenient method developed by Stevens' is widely spread for calculating the K_D-value using indirect competitive ELISAs.[20] The basic equation for this analysis is as follows:

$$1/f = 1 + K_D/a_0 \tag{2}$$

Here, f is the square root of $(A_0-A_i)/A_0$ (A_i and A_0 are the absorbance with and without competing antigen, respectively), and a_0 stands for the initial concentration of competing antigen. We tried firstly to evaluate the K_D-value with the TNP-aha inhibition curve obtained in indirect competitive ELISA using Stevens' method. The K_D-value obtained was 2.1×10^{-8} M ($K_A=4.7\times10^7$ M^{-1}).

Next, we attempted to evaluate the affinity constants of the anti-TNP antibody to immobilized TNP-OVA and TNP-aha using SPR data. The analyses were performed according to the method reported by Sakai *et al.*[21,22] They evaluated affinity constants relevant to an immunoassay system by assuming a Langmuir-type adsorption model for the immunoreaction. The present system involves two competitive immunoreactions as follows:

$$\text{Ab} + \text{TNP-OVA}_{\text{bound}} \xrightarrow{K_1} \text{Ab-TNP-OVA}_{\text{bound}} \tag{3}$$

$$\text{Ab} + \text{TNP-aha} \xrightarrow{K_2} \text{Ab-TNP-aha} \tag{4}$$

Here, Ab indicates anti-TNP antibody, and K_1 and K_2 are affinity constants. Three assumptions are required to correlate the incidence angle shift with each immunoreaction. First, the antigen-antibody reaction proceeds by monovalency. Second, a Langmuir-type adsorption model can be adopted. Third, the incidence angle shift is proportional to the coverage of adsorption sites (immobilized TNP-OVA) by anti-TNP antibody. Based on these assumptions, we obtain the following equation for the first immunoreaction.

$$[\text{Ab}]/\Delta\theta_0 = [\text{Ab}]/\Delta\theta_{0,\,\max} + 1/\Delta\theta_{0,\,\max} K_1 \tag{5}$$

Here, [Ab] is the molar concentration of anti-TNP antibody, $\Delta\theta_0$ is the equilibrium incidence angle shift for a given concentration of antibody, and $\Delta\theta_{0,\,\max}$ is the maximum incidence angle shift at full coverage. Assuming the latter immunoreaction to also be equilibrium, eq. (6) can be derived.

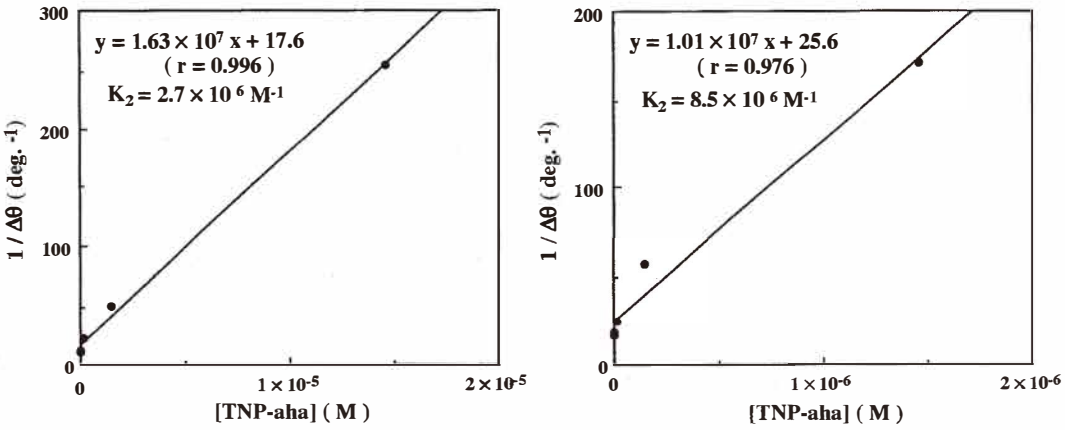
$$1/\Delta\theta = 1/\Delta\theta_0 + K_2[\text{TNP-aha}]/\Delta\theta_{0,\,\max} K_1[\text{Ab}]_0 \tag{6}$$

Here, $\Delta\theta$ and $\Delta\theta_0$ are the angle shifts in the presence and absence of TNP-aha, respectively. $[\text{Ab}]_0$ is the initial molar concentration of anti-TNP antibody.

From eq. (5), $[\text{Ab}]/\Delta\theta_0$ should be linear to [Ab], the slope and intercept giving the values of $\Delta\theta_{0,\,\max}$ and K_1, respectively. The data points in Fig. 5 are replotted in this way, and a linear correlation (correlation coefficient r=0.978) was obtained, giving $\Delta\theta_{0,\,\max}$=0.197 deg. and K_1=6.4×10^6 M^{-1}. From eq. (6), the inverse $\Delta\theta$ should be linear to [TNP-aha], K_2 being obtained from the slope and the known values of $\Delta\theta_{0,\,\max}$, K_1 and $[\text{Ab}]_0$. The data points of the SPR method using 20 μg/ml of antibody (closed circles) in Fig. 7(a) were replotted in Fig. 8(a). The linear correlation obtained gave K_2=2.7×10^6 M^{-1}. In the same way, the data obtained using 10 μg/ml of antibody (open circles) in Fig. 7(a) were replotted in Fig. 8(b), giving K_2=8.5×10^6 M^{-1}. The evaluated K_2 values by SPR are the affinity constant between anti-TNP antibody and free TNP-aha, and correspond to K_A evaluated from ELISA. The value of K_2=8.5×10^6 M^{-1} from SPR (antibody concentration 10 μg/ml) is approximately 1/5 smaller than that (4.7×10^7 M^{-1}) obtained from ELISA data using Stevens' method, but the K_2 does not take into account the bivalency of the IgG.

The K_2 value obtained from SPR using 5 μg/ml of antibody was 7.4×10^7 M^{-1} (data were not shown), so we conclude that the lower the antibody concentration used, the higher the affinity between antibody and free antigen.

The anti-TNP antibody used here is a polyclonal antibody and may contain several kinds of IgG. This was the cause for the very slow inhibition curve in both ELISA and SPR measurements. The preparations of monoclonal antibodies are now in progress. For the



(a) (b)

Fig. 8. Analyses of K_2 values from the result by indirect competitive SPR measurement of TNP-aha using 20 μg/ml antibody (a) and 10 μg/ml antibody (b).

purpose of searching for explosives, however, polyclonal antibodies are useful if inhibition occurs in low concentrations. We are now studying both approaches using monoclonal and polyclonal antibodies.

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References

1 J. MacDonald, J. R. Lockwood, J. McFcc, T. Altshuler, T. Broach, L. Carin, R. Harmon, C. Rappaport, W. Scott and R. Weaver: Rand (2003) 1.
2 A. Goth, I. G. MacLean and J. Trevelyan: Geneva International Centre for Humanitarian Demining (2002).
3 P. Y. Piece and O. Carmona: J. Applied Geophysics **40** (1998) 59.
4 R. J. Stanley, P. D. Gader and K. C. Ho: Information Fusion **3** (2003) 215.
5 J. V. Goodpaster and V. L. McGuffin: Anal. Chem. **73** (2001) 2004.
6 A. Hilmi and J. H. Luong: Electrophoresis **21** (2000) 1395.
7 A. Hilmi, J. H. Luong and A. L. Nguyen: J. Chromatogr. A **844** (1997).
8 E. R. Goldman, A. Hayhurst, B. M. Lingerfelt, B. L. Iverson, G. Georgiou and G. P. Anderson: J. Environ. Monit. **5** (2003) 380.
9 T. M. Green, P. T. Charles and G. P. Anderson: Anal. Biochem. **310** (2002) 36.
10 R. Wilson, C. Clavering and A. Hutchinson: Analyst **128** (2003) 480.
11 F. Deckert and F. Legay: Anal. Biochem. **274** (1999) 81.

12 T. P. Vikinge, A. Askendal, B. Liedberg, T. Lindahl and P. Tengvall: Biosens. Bioelectron. **13** (1998) 1257.
13 R. Haynes, D. T. Osuga and R. E. Feeney: Biochemistry **6** (1967) 541.
14 A. Zeck, M. G. Weller and R. Niessner: Fresenius J. Anal. Chem. **364** (1990) 113.
15 E. W. Weiler and M. H. Zenk: Phytochemistry **15** (1976) 1537.
16 K. V. Gobi, S. J. Kim, R. Harada and N. Miura: Electrochem. **71** (2003) 430.
17 N. Miura, H. Higobashi, G. Sakai, A. Takeyasu, T. Uda and N. Yamazoe: Sens. Actuators B **13** (1993) 188.
18 N. Miura, M. Sakai, K. V. Gobi, C. Kataoka and Y. Shoyama: Biosens. Bioelectron. **18** (2003) 953.
19 T. F. Jenkins, M. E. Walsh, P. H. Miyares, J. A. Kopczynski, T. A. Ranney, V. George, J. C. Pennington and T. E. Berry Jr.: "Release of Explosive-Related Vapors from Land Mines." Cold Regions Research and Engineering Laboratory, Technical Report ERDC TR-00-5, August 2000.
20 F. Stevens: Mol. Immunol. **24** (1987) 1055.
21 G. Sakai, K. Ogata, T. Uda, N. Miura and N. Yamazoe: Sensors and Actuators B, **49** (1998) 5.
22 G. Sakai, S. Nakata, T. Uda, N. Miura and N. Yamazoe: Electrochim. Acta, **44** (1990) 3849.