Development of an ELISA Method for Detecting Immune Complexes Between Tissue-Nonspecific Alkaline Phosphatase and Immunoglobulin G

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A convenient method for measuring immune complexes between tissue-nonspecific alkaline phosphatase (TNSALP) and immunoglobulin G (IgG) (i.e., TNSALP-IgG) would be highly useful for routine analyses. Here, we identified a surface-active agent that would dissolve membrane but not dissociate TNSALP-IgG complexes. Next, we developed an enzyme-linked immunosorbent assay (ELISA) method for detecting TNSALP-IgG complexes with two monoclonal antibodies (MoAbs): 3-29-3R was coated on assay plates and captured TNSALP-IgG from a specimen; an horseradish peroxidase (HRP)-conjugated anti-human IgG1 then reacted with captured TNSALP-IgG to form an “immunocomplex sandwich.” The immunocomplex was detected via the absorbance of an HRP substrate, resulting in a semiquantitative assay. The mean absorbance of 0.195

($n = 5$) measured in sera from healthy donors was designated as an arbitrary unit (AU/mL) of TNSALP-IgG concentration. The ELISA values of patient sera known to contain TNSALP-IgG complexes were greater than those of normal sera (normal, 1.86 plusmn; 0.61; patient, 9.30 plusmn; 5.44), and these data were confirmed by electrophoresis. Thus, the ELISA could detect TNSALP-IgG complexes. The intraassay coefficient of variation (CV) was within 7.4% and analytical recovery was excellent. There was no significant interference from hemolytic, lipemic, or icteric serum. In summary, an ELISA using 3-29-3R MoAb and HRP-conjugated anti-human IgG1 constitutes a reliable and convenient method for the semiquantitative detection of TNSALP-IgG complexes in human serum. *J. Clin. Lab. Anal.* 21:322–329, 2007.

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Abbreviations: ELISA, enzyme-linked immunosorbent assay; HRP, horseradish peroxidase; CV, coefficient of variation; ALP, alkaline phosphatase; SNP, single nucleotide polymorphism; PAGE, polyacrylamide gel electrophoresis; BSA, bovine serum albumin; SDS, sodium dodecyl sulfate; SD, standard deviation.

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INTRODUCTION

Complexes formed between tissue-nonspecific alkaline phosphatase (TNSALP) and immunoglobulin G (IgG) (i.e., TNSALP-IgG complexes) have been found not only in patients with multiple epiphyseal dysplasia (1) but also in the serum of patients suffering from malignancies, liver diseases, ulcerative colitis, and autoimmune diseases (1–4). In autoimmune etiologies, these TNSALP-IgG complexes contain autoantibodies (3,5). Furthermore, TNSALP-IgG complexes have been frequently observed in ulcerative colitis, and this immunological abnormality has often been associated with liver diseases (6). Alkaline phosphatase (ALP) isoforms in TNSALP-IgG complexes consist of liver or bone variants of ALP (2–4). In addition, soluble and insoluble types of TNSALP are found in human serum. Soluble TNSALP is present in liver and bones, mainly as a dimer and/or tetramer. The insoluble type, termed membrane-TNSALP, is found in the serum of individuals with liver disease (5,7).

To detect TNSALP-IgG complexes, methods such as immunoelectrophoresis (1,8), immunofixation electrophoresis (9), affinity electrophoresis (10), and electrosyneresis (11) were developed in the 1970s and 1980s. However, these methods involve tedious assay procedures and are unsuited to the simultaneous assay of multiple specimens. In particular, it is difficult to confirm some data from disk electrophoresis, such as overlap or minor shifts of bands. The possibility that IgG can combine with TNSALP on membranes to form a membrane-TNSALP-IgG complex also makes clinical assessment difficult (Fig. 1). One other reason may be that single nucleotide polymorphisms (SNPs) in ALP

can affect the results of electrophoresis by leading to different ALP variants (12). Furthermore, posttranscriptional modification of TNSALP dependent on disease can also give rise to multiple bands on electrophoresis. Thus, even though it would have clinical utility, a simple method of measuring TNSALP-IgG with easy interpretation of the data has not been developed, and these early analytical methods have not been advanced until recently. At present, although methods to detect TNSALP-IgG complexes exist, research into TNSALP-IgG is ongoing. We therefore wanted to develop a simple method of isolating TNSALP-IgG using a surface-active agent that could dissolve a membrane. Initially, we identified a surface-active agent that would dissolve a membrane but not dissociate TNSALP-IgG complexes. Subsequently, we applied this surface-active agent to enzyme-linked immunosorbent assay (ELISA). Here, we report a novel ELISA method that will aid routine screening for TNSALP-IgG complexes (Fig. 2).

MATERIALS AND METHODS

Serum Specimens

Sera from healthy volunteers ($n = 5$) taken with their informed consent were used as normal serum. Sera containing TNSALP-IgG complexes ($n = 5$) and membrane-ALP were obtained from the Laboratory of Medicine at Niigata Cancer Center Hospital, where serum specimens are collected routinely for ALP isozyme analysis. Although the sera contained high amounts of TNSALP-IgG complexes, the clinical conditions of the patient sera supplied were unknown because the sera were collected anonymously.

Preparation and Characterization of TNSALP From SaOS-2 Cells

The human osteosarcoma cell line (SaOS-2; HTB-85) was purchased from the American Type Culture Collection (ATCC) and cultured in alpha minimum essential medium (Dainippon Pharmaceutical Co., Osaka, Japan) containing 10% fetal calf serum (13). TNSALP was purified from proliferated SaOS-2 cells according to a previously described method (14,15). To characterize SaOS-ALP, a heat inactivity test and an inhibition test with amino acids were performed. Enzyme activity was measured after incubation of the enzyme at 56°C for 5 min, at 65°C for 10 min, or with L-phenylalanine, L-leucine, or L-homoarginine at 5 mM. ALP activity of the specimens was measured with a Hitachi 7150 automatic analyzer (Hitachi Ltd., Tokyo, Japan) using the Quick Auto Neo ALP-JS kit (Shinotest Co., Tokyo, Japan).

Fig. 1. Schematic of TNSALP-IgG. TNSALP and some immunoglobulin can bind in serum (free TNSALP-IgG complex). Furthermore, TNSALP that binds to membrane (membrane-TNSALP) with an anchor becomes “macro ALP” in serum. It is also predicted that some immunoglobulin may bind to TNSALP on the membrane (membrane-TNSALP-IgG complex). Thus, for accurate measurement of TNSALP-IgG, TNSALP-IgG must be separated from the membrane.

Fig. 2. Semiquantitative method of TNSALP-IgG measurement. The immunocapture enzymatic assay measured TNSALP activity, whereas the ELISA measured TNSALP-IgG concentration in serum. The mean absorbance value of five different normal sera was temporarily set as the standard concentration of the complex and was thus defined as 1 arbitrary unit (AU)/mL. The absorbance value of the specimens measured by ELISA was directly divided by this mean absorbance value, and the ratios obtained were multiplied by 1,000 and used as a relative measure of the concentration of TNSALP-IgG complexes in specimens.

Development of Monoclonal Antibodies to TNSALP

Monoclonal antibodies (MoAbs) showing strong binding ability to SaOS-ALP were prepared in mouse ascites according to a conventional procedure (14). The class and subclass of the MoAbs were determined using a mouse MoAb isotyping kit (Amersham Biosciences, Piscataway, NJ).

Measurement of ALP Activity

The total ALP activity was measured with a Hitachi 7600-110 automatic analyzer (Hitachi, Ltd.) using the Quik Auto Neo ALP-JS kit (Shinotest Co.). Liver-ALP, Bone-ALP, Intestine-ALP, and Placenta-ALP were adjusted to 25 IU/L for the experiments. ALP liver marker at 100 IU/mL (Lot33101; Jokoh Co., Ltd., Tokyo, Japan) was used as a standard for the measurement of ALP activity.

Surface-Active Agent Treatment

In order to digest the membrane-TNSALP, the following surface-active agents were tested: sucrose monolaurate (16), sodium cholate and 3-[(3-cholamidopropyl) dimethylammonio] propanesulfonic acid (CHAPS) (Wako Pure Chemical Industries, Ltd., Tokyo, Japan). Each detergent was dissolved in 10 mM Tris -HCl buffer (pH 7.5) to make a 10% suspension according to the described previously preparation procedure (13). A total of 50 microliters of detergent solution was mixed with 50 μ L of 10 mM Tris -HCl buffer (pH 7.5) and 200 μ L of serum, and the mixture (final detergent concentration

1.7%) was used for polyacrylamide gel electrophoresis (PAGE) and ELISA.

Electrophoresis

We used electrosynthesis and PAGE to detect TNSALP-IgG complexes. Essentially, the same electrosynthesis method as described previously (7,11) was used for the detection of TNSALP-IgG complexes in serum. For electrophoresis, agarose (ICN ImmunoBiologicals, Lisle, IL) was prepared at 1.5%, and rabbit anti-human polyclonal IgG antibody (Dako Cytomation, Japan Co. Ltd., Kyoto, Japan) and Beronal Buffer (Jokoh Co., Ltd.; $\mu = 0.05$, pH 8.6) were used. PAGE was carried out with a polyacrylamide disc gel kit (AlkPhor; Jokoh Co., Ltd.). To evaluate the stability of TNSALP-IgG complexes, 50 μ L of serum, or serum treated with surface-active agent, was incubated with rabbit anti-human IgG polyclonal antibody (6 μ g/mL; Dako Cytomation Japan Co. Ltd., Kyoto, Japan) or anti-human TNSALP MoAb (6 μ g/mL) for 24 hr at 4°C. The mixture was then applied to PAGE. To evaluate ALP activity after PAGE analysis, the gels were stained with 5-bromo-4-chloro-3-indolylphosphate, p-toluidine salt (BCIP) (17).

Construction of Immunocapture Enzymatic Assay

To measure TNSALP activity by immunocapture enzymatic assay, the wells of a plastic microplate (Nalgen Nunc, Rochester) were coated with 100 μ L of anti-human TNSALP MoAb at 20 μ g/mL in phosphate-buffered saline (pH 7.0) for 12 hr at 4°C. After three

washes with 200 μ L of wash buffer (20 mM Tris-HCl buffer, pH 7.5, containing 0.05% Tween 20), the wells were blocked with 200 μ L of 20 mM Tris-HCl buffer (pH 7.0) containing 1.5% bovine serum albumin (BSA) for 24 hr at 4°C. For the assay, 10 μ L of serum was premixed with 90 μ L of 100 mM Tris-HCl buffer (pH 7.5), and the mixture was added to the plate wells and incubated for 1 hr at room temperature. After the plate was washed three times with 200 μ L of washing buffer, 100 μ L of p-nitrophenylphosphate (Nitto Boseki Co., Ltd., Tokyo, Japan) substrate solution (5.5 mM 2-ethylaminoethanol buffer, pH 9.8) was added and incubated for 1 hr at 37°C. The enzyme reaction was then stopped by adding 100 μ L of 1 N NaOH solution and absorbance at 405 nm was measured on a Model 550 microplate reader (Bio-Rad Laboratories, San Jose, CA).

Construction of ELISA for Measuring TNSALP-IgG Complexes

To detect TNSALP-IgG complexes by a two-step sandwich ELISA, a standard ELISA assay plate was prepared. The method for the immunocapture enzymatic assay was then followed to the addition of serum. After the primary reaction, the plate was washed three times with 200 μ L of washing buffer and 100 μ L of horseradish peroxidase (HRP)-labeled mouse anti-human IgG1 MoAb (Dainippon Pharmaceutical Co.) was added. After incubation for 1 hr at 37°C, the plate was washed three times and 100 μ L of O-phenylenediamine at 3 mg/mL in citrate buffer was added and incubated for 10 min at 25°C. The enzyme reaction was stopped by adding 100 μ L of 1 N sulfuric acid and absorbance at 492 nm was measured on a Model 550 microplate reader (Bio-Rad Laboratories).

Characterization of ELISA

Linearity

The linearity of the ELISA was tested with five different patient serum samples that were observed to contain TNSALP-IgG by electrophoresis. Each serum was diluted 10-, 20-, 40-, and 80-fold with 100 mM Tris-buffer (pH 7.5) solution containing 2% BSA. The diluted samples were used for the ELISA assay.

Precision

Analytical reproducibility in one plate (8 [A–H] $\times$ 12 [1–12] = 96 wells) was simultaneously evaluated with a TNSALP-IgG-positive specimen.

Interference on the ELISA

The effects of hemolytic serum (hemoglobin concentrations at 0, 100, 200, 300, 400, and 500 mg/dL), lipemic

serum (fatty acid concentrations at 0, 500, 1000, 1500, and 2000 mg/dL) and icteric serum (bilirubin C and F concentrations each at 0, 5, 10, 15, and 20 mg/dL) on the ELISA were studied with a specimen containing the TNSALP-IgG complex at 3.6 AU/mL (absorbance $\sim$ 0.7 at 492 nm).

RESULTS

Preparation and Characterization of SaOS-ALP

The apparent molecular size of purified SaOS-ALP was estimated to be 155 kDa on sodium dodecyl sulfate PAGE (SDS-PAGE) (data not shown). Purified SaOS-ALP was characterized by an inhibition test with amino acids and by heat treatment. The activity of the SaOS-ALP was inhibited to 27.0%, 41.3%, and 63.6% of the original activity in the presence of L-phenylalanine, L-leucine, and L-homoarginine, respectively. Approximately 65% of the original SaOS-ALP activity was lost at 56°C, and a complete loss of activity occurred at 65°C. For liver-ALP, bone-ALP, and intestine-ALP, loss of activity similar to that measured for SaOS-ALP was observed at both 56°C and 65°C.

Selection of MoAb for ELISA

The immunoreactivity of established MoAbs against liver-ALP, bone-ALP, intestine-ALP, placenta-ALP, and SaOS-ALP was evaluated. The 3-29-3R (Class IgG, Light Chain κ ; Nalge Nunc, Rochester, NY) showed stronger binding to liver-ALP and bone-ALP than to SaOS-ALP and no binding to intestine-ALP or placenta-ALP.

Electrophoresis

On electrosynthesis, a line of precipitin between serum specimens and anti-human IgG antibody was observed (Fig. 3). ALP activity was also observed in the same region of precipitin. On PAGE analysis, all specimens of normal sera showed strong bands of liver-ALP and bone-ALP and weak or no bands of membrane-ALP at the top of the gel (Fig. 4a), whereas in patient sera intestine-ALP and strong membrane-ALP bands were observed in addition to the liver-ALP and bone-ALP bands (Fig. 4b). Furthermore, the membrane-ALP bands partially disappeared and new, distinct ALP bands (soluble-ALP) emerged after treatment with sucrose monolaurate (Fig. 5, Lane 2). On the mixture of surface-active agent-treated serum and 3-29-3R MoAb, the soluble-ALP bands disappeared completely, and new bands emerged on the gel between the membrane-ALP and the intestine-ALP positions (Fig. 5, Lane 3). When the sera were mixed with rabbit anti-human IgG polyclonal antibody, a similar partial disappearance of the soluble-ALP bands occurred (Fig. 5, Lane 4).

Fig. 3. Electrosyneresis of patient sera using anti-human IgG antibody. Five patient serum samples, Nos. 6–10, were analyzed by electrosyneresis. Rabbit anti-human IgG antibody and serum specimens were placed in the well and the sample holes, respectively. ALP activity was observed on the precipitate lines. The arrow indicates the direction of electrophoretic flow.

Fig. 4. PAGE of TNSALP in normal and patient sera. **a:** Lanes 1–5 contained the five normal serum specimens. **b:** Lanes 6–10 contained the five patient serum specimens. Membrane-ALP has a high molecular weight. Arrows indicate the direction of electrophoretic flow. ALP activity was observed as described in Materials and Methods using BCIP.

Performance of ELISA for the Detection of TNSALP-IgG Complexes

Effect of Surface-Active Agent

In the ELISA, the absorbance at 492 nm of the specimens treated with sucrose monolaurate

was increased to 149% of the value without surface-active agent treatment. No significant changes in absorbance at 492 nm in the ELISA or the pattern of bands on PAGE occurred after specimen treatment with sodium cholate or CHAPS.

Calculation of TNSALP-IgG

As shown in Table 1, the absorbance values of TNSALP-IgG complexes for normal serum ranged from 0.130 to 0.253 and the mean value was 0.195 plusmn; 0.046 standard deviation (SD) (n = 5). This mean value was temporarily defined as a TNSALP-IgG complex concentration of 1 AU/mL. For the patient sera, higher absorbance values surpassing 1.000 were obtained for all

samples. These absorbance values were directly divided by the mean absorbance value of normal sera, resulted a mean assay value of 9.28 plusmn; 5.09 AU/mL for patient sera.

The TNSALP activity of normal and patient sera was also measured. All patient sera showed higher ALP activities as compared with normal sera (Table 1). The ratio of TNSALP-IgG complexes (AU/mL) to ALP activity (IU/L) was then calculated for each serum and

Fig. 5. PAGE of serum treated with sucrose monolaurate. Lane 1: no treatment; Lane 2: serum treated with sucrose monolaurate at 1.7%; Lane 3: sucrose-monolaurate-treated serum mixed with 3-29-3R MoAb; Lane 4: sucrose-monolaurate-treated serum mixed with anti-human IgG polyclonal antibody. The arrow indicates the electrophoretic flow. Soluble-ALP was observed in specimens treated with surface-active reagent and confirmed to react with the TNSALP MoAb 3-29-3R. The immunocomplex between TNSALP and 3-29-3R migrated slowly.

TABLE 1. Measurement of TNSALP-IgG complex*

	(A) TNSALP-IgG complex		(B) TNSALP activity		(C) Ratio × 1,000
	A492	AU/mL	A492	IU/mL	(A)/(B) × 1,000
Normal serum					
1	0.130	0.67	0.123	120.30	5.57
2	0.253	1.30	0.160	156.49	8.31
3	0.222	1.14	0.140	136.93	8.33
4	0.194	0.99	0.202	197.57	5.01
5	0.177	0.91	0.245	239.62	3.80
Mean	0.195	1.00	0.174	170.18	6.20
SD	0.046	0.24	0.049	48.36	2.03
Patient serum					
6	1.000	5.10	0.339	331.56	15.38
7	3.460	17.70	0.386	377.53	46.88
8	2.000	10.30	0.603	589.76	17.46
9	1.382	7.10	0.134	131.06	54.17
10	1.200	6.20	0.301	294.39	21.06
Mean	1.808	9.28	0.353	344.86	30.99
SD	0.996	5.09	0.169	165.44	18.13

* (A) A492: Absorbance at 492 nm, (B) AU/mL: arbitrary units/mL, IU/mL: international units/mL, (C) Ratio × 1000: (A)/(B) × 1,000.

TABLE 2. Intraassay CV for ELISA of TNSALP-IgG complex*

	Lanes								Total
	A	B	C	D	E	F	G	H	
n	11	11	12	12	12	12	12	12	94
Mean	0.570	0.565	0.570	0.558	0.558	0.559	0.550	0.549	0.560
SD	0.016	0.017	0.022	0.016	0.041	0.024	0.036	0.031	0.027
CV (%)	2.8	3.0	3.9	2.8	7.4	4.3	6.6	5.7	4.9

*Each lane (A–H) of the plastic microplate contained 12 wells. The mean absorbance and SD at 492 nm of 11–12 samples were calculated and CV was expressed as a percentage of 1 SD against the mean absorbance value of each lane.

multiplied by 1,000. The results showed that patient sera (mean 9.30 plusmn; 5.44) had higher concentrations of TNSALP-IgG complexes than normal sera (mean 1.86 plusmn; 0.61).

Linearity

A typical standard curve of the immunocapture enzymatic assay and ELISA showed an almost linear relationship. All five patients sera (Nos. 6–10) showed excellent dilution linearity.

Precision

The analytical reproducibility was evaluated and the results obtained are listed in Table 2. The intraassay coefficients of variation (CVs) ranged from 2.8% (n = 12) in lane D to 7.4% (n = 12) in lane E of the plastic plate. The total assay CV was 4.9% (n = 94).

Interference

Hemolytic, lipemic, and icteric sera were tested, and the composition of these samples did not interfere at all with the assay results.

DISCUSSION

From the results of heat inactivation and amino acid inhibition, SaOS-2 TNSALP showed the characteristics of bone- or liver-type ALP and was suitable for generating MoAb. The MoAb 3-29-3R recognized TNSALP, but not intestine-ALP or placenta-ALP, and was used in two types of assay system, an immunocapture enzymatic assay and ELISA. The immunocapture enzymatic assay detected the TNSALP activity and ELISA detected the amount of TNSALP-IgG complexes. Assuming that ALP activity per mole of ALP was equal in all sera, the ratio of TNSALP-IgG complexes to ALP activity (C in Table 1) can be taken as an index for comparing the molar ratio of IgG to ALP in TNSALP-IgG complexes. Taking these ratios into consideration, the patient sera contained more IgG molecules per ALP molecule of TNSALP-IgG complex than did normal sera. It seems likely that there is a

heterogeneous population of TNSALP-IgG complexes in serum. Thus, our ELISA method should be semiquantitative. Needless to say, the complex formed between ALP and IgG is specific, whereby ALP is bound to IgG through the F(ab)' of IgG (2).

Using normal sera, we observed significant absorbance at 492 nm on ELISA (Table 1), and the mean value was temporarily assigned as the concentration of TNSALP-IgG complexes indicated by an arbitrary unit (1 AU/mL) to estimate the apparent concentrations of TNSALP-IgG complexes. Although TNSALP-IgG complexes could be detected in human patient sera by electrosynthesis (Fig. 3), the presence of TNSALP-IgG complexes in normal sera was not confirmed. By contrast, the sensitivity of the ELISA could detect TNSALP-IgG complexes in normal serum. The higher absorbance values obtained in the ELISA seemed to correspond to an increase in membrane-ALP concentrations in patient sera (Table 1; Fig. 4), indicating that the amount of TNSALP-IgG complexes was increased. When serum containing membrane-ALP was treated with the surface-active agent sucrose monolaurate, the increase in ELISA absorbance was accompanied by a disappearance in membrane-ALP and the appearance of a new soluble-ALP in electrophoresis (Fig. 5, Lane 2). It is reasonable to speculate that the soluble-ALP becomes available for immunoreaction in an ELISA in the presence of sucrose monolaurate and, therefore, that the ELISA shows an increase in absorbance. In fact, the soluble ALP completely disappeared on the addition of 3-29-3R MoAb (Fig. 5, Lane 3). By contrast, the rabbit anti-human IgG polyclonal antibody caused only a very small reduction in soluble-ALP as compared with 3-29-3R MoAb (Fig. 5, Lane 4). These results suggest that soluble-ALP does in fact react with the antibody but weakly. Further studies are needed to confirm the effect of surface-active agents on the ELISA, but these results suggest that sucrose monolaurate is very effective for the digestion of membrane-ALP.

In terms of the performance of this novel ELISA, the intraassay CV was within 5% and analytical recovery was excellent. There was no significant interference from

the components of hemolytic, lipemic, or icteric serum. Although only semiquantitative, this ELISA using 3-29-3R MoAb and HRP-anti-human IgG MoAb is reliable and may be useful for the diagnosis of diseases, such as ulcerative colitis (5), because it enables the simultaneous assay of multiple samples and readily detects TNSALP-IgG complexes in human sera. Such a novel ELISA method may be of great utility for routine initial screening for TNSALP-IgG.

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